EFFECTS OF MILK PROTEIN INGREDIENTS ON PHYSICO-CHEMICAL PROPERTIES OF RICE STARCH

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ABSTRACT

The overall aim of this thesis is to determine if the interactions between normal and waxy rice starch and milk proteins from four milk protein ingredients, namely skim milk powder (SMP), milk protein concentrate (MPC), sodium caseinate (NaCAS) and whey protein isolate (WPI) do occur, and to identify the mechanisms underlying these interactions.

Different milk protein ingredients at various concentrations (0 to 10%, w/w) affected markedly and differently the pasting behaviour of 10% (w/w) rice starches. SMP delayed the pasting of both rice starches by increasing the onset temperature (T_{onset}) and the peak viscosity temperature (T_{peak}) of pasting. This was mainly due to the presence of lactose and ions, which was further supported by the investigation of the effects of UFSMP (a solution of salts and lactose present in SMP at their proper concentration) and lactose. The addition of NaCAS also delayed the pasting of rice starch; T_{peak} in the case of both starches was increased. For normal rice starch paste, MPC and WPI decreased the T_{peak}. MPC had no affect on T_{peak} of waxy rice starch paste.

The qualitative viscoelastic behaviour of rice starch/milk protein ingredient gels obtained from the above pastes was dominated by the continuous phase made of the starch molecules. There was evidence, as indicated by confocal microscopy, of phase separation between the milk proteins of SMP and MPC and the two starches. The phase separation was not observed in the addition of either NaCAS or WPI.

Studies on the thermal behaviour of rice starch/milk protein ingredient mixtures by differential scanning calorimetry (DSC) showed that SMP, similarly to UFSMP, delayed the gelatinization of both starches. NaCAS also delayed the gelatinisation of both starches but had a greater effect on waxy than normal rice starch. The addition of NaCAS did not affect T_{onset} but increased T_{peak} for normal rice starch, whereas the gelatinisation temperature of waxy rice starch was highly affected by the addition of NaCAS with both T_{onset} and T_{peak} shifted to higher temperatures. MPC had no affect on

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the gelatinization temperature of normal rice starch, whereas the gelatinization temperature of waxy rice starch was increased by the addition of MPC. The addition of WPI to both rice starches showed two thermal transitions. The first of these was due to the gelatinisation of the starches and the second to the denaturation of β-lactoglobulin (β-lg). The addition of WPI to normal rice starch showed that the thermal behaviour of normal starch and protein were independent from each other. In contrast, the thermal behaviour of waxy rice starch was modified by the addition of WPI; both T_{onset} and T_{peak} were increased.

SMP decreased the T_{onset} of swelling, swelling ratio and the amount of starch leaching from both starches. These observed changes were due to the presence of lactose and ions in SMP. NaCAS slightly increased T_{onset} of swelling but the amount of starch leaching was reduced for both rice starches. The rigidity of both starches tended to increase in the presence of NaCAS. MPC and WPI affected the swelling behaviour of normal and waxy rice starch differently. A dramatic increase in the swelling of normal rice starch/MPC or WPI mixtures was observed, whereas this trend was not evident for waxy rice starch/ MPC or WPI mixtures. The difference in the water holding ability and gelatinization peak temperatures of the two starches over the temperature range at which whey proteins denature and form gels are believed to be responsible for the observed differences.

The results from confocal microscopy showed that milk proteins, such as α-casein, β-casein, β-lg and α-lactalbumin (α-la), were adsorbed onto the granule surface of both normal and waxy rice starch. The mechanism for this adsorption is the hydrophilic interactions; hydrogen bonds between hydroxyl group from terminated glucan molecule that protrude around starch granule surface-hydroxyl; amino, or other electron-donation or electron-accepting groups of the added proteins. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) it was found that for SMP and MPC the adsorbed α_s- to β-casein ratio on both starches was similar to the α_s-casein to β-casein ratio in the casein micelle at low SMP and MPC concentrations. But at high concentrations of SMP or MPC, this ratio decreased indicating that more β-casein was adsorbed preferentially to α_s-casein. In the case of NaCAS, α_s-casein was adsorbed

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preferentially to β-casein. Moreover, there was evidence of multilayer adsorption of α_s-casein into the surface of rice starch granules. Compared to the other milk protein ingredients, very small amounts of the β-lg and α-la from WPI were adsorbed onto starch granules. However, the adsorbed amounts of β-lg and α-la from WPI continuously increased with increasing WPI concentration, suggesting that these two proteins, particularly β-lg, adsorbed in multilayers too.

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Chapter 1

INTRODUCTION

Starches are widely used in dairy-based food products, such as dairy desserts, yoghurt, processed cheese and imitation cheese, where they are added as a functional ingredient to obtain desired rheological and sensory properties. However, starch-milk protein interactions are not utilised specifically to obtain these desired rheological and sensory properties. They are also added to the dairy-based food products to reduce the amount of the costly dairy ingredients needed and hence provide significant cost-savings (Keogh and O'Kennedy, 1998; MacDougall, 1998; Tamime, Muir, Shenana, Kalab and Dawood, 1999; Mounsey and O'Riordan, 2001; Guinee, Caric and Kalab, 2004; Sandoval-Castilla, Lobato-Calleros, Aguirre-Mandujano and Vernon-Carter, 2004; Montesinos-Herrero, Cottell, O'Riordana and O'Sullivan, 2006; Oh, Anema, Wong, Pinder and Hemar, 2007; Zuo, Hemar, Hewitt and Saunders, 2007; Doublier and Durand, 2008; Mounsey and O'Riordan, 2008b; Mounsey and O'Riordan, 2008c; Mounsey and O'Riordan, 2008a).

Gelatinized rice starches have a bland flavor and produce a smooth, creamy spreadable gel, which make them suitable for the use in dairy based food products (Champagne, 1996). Rice starch granules are extremely small (1.5 to 15 μm in diameter), rice starches have a more homogenous size distribution than other cereal grain starches (Blanshard, 1987; Champagne, 1996; Bao and Bergman, 2004), with particle size comparable to fat globules, making them ideal fat replacers (Champagne, 1996). In addition, starches from waxy rice varieties have much better freeze-thaw characteristics than other starches, and hence they are ideal for use in frozen products such as desserts, and frozen gravies (Champagne, 1996).

Despite their industrial importance, and the fact that individually starch and milk proteins have been extensively studied, studies on their interactions are relatively scarce compared to the mixtures of milk proteins with other biopolymers. For this

reason, a systematic study of mixtures of the normal and the waxy rice starches and different dairy ingredients would advance our understanding of how starches and milk proteins interact during processing of typical dairy product formulations.

1.1 Research objectives

The overall aim of this thesis is to determine if milk proteins-rice starch interactions do occur, and to identify the mechanisms underlying these interactions.

1.2 Research outline

The first step of the research was a set of experiments to demonstrate the interaction of milk protein and rice starch. Chapter 3, investigates how the pasting behaviour of rice starches (both normal and waxy) is affected by milk proteins. The viscoelastic behaviour and microstructure of gels resulting from the pasting of rice starch-milk protein ingredients, are studied in Chapter 4. Subsequently, the effects of milk proteins on the thermal behaviour of rice starch were studied in Chapter 5. Chapter 6 and 7, were conducted in order to identify the mechanisms that explain the interactions between rice starch and milk proteins.

Chapter 2

LITERATURE REVIEW

2.1 Rice starch

Rice (*Oryza sativa* L.) which belongs to the *Porceae Gramineae* or grass family, is one of the most important staple foods of the world (Bao and Bergman, 2004). Kennedy and Burlingame (2003) recognize three sub-species of *Oryza sativa*; *indica, japonica* and *javanica*. These three sub-species consist of genotypes which have different starch properties. They can be divided into two types depending on their amylose content; the waxy varieties consist of only amylopectin or amylopectin with little amylose and the normal varieties have varying concentration of amylose >5%) (Juliano, Perez, Blakeney, Castillo, Kongseree, Laignelet, Lapis, Murty, Paule and Webb, 1981). The normal rice varieties were divided into four types by Juliano (1992); very low amylose (5-12% amylose), low amylose (12-20% amylose), intermediate amylose (20-25% amylose) and high amylose (25-33% amylose).

In general, rice is milled and then consumed in cooked form, but there is a rapid growth in the rice starch industry, mainly due to the unique characteristics of rice starch. These characteristics include, for example their small granule size, hypoallergenicity, freedom from gluten, bland flavour, smooth texture when cooked and tendency to form soft gels, make rice starch highly desirable for use in food systems (Bao and Bergman, 2004).

2.1.1 General characteristics

Rice starch is one of the smallest starch granules among the cereal grains, ranging in size from 1.5 to 15 μm. They are polyhedral and irregular in shape but present a smooth surface (BeMiller and Whistler, 1996; Champagne, 1996; Belitz and Grosch, 1999; Qi,

Tester, Snape and Ansell, 2003; Bao and Bergman, 2004). As with other cereal starch, rice starch shows an A-type X-ray diffraction pattern. The density of waxy and normal rice starch granules are 1.49-1.51 g/cm^3, and 1.48-1.50 g/cm^3, respectively (Juliano, 1984). However, both waxy and normal rice starches show similar granule size and size homogeneity (Champagne, 1996).

2.1.1.1 Composition and structure

2.1.1.1.1 Composition

The major components of rice starch are amylose and amylopectin, and the minor components include proteins, lipids and phosphorus.

2.1.1.1.1.1 Major components

Amylose (Figure 2.1) is a heterogeneous mixture of molecules having variable molecular size and branching; the molecules are essentially linear polymers of (1→4)-linked α-D-glucopyranosyl units with short (<0.1%) α-D-(1→6) branches (Buleon, Colonna, Planchot and Ball, 1998; Tester, Karkalas and Qi, 2004; Vandeputte and Delcour, 2004). It has a molecular weight (M_W) of approximately 10^5-10^6 Da (BeMiller and Whistler, 1996; Tester *et al*., 2004). Rice starch amylose has a degree of polymerisation by number (DPn) of 920-1110, each chain consists of an average chain length (CL) of 250-370 and β-amylolysis limit of 70-85% (Hizukuri, 1996; Tester *et al*., 2004; Vandeputte and Delcour, 2004).

Amylopectin (Figure 2.1 and 2.2) is a large molecule with a molecular weight of 1×10^7 - 1×10^9 Da (Hizukuri, 1996; Tester *et al*., 2004). It is a highly branched structure built from chains of α-D-glucopyranosyl residues linked together mainly by α- (1→4) and 5-6% α-(1→6) linkages (Buleon *et al*., 1998; Tester *et al*., 2004). Rice amylopectins have a DPn of 4700 – 12,800, an average chain length of 17.5-23 and a β-amylolysis limit of 49-59% (Tester *et al*., 2004; Vandeputte and Delcour, 2004). Rice

amylopectin (Peat, Wheland, and Thomas in 1956 defined the structure of the amylopectin molecule) as having three different basic side-chains (Buleon *et al*., 1998; Vandeputte and Delcour, 2004): the A-, B- and C-chains. From Figure 2.2, the A-chains or the outer chains are glycosidically attached through their potential reducing end to the B-chains. The B-chains or the inner chains are linked in the same way as the A-chains and contain other chains as branches. The single C-chain per amylopectin molecule contains the sole reducing group of the amylopectin molecule and carries other chains as branches.

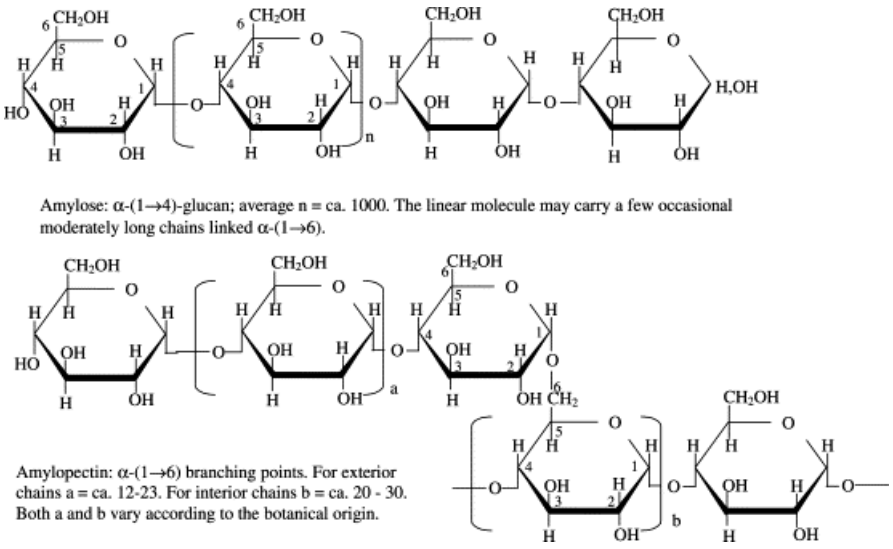

Figure 2.1 Structure of amylose and amylopectin, from Tester and Karkalas (2002) as redrawn by Tester *et al*. (2004).

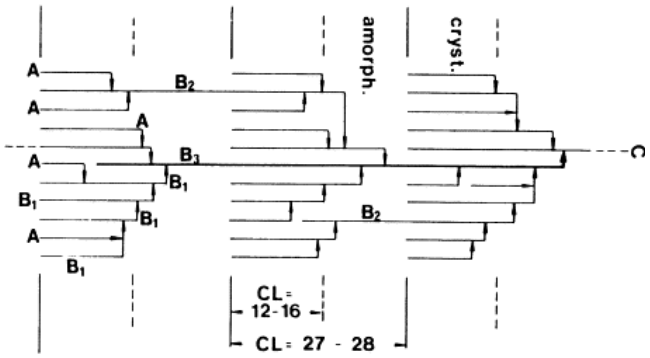

Figure 2.2 Schematic representation of the cluster model of amylopectin proposed by Hizukuri (1986) and redrawn by Morrison and Karkalas (1990). φ, reducing chain-end; solid lines, (1→4)-α-D-glucan chain; arrows, α-(1→6) linkage; C.L., average chain length (Vandeputte and Delcour, 2004).

2.1.1.1.1.2 Minor components

The components, present in addition to amylose and amylopectin molecules, are usually described as minor components as they exist in very low amounts (Eliasson and Gudmundsson, 2006). These minor components, for example, proteins, lipids, and phosphorus are present in very small amounts in rice starches (Table 2.1), however they significantly affect the physico-chemical properties of rice starch (Morrison, 1988; Baldwin, 2001; Debet and Gidley, 2006; Eliasson and Gudmundsson, 2006).

Table 2.1 Composition (minor components) of normal and waxy starch

Composition (%)	Starch	
	Normal rice	Waxy rice
Protein	0.45-0.9[1]	0.2[1]
		0.31[2]
Lipids	0.63-1.11[3]	0.041[2]
	0.3-0.4[4]	0.03[4]
		0-0.05[5]
Phosphorus	0.046-0.056[6]	0.003-0.005[6]
Ash	0.20-0.22[1]	0.10[1]
	0.12-0.17[6]	0.06-0.09[6]

[1] Singh *et al*. (2000)

[2] Qi *et al*. (2003)

[3] Eliasson and Gudmundsson (2006)

[4] Champagne (1996)

[5] Tester and Morrison(1990b)

[6] Vandeputte *et al*. (2003b)

2.1.1.2 Starch structure

When observed under polarized light microscopy, starch granules exhibit a birefringence cross (Maltese cross) which is a refraction of polarized light by the crystalline regions in the starch granules (Vandeputte *et al*., 2003b referred to Buleon *et al*., 1998). The diagrammatic representation (Figure 2.3a) of the starch granule shows that starch granules are composed of alternating semi-crystalline and amorphous growth rings, as observed under the optical microscope (Vandeputte *et al*, 2003; Donald *et al*., 1997; French, 1972). The semi-crystalline growth rings are the radial growth of amylopectin, each growth ring having a thickness between 120 and 400 nm (French, 1974).

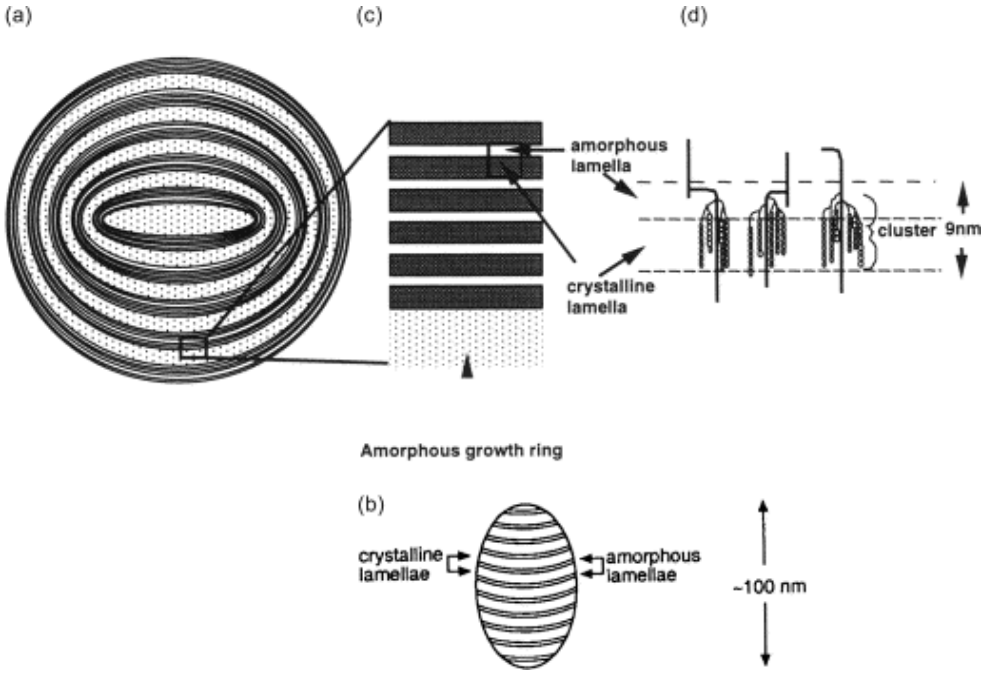

Figure 2.3 Schematic representation of the lamellar structure of the starch granule: (a) amorphous and semi-crystalline growth rings in a starch granule, (b) a blocklet, (c) amorphous and crystalline lamellae and part of an amorphous lamella in a repeating stack, (d) aligned double helices structure (from amylopectin side chains) within a crystalline lamella and amylopectin branch points within an amorphous lamella. The schematic was redrawn by Vandeputte and Delcour, (2004), and are adapted from Donald *et al*. (2001) for (a), (c) and (d), and Gallant *et al*., (1997) and Ball and co worker (Myers *et al*., 2000) for (b).

Each semi-crystalline growth ring (120-400 nm thick) consists of approximately 16 lamellae. Each lamellae consists of a 2-5 nm thick amorphous lamellae region and a 5-6 nm thick crystalline lamellae region with a repeat distance of the amorphous and crystalline lamellae being about 9 nm; Figure 2.3(c) and Figure 2.3(d) (Vandeputte and Delcour, 2004, Tester, Karkalas and Qi, 2004). The crystalline lamellae are constituted of double helical structures formed by adjacent chains of amylopectin, whereas the amorphous lamellae constitute branch points of the amylopectin side chains; Figure 2.3(d) (Tester *et al.*, 2004; Vandeputte and Delcour, 2004).

The next level of starch structure, on the 100 nm scale of the level of order between the growth rings and the lamellae are the "blocklets" (Gallant *et al*, 1997) (Figure 2.3(b)). This is based on their observation of starch granules under transmission electron microscopy, which found crystalline and amorphous lamellae formed into discrete, elongation structures surrounded by large, non-crystalline regions (Gallant *et al.*, 1997; Myers, Morell, James and Ball, 2000). They found that the blocklets within the starch granules vary in size and shape and proposed that both the semi-crystalline and amorphous growth rings are subdivided into large (20 – 500 nm in diameter) and small (25 nm in diameter in wheat starch) spherical blocklets, respectively (Gallant *et al*., 1997; Myers *et al*., 2000; Vandeputte and Delcour, 2004).

2.1.1.3 Starch granule surface

The starch granule surfaces contain the nonreducing end-groups from amylose and amylopectin (Stark and Lynn, 1992; Morrison, Tester and Gidley, 1994). The arrangement of the starch molecules at the surface is unknown, but the surface has been described in 1986 by Lineback as a "hairy billiard ball" (Stark and Lynn, 1992; Eliasson and Gudmundsson, 2006). Recently, Baldwin *et al*. (1997; 1998) studied starch granule surfaces by time-of-flight secondary ion mass spectrometry and atomic force microscopy and found that the surface of a starch granule is composed of a flat surface and protrusions. These protrusions are believed to be the ends of the amylose or amylopectin within the crystalline amylopectin side chain clusters of the granule surface (Baldwin *et al*., 1998). Moreover, the studies of Baldwin *et al* (1997; 1998) also

confirmed the "blocklet" structure of a starch granule reported by Gallant *et al*.(1997). Apart from the blocklet structure (From Figure 2.4,), Tang *et al*.(2006), based on the study of Gallant *et al*.(1997), speculated the existence of pores on the surface of starch granules. These starch pores were also reported by a number of researchers (Fannon, Hauber and Bemiller, 1992; Fannon, Shull and Bemiller, 1993; Baldwin, Adler, Davies and Melia, 1994; Huber and BeMiller, 2000). It is well-known that the starch granule surface is freely permeable to most ionic and low molecular weight; M_W < 1000 mol. wt. (Galliard and Bowler, 1987). Planchot *et al* (2000) reported that wet starch granules can be considered as a porous substance molecule unit, which is penetrable by low – molar –mass solutes but carbohydrates with a molar mass ≥ 1,000 g/mol or proteins with a hydrodynamic radius of 0.6 nm are excluded.

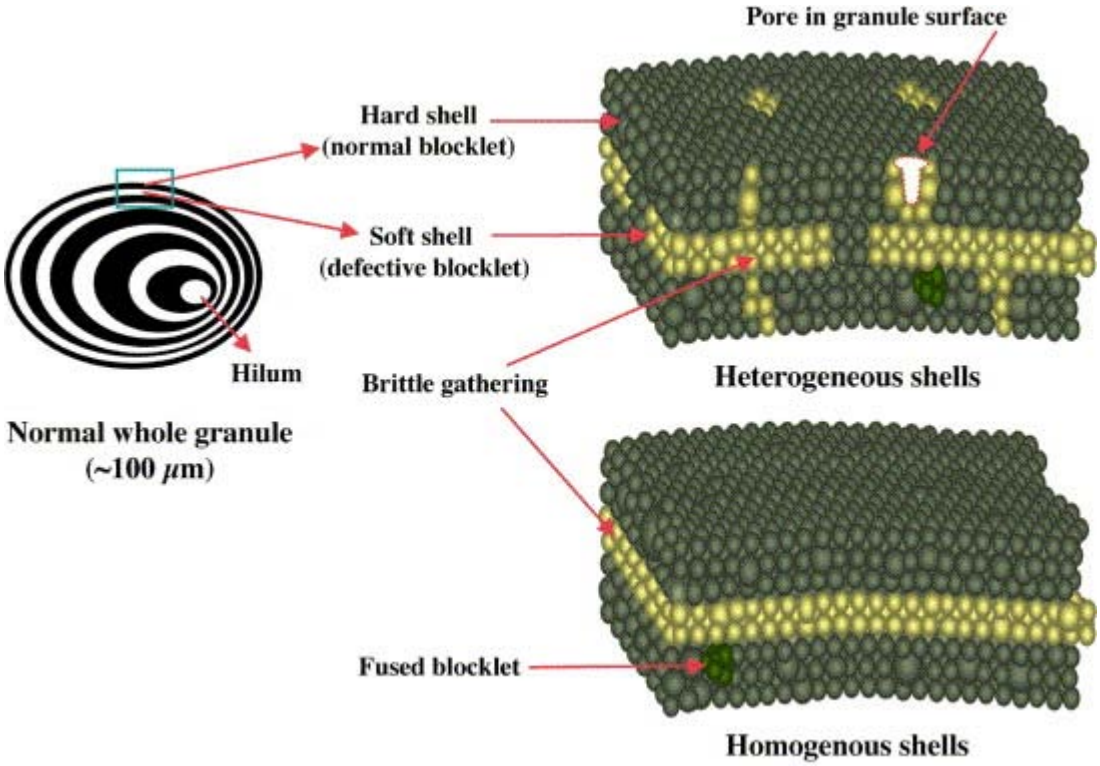

Figure 2.4 Schematic representation of starch granule structure, surface granule and surface pores of blocklet structure (Tang *et al*., 2006).

Starch granule surfaces are mainly made of carbohydrate (~90 to 95%), but also contain the minor constituents: proteins, lipids, pentosan, phosphorus, and silicon (Russell, Gough, Greenwell, Fowler and Munro, 1987; Baldwin *et al.*, 1997; Buleon *et al.*, 1998; Eliasson and Gudmundsson, 2006). Among the minor components, proteins and lipids are the most abundant components and well known for their effect on the physico-chemical properties of starches (Banks and Greenwood, 1975; Seguchi, 1984; Tester and Morrison, 1990a; Baldwin *et al*., 1997; Baldwin, 2001; Debet and Gidley, 2006; Eliasson and Gudmundsson, 2006).

The starch surface lipids contain mainly triglycerides, followed by free fatty acids, glycolipids and phospholipids, consistent with the composition of storage lipids (Vasanthan and Hoover, 1992; Debet and Gidley, 2006). The molecular weight of the lipids is less than 1000 Da. As mentioned earlier, soluble compounds with molecular weights below 1000 Da are able to penetrate a starch granule. However, it is believed that lipids cannot penetrate starch granules, but instead form multimolecular droplets or micellar forms, distributed unevenly and loosely associated or absorbed, onto the surface layers of the starch granule (Galliard and Bowler, 1987; Morrison, 1995).

There is evidence of the presence of surface proteins on starch granule surfaces; dye-binding experiments have shown that proteins are present on the starch granule surface as was recently observed under confocal laser scanning microscopy (Seguchi, 1986; Han and Hamaker, 2002; Eliasson and Gudmundsson, 2006). The starch surface proteins are mainly storage proteins with molecular weights ranging from 5 to 97 kDa (Eliasson and Gudmundsson, 2006). In cereal starches approximately 4 to 12.5% of the granule surface is made of protein. In rice starch the granule surface contains about 5 to 6% protein (Baldwin, 2001). These surface proteins are known to contain high quantities of basic and hydrophobic amino acids (Baldwin, 2001). The way in which native protein is distributed or interacts with the starch granule surface is not known, but it is believed that the surface proteins are adsorbed onto the surface layer of the starch granules and are highly associated with surface lipids (Oda and Schofield, 1997; Baldwin, 2001; Debet and Gidley, 2006). Oda and Schofield (1997) proposed the involvement of surface lipids in the binding of friabilin (molecular weight of 15 kDa) to the starch granule surface and that lipids act either like a bridge between proteins and

the starch or lipids to cause a change in conformation of the proteins and allow them to bind with amylose/or amylopectin on the starch surface layer. Recently, Ryan and Brewey (2006) removed the native wheat starch granule surface protein and found that it decreased the binding of added proteins, suggesting that native granule proteins might mediate the binding of exogenous protein.

2.1.2 Gelatinisation

The starch granule has been well documented as being semicrystalline, exhibiting birefringence under polarized microscopy (Lund, 1984; Jenkins and Donald, 1998). When starch is heated in excess water it undergoes a process called gelatinisation. Broad mechanisms have been suggested for the gelatinisation of starch, and the earliest mechanism proposed by Donovan (1979) is the most accepted and has been supported by a number of studies such as Blanshard (1987) and Jenkins and Donald (1998). Donovan (1979) suggested that gelatinisation is a swelling-driven process, which results in an irreversible order-disorder of the molecules within the starch granule, also observed as irreversible granule swelling, loss of birefringence, loss of crystallinity, and leaching of amylose and/or amylopectin (BeMiller and Whistler, 1996; Jenkins and Donald, 1998; Bao and Bergman, 2004). The first step of gelatinisation is the uptake of water by the amorphous growth rings resulting in a rapid expansion in granule size (Donovan, 1979; Lund, 1984; Biliaderis, Page, Maurice and Juliano, 1986; Jenkins and Donald, 1998).

The destabilization and swelling of the amorphous growth ring imposes a stress upon the crystalline region, destabilizing the crystalline region and eventually causing the amylopectin double helices within the crystallites to dissociate, thus leading to the breakdown of the integrity of the starch granule (Donovan, 1979; Lund, 1984; Waigh, Gidley, Komanshek and Donald, 2000; Donald, 2004).

The temperature required for gelatinisation varies. For an individual granule observed under excess water conditions, the gelatinisation temperature range is small (1 to 2°C),

whereas for the bulk sample, the gelatinisation temperature range is larger (10 to 15 °C) (Donald, 2004; Eliasson and Gudmundsson, 2006).

2.1.2.1 Swelling and polysaccharides leaching

Starch granules are insoluble in cold water as a result of their semicrystalline structure and the hydrogen bonds formed between hydroxyl groups in the starch molecules, but they can swell to a limited extent (10-20%) due to diffusion and absorption of water in the amorphous regions (Collison, 1968; Appelqvist and Debet, 1997; Eliasson and Gudmundsson, 2006). This swelling in cold water is reversible, but on heating to a certain temperature, the structure is altered significantly and the swelling becomes irreversible (Lund, 1984). The starch granule crystalline structure is disrupted because of the breakage of the hydrogen bonds, water molecules become linked by hydrogen bonding to the exposed hydroxyl groups of amylose and amylopectin, and substantial swelling occurs concomitantly with the loss of crystallinity (Jenkins and Donald, 1998; Bao and Bergman, 2004). The swelling of starch granules can be characterised by an initial phase of slight swelling, a second phase of rapid swelling and a final stage in which maximum swelling is reached. The final stage cannot be observed if starch granules disintegrate or in the case of high-gelatinizing starches (Tester and Morrison, 1990a). Vandeeputte *et al*. (2003a) investigated the effect of the swelling behaviour of rice starches and found that amylopectin chains with degrees of polymerisation (DP) 6–9 led to increased swelling power (SP) of starches at 55°C and 65°C. They also reported that waxy starches had higher SP than normal starches at temperatures between 55°C and 85 °C.

As the starch granules expand, amylose and/or amylopectin is/are leached out. The leaching out of amylose and amylopectin molecules has been found to be highly linked to the swelling of the starch granules (Tester and Morrison, 1990a). The leaching material is mainly amylose, however amylopectin is also reported to leach out, the amount depending on the starch source and the conditions applied during the gelatinisation (Eliasson and Gudmundsson, 2006). It has been reported that the starch molecules that leach out at low temperature are amylose and at higher temperature low

molecular weight amylopectins (Greenwood and Thomson, 1962; Ghiasi, Hoseney and Varrianomarston, 1982). However, Doublier et al (1987) reported that for oat starch, amylose was leached together with amylopectin. Tsia and Lii (2000), studied the leaching of rice starches and found that amylose leached out along with amylopectin at 70°C. Note that amylose does not leach out of all of the starch granules during the gelatinisation process. After heating high-amylose starches and normal starch at 90°C, the solubilized material corresponds to only 6 to 9% and 60 to 76% of total starch in high-amylose starches and normal starch, respectively (Eliasson and Gudmundsson, 2006).

Both the swelling of starch and the leaching of amylose have been related to the pasting and rheological properties of starch solutions during heating (Miller, Derby and Trimbo, 1973; Choi and Kerr, 2004).

There are many factors that affect the swelling and leaching out of polysaccharides from starch granules. Starch granules that contain fewer lipids and proteins tend to swell rapidly during heating and are also more shear sensitive (Tester and Morrison, 1990a; Debet and Gidley, 2006). Not only the bulk content of lipids and proteins, but the swelling was reported to increase as a result of the depletion of surface lipids and proteins (Debet and Gidley, 2006). The presence of electrolytes also has a marked effect on the swelling of starch granule; anions, for example sulphate, acetate, chloride, nitrate, chlorate, bromide iodide, and thiocyanate tend to increase swelling and decrease the gelatinisation temperature of starch, whereas cations, for example, magnesium, lithium, sodium, potassium, and ammonium decrease the degree of swelling and increase the initial gelatinisation temperature of starch by stabilizing the structure of the starch granule (Collison, 1968; Lund, 1984; Kelly, vanWagenberg, Latham and Mitchell, 1995).

2.1.2.2 Loss of birefringence

As reported earlier starch granules under polarized light microscopy exhibit birefringence or a Maltese cross, due to the semi-crystalline structure of the granules. When starch granules are heated in excess water the starch granules swell and this birefringence is lost, as the order of the starch granule structure is disrupted (Donald, 2004). The loss of birefringence is a basic method to determine the gelatinisation temperature range. Many studies have attempted to observe starch granules or starch-containing food samples under polarized light microscopy, in order to obtain some indications of the heat treatment that the sample has undergone (Donald, 2004; Eliasson and Gudmundsson, 2006). The loss of birefringence occurs over a large temperature range (~8°C) for a bulk sample, but a much smaller temperature range for a single granule, generally less than 1°C (Jenkins and Donald, 1998).

2.1.2.3 Endothermic Transition and Differential Scanning Calorimeter (DSC) determination of starch gelatinisation

The gelatinisation of starch is recognized as an endothermic process and exhibits enthalpy values in the range of 10 to 20 J/g (Eliasson and Gudmundsson, 2006). DSC has become an important tool for studying starch gelatinisation as it is particularly well suited to investigate the thermal behaviour of starch/water systems. It allows the study of gelatinisation over the range of transition temperatures required for gelatinisation to occur, and allows the study of gelatinisation over a wide range of starch/water ratios and provides a method for estimating the transition enthalpies of gelatinisation (Biliaderis, Maurice and Vose, 1980; Eliasson and Gudmundsson, 2006).

Thermal properties reported using DSC, include the enthalpy (ΔH) and the range in transition temperature required for the gelatinisation to occur; the gelatinisation onset (T_{onset}), peak (T_{peak}) and conclusion (T_c) (Bao and Bergman, 2004). A typical DSC thermogram of normal rice starch is given in Figure 2.5. The thermal properties of starch measured by DSC are influenced by various factors, for example moisture content, non-ionic solutes such as sugars, and ionic solutes (Evans and Haisman, 1982;

Chungcharoen and Lund, 1987; Lund, 1989; Slade and Levine, 1989; Biliaderis, 1990; Kim and Walker, 1992b; Slade and Levine, 1993; Erdogdu, Czuchajowska and Pomeranz, 1995; Hoover and Senanayake, 1996; Ahmad and Williams, 1999b; Ahmad and Williams, 1999a; Perry and Donald, 2002).

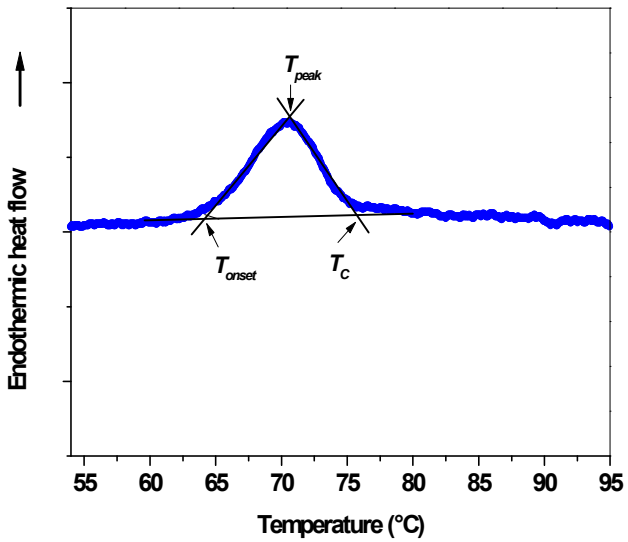

Figure 2.5 Thermal property of normal rice starch measured by DSC; T_{onset} = onset temperature, T_{peak} = peak temperature, and T_c = conclusion temperature.

The effect of moisture content on starch gelatinisation has been extensively studied (Lund, 1989; Slade and Levine, 1989; Slade and Levine, 1993; Perry and Donald, 2002). Biliaderis *et al*. (1980) studied starch gelatinisation phenomena by DSC and suggested a possible explanation for the overall gelatinisation process. In a system of excess water, on heating, extensive hydration and swelling of the amorphous regions facilitate melting of the starch crystallites over a narrow temperature range, resulting in a single endothermic transition (referred to as a *G* endotherm). The extensive swelling of starch granules is associated with a disruption of the starch crystallites, resulting in an increase in viscosity and loss of starch granule birefringence. In system of limited water, DSC thermograms display two endothermic transitions. The first endotherm (*G* endotherm) occurs as a result of the increased destabilization of the amorphous regions, and as there is insufficient water to disrupt all the starch granule crystallites, only partial melting of the crystallites occurs. Upon further heating to higher temperatures,

unmelted starch granule crystallites are melted, and thus result in the second endotherm (*M1* endotherm). They also found that the *G* endotherm of starch gelatinization occurred at a constant temperature, regardless of the amount of water present. Recently, Waigh *et al*. (2000) proposed that gelatinisation is coupled with the amylopectin helix-helix dissociation and the helix-coil transition. In excess water (>40%, w/w), the DSC endotherms of the helix dissociation and the helix-coil transition are merged together, which is due to the loss of crystallites (the helix-coil transition) and occurs simultaneously with the helix dissociation. In limited water conditions (5-40%, w/w), the temperature of the amylopectin helix-coil transition is higher than the temperature for the dissociation of the amylopectin helix-helix. Therefore two endotherms are displayed; the *G* endotherm which is considered to reflect the helix-helix dissociation and the *M1*endotherm which represents the helix-coil transition.

2.1.3 Pasting behaviour

When starch granules are heated in the presence of water up to the gelatinisation temperature, the granule lose their crystalline order and are able to adsorb a large amount of water, resulting in swelling of the granules to several times their initial size (Eliasson and Gudmundsson, 2006). Starch granules swell over a range of temperatures and there is a concurrent leaching out of amylose/amylopectin which leads to an increase in viscosity. As the temperature increases further the starch granules are disrupted, particularly when shear force is applied, which results in the formation of a starch paste (BeMiller and Whistler, 1996). This starch paste consists of a continuous phase of solubilized amylose/amylopectin and a discontinuous phase of swollen starch granules/granule remnants; granule ghosts and fragments (BeMiller and Whistler, 1996). After practically all of the granules have been disrupted the viscosity of the paste decreases (Lund, 1984). The increase and decrease in viscosity of a starch paste can be recorded using viscometers, such as the Rapid Visco Analyzer (RVA), which records the viscosity continuously as the temperature is increased. As shown in Figure 2.6, in the early stage of heating the swelling of starch granules results in a rapid increase in viscosity of the starch/water system with increasing temperature. With further heating of the starch/water system there is a balance between the swelling and

the disruption of the starch granules, and the viscosity reaches a peak value. With continued heating and stirring, more granules are disrupted and the viscosity of the paste is decreases.

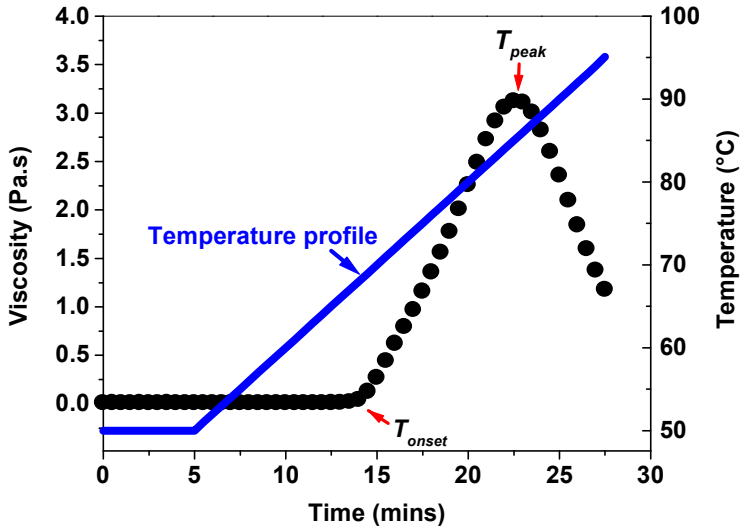

Figure 2.6 Pasting behaviour of 10% normal rice starch determined by Parr Physica Rheometer using the starch cell geometry TC 20. T_{onset} = Temperature at which the viscosity start to increase, T_{peak} = Temperature at which the viscosity reaches its maximum.

2.1.4 Rheological behavior of starch gels

A starch gel is generally known as a composite system in which gelatinized starch granules and/or fragments are embedded in, and reinforced by an amylose gel matrix, which has leached out from the starch granules during the gelatinisation and form an aggregate of a three-dimensional polymer network when the starch paste is cooled to room temperature (Miles, Morris and Ring, 1985; Ring, 1985; Morris, 1990; Noel, Ring and Whittam, 1993). This complex polymer network is a viscoelastic gel in which the associations of the molecules is mainly physical, involving hydrogen bonding between chains rather than covalent cross-links (Appelqvist and Debet, 1997). The structure of a starch gel network can be characterized by dynamic rheological measurements (Ross-Murphy, 1984).

In a dynamic rheological experiment, the viscoelastic properties of a starch gel can be characterized by two rheological parameters, the elastic or storage modulus (G') and the viscous or loss modulus (G'') obtained when small deformations are applied. During measurement, a harmonic, low amplitude shear strain, γ is applied (Ferry, 1980; Whorlow, 1992).

$$\gamma(t) = \gamma_0 \sin(\omega t) \qquad \text{Equation 2.1}$$

Where γ is the shear strain, γ_0 is the strain amplitude, ω is the angular frequency $\omega = 2\pi f$, f is the oscillation frequency in Hz, and t is the time.

For an ideal elastic solid, when a shear strain is applied to the sample the shear stress responds instantly and is proportional to the strain. The constant of proportionality is the shear modulus:

$$\sigma = G\gamma \qquad \text{Equation 2.2}$$

Where σ is the stress, γ the strain and G is the shear modulus.

Thus for an oscillating strain;

$$\sigma(t) = G'\gamma_0 \sin(\omega t) \qquad \text{Equation 2.3}$$

Where G' is the elastic or storage modulus.

In contrast, an ideal viscous liquid has a stress response that is out of phase, by the angle δ, with the strain:

$$\sigma(t) = G''\gamma_0 \cos(\omega t) \qquad \text{Equation 2.4}$$

Where G'' is the viscous or loss modulus.

A viscoelastic material has a stress response that can be divided into an elastic part and a viscous part:

$$\sigma(t) = \gamma_0 \left[G' \sin(\omega t) + G'' \cos(\omega t) \right] \qquad \text{Equation 2.5}$$

Thus the stress response of a viscoelastic material and the phase angle δ between the stress and the strain is:

$$\sigma(t) = \sigma_0 \sin(\omega t + \delta) = \sigma_0 \cos\delta \sin\omega t + \sigma_0 \sin\delta \cos\omega t \qquad \text{Equation 2.6}$$

A comparison of equations 2.4 and 2.5 for the two convenient dynamic moduli gives:

$$G' = \frac{\sigma_0}{\gamma_0} \cos\delta \qquad \text{Equation 2.7}$$

and

$$G'' = \frac{\sigma_0}{\gamma_0} \sin\delta \qquad \text{Equation 2.8}$$

The elastic or storage modulus G', which is a measure of the energy stored and recovered per oscillation cycle, this is the stress in phase with the strain in a sinusoidal shear deformation divided by the strain. The viscous or loss modulus G'', a measure of the energy dissipated as heat per cycle of sinusoidal deformation is the stress 90° out-of-phase with the strain divided by the strain.

The overall resistance to deformation is expressed as a complex quantity; the complex modulus (G^*) and is given by:

$$G^* = G' + iG'', \qquad \text{Equation 2. 9}$$

$$|G^*| = \sqrt{(G')^2 + (G'')^2} \qquad \text{Equation 2. 10}$$

Starch gels characterized at room temperature after gelatinisation show a shear thinning behaviour. Dynamic measurements show that the frequency behaviour of the viscosity and storage modulus are typical of a weak gel. The rheological behaviour of starch gels are affected by many factors, for example starch source, starch concentration, temperature and heating rate. Normal and waxy starch gels exhibit significant differences in their viscoelastic behaviours. Starch gels of waxy starch with lower amylose content in the gel show lower G'; that is they exhibit more liquid-like behaviour than normal starch gel (Eliasson and Gudmundsson, 2006).

2.1.5 Effects of additional components on the physico-chemical properties of starch.

It is generally accepted that the physico-chemical properties of starch can be affected by the presence of components such as soluble low-molecular-weight components such as sugars, salts etc., or to the presence of macromolecules, such as lipids and proteins (Lund, 1984; Eliasson and Gudmundsson, 2006).

2.1.5.1 Sugars

It has been known for a long time that the addition of sugars in varying concentrations affects the gelatinisation of starch, by for example increasing the onset temperature of gelatinisation. There are a number of explanations for these effects; the antiplasticising effect of sugars compared to water; specific starch-sugar interactions; and competition for water with starch (Spies and Hoseney, 1982; Slade and Levine, 1989; Kim and Walker, 1992b; Slade and Levine, 1993; Ahmad and Williams, 1999b; Perry and Donald, 2000; Perry and Donald, 2002). However, Perry and Donald (2000; 2002) have suggested that this observed phenomena is unlikely to be due to the sugars out-competing starch for the available moisture in the system. They base this suggestion on their observations that the addition of sugar, up to the regime of limiting water condition, did not modify qualitatively the gelatinisation behaviour. Their DSC thermograms displayed single endotherms for all concentrations of sugar. Although the

gelatinisation temperature increased with sugar addition, the peak shape or breadth of the endotherm remained unchanged. They also suggested that the alteration of the peak gelatinization temperature could be best explained by the plasticisation model, suggested by Slade and Levine (1989). Slade and Levine (1989) argued that compared to water, sugars acted as anti-plasticising agents because they had a higher average molecular weight than water, hence would have less influence on depressing the glass transition temperature.

The swelling of starch granules tends to increase with increasing concentration of sugars up to a certain concentration, and then further increases in sugar concentration decreased the swelling of the starch granules (Hester, Briant and Personius, 1956; Cheer and Lelievre, 1983; Kim and Walker, 1992a; Hoover and Senanayake, 1996; Ahmad and Williams, 1999b; Richardson, Langton, Bark and Hermansson, 2003). Cheer and Lelievre (1983) found that the swelling of wheat starch granules increased with increasing sucrose concentration below 25%, but at higher concentrations the swelling of granules was decreased. Similar results were found in studies on sago starch by Ahmad and Williams (1999b), where the swelling factor increased up to a sugar concentration of 20% and then decreased. Hoover and Senanayake (1996) found that the swelling factor of oat starch was decreased in the presence of a 36% sugar solution. Hester *et al*. (1956) proposed that at low sugar concentrations the sugars retarded the degree of granule disintegration, and hence increased the swelling ability of the starch granules. The osmotic effect might be the mechanism responsible for the suppression of starch granule swelling at high sugar concentrations (Cheer and Lelievre, 1983). Note that, both Ahmad and Williams (1999b) and Hoover and Senanayake (1996) reported a decrease in the amount of amylose that leached out from starch granules in the presences of sugars.

The apparent viscosity of a starch paste has been reported to increase with increasing sucrose concentration up to a certain value, and then the viscosity decreased (Cheer and Lelievre, 1983). Generally, in the presence of sugars, the amylose concentration in the continuous phase of starch pastes is low, thus the storage modulus (G') and the gel strength is decreased (Ahmad and Williams, 1999b).

2.1.5.2 Salt

The effects of ionic agents on starch physico-chemical behaviour are more complicated than the affect of sugars. It is found that the influence of salt depends on the type of salt as well as on the concentration. According to Wootton and Bamunuarachchi (1980) cited by Lund (1984), the peak gelatinisation temperature of starch increased with increasing concentration of NaCl from 0 to 9%, but then dropped with further increases in salt concentration up to a salt concentration of 30%. Evan and Haisman (1982) also found the same trend with the addition of NaCl to starch. Moreover, they reported the effects of other salts on starch gelatinisation, for example sodium sulphate and sodium hydrogen phosphate which also raised the gelatinisation temperature of starch, while calcium chloride at low concentrations decreased the gelatinisation temperature, but it rose with further increases in $CaCl_2$ concentration.

Oosten (1982) proposed an hypothesis to explain these phenomena: that starch is a weak acid ion exchanger and cations tend to protect and to stabilize the starch granule structure, whereas anions act as gelatinising agents by rupturing hydrogen bonds. Lund (1984) explained the effects of NaCl and NaOH on the basis of Oosten's hypothesis: that in the case of the addition of NaCl to a starch slurry, some alcoholic groups in the starch granules are converted to sodium alcoholate groups, which are better dissociated, thereby increasing the Donnan potential and thus reducing the diffusion of chloride ions that act as gelatinising agents. As there are limited agents to bind the hydrogen ions released from the starch granules, so the extent of absorption of sodium ions was limited, hence the increase of starch gelatinisation is limited to a maximum of 6 to 9% NaCl.

2.1.5.3 Lipids

The physico-chemical properties of starch pastes and starch-containing foods are altered by the presence of polar lipids (Lund, 1984; Eliasson and Gudmundsson, 2006). Both the lipids present in cereal starch and the added lipids have similar effects on their physico-chemical properties. This is thought to be due to the ability of the polar lipids

(e.g., monoglycerides, fatty acids, and similar compounds) to form a helical inclusion complex with the amylose molecule (Eliasson and Gudmundsson, 2006). During the gelatinisation of starch, amylose leaching out from the granules might form complexes with either native or added lipids and deposit onto the granule's surface, hence retarding the swelling of starch (Lorenz, 1976; Larsson, 1980). Starch paste viscosity is affected by the addition of lipids. At low concentrations of starch, lipids retard the swelling and solubility of starch, and hence decrease the viscosity of the starch paste. At high concentrations of starch, the addition of lipids results in a higher viscosity starch paste than is the case when lipid is absent. This is due to lipids making the starch granule more rigid.

2.1.5.4 Proteins

The influence of proteins on the physico-chemical properties of starch has been widely studied and it is generally agreed that their effect is rather complex. Proteins are amphiphilic molecules. Moreover, some types of proteins have the ability to form gels, which leads to more complicated interactions as the compatibility between the components governs the rheological properties of the starch/proteins systems (Eliasson and Gudmundsson, 2006).

The effects of added protein on gelatinisation of starches have been studied by DSC. The addition of starch to fish protein does not affect the thermal transition of the mixed system, but have been shown to proceed independently of each other (Eliasson and Gudmundsson, 2006). In contrast, the addition of gluten to wheat starch decreased the starch gelatinisation enthalphy but increased the onset temperature of the mixed system with increased gluten (Appelqvist and Debet, 1997; Eliasson and Gudmundsson, 2006). Muhrbeck and Eliasson (1991) studied the rheological properties of starch-gelatin and starch-bovine serum albumin (BSA) mixed gels and found that both the transition temperature and the rates of gelation of the component were critical for the behaviour of the mixed gels. When the continuous network was first formed by starch, the sum of the polynomes of the single-component systems could be used to predict G' and G'' of the mixed components systems. In contrast, if the protein network was formed before

that of the starch, the mixed gel was stronger than predicted by the sum of the polynomes for the single-component systems. Lindahl and Eliasson (1986) studied the effect of the addition of gluten on the viscoelastic behaviour of starches and found that the addition of gluten increased G' of wheat and rye starches, decreased G' of maize but did not affect the viscoelastic behaviour of potato starch. Moreover, in the case of wheat and rye starches, a decrease in phase angle (δ) was found. This shows that gluten might facilitate the granule-granule contact in wheat and rye starches; thus enhancing the formation of a transient network. Lindahl and Eliasson (1986) proposed from these results that the gluten was interacting either with the starch molecules or proteins at the surface of the granules. But this effect was not observed in all the starch gels studied. It is possible that specific groups of protein must be present on the surface of the granule for these interactions to occur (Lowy *et al*., 1981).

Eliasson and Tjernald (1990) found that wheat proteins and BSA do adsorb onto wheat starch surface granules, at least at low protein concentrations. The globular protein BSA and low molecular weight wheat proteins adsorbed in very low amounts, but for the high molecular weight proteins the amount of adsorbed protein was much higher. The adsorption of protein onto starch granules was dependant not only on the type of proteins, but also on the type of starch.

2.2 Milk protein ingredients

2.2.1 General characteristics

The major constituents of milk are proteins, fat, lactose and minerals, which together constitute about 12.6% wet weight. The general composition of commercial raw milk is shown in Table 2.2. The fat component of milk is mainly triglycerides (97 – 98%), 0.2 – 1% is phospholipids, 0.2 – 0.4% sterols and traces of fatty acids. The minerals of milk are classified as in solution or in association with the proteins, as either undissolved salts or bond ions. Lactose, the major carbohydrate of milk, is a soluble carbohydrate molecule, a disaccharide of glucose and galactose. The protein content of milk, about 3.3%, can be classified as either caseins or whey proteins.

2.2.1.1 Caseins

Casein, a mixture of several components, can be fractionated into four distinct groups: α_{s1}-, α_{s2}-, β-, and κ-casein. The various chemical properties of the casein proteins have been reviewed by Kinsella *et al*. (1989), Swaisgood (2003), and Walstra *et al*.(2006) and are given in Table 2.3. The caseins are amphipathic as they contain both polar and non-polar regions, hence caseins were generally reported as having excellent emulsifying and emulsion stabilizing properties (Dickinson, 1999b). Many hydrophobic groups are exposed; hence casein molecules readily form hydrophobic bonds, so that the casein is extensively associative, both self-associative and associating with each other (Walstra *et al*., 2006). Casein is not a globular protein. It appears to have some secondary structure as determined by spectral methods and various predictive methods, and a definite unordered tertiary structure (Swaisgood, 2003). Caseins undergo post-translational modifications; phosphorylation, glycosylation and proteolysis, giving each a unique character. All caseins are phosphorylated to varying extents at seryl and occasionally threonyl residues. Most of the κ-casein molecules are glycosylated. This occurs mainly in the polar domain and the availability of threonyl and seryl glycosylation sites is determined by the secondary and/or tertiary structures. On the other hand, there are no threonyl residues in α_{s1}-, α_{s2}-, (Swaisgood, 1992;

Walstra *et al*., 2006). β-casein is particularly susceptible to proteolysis by proteolytic enzymes into γ-casein and proteose peptones.

The binding of Ca^{2+} to caseins is mainly through the phosphoseryl residues. The α_s-, and β-casein are phosphoproteins, which have a number of phosphate groups esterified to serine residues (Walstra *et al*., 2006), hence they can bind and precipitate with Ca^{2+}. The binding capacity of α_s- and β-casein follows the order; α_{s2}-> α_{s1}-> β-casein.

The structures of α_{s1}- and α_{s2}-caseins are characterized by charged polar domains and hydrophobic domains. α_{s1}-caseins have a high net negative charge and high phosphate content; possesses eight sites of post-translational phosphorylation (Table 2.3). The polar domain of α_{s1}-caseins is flexible in nature and exhibits random coil type behaviour, whereas the hydrophobic domain is composed of a mixture of α-helix, β-structure, β-turns and unordered structure. The intermolecular interactions between the hydrophobic domains of α_{s1}-caseins lead to self-association, or association with other caseins. α_{s2}-caseins is characterized by a charged polar domain and a hydrophobic domain, which contain two cysteine residues and several phosphoseryl clusters, thus it is the most hydrophilic casein.

β-casein is the most hydrophobic casein and is highly charged at the N-terminal domain containing the anionic phosphoserine cluster. It contains several proline residues and the charge is unevenly distributed. β-casein has a soap-like molecule with a polar head and a long-chain, apolar tail. The association of β-casein is strongly dependant on temperature and ionic strength.

κ-casein differs from the other caseins. It does not bind Ca^{2+} like the other caseins as it does not contain clusters of phosphoseryl residues in its polar domain. However, it contains two cysteine residues that can form intermolecular disulfide bonds; thus it occurs as an oligomer containing 5 to 11 monomers in milk (Walstra *et al*., 2006). κ-casein strongly associates and exists in the form of large spherical aggregates

resembling soap micelles, which contain over 30 molecules linked together by interactions in the hydrophobic domain.

Table 2.2 Approximate composition of commercial raw milk (Walstra *et al*., 2006)

Component	**Average content (%, w/w)**	**Average content in dry matter (%, w/w)**
Water	87.1	-
Solids-non-fat	8.9	69
Fat	4.0	31
Protein	3.3	25
Casein	2.6	20
Lactose	4.6	36
Minerals	0.7	5.4
Organic acid	0.17	1.3
Miscellaneous	0.15	1.2

Table 2.3 General characteristics of casein proteins (Kinsella *et al*., 1989; Swaisgood, 2003; Walstra *et al*., 2006)

Attribute	Casein			
	α_{s1}-casein	α_{s2}-casein	β-casein	κ-casein
Concentration in milk ($g.l^{-1}$)	12-15	3-4	9-11	3-4
Molecular weight (Da)	23,623	25,238	23,988	19,006
Total amino acids	199	207	209	169
Isoelectric pH3	4.5	5.0	4.8	5.6
Proline residue (mole/mole)	17	10	35	20
Cysteine (mole/mole)	0	2	0	2
Lysine (mole/mole)	14	24	11	9
Phosphoseryl residues(mole/mole)	8	11	5	1
Hydrophobicity	25	23	29	22
Net charge/residue	-0.10	-0.07	-0.06	-0.02
Disulphide	0	1	0	-
α-helix	22	-	9-16	23
β-sheet	7.6	-	20-26	31
β-turns	-	-	15-31	24

2.2.1.1.1 Caseins associations

Self-association of casein is mainly through electrostatic and hydrophobic interactions. α_{s1}-casein association is initiated by ionic strength. The degree of association depends on the pH and ionic strength, and temperature has little effect; at low ionic strength (0.003-0.01 M) and neutral pH the protein exists as a monomer (Swaisgood and Timasheff, 1968; Schmidt, 1970; Rollema, 1992).

α_{s2}-casein is amphipathic with a highly charged structure. The N-terminal is a negatively charged hydrophilic domain and the C-terminal is a positively charged

hydrophobic domain, hence the extent of α_{s2}-casein association is highly depending on ionic strength. The polymerization of both α_{s1}- and α_{s2}-casein proceeds by a series of consecutive steps; dimmers, tetramers and hexamers (Payens and Schmidt, 1966; Snoeren, Vanmarkwijk and Vanmontfort, 1980).

$$(\alpha_{s1})_i + \alpha_s \leftrightarrow (\alpha_s)_{i+1}$$

Figure 2.7 Polymerization of α_{s1}- or α_{s2}-casein, where α_s represent either of the caseins (Rollema, 1992).

The molecular association of β-casein is dominated by hydrophobic interactions (Swaisgood, 1992). β-casein association is strongly dependent on temperature and ionic strength, but compared to α_{s1}- and α_{s2}-casein, the β-casein association is less sensitive to ionic strength (Schmidt and Payens, 1972; Swaisgood, 1992). At 4°C, β-casein exists in solution as monomers and an increase in temperature leads to increased association (Payens and Vanmarkw.Bw, 1963; Schmidt and Payens, 1972). The monomer forms polymers with long thread-like chains (micelle) of approximately 20 units at 8.5°C and extends to larger aggregates at higher temperatures (Fox and McSweeney, 1998). The degree of association is dependent on protein concentration (Fox and McSweeney, 1998). There is a critical concentration to form the micelle which ranges from <0.5 mg/ml to about 2 mg/ml depending on ionic strength, pH and temperature (Schmidt and Payens, 1972; Niki, Takase and Arima, 1977; Evans, Phillips and Jones, 1979). The size of β-casein micelle polymerization (η) depends on temperature, ionic strength and pH (Rollema, 1992)(Rollema, 1992).

κ-caseins form micelles similar to that of β-casein but κ-casein micelles are not highly sensitive to temperature or ionic strength. According to Vreeman *et al*. (1981), the polymerisation of κ-casein between 4 and 20°C, is relatively independent of temperature and ionic strength (between 0.1 to 1.0 M) and the number of monomers in a micelle is approximately 30 molecules. The self-association mechanism of β-casein and κ-casein can be depicted as a monomer-polymer equilibrium (Figure 2.8).

$$\eta\beta \leftrightarrow \beta_n$$

Figure 2.8 Association of β-casein or κ-casein as a monomer-polymer equilibrium (Rollema, 1992).

2.2.1.1.2 Casein structure in milk

In milk, about 95% of the caseins associate together to form large colloidal particles, known as micelles. Each micelle is composed of approximately 94% protein and 6% inorganic salts, largely in the form of calcium phosphate consisting of calcium, magnesium, phosphate and citrate (Fox and McSweeney, 1998).

The structure of the casein micelle is not entirely clear, and there is continuing debate regarding sub-micelle models and non sub-micelle models. The sub-micelle model was first proposed by Schmidt (1982) and was modified by Walstra and Jenkin (1984). In general, it is believed that the casein micelle consists of a number of sub-micelles or sub-units linked together by the colloidal calcium phosphate (CCP), with hydrophobic and hydrogen bonds contributing to the relatively stable structure (Figure 2.9) (Walstra and Jenness, 1984; Walstra, 1999; Walstra *et al*., 2006). The non-submicelle or the gel matrix models were proposed by Holt (1992) to refute the notion of discrete submicellar structures within the casein micelle (Farrell, Malin, Brown and Qi, 2006). Holt (1992) proposed that the sub-structure of the casein micelle consists of microgranules of calcium phosphate that are incorporated into a protein matrix that has an outer region displaying hairy layers which are considered to provide steric stability to the micelle. Recently, Horne (1998) proposed the new non-submicelle; the dual binding model of casein micelle assembly and structure (Figure 2.10). He considered the surface chemistry of the individual caseins, for example β-casein in which the amphiphilic nature of β-casein lead them to act like block copolymers of alternating charge and hydrophobicity; a charged phosphopeptide loop and a hydrophobic train (Horne, 1998; Farrell *et al*., 2006). The polymerization pathway is via intermolecular binding of hydrophobic blocks. The other polymerization pathway is the bridging across calcium phosphate nanoclusters. The growth of calcium phosphate nanoclusters is limited by binding to the phosphopeptide loop regions of κ-casein. κ-casein has a

hydrophobic N-terminal peptide but does not contain a cluster of phosphoserine residues in its sequence, thus it is unable to continue the polymer growth (Horne, 1998; Horne, 2002).

Casein micelles are roughly spherical in shape, ranging from 40 to 300 nm in diameter, with mass ranging from 10^6-10^9 Da and the particle is made up from about 10^4 casein molecules (Fox and McSweeney, 1998; Walstra *et al*., 2006). The outer surface layer of the micelle is thought to be composed of equimolar amounts of α_s- and κ-casein with a small amount of β-casein, while the interior contains β- and α_s-casein in equimolar amounts and only a minor amount of κ-casein (Dalgleish, Horne and Law, 1989). The micelle surface area is very large ($5x10^4$ cm^2 ml^{-1}), thus the surface properties of the micelles are critical to their behaviour (Fox and McSweeney, 1998). The stability of casein micelles is believed to be due to κ-casein, as the hydrophilic C-terminal of κ-casein protrudes into the surrounding solution, reducing surface hydrophobicity and providing electrostatic and steric stabilization (Swaisgood, 1992).

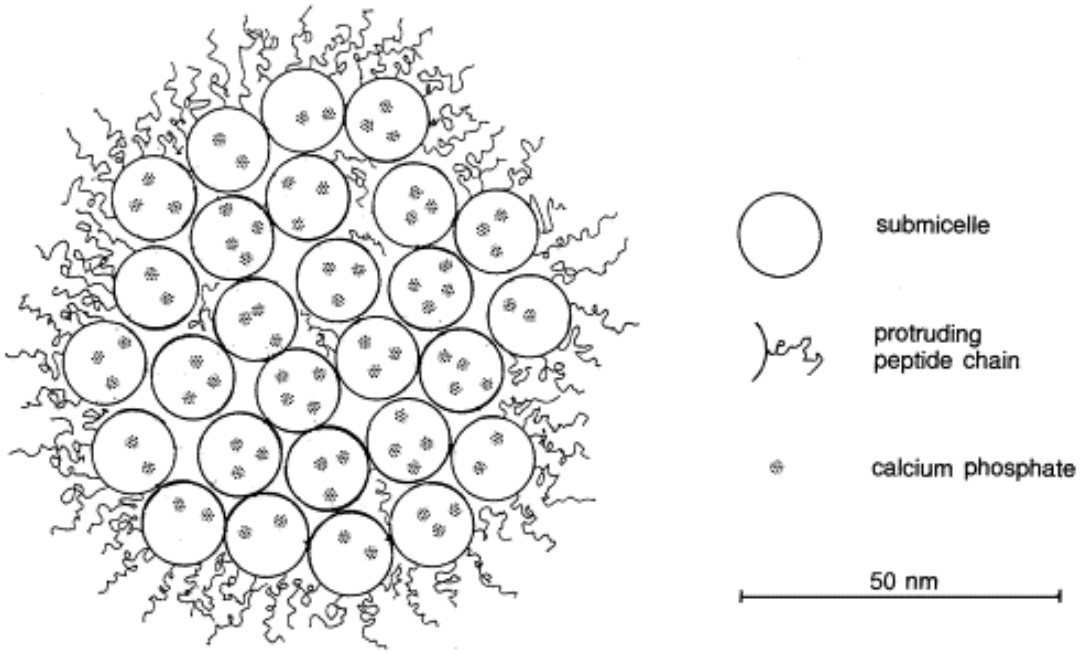

Figure 2.9 The sub-micelle model of the casein micelle (Walstra, 1999).

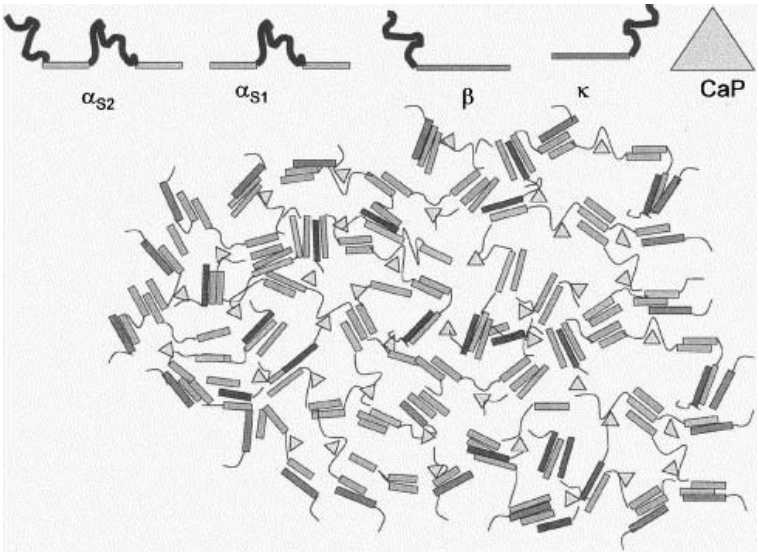

Figure 2.10 The dual bonding model of casein micelle structure using casein monomers as indicated. Protein–protein interactions occur between hydrophobic regions (rectangular bars) while the protein hydrophilic regions (loops) bind to calcium phosphate clusters (triangles). κ-casein is monomeric and is present at the surface, limiting further growth of micelle; Horne (1998) modified by Farrell *et al*. (2006).

2.2.1.2 Whey proteins

Generally, whey proteins are globular to ellipsoid in structure. They have relatively high hydrophobicity and are composed of compactly folded peptide chains. Most of them contain an appreciable proportion of α-helix and β-sheet with homogeneous charge distribution and are soluble and heat labile (except the proteose peptones). The five major groups of whey proteins are, β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA), immunoglobulins (Ig) and proteose peptones (PP). The structure and properties of the whey proteins are shown in Table 2.4.

β-lg is the major whey protein of bovine milk and represents 50% of the total whey proteins. In its natural state, β-lg is a dimer of two monomeric subunits that are non-covalently linked, mainly by hydrophobic interaction (hence, M_W = 36.6 kDa) (Walstra *et al*., 2006). The dimer is stable between pH 5.5 and 7.5. Below pH 5.5, the dimers

tetramerize to form octomers, and smaller aggregates might also form. Above pH 7.0, only monomers occur (Walstra *et al*., 2006). The hydrodynamic radius of β-lg is 2.04, and 3.19 nm for the monomer and dimer respectively (Aymard, Durand and Nicolai, 1996)

The functional properties of β-lg are mainly affected by the presence of a sulphydryl-group and disulfide bond. There are five cysteine residues per molecule of β-lg, but only four of these are able to form two disulphide linkages (From Table 2.4,). This leaves one free thiol group which is important for the changes occurring between β-lg and the other proteins during heating, mainly κ-casein and α-la (Walstra and Jenness, 1984). Because of its high hydrophobicity, β-lg tends to bind some apolar molecules, for example retinol and some fatty acids (Puyol, Perez, Ena and Calvo, 1991; Walstra *et al*., 2006).

Table 2.4 General characteristics of whey proteins (Kinsella *et al*., 1989; Walstra *et al*., 2006)

Attribute	Whey proteins			
	β-lg	**α-la**	**BSA**	**Ig**
Concentration in milk(g.kg^{1})	3.2	1.2	0.4	0.8
Molecular weight (Da)	18,283	14,176	66,267	(1.5-10)x105
Total amino acids	162	123	582	
Isoelectric pH	5.2	~4.3	4.8	5.5-8.3
Cysteine (mole/mole)	5	8	35	-
Phosphoseryl residues (mole/mole)	0	0	0	-
Hydrophobicity	29	28	24	-
Disulphide bonds	2	4	17	21
α-helix	15	26	54	-
β-sheet	50	14	18	-
β-turns	18	-	20	-

Whey proteins are composed of about 20% of α-la; a small, compactly folded and more or less spherical molecule that does not associate except at low ionic strength (Walstra *et al*., 2006). There are four inter chain disulphides, but no sulphydryl groups. The disulphide bridges are located between amino acids 6 and 120, 28 and 111, 61 and 77 and between 73 and 91 in α-la (Walstra and Jenness, 1984). The amino acid sequence of α-la is similar to lysozyme, and that has led to the development of a three dimensional structure for α-la based on the main chain conformation of lysozyme (Browne, North and Phillips, 1969; Walstra and Jenness, 1984).

Bovine serum albumin (BSA) is a minor protein that is identical to the serum albumin found in the blood stream, and represents about 5% of the total whey proteins. It is a large molecule, the structure of which is thought to be elongated in shape with three globular domains, about 3 x 12 nm in size. There is one free thiol group and 17 disulphide bonds (Table 2.4), which act to form the protein into a multi-loop structure. BSA appears to function as a carrier of small molecules via hydrophobic interaction, such as fatty acids, however the specific role of BSA in milk and milk products is unknown (Walstra and Jenness, 1984).

Immunoglobulins (Ig) are large glycoprotein molecules of heterogeneous composition and comprise about 10% of the whey proteins. They are polymers of two kinds of polysaccharide chains, light chains (M_W of 22,400 Da) and heavy chains which are antibodies synthesized in response to stimulation by specific antigens (Walstra *et al*., 2006). The heavy chains consist of several types; γ (M_W 52,000), α (M_W 52,000-56,000) and μ (M_W 69,000) (Walstra and Jenness, 1984). The physiological function of Ig is to provide various types of immunity in the body. There are five classes of Ig: IgA, IgG, IgD, IgE and IgM, but only IgA, Ig G, and IgM and all are present in milk (Fox, 2003).

Proteose peptones (PP) are different from other whey proteins as they are non-heat-sensitive and do not precipitate at pH 4.6 but are precipitated by 12% trichloroacetic acid. These proteins are not considered to be real whey proteins as they are the degraded products of β-casein. The molecular weight of proteose peptones is in a range

of 4 to 22 kDa. There are four principal groups of components of PP, which are distinguishable by their electrophoretic mobility: PP-3, PP-5. PP-8-fast and PP-8-slow (Fox, 2003). There is no disulphide or sulphydryl residue, but one of the proteose peptones, PP-3, is reported to contain a glycoprotein. In whey protein concentrate (WPC) and whey protein isolate (WPI), the amount and composition of the proteose peptones may have some significant effect on functionality as they are not denatured by heat and can bind calcium (Kinsella *et al*., 1989)

2.2.1.2.1 Denaturation of whey proteins

The denaturation of proteins is described as the unfolding and uncoiling of the tertiary and secondary structures without rupture of the covalent peptide bonds of the primary structure. The denaturation of protein is caused by many physical and chemical treatments such as high temperature, high and low pH, organic solvents, high pressure etc. In the denaturation of β-lg, the free thiol group is exposed and reacts with one of the -S-S groups from another molecule to form a dimer. Trimers and tetramers are formed in the same way (Walstra *et al*., 2006). The aggregates at this step may remain quite small and become poorly soluble. At high concentrations, these aggregates eventually form a gel. Thermal denaturation temperature and enthalpies of the whey proteins are shown in Table 2.5

Table 2.5 Thermal denaturation temperature and enthalpies of whey proteins

Protein	***Td* (°C)**	***Tpeak* (°C)**	***ΔH***	
			(kJ/mol)	**(J/g)**
β-lg	78[a]	83[a]	311[a]	
	75[b]			44[b]
α-la	62[a]	68[a]	253[a]	
	63[c]			18[c]
BSA	64[a]	70[a]	803[a]	
	65[c]			7[c]
Ig	72[a]	89[a]	500[a]	

[a] Singh and Havea (2003)

[b] deWit and Swinkels (1980)

[c] deWit *et al*. (1983)

2.2.1.3 Minerals

The salts in milk are divided mainly between the colloidal and soluble phases, with a limited amount bound to the fat globules (Walstra and Janness, 1984). Milk is supersaturated with calcium and phosphorous in the form of phosphate. This allows the formation of insoluble colloidal calcium phosphate (CCP) complexes which stabilize the micelle. The total amount of the insoluble colloidal calcium phosphate is roughly 7 g per 100 g of dry casein. Colloidal calcium phosphate contains 67% of the total calcium and 57% of the total phosphate. Although it is named as colloidal calcium phosphate, it also contains some citrate and other components, such as K^+ (110 mgl^{-1}), Na^+ (30 mgl^{-1}) and Mg^{2+} (30 mgl^{-1}). Part of the colloidal calcium phosphate is thought to act as counter-ions, which are probably associated with the negatively charged organic phosphate and carboxylic acid groups of the casein (Fox and McSweeney, 1998; Walstra *et al*., 2006).

Salts in the serum phase are present in various ionic forms and unionized complexes; the monovalent ions Na^+, K^+ and Cl^- exist in milk mainly as free ions, whereas the multivalent ions such as Ca^{2+}, Mg^{2+}, PO_4^{3-} and Cit^{3-} form complexes such as HPO_4^{-2}, $CaCit^-$ and $MgHPO_4$ (Holt, 1985). Free ion concentrations in the serum phase determined calorimetrically, by ion-selective electrode, by ion exchange methods and by calculation, indicate that 20-30% of Ca^{2+} and Mg^{2+} are free ions and that some exist as complex undissociated ions with citrate, phosphate and bicarbonate, such as $CaCit^-$, $CaPO_4^-$, $CaHO_3^+$ (Holt, 1985). The salt balance between the colloidal and soluble phases largely determines the physico-chemical state of milk and hence the functionality of the proteins. The milk salts are of particular interest as they have been shown to have a major effect on the rheology of milk protein solutions (Carr, 1999).

2.2.1.4 Lactose

Lactose is the principle carbohydrate in bovine milk, and accounts for over 50% of the solids in skim milk. It is a disaccharide composed of one residue each of D-glucose and D-galactose linked in a β-1, 4-glycosidic linkage and occurs in both α and β crystalline forms, with an equilibrium mixture at 20°C composed of 62.9% β- and 37.3% α-lactose and the equilibrium constant ratio of β/α at 20°C is 1.68 (Fox and McSweeney, 1998).

The solubility and stability characteristics of α- and β-isomers are very different. The β-isomer is more soluble than the α-isomer, and its rate of mutarotation is slow, hence it is possible to form more highly concentrated solutions by dissolving β- rather than α-lactose (Fox and McSweeney, 1998).

2.2.2 Milk protein ingredients

Figure 2.11 gives a flow sheet for the manufacture of skim milk powder (SMP), milk protein concentrate (MPC), sodium caseinate (NaCAS) and whey protein isolate (WPI). The approximate chemical composition of typical SMP, MPC, NaCAS and WPI are shown in Table 2.6.

SMP is manufactured after the cream has been removed from whole milk by centrifugal separation, then the skim milk is pre-heated. This pasteurizes the product and also produces the desired functionality and storage stability in the final product. Evaporation removes approximately 90% of the water from milk. Spray drying then further reduces moisture to 4% or less.

A general process for manufacturing MPC is to produce skim milk concentrates using a membrane process (ultrafiltration or diafiltration) that concentrates the casein and whey proteins without precipitation. Thus the casein in MPC, like the casein in SMP, generally retains the same micelle form as is present in milk, though minor changes may occur as a consequence of processing (Mulvihill, 2003).

In the manufacture of NaCAS from whole milk, fat is first removed by centrifugal separation to obtain the skim milk. Acid is either added to or generated within the skim milk solution by bacteria to destabilize the casein in the milk. There are two methods used to destabilize the casein in industry; (i) isoelectric precipitation (acid casein), and (ii) Proteolytic coagulation (rennet casein). The insoluble casein is separated from the soluble whey proteins, lactose and salts, in the case of acid casein; it is followed by neutralization with an alkali to solubilise the casein. It is then washed to remove residual soluble solids, and spray-dried to obtain caseinates. Unlike SMP and MPC, the casein micelles in NaCAS are destabilized and neutralized, thus its caseins are not in micelle form. However, when reconstituted in water, it exists as small self-assembled protein particles of casein sub-micelles (diameter 10 – 20 nm) in equilibrium with free casein molecules (Creamer and Berry, 1975).

Generally, in the final WPI product, 90% or more of the dry matter consists of whey protein. Solutions of WPI are generally obtained from the manufacture of caseins or cheese. The whey used for WPI manufacture is treated by ion-exchange chromatography, in which proteins are adsorbed onto an ion exchanger. It is then freed of salts by ultrafiltration before spray-drying to yield WPI (Fox and McSweeney, 1998). WPI consists of about 80% β-lg, 15% α-la, a little of the other serum proteins but no caseinomacropeptide. However, the protein composition of WPI can vary widely depending on the whey source and process (Walstra *et al*., 2006). In WPI, the whey proteins exist largely as non-aggregated forms of free globular molecules (Segall and Goff, 1999).

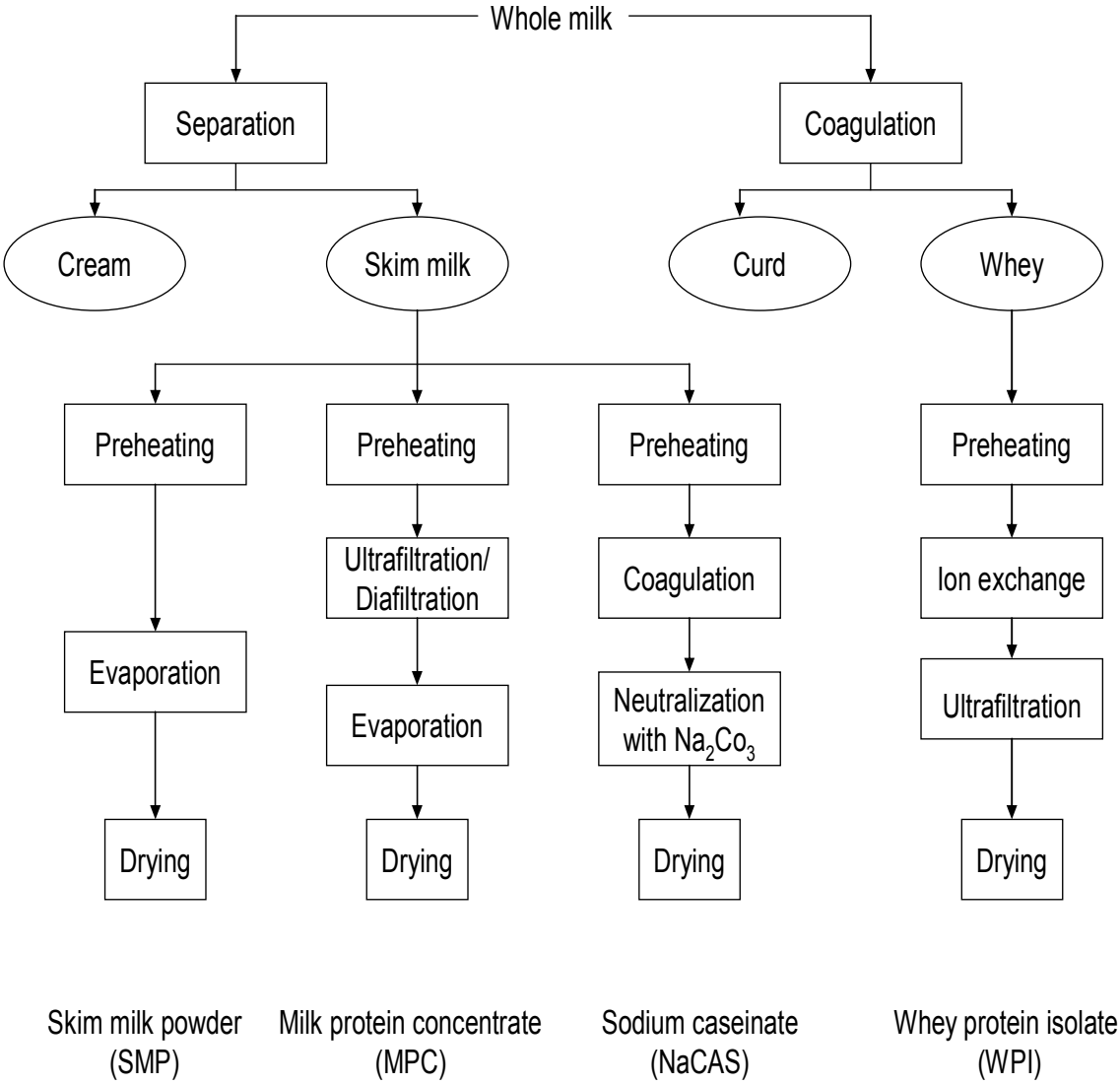

Figure 2.11 Schematic diagram of the milk protein ingredients manufacture.

Table 2.6 Approximate composition of milk proteins ingredients

Products	Protein	Lactose	Fat	Ash
SMP[a]	33.6	51.0	1.0	8.5
MPC[b]	85.4	4.6	1.7	7.3
NaCAS[c]	91.4	0.1	1.0	4.0
WPI[c]	93.0	1.0	0.5	2.0

[a] Walstra *et al*. (2006)

[b] Car (1999)

[c] Chandan (1997)

2.2.3 Adsorption properties

Milk proteins are large complex amphipathic molecules having a combination of ionic, polar and non-polar regions, hence they are surface-active macromolecules that strongly adsorb onto interfaces (Dickinson, 1999a). Generally, there are four steps in the adsorption of proteins onto an interface

(i) Transportation of protein from the aqueous phase towards the interface
(ii) Deposition and attachment at the interface, resulting in reduction in the interfacial tension
(iii) Protein unfolding and spreading at the interface
(iv) Structural rearrangement of the adsorbed protein layer

The first step of adsorption begins with the migration of proteins molecules from the aqueous phase to the interface, this adsorption of protein onto the interface lowers the surface free energy of the system, hence lowering the surface tension (Phillips, 1981). Once adsorbed at the interface, the protein molecules undergo a process of unfolding and spreading out at the interface in order to attain the lowest free energy with its new environment (Dickinson and McClements, 1995). Soft (flexible) proteins, for instance

caseins that are substantially amphiphilic with a high proportion of accessible non-polar residues, are more effective in reducing the interfacial tension than "hard" proteins such as globular proteins, which contain fewer non-polar residues (Dickinson, 1999a). The final step of protein adsorption is a rearrangement of the adsorbed protein layer. This final conformation of the protein at the interface is dependent on various factors, for example type and concentration of protein and the state of aggregation of the protein in the bulk phase. In general, the conformational changes that the proteins undergo takes longer than needed for adsorption, for instance 10 seconds for β-casein but 10 minutes for β-lg (Walstra *et al*., 2006).

In practice, it has been found that during the adsorption process of proteins onto the interface there is also a desorption process that eventually reaches an equilibrium between the two processes. Figure 2.12, schematically illustrates the consecutive steps of adsorption and desorption of proteins,

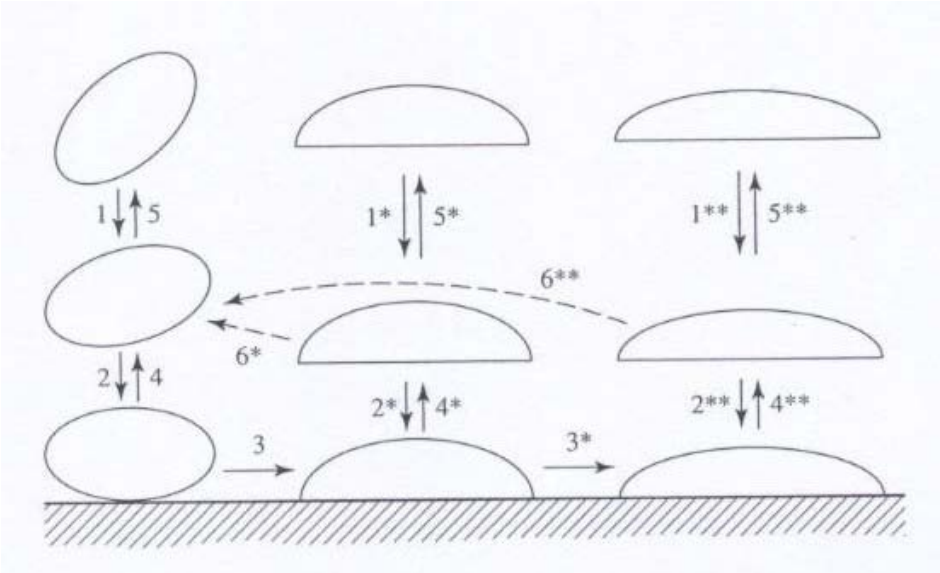

Figure 2.12 Schematic illustration of the protein adsorption process. (1) transport towards the interface (2) deposition and attachment at the interface (3) structural rearrangement of the adsorbed molecule (4) detachment from the interface (5) transportation away from the interface, and (6) possible restructuring of the desorbed molecule. The * indicate the degree of relaxation of the adsorbed protein (Norde, 2003).

2.2.3.1 Kinetics of adsorption

Diffusion and convection by laminar or turbulent flow are the basic mechanisms of transport of proteins from bulk solution to the surface. According to the theory of diffusion-controlled adsorption, the adsorption rate can be determined by the equation below (Norde, 2003):

$$J = (C - C_s)\left(\frac{D}{\pi t}\right)^{1/2} \qquad \text{Equation 2.11}$$

Where J = the flux from the bulk solution towards the surface region

C = protein concentrations in the bulk solution

Cs = protein concentrations in the subsurface area

D = the diffusion coefficient

t = the contact time between the solution and the surface

However, this equation is only applicable in the early stages of adsorption, when the protein concentration in the subsurface area is much less than the protein concentration in the bulk solution.

In a system with high dynamic flow, proteins tend to be transported to the surface by convection rather than by diffusion, hence the kinetics of adsorption are strongly dependant on the adsorption and desorption rates. The velocity of adsorption depends on the rate of which protein molecules crash into the surface, the probability of striking a vacant site (1- Γ/Γ_{max}), and an activation term exp[-E/RT], where E is the activation energy for adsorption. The velocity of desorption depends on the fraction of the covered surface, and an activation term exp[-E/RT], where E is the activation energy for desorption (Shaw, 1992). Thus, when adsorption equilibrium is established,

$$C\left(1 - \frac{\Gamma}{\Gamma_m}\right)\exp\left[\frac{-E}{RT}\right] = k\left(\frac{\Gamma}{\Gamma_m}\right)\exp\left[\frac{-E'}{RT}\right] \qquad \text{Equation 2.12}$$

a_x a_x

Where Γ = the surface protein concentration

Γ_{max} = the monolayer coverage value of surface protein concentration

C = solute concentrations in the bulk solution

k = a proportionality constant

E = activation energy

R = gas constant

T = absolute temperature

$$C = k \exp\left[\frac{\Delta H_{ads}}{RT}\right] \frac{\Gamma / \Gamma_m}{\left(1 - \Gamma / \Gamma_m\right)^a} \qquad \text{Equation 2.13}$$

Where $\Delta H_{ads} = E\text{-}E' =$ heat of adsorption

Assuming that the heat of adsorption is independent of surface coverage,

$$k \exp\left[\frac{\Delta H_{ads}}{RT}\right] = \frac{1}{A_{BS}} \qquad \text{Equation 2.14}$$

Where A_{BS} is the affinity of the adsorbed molecules and is related to the enthalpy of adsorption, and is dependent on the temperature, but is independent of surface coverage. Thus,

$$A_{BS} C = \frac{\Gamma / \Gamma_m}{\left(1 - \Gamma / \Gamma_m\right)^a} \qquad \text{Equation 2.15}$$

or $$\Gamma = \frac{\Gamma_m \ A_{BS} C}{\left(1 + A_{BS} C\right)} \qquad \text{Equation 2.16}$$

2.2.3.2 Adsorption isotherm model

2.2.3.2.1 Langmuir isotherm

The Langmuir adsorption isotherm is based on the assumptions that only monolayer adsorption takes place, adsorption is localized and the heat of adsorption is independent of surface coverage. Given these assumptions, the Langmuir adsorption isotherm model can be derived and is expressed as shown below (Weber, McGinley and Katz, 1991),

$$\Gamma = \frac{\Gamma_m \ A_{BS} C}{\left(1 + A_{BS} C\right)} \qquad \text{Equation 2.17}$$

Where Γ = the surface protein concentration

Γ_{max} = the monolayer coverage value of surface protein concentration

C = solute concentrations in the bulk solution

A_{BS} = The affinity of the adsorbed molecules related to the enthalpy of adsorption

2.2.3.2.2 BET

Physical adsorption is not always limited to a monolayer and in many cases is shown as multilayer adsorption. The theory of Brunauer, Emmett and Teller (Brunauer, Emmett and Teller, 1938) is applied when multilayer adsorption on a solid surface is expected. With this theory, it is suggested that the forces acting in multilayer adsorption are similar to those acting in balancing the rates of evaporation and condensation of vapor. It is based on the simplified assumption that only the first monolayer is bound by the adsorption force originating from the interaction between the adsorbate molecules and the adsorbent surface. The second and subsequent layers have the same characteristics as in the liquid. The BET equation is given as:

$$\Gamma = \frac{\Gamma_m \ A_{BS} C}{\left[(C_S - C)(1 + (A_{BS} - 1)(C / C_S)\right]} \qquad \text{Equation 2.18}$$

Where Γ = the surface protein concentration

Γ_{max} = the monolayer coverage value of surface protein concentration

C = solute concentrations in the bulk solution

Cs = solute concentrations in the subsurface area

A_{BS} = The affinity of the adsorbed molecules related to the enthalpy of Adsorption

2.2.3.3 Adsorption of milk proteins at the oil/water interface

Milk proteins are large complex amphipathic molecules containing both polar and non-polar regions (Swaisgood, 1992; Dickinson, 1999a). At relatively low concentration milk proteins adsorb at the surface or interface and this adsorption reduces the free energy of the system, reducing surface or interfacial tension. Once adsorbed at the surface or interface, the milk protein unfolds, orientates, rearranges itself and spreads to form an adsorbed film which is in contact with the interface; the hydrophobic loops are oriented in the oil phase, whilst the polar groups extend into the aqueous phase (Phillips, 1981; Dalgleish, 1996). The proteins that can spread out extensively to form a cohesive film and have a significant proportion of non-polar groups are effective, due to the ability of the protein to reduce the surface tension depending on the number and type of interactions between the protein and the interface. Therefore with milk proteins the caseins have been reported to be more effective in their adsorption at the oil/water interface than the rigid globular proteins with fewer non-polar groups (Dickinson and McClements, 1995). The order of surface activity for milk proteins is: β-casein > monodispersed casein micelles > serum albumin > α-la > α_S-caseins = κ-casein > β-lg (Mulvihill, 1989).

Dickinson(1999b) suggested that β-casein has a highly hydrophilic region of 40-50 residues and includes many charge residues at the N-terminal and a distinctly non-random distribution of hydrophilic charges. Therefore, β-casein adsorbs at a hydrophobic surface in a mix of 20-25% of the chain existing as a highly charged tail dangling away from the surface, 75-80% of the flattened train and small loop that is

predominantly hydrophobic, whereas α_{S1}-casein's primary sequence has three distinct hydrophobic regions; one in the middle (about 90-110), and two at the ends (1-40 and 130-199). Hence, α_{S1}-casein tends to adsorb at a hydrophobic surface in a loop type configuration (Figure 2.13). The monolayer of β-lg can be regarded as a close-packed monolayer and has been reported to provide a dense and thin adsorbed layer (Dickinson, 2001).

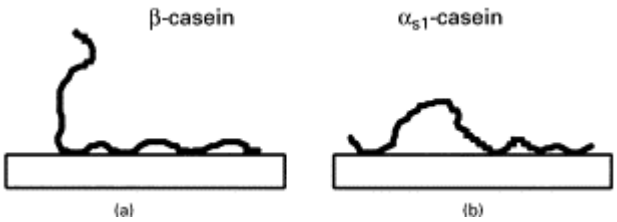

Figure 2.13 The adsorbed configurations at solid hydrophobic surface for (a) β-casein and (b) α_{s1}-casein (Dickinson, 1999b).

There are a number of studies on the adsorption behaviour of milk proteins onto oil/water interface (Hunt and Dalgleish, 1994a; Hunt and Dalgleish, 1994b; Hunt and Dalgleish, 1996; Sharma, Singh and Taylor, 1996a; Sharma, Singh and Taylor, 1996b; Srinivasan, Singh and Munro, 1996; Sharma and Singh, 1998; Euston and Hirst, 1999; Segall and Goff, 1999; Srinivasan, Singh and Munro, 1999; Euston and Hirst, 2000). Sharma et al. (1996b) studied the effect of low heat skim milk powder concentration on the amount and composition of protein surface layer covering the fat globule and found that caseins were adsorbed in preference to whey proteins at all concentration, and the preference was greater at low concentration. They also found that the amount of adsorbed protein onto the fat surface increased with increasing the protein concentration (from 50 to 200 mg/ml fat) but the amount of adsorbed protein showed little change at protein concentrations above 200mg/ml fat. They reported that the maximum amount of adsorbed total protein was ~6 mg/m^2.

Hunt and Dalgleish (1994a) reported that at a protein concentration higher than 2.5%, both caseins and whey proteins exhibited similarity in the amount of adsorbed protein (~3.2 mg/m^2). However, WPI behaved as though it was adsorbing in a multilayer. In

the studies on competitive adsorption of caseins and whey proteins mixtures, it was found that under a limiting concentration whey proteins and caseins adsorbed to the same extent, but casein was adsorbed in preference to whey proteins when protein concentration was in excess of that needed to form a monolayer. It is possible that whey protein cannot form a binary adsorbed layer in the presence of adsorbed caseins, even when protein concentration is quite small. It has also been observed that there is no preferential adsorption of β-lg to α-la.

Srinivasan *et al*. (1996; 1999) studied factors that affected the amount of caseins that were adsorbing from a solution of NaCAS onto oil droplets. They reported that the amount of caseins adsorbed on to the oil droplet increased with increasing NaCAS concentration from 0.5 to 4.0%, but it levelled off at higher concentrations. In NaCAS emulsions, β-casein was adsorbed preferentially at NaCAS concentrations below 1%. Euston *et al*. (1995) also reported the preferential adsorption of β-casein in NaCAS over other caseins at low concentration of NaCAS. Srinivasan *et al*. (1996; 1999) and Euston *et al*. (1995) suggested that it might be due to the self-association of β-casein micelles at higher protein concentration. As the self association is driven by hydrophobic interactions there will less hydrophobic residues available for adsorption onto surfaces and so less β-casein will adsorb onto the oil droplet. Euston (1996) proposed an alternative explanation for the differences found at high protein concentration. He proposed that α_S-caseins and β-casein were adsorbed in the form of a complex containing almost equal proportions of α_S-caseins and β-casein.

Mulvihill and Murphy (1991) and Euston and Hirst (1999) found that the amount of proteins adsorbed in emulsion was dependent on the aggregation state. The amount of proteins adsorbed showed the highest value for highly aggregated micelle casein-stabilized emulsion (Mulvihill and Murphy, 1991). Euston and Hirst (1999) reported that the amount of proteins adsorbed in emulsion made from MPC and SMP, where caseins exist in casein-micelle form, was 10 times higher than in emulsion made from NaCAS and whey protein concentrate (WPC). This suggested that the size of the protein aggregates was one of critical factors that affected the amount of protein that was adsorbed in emulsion.

2.3 Interaction of starch and milk proteins ingredients

Despite starches being extensively studied, little is found in the literature about their interaction with milk protein ingredients. In these few studies it has been found that the structure, gelatinisation, pasting, rheological, and other physico-chemical properties of starch were influenced by the addition of milk protein ingredients.

In general, for the same amount of starch, the viscosity of the starch/milk or skim milk systems is higher than the starch/water system (Bradley, 1993; Matser and Steeneken, 1994). Matser and Steeneken (1997) investigated the rheological properties of highly cross-linked waxy maize starch in aqueous suspensions of skim milk components. It was found that, although the salts present in milk did not increase the final gel strength of the starch gels, lactose did affect the gelatinisation temperature of the starch. The authors suggested that the diffusion of lactose into the starch granules affected their swelling behaviour. They also showed that whey proteins did not affect the final elastic modulus of the whey protein/starch system. This could have been due to the low whey protein concentration used in this work (maximum concentration 4.2%). However, the casein micelles were found to significantly increase the elasticity of the gels. A possible explanation is the increase in the local concentrations of both the casein micelles and the starch as a result of their phase separation.

Lelievre and Husbands (1989) studied the effect of sodium caseinate on the rheological properties of starch pastes and reported that in the presence of sodium caseinate the mixed gel showed a shear-thinning behaviour, similar to the starch gel. There appeared to be a synergistic effect, as the mixed gel exhibited a higher apparent viscosity than the starch gel or sodium caseinate alone. They also found an increase in the starch swelling volume up to a specific concentration of protein and proposed that the high viscosity of the sodium caseinate solution in which the pastes were formed may have influenced the swelling volume. Normally swollen starch granules rupture when they reach some maximum size during pasting as a consequence of the shearing action, and this leads to the leaching out of amylose/amylopectin from the gelatinised granule to the bulk solution. It would appear that the protein in the continuous phase, by reducing

the disintegration of the granules, may have allowed the granules to swell more than would be the case if they were in solution alone. Although sodium caseinate was found to decrease the viscosity of potato starch, its addition to maize starch resulted in an increase in viscosity (Kelly et al 1995). These studies showed that, in distilled deionized water, sodium caseinate generally increased the swelling properties of the starch granules. In contrast, Doublier *et al.* (1994) studied the swelling and pasting behaviour of wheat, potato and tapioca starches in the presence of NaCAS and reported a large decease in the volume fractions occupies in the paste by the swollen starch granules. On the basis of this swelling result, a large decrease in the viscosity of starch/NaCAS system was expected, however only the tapioca starch/NaCAS system was found to show a decrease in the viscosity. More recently, Bertoloini *et al*. (2005) studied the rheological properties of sodium caseinate–starch gels. They reported that, when added to cassava, amylomais corn, waxy corn, wheat or rice starch, sodium caseinate increased the elastic modulus and the viscosity of the gel, compared with the gel made using starch alone. However, when added to potato starch, sodium caseinate decreased the viscosity of the sodium caseinate/potato starch mixture. Although in the case of potato starch no clear explanation for the decrease in viscosity was proposed. The authors suggested that minerals, such as phosphorus, calcium, and sodium, could have an important role and thus should be investigated.

Compared to the other milk protein ingredients, mixtures of WPI and starch are by far the most studied from a fundamental view point. However, most of the studies have concentrated on the gel systems. Appelqvist and Debet (1997), in their review on starch–biopolymer interactions, reported that a study by van der Kamp (1976) had shown that the addition of NaCAS to modified starch (Colfo) led to a decrease in the viscosity, whereas the addition of whey powder and skimmed milk powder increased the viscosity of the gelatinising starch. With the addition of whey protein to wheat starch, a decrease in the gelatinisation temperature was observed. They proposed that the denaturation and/or gelation of whey protein influenced the gelatinisation of starch. WPI can form gels under certain conditions of temperature, salt and concentration. Thus WPI offers further interests when heated in the presence of starch dispersions, since mixed gels where either the continuous phase is starch-rich or whey protein-rich, or made of interpenetrating whey protein and starch networks. Aguilera and Rojas

(1996) studied the rheological, thermal and microstructural properties of WPI/starch gels and found that WPI/cassava starch mixed gels (10% total solid, pH 5.7) at a starch fraction < 0.4 showed higher G' than pure WPI and cassava starch. Muchrbeck and Eliasson (1991) reported in the case of either a starch/gelatin or starch/BSA gel, a synergistic effect when a protein network was formed before the starch underwent gelatinisation. In contrast, Aguilera and Baffico (1997) proposed that in a protein/starch gel, the synergistic effect was found only when the starches undewent gelatinisation prior to protein gelation. They studied the structure and mechanical properties of WPI/cassava starch mixed gels and found that the starch granules took up water and swelled first, thus water from the system was removed and this concentrated the WPI solution, which gelled later. The synergistic effect found in the WPI/cassava gel was believed to occur because the continuous WPI phase was filled with active filler, i.e. swollen cassava starch granules. However this synergistic effect was not detected in WPI/corn starch gels under the same conditions, which might be due to corn starch gelatinising after the gelation of the whey proteins (Aguilera and Rojas, 1996). Shim and Mulvaney (2001) studied the viscoelastic properties of mixed gels made with whey protein and corn starch at different protein and corn starch concentrations and at different pHs. Using scanning electron microscopy, they showed that only gels made at pH 9 formed compatible networks, which resulted in a synergistic increase in the elasticity of the gels. At lower pHs (pH 5 and pH 7), the gels were made of separate whey protein and starch networks. Ravindra, Genovese, Foegeding and Rao (2004) used dynamic rheological measurements to investigate WPI/cross-linked waxy maize starch dispersions containing 75 mM NaCl, at pH 7 and a total solid concentration of 5%. They identified three different networks, a WPI network weakened by the presence of the starch at starch concentrations < 0.5%, a two-continuous phase at starch concentrations between 0.4 to 0.8% , and a continuous starch network weakened by starch aggregates at starch concentrations ≥0.8%. Recently, Fitzsimons, Mulvihill and Morris (2008) conducted a systematic rheological study on co-gels of WPI/cross-linked waxy maize starch, with concentration ranging from 0 to 15% for WPI and 0 to 28% for cross-linked waxy starches. They showed that a continuous WPI network containing a dispersed starch phase was obtained at high WPI concentration and, that at low WPI concentrations, a continuous starch network was formed, and then upon

further heating this network fragmented into a dispersed starch phase in a continuous WPI matrix.

2.3.1 Mixing behaviour of biopolymer

On mixing protein and polysaccharides in solution, the interaction between them could be discussed in terms of the separation between the biopolymer. This interaction, which is of enthalpic and entropic dominated types, is primarily controlled by enthalpic effects, given by the relative strength of the interactions of the polymers between each other and with the solvent, and include entropic effects, such as, the release of bound water or counterions (Syrbe, Bauer and Klostermeyer, 1998; de Kruif and Tuinier, 2001).

There are three different systems that can result from mixing aqueous solutions of proteins and polysaccharides (Tolstoguzov, 1986; Syrbe *et al*., 1998; de Kruif and Tuinier, 2001), as depicted in Figure 2.14.

(i) Incompatibility: two distinct immiscible aqueous phases are formed and are mainly in different phases. This is due to the limited thermodynamic compatibility of proteins and polysaccharides in aqueous media; hence segregation of the biopolymers takes place.

(ii) Complexation or complex coacervation: This phenomenon is attributed to the formation of an insoluble electrostatic biopolymer-biopolymer complex. The net attraction between different biopolymer molecules causes polymer complex formation, and then the two distinct aqueous phases are formed. One phase is loaded with both polymers in the same single concentrated phase, while the other phase is depleted of polymer.

(iii) Co-solubility: Homogeneous stable solutions in which the two polymers components do not interact or alternatively exist as soluble complexes. This system is the least typical situation in view of the polymeric nature of proteins and polysaccharides.

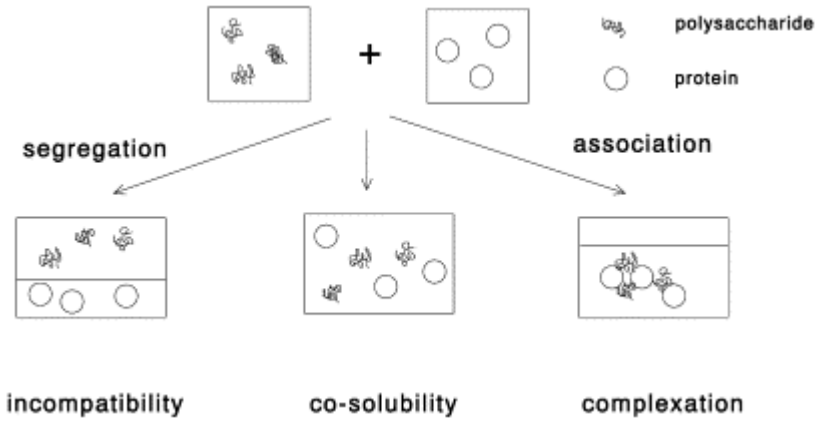

Figure 2.14 Classification of the mixing behaviour of aqueous solutions of protein/polysaccharide mixtures(de Kruif and Tuinier, 2001).

According to Syrbe *et al*.(1998), polymer additions to a colloidal system tend to induce flocculation of either the bridging or the depletion type.

For non-adsorbing, non-gelling polymer, the addition of free polymer at low concentrations, results in a stable colloidal dispersion (Figure 2.15a), but the addition of polymer at moderate concentrations leads to destabilization of the colloidal dispersion, resulting in flocculation or separation into two phases, one concentrated and the other one depleted in colloidal particles (Figure 2.15b). The addition of polymer at high concentrations restabilizes the colloidal dispersion (Figure 2.15c). In the colloidal dispersion systems with high volume ratios of colloid particles, strong destabilization results in the particles aggregating into a particle network, and this could turn the colloidal dispersion systems into a pseudostable system (Figure 2.15b*).

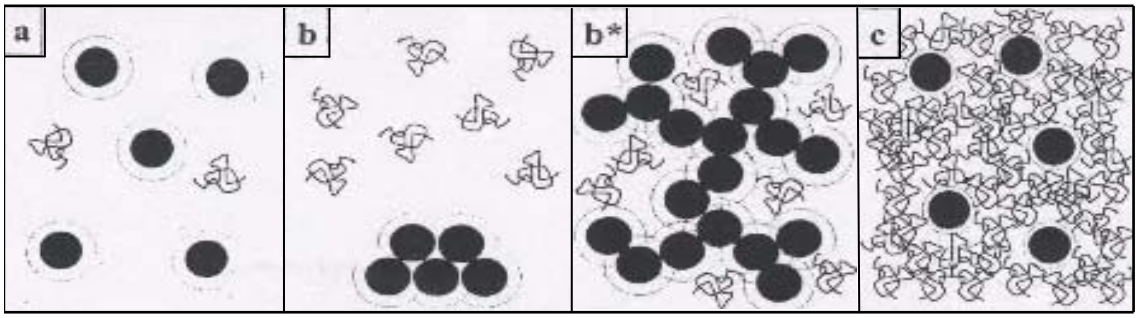

Figure 2.15 Non-absorbing, non-gelling polymer in colloidal dispersions. With increasing free polymer concentrations to the colloidal system, the system underwent the transition: (a) stable → (b) depletion flocculation → (c) restable. At high-dispersed phase volume fractions, a particle network could form which would need a long delay time for reorganisation into a close packing ('pseudostability', b*). The region of stability at high non-absorbing polymer concentration was linked to high viscosities and might be difficult to attain (Syrbe *et al*., 1998).

Adsorbing polymers, can cause bridging flocculation or polymeric stabilization depending on their concentration (Figure 2.16). At low concentration of a polymer, bridging flocculation occurs. This is due to multiple anchor sites and a spatial extension beyond the range of the repulsive barrier between the colloid particles, the surface of the colloidal particles are partially covered and approaching particles easily become linked by polymer bridges at distances that prevent any impact of the repulsive barrier (Figure 2.16a). Excess of adsorbing polymer (high surface coverage adsorbing polymers) affects the stability of the colloidal dispersion as it is turned into free, non-absorbing polymer in colloidal dispersion (Figure 2.16b), and then could result in depletion flocculation (Figure 2.16c).

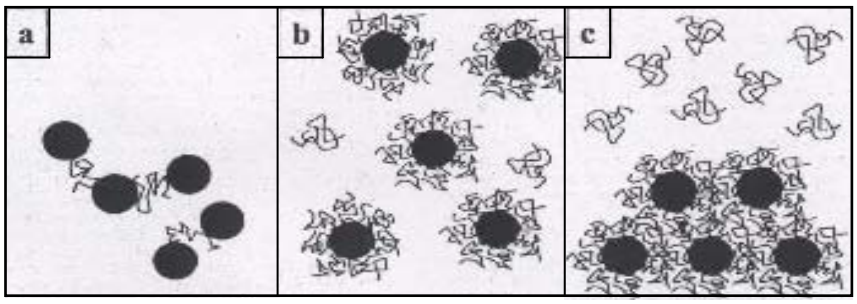

Figure 2.16 Adsorbing, non-gelling polymer in colloidal dispersion, with increasing polymer concentration in the colloidal system. The system undergoes the transition (a) bridging flocculation → (b) polymeric stabilization → (c) depletion flocculation. Bridging set in at very low polymer concentration, but flocculation became more and more effective up to about half saturation surface coverage. Excess adsorbing polymer turned into free, non-absorbed polymer and could also lead to depletion flocculation (comparable to Figure 2.15b) (Syrbe *et al*., 1998).

For a gelling polymer, the stability of the colloidal dispersion occurs when the soluble polymer forms a gel network on its own, in which the colloidal particles are trapped (Figure 2.17).

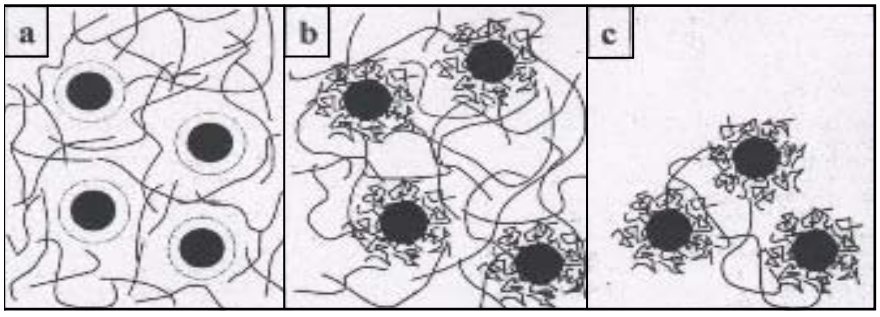

Figure 2.17 Gelling polymer in colloidal dispersions. (a) Non-absorbing polymer generated a network around the colloidal particles. (b) Adsorbing polymer integrates the particles into the gel structure. In both case, the particles are stabilized against flocculation. At sub-gelling polymer concentration, adsorbing polymer could cause indirect bridging when anchored polymers self-associate with their dangling ends (c) (Syrbe *et al*., 1998).

2.3.2 Depletion flocculation

The mixing of milk proteins and polysaccharides can result in a phase separation into polysaccharide-enriched and casein-enriched phases. This is often due to a segregative interaction between these biopolymers. The segregative interaction between polysaccharides and proteins causes an effective attraction between the proteins through a depletion flocculation mechanism. The fundamental explanation for depletion flocculation was first given on the basis of a theoretical expression in 1954 by Asakura and Oosawa. Later it was generalized in 1976 by Vrij. According to Tuinier *et al*. (2000) Lekkerkerker *et al*. (1992) have developed a more sophisticated theoretical model for depletion interaction. Figure 2.18 schematically represents the ideas of Asakura and Oosawa, and Vrij, which has been redrawn by de Bont *et al*. (2002).

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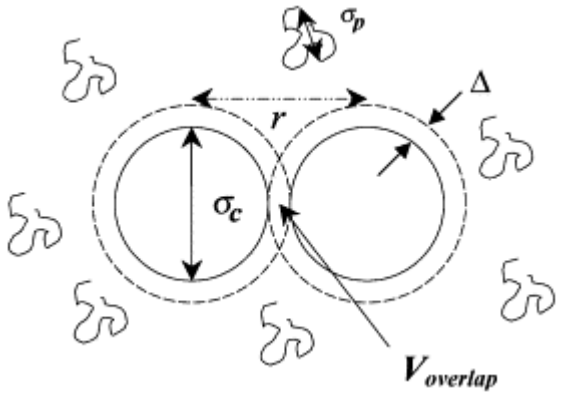

Figure 2.18 Schematic representation of depletion interaction mechanism (de Bont *et al*., 2002).

From a qualitative point of view, the depletion interaction is a repulsive interaction between the colloidal particles and the polymers (de Bont *et al*., 2002). The polymers with a diameter of σ_p are excluded from the surface of the colloidal particles (diameter $=\sigma_c$) (Figure 2.18) as there is no specific interaction besides excluded volume of the polymers and the colloidal particles (de Kruif and Tuinier, 2001). This causes an effective depletion layer (thickness $=\Delta$, is order of the radius of gyration of the polymer) where the osmotic pressure, Π, that is created by the polymer segments, is smaller than in the bulk (de Kruif and Tuinier, 2001). When two colloidal particles

approach each other as a result of Brownian motion, they share this depleted volume. Overlap of depletion layers ($V_{overlap}$) results in increased available volume for the polymers. The volume which was increased by $V_{overlap}$ causes the total entropy to increase, hence decrease the free energy of the system by $\Delta G = - \Pi V_{overlap}$. This results in an effective attraction between the colloidal particles (de Bont *et al*., 2002).

2.3.3 Interaction of starch and milk protein in dairy-based food products

Starches are widely used in dairy-based food products, such as dairy desserts, imitation cheese, yoghurt and processed cheese either to reduce formulation cost by replacing costly dairy ingredients, or obtain desired characteristics such as the rheological and sensory properties of the dairy-based product (Mounsey and O'Riordan, 2001; Guinee et al., 2004; Sandoval-Castilla et al., 2004; Bennett, Trivedi, Hemar, Reid, Illingworth and Lee, 2006; Montesinos-Herrero et al., 2006; Oh et al., 2007; Zuo et al., 2007; Mounsey and O'Riordan, 2008b; Mounsey and O'Riordan, 2008c; Mounsey and O'Riordan, 2008a; Mounsey and Oriordan, 2008).

Starch has been extensively studied for its use in imitation cheese and it was found that the physical properties, such as the rheological properties and microstucture were influenced by the interaction between starch added and milk protein (Mounsey and O'Riordan, 2001; Montesinos-Herrero *et al*., 2006; 2008b; 2008c; 2008a; 2008). Mounsey and O'Riordan(2001; 2008b; 2008c; 2008a; 2008) investigated native and modified starches of different botanical origins and reported that the physical properties of imitation cheese was affected by the amylose content, swelling ability, the shape and size of starch granules, and the concentration of starch. High amylose starch increases the hardness of imitation cheese more than low amylose starch, due to the fact that high amylose starch undergoes gelation more readily during imitation cheese storage, than low amylose starch (Mounsey and O'Riordan, 2001; Guinee *et al*., 2004). In addition, the hardness of imitation cheese increases linearly with concentration of starch added (Montesinos-Herrero *et al*., 2006). The G' and G'' of imitation cheese were also found to increase, as a consequence of high levels of starch added, this is likely due to the binding of water by the starch, G'' (Montesinos-Herrero *et al*., 2006). The addition of

starch affects the microstructure of imitation cheese as the interactions between casein and starch results in the mutual exclusion of each component (Montesinos-Herrero *et al*., 2006; Mounsey and O'Riordan, 2008a; 2008). The increase in starch concentration, results in a less homogeneous protein structure due to the exclusion effect, and the increase in the local concentrations of each component, resulting in a phase separation of the system (Mounsey and Oriordan, 2008).

Bennet *et al*. (2006) studied the influence of the addition of six different types of starches (potato starch, wheat starch, acid converted starch, high amylose cornstarch, waxy cornstarch and rice starch) on the rheological characteristics of model processed cheese and found that the addition of starch modified the cheese rheological characteristics by increasing the number of the different interactions, such as interaction between starch/protein and starch/fat. The addition of starch increases the firmness of a model processed cheese, and the degree of increase depends on the type and the concentration of starch added. Due to the ability of the addition of starch to increase the firmness of the cheese, a reduction in the concentration of protein could be offset by starch addition.

Starch has also been used in yoghurt (Keogh and O'Kennedy, 1998; Schmidt, Herald and Khatib, 2001; Williams, Glagovskaia and Augustin, 2003; Williams, Glagovskaia and Augustin, 2004; Oh *et al*., 2007; Zuo et al., 2007). William *et al*. (2004) found that the addition of 1% (w/w) modified waxy corn starch to stirred yoghurt made from skim milk powder (SMP), at 10% dairy solid, markedly increased the viscosity of the yoghurt but developed a grainy texture. In combination with wheat starch, increasing the concentration of SMP or the level of SMP replacement with whey protein concentrate, decreased the graininess of the yoghurt but had little or no effect on the viscosity of the yoghurt. In the case of the addition of starch to acidified skim milk, it was found that the rheological behaviour of the acid milk gel was dominated by the protein network, where the swollen starch granules are embedded (Oh *et al*., 2007; Zuo *et al*., 2007). Oh *et al*. (2007) reported a linear increase in the storage modulus of the acid milk gel when potato starch concentration was increased, which is due to the reinforcement of the protein network through the increase in the density of the protein network. This results firstly from the ability of starch granules to imbibe water during

the gelatinization process; and secondly from the starch granules acting as active fillers and contributing to the gel strength of the acid milk gels. Sandoval-Castilla *et al*. (2004) investigated the replacement of fat by modified tapioca starch and whey-protein-based fat replacers in yoghurt and reported that although the addition of modified tapioca starch provided a more loose structure, it provided greater firmness to the yoghurt than full-fat yoghurt. However, they suggested that the loss of the yoghurt network strength might be due to the phase separation between the starch molecules and the milk protein.

2.4 Summary of literature

Waxy and normal rice starch have extremely small and homogeneous granule sizes (1-15 μ diameter) compared to other cereal grains. The two rice starches differ in their composition. Waxy rice starch has less amylose, protein, lipid and phosphorus than normal rice starch. They also differ in their structural composition, starch structure and the the components on the surfaces of their respective granules. These differences are believed to lead to the observed differences in their physico-chemical properties, such as their gelatinisation, pasting and viscoelastic behaviours.

Although the physico-chemical behaviours of starches are influenced by their chemical, and structural composition and also starch structure and the components on the surface of the granules, it is generally accepted that these behaviours are influenced more by the added proteins to starch/protein systems than by the compositional and structural properties of the starches. The magnitude of the protein's influence appears to depend on its complexity and whether it can form an independent gel. Proteins with an ability to form a gel result in more complicated starch/protein interactions, which depend on their compatibility. As a consequence the rheological properties of the starch/proteins systems can vary quite markedly from systems with no compatability to systems with complete compatability.

Interactions occur between exogenous protein and the components on the surface of the starch granules. The mechanism by which the exogenous proteins interact with the surface of the starch granule is not known. But it is possible that the starch surface lipid might act either like a bridge between proteins and the starch or lipid to cause a change in conformation of the proteins, hence the proteins can bind with amylose/amylopectin on the granule surface. Or it might occur by starch surface proteins mediating the binding of the exogenous protein.

The four milk protein ingredients; skim milk powder (SMP, milk protein concentrate (MPC), sodium caseinate (NaCAS), and whey protein isolate (WPI), are present different physico-chemical properties and functionality. WPI consists of about 80% β-lactoglobolin, 15% α-lactalbumin, a little of the other serum proteins including proteose peptones (PP); the small MW proteins that are degraded products of β-casein, but no caseinomacropeptide. The whey proteins in WPI are present as largely non-aggregated free globular molecules. When a WPI solution is heated above 75°C, a particulate gel with network strands of protein aggregates is formed. NaCAS consists mainly of the casein proteins; α- and β-casein. When NaCAS is reconstituted in water, the caseins are present as small self-assembled protein particles of casein sub-micelles in equilibrium with disordered casein molecules. The casein proteins are non-heat-sensitive. They are amphipathic, thus they have excellent emulsifying properties. The caseins tend to adsorb at an oil-water interface by orientating their molecules; non-polar amino acids are in contact with the oil phase, and the polar groups are in contact with the aqueous phase. MPC consists of both casein and whey proteins, milk salts and small amount of lactose (4.6%). The casein in MPC exists as the micelle form which is found in milk. This it is similar to the caseins in SMP that still retain their micelle form. However, SMP contains about three times less milk protein than WPI, NaCN and MPC. There are very high amounts of lactose in SMP (about 51%).

As with other proteins, milk proteins have been reported to have quite complex interactions with starch and these interactions depend on which of the milk proteins is being investigated. Whey proteins, for instance, have quite complex effects on the physico-chemical behaviours of starch due to their ability to form gels. In WPI/starch

gel, WPI can have a synergistic effect on the viscoelastic behaviours of the gels depending on the temperature that the system is heated to. If the system is heated above 75^{o}C the rice starch undergoes gelatinisation prior to WPI gelation. In this situation the starch granules swell first, imbibe water from the system into their granules, thus resulting in a higher concentration of whey protein in the remaining water. This resulting gel is a reinforced gel that has a continuous whey protein phase filled with swollen starch granules; that act as an active filler. The effects of NaCAS on starch are still not fully understood. In general NaCAS increases the viscosity of starches but the mechanism for this result is still in question. There is disagremment between researchers as to how NaCAS alters the swelling behaviour of starch. However, it is believed that NaCAS increase the swelling ability of starch granules, which in turn increase the viscosity of the system. There are no reports about the effects that MPC have on the physico-chemical properties of starch. The effects of SMP on the physico-chemical properties of starch are well known and these effects are mainly due to the lactose and salts in SMP. Lactose is reported to decrease the swelling ability of starch granules by three possible mechanisms; the antiplasticising effect of sugars compared to water, specific starch-sugar interactions and competition for water with starch. The effects of salts on starch are more complicated than the effect of sugars. But it is believed that cations tend to protect and to stabilize the starch granule structure, which is a weak acid ion exchanger. In contrast, anions act as gelatinising agents by rupturing hydrogen bonds.

In summary, although starches are widely used in dairy based food products and interactions between individual starches and milk proteins have been extensively studied, there have been no systematic, comprehensive studies that have looked at the interactions between milk protein ingredients and rice starch. Therefore the aims of the present study were to determine if milk proteins-rice starch interactions did occur and if they did to identify the mechanism for the interactions identified. This was achieved by a systematic study with focus on the interaction of four milk proteins ingredients; SMP, MPC, NaCAS and WPI, on the physico-chemical behaviours of normal and waxy rice starch from the ungelatinised starches to gels. As it is difficult to distinguish the effects of the milk proteins in the milk protein ingredients from the effects of the other constituents of milk protein ingredients such as lactose and the salts, the effects of

ultrafiltrated skim milk powder permeated (UFSMP); a solution of salts and lactose present in SMP at their proper concentration, and lactose were also included in the study. Their inclusion would better help explain the effects that the milk ingredients had on the physico-chemical properties of normal and waxy rice starch.

Chapter 3

PASTING BEHAVIOUR OF STARCH/MILK PROTEIN INGREDIENT MIXTURES

3.1 Introduction

The pasting and viscoelastic behaviours of starch have a major impact on the texture and quality of dairy-based food. Investigations by various groups (Lelievre and Husbands, 1989; Kelly *et al*., 1995; Aguilera and Rojas, 1996; Aguilera and Baffico, 1997; Matser and Steeneken, 1997; Shim and Mulvaney, 2001; Bertolini, Creamer, Eppink and Boland, 2005), have found that adding different milk protein ingredients affected the pasting and viscoelastic properties of starches. However, despite the industrial importance and the relevance of starch-milk protein interactions, the published literature on the effects on the visco-elastic properties is very scarce.

The different studies on the effect on milk protein ingredients on starch are reported in the literature review section, and these are briefly summarized below. However, to the best of our knowledge, there is no published work on the effect of MPC on starch, except our own work published recently (Noisuwan, Bronlund, Wilkinson and Hemar, 2008). In general, it was found that the viscosity of the starch/milk or the starch/skim milk systems is higher than the starch/water system (Bradley, 1993; Matser and Steeneken, 1994). Carvalho, Onwulata and Tornasula (2007) reported the increase in the onset temperature of pasting of corn, Amioca (low amylose corn starch), and Hylon VII (high amylose corn starch) in the presence of WPI, but for tapioca starch, the onset temperature of pasting was not affected. However, they found that in the presence of WPI, all starches display higher peak viscosity compared to starches in the absence of WPI. Effects of NaCAS on the viscosity of starch are varied and there have been conflicts among the literatures, particularly for potato starch, but in general NaCAS was reported to increase the viscosity of starches (Lelievre and Husbands, 1989;

Doublier *et al*., 1994; Kelly *et al*., 1995; Marzin, Doublier and Lefebvre, 1995; Bertolini *et al*., 2005).

For the effects of SMP on starch pasting, in addition to the effects of the milk proteins present in SMP, the pasting behaviour of starch is strongly affected by lactose and salts (Noisuwan *et al*., 2008). Lactose is reported to decrease the swelling ability of starch granules, resulting in the decrease in the viscosity of starch/lactose pastes. All sugars act as anti-plasticizer when added to starch, whereas the effects of salts are more complicated because they depend on the type of salt and also its concentration. They can cause an elevation or depression of the gelatinization temperature but it is believed that cations; the main salts in bovine milk, tend to protect and to stabilize the starch granule structure, as the granule acts as a weak acid ion exchanger (Evans and Haisman, 1982; Chuncharoen and Lund, 1987; Jane, 1993).

We hypothesised that the four dairy ingredients would have a positive, though possibly a varied effect on the pasting behaviour of the two rice starches. As a consequence in this chapter a systematic investigation of the effect of the milk protein ingredients; SMP, MPC, NaCAS and WPI, and their concentrations on the pasting properties of normal rice starch and waxy rice starch was carried out. These four milk ingredients range in complexity from a simple whey protein based ingredient (WPI) to SMP in which all the milk components, except fat, are present. In the case of SMP, lactose and salts are present in SMP in quantities that could affect the pasting behaviour of starches. For this reason ultrafiltrated reconstituted skim milk permeate (UFSMP) and lactose were also investigated to understand their individual effects on the pasting behaviour.

3.2 Materials and Methods

3.2.1 Materials

Normal rice starch and waxy rice starch, donated by National Starch and Chemical Co., Thailand, were used.

Four different milk protein ingredients were used; low heat skim milk powder (SMP); milk protein concentrate, ALAPRO 4850, (MPC); sodium caseinate, ALANATE 180 (NaCAS); and whey protein isolate, WPI 895, (WPI). These milk ingredients were obtained from Fonterra Co-operative Group Ltd, New Zealand. UFSMP was obtained using a Fonterra-designed and -built ultrafiltration unit. Reconstituted skim milk (10% by weight) was pumped through a hollow fibre membrane cartridge (molecular weight cut-off 5 kDa) (Amicon Div. Inc., Beverly, MA, USA) using a pumping system with adjustable pressure and flow control (ISMATIC-MV pump system, Zurich, Switzerland).

3.2.2 Sample preparation

Mixtures with a constant rice starch to water ratio but with varying concentrations of milk proteins were prepared as follows. Stock solutions of 10% (w/w) milk protein ingredients were made by dissolving the powder in MilliQ water at ambient temperature with stirring for 1 hour. These solutions were kept overnight at 4°C. Before measurements, 10% (w/w) rice starch and 0, 2.5, 5, 7.5, or 10% (w/w) milk protein solutions were made by weighing rice starch, stock solution of 10% (w/w) milk protein ingredients and MilliQ water as show in Table 3.1 and gently mixing the ingredients for 5 minutes using a magnetic stirrer. A sample of 21 ml was transferred to the rheometer to measure the pasting behaviour as describe in section 3.2.3.2. This sample preparation method enables the concentration of starch in water to be kept constant at 10% (w/w) even when the amount of milk protein ingredient is varied. This ensured that any changes in the rheological behaviour of the mixtures were due to the presence of the milk proteins and not to the availability of water.

Table 3.1 Weight used in the preparation of the starch-milk protein samples.

Concentration of milk protein ingredients (%)	Weight used in the preparation (g)			Composition of 21 ml. of the starch/milk protein sample			
	Starch	Milk protein stock solution	Water	Starch (g)	Water (g)	Milk protein ingredients (g)	Starch/ water (%)
0.0	3.0	0.00	27.00	2.1	18.9	0.0	10.0
2.5	3.25	7.50	22.50	2.1	18.5	0.5	10.0
5.0	3.17	15.00	15.00	2.0	18.0	1.0	10.0
7.5	3.08	22.50	7.50	2.0	17.6	1.4	10.0
10.0	3.00	30.00	0.00	1.9	17.2	1.9	10.0

3.2.3 Methods

3.2.3.1 Chemical analysis

The chemical compositions, except the amylose content, of normal rice starch and waxy rice starch were analysed by the AACC method (1995). The total protein content was determined using the Kjeldahl method, AACC method 46-11A with a nitrogen conversion factor of 5.7 for flour and starch as recommended by AACC (AACC, 1995). The fat content was determined by the Mojonnier method, AACC method 30-10; moisture content was determined by the air-oven method at 103±1°C for 1 hour, AACC method 44-15A; ash content was determined using a muffle furnace, AACC method 08-01. The amylose content of both starches was determined by using an amylose/amylopectin assay kit (Megazyme, Ireland), according to the method described by Gibson, Solah, & McCreary (1997), which is based on the principle of specific binding of non-reducing end-groups of amylopectin with concanavalin A (Appendix A).

For milk protein ingredients, the chemical compositions were determined using the AOAC (2000) or IDF method. The total protein content was determined using the Kjeldahl method, AOAC official method 930.29 with a nitrogen conversion factor of

6.38 for proteins in dried milk as recommended by AOAC (AOAC, 2000). As MPC, NaCAS and WPI do not readily dissolve in ammonia, the Roese-Gottlieb method is not suitable for the determination of their fat contents. The Schmid-Bondzynski-Ratzlaff gravimetric method, IDF standard 127A (IDF, 1988), recommended for low lactose content milk ingredients (lactose less than 5% of the dry matter), was used for these three ingredients; moisture content was determined by air-oven method, IDF standard 78C: 1991(IDF, 1991); ash content was determined using muffle furnace, AOAC official method 930.30; and lactose was determined by the photometric method, IDF standard 106:1982 (IDF, 1982).

3.2.3.2 Pasting behaviour measurements

The pasting behaviours measurements of milk protein/rice starch mixtures and 10% milk protein ingredient solutions alone were measured on a stress-controlled rheometer (Parr Physica UDS 200; Physica, Stuttgart, Germany) using the starch cell geometry TC 20.

From preliminary experiments at various heating rates (1°C min^{-1} to 10°C min^{-1}) and shear rates (10 rev min^{-1} to 500 rev min^{-1}) for both normal and waxy rice starches, it was found that the pasting temperature was lower at low heating rate because the low heating rate allowed longer duration for starch granule swelling. The shear rate did not affect the pasting temperature but the lower shear rate did results in a higher peak viscosity due to less disruption of the swollen starch granules. However, fluctuation in the temperature profile curve at the lower shear rate was observed and was probably due to the inefficiency of the sample mixing. From the results, the lowest heating rate and lowest shear rate, which do not influence the pasting profile were selected. In this study the heating rate of 2°C min^{-1} and a shear rate of 100 rev min^{-1} were chosen.

Other important parameters for the pasting measurement are the initial temperature. When heating at 2°C min^{-1} and at shear rate of 100 rev min^{-1}, the rice starches used in this study start to gelatinize at temperatures >55°C. Hence, in this study an initial

temperature of 50°C, and a holding time of 5 minutes were chosen to ensure that the whole sample was equilibrated at 50°C. Thus, the measurements were performed as follows:

The sample was equilibrated at 50°C for 5 min under a constant shear rate at 100 rev min^{-1} (*step 1*) then a temperature ramp from 50°C to 95°C was applied, at a heating rate of 2°C min^{-1} and under a constant shear rate of 100 rev min^{-1} (*step 2*) before cooling the sample at a rate of 2°C min^{-1} without shear until the temperature was reduced to 25°C (*step 3*). In this step 3, the shear was removed to ensure uninterrupted gel formation during cooling. From the pasting behaviour of normal rice starch/milk protein ingredients systems, i.e., curves of apparent viscosity as a function of temperature, a key pasting parameters can be determined (Figure 3.1). These include:

- *The onset temperature* (T_{onset}) at which the viscosity starts to increase. From the pasting curve shown in Figure 3.1, a baseline was drawn through the early temperatures where the viscosity is constant. Then a straight line was drawn down the leading edge of the region where the viscosity starts to increase. T_{onset} is defined as the intersection between these two lines (point T_{onset} on Figure 3.1).
- *The peak viscosity temperature* (T_{peak}), which is the temperature at which the viscosity reaches its maximum. It is determined by drawing straight lines from either side of the peak viscosity. The intersection point of these two lines gives T_{peak}.
- *The peak viscosity*, the maximum viscosity of the paste (point Peak vis on Figure 3.1) and
- *The final viscosity*, which in the current study, corresponds to the viscosity at 50°C (point Final vis on Figure 3.1).

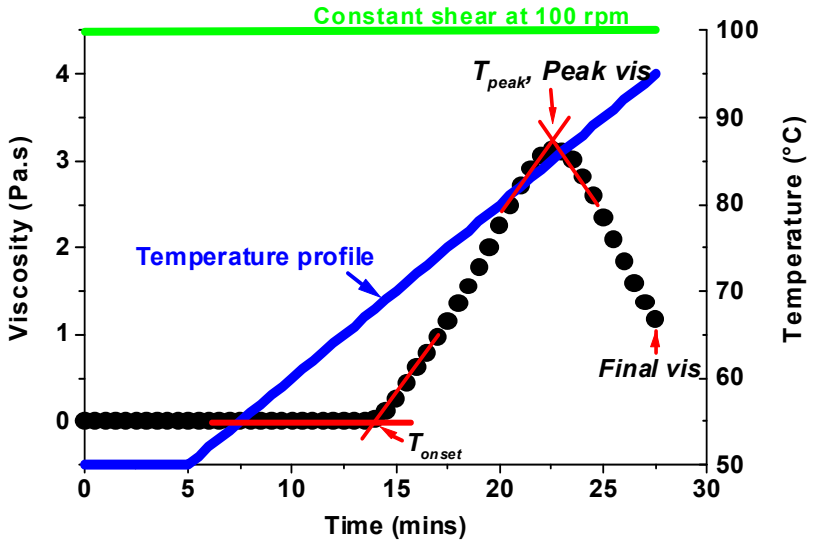

Figure 3.1 Temperature profile (blue line) used and different parameters (indicated by arrows) obtained from the pasting curve (solid symbols).

3.2.4 Statistical analysis

All statistical analyses were performed using the Statistical Analysis System (version 8.2, SAS Institute, Cary, NC). Data were analysed using the general linear model (GLM) approach. The pasting behaviour data were analysed for the T_{onset}, T_{peak}, peak viscosity, and final viscosity. All the parameters were measured in duplicate and assessed to consider the effects that the milk protein ingredients at varying concentration had on the rheological properties of normal and waxy rice starch. Significance was accepted at the 5% confidence level.

3.3 Results

The chemical compositions of normal and waxy rice starch are shown in Table 3.2. The protein and lipid contents form this study were in agreement with values reported by Singh *et al*. (2000), for normal rice starch; protein 0.45-0.9% and fat 0.20-0.22%, waxy rice starch; protein 0.2% and fat 0.1%. Qi *et al*. (2003) studied waxy rice and reported values of 0.31% and 0.041% for protein and fat, respectively. In the present study, the amount of amylose in the waxy rice starch is 3.25% and in the normal rice starch is 11.19%. According to Juliano *et al*. (1981), this normal rice can be classified as a low amylose content normal rice.

The chemical composition of SMP, MPC, NaCAS and WPI used in this study are shown in Table 3.3. The results are in agreement with those reported in the literature; SMP, protein 33.6%, lactose 51.0%, fat 1.0% and 8.5% ash (Walstra *et al*., 2006); MPC, protein 85.4%, lactose 4.6%, fat 1.7% and ash 7.3% (Carr, 1999); NaCAS, protein 91.4%, lactose 0.1%, fat 1.0% and ash 4.0% (Chandan, 1997), and WPI, protein 93.0%, lactose 1.0%, fat 0.5% and ash 2.0% (Chandan, 1997).

Table 3.2 Chemical compositions of normal rice starch and waxy rice starch

Starch	Component (%)					Amylose
	Carbohydrate	Protein	Fat	Ash	Moisture	Content (%)
Normal	87.63	0.42	0.58	0.17	11.20	11.19
Waxy	87.92	0.30	0.11	0.17	11.50	3.25

Table 3.3 Chemical compositions of milk protein ingredients.

Milk protein ingredients	Component (%)			
	Protein	**Fat**	**Lactose**	**Ash**
SMP	33.40	0.80	54.10	7.90
MPC	83.10	1.60	4.30	7.10
NaCAS	93.00	0.70	0.10	3.60
WPI	93.20	0.30	0.60	2.10

3.3.1 Milk protein ingredient solutions.

The results of the apparent viscosity changes for the milk protein ingredients on their own during the pasting experiment conditions are reported in Figure 3.2, where it can be clearly seen that the apparent viscosities of the SMP and UFSMP solutions decreased with increasing temperature in a similar fashion to water (open symbols). The apparent viscositiy of NaCAS solution was rapidly decreased with increasing temperature up to ~80°C, and then the decrease was slowly. The anomalous decrease in the apparent viscosity of the MPC solution with temperature was possibly due to the fact that MPC is not fully solubilised until the temperature reaches 50°C and even at 50°C it takes time for this to occur (Carr, 1999). The apparent viscosity of this solution was higher than it should be at temperature below 75°C and then dropped when the protein went into solution. The apparent viscosity of the WPI solution decreased up to 72°C, and then started to increase due to the heat denaturation and aggregation of the whey proteins present in WPI (Paulsson and Dejmek, 1990). Note that the values of viscosity obtained for water are larger than that expected. This is due to the complex design of the stirrer and the wide-gap between the stirrer blades and the cup used to measure the rheological properties of starch, and to the limitations of rotational viscometers when measuring systems having low viscosity.

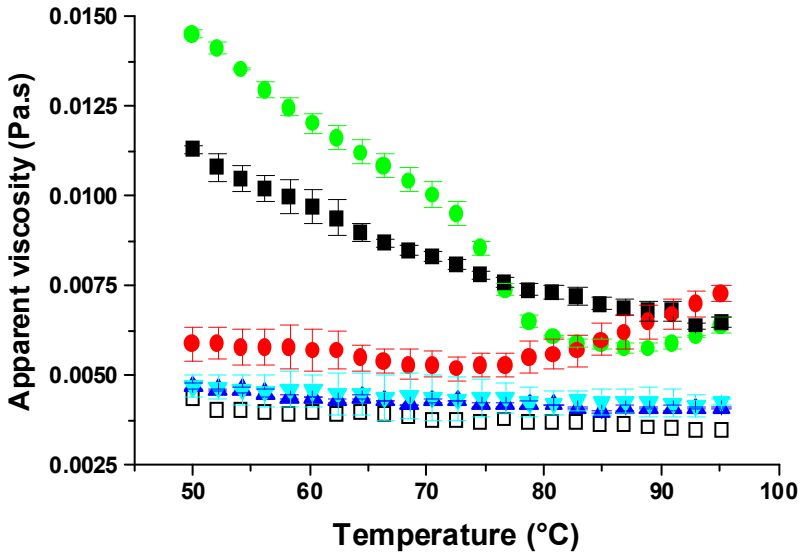

Figure 3.2 Viscosity as a function of temperature for 10% milk protein ingredients in water. UFSMP was measured without further dilution. The milk protein ingredients are: (▲) SMP; (●) MPC; (■) NaCAS; (●) WPI; (▼) UFSMP; (□) is water alone. The error bars show the SD across measurements (n=2).

3.3.2 Normal rice starch/milk ingredients mixtures

The different pasting curves for the addition of SMP, MPC, NaCAS, WPI, UFSMP, and lactose to normal rice starch are plotted in Figure 3.3 to 3.8 respectively. The key pasting parameters extracted from these curves are reported in Table 3.4. These results clearly show that each different type of milk protein ingredient affected markedly and differently the pasting behaviour of normal rice starch.

The addition of SMP increased the T_{onset} and T_{peak} of normal rice starch/SMP mixture (Figure 3.3). The value of the peak viscosity was slightly decreased, but the final viscosity of the mixture increased with increasing SMP concentration. UFSMP showed a similar but greater effect than SMP on the pasting behaviour of normal rice starch (Figure 3.7). The addition of UFSMP increased the T_{onset} and T_{peak} of normal rice starch/UFSMP paste. The peak viscosity was decreased, but the final viscosity of the pastes increased with increasing UFSMP concentration. Lactose delayed the pasting of

normal rice starch, shifting the whole pasting curves to higher temperature. The shift in temperature increased with increasing lactose concentration (Figure 3.8). The final viscosity of the pastes slightly increased with increasing lactose concentration.
In contrast to SMP, the addition of MPC to normal rice starch decreased the T_{onset}. This decrease in the T_{onset} was larger when the MPC concentration was increased (Table 3.4). The T_{peak} of normal rice starch was decreased by the addition of MPC (Figure 3.4). However, the peak viscosity was markedly increased at high MPC concentrations while the final viscosity increased only slightly.

The pasting behaviour of normal rice starch containing different NaCAS concentrations is shown in Figure 3.5. The T_{onset} of normal rice starch was increased only slightly (<1°C) when NaCAS was added (Table 3.4). However, T_{peak} increased from 84.8°C, for the normal rice starch without NaCAS, to up to 92°C for the addition of NaCAS to normal rice starch, (Table. 3.4). Peak viscosity decreased when low (2.5 and 5%) NaCAS concentrations were added and increased on the addition of higher (7.5 and 10%) NaCAS concentrations. The final viscosity of the normal rice starch/NaCAS mixtures increased with increasing NaCAS concentrations (Figure 3.5 and Table 3.4).

The addition of WPI affected the pasting behaviour of normal rice starch by decreasing the T_{onset}. However, T_{peak} first increased from 84.8 to 88.9°C when 2.5 or 5% WPI were added (Table 3.4), and then decreased back to 84.8°C when 10% WPI was added (Figure 3.6). The peak viscosity also decreased at low WPI concentrations (2.5 and 5%) and markedly increased for the higher WPI concentrations (7.5 and 10%). The final viscosity of the WPI/normal rice starch mixtures increased with the addition of WPI. Whilst the peak viscosity decreased slightly with the addition of low concentration of WPI, the final viscosity increased, particularly at 10% WPI where a marked increase in final viscosity was obtained (Figure 3.6).

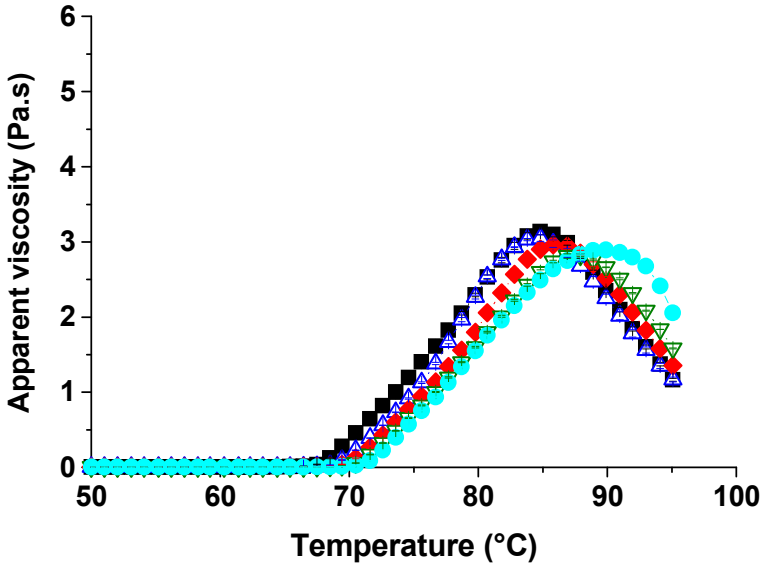

Figure 3.3 Apparent viscosity as a function of temperature for normal rice starch/ SMP. Concentration of SMP: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●)10%.

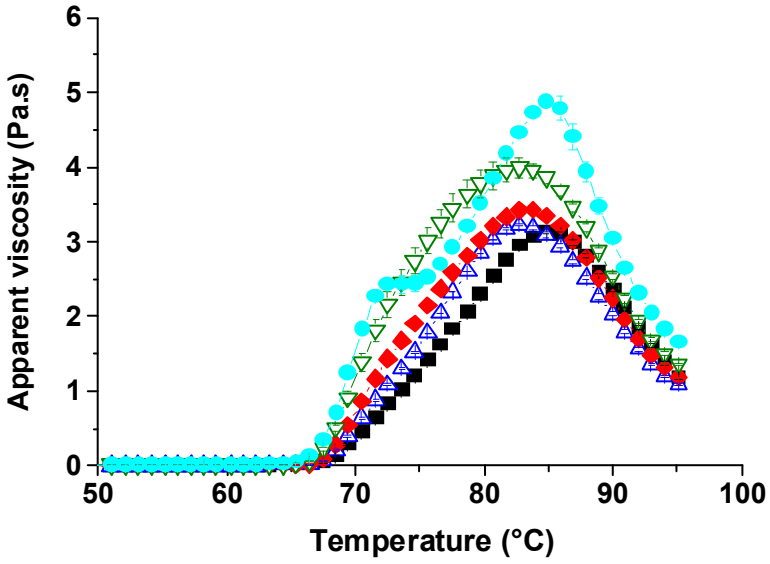

Figure 3.4 Apparent viscosity as a function of temperature for normal rice starch/ MPC. Concentration of MPC: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●)10%.

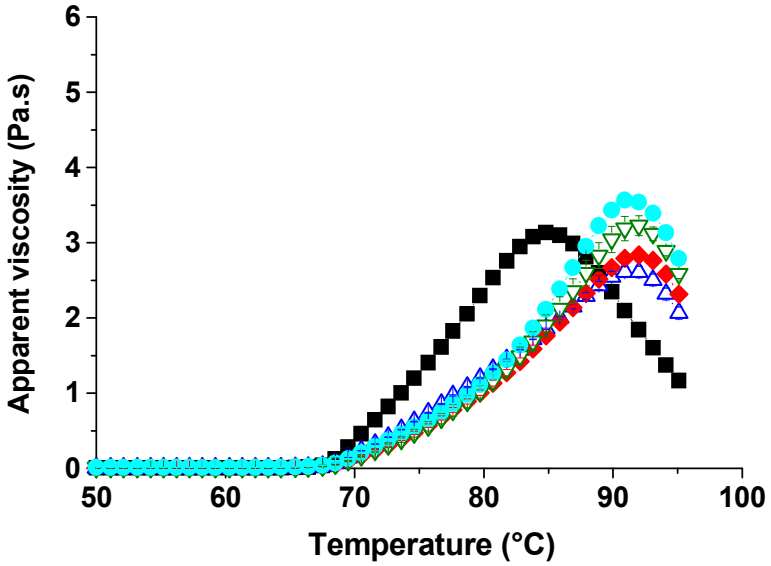

Figure 3.5 Apparent viscosity as a function of temperature for normal rice starch/ NaCAS. Concentration of NaCAS: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

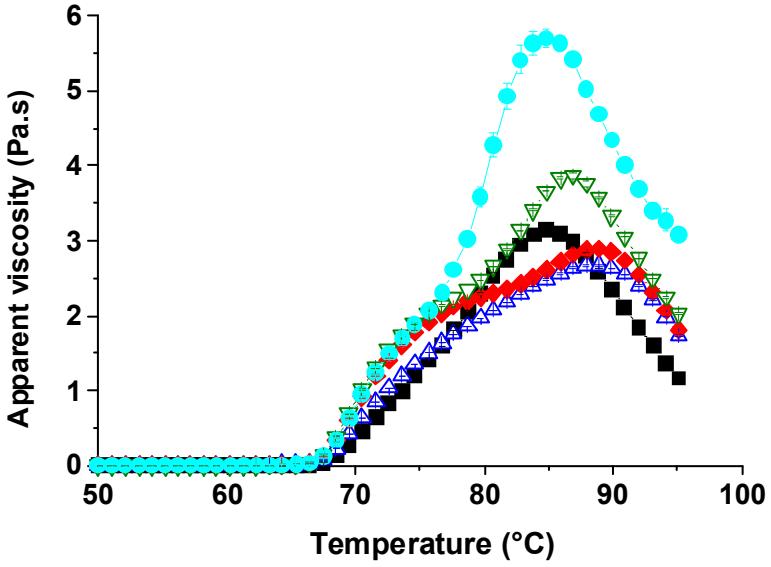

Figure 3.6 Apparent viscosity as a function of temperature for normal rice starch/ WPI. Concentration of WPI: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

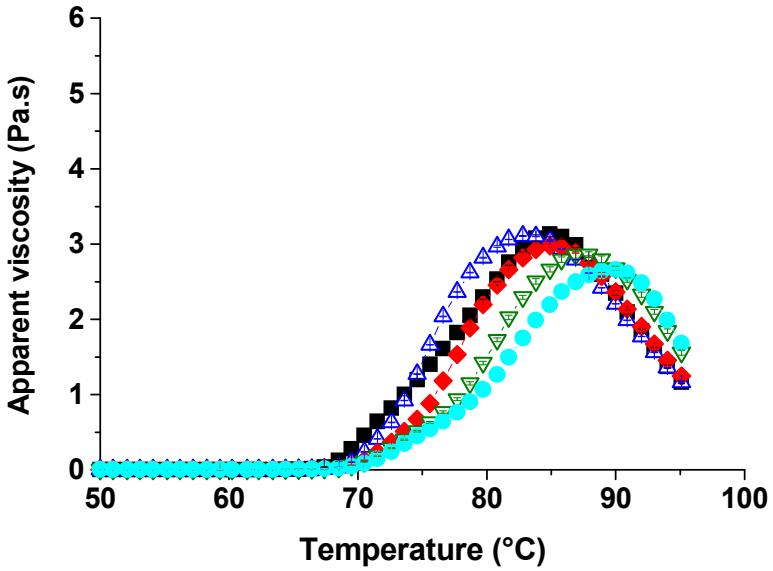

Figure 3.7 Apparent viscosity as a function of temperature for normal rice starch/ UFSMP. Concentration of UFSMP: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●)10%.

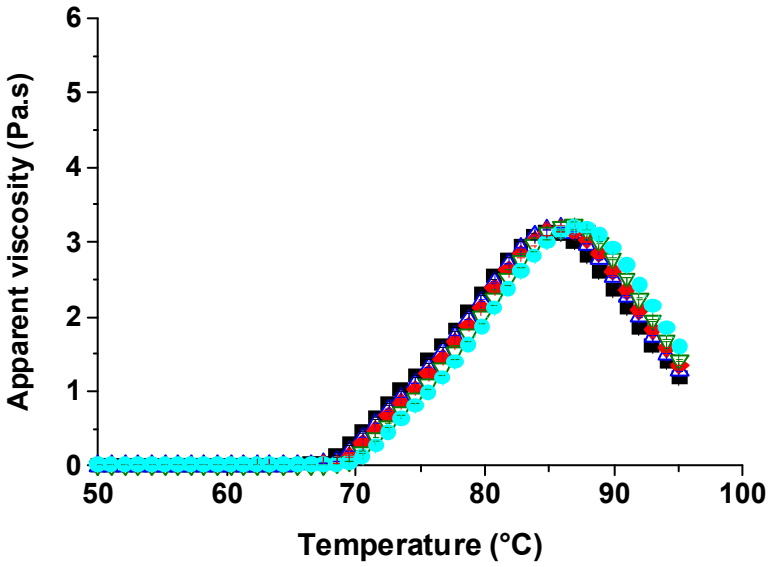

Figure 3.8 Apparent viscosity as a function of temperature for normal rice starch/ lactose. Concentration of lactose: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

Table 3.4 The pasting parameters[*, **] for 10% normal rice starch/water mixtures and 10% normal rice starch/milk protein ingredient mixtures.

Dairy ingredient	Conc. (%)	T_{onset} (°C)	T_{peak} (°C)	*Peak viscosity* (Pa.s)	*Final viscosity* (Pa.s)
None	0	67.7±0.0i	84.8±0.0i	3.13±0.04ghi	1.17±0.02no
SMP	2.5	68.8±0.0f	84.3±0.7j	3.05±0.04ij	1.17±0.03o
	5	69.6±0.0c	85.9±0.0h	2.96±0.05jklm	1.36±0.00jk
	7.5	70.3±0.0b	87.9±0.0e	2.82±0.02n	1.58±0.01i
	10	70.9±0.0a	89.9±0.0c	2.89±0.01lmn	2.06±0.03e
MPC	2.5	67.1±0.1j	82.8±0.0k	3.23±0.02g	1.09±0.01p
	5	66.7±0.0kl	82.7±0.0k	3.42±0.03f	1.19±0.00no
	7.5	66.0±0.0m	82.8±0.0k	3.99±0.13c	1.37±0.02jk
	10	65.4±0.1n	84.8±0.0i	4.88±0.03b	1.66±0.04h
NaCAS	2.5	68.0±0.0h	90.9±0.0b	2.61±0.07o	2.06±0.08e
	5	68.3±0.1g	92.0±0.0a	2.84±0.05n	2.31±0.04d
	7.5	68.3±0.1g	92.0±0.0a	3.22±0.13g	2.58±0.06c
	10	68.2±0.0gh	91.0±0.0b	3.57±0.00e	2.79±0.01b
WPI	2.5	66.9±0.0k	88.9±0.0d	2.67±0.00o	1.74±0.01g
	5	66.6±0.0l	88.9±0.0d	2.90±0.02klmn	1.82±0.03f
	7.5	66.6±0.1	86.9±0.0f	3.86±0.03d	2.03±0.00e
	10	66.6±0.1l	84.8±0.0i	5.71±0.11a	3.09±0.05a

Continued-

Table 3.4-continued

Dairy ingredient	**Conc. (%)**	T_{onset} **(°C)**	T_{peak} **(°C)**	***Peak viscosity* (Pa.s)**	***Final viscosity* (Pa.s)**
UFSMP	2.5	68.8±0.1f	82.8±0.0k	3.11±0.01hi	1.17±0.01o
	5	69.3±0.1d	84.8±0.0i	2.98±0.01jkl	1.25±0.010mn
	7.5	69.7±0.1c	87.9±0.0e	2.86±0.04mn	1.56±0.0314j
	10	70.2±0.1b	89.9±0.1c	2.66±0.01o	1.69±0.0211gh
Lactose	2.5	68.1±0.0h	85.9±0.0h	3.19±0.00gh	1.28±0.0010lm
	5	68.7±0.0f	85.8±0.1h	3.18±0.05gh	1.34±0.0015kl
	7.5	69.1±0.1e	86.4±0.7g	3.22±0.05gh	1.42±0.01j
	10	69.8±0.0c	86.9±0.0f	3.19±0.00gh	1.59±0.01i

* Mean value ± SD (n=2).

** Different letters within the same column indicate significant difference at $P < 0.05$ different through the Duncan test.

3.3.3 Waxy rice starch/milk ingredients mixtures

Compared to normal rice starch, waxy rice starch showed lower pasting temperatures and more rapid but smaller increases in viscosity i.e. the temperature range from T_{onset} to T_{peak} for waxy rice starch was only 5.7°C but was 17.1°C for normal rice starch.

As with normal rice starch, the addition of the different milk ingredients affected the pasting behaviour of waxy rice starch. However, the extent of these effects was more dramatic in the case of normal rice starch than in the case of waxy rice starch. The effects of SMP, UFSMP and lactose on waxy rice starch was similar to those when added to normal rice starch, as they delayed the pasting of starch (Figure 3.9, Figure 3.13, Figure 3.14 and Table 3.5). The addition of MPC to waxy rice starch showed different effects to those on normal rice starch. At any concentration it was found that MPC did not affect the T_{peak} of waxy rice starch (Figure 3.10, Table 3.5) but the peak viscosity was markedly increased. Note that the two peaks observed in the addition of 10% MPC to normal rice starch could not be observed in waxy rice starch paste. However, the final viscosity of waxy rice starch increased with the increase in MPC concentration.

Similar to normal rice starch, NaCAS slightly increased the T_{onset} and T_{peak} of waxy rice starch (Figure 3.11 and Table 3.5). The final viscosity also increased with an increase in the NaCAS concentration. In contrast to normal rice starch, the value of the peak viscosity was increased with an increase in the NaCAS concentration.

The addition of WPI increased the T_{onset} and T_{peak} of waxy rice starch (Figure 3.12 and Table 3.5). The peak viscosity decreased with the addition of WPI. The final viscosity of the WPI/waxy rice starch mixtures increased with the addition of WPI. Whilst the peak viscosity decreased slightly with the addition of WPI, the final viscosity increased markedly, particularly when 10% WPI was added.

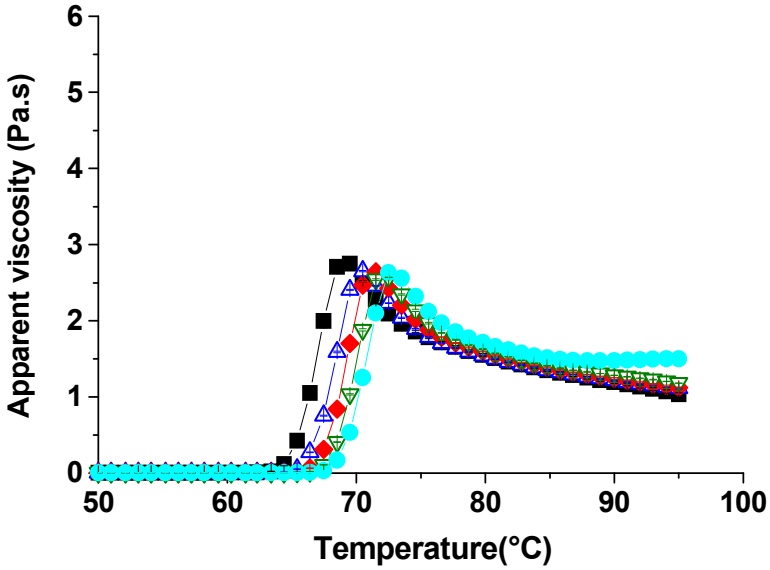

Figure 3.9 Apparent viscosity as a function of temperature for waxy rice starch/SMP. Concentration of SMP: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

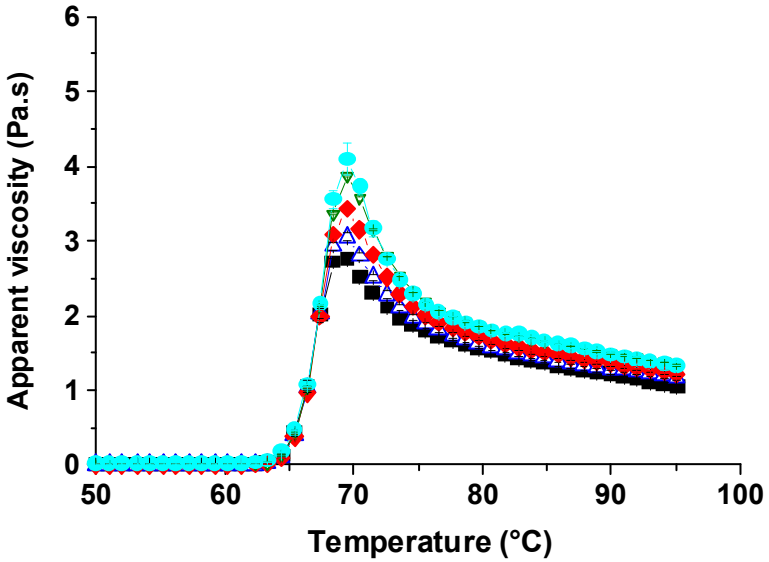

Figure 3.10 Apparent viscosity as a function of temperature for waxy rice starch/MPC. Concentration of MPC: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

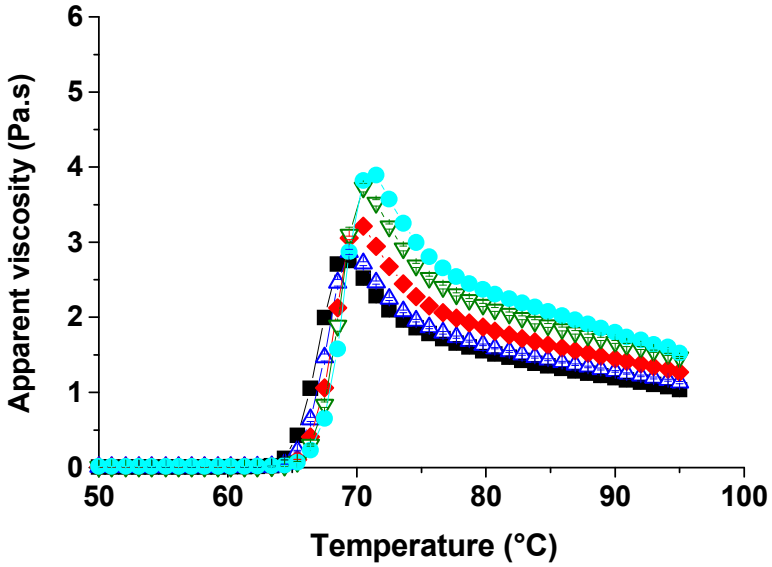

Figure 3.11 Apparent viscosity as a function of temperature for waxy rice starch/ NaCAS. Concentration of NaCAS: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

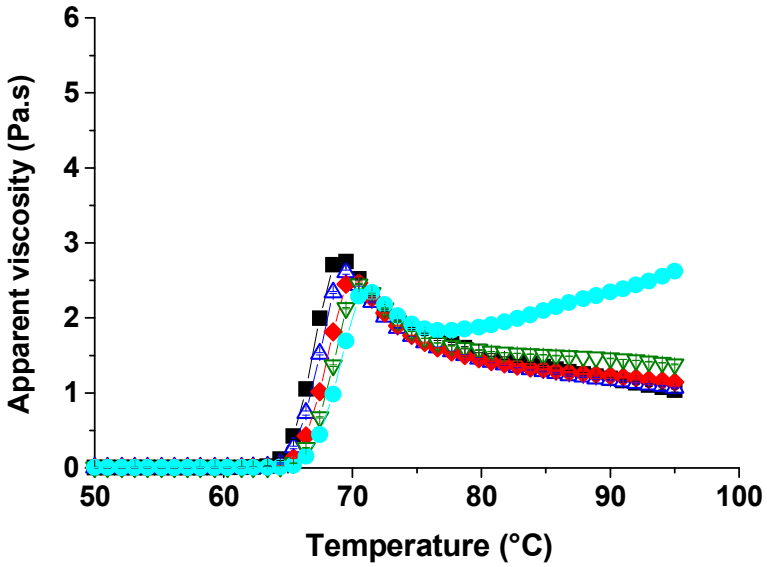

Figure 3.12 Apparent viscosity as a function of temperature for waxy rice starch/ WPI. Concentration of WPI: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

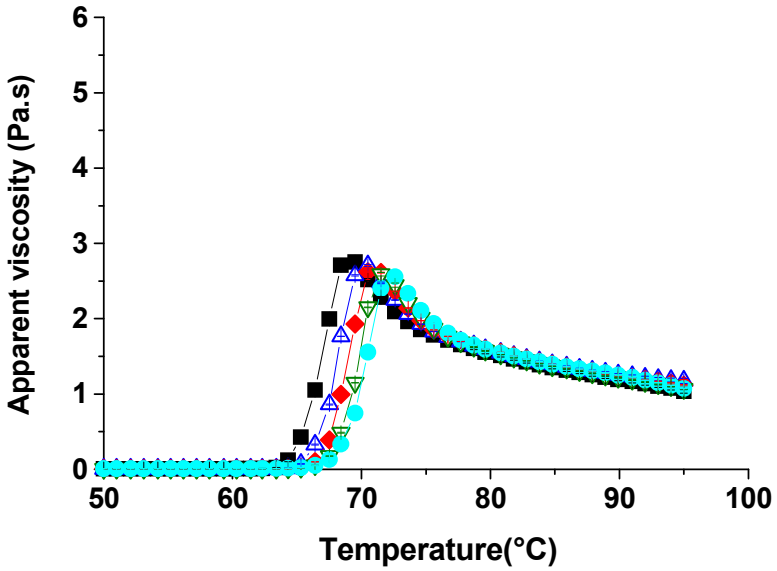

Figure 3.13 Apparent viscosity as a function of temperature for waxy rice starch/ UFSMP. Concentration of UFSMP: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

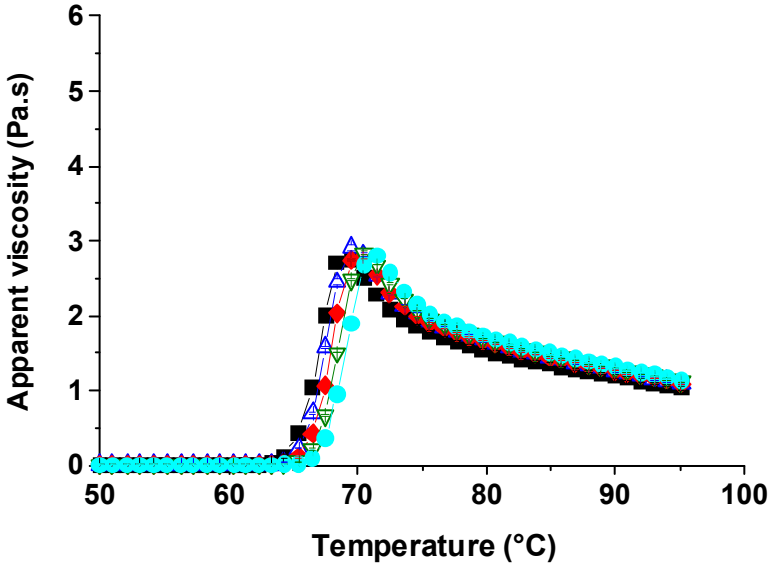

Figure 3.14 Apparent viscosity as a function of temperature for waxy rice starch/ lactose. Concentration of lactose: (■) 0%; (△) 2.5%, (◆) 5%, (▽) 7.5%, and (●) 10%.

Table 3.5 The pasting parameters[*, **] for 10% waxy rice starch/water mixtures and 10% waxy rice starch/milk protein ingredient mixtures.

Dairy ingredient	Conc. (%)	T_{onset} (°C)	T_{peak} (°C)	*Peak viscosity* (Pa.s)	*Final viscosity* (Pa.s)
None	0	63.6±0.0n	69.2±0.5e	2.76±0.01ij	1.03±0.02p
SMP	2.5	65.3±0.0h	70.5±0.0d	2.65±0.01klm	1.11±0.01klm
	5	66.1±0.0e	71.5±0.0c	2.64±0.00lm	1.12±0.01jkl
	7.5	66.7±0.0c	72.5±0.0b	2.57±0.00mn	1.18±0.00gh
	10	67.6±0.0a	72.5±0.0b	2.63±0.00lm	1.50±0.01bc
MPC	2.5	63.7±0.0mn	69.5±0.0e	3.05±0.05f	1.17±0.03hij
	5	3.8±0.1mn	69.5±0.0e	3.42±0.02d	1.22±0.00g
	7.5	63.6±0.0n	69.4±0.0e	3.86±0.01b	1.34±0.01e
	10	63.3±0.0no	69.5±0.0e	4.09±0.21a	1.32±0.05ef
NaCAS	2.5	64.3±0.0k	69.5±0.0e	2.87±0.04gh	1.13±0.01hijkl
	5	64.7±0.0j	70.5±0.0d	3.21±0.03e	1.27±0.01f
	7.5	64.9±0.1i	70.5±0.0d	3.73±0.06c	1.46±0.01c
	10	64.9±0.0i	71.5±0.0c	3.89±0.03b	1.52±0.02b
WPI	2.5	63.9±0.3lm	69.4±0.0e	2.61±0.03lm	1.07±0.01nop
	5	64.6±0.0j	70.5±0.0d	2.47±0.01no	1.14±0.00hijk
	7.5	65.1±0.0hi	70.5±0.0d	2.45±0.01op	1.38±0.01d
	10	65.5±0.0g	71.5±0.1c	2.34±0.01p	2.62±0.01a

Continued-

Table 3.5-continued

Dairy ingredient	**Conc. (%)**	T_{onset} **(°C)**	T_{peak} **(°C)**	***Peak viscosity* (Pa.s)**	***Final viscosity* (Pa.s)**
UFSMP	2.5	65.2±0.0h	70.5±0.0d	2.71±0.01	1.17±0.03
	5	66.1±0.0e	70.5±0.0d	2.62±0.01	1.12±0.01
	7.5	66.4±0.1d	71.5±0.0c	2.59±0.02	1.07±0.00
	10	67.4±0.2b	72.6±0.0a	2.56±0.00	1.07±0.00
Lactose	2.5	64.2±0.0kl	69.4±0.1e	2.91±0.03	1.09±0.01
	5	64.7±0.0j	70.4±0.1d	2.78±0.02	1.10±0.00
	7.5	65.2±0.0h	70.5±0.0d	2.84±0.01	1.12±0.01
	10	65.7±0.0f	71.5±0.0c	2.80±0.02	1.14±0.01

* Mean value ± SD (n=2).

** Different letters within the same column indicate significant difference at $P < 0.05$ different through the Duncan test.

3.4 Discussion

As shown in Figure 3.2, the apparent viscosities of the milk ingredients alone, except MPC and WPI, showed an expected reduction in viscosity with temperature. MPC and WPI did show a slight increase in viscosity at temperatures higher than 85 and 72°C, respectively, due to the aggregation of the whey proteins; α-la and β-lg, which is caused by heat denaturation and subsequent aggregation (Paulsson and Dejmek, 1990). However, these milk ingredients affected the pasting behaviour of both normal and waxy rice starch differently.

SMP increased the T_{onset} and the T_{peak}, when added to both normal rice starch (Figure 3.3) and waxy rice starch (Figure 3.9). It also slightly decreased the peak viscosity and increased the final viscosity of these two starches (Figure. 3.3 and 3.9). This is certainly due to the presence of large amounts of lactose and free cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} etc.) in SMP as both sugars and salts are well known for their effect on the pasting and gelation behaviour of starches. Kelly *et al*. (1995), Muhrbeck and Eliasson (1987), and Paterson *et al*. (1994) have reported that sodium chloride caused a large reduction in viscosity and swelling volume of potato starch. Sugars were reported to increase the starch gelatinization temperature by reducing the level of solvent plasticization (Perry and Donald, 2002). Furthermore, Perry and Donald (2002) pointed out that the mechanism of starch gelatinization is not affected by the addition of sugars but that the kinetics of gelatinization are simply translated further up the temperature axis. This translation to a higher temperature could be seen in the pasting curves of the normal and waxy rice starches containing SMP (Figures 3.3 and 3.9). This effect on the pasting behaviour was also confirmed when only the UFSMP (Figures 3.7 and 3.13), which consisted of lactose and salts, or lactose alone (Figures 3.8 and 3.14) was added to normal and waxy rice starch.

The decrease in the value of the peak viscosity when SMP is added is also due to the salts present in SMP, as this has been also observed when UFSMP has been added. The reduction in the value of the peak viscosity is probably due to the reduction in the swelling volume of the starch granules (Muhrbeck and Eliasson, 1987; Paterson *et al*.,

1994; Kelly *et al*., 1995). Finally, the increase in the final viscosity is expected, as it is the result of the increase in the total biopolymer concentration.

The different effects that were observed on the addition of MPC to rice starches compared to those of SMP are due to two factors. Firstly, MPC has substantially lower concentrations of lactose and minerals than SMP. Secondly, the concentration of milk proteins in MPC is approximately three times that in SMP. For normal rice starch, although the peak temperature did not change greatly, the increase in viscosity, was shifted towards lower temperatures. This could have been due to the denaturation of the whey proteins, particularly at high MPC concentrations where two peaks were observed. In fact at 10% MPC, a first temperature peak was seen at 72°C and a second peak was seen at 85°C. The temperature of 72°C is the same as that found for the increase in viscosity of WPI solution alone (Figure 3.2). Using DSC, Paulsson and Dejmek (1990) showed that the whey proteins α-la and β-lg denature at temperatures of 66.9 ± 0.9 and 76.6 ± 0.9 °C respectively.

Some of the effects observed in the MPC/normal rice starch mixtures were observed when WPI is added to normal rice starch. That is, the increase in viscosity during pasting also shifted towards lower temperatures. In addition, a first pseudo-peak is observed at ~75°C and a second peak, corresponding to the swelling of the starch granules, is observed at much higher temperatures. The presence of the first pseudo-peak confirms that, as in the case of MPC, the shift of the pasting curves to lower temperatures is due to whey proteins. The normal rice starch/WPI mixture, as in the normal rice starch/MPC, the peak viscosity increased markedly due to the aggregation and gelification of the whey proteins which holds water and which in turn, results in an increase in the local concentration of starch molecules. This also markedly increased the final viscosity due to the increase in biopolymer concentration and particularly to the aggregation of the whey proteins.

Despite having a large effect on the pasting of normal rice starch, the effect of MPC or WPI was less noticeable when added to waxy rice starch (Figures 3.10 and 3.12). When MPC is added to waxy rice starch only the increase in the peak and final viscosities are

observed. These are expected as the concentration of the biopolymers present, i.e. starch molecules and MPC proteins, increased. Similar behaviour is observed when WPI is added to waxy rice starch, along with a slight shift of the pasting curves toward higher temperatures and a high final viscosity at high WPI concentrations.

The difference see in the behaviours of normal rice starch and waxy rice starch is due to the differences in chemical composition, the amylose/amylopectin ratios (Table 3.2), the swelling ability, and water uptake and gelatinisation temperature. From Table 3.4, normal rice starch has a T_{onset} of 67.7°C and a T_{peak} of 84.8°C so when normal rice starch is heated in the presence of MPC or WPI, the swelling of the normal rice starch granules in this temperature range, is concomitant with the thermal gelation of the whey proteins; which are reported to denature at temperatures of 66.9 ± 0.9 and 76.6 ± 0.9 °C for α-la and β-lg respectively (Paulsson, Dejmek and Vanvliet, 1990). Thus the swollen starch granules, holding a large amount of water, will result in the increase in the local concentration of the milk proteins, in turn, leading to the increase in the viscosity of the mixture.

In the case of waxy rice starch, T_{onset} is 63.6°C and the T_{peak} is 69.2°C, so that the waxy rice starch solution is nearly fully gelatinised before the whey proteins starts to denature. In this case the competition for water between the starch granules and the whey protein is minimal compared to normal rice starch and consequently the local concentration of the whey protein is not greatly increased. The difference in the gelatinisation of these two different rice starches in the presence of whey proteins is schematically represented in Figure 3.15 below. Note that the kinetics of the aggregation and the size and the shape of the whey protein aggregates should also be affected differently when the aggregation takes place in the presence of normal rice starch or waxy rice starch. In addition, because normal rice starch has a higher peak viscosity (3.13 Pa.s) than waxy rice starch (2.76 Pa.s) it is expected that the effects of MPC and WPI will have a greater effect when added to normal rice starch.

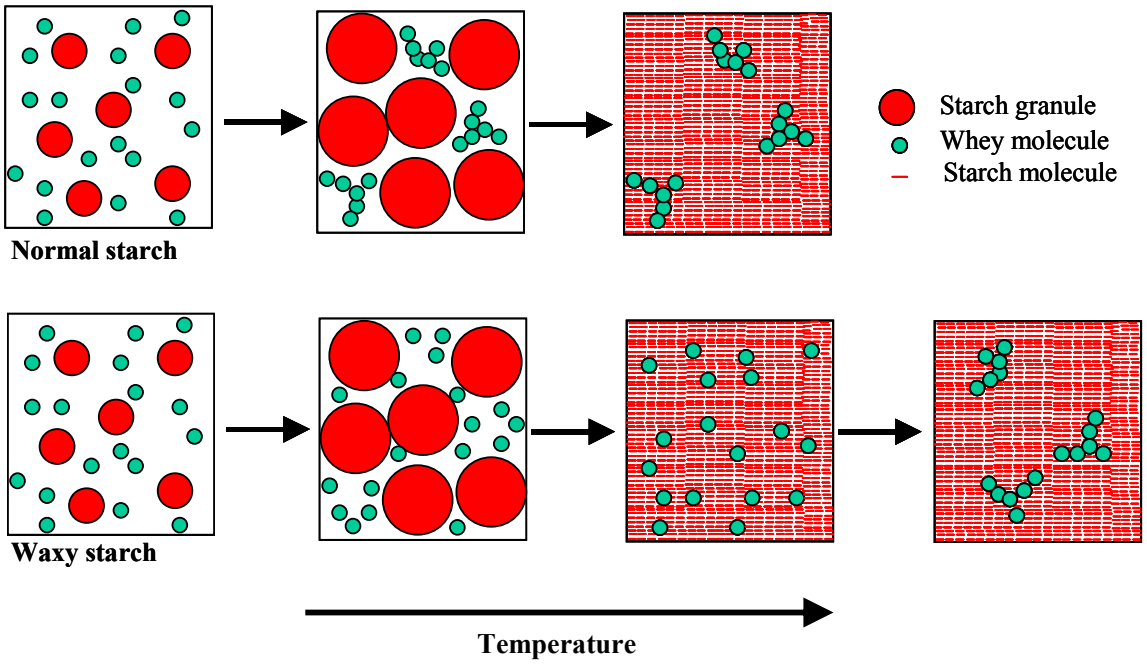

Figure 3.15 Schematic representation of the proposed routes for the pasting of 10% (w/w) rice starches in the presence of milk protein ingredients solution containing whey proteins.

From the results we can see clearly that the effect of WPI on the pasting behaviour of normal rice starch was different from that of the other milk protein ingredients. Particularly, a first pseudo-peak viscosity was observed at 77 °C followed by a second peak viscosity at much higher temperatures. However, both the temperature and the value of the peak viscosity were dependent on the WPI concentration (Figure 3.6), because the aggregation of WPI and the gelatinisation of normal rice starch take place in the same range of temperatures. The addition of WPI decreased the temperature at which the viscosity of the mixture increased, as in the case of MPC, due to whey protein denaturation and aggregation.

Finally, NaCAS also affected the pasting behaviour of normal rice starch (Figure 3.5) and waxy rice starch (Figure 3.11). When added to normal rice starch at low concentration, the peak viscosity decreased, but increased again when higher NaCAS concentrations were added. Previous studies have reported the decrease in viscosity of starch containing NaCAS. Appelqvist and Debet (1997), in their review on starch–

biopolymer interactions, reported that a study in 1976 by van der Kamp had shown that the addition of NaCAS to modified starch (Colfo) led to a decrease in the viscosity. Similar findings were observed on the addition of NaCAS to potato starch (Bertolini *et al*., 2005). The present study shows clearly that, although the peak viscosity decreased at low NaCAS concentrations, the final viscosity at 95°C increased with the addition of NaCAS. This is in agreement with Lelievre and Husbands (1989), who showed that the viscosity of NaCAS/starch pastes heated to 95°C increased with increasing NaCAS addition.

When NaCAS was added to waxy rice starch the peak viscosity always increased. As expected, the final viscosity of both normal and waxy rice starch containing NaCAS increased due to the increase in the total biopolymer concentration, however the shift in the pasting curves toward higher temperatures was far more pronounced in normal rice starch. For example, the T_{peak} of normal rice starch alone is 84.8 °C but 92.0 °C when 5% NaCAS is added. This effect has not been reported previously, but may be explained by the highly amphiphilic nature of the caseins (Dickinson, 1999b) behaving similar to soap, which is well known to raise the gel point of starches and, if added in large concentrations it can inhibit completely the swelling of the starch granules (Radley, 1953).

3.5 Summary to chapter

The addition of the different milk protein ingredients and components; SMP, MPC, NaCAS, WPI, UFSMP, and lactose affected the pasting behaviour of normal rice starch and waxy rice starch. SMP, UFSMP, and lactose increased the T_{onset} and the T_{peak} of both normal rice starch and waxy rice starch paste. This is due to the presence of lactose and ions, in the case of SMP and UFSMP, which are well known to increase the gelatinisation temperatures. The addition of NaCAS increased the T_{peak} in both starches. For normal rice starch paste, MPC and WPI reduced the T_{peak}. MPC did not affect the T_{peak} of waxy rice starch paste. Furthermore, the addition of milk protein ingredients had a greater effect on normal rice starch than on waxy rice starch.

Briefly, there are significant effects of milk proteins on pasting behaviour of normal and waxy rice starches. Although, some have been demonstrated previously in the literature but there is not a good understanding of the mechanisms involved. Hence, there is a need to explain the mechanisms of milk protein and starch interaction on pasting behaviour of normal and waxy rice starch. It is also of interest to characterize the dynamic rheological behaviour and microstructure of gels resulting from the pasting of rice starch in the presence of milk protein ingredients. In fact, in almost all industrial applications starch-milk proteins are used in the gel state. The dynamic rheological behavior of the starch-milk protein gels will be the object of the following chapter.

Chapter 4

THE EFFECTS OF MILK PROTEIN INGREDIENTS ON RICE STARCH GELS

4.1 Introduction

Starch gels are obtained upon cooling starch pastes. Results of the the previous chapter, showed that the pasting behaviours of normal and waxy rice starches were affected by the addition of different milk protein ingredients. Since the pasting behaviour of normal and waxy rice starch changes with milk protein addition, this might also affect the viscoelastic properties of their resulting gels upon cooling. These changes in the viscoelastic properties of starch gels can cause important changes in the physical properties, such as texture, of dairy-based food products.

The hypothesis of this chapter was that the four dairy ingredients would have an affect on the rheological behaviour of normal and waxy rice starch gels. Therefore the objective of this chapter was to investigate the effect of the addition of different milk protein ingredients on the viscoelastic properties of the two rice starch gels. The viscoelastic properties of the rice starch gels can be measured in a dynamic measurements mode; and by using both small and large deformation rheology. The small deformation rheological properties are described as the storage modulus (G') and the loss modulus (G'') as a function of the frequency. At a particular frequency, the G' and G'' of the gels will indicate the elastic and viscous properties of the fully structured material. The complex modulus (G^*), which is the overall resistance to deformation was also determined. G^* is given by;

$$|G^*| = \sqrt{(G')^2 + (G'')^2} \qquad \text{Equation 4.1}$$

The large deformation rheology properties were performed by the amplitude sweep test to observe the breaking-down of the gel network. They are described by the maximum

stress (σ_{max}), and maximum strain (γ_{max}), which were derived from the curves of G' and G'' as a function of the strain. In brief, small-deformation rheology gives an insight to the characteristics of the gel network, such as the fractal dimension of particulate-gels, whilst large deformation-rheology gives information about the ability (i.e., gel strength) of the gel to withstand large loads, as in the case of chewing for example. Confocal microscopy was also performed to investigate the microstructural state of the gels, in order to better understand the results of the rheological measurements.

4.2 Materials and Methods

4.2.1 Materials

Normal rice starch, waxy rice starch, milk protein ingredients (SMP, MPC, NaCAS, and WPI), UFSMP and lactose as described in section 3.2.1.

4.2.2 Methods

4.2.2.1 Rheological measurements

4.2.2.1.1 Small-deformation rheological measurements

The rheological measurements were performed on the sample at the end of the pasting regime described in section 3.2.3.2 of the previous chapter. The frequency sweep measurements were performed on the stress-controlled rheometer (Parr Physica UDS 200; Physica, Stuttgart, Germany) using the starch cell geometry TC 20. Before the frequency sweep test was performed, firstly, an amplitude sweep test with an applied strain from 0.01% to 10,000% was carried out at a frequency of 1 Hz to determine the linear viscoelastic region; a region that the measured rheological parameters are independent of the magnitude of the applied strain or stress. A strain of 1% was selected for the measurement to be carried out within the linear viscoelastic region. The frequency sweep was performed for a frequency range 10^{-2} Hz to 10 Hz, with an applied constant strain of 1%. In this measurement G', G'' and G^* as a function of the

frequency were obtained. All the measurements were performed in duplicate at a constant temperature of 25°C.

4.2.2.1.2 Large-deformation rheological measurements

A strain-sweep measurement was performed after the frequency sweep measurement. A frequency of 1 Hz was selected to measure the large deformation properties by varying the applied strain from 10^{-2}% to 10^{4}%. All the measurements were performed in duplicate at the constant temperature of 25°C. In this measurement G', G'' and G^* at 1 Hz were obtained. The γ_{max} and σ_{max} were derived from the curves of G' and G'' as a function of the strain. Where the γ_{max} can be defined as the strain at which $G' = G''$, and from the value of the γ_{max} the equivalent σ_{max} can also be determined.

4.2.2.2 Confocal Scanning Laser Microscopy (CSLM)

CSLM was performed on a Leica TCS 4D confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a 100x oil immersion objective lens. The microscope was used in a fluorescent mode, with excitation using an air-cooled Ar/Kr laser, and CSLM was performed at 488 nm. Fast Green CFC dye (Merck, Darmstadt, Germany) was used to stain the milk proteins. The dye was added to Milli-Q water or milk protein solution prior to mixing with the starch. After pasting in the rheometer at 95°C (end of step 3 in section 3.2.3.2), a portion of each sample was placed on a glass slide with a cavity and was covered by a cover slip. The sample was left at room temperature (~25°C) for 30 minutes before observation under the CSLM.

4.2.3 Statistical analysis

All statistical analyses were performed as described in 3.2.4. The rheological data: the G', the G'', the G^*, theσ_{max}, and the γ_{max} were analysed. All the measurements were performed in duplicate and the significance was accepted at the 5% confidence level.

4.3 Results

The frequency and amplitude sweep measurements for gels of normal rice starch and waxy rice starch alone are reported in Figure 4.1. The frequency sweep measurements for the small-deformation rheological measurements of normal or waxy rice starches/ milk ingredients gels are reported in Figure 4.2 and Figure 4.3, respectively. *G*,* as a function of the concentration of milk proteins ingredient for normal and waxy rice starch is shown in Figure 4.4. For the large-deformation rheological measurements, the strain-sweep measurements of normal or waxy rice starch/milk protein ingredients are shown in Figure 4.5, and 4.6, respectively. The σ_{max}, and the $\gamma_{max,}$ as a function of the concentration of milk proteins ingredients from normal and waxy rice starch are shown in Figure 4.7.

The frequency sweep measurement of normal starch gel alone (square symbols in Figure 4.1A) showed that G' increased slightly with the frequency (solid symbols). G'' (open symbol) decreased at low frequencies (0.04 Hz) and increased again slightly with an increase in frequency. $G'$was higher than $G''$for all the measured samples, which indicates its elastic nature. In addition, the difference in the value of these two moduli is less than one decade, indicating that the sample behaves as a weak-gel (Clark and Ross-Murphy, 1987; Lapasin and Pricl, 1995). For the waxy rice starch gel alone, the frequency behaviour of $G'$and $G''$was also that of a very weak-gel, with both $G'$and G''increasing with the frequency and G'higher than G''at all the measured frequencies, by less than a decade (circle symbols in Figure 4.1A). Note that the dependence in frequency of $G'$and G''in the case of waxy rice starch was more noticeable than in the case of normal rice starch, indicating that the gel made with waxy rice starch was weaker than that made with normal rice starch.

Figure 4.1B, the amplitude sweep measurement of normal and waxy rice starch gels alone showed that at low strains both the normal and waxy rice starches were elastic, with the G'higher than the G''. But at high strain values (>600) the rice starch gels behaved as a viscous material. At 1 Hz and 1% strain, the $G'$and the $G''$of the normal

rice starch gel were higher than waxy rice starch; the G' 52.95 Pa and 17.61 Pa, the G'' 10.85 Pa, and 7.67 Pa, for normal and waxy rice starch, respectively.

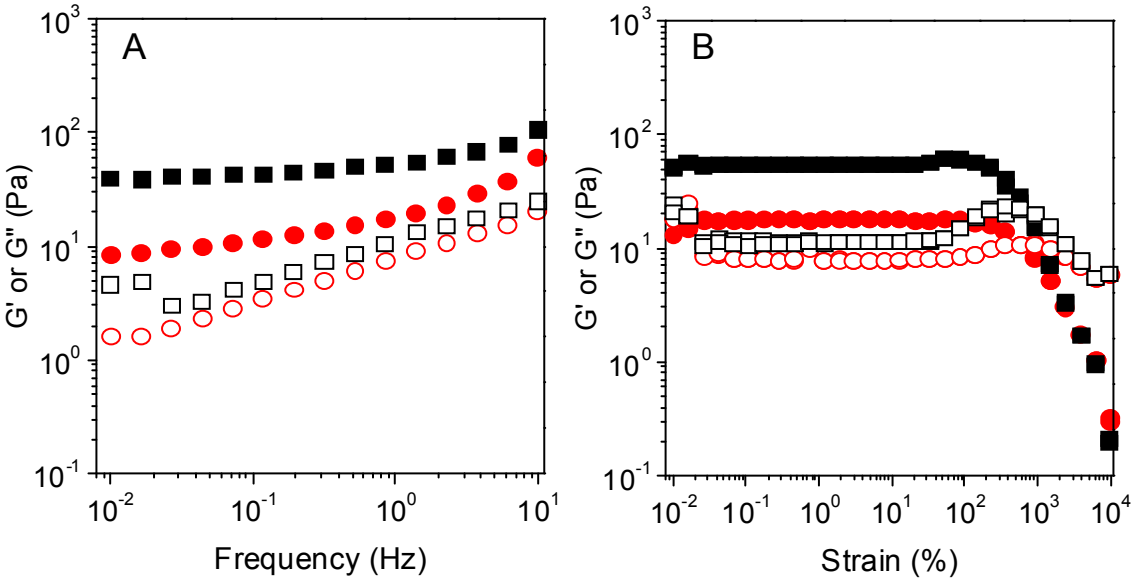

Figure 4.1 G' (closed symbol) and G'' (open symbol), as a function of frequency (A) and the strain (B) for normal rice starch (square) and waxy rice starch (circle). The data are presented as a mean; n = 2.

4.3.1 Small-deformation rheological measurements for rice starch/milk ingredients gels.

From Figure 4.2 it can be clearly seen that the addition of the different milk ingredients affected only the magnitude of the values of G' and G'' (vertical shift), without affecting the overall behaviour. In other words all milk ingredients-normal rice starch mixtures also behaved as weak-gels. However, the values of G' and G'', and consequently of G^* were affected differently by the different milk ingredients. The same observation was found in the case of waxy rice starch containing milk protein ingredients, where the frequency behaviour of G' and G'' was also that of a very weak-

gel, with both G' and G'' increasing with the frequency with G' higher than G'' at all the measured frequencies, by less than a decade (Figure 4.3).

To quantify the effect of the milk protein ingredients on normal and waxy rice starches, the value of G^* at 1 Hz and 1% strain was plotted as function of the concentration of the added milk protein ingredient (Figure 4.4). In addition to SMP, MPC, NaCAS and WPI the effects of UFSMP and lactose were also included. In the case of normal rice starch, the addition of SMP when added at the level of 2.5% decreased G^* compared with normal rice starch in the absence of SMP from 53.79 Pa to 49.19 Pa. At higher SMP concentration (10%) G^* increased to 111.55 Pa. The addition of MPC up to 5 % also decreased the G^*, to 40.33 Pa, and 43.53 Pa for the addition of 2.5 and 5% MPC respectively. However, G^* increased when higher MPC concentration were added. At 10% that G^* was increased to 86.48 Pa. The addition of NaCAS and WPI increased both G' and G'' at all the concentrations investigated here (Figure 4.4A), and the 10% WPI addition resulted in the highest increase in G^*. The addition of 2.5 % UFSMP decreased the value of G^*, however upon addition of more UFSMP G^* increased. The addition of lactose increased G^*, and this increase was nearly linear with respect to the increase in lactose concentration.

As with normal rice starch, the addition of SMP to waxy rice starch up to a concentration of 5% lowered the complex modulus. However at higher added SMP concentration (≥7.5%), G^* increased (Figure 4.4B). The addition of MPC increased G^* slightly for all concentrations, from 20.08 Pa when 2.5 % was added to 30.19 Pa when 10% MPC was added. The addition of NaCAS also increased G^* at all concentrations, from 20.98 Pa for 2.5 % to 35.68 Pa for 10% NaCAS addition. The addition of WPI slightly decreased G^* (from 19.20 Pa at 0% to 18.74 Pa at 2.5%) at very low added WPI concentration, and slightly increased G^* when the WPI was added up to a concentration of 7.5% (G^*=26.05 Pa). However, when 10% WPI was added, as was observed in the case of normal rice starch, a large increase in G^* (=116.95 Pa) was obtained. The addition of UFSMP and lactose did not affect G^* markedly.

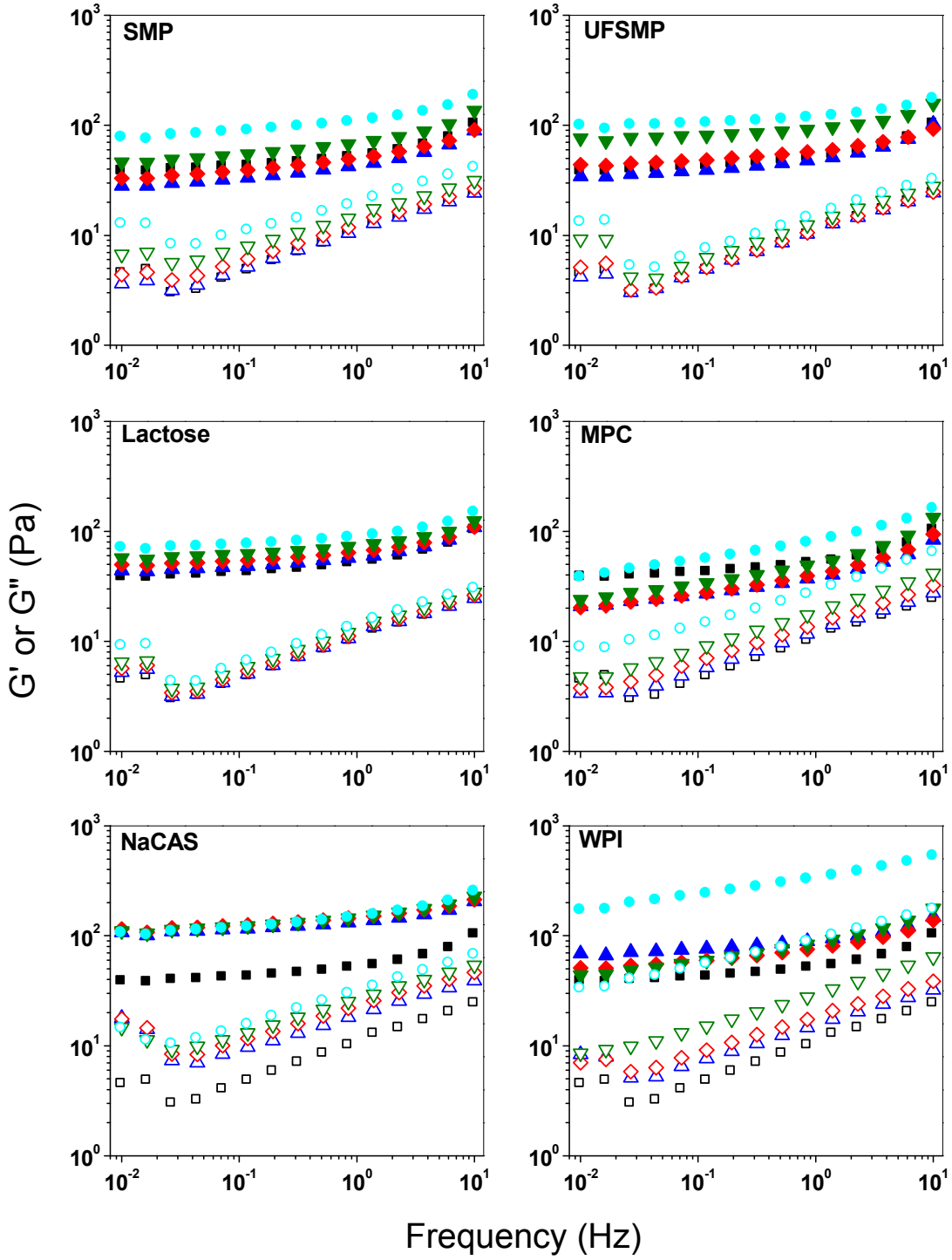

Figure 4.2 *G′*(closed symbol) and loss modulus; *G″*(open symbol), as a function of frequency for normal rice starch in the addition of milk protein ingredients at various concentrations: (□,■) 0%; (△,▲) 2.5%, (◇,◆) 5%, (▽,▼) 7.5%, and (○,●) 10%.

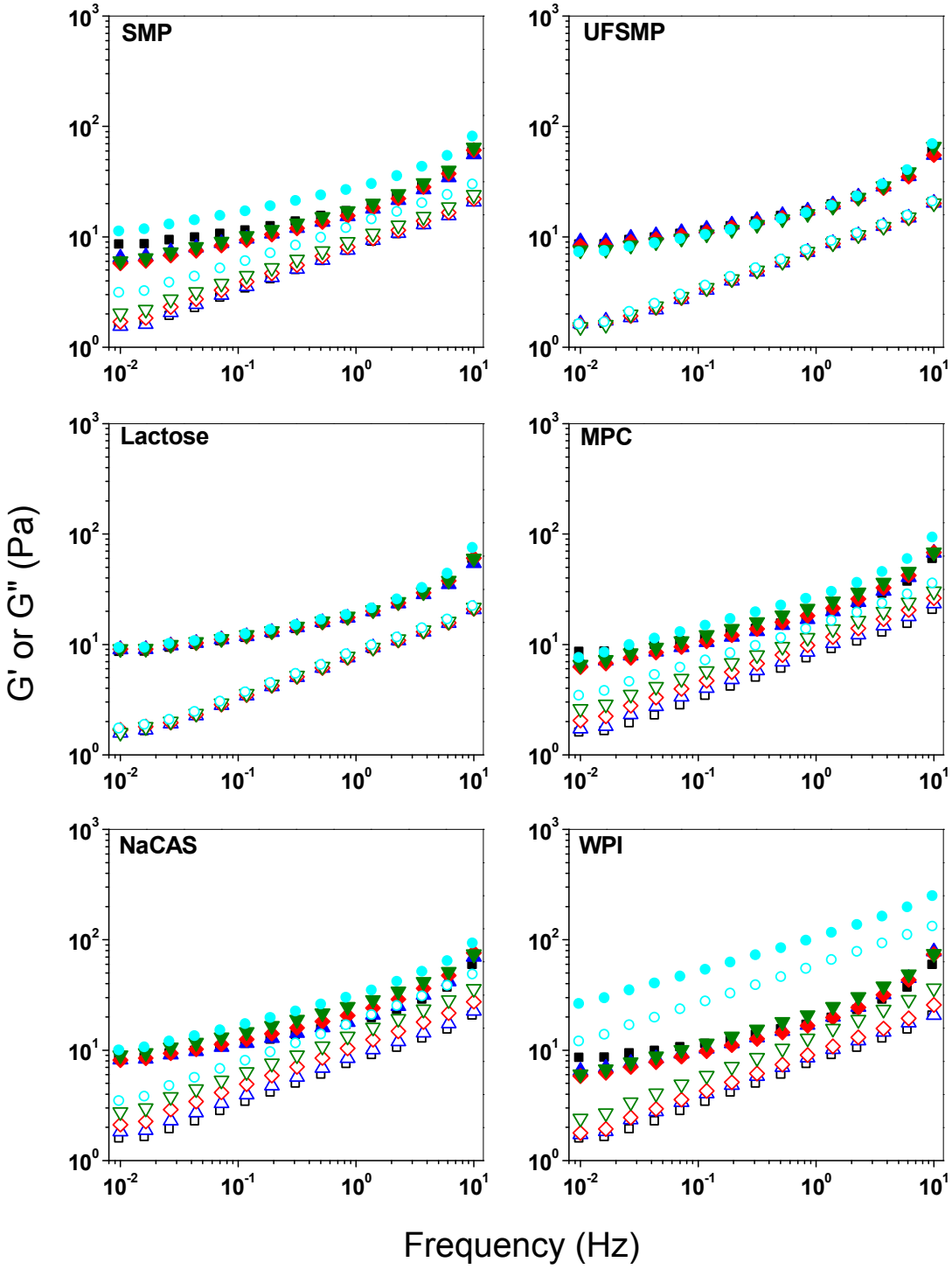

Figure 4.3 *G′*(closed symbol) and *G″*(open symbol), as a function of frequency for waxy rice starch in the addition of milk protein ingredients at various concentrations: (□,■) 0%; (△,▲) 2.5%, (◇,◆) 5%, (▽,▼) 7.5%, and (○,●) 10%.

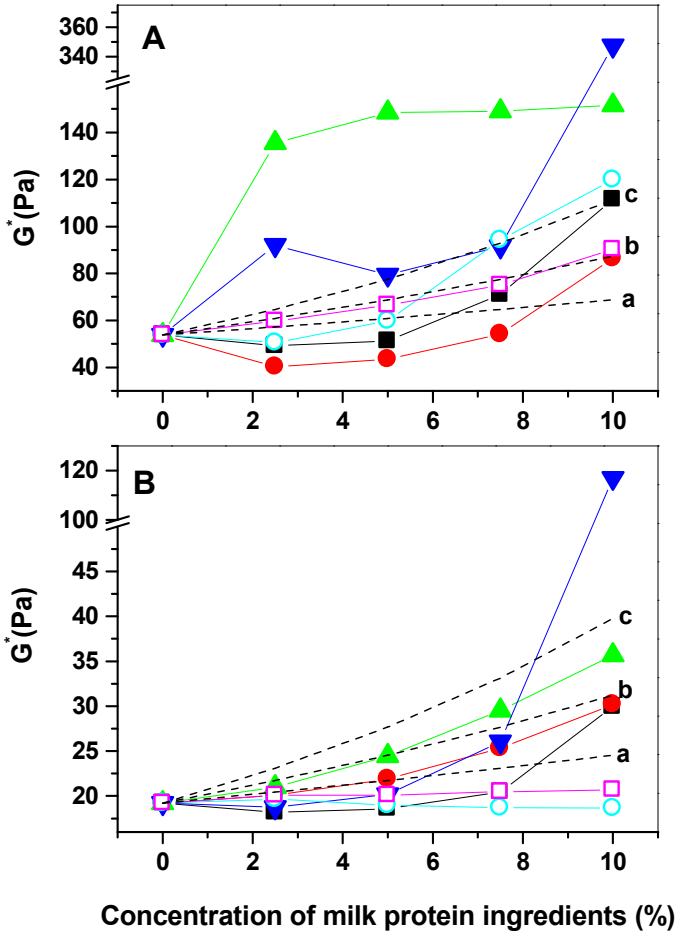

Figure 4.4 *G** at 1 Hz and 1% strain, as a function of the concentration of milk ingredients added for normal (A) and waxy (B) rice starches. Symbols are for SMP (■), MPC (●), NaCAS (▲), WPI (▼), UFSMP (○) and lactose (□). Dashed lines are the results of the Palierne's model, and the details are given in discussion section.

4.3.2 Large-deformation rheology for rice starch/milk ingredients gels.

The large deformation, or strain sweep measurements, also showed that the addition of the different milk ingredients to normal and waxy rice starches did not dramatically affect the overall behaviour of G' and G'' as a function of the strain (Figure 4.5, and 4.6). G' and G'' were independent of the strain at very low strain (<30%), with G' higher than G''. This region were the G' and G'' are independent of the strain is known as the linear viscoelastic region. This confirmed that at low strains the normal rice starch-milk protein ingredients were elastic ($G'>G''$) and that the frequency-sweep measurements in the previous section, performed at 1% strain, were within the linear viscoelastic region. At high strain values (>600%) G'' became higher than G' indicating that the samples behaved as a viscous material, i.e. they flowed under the high strains. To compare the differences between the different milk protein ingredients, the maximum strain, γ_{max}, was defined as the strain at which $G'= G''$, and from this value of the γ_{max} the equivalent maximum stress, σ_{max}, could be determined. The results of the γ_{max} and σ_{max} for normal or waxy rice starch-milk protein ingredients gels are reported in Figure 4.7.

The γ_{max} for 10% normal rice starch alone was found to be 763% (Figure 4.7A(1)). The addition of SMP did not increase this significantly; only at 10% addition when the γ_{max} reached 839%. When MPC was added, the γ_{max} deceased with increasing in MPC concentration, to 620% at 2.5% MPC to 500% at 10% MPC. The addition of NaCAS also decreased the γ_{max}, but unlike MPC, a sharp decrease occurred from 763% to 510% at 2.5 % NaCAS. At NaCAS concentrations above this the value of the γ_{max} was not affected noticeably. Low concentrations (2.5%) of WPI decreased the γ_{max} to 666%. However, higher concentrations of WPI (≥5%) all increased γ_{max}. The highest γ_{max} was obtained for a concentration of WPI of 7.5%. The addition of UFSMP up to a level of 5% had no affect on the γ_{max}. However, when the addition level was higher the γ_{max} decreased to reach a value of 541%. A slight decrease was observed when lactose was added, with the γ_{max} decreasing from 770% when 2.5% lactose was added to 702% when 10% lactose was added.

In the case of waxy rice starch, except at the highest concentrations (10%) of SMP and WPI, all the milk ingredients decreased γ_{max} by approximately 2.5 times (Figure 4.7A(2)). The addition of UFSMP also decreased γ_{max} but not to the extent of the milk proteins, with γ_{max} decreasing to 477% when 10% UFSMP was added. Lactose had no effect on the γ_{max} of waxy rice starch.

The σ_{max} changes for both normal rice starch-milk protein ingredients and that of waxy rice starch-milk protein ingredients were very similar to those observed for G^*, which was discussed in the previous section. In fact, an equivalent maximum elastic modulus could be calculated as the ratio of σ_{max} to γ_{max}. The result of this ratio as a function of G^* is reported in Figure 4.8, where a very good correlation ($R^2 = 0.9765$) could be observed for all the measured samples and for both normal and waxy rice starches.

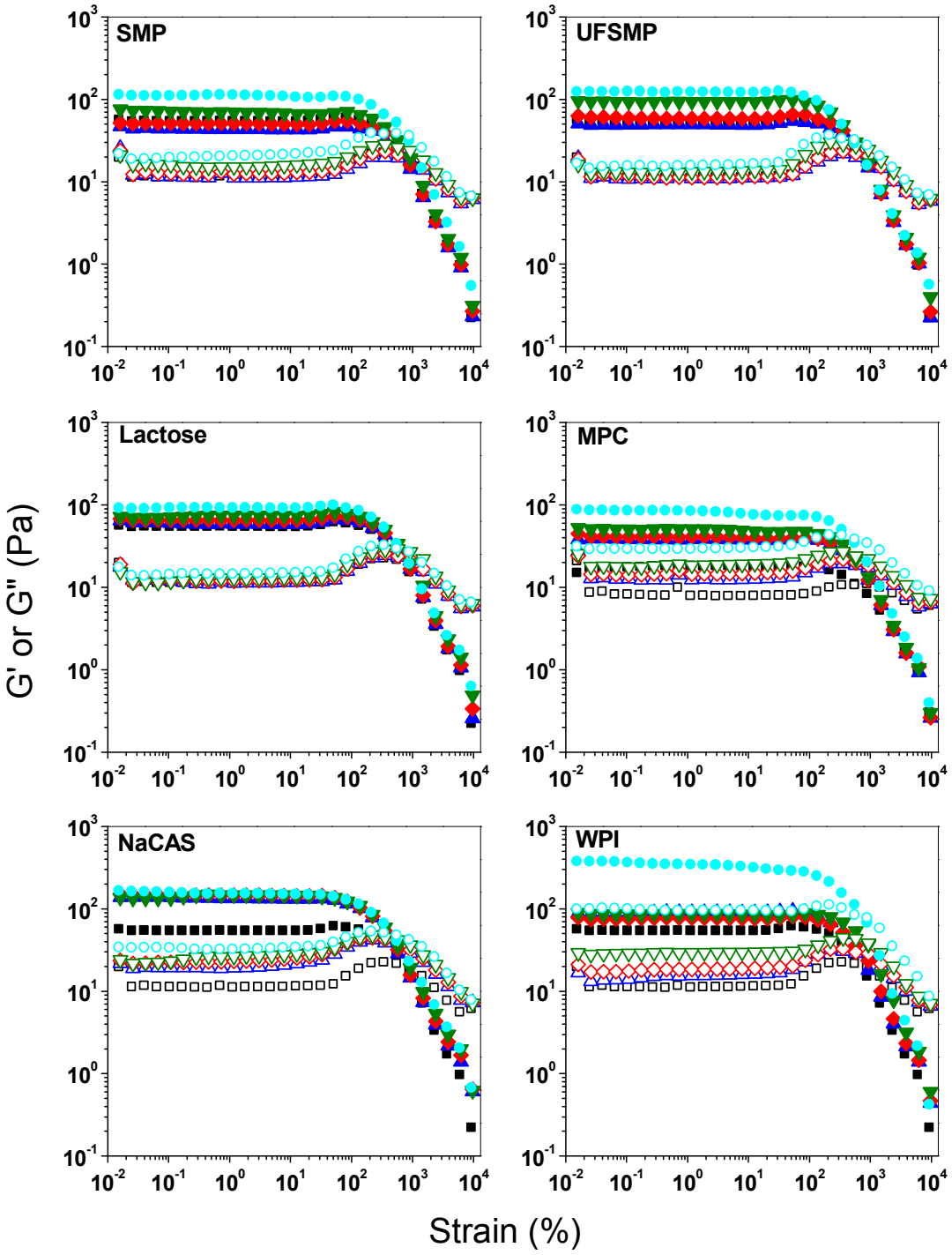

Figure 4.5 *G′*(closed symbol) and *G″*(open symbol) at 1 Hz, as a function of the strain for normal rice starch in the addition of milk protein ingredients at various concentrations: (□,■) 0%; (△,▲) 2.5%, (◇,◆) 5%, (▽,▼) 7.5%, and (○,●) 10%.

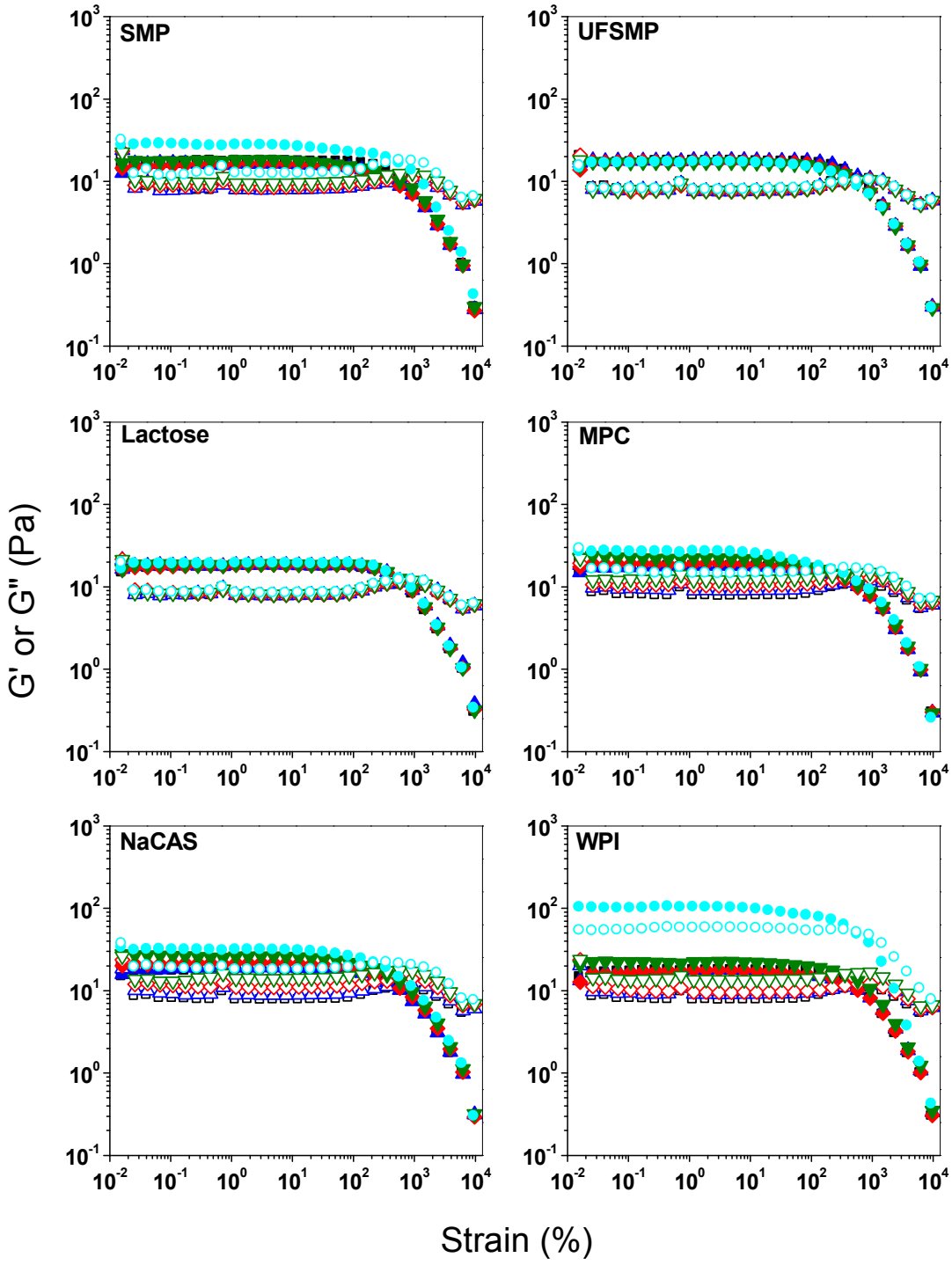

Figure 4.6 *G′*(closed symbol) and *G″*(open symbol) at 1 Hz, as a function of the strain for waxy rice starch in the addition of milk protein ingredients at various concentrations: (□,■) 0%; (△,▲) 2.5%, (◇,◆) 5%, (▽,▼) 7.5%, and (○,●) 10%.

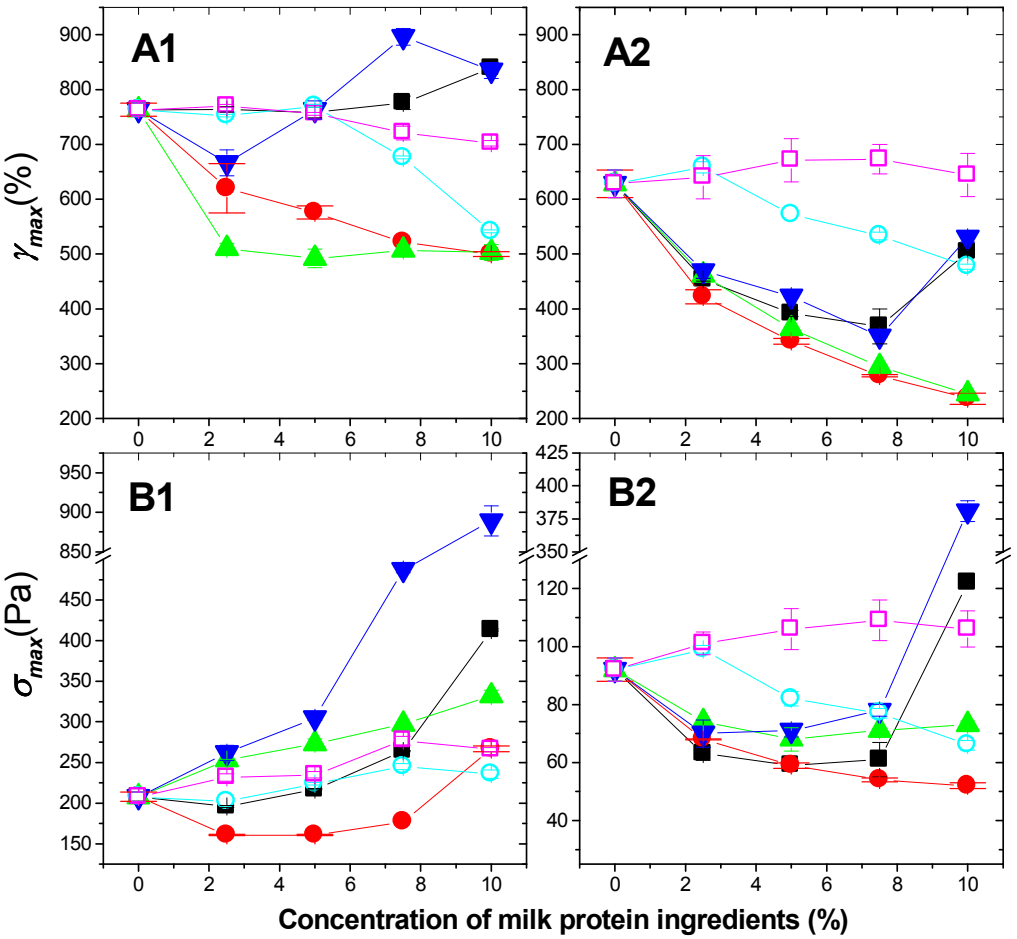

Figure 4.7 γ_{max} (A) and σ_{max} (B) at 1 Hz, as a function of the milk ingredients concentrations, for normal (1) and waxy (2) rice starches. Symbols are for SMP (■), MPC (●), NaCAS (▲), WPI (▼), UFSMP (○) and lactose (□). The error bars show the SD across measurements (n=2).

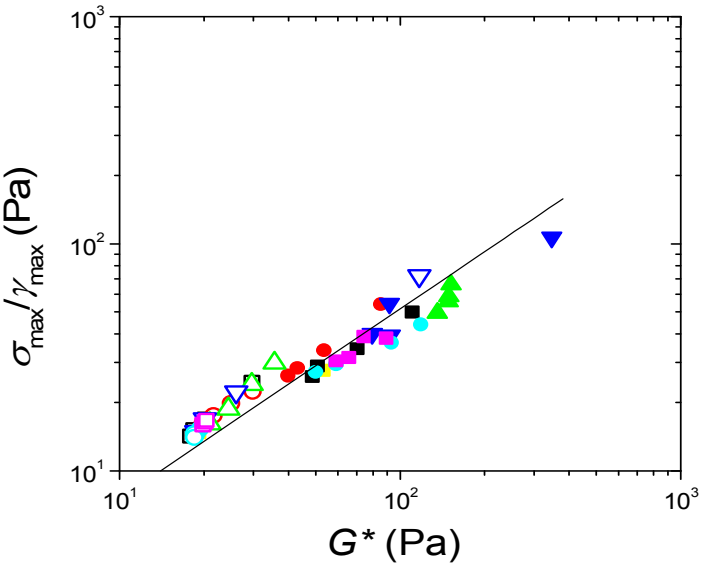

Figure 4.8 Ratio of the σ_{max} to γ_{max} as a function of the G^* for all the measured samples. Normal rice starch-milk ingredients samples (solid symbols) and waxy rice starch-milk ingredients samples (open symbols). The different milk protein ingredients are: SMP (■,□), UFSMP (●,○), Lactose (■,□), MPC (●,○), NACAS (▲, △) and WPI(▼,▽). Normal rice starch alone (■) and Waxy rice starch alone (□). The continuous line is a linear regression line with n = 50, R = 0.976, SD = 0.049 and P< 0.0001.

4.3.3 Confocal scanning laser microscopy (CSLM) observations

As the proteins were stained with fast green they appear white on the CSLM image. Figure 4.9 shows the CSLM image of 10% normal rice starch, either alone, or with the addition of 5% or 10% milk proteins. The CSLM image of the starch alone (Figure 4.9A(1)), was as expected, mostly dark due to the very low concentrations of protein in the starch ingredient (see Table 3.3). Some white spot regions (indicated by the arrow), indicating starch protein, could be found. All the milk protein ingredient CSLM images showed that the proteins in solution were homogeneously distributed (Figure 4.9A(2) to A(5)). When SMP was added to normal rice starch (Figure 4.9B(2) and 4.9C(2)), the SMP proteins were not homogeneously distributed, and voids, or dark regions, appeared in the CSLM images. The protein aggregates (white region) increased in size when the concentration of SMP increased.

A similar behaviour was observed for the normal starch/MPC gels (Figure 4.9B(3) and 4.9C(3)). The MPC proteins aggregated in the presence of the starch molecules and voids regions containing only starch molecules appeared. The MPC protein aggregates also increased in size with increasing in MPC concentration. When NaCAS (Figure 4.9B(4) and 4.9C(4)) or WPI (Figure 4.9B(5) and 4.9C(5)) were added the CSLM images remained similar to the CSLM images of the NaCAS (Figure 4.9A(4)) or WPI (Figure 4.9A(5)) alone, indicating that the milk proteins were distributed throughout the gel. However, although not fully conclusive, at high WPI concentration some milk protein aggregates could be seen (indicated by arrows on Figure 4.9C(5)).

The CSLM images of waxy rice starch/milk protein ingredient gels were very similar to those obtained for normal rice starch/milk protein ingredient gels (Figure 4.10). In fact, both the addition of SMP (Figure 4.10B(2) and 4.10C(2)) and MPC (Figure 4.10B(3) and 4.10C(3)) showed protein phase separation. However, compared to normal rice starch, the protein aggregates were much smaller. The addition of NaCAS (Figure 4.10B(5) and 4.10C(5)) and WPI (Figure 4.10B(5) and 4.10C(5)) did not show phase separation, although at high WPI concentration the CSLM images seemed to show some small spherical protein aggregates, indicated by the arrows in Figure 4.10C(5).

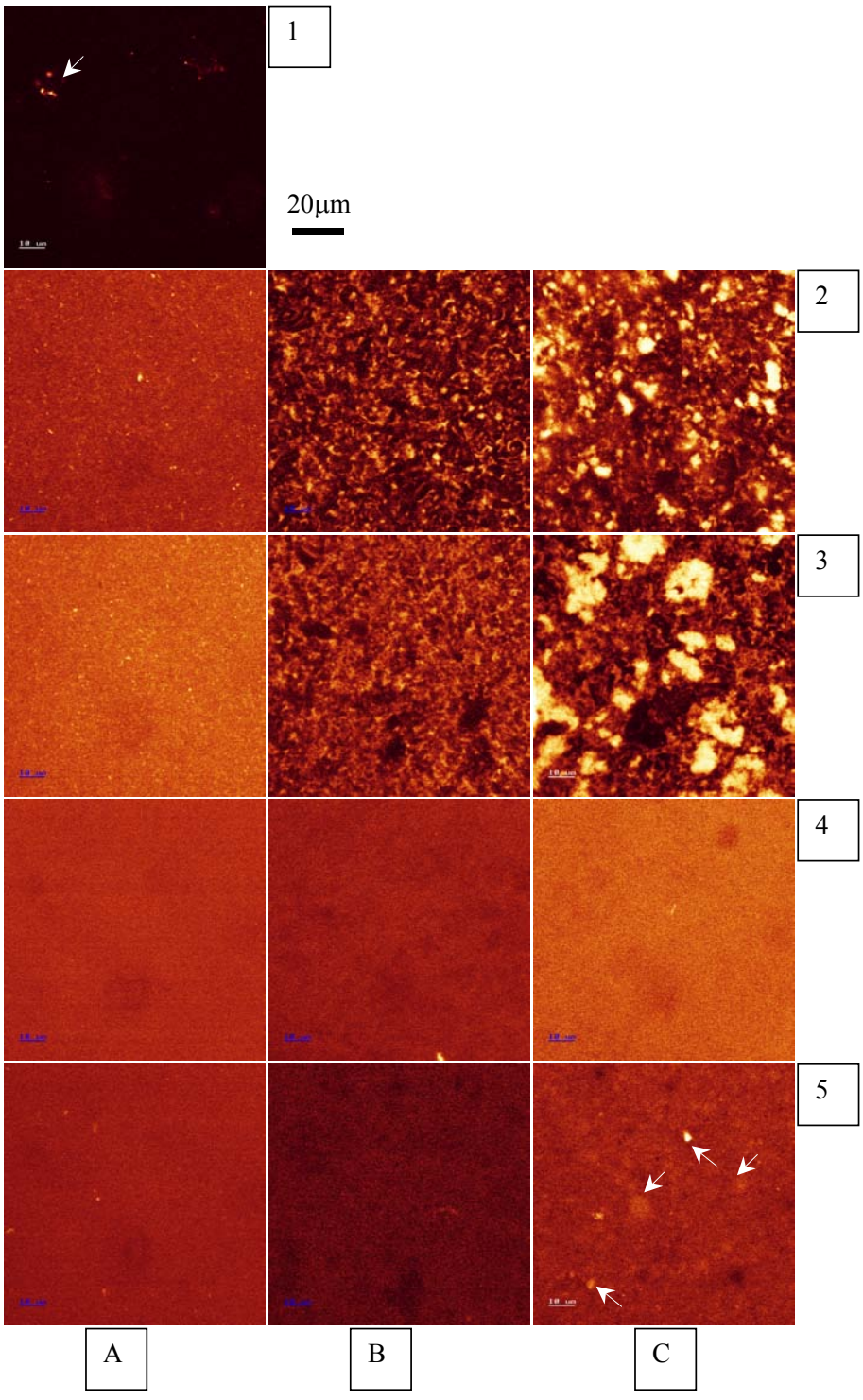

Figure 4.9 CLSM micrographs of milk protein ingredients/normal rice starch gels: (A) 10% normal rice starch or 10% milk proteins without starch; (B) 10% normal rice starch/5% milk protein ingredients; (C) 10% normal rice starch/10% milk protein ingredients: (1) without milk proteins ingredients; (2) SMP; (3) MPC; (4) NaCAS and (5) WPI.

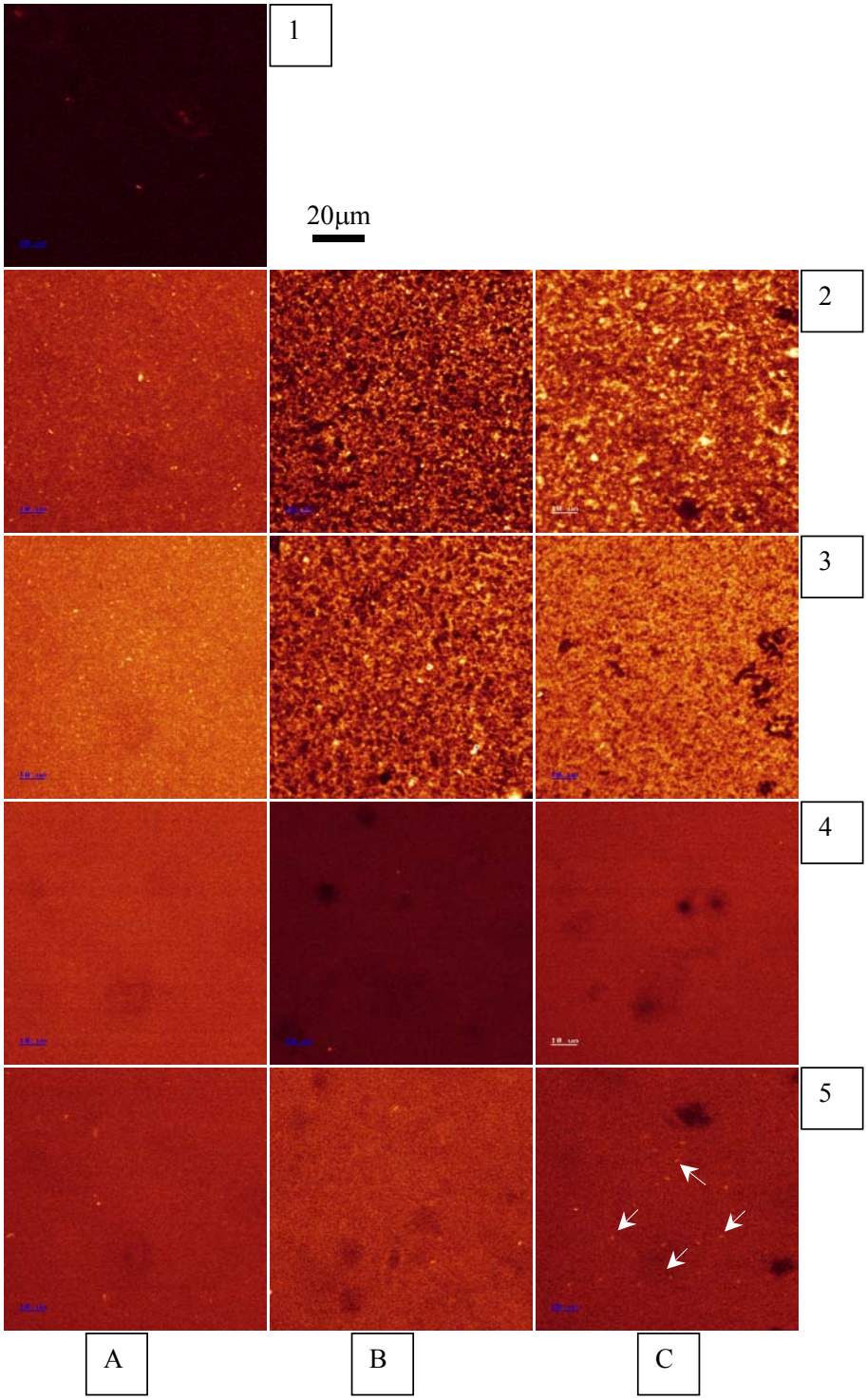

Figure 4.10 CLSM micrographs of milk protein ingredients/waxy rice starch gels: (A) 10% waxy rice starch or 10% milk proteins without starch; (B) 10% waxy rice starch/5% milk protein ingredients; (C) 10% waxy rice starch/10% milk protein ingredients: (1) without milk proteins ingredients; (2) SMP; (3) MPC; (4) NaCAS (5) WPI.

4.4 Discussion.

The CSLM gel observations clearly showed that there was phase separation between SMP and both normal (Figure 4.9B(2), and 4.9C(2)) and waxy rice starch (Figure 4.10B(2), and 4.10C(2)), and MPC and normal (Figure 4.9B(3), and 4.9C(3)) and waxy rice starch (Figure 4.10B(3), and 4.10C(3)). It is well known that casein micelles, the major protein component in SMP and MPC, and amylopectin phase separate through a depletion flocculation mechanism (de Bont *et al*., 2002). It is highly-likely that, during the pasting of rice starch in the presence of SMP and MPC, this phase separation was not possible at high temperatures, firstly because at these high temperatures the viscosity of the continuous phase (made of amylose and amylopectin) is very low, and secondly because the applied shear would ensure that the paste was a homogeneous mixture. However, once the shear was removed and the paste was allowed to cool phase separation would be possible and a continuous starch phase with discontinuous aggregated casein micelles phase would result. This phase-separation mechanism is schematically depicted in Figure 4.11 below.

The phase separation in the case of SMP and MPC resulted in a continuous starch molecule phase and an SMP- or MPC-rich discontinuous phase (Figure 4.9B(2), 4.9C(2), 4.9B(3) and 4.9C(3)). The two other ingredients, NaCAS and WPI did not show any extensive phase separation. Except in the case of high WPI concentrations, where a few proteins aggregates, dispersed in the starch continuous phase could be seen (arrows in Figure 4.9C(5) and Figure 4.10C(5))

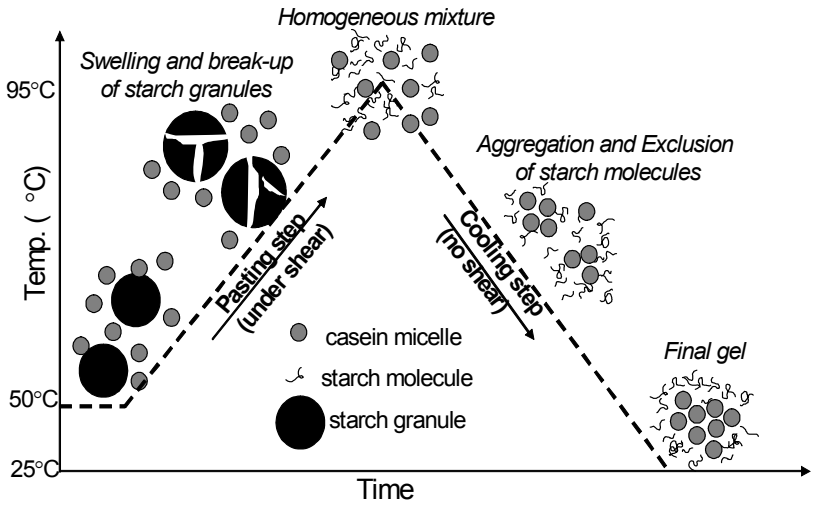

Figure 4.11 Schematic representation of the phase separation occurring during the making of the starch/SMP or starch/MPC gels. In the pasting step, the starch granules undergo swelling and break-up. At the end of this step a homogeneous paste made of starch molecules and casein micelles. This paste of low viscosity, due to heating, is homogeneous due to shear. During the cooling step, the shear is removed, but the starch molecules are still in an individual form, inducing phase separation through a depletion-flocculation mechanism. When the paste is cooled down to room temperature, the aggregated casein micelles are entrapped inside the continuous starch phase of the gel. Dashed line represents the temperature profile.

The rheological measurements, both at small and large deformation (as for the pasting behaviour reported in section 3.3.2, and 3.3.3), showed that the various milk ingredients had different effects, and that these effects are also different for different starches. This is partly due to the major known differences in the behaviour of normal and waxy starches gels, and the differences between the milk protein ingredients. Normal rice starch gels are made of swollen gelatinised starch granules embedded in a continuous phase of amylose-gel matrix. The amylose molecules leach out of the starch granules during gelatinisation and form aggregates of a three-dimensional polymer network when the starch paste is cooling (Miles *et al*., 1985; Ring, 1985; Morris, 1990). Waxy starches, which have a very small amount of amylose, do not gellify, but form viscoelastic liquids (Eliasson and Gudmundsson, 2006), this could be seen through the relatively large frequency dependence of G' and G'', compared to

normal rice starch. It is expected that the effects of the milk ingredients will be more evident in starch with high amylose content (Marzin *et al*., 1995).

Despite these differences, both the small and large deformation rheological experiments clearly showed the rheological behaviour of normal rice starch-milk ingredients mixtures (Figure 4.2 and Figure 4.5) and waxy rice starch-milk ingredients mixtures (Figure 4.3, and Figure 4.6) were qualitatively similar to that of the starch solutions alone. This is an indication that the rheological behaviour of the mixtures was dominated by that of the continuous phase, which in this case was made of starch molecules, as shown by CSLM even in the phase separated samples. Thus, it is tempting to consider normal and waxy rice starch gels containing milk protein ingredients as composite materials, where the continuous phase is made of the starch and the milk ingredients act as fillers.

A theory which has been proven effective in modelling such systems, particularly mixtures of synthetic polymer melts, is the Palierne equation (Palierne, 1990). In the case of solid inclusions, where the surface tension is neglected, this equation reduces to:

$$G^* = G^*_{mat}\left(\frac{1+\frac{3}{2}\phi_m}{1-\phi_m}\right) \qquad \text{Equation 4.2}$$

Where G^*_{mat} is the complex modulus of the matrix at 1 Hz and 1% strain at 25°C, extracted from Figure 4.4 (i.e. G^*_{mat} = 53.79 Pa for normal rice starch and G^*_{mat} = 19.20 Pa for waxy rice starch) and ϕ_m is the volume fraction of the milk protein ingredients.

By comparing the results of the rheological measurements to the theoretical predictions it is possible to infer whether the milk proteins behaved as simple fillers. However, since we do not know the exact volume fraction occupied by these ingredients, equation 4.2 is used to calculate G^* for normal or waxy rice starch

matrices containing milk protein ingredients for fillers having a volume 1, 2 or 3 times their weight. The results of these calculations are reported in Figure 4.4A and 4.4B as line a (1 time), b (2 times) or c (3 times).

Due to the differences in the rheological behaviour of normal and waxy rice starch, the effects of the protein ingredients are discussed separately for these two starches.
For normal rice starch, up to a concentration of 5%, the addition of SMP and MPC had a weak and decreasing effect on G^* (Figure 4.4A). The measured value of G^* was also lower than the value of G^* predicted by the theory. For instance at 5% SMP, G^* decreased by 16% compared to the starch alone. This indicates that at these concentrations, for both MPC and SMP, the ingredients acted at best as inert fillers. This slight drop in G^* could have been due to the small decrease in starch concentration used during the sample preparation to compensate for the water loss due to the addition of the milk protein (section 3.2.2). When the concentration of SMP and MPC was higher than 5%, G^* was higher than that of the normal rice starch alone. This could have been due to phase separation between these two milk ingredients and the starch molecules as seen in the confocal microscopy images (Figures 4.9C(2) and 4.9C(3)). The effect of phase separation on the increase of G^* could be viewed as follows. The casein micelles in MPC and SMP form aggregates, from the inside of which the starch molecules are excluded, and water is held. This will have a double effect, firstly it will increase the local concentration of the starch molecules in the continuous phase, and secondly the volume occupied by the protein aggregates will be much higher than if the casein micelles were homogenously distributed in the continuous phase. These effects are depicted in Figure 4.11. Theoretically the increase in the local concentration of the starch molecules will result in an increase in the value of G^*_{mat} and the increase in the volume fraction occupied by the aggregated casein micelles will result in an increase in ϕ. Thus the measured G^* should be also higher than that predicted by Palierne's model, which assumes homogenously distributed spherical particles in the matrix.

In the case of SMP, in addition to the effect of phase separation, further contributions due to salt and lactose present in this ingredient are expected. MPC does not contain

large amounts of lactose, and the only salts present in MPC are mainly in the form of colloidal calcium-phosphate inside the casein micelles. In fact these results show that lactose, at the concentrations investigated here, increased the G^* of normal starch nearly linearly, and that UFSMP also increased the G^* of normal starch markedly, particularly at 10% UFSMP where the G^* increased more than two-fold. The effect of salts and lactose could be the key reason for the differences in the maximum strain γ_{max} observed. In fact as for MPC, γ_{max} was expected to decrease, since the casein micelles in these milk ingredients would act as defects contributing to the weakening of the starch matrix, and thus breaking at much lower strains.

The addition of NaCAS and WPI to normal starch resulted in the highest increase in G^* and in a behaviour very different to that due to the addition of SMP and MPC. These ingredients showed a plateau value for G^*, between 2.5% and 10% for NaCAS, and between 2.5% and 7.5% for WPI. As confocal microscopy did not show any phase separation in the mixtures of normal rice/NaCAS or normal rice/WPI, this peculiar effect is not yet fully-understood and further investigation is needed. However, it is noteworthy to report here that Kelly *et al*. (1995) has previously reported that NaCAS had no effect on the viscosity of 4% cornstarch in pH 7 phosphate buffer. They explained this in terms of the decrease in swelling volume of the starch granules, which would compensate for the increase in the viscosity of the continuous phase with the increase in NaCAS concentration. The pasting behaviour reported in this work (Chapter 3) also showed a delay in the increase in viscosity of normal starch with the addition of NaCAS (see Figure 3.5, section 3.3.2).

Note that WPI was also reported to have an effect on the swelling of cassava starch granules (Aguilera and Baffico, 1997). Shim and Mulvaney (2001) studied the viscoelastic and the fracture properties of mixed gel of cornstarch and WPI at pH 5, pH 7 and pH 9. They reported that at lower solid contents (15% total solid) and pH 7, WPI acted as an inactive diluent of the cornstarch fraction.

Another possible explanation in the case of the experimental conditions used in this work, is the effect of these proteins during pasting. Both WPI and NaCAS are made of

small molecules, with an average molecular weight value of 23.6 to 25.2 kDa for caseins in NaCAS (Walstra *et al*., 2006) and of 18 kDa for β-lg the major component of WPI (de la Fuente, Singh and Hemar, 2002), compared to rice amylose (M_W = 10-100 kDa) and amylopectin (M_W 1,000 – 100,000 kDa) (Hizukuri, 1996; Tester *et al*., 2004). During pasting, both these molecules are homogenously distributed in the matrix made of the starch molecules, and remain homogeneously distributed once the gel has been cooled down. Because the amount of starch to water used was constant (see section 3.2.2), at each level of protein addition, no change in the G^* of the matrix was observed. However, the proteins appeared to have no affect on the final gel. The difference in the values of the plateau could be due to the difference in the water-holding ability and size of NaCAS and WPI. For instance the hydrodynamic radius of NaCAS is known to have a bimodal distribution with ~9 nm for the individual casein and ~75 nm for the aggregated casein due to hydrophobic interactions (Chu, Zhou, Wu and Farrell, 1995; Nash, Pinder, Hemar and Singh, 2002), and the hydrodynamic radius of WPI molecules is 2.04, and 3.19 nm for the monomer and dimer, respectively (Aymard *et al*., 1996). The high increase in G^* observed at 10% WPI addition, is due to the gelation of the WPI at high concentrations.

Similarly upon addition of SMP and MPC, γ_{max} of the normal rice/NaCAS gels decreased with increasing in concentration of NaCAS, as the NaCAS has also a weakening effect on the starch matrix. This was expected since all these ingredients, except gelled WPI, as solutions would flow under very small γ_{max}. Conversely the effect of WPI was not constant, with γ_{max} decreasing at 2.5% WPI, increasing at 7.5% and decreasing again at 10% WPI (see Figure 4.7A1). This is believed to be due to the state of aggregation of the WPI proteins which occurred on heating during the pasting step, with the size and shape of the WPI aggregates depending on the WPI concentration. Note that Shim and Mulvaney (2001) also reported that at pH 7 the fracture strain, which is equivalent to γ_{max}, was constant at low concentration of WPI (less than 50% of total solids) but increased when WPI concentration was increased.

As mentioned above, waxy starch did not form gels, but rather viscoelastic solutions. Furthermore, it was also expected that the milk proteins would have less of an effect

than in the case of normal rice starch. This could be seen in Figure 4.4B were apart from for 10% WPI, the G^* of the mixture increased very consistently with increasing milk protein concentration. A small decrease in G^* at 2.5% and 5% SMP was observed, which is believed to be due to the salts present in SMP, as discussed in the case of normal rice starch. In fact the measurements showed that the addition of UFSMP to waxy rice starch also decreased G^* slightly (a decrease of 2.7%). Only at concentrations higher than 5% for both SMP and MPC, did we see G^* increase markedly for the waxy starch solutions. Here again, as for normal rice starch, this observation was due to the phase separation between these milk ingredients and waxy rice starch molecules (Figure 4.10B(2), 4.10C(2), 4.10B(3), and 4.10C(3)).

WPI, at concentrations higher than 5%, affected the G^* of the waxy rice starch gels, due to its heat-induced-aggregation during pasting. Finally, NaCAS was the milk ingredient which imparted the highest G^* to the waxy rice starch. NaCAS's are known to achieve high viscosities and high elasticities at high concentrations, as has been shown with entangled polymer systems (Farrer and Lips, 1999). Thus it is expected that the behaviour of a NaCAS/waxy rice starch mixture would be similar to a solution of homogeneous entangled molecules of NaCAS and starch, and that would result in a constant increase in G^* with the increasing in NaCAS concentration.

All milk protein ingredients, except 10% SMP and 10%WPI, decreased the value of γ_{max} of waxy rice starch gels. Again this was expected, as these milk ingredients alone form solutions and thus have very small γ_{max}. Thus their addition to the waxy rice starch matrix will result in a decrease to γ_{max}. The increase in γ_{max} at 10% WPI was due to the gelation of WPI during the pasting step. For the waxy rice starch/10% SMP gel, it is likely that the increase in γ_{max} was a combination of both the phase separation of SMP, and possibly of the lactose present in SMP, since the results showed that lactose slightly increase γ_{max} of waxy rice starch.

4.5 Summary to chapter

The differences in the physico-chemical properties of the milk protein ingredients and normal and waxy rice starch resulted in different rheological behaviour of the resulting pasted gel structures. However, despite these differences, it was found that qualitatively the rheological behaviour was mainly dominated by the rice starch. Further more, the effect of the milk protein ingredients could be divided into three categories; one which resulted from the addition of SMP and MPC where the proteins were mainly organized in a casein micelle form; and a second due to the addition of NaCAS where the individual caseins are alone or form casein aggregates; and finally WPI a which forms gels at high concentration under heat-treatment.

At low concentrations (≤5%), in the case of both normal and waxy rice starch, when compared to the theory these milk protein ingredients could be considered at best as inert fillers. In the case of normal rice starch at high concentrations (>5%), SMP and MPC increased the G^* as a consequence of the phase separation which was observed by CSLM. WPI also increased G^* at high concentrations due to its heat-induced aggregation. NaCAS, had the most peculiar behaviour as it increased G^* to a plateau value independent of the NaCAS concentration. This is could be due to the water holding ability of NaCAS molecules, and to the effect of small molecular size of NaCAS molecules, which are homogeneously distributed in a gel matrix made of the normal starch molecules.

In the case of waxy rice starch, the effect of the milk ingredients, as expected, was less dramatic than that exhibited in the case of normal rice starch. All the ingredients increased G^* of the mixtures linearly with increasing protein concentration, with the exception of 10% WPI which markedly increased G^* due to the heat-induced aggregation of WPI. NaCAS was the milk ingredient which showed the most increase, in G^*, most likely due to its water-holding and viscosity enhancing abilities.

Regarding the γ_{max}, for both normal and waxy rice starch, most ingredients decreased the value of the γ_{max}. This was mainly because the γ_{max} of the ingredients alone in

solution is very low. The exceptions were WPI which gelled at high concentration, and MPC and SMP which phase separated on cooling, particularly SMP at high concentration where the effect of lactose and salt were also thought to be playing a role.

It was clearly that the rheological behaviour of rice starch/milk ingredients gels was dominated by the starch, and that the observed effects of the different proteins could be largely explained. However, the need for further understanding lies with the pasting behaviour. For this reason the remainder of the thesis focuses on explaining the mechanisms for the observed effects of the milk proteins on pasting. Gelatinisation is a precursor for the pasting of starch, therefore in the next chapter, a study of the effects of milk protein ingredients on the gelatinisation of starch will be investigate.

Chapter 5

THERMAL BEHAVIOUR OF STARCH/MILK PROTEIN INGREDIENTS MIXTURES

5.1 Introduction

Starch is a semicrystalline solid that consists of amorphous and crystalline regions (Lund, 1984; Jenkins and Donald, 1998). The gelatinization is an irreversible order-disorder of molecules within the starch granule, which is mainly a swelling driven process and occurs during the heating of starch in excess water - water is initially taken up by the amorphous region due to its lower order of crystallinity and is followed by the crystalline regions once the crystalline regions have been disrupted (Donovan, 1979; Lund, 1984; Biliaderis *et al*., 1986; Donald, 2004). As a consequence of the melting of the crystalline regions, a large extent of starch granules swelling occurs result in a change of rheological behaviours of the system; which is importance to texture and sensory properties of starch-based food products. Therefore, gelatinisation is a critical precursor for physical phenomenon of starch.

Gelatinisation is recognized as an endothermic process and can be examined by differential scanning calorimetry (DSC) (Whistler and BeMiller, 1997; Eliasson and Gudmundsson, 2006). DSC determines the temperature and the heat flow changes associated with second-order (glass transition) and first-order (melting) transitions of polymeric materials that respond for the order-disorder phenomena of starch granules (Donovan, 1979; Biliaderis *et al*., 1980; Evans and Haisman, 1982; Biliaderis *et al*., 1986). It provides the thermal behaviour critical parameters associated with the gelatinisation of starch; the onset gelatinization temperature (T_{onset}), the peak gelatinization temperature (T_{peak}), and the enthalpy of gelatinization (ΔH).

Starch gelatinization is influenced by numerous factors such as moisture content, non-ionic solutes (sugars), proteins, and ionic solutes to name a few (Evans and Haisman, 1982; Chungcharoen and Lund, 1987; Lund, 1989; Slade and Levine, 1989; Biliaderis, 1990; Kim and Walker, 1992b; Slade and Levine, 1993; Erdogdu *et al*., 1995; Hoover and Senanayake, 1996; Ahmad and Williams, 1999b; Ahmad and Williams, 1999a; Perry and Donald, 2002). When starch was heated in excess water system, extensive hydration and swelling of the amorphous regions facilitate melting of the starch crystallites over a narrow temperature range; hence the DSC thermogram of starch displayed a single endotherm. In system of limited water, the DSC thermograms displayed two endothermic transitions. The first endotherm occured at the same temperature as the endotherm in excess water. The second endotherm was observed at higher temperature due to more energy was required to disrupt the remain unmelted starch granule crystallites in a condition of limited available water (Donovan, 1979; Biliaderis *et al*., 1980; Liu and Lelievre, 1992).

Sugars were reported for their ability to increase the gelatinization of starch and there are several mechanisms have been proposed to explain this phenomenon such as the competition of available water with starch, the antiplasticising effect of sugars compared to water and specific starch-sugar interactions (Hansen, Setser and Paukstelis, 1989; Slade and Levine, 1989; Hoover and Senanayake, 1996; Ahmad and Williams, 1999b; Perry and Donald, 2000; Perry and Donald, 2002; Donald, 2004).

Compare to sugars, the effects of salts on starch gelatinization are more complicated depending on nature and concentration of salts. It was found that salts can cause either increase or decrease the gelatinization temperature and enthalpy of starch because of their ability to attributing to water structure and electrostatic interaction between hydroxyl groups of starch and ions (Jane, 1993; Chiotelli, Pilosio and Le Meste, 2002).

Proteins, which are amphiphilic molecules, have been reported for their complex effects on gelatinization of starch (Liang and King, 2003; Eliasson and Gudmundsson, 2006). Some functional properties of proteins such as the water binding capacity, the surface activity and the ability to form gels are known to affect starch-protein

interactions and the nature of the interactions is quite complex and depends on which of the properties has the major influence over the interaction (Eliasson and Gudmundsson, 2006).

Starches are added to food systems because they are known to affect the structure and texture of starch based foods. Their major effect occurs when the starches have been gelatinized during the manufacture of the foods. Therefore, with this portion of the study, attempts were made to understand how the gelatinisation of normal and waxy rice starches was affected by milk proteins from four different milk ingredients products; SMP, MPC, NaCAS and WPI. To achieve this objective, the gelatinisation of normal and waxy rice stach in the presence of the above milk protein ingredients were monitored by the DSC via three parameters; T_{onset}, T_{peak}, and ΔH. To elucidate the effect of lactose and salts in SMP, the effect of UFSMP was also investigated. In addition to these parameters the loss of birefringence of the two starches in the presence of the four milk protein ingredients was also carried out using polarized microscopy.

5.2 Materials and Methods

5.2.1 Materials

Normal rice starch, waxy rice starch, milk protein ingredients (SMP, MPC, NaCAS and WPI) and UFSMP as described in section 3.2.1.

5.2.2 Methods

5.2.2.1 Thermal behaviour measurements

Thermal behaviour measurements were performed on a Perkin-Elmer DSC-6 (Perkin-Elmer, Corp., Norwalk, CT Germany) using large volume stainless steel pans (catalogue number 03190029, capacity 60 μl). Approximately 60 mg of a thoroughly mixed rice starch/milk protein ingredient mixture was weighed into a pre-weighed stainless steel pan and weighed to 4 decimal places. The pans were hermetically sealed

and the measurements were performed as follows. First, the sample was heated to 50°C and allowed to equilibrate for 5 min, and then a temperature ramp from 50 to 95°C was applied, at a heating rate of 2°C/min. The DSC was calibrated with indium (T_{onset} = 156.6°C, ΔH= 28.45 J/g) and n-Octadecane (T_{onset} = 28.24°C, ΔH= 241.42 J/g), and an empty pan was used as a reference. All the measurements were performed at least in duplicate.

The T_{onset}, T_{peak}, and ΔH were calculated from the DSC thermograms. The determination of these values was based on the method proposed by Lund (1984) and Jerkins and Donald (1998). From the recorded thermogram schematically shown in Figure 5.1, a baseline was drawn across the peak. Then a straight line from each side of the peak was drawn down the leading edge to intercept the baseline on each respective side. The T_{onset} was defined as the intersection (point T_{onset} on Figure 5.1) and this represents the onset temperature of transition. The T_{peak} was defined as the maximum endothermic heat flow relative to the baseline and was determined by drawing straight lines from the baseline on either side of the peak that were parallel to the straight line portion of the thermogram and their intersection point defined T_{peak} as shown on the figure below. The apparent transition enthalpy of gelatinization (ΔH) was calculated from the shaded area in Figure 5.1 (i.e. area divided by scanning rate divided by sample dry mass).

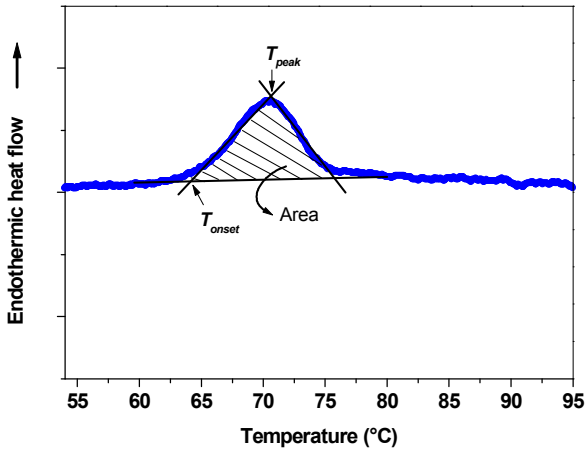

Figure 5.1 Measurement of T_{onset}, T_{peak} and area from a DSC thermogram (adapted from Lund 1984).

5.2.2.2 Polarized light microscopy observations

Rice starch samples at 10% (w/w) in the presence of various concentration of milk protein ingredients (0, 2.5, 5, 7.5, or 10%, w/w) were prepared as described in section 3.2.2. After gently mixing the rice starch/milk ingredients for 5 min using a magnetic stirrer, the samples were transferred to the rheometer as described in section 3.2.3.2 to affect the same heating rates previously used in pasting experiments. When the mixtures reached a specific temperature a sample (100 µl) was removed from the rheometer. The small amount from this 100 µl sample was placed on a glass slide and covered with a cover-slip, the sides of which were sealed with glue. The starch granules were observed under 50x magnification using a Nikon polarized light microscope (Nikon Eclipse E600POL, Nikon Corporation, Tokyo, Japan). Images were attained using a Nikon Digital camera (DXM 1200F, Nikon Corporation, Tokyo, Japan), which was equipped with the polarized light microscope. The images were subsequently captured using ACT-2U software version 1.4 (Nikon Corporation, Tokyo, Japan).

5.2.3 Statistical analysis

All statistical analyses were performed as described in 3.2.4. The following thermal behaviour data: T_{onset}, T_{peak}, and ΔH, were analysed. All the measurements were performed in duplicate and the data are presented as mean ± standard deviation (SD). The significance was accepted at the 5% confidence level.

5.3 Results

5.3.1 Thermal behaviour

The effects of the addition of milk proteins ingredients (SMP, MPC, NaCAS, WPI, and UFSMP) to the T_{onset}, the T_{peak}, and the ΔH are reported in the sections below.

5.3.1.1 Normal rice starch/milk ingredients mixtures

The DSC thermograms of normal rice starch alone (10% normal rice starch in water) and with the added milk protein ingredients are shown in Figure 5.2 and T_{onset}, T_{peak} and ΔH are displayed in Table 5.1. The figure shows one symmetric endothermic peak for the starch/water mixture.

The solution of 10% SMP alone showed a broad but very tiny endothermic transition between 58 to 90°C in the condition used in this study. The addition of 5 and 10% SMP to normal rice starch showed a similar thermogram to the starch/water system, except the whole thermogram was shifted to the right by an amount that was proportional to the amount of added SMP (Figure 5.2). A 2.52°C increase of T_{onset} and 1.74°C for T_{peak} was observed due to the addition of 5% SMP compared to just a starch solution. Increasing the SMP concentration from 5-10% resulted in an approximately 1.4 – 1.5°C increase of T_{onset} and T_{peak}. There was also an increase in ΔH due to the addition of 5% SMP but no observed change upon increasing SMP concentration to 10%.
UFSMP showed a similar but greater effect than SMP on the thermal behaviour of normal rice starch. The addition of 5 and 10% UFSMP increased the measured T_{onset}, and T_{peak} of normal rice starch (Figure 5.2 and Table 5.1) with the increase was proportional to the concentration of added UFSMP. T_{onset} increased by 3.34°C and T_{peak} by 2.95°C on the addition of 5% UFSMP compared to no UFSMP. Increasing the UFSMP concentration from 5-10% resulted in T_{onset} and T_{peak} increasing by about 1.7 - 1.9°C. The value of ΔH of normal rice was increased by the addition 5% UFSMP, and increased again on the addition of 10% UFSMP. A 10% solution of UFSMP on its own showed no endothermic transition.

It would appear that the MPC had little or no effect on the thermal properties of starch in this system irrespective of MPC concentration (Figure 5.2 and Table 5.1). The difference between normal rice/water and normal rice/SMP mixtures was the fact that the thermogram of the MPC/starch mixtures, unlike the starch/water mixtures and starch/SMP mixtures, failed to drop completely back to the baseline at temperatures above T_{peak}. The endotherm's peak of the starch/MPC mixtures was broader than the starch/water mixture. As with UFSMP the solution of 10% MPC alone showed no endothermic transition.

Althought, NaCAS did not significantly increase T_{onset} as was the case for SMP and UFSMP (only increased T_{onset} by 0.42°C on the addition of 10% NaCAS) it did slightly shift the T_{peak} to a higher temperature. This increase in T_{pea} was increased by increasing NaCAS concentration from 5-10%. Like the MPC thermogram the samples containing the added protein failed to drop back to the baseline, unlike starch/water mixture which did drop back to the baseline. The NaCAS endothermic peak was clearly broader than for the starch/water mixture. From the thermogram, there was no an endothermic transition for a pure solution of 10% NaCN.

Figure 5.2 and Table 5.1 shows the thermograms and thermal properties for normal rice starch and mixtures of WPI-starch. An endothermic peak for the 10% WPI solution without starch was observed. It started at T_{onset} of 72.57°C, and peaked at T_{peak} of 75.51°C. Moreover, there were observed shoulders of the 10% WPI solution DSC thermogram, the biggest shoulder displayed T_{onset}, and T_{peak} at 58.19 and 64.53°C, respectively. The DSC thermograms obtained from normal rice starch/5% or 10% WPI displayed two endothermic transitions; the first endothermic transition exhibited peak temperatures of 67.16^{o}C and 67.66°C for the addition of 5% and 10% WPI, respectively. The second endothermic transition occurred at a higher peak temperature; 77.91^{o}C, and 75.95^{o}C for the addition of 5% and 10% WPI, respectively. This second endothermic peak is coincident with the whey protein denaturation peak. The remarkable increase in enthalpy of the normal rice starch in the addition of 10% WPI could be a result of the uptake of significant amounts of water by the swelling rice granules thus leading to an effective increase in the WPI concentration above 10% in the remaining water. This increase in effective concentration should lead to marked

increase in the amount of heat needed to effect the necessary conformational changes in the WPI proteins. .

In summary, SMP and UFSMP increased the onset and peak gelatinization temperature of normal rice starch and the respective temperatures increased when protein concentration was increased from 5-10%. Whereas, MPC, NaCAS and WPI had no affect on the gelatinization onset temperature of normal rice starch. There were two endothermic peaks observed in normal rice starch/WPI mixtures, the first peak represented the peak of normal rice starch gelatinisation and the second peak corresponded with the endothermic transition of a 10% WPI solution alone. Higher enthalpies were observed on the addition of 10% UFSMP and 10%WPI to normal starch.

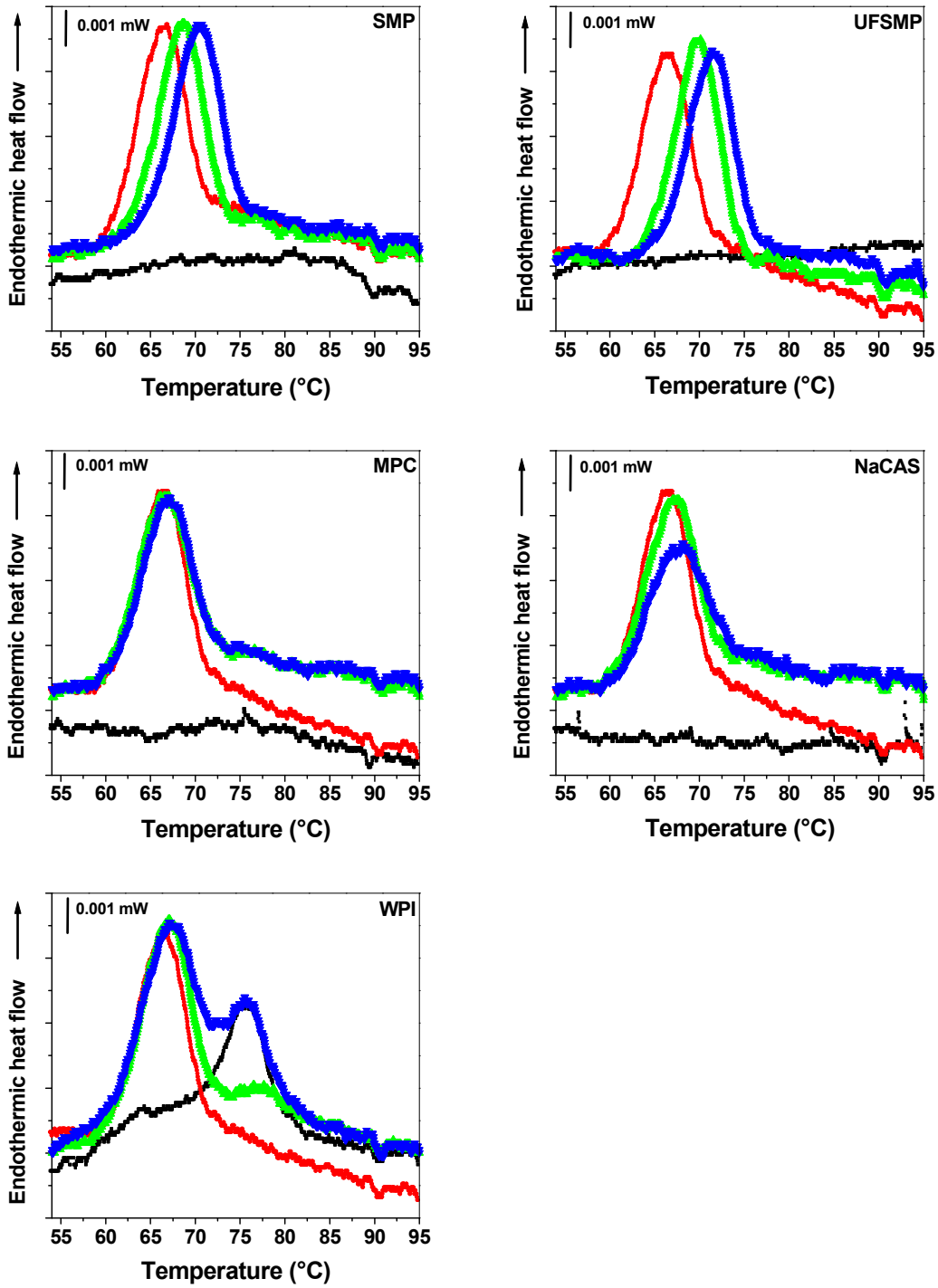

Figure 5.2 DSC thermograms of 10% normal rice starch/ milk protein ingredients mixtures (n ≥ 2). ◆: 10wt% normal rice starch in water, ▲: 10% normal rice starch/5% milk protein ingredient mixtures, ▼:10% normal rice starch/10% milk protein ingredient mixtures, ■: 10% milk protein ingredient solution.

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Table 5.1 Effect of milk protein ingredients on the thermal behaviour normal rice starch.

Sample	Thermal behaviour[*,**]		
	T_{onset} (°C)	T_{peak} (°C)	ΔH (J/g)
Milk protein ingredient			
10% SMP	ND[***]	ND	ND
10% UFSMP	ND	ND	ND
10% MPC	ND	ND	ND
10% NaCAS	ND	ND	ND
10% WPI	72.57±0.03	75.51±0.00	12.04±0.01
Normal rice starch	62.20±0.18de	66.97±0.10h	13.25±0.06f
Normal rice/milk protein ingredient mixtures			
5% SMP	64.72±0.21b	68.71±0.24c	13.94±0.47de
10% SMP	66.11±0.02a	70.24±0.00a	14.05±0.66d
5% UFSMP	65.54±0.28	69.92±0.26b	13.54±0.49c
10% UFSMP	67.46±0.16b	71.59±0.24a	15.25±0.60b
5% MPC	62.43±0.36de	66.82±0.31h	13.59±0.76def
10% MPC	62.36±0.26de	67.07±0.06h	13.00±0.47g
5% NaCAS	62.48±0.10de	67.29±0.16h	13.50±0.49ef
10% NaCAS	62.62±0.39d	68.14±0.05d	13.21±0.44f
5% WPI	62.58±0.25d	67.16±0.02gh	13.34±0.44f
10% WPI	62.12±0.25de	67.66±0.73ef	29.60±0.80a

[*] Mean value ± SD (n ≥ 2).

[**] Different letters within the same column indicate significant difference at $P < 0.05$ different through the Duncan test.

[***] None detected

5.3.1.2 Waxy rice starch/milk ingredients mixtures

The DSC thermograms and thermal properties of waxy rice starch alone (10% waxy rice starch in water) and with the added milk protein ingredients are shown in Figure 5.3 and Table 5.2. As with the normal rice starch/water mixture, the thermogram for the waxy rice starch/water mixture displayed a single symmetric endothermic peak.

The addition of SMP to waxy rice starch resulted in an increase to the gelatinization temperature of waxy rice starch and this increased with increasing concentration of SMP (Figure 5.3 and Table 5.1). However, apart from a shift of the whole thermogram to the right, SMP did not change the other characteristics of the waxy rice starch thermogram. T_{onset} was increased by 1.21°C and 4.42°C on the addition of 5% and 10% SMP, respectively. T_{peak} was increased by 4.35°C and 5.35°C on the addition of 5% and 10% SMP, respectively compared to starch and water alone. Similar to the effect of SMP on normal rice starch, the value of ΔH of waxy rice starch was slightly increased by the addition SMP but there was no observed change upon increasing SMP concentration.

The addition of UFSMP increased the measured T_{onset} and T_{peak} of waxy rice starch (Figure 5.3 and Table 5.2) compared to starch and water alone and there was also an increase again on the addition of 10% UFSMP compared to 5% UFSMP. T_{onset} increased by 3.60°C and T_{peak} by 5.23°C on the addition of 5% UFSMP compared to starch alone. On the addition of 10% UFSMP, T_{onset} and T_{peak} increased by 4.87°C and 7.05°C, respectively. There was an increase in ΔH due to the addition of UFSMP and this too increased with increasing UFSMP concentration.

The thermograms and thermal properties for waxy rice starch and MPC are shown in Figure 5.3 and Table 5.2, respectively. MPC increased T_{onset} by 0.19°C and 1.91°C on the addition of 5 and 10% MPC, respectively. T_{peak} increased by 2.07°C and 3.13°C when MPC concentration was increased from 5-10% compared to starch and water alone. A slightly higher enthalpy was observed on the addition of 5% MPC but no observed change upon increasing MPC concentration.

The addition NaCAS to waxy rice starch showed a clearly shorter, but broader thermogram than the starch/water system. The width of the thermogram increased with increasing. NaCAS concentration, whilst the height decreased. The whole thermogram was shifted to the right by an amount that was proportional to the amount of added NaCAS (Figure 5.2). T_{onset} increased by 1.68°C and T_{peak} by 2.50°C on the addition of 5% NaCAS to 5% NaCAS compared to starch and water alone. There was a 2.06°C and 4.19°C increase in T_{onset} and T_{peak} on the addition of 10% NaCAS. There was also an increase in ΔH due to the addition of 5% NaCAS but this dropped down upon increasing NaCAS concentration.

Figure 5.3 and Table 5.2, shows the thermograms and thermal properties of waxy rice starch when WPI was added. Similar to the addition of WPI to normal rice starch, the DSC thermograms of waxy rice starch also displayed two endothermic transitions. The first endothermic transition, which corresponded to endothermic transition of waxy rice starch exhibited peak temperatures of 66.74°C and 67.14°C for the addition of 5 and 10% WPI, respectively. The second endothermic transition, which was coincident with the whey protein denaturation endothermic transition displayed at 77.53°C, and 75.87°C for the addition of 5 and 10% WPI, respectively. As seen in normal rice starch, the remarkable increase in enthalpy of the waxy rice starch in the addition of 10% WPI was also observed.

In summary, all of milk protein ingredients increased the gelatinization temperatures of waxy rice starch. Two endothermic peaks were observed in the waxy rice starch/WPI mixtures and the frst corresponded to the gelatinization of starch and the second the corresponded with the endothermic transition of the 10% WPI solution alone. In general (except 10% NaCAS and 5% WPI), waxy rice starch/milk protein ingredients mixtures exhibited higher enthalpy than a waxy/water mixture. Enthalpy was extraordinary high in case of 10% WPI.

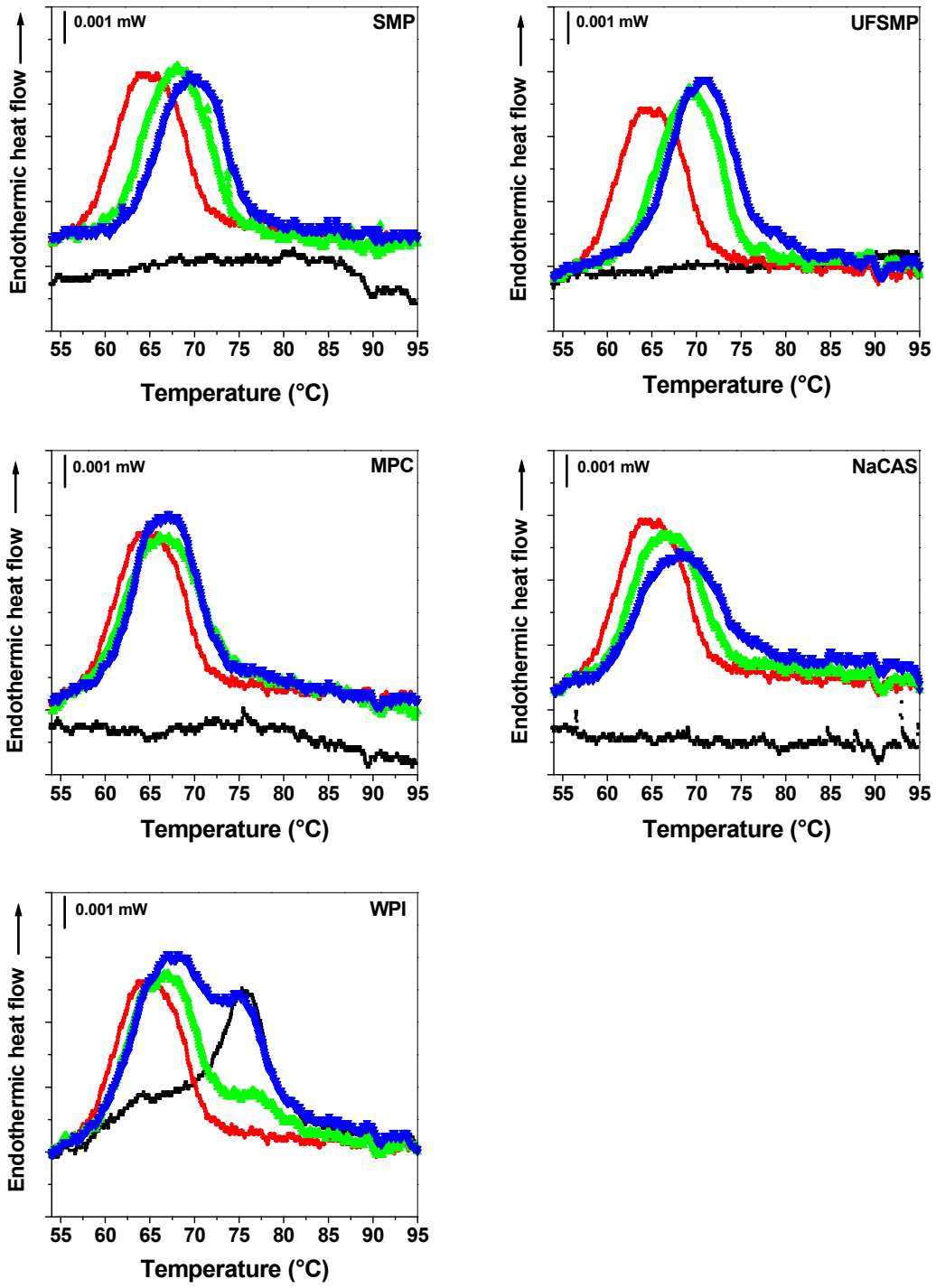

Figure 5.3 DSC thermograms of 10% waxy rice starch/milk protein ingredients mixtures (n ≥ 2). ◆: waxy rice starch in water, ▲: waxy rice starch/5% milk protein ingredient mixtures, ▼:waxy rice starch/10% milk protein ingredient mixtures, ■: 10% milk protein ingredient solution.

Table 5.2 Effect of milk protein ingredients on the thermal behaviour waxy rice starch.

Sample	Thermal behaviour*, **		
	T_{onset} (°C)	T_{peak} (°C)	ΔH (J/g)
Milk protein ingredient			
10% SMP	ND***	ND	ND
10% UFSMP	ND	ND	ND
10% MPC	ND	ND	ND
10% NaCAS	ND	ND	ND
10% WPI	72.57±0.03	75.51±0.00	12.04±0.01
Waxy rice starch	60.26±0.33f	63.99±0.07g	13.71±0.16e
Waxy rice/milk protein ingredient mixtures			
5% SMP	61.47±0.51de	68.34±0.28c	14.29±1.16d
10% SMP	64.68±0.12a	69.34±0.09b	14.41±0.83d
5% UFSMP	63.86±0.68b	69.22±0.02b	16.60±0.85c
10% UFSMP	65.13±1.29a	71.04±0.33a	18.26±0.92b
5% MPC	60.45±0.78f	66.06±0.07f	14.51±1.72d
10% MPC	62.17±0.23f	67.12±0.02d	14.26±1.09d
5% NaCAS	61.94±0.56cd	66.49±0.59e	14.21±0.67d
10% NaCAS	62.32±0.08c	68.18±0.00c	13.03±0.26f
5% WPI	60.49±0.22f	66.74±0.24e	13.29±0.29ef
10% WPI	61.26±0.89e	67.14±0.00d	26.06±1.44a

* Mean value ± SD (n ≥ 2).

** Different letters within the same column indicate significant difference at $P < 0.05$ different through the Duncan test.

*** None detected

5.3.2 Correlation between the pasting and thermal behavior of normal or waxy rice starch/milk protein ingredients mixtures.

The thermal behavior of normal or waxy rice starch/milk protein ingredients mixtures measured by the DSC (Figure 5.2, and Figure 5.3, respectively) paralleled the effects that the different milk protein ingredients had on the onset of pasting behaviour of normal rice starch and waxy rice starch as described in Chapter 3. The correlation between the onset temperature measured by DSC and that the onset temperature of pasting measured by rheometer is shown in Figure 5.4A. It can be seen that there is a good correlation between these two independent measurements (R^2 =0.842). However, there was no correlation between the peak temperatures measured by the two methods (Figure 5.4B).

The peak temperature as measured by DSC corresponds to the point during the heating of the starch where the maximum rate of gelatinization occurred. Conversely the temperature at the point of peak viscosity is the point during the heating that the maximum granule volume fraction is reached. This is a function of the rate of volume increase due to swelling and the rate of volume fraction reduction due to breakage of the granules. Because of the quite different reasons for the peaks in responses, it is not expected that the DSC and pasting curve peak temperatures would be correlated. During the initial stages of heating the starch solution the onset of both viscosity increase and thermal response are both due to starch gelatinization. As such the observed correlation between onset temperatures measured by the two experimental techniques is understandable. The consistently lower onset temperature observed by DSC is due to the sensitivity of the thermal response. A significant extent of the gelatinization process must be completed before enough swelling can occur to cause a viscosity change.

A possible explanation for this discrepancy could be that the rheometer pasting measurements were performed under shear whereas DSC measurements were performed without shear. This could have at least three consequences. Firstly, the peak temperature measured by the pasting of the starch in the rheometer corresponds to the

temperature at which a maximum viscosity is attained. This peak viscosity is an optimum between the swelling and the breakdown of the starch granules due to shear. Secondly, when not mixed continuously, as during DSC measurements, rapid sedimentation can occur. This will result in an increase in the local concentration of the starch granules, and it is well established that the results obtained by DSC depends on the starch concentration (Biliaderis, Maurice and Vose, 1980). Thirdly, phase separation between the starch molecules and the milk protein, if it occurs, will be affected by the presence of shear, which in turn will affect the measurements obtained by the pasting of rheometer and DSC.

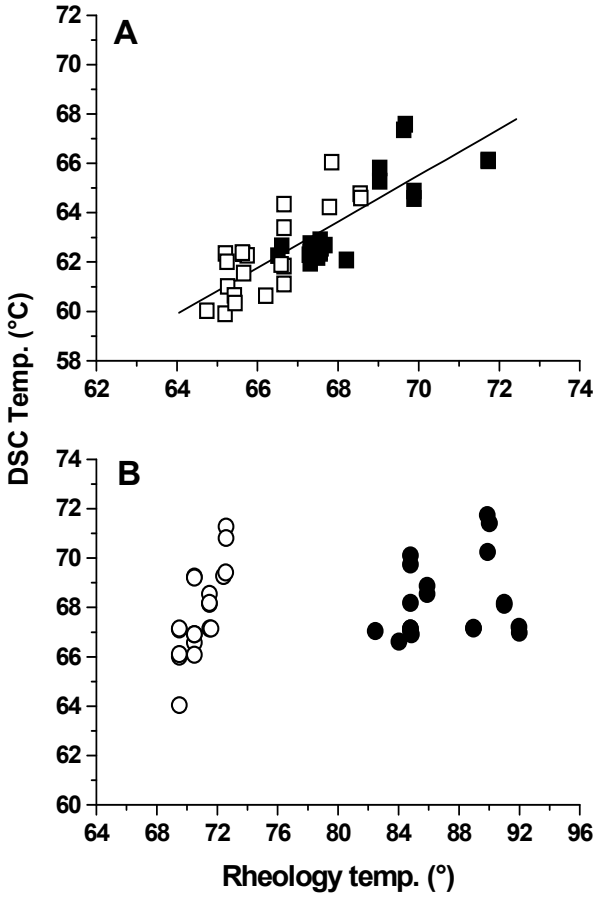

Figure 5.4 Relationships between T_{onset} (A) and T_{peak} (B) as determined by DSC and rheological methods. Normal rice starch/milk protein ingredient mixtures (solid symbols) and waxy rice starch/milk protein ingredient mixtures (open symbols). (The continuous line is a linear fit with intercept = 1.111, slope = 0.919 and R^2 = 0.842.).

5.3.3 The loss of birefringence

The mixtures of normal or waxy rice starches in water, 5%, or 10% milk protein ingredients were heated from 50°C to 95°C in the rheometer. A very small amount of sample was transferred from the rheometer to a glass slide and immediately cooled down to room temperature (~20°C) to ensure that the starch sample would not continue to gelatinise by the heat retained in the sample. The normal or waxy rice starch/milk protein ingredient mixture was collected at various temperatures and observed under the polarized light microscope. Polarized light microscopy images for the mixtures of normal rice starches/water, SMP, MPC, NaCAS, or WPI are shown in Figures 5.5, 5.6, 5.7, 5.8, and 5.9. For the mixtures of waxy rice starch/water, SMP, MPC, NaCAS, or WPI, the images for polarized light microscopy are shown in Figures 5.10, 5.11, 5.12, 5.13, and 5.14, respectively. The conclusions drawn from the above figures are presented below.

5.3.3.1 Normal rice starch/milk ingredients mixtures

Images for polarized light microscopy of normal rice starch alone (10% normal rice starch in water), 10% SMP, 10% MPC, 10% NaCAS, and 10% WPI are presented in Figures 5.5 to 5.9, respectively. At 50°C, all samples showed birefringence. For normal rice starch in water at 58° C the starch granules were swollen and a few granules had started to lose their birefringence, but there was no disruption of the starch granules. The loss of birefringence increased with increasing temperature. From Figure 5.5A at 62°C, the normal rice starch granules were swollen, deformed, some granules were disrupted, and one third of the starch granules had lost their birefringence, and finally some of the molecules were embedded in a matrix; at 70°C most of birefringence was lost; however there were granules that still retained their shape and showed birefringence (Figure 5.5B). By the time the temperature reached 76°C (Figure 5.5C), almost all of the starch granules had lost their birefringence.

The addition of 10% SMP decreased the loss of birefringence at the higher temperatures consistent with DSC observation. From Figure 5.6A, normal rice

starch/10% SMP mixture started to lose birefringence at 62°C. By 66°C, one third of the starch granules had lost their birefringence (Figure 5.6B); birefringence loss continued with rising temperature and by 76°C the loss was quite significant, though noticeably less when 10% SMP was present compared to starch/water (Figure 5.6C), i.e., the results suggest that there is less loss in crystallinity when SMP is present in solution with the normal starch. These findings parallel the results obtained from the DSC which showed that the gelatinization temperature of normal starch was increased by approximately 4^{o}C when SMP was present compared to an absence of SMP.

The addition of 10% MPC had no affect on the birefringence loss of normal rice starch granules compared to the starch and water system. This is in agreement with the DSC results which showed that the addition of MPC had no affect on the gelatinization temperature of normal rice starch.

Figure 5.8, the loss of birefringence in the presence of NaCAS for normal rice starch. Some starch granules had lost their birefringence at 62°C, and by 70°C (Figure 5.8B) and 76°C (Figure 5.8C) a proportion of the starch granules had lost their birefringence.

According to Figure 5.9A the starch granules on the addition of 10% WPI started to lose their birefringence at 58°C and by 70°C (Figure 5.9B) a high proportion of the granules of normal starch had lost their birefringence. When the temperature reached 76°C (Figure 5.9C) practically all the granules had lost their birefringence, though the loss of birefringence was not quite as high as it was for normal starch and water where the loss of birefringence was greater for all of the studied temperatures.

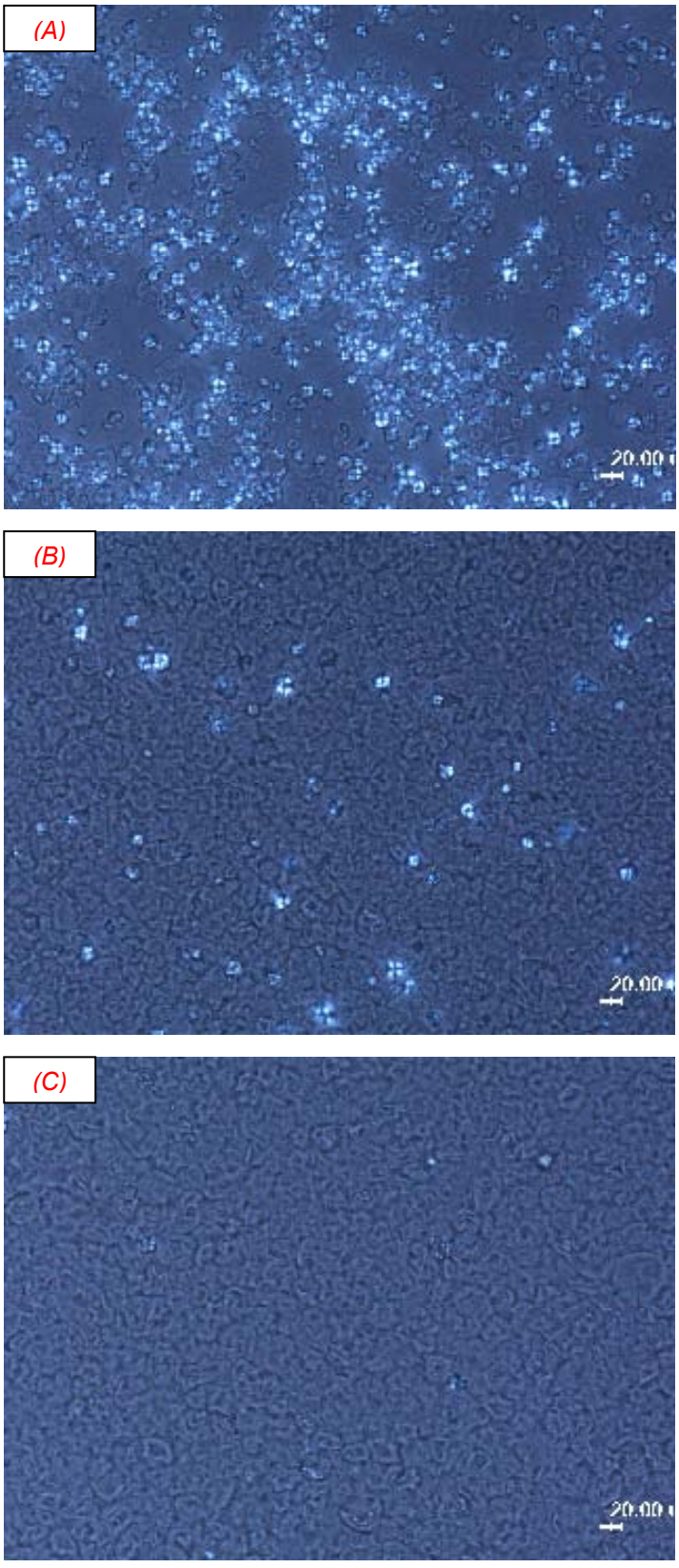

Figure 5.5 Images for polarized light microscopy for the birefringence loss of normal rice starch at (A) 62°C, (B) 70°C, and (C) 76°C, respectively.

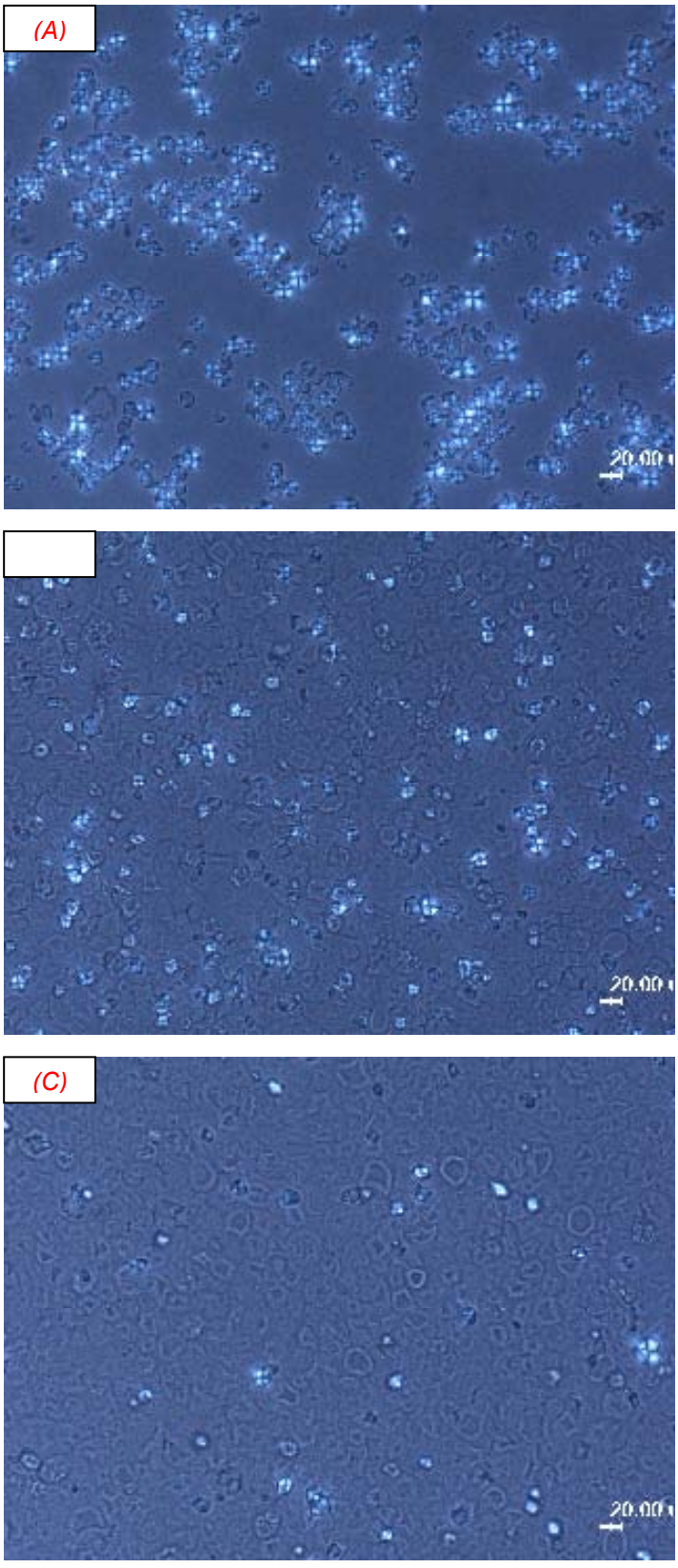

Figure 5.6 Images for polarized light microscopy for the birefringence loss of normal rice starch/10% SMP at (A) 62°C, (B) 70°C, and (C) 76°C, respectively.

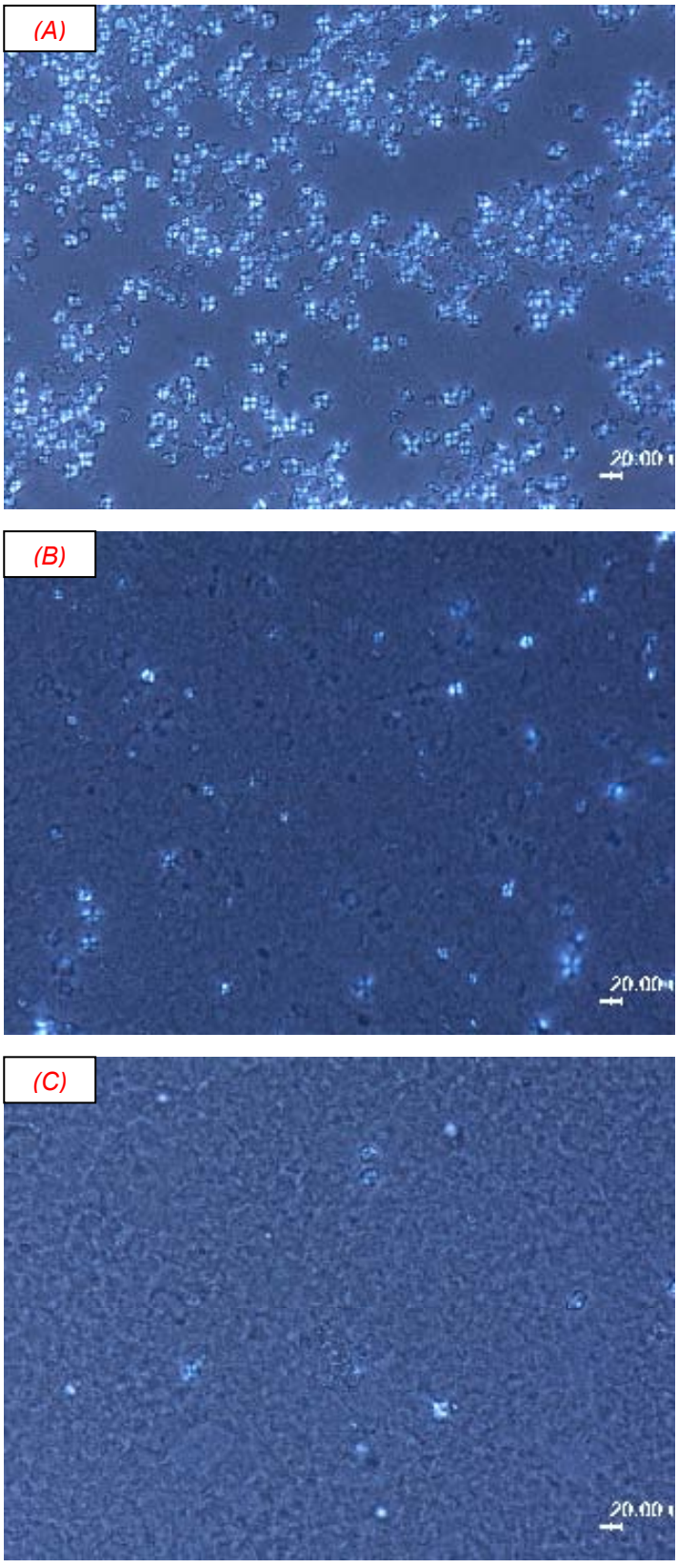

Figure 5.7 Images for polarized light microscopy for the birefringence loss of normal rice starch/10% MPC at (A) 62°C, (B) 70°C, and (C) 76°C, respectively.

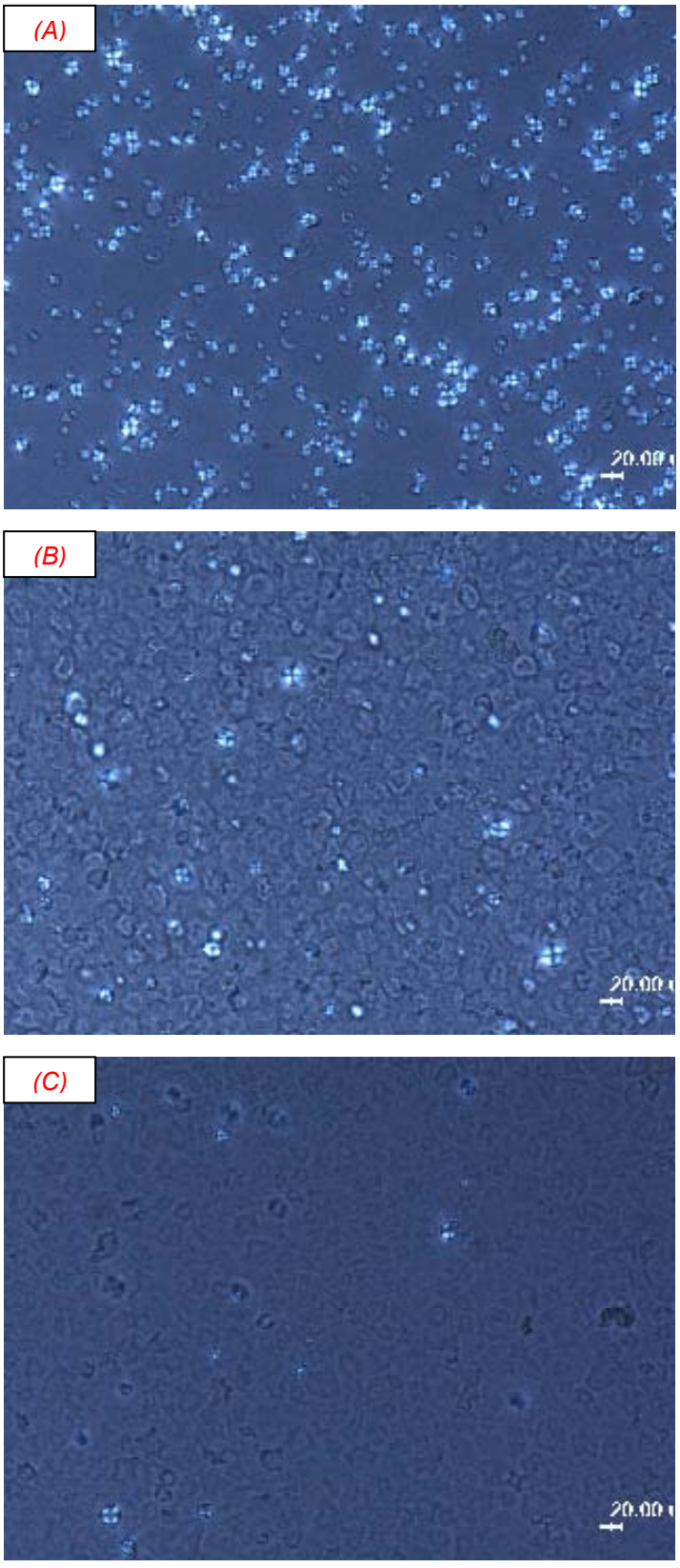

Figure 5.8 Images for polarized light microscopy for the birefringence loss of normal rice starch/10% NaCAS at (A) 62°C, (B) 70°C, and (C) 76°C, respectively.

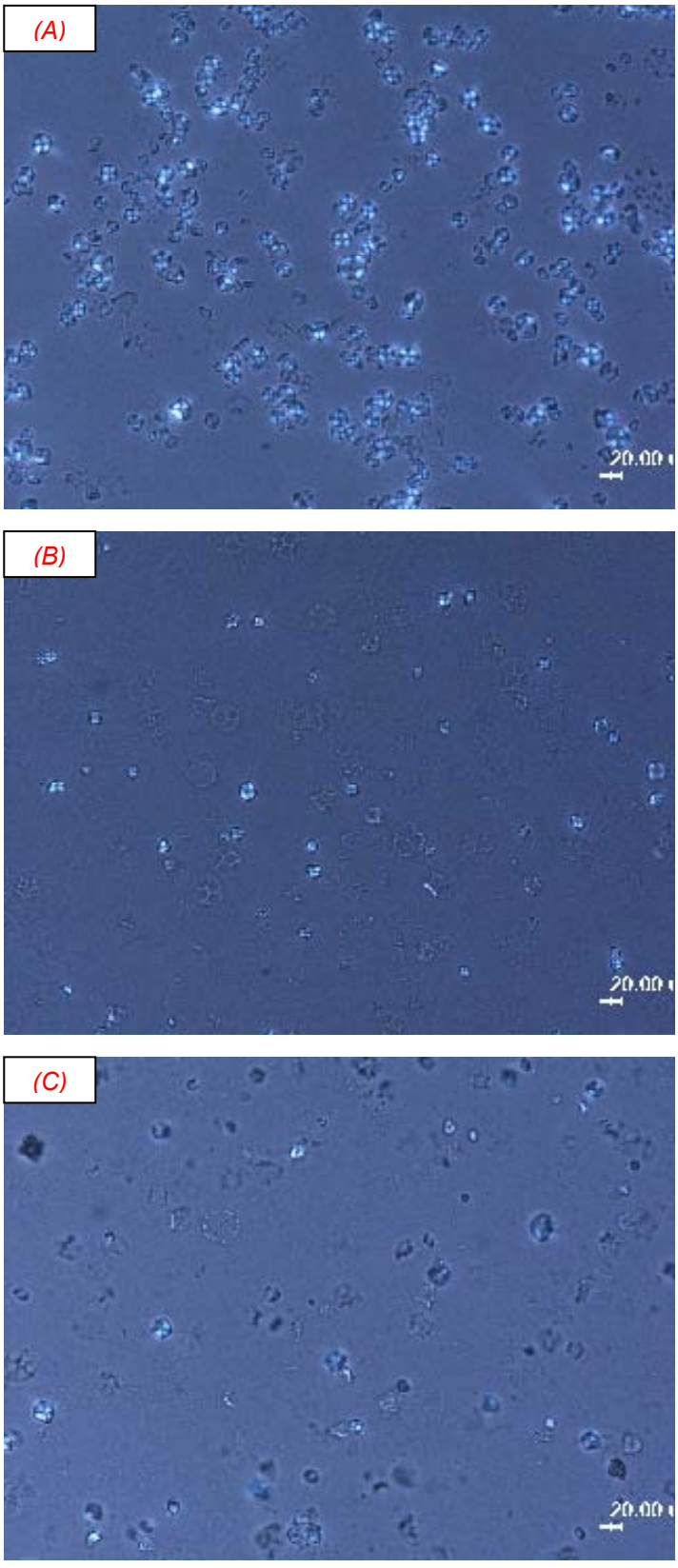

Figure 5.9 Images for polarized light microscopy for the birefringence loss of normal rice starch/10% WPI at (A) 62°C, (B) 70°C, and (C) 76°C, respectively.

5.3.3.2 Waxy rice starch/milk ingredients mixtures

Images for polarized light microscopy of waxy rice starch/water and 10% milk protein ingredients mixtures are presented in Figures 5.10 to 5.14, respectively. At 50°C, all samples showed birefringence. For waxy rice starch alone, the first detectable loss of birefringence occurred at 56°C and naturally birefringence loss increased with increasing temperature as shown in Figures 5.10A-C. The proportion of swollen and deformed granules increased with increasing temperature, and at 60°C some of the starch granules had actually ruptured (Figure 5.10B). Most of waxy starch granules lost their birefringence at 70°C (Figure 4.10C). In contrast to normal rice starch, there were no swollen, deformed or disrupted waxy rice starch granules embedded in a matrix and after the complete disruption of all the granules the components blended together to form a homogenous paste. This extreme fragility of waxy rice starch granules compared to normal rice starch granules has been reported in the literature by Rani and Bhattacharya (1995).

Like normal rice starch, 10% SMP increased the temperature at which birefringence was lost compared to waxy starch/water mixture. From Figure 5.11A, waxy rice starch/10% SMP heated at 58°C showed no loss of birefringence of starch granules. The loss of birefringence only started at 60°C (Figure 5.11B).

At 58°C there was no sign of birefringence loss in either a 10% MPC (Figure 5.12) or a 10% WPI (Figure 5.14) waxy rice starch/water system, but the loss of birefringence started at 60°C. The loss of birefringence increased with increasing temperature, but it was less at each of the observed temperatures than the starch/water system. The DSC results showed that these two milk proteins ingredients increased the gelatinization temperature when 10% was added to waxy rice starch, and these results are coincident with the result obtained from this birefringence study.

The addition of 10% NaCAS also increased the temperature at which the loss of birefringence occurred. At 58°C, waxy rice starch granules did not lose their birefringence (Figure 5.13A), by 60°C the first few starch granules had lost their

birefringence (Figure 5.13B) and by 70°C (Figure 5.13C) a large proportion of the starch granules showed loss of birefringence, though the proportion was once again less at each temperature than in the starch/water system.

To summarize, the addition of 10% milk protein ingredients (SMP, MPC, NaCAS, and WPI) increased the temperature at which the waxy rice starch granules lost their birefringence. Observed trends were all consistent with DSC observation for all milk protein ingredients. The DSC study showed that all the protein ingredients increased the gelatinization temperatureof waxy rice starch.

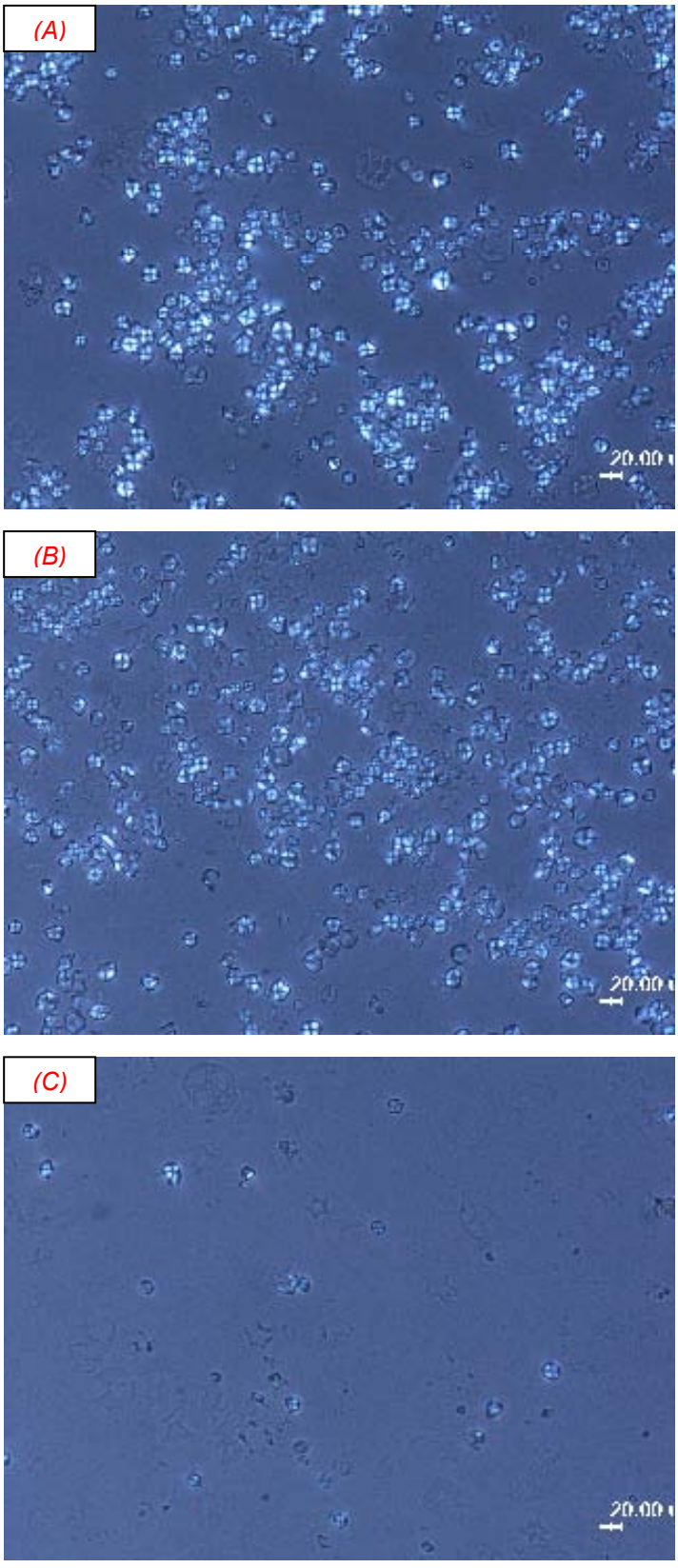

Figure 5.10 Images for polarized light microscopy for the birefringence loss of waxy rice starch at (A) 58°C, (B) 60°C, and (C) 70°C, respectively**.**

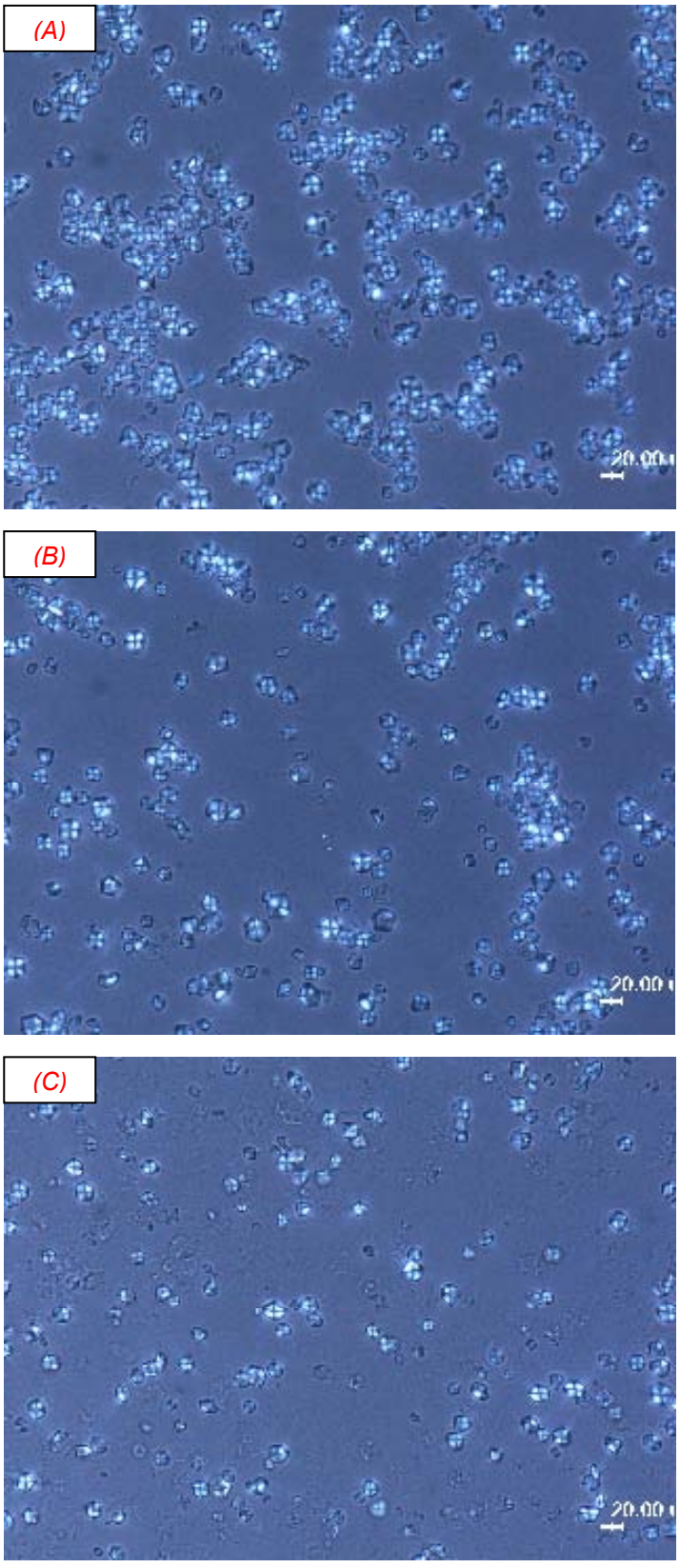

Figure 5.11 Images for polarized light microscopy for the birefringence loss of waxy rice starch/10% SMP at (A) 58°C, (B) 60°C, and (C) 70°C, respectively.

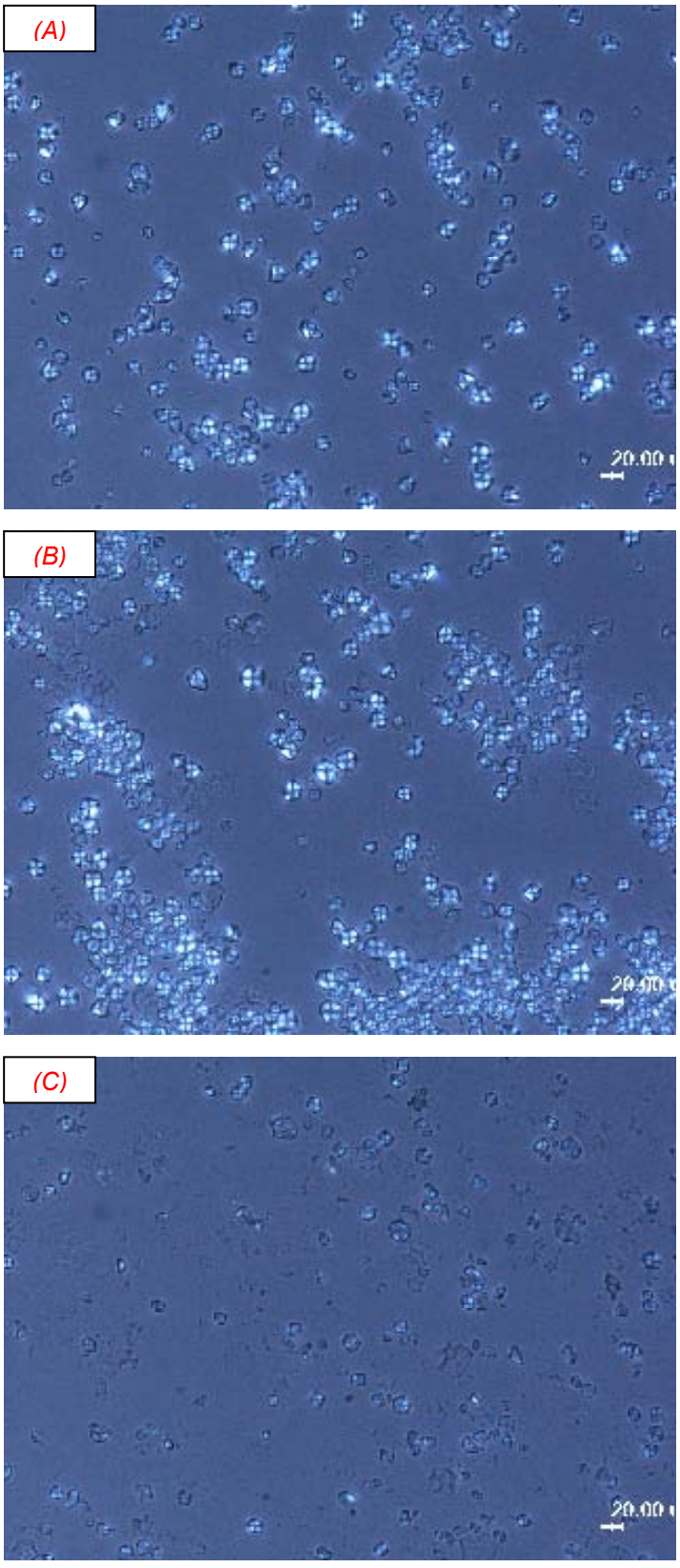

Figure 5.12 Images for polarized light microscopy for the birefringence loss of waxy rice starch/10% MPC at (A) 58°C, (B) 60°C, and (C) 70°C, respectively.

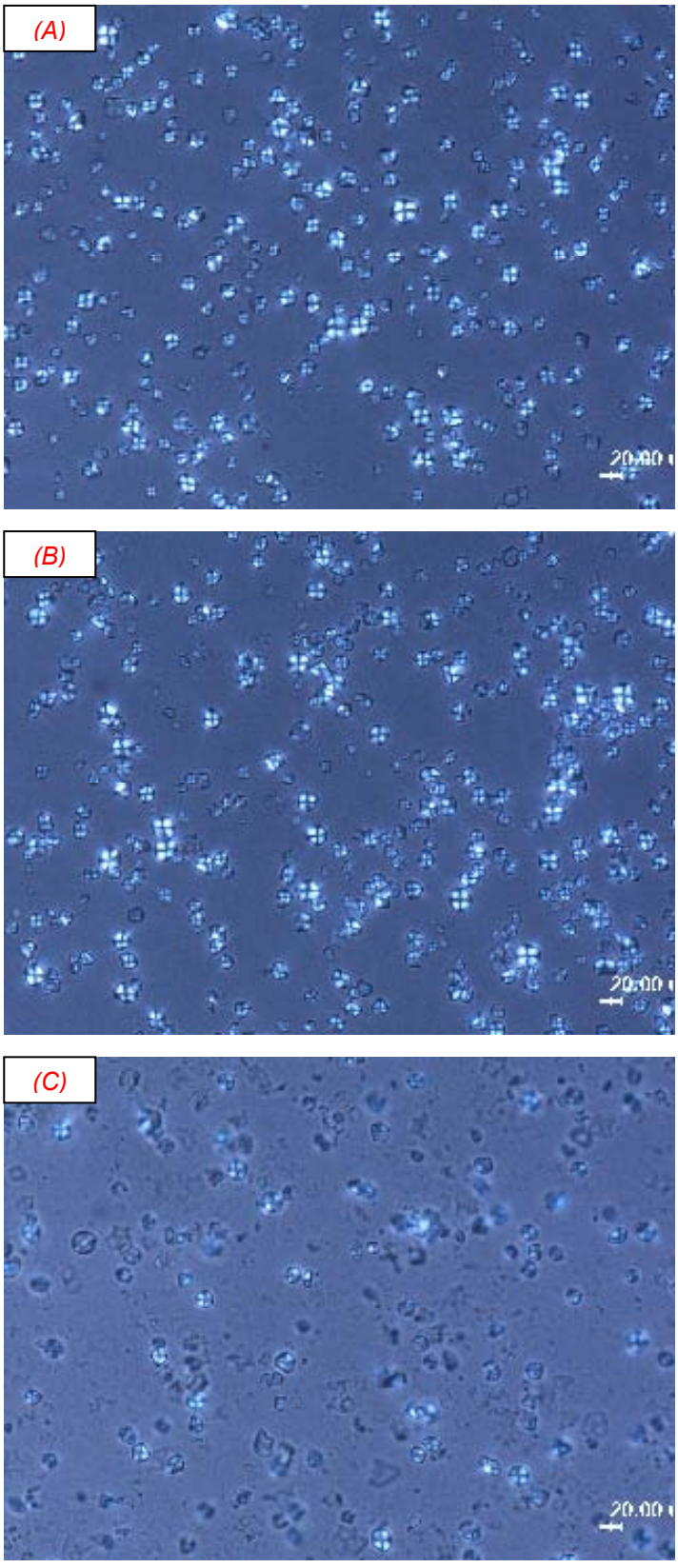

Figure 5.13 Images for polarized light microscopy for the birefringence loss of waxy rice starch/10% NaCAS at (A) 58°C, (B) 60°C, and (C) 70°C, respectively.

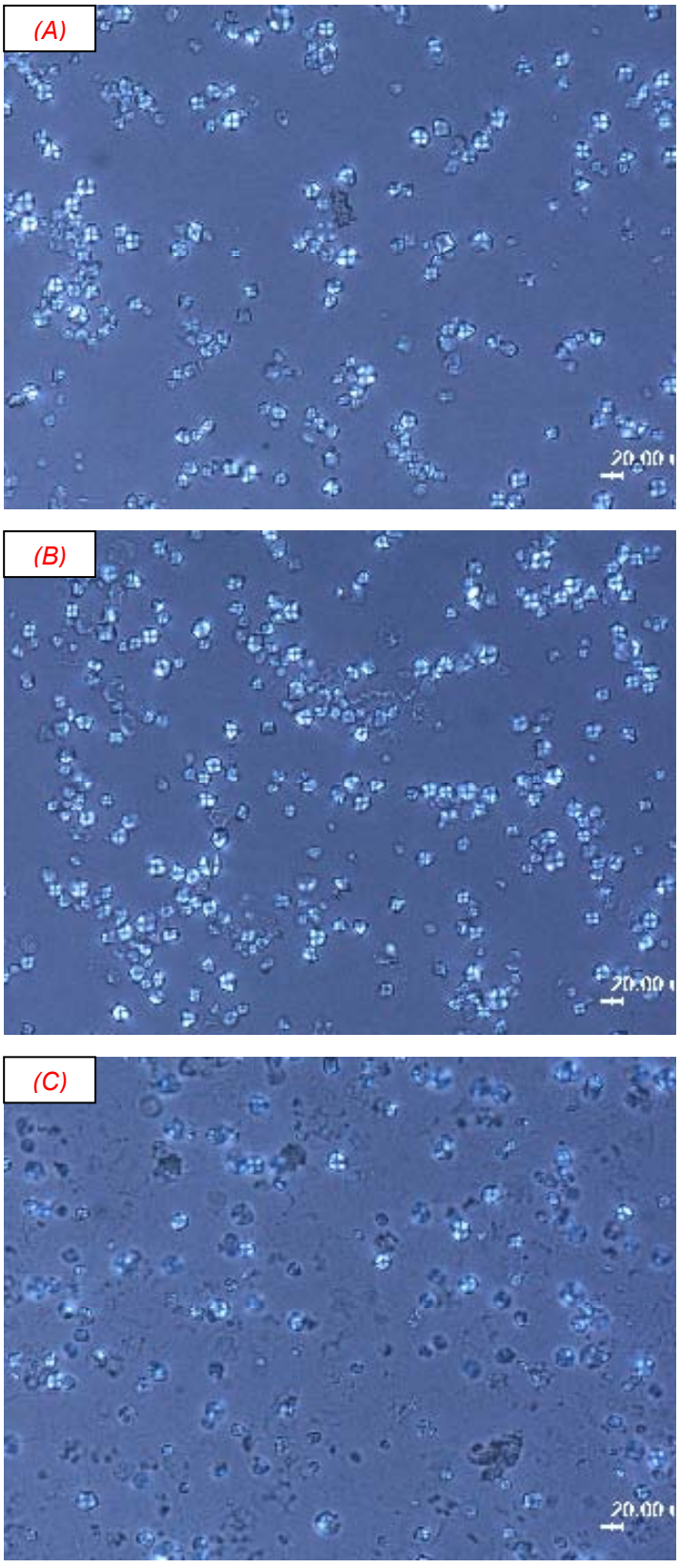

Figure 5.14 Images for polarized light microscopy for the birefringence loss of waxy rice starch/10% WPI at (A) 58°C, (B) 60°C, and (C) 70°C, respectively.

5.4 Discussion

In this study the starch concentration was fixed at 10% by weight of water for the starch/water and starch/milk protein ingredient/water systems because water content is known to have a major impact on the gelatinization temperature of starch. Many studies have confirmed that in the presence of excess water (>60%), as is the case in the present study, water uptake by the amorphous regions is accompanied by hydration and swelling and that this facilitates the melting of the starch crystallites during heating. In systems with excess water this happens over a very narrow temperature range and therefore results in a single endothermic transition. On the other hand, in the presence of limited amounts of water, two endothermic transitions have been observed; the first endothermic transition mainly occurring at the same temperature as the single endothermic transition observed in excess waters with the second endothermic transition ocurring at higher temperature (Donovan, 1979; Biliaderis *et al*., 1980; Lund, 1984; Biliaderis *et al*., 1986; Lund, 1989).

In the present study, the normal and waxy rice starches were evaluated when in the presence of excess water (10% starch in water). In this case with the DSC a single endothermic transition occurred as expected. The DSC results, T_{onset}, T_{peak}, and ΔH of normal rice starch/water and waxy rice starch/water mixtures were compared to values reported by previous studies (Table 5.3). It was found that the DSC results from the present study are in agreement with the results obtained by those studies.

Table 5.3 The thermal properties of normal and waxy rice starch.

Starch	Values from this study			Values from literature		
	T_{onset}(°C)	T_{peak}(°C)	ΔH (J/g)	T_{onset}(°C)	T_{peak}(°C)	ΔH (J/g)
Normal	62.20	66.97	13.25	56.6 – 62.1[a]	62.8 – 67.0[a]	17.3 – 19.8[a]
				57.7[b]	65.1[b]	11.5[b]
				62.0[c]	67.4[c]	11.0[c]
Waxy	60.26	63.99	13.71	57.9 – 59.6[a]	65.2 – 65.8[a]	18.4 – 19.6[a]
				60.0[d]	68.9[d]	13.8[d]

[a] Vandeputte *et al*. (2003b)

[b] Li and Yeh (2001)

[c] Jenkins and Donald (1998)

[d] Biliaderis *et al*. (1986)

In the present study, the addition of SMP to normal and waxy rice starches clearly affected the thermal behaviour of both rice starches in that the gelatinization temperature increased with increasing concentration of SMP. This result was confirmed by the images from polarized light microscopy of normal and waxy rice starch/water mixtures and normal and waxy rice starches with 10% SMP. These images showed that the temperature at which birefringence loss occurred was increased by the addition of 10% SMP. From the chemical composition of milk protein ingredients (Table 3.3), SMP contains 54.1% of lactose plus a range of divalent cations (Mg^{2+} and Ca^{2+}) associated with the proteins and also present as free salts. Both sugar and salts are reported by a number of researchers to have an influence on the thermal behaviour of starch (Evans and Haisman, 1982; Spies and Hoseney, 1982; Lund, 1984; Biliaderis, 1990; Kohyama and Nishinari, 1991; Kim and Walker, 1992b; Hoover and Senanayake, 1996; Matser and Steeneken, 1997; Aee, Hie and Nishinari, 1998; Ahmad and Williams, 1999b; Perry and Donald, 2002; Sopade, Halley and Junming, 2004). Sugars tend to increase the gelatinization temperature of starch. Whereas salts exhibit a more complicated effect on starch gelatinization than sugars. They can cause either an increase or decrease in both the gelatinization temperature and the enthalpy of starch

depending upon their nature (Jane, 1993; Chiotelli *et al*., 2002). Cations tend to stabilize the starch granule structure due to the weak acid ion exchange property of the starch (Oosten, 1982). It was not surprising therefore that SMP, consisting of large amounts of lactose and a range of divalent cations, shifted the gelatinisation of normal and waxy rice starches to higher temperatures.

UFSMP was used to elucidate the effects of the lactose and minerals that are present in SMP on the thermal behaviour of normal and waxy rice starch (Figure 5.2 and 5.3 for normal and waxy rice starch, respectively). The solution of 10% UFSMP alone showed no change in enthalpy. The thermograms for the starch/10% SMP mixture and the starch/10% UFSMP mixture were virtually identical suggesting that the SMP proteins had little effect on either the T_{onset} or T_{peak}. Consequently, it appears that the observed changes to these values can be attributed to either the lactose and/or the salts present in SMP.

Kim and Walker (1992b), and Erdogdu *et al*. (1995) reported an increase in T_{onset}, and T_{peak} of starch in the presence of lactose and proposed, by referring to Spies and Honey (1982), that this might be due to the formation of hydrogen bonds between the lactose and the glucose groups on the amylose and amylopectin chains in the amorphous region of the starch granule and that these extra hydrogen bonds effectively stabilize the amorphous region with a consequential rise in the gelatinization temperature and enthalpy. They also hypothesised that the lactose successfully competed with the amorphous regions for water thus limiting the availability of water to the starch chains (i.e. reduced the water activity) and as a consequence the gelatinization temperature was raised. Another explanation was advanced by several researchers (Slade and Levine, 1989; Perry and Donald, 2000; Perry and Donald, 2002; Donald, 2004), who argued that it is unlikely that lactose outcompetes the amorphous regions of the granule for the available water, as lactose does not change the kinetics of starch gelatinization. Instead they proposed that lactose acts as an anti-plasticiser, slowing or inhibiting the plasticisation of the amorphous region and decreasing the free volume of the amorphous region, thereby increasing the gelatinization temperature of the system.

MPC had no affect on the thermal behaviour of normal rice starch (Figure 5.2), nor did it have any affect on the temperature at which birefringence was lost (Figure 5.7). MPC, which is essentially SMP with much of the lactose and salts removed (contains three times more protein and has far less lactose and salts than SMP), had a very slight affect on the endotherm of normal rice starch (slightly broader peak) compared to starch alone but the differences were so small as to suggest that the proteins in MPC had a negligible effect on the thermal behaviour of normal rice starch. This supports the results from the SMP and UFSMP study which suggest that it was lactose and salts that influenced the thermal behaviour of normal rice starch when they were present in reasonable concentrations.

In contrast to normal rice starch, the thermal behaviour of waxy rice starch was modified by the addition of MPC in that the gelatinization temperature increased. This could have been due to the denaturation of the α-la in MPC, which occurs in the same temperature range that waxy rice starch granules take up water and swell. The competition for water between α-la and waxy rice starch granules results in decreased water availability for the gelatinization of starch and hence the increase in the temperature of gelatinization when MPC is present. This effect was also noticeable on the addition of WPI to waxy rice starch, discuss further below.

NaCAS also showed different effects on the thermal behaviour of the two rice starches. The addition of 10% NaCAS to normal rice starch increased the T_{onset} by only 0.42°C and T_{peak} by 1.17°C (Figure 5.2 and Table 5.1). However, despite having a small effect on the thermal behaviour of normal rice starch, the effect of the addition of NaCAS to waxy rice starch was more noticeable (Figure 5.3 and Table 5.2). The gelatinization temperature of waxy rice starch was increased with increasing concentration of NaCAS. The addition of 10% NaCAS to waxy rice starch increased T_{onset} by 2.06°C and T_{peak} by 4.19°C. Erdogdu *et al*. (1995), who studied the thermal behaviour of a mixture of casein and wheat starch, similarly found that casein increased the T_{onset}, and T_{peak} values but unlike the results in this study, they found that it decreased the ΔH. Their results were in agreement with those of Bertolini *et al*. (2005), who after studying the thermal properties of NaCAS and various kinds of starch, including normal rice

starch, reported the difference in T_{onset}, and T_{peak} between normal rice starch alone and normal rice starch/10% NaCAS was 5.6°C and 4.3°C, respectively. Although the ΔH values in this study did not change due to NaCAS content, the DSC thermogram of normal and waxy rice starch on the addition of 10% NaCAS displayed clearly shorter but broader endothermic peaks than starch alone.

There are at least two mechanisms by which NaCAS can delay starch gelatinisation: water competition between NaCAS and starch granules and adsorption of the NaCAS molecules to the starch granule surfaces. Both mechanisms are concentration dependent. Firstly, NaCAS was reported to have a high hydration capacity of 3.4 g water/ g protein (Korolczuk, 1984). Thus it might compete with starch for water according to the mechanism proposed by Tester and Sommerville (2003) where non-starch polysaccharides due to their hydration ability limit the water available for the starch hydrate. As water plasticises the amorphous regions of the starch, any restriction to the hydration of this region by the addion of non-starch polysaccharides probably results in an anti-plasticising effect and thus contributes to a delay in gelatinisation. The effect was more pronounced at the end point of gelatinisation with higher temperatures required to reach the end point due to the limited availability of water. This is in agreement with the results in this study for the addition of NaCAS to normal rice starch. NaCAS did not affect T_{onset} but extended the end point of gelatinisation as seen by the broader DSC thermogram ending at a higher temperature (Figure 5.2 and 5.3).

The other mechanism for NaCAS delaying starch gelatinisation is adsorption of the caseins onto the starch granule surfaces. The main proteins in NaCAS; α-casein and β-casein, are flexible, linear, amphiphilic polyelectrolytes, with molecular weight ~24 kDa. Thus they are highly surface active proteins (Dickinson, 1999a). It might be possible for α- and β-casein to adsorb onto starch granule surfaces as it has been reported that other proteins adsorbed onto starch granules (Eliasson and Tjerneld, 1990; Larsson and Eliasson, 1997; Ryan and Brewer, 2005b; Ryan and Brewer, 2006). Consequently, the adsorbed proteins might restrict water penetration into the starch granules delaying the gelatinisation.

The DSC thermograms of 10% WPI solution without starch displayed an endothermic transition of T_{onset} at 72.57°C and T_{peak} at 75.51°C (Figure 5.2 and Figure 5.3), corresponding to the endothermic transition of β-lg. Aguilera and Rojas, (1996) reported that the denaturation temperature (T_d) of WPI was 74.5°C for a 10% WPI/water system at pH 5.75. Bernal and Jelen (1985) reported a WPC denaturation temperature range of 75.9-81.2°C depending on the pH, heating rate, and relative proportion of β-lg; and Paulsson and Dejmek (1990) showed that the whey proteins α-la and β-lg denatured at temperatures of 66.9 and 76.6 °C, respectively. The shoulders of the DSC thermogram (Figure 5.2) for the 10% WPI solution reported in this study were observed also by Relkin *et al*. (1998) and they proposed that these shoulders might correspond to the heat transitions of minor components in WPI, such as BSA, lactoferrin, and α-la. Note that the biggest shoulder found in this study occurred at T_{onset} and T_{peak} for denaturation at 58.19 and 64.53°C, respectively. These temperatures were in good agreement with the denaturation temperatures of α-la; 59-62°C and 66.9°C reported by Bernal and Jelen (1985) and Paulsson and Dejmek (1990), respectively.

The DSC thermograms of normal and waxy rice starches, on the addition of 5% and 10% WPI, showed two endothermic transitions (Figure 5.2 and Figure 5.3). The first transition temperature was identical to that of normal rice starch and thus probably corresponds to the gelatinization of normal starch with values of T_{onset} and T_{peak} that were virtually identical to that of a pure starch solution. The second DSC transition represents the endothermic transition of β-lg. They also showed an overlap between the first and second endothermic transitions. T_{onset} for the denaturation of β-lg is about 72.5°C and T_{peak} for the 5 and 10% WPI addition levels were 77.91^{o}C and 75.95°C, respectively. This latter value is identical to the T_{peak} value for a pure 10% WPI system. The drop in the T_{peak} value of the second endorthermic transition with increasing WPI concentration can probably be attributed to a WPI concentration effect, rather than any influence from the starch.

Fitzsimons *et al*. (2007) reported for WPI at pH 7.0 in an NaCl solution that the peak transition temperature increased with decreasing WPI concentration. Moreover, Relkin

(2004) found that when the concentration of WPI in skimmed milk permeate decreased, the endothermic transition shifted to a higher temperature.

These results tend to indicate that the thermal behaviour of normal rice starch was not affected by the addition of WPI and the denaturation of β-lg was not affected by normal rice starch. This finding is in agreement with results from a study of Aguilera and Rojas (1996), who reported that the thermal behaviour of cassava starch was not influenced by the addition of WPI and that whey proteins' thermal behaviour was not modified by starch. They proposed that the thermal behaviour of WPI/cassava starch solution of 10% total solid might be considered as typical of pure WPI or pure cassava starch.

WPI influenced the thermal properties of waxy rice starch, in complete contrast to the effect on normal rice starch. The addition of 10% WPI increased the T_{onset} of waxy rice starch by 1°C. The T_{peak} of waxy rice starch was also significantly increased on the addition of 5% and 10% WPI to 2.75°C and 3.15°C, respectively. Moreover, there was a shoulder with a peak temperature at 64.55°C on the addition of 5% WPI and 64.61°C on the addition of 10% WPI to waxy rice starch but there was no sign of this peak in normal rice starch. The difference between the thermal behaviour on the addition of WPI to normal and waxy rice seen here might be due to the difference in their swelling and water uptake behaviours. The temperature at which waxy rice starch granules imbibe water and swell (from chapter 3; T_{onset} and T_{peak} of pasting are 63.59°C and 69.25°C, respectively) is concomitant with the denaturation temperature of α-la. The swelling of waxy rice starch granules holding a large amount of water results in an increase in the local concentration of α-la, significant enough to produce the observed denaturation peak; at 64.55°C and 64.61°C on the addition of 5% and 10% WPI, respectively. The pasting temperature of normal rice starch (from chapter 3; T_{onset} and T_{peak} of pasting are 67.66°C and 84.80°C, respectively) being higher than the denaturation temperature of α-la means the amount of water imbibed by normal rice starch granules is not large enough to increase the local concentration of α-la before its denaturation temperature. Thus it does not display a noticeable denaturation peak in normal rice starch mixtures.

The increase in T_{onset}, and T_{peak} of waxy rice starch in the presence of WPI and also in the presence of MPC might be due to the effect of the denaturation of α-la. The competition for water between waxy rice starch gelatinisation and α-la denaturation result in a slight increase in the starch gelatinisation temperature. This is in agreement with Lupano and Gonzalez (1999) who studied the thermal behaviour of a mixture of 7.5% or 10% whey protein concentrate (WPC) and cassava starch at pH 4.2 and found that the cassava starch gelatinization temperature increased on the addition of WPC. They explained these anomalies by the fact that the WPC proteins reduced the availability of water for the gelatinization of starch.

Waxy rice starch had no affect on the thermal behaviour of β-lg. The T_{peak} for the denaturation of β-lg was 77.12°C and 75.05°C in 5 and 10% WPI, respectively (Figure 5.3). From Table 5.2, it is clear that the addition of 5% WPI had no affect on the ΔH value of waxy rice starch but an increase in concentration of WPI to 10% had the effect of increasing ΔH to 26.06 J/g, where ΔH represents the heat required to both gelatinize the starch and denature the β-lg.

5.5 Summary to chapter

The addition of the different milk protein ingredients; SMP, MPC, NaCAS, WPI, and UFSMP affected the thermal behaviour of normal rice starch and waxy rice starch. SMP strongly influenced the thermal behaviour of normal and waxy rice starches by increasing the gelatinization temperature. This was confirmed by images from polarized light microscopy which showed that SMP delayed the loss of birefringence in both rice starches. The lactose and salts in SMP are most likely responsible for the modification of rice starch thermal behaviour as UFSMP comprising lactose and salts from SMP, showed a similar effect as the addition of SMP. The addition of MPC did not affect the gelatinization temperature of normal rice starch, but clearly increased the gelatinization temperature of waxy rice starch. In normal rice starch, the addition of NaCAS did not affect T_{onset} but increased T_{peak}, while waxy rice starch was highly affected by it with both T_{onset} and T_{peak} shifting to higher temperatures. The addition of WPI in both rice starches exhibited two transitions due to starch gelatinization and the denaturation of β-lg. In normal rice starch, the thermal behaviour of starch and protein was shown to be independent of each other but not in waxy rice starch where both T_{onset} and T_{peak} were increased. In general, the addition of 10% milk protein ingredients had more effect on the thermal behaviour of waxy than normal rice starch.

In all cases there was a good correlation between the onset of gelatinisation and the onset of pasting. It was also clear that there was very little agreement between peak DSC thermogram temperatures and peak viscosity temperatures. This could be in part be due to the differences in application of shear in the two measurement systems but was most likely due to the different physical processes being measured by each technique.

Overall it is clear that plasticisation by milk sugars and effects of milk salts can explain some of the differences in pasting behaviour of rice starch with added milk protein ingredients. It can not, however, explain the observed differences in peak viscosity or rate of viscosity increase. It is possible that the proteins in the system are affecting the rate of swelling of the starch granules. This is the focus of the following chapter.

Chapter 6

SWELLING BEHAVIOUR OF STARCH/MILK PROTEIN INGREDIENTS MIXTURES

6.1 Introduction

The results presented in the previous chapters showed that the addition of milk protein ingredients affected the physico-chemical behaviour of normal and waxy rice starches markedly and differently. SMP strongly influenced the pasting and thermal behaviour of normal and waxy rice starches by increasing the pasting and gelatinization temperatures. This was mainly due to the lactose and ions present in SMP. MPC and WPI did not have as strong an affect as SMP on the T_{onset} of pasting and gelatinisation of the starches. However, the whey proteins in MPC and WPI, which have the ability to form a gel, displayed far more complicated interactions, such as dramatically increasing the pasting viscosity of the rice starch/MPC or WPI mixtures than SMP. These increases in pasting viscosity were not as great with MPC as with WPI. At high concentrations of WPI the G^* of rice starch/WPI gels markedly increased as a result of the heat-induced aggregation of the whey proteins. NaCAS exhibited less understood effects on the pasting and gelatinisation of rice starch. Compared to SMP, NaCAS showed less effect on the T_{onset} of both pasting and gelatinization but the effects were more pronounced once the onset of gelatinisation of the starches had commenced even to the end of the pasting stage. The T_{peak} of rice starch pasting and gelatinisation shifted to a higher temperature on the addition of NaCAS.

It is clear that the physico-chemical behaviours of rice starch were affected by the presence of the milk protein ingredients. But how the milk protein ingredients altered these physico-chemical behaviours of rice starch is not yet known.

Gelatinization has been known to be initiated by moisture, which is initially taken up by the amorphous region of the starch granule and then by the crystalline regions once the crystalline regions have been disrupted (Donovan, 1979; Lund, 1984; Biliaderis *et al*., 1986; Donald, 2004). Thus adsorption of water into starch granules is a critical precursor for gelatinisation of starch.

After the melting of the crystalline structure, starch granules rapidly expand their size due to the breakage of the hydrogen bonds in the starch granule crystalline structure. Water molecules become linked by hydrogen bonding to the exposed hydroxyl groups of the amylose and amylopectin molecules, hence starch granules swell and this swelling occurs concomitantly with leaching of granule constituents, predominantly amylose (Steeneken, 1989; Jenkins and Donald, 1998; Bao and Bergman, 2004; Choi and Kerr, 2004). The swelling and the leaching of starch molecules from the starch granules are responsible for the rheological behaviours of starch pastes during heating (Choi and Kerr, 2004)

A number of studies have reported a strong relationship between swelling, the leaching out of the starch molecules (amylose and/or amylopectin) during pasting, and the rheological behaviour of starch granules (Miller *et al*., 1973; Bagley and Christianson, 1982; Cheer and Lelievre, 1983; Lelievre and Husbands, 1989; Kelly *et al*., 1995; Appelqvist and Debet, 1997; Matser and Steeneken, 1997; Choi and Kerr, 2004; Bertolini *et al*., 2005). It is hypothesied that the addition of milk proteins alters the swelling, and/or leaching out of starch molecules, and thereby affects the pasting, gelatinisation and rheological behaviours of the rice starch. The aim of the current chapter was to study the mechanism(s) responsible for the observed effects. Therefore in this chapter, the swelling of starch granules and the leaching of starch molecules (amylose and amylopectin) from normal and waxy rice starch as affected by the milk protein ingredients are investigated. Furthermore, a simple model relating the swelling to the viscosity of the mixtures is also proposed.

6.2 Materials and Methods

6.2.1 Materials

The details on normal rice starch, waxy rice starch, milk protein ingredients (SMP, MPC, NaCAS, WPI), and UFSMP are given in section 3.2.1.

6.2.2 Methods

6.2.2.1 Swelling measurement

An elegant and simple centrifugation method, based on a theory proposed by Hemar and Horne (1998), was developed for measuring swelling of the starch using very small samples. Stock solutions (5 % and 10%) of milk protein ingredients were made as described in section 3.2.2. The rice starch/milk protein ingredient mixtures were pasted using a stress-controlled rheometer (Parr Physica UDS 200; Physica, Stuttgart, Germany) with the starch cell geometry TC 20, as described in section 3.2.3.2. The apparent viscosity of the rice starch/milk protein ingredient mixtures was recorded during the pasting process. Sample aliquots (250 μL) were collected from the rheometer every 2°C (from 50°C to 95°C) and transferred to a heparinized micro-hematocrit capillary tube; 75 mm long and 1 mm internal diameter (Oxford Labware, St. Louis, MO, USA), to fill approximately two third of the capillary tube. One end of the capillary was flame sealed, as the swelling of starch is a dynamic process, hence this sealing step was done in a short time with great care to prevent heat transfer to the sample inside the heparinized micro-hematocrit capillary tube. The capillary was then centrifuged at 12,000 *g* for 10 minutes using a Haemofuge microcentrifuge (Heraeus-CHRIST, Osterode, Germany).

The swelling ratio ϕ is defined here as:

$$\phi = x/y \qquad \text{Equation 6.1}$$

where; *x* is the height of the sediment in the capillary tube after centrifugation and *y* is the total height of the sample in the capillary (Figure 6.1). The X and Y values were determined as follows. The centrifuged capillary tubes were scanned (Epson Perfection 3200 Photo, Seiko Epson Corporation, Nagano, Japan); the scanner software was the Epson software package provided with the scanner. The generated images were used to obtain the values of *x* and *y* using Microsoft Photo Editor 3.0.2.3 (Microsoft Corporation, USA). The onset temperature of the swelling can be obtained from the plot of the swelling ratio as a function of temperature similar to the method described in section 3.2.3.2.

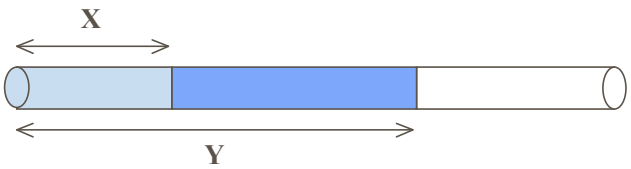

Figure 6.1 Schematic representation of the capillary tube containing starch/milk protein sample after centrifugation. X is the height of the sediment and Y is the total height.

6.2.2.2 Total starch and amylose/amylopectin measurements

For the quantification of leached total starch and amylose/amylopectin, the starch/milk protein ingredient mixtures were prepared and pasted as described in section 3.2.2 and 3.2.3.2, respectively. For each study temperature (every 2°C from 50°C to 95°C), the mixtures were heated in a stress-controlled rheometer (Parr Physica UDS 200; Physica, Stuttgart, Germany) and heating was stopped when the sample reached a certain temperature. The sample (2 g) was collected and centrifuged at 2000 *g* for 10 min at 20°C using an Eppendorf centrifuge (5417R Eppendorf, Germany). 1 ml of the supernatant was used to determine the amount of amylose/amylopectin and total starch that had leached from the starch granules. An amylose/amylopectin assay kit (Megazyme, Wicklow, Ireland), based on the method developed by Gibson *et al*. (1997), was used for the determinations. The measured amount of leached total starch and amylose/amylopectin are given as mg per g of starch (dry basis).

6.2.2.3 Determination of the onset temperature at the early stage of swelling and apparent viscosity, using the swelling measurements

The onset temperature for swelling at the early stage (T_S) of normal or waxy rice starch/ milk protein ingredient mixtures was determined from a curve of the swelling ratio (ϕ) as a function of temperature. The T_S is defined here as the temperature at which ϕ started to increase. Thus T_S is different from the onset temperature of swelling determined in section 6.2.2.1, which was obtained from the swelling curve as a function of temperature.

The onset temperature at the early stage obtained from the apparent viscosity is also defined as the temperature at which the apparent viscosity first started to increase. There are 2 types of onset temperature at the early stage for apparent viscosity; the value obtained by the rheometer (T_R) and the value which was calculated by using equation 6.3 (T_{CS}).

6.2.3 Statistical analysis

All statistical analyses were performed as described in 3.2.4. The total amount of leached starch and amylose/amylopectin leaching from the various treatments were performed in duplicate. The swelling measurements were performed also in duplicate, and two capillaries were centrifuged for each sample. The data are presented as mean ± standard deviation (SD) and the significance was accepted at the 5% confidence level.

6.3 Results

6.3.1 Normal and waxy rice starch in water mixtures

6.3.1.1 Swelling behaviour

The swelling ratio and the apparent viscosity of a normal rice starch/water and a waxy rice starch/water mixture as a function of temperature is reported in Figure 6.2. The swelling ratio of normal or waxy rice starches/water mixtures remained mostly constant until the mixture reached a specific temperature, when the granules started to swell. The initial swelling was slight, but then increased steeply over a narrow temperature range to reach a maximum value of ϕ of 1. This maximum value occurred when the capillary tube was completely filled by the sediment. This sediment might contain fully swollen granules or disrupted/dissolute granules that formed a paste. In both cases, this indicated that water could not be separated from the paste by the centrifugation method used in our experiment. Note also that the initial value of ϕ was approximately 0.16. Although normal rice started to swell at a slightly higher temperature than waxy rice, swelling occurred over a greater temperature range for normal rice (59.8 to 82.8°C, i.e. 23°C) than waxy rice (58.6 to 68.4°C i.e. 9.8°C). The swelling of both normal and waxy rice starches clearly occurred before the apparent viscosity started to increase. In fact, at the T_{onset} measured by the rheometer, ϕ for waxy rice was approximately 0.43 and 0.48 for normal rice starch (Figure 6.2).

6.3.1.2 Total starch and amylose leaching from rice starch/water mixtures

The amount of total starch and amylose leached out of the rice starches/water mixtures at 50, 58.3, 62.3, 64.4, 66.5, and 68.5°C for normal rice starch and 50, 56.2, 60.3, 62.4, 64.4, and 66.5°C for waxy rice starch is reported in Figure 6.3. The amount of total starch and amylose leached out from the granules at each temperature for both normal and waxy rice starch followed the swelling results. Up to a certain temperature, no starch was detected in the continuous phase, and then an increasing amount of total starch and amylose were detected as the temperature was increased. For normal rice starch, the total starch leached at 58.3°C was 0.56 mg/g starch, and increased to 8.15

mg/g starch when the temperature was increased to 68.5°C. Similar to total starch, the amount of amylose leached out at 58.3°C was only 0.12 mg/g and then increased up to 3.92 mg/g starch at 68.5°C. For waxy rice starch, there was 0.85 mg of total starch per gram of starch leached at 56.2°C, and 7.22 mg at 66.5°C. Although the original amount of amylose present in waxy rice starch is very small (3.25%), only 0.71 mg of amylose per gram of starch leached out from waxy rice starch granules at low heating temperatures (≤56.2°C). The amount of amylose leached increased with temperature, at 66.5°C there was 1.47 mg of amylose leached per gram starch, i.e. 4.52% of the total amylose content of the starch granules.

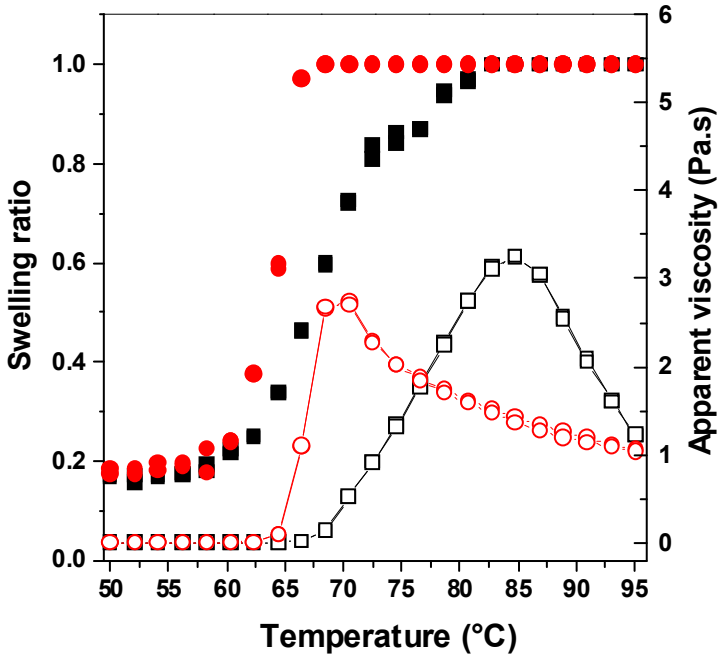

Figure 6.2 Swelling ratio (solid symbols) and apparent viscosity (open symbols) for 10% normal rice starch/water mixture (■,□) and 10% waxy rice starch/water mixture as a function of temperature (●,○).

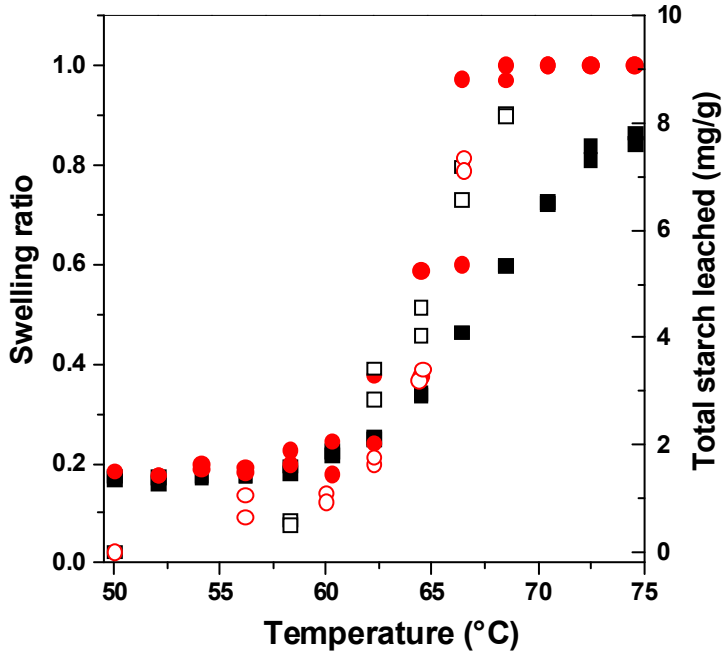

Figure 6.3 Swelling ratio (solid symbols) and total starch leached (open symbols) for 10% normal rice starch/water mixture (■,□) and10% waxy rice starch/water mixture as a function of temperature (●,○).

6.3.2 Normal rice starch/milk ingredients mixtures

6.3.2.1 Swelling behaviour

The swelling ratio and the apparent viscosity of normal rice starch/milk protein ingredients mixtures as a function of temperature are shown in Figure 6.4.
On the addition of 5% and 10% SMP T_{onset} of swelling increased by 1.4°C and 2.2°C, respectively. The addition of UFSMP also increased the T_{onset} of swelling by 1.4°C and 2.2°C when 5 and 10% UFSMP were added, respectively. The temperatures at which the swelling ratio reached the maximum plateau values (the final phase of swelling) decreased from 82.8°C to 80.7°C and 76.6°C when 5 and 10% SMP were added, respectively.

Whilst MPC had no effect on the T_{onset} of swelling of normal rice starch (59.4°C and 60.0°C for 5 and 10% MPC, respectively) it had a marked effect on the degree and rate of swelling of normal rice starch. The rate of swelling was markedly increased when both 5 and 10% MPC were added. The temperature for the maximum plateau was 72.5°C for the addition of 5% MPC and 68.5°C for 10% MPC.

The addition of 5 and 10% NaCAS to normal rice starch showed a similar effect to normal rice starch in water for the early stage of swelling. The T_{onset} of swelling increased by about 0.8°C and 1.3°C on the addition of 5 and 10% NaCAS, respectively. However, 10% NaCAS markedly slowed the rate of swelling compared to starch alone or starch/SMP mixtures and the swelling ratio of normal rice starch reached maximum plateau values at 82.8°C and 72.5°C for the addition of 5 and 10% NaCAS, respectively. Note that, the peak viscosity of normal rice starch decreased when 5% NaCAS was added but increased on the addition of 10% NaCAS. Note also that the apparent viscosity was not detectable until ϕ was between 0.43 and 0.48 for all protein ingredient/starch systems.

The addition of 5 and 10% WPI to normal rice starch did not alter T_{onset} of swelling; 59.9°C and 59.8°C for 5 and 10% WPI, respectively. The swelling of normal rice starch was sharply increased with temperature when both 5 and 10% WPI were added. The

temperatures at which the maximum plateau value of swelling was reached were decreased to 74.6°C and 72.5°C when 5 and 10% WPI respectively were added.

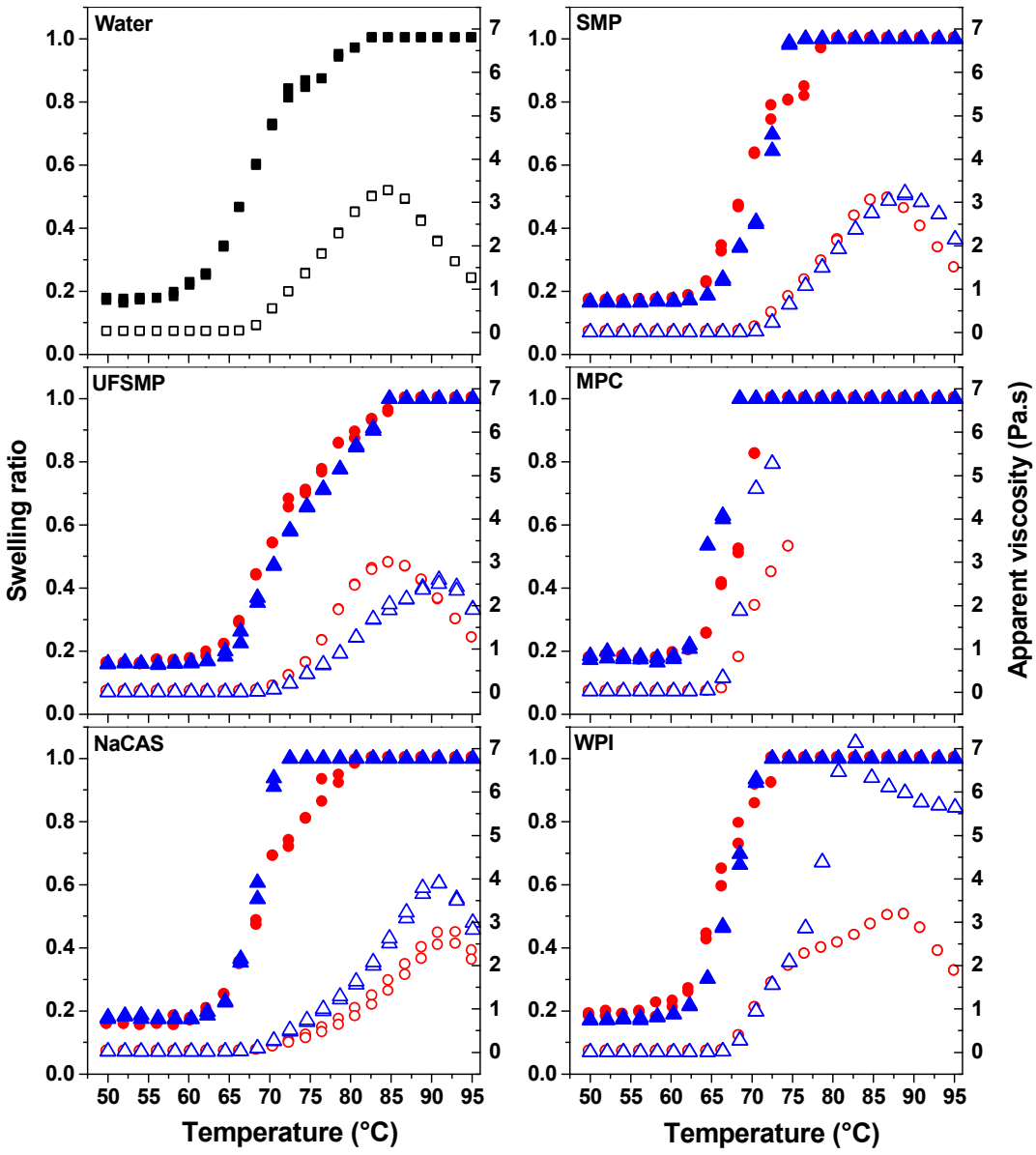

Figure 6.4 Swelling ratio (solid symbols) and apparent viscosity (open symbols) for normal rice starch/water mixture and normal rice starch/milk protein ingredients mixtures as a function of temperature. Concentration of milk protein ingredients: 5% (●,○) and 10% (▲,△).

6.3.2.2 Total starch and amylose leaching from normal rice starch/milk protein ingredient mixtures

The amount of total starch and amylose leached out of normal rice starch/milk protein ingredients mixtures were measured at the same temperature as that for the swelling behaviour (50, 58.3, 62.3, 64.4, 66.5, and 68.5°C). The amount of amylose leached out of normal rice starch/milk protein ingredients mixtures is shown in Table 6.1. The temperatures at which amylose and amylopectin, and thus total starch, leached out of the starch granules in normal rice starch/milk protein ingredients mixtures was similar to those reported for their swelling behaviour (Figure 6.5) and the trends were very similar to those reported for normal rice/water mixtures, i.e., starch was not detected until some minimum temperature that appeared to depend on the milk protein ingredient used, and then there was a rapid increase in the amount of solubilised starch and this too was affected by milk ingredient. The milk ingredients also had differing effects on the total amount of starch solubilised.

The addition of SMP and UFSMP to normal rice starch significantly decreased the amount of total starch leached out. On the addition of 10% SMP and UFSMP, there was no detectable starch in solution until the starch/protein ingredient mixture reached 58.3°C. Even at 62.3°C the total amount of starch in solution was only 0.83 and 0.68 mg/g starch at 10% SMP and UFSMP, respectively. At 68.5°, the addition of 5 and 10% SMP to normal rice starch had decreased the total amount of starch that had leached out from 8.15 mg/g starch to 6.69 and 5.82 mg/ g starch, respectively. The amount of amylose too that leached out was decreased by the addition of SMP and UFSMP. The amount of amylose leaching at 68.5°C decreased from 3.92 mg/g starch in the absence of milk protein to 2.91, 2.83, 3.07 and 2.66 mg/g starch on the addition of 5 and 10% SMP and 5 and 10% UFSMP, respectively.

The addition of 5% MPC to normal rice starch also decreased the total amount of starch leached out of the granules to 0.44 and 6.87 mg/g starch at 58.3^{o}C and 68.5°C, respectively. At 68.3°C, the amount of amylose leaching out was 3.37 mg/g starch.

NaCAS also reduced the amount of amylose and total starch that leached from the granules. Starch was first detected in solution at 58.3°C, and the amounts of solubilised total starch were 0.33 and 0.32 mg/g starch for 5 and 10% NaCAS, respectively. At 68.5°C, the amount of total starch in solution increased to 6.31 and 5.19 mg/g starch for 5 and 10% NaCAS, respectively. Note that, the addition of 10% NaCAS significantly decreased the amount of amylose leaching from normal rice starch. At 68.5°C, there was only 1.72 mg of amylose per g of starch leaching out from starch granules.

The amount of total starch leached out of normal rice starch on the addition of 5 and 10% WPI was less than in the absence of milk protein ingredient except on the addition of 10% WPI at 68.5°C, which showed a higher total starch leachate value at 11.19 mg/g starch. However, at temperatures <66.5°C the addition of 10% WPI decreased the leaching of both total starch and amylose. Moreover, WPI inhibited starch molecule loss until the temperature reached 58.3°C and it was not until the temperature had reached 62.3°C that the first measurable quantity of leachate could be determined (1.02 mg/g starch).

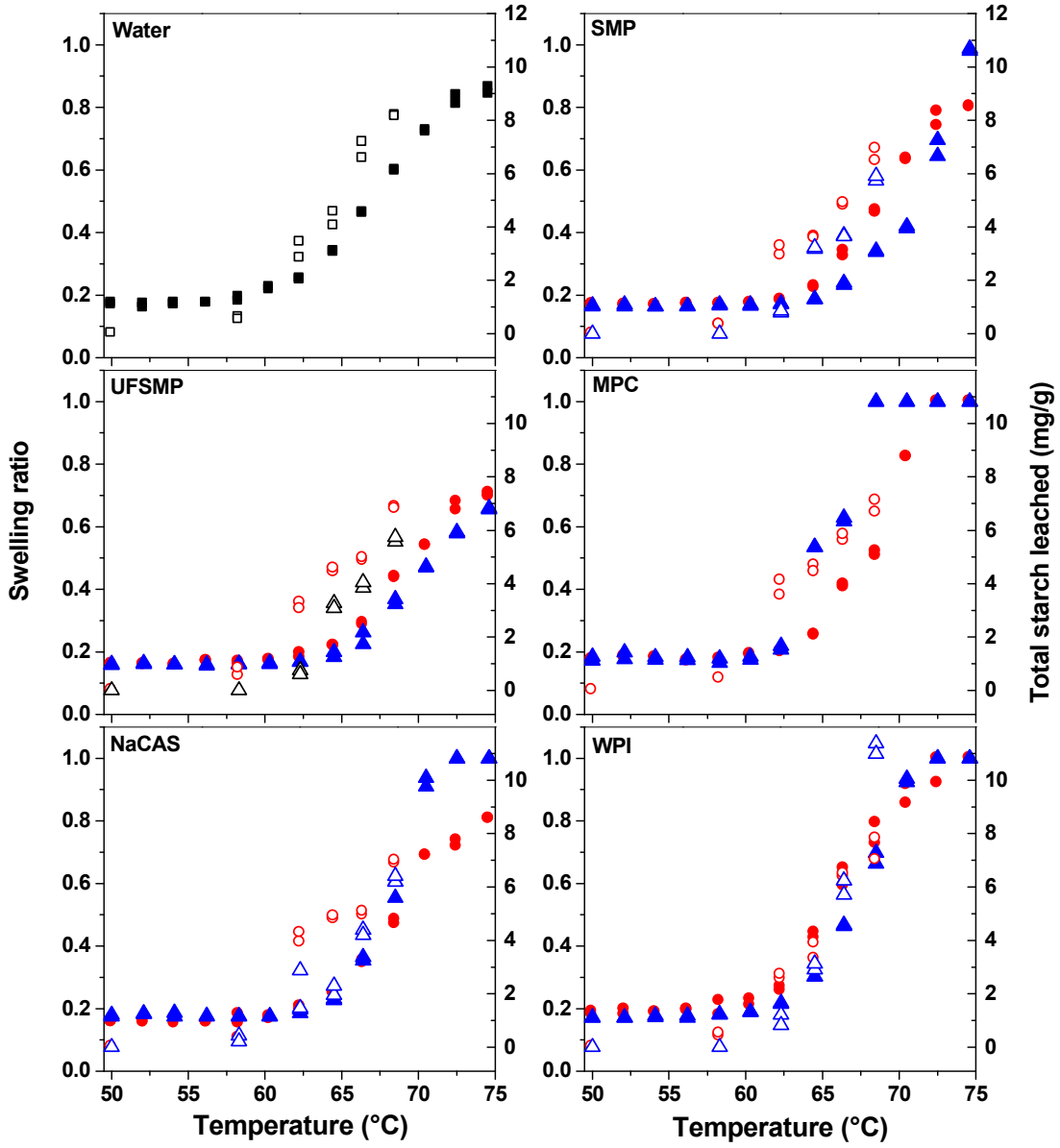

Figure 6.5 Swelling ratio (solid symbols) and total starch leached (open symbols) for normal rice starch/water mixture and normal rice starch/milk protein ingredients mixtures as a function of temperature. Concentration of milk protein ingredients: 5% (●,○) and 10% (▲,△).

Table 6.1 Amylose leached (mg/g starch) for normal rice starch/water mixture and normal rice starch/milk protein ingredient mixtures at various temperatures. The values of the duplicate measurements are given, with their mean and standard deviation given inside the brackets.

Dairy ingredient	Conc. (%)	Temperature (°C) [*,**]					
		50ns	58.3	62.3	64.4	66.5	68.5
None	0	0.00±0.00	0.12±0.00fg	2.71±0.13a	2.58±0.04bc	4.24±0.00b	3.92±0.09a
SMP	5	0.00±0.00	0.33±0.01cde	2.59±0.02a	2.52±0.18bc	2.74±.03c	2.91±0.12cd
	10	0.00±0.00	0.00±0.00g	0.80±0.05f	2.43±0.003c	2.39±0.02e	2.83±0.23de
UFSMP	5	0.00±0.00	0.48±0.16a	2.34±0.02b	2.70±0.07b	2.85±0.04c	3.07±0.14c
	10	0.00±0.00	0.00±0.00g	0.40±0.11g	2.00±0.07e	2.27±0.02e	2.66±0.02e
MPC	5	0.00±0.00	0.37±0.02abc	2.08±0.21c	2.93±0.17a	2.74±0.08c	3.37±0.11b
	10	0.00±0.00	0.19±0.04ef	1.10±0.04e	1.96±0.12f	2.45±0.16de	2.88±0.04cd
NaCAS	5	0.00±0.00	0.22±0.00def	1.64±0.02d	2.41±0.04c	2.65±0.07cd	3.02±0.09cd
	10	0.00±0.00	0.29±0.17cde	0.94±0.00ef	1.16±0.01g	1.56±0.34f	1.72±0.10f
WPI	5	0.00±0.00	0.33±0.04bcd	1.88±0.23d	2.01±0.07de	3.82±0.16a	3.82±0.03a
	10	0.00±0.00	0.11±0.00fg	0.76±0.29f	2.00±0.19e	3.60±0.18a	3.99±0.16a

[*] Mean value ± SD (n ≥ 2).

[**] Different letters within the same column indicate significant difference at $P < 0.05$ different through the Duncan test.

6.3.3 Waxy rice starch/milk ingredients mixtures

6.3.3.1 Swelling behaviour

The swelling ratio and the apparent viscosity of waxy rice starch/milk protein ingredients mixtures as a function of temperature are reported in Figure 6.6.

Compared to a waxy rice starch/water mixture, SMP increased the T_{onset} of swelling by 1.5°C and 3.3°C on the addition of 5 and 10% SMP, respectively. The temperatures at which the swelling ratio of waxy rice starch reached the maximum plateau values for the final phase of swelling was not affected by the addition of 5% SMP, but increased to 70.5°C on the addition of 10% SMP. UFSMP also increased the T_{onset} of starch swelling; an increase of 1.9°C, and 3.2°C for 5 and 10% UFSMP, respectively compared to starch alone. UFSMP at 5% addition, like SMP, had no effect on the temperature at which the starch reached a value of $\phi = 1$, but the addition of 10% UFSMP increased this temperature to 72.5°C.

The addition of 5% MPC had no affect on either the T_{onset} of swelling or the temperature at which the waxy rice starch granules reached their swelling maxima. However, 10% of MPC decreased the temperature at which maximal swelling was observed (66.4°C), even though the higher MPC concentrations failed to change the onset swelling temperature.

NaCAS slightly increased the T_{onset} of swelling for waxy rice starch; an increase of 0.9°C and 1.3°C on the addition of 5 and 10% NaCAS, respectively compared to waxy rice alone. However, the temperature at which swelling reached its maximum plateau values was not affected by the addition of NaCAS.

As with NaCAS, the addition of WPI to waxy rice starch slightly increased the T_{onset} of swelling by 1.1°C and 1.5°C for 5 and 10% WPI, respectively. The temperature at which the maximum plateau value of swelling was reached was not affected by the addition of 5% WPI, but increased to 70.5°C on the addition of 10% WPI.

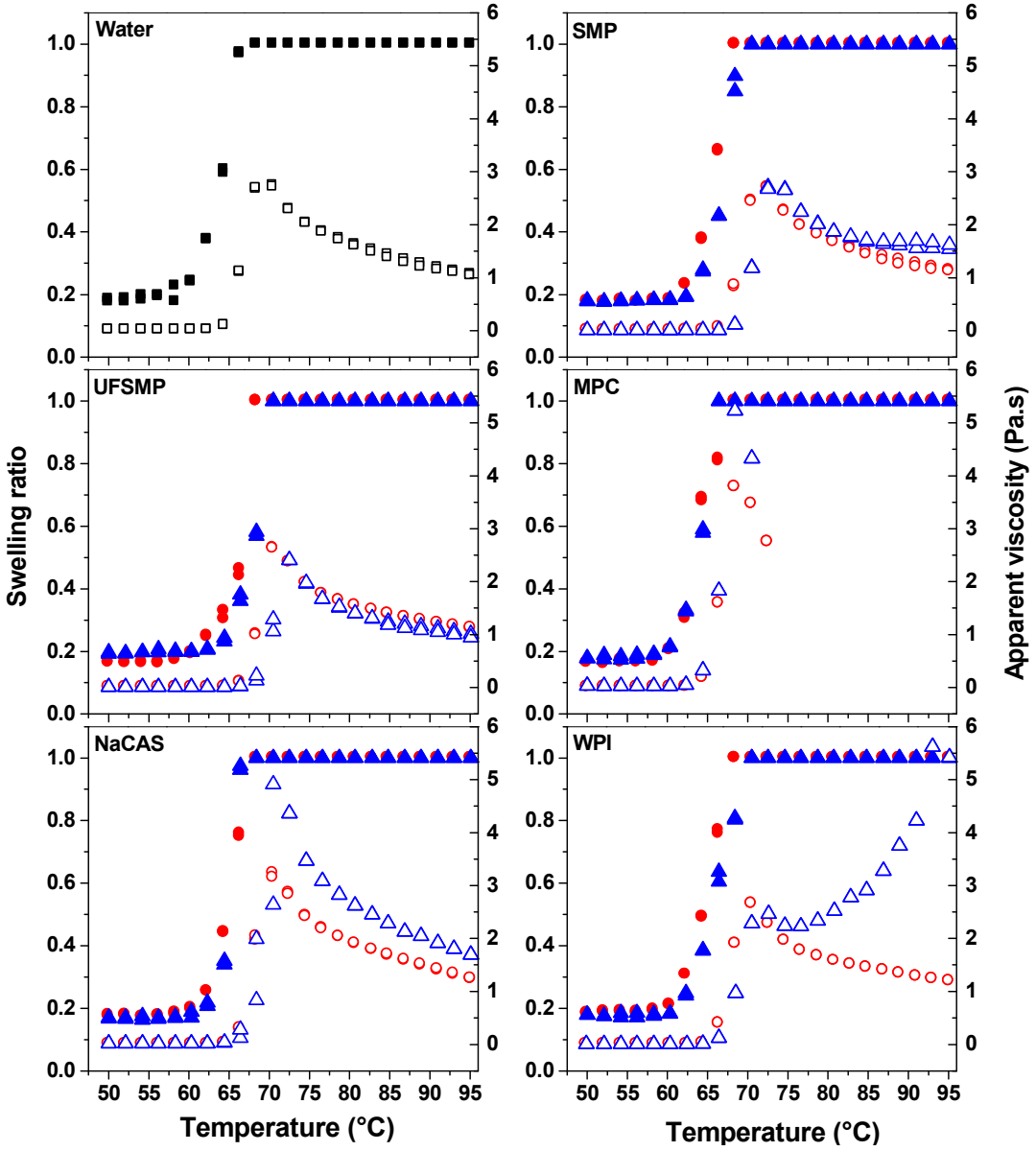

Figure 6.6 Swelling ratio (solid symbols) and apparent viscosity (open symbols) for waxy rice starch/water mixture and waxy rice starch/milk protein ingredients mixtures as a function of temperature. Concentration of milk protein ingredients: 5% (●,○) and 10% (▲,△).

6.3.3.2 Total starch and amylose/amylopectin leached from waxy rice starch/milk protein ingredient mixtures

The amount of total starch and amylose leached out of waxy rice starch/milk protein ingredients mixtures were measured at the same temperatures that were used in the swelling experiments (50, 56.2, 60.0, 62.3, 64.4, and 66.5°C). The amount of amylose that was leached out of waxy rice starch/milk protein ingredients mixtures is shown in Table 6.2. The swelling ratio and total starch leached as a function of temperature for waxy rice starch/milk protein ingredient mixtures is reported in Figure 6.7. It is clear from the respective figures that the loss of amylose and amylopectin closely followed the swelling behaviour of the starch granules for each of the dairy ingredients, which was not the case for some of the normal rice starch/dairy ingredient mixtures.

As with normal rice starch/milk ingredient, no starch was detected in the centrifugate until a certain temperature, but then total starch and amylose leached from the granules increased with increasing temperature for waxy rice starch and milk protein ingredient mixtures. The addition of 10% SMP significantly decreased the total starch leached from waxy rice starch. At 66.5°C the total starch leached out was only 3.06 mg/g of starch when 10% SMP was added compared to 7.22 mg/g of starch in the absence of SMP.

The addition of 5% MPC decreased the amount of total starch leaching from waxy rice starch if the temperature of heating was ≤ 60.3°C (0.25 and 0.96 mg/g starch for 56.2 and 60.3°C, respectively). However, at temperatures >60.3°C, e.g. 66.5°C, the total amount of starch leached from waxy rice starch was not decreased by the addition of 5% MPC (7.54 mg/g starch). Like SMP the addition of 5% MPC resulted in an increased loss of amylose.

The addition of 5 and 10% NaCAS tended to decrease both the amount of total starch and amylose leaching from waxy rice starch granules. The amount of total starch and amylose that leached from the granules seemed to decreases with increasing NaCAS concentration. At 66.5°C there were 6.28 and 6.20 mg/g of total starch lost from the

granules on the addition of 5 and 10% NaCAS, respectively. The amounts of amylose that leached out at 66.5°C were 1.06 and 0.97 mg/g starch for the addition of 5 and 10% NaCAS, respectively.

The addition of WPI significantly decreased the amount of total starch leached from the granules. The total starch leached at 66.5°C was 6.06 and 5.87 mg/g for 5 and 10% WPI, respectively. The amount of amylose leached at 66.5°C was also decreased by the addition of WPI (1.00 and 1.06 mg/g starch on the addition of 5 and 10% WPI, respectively).

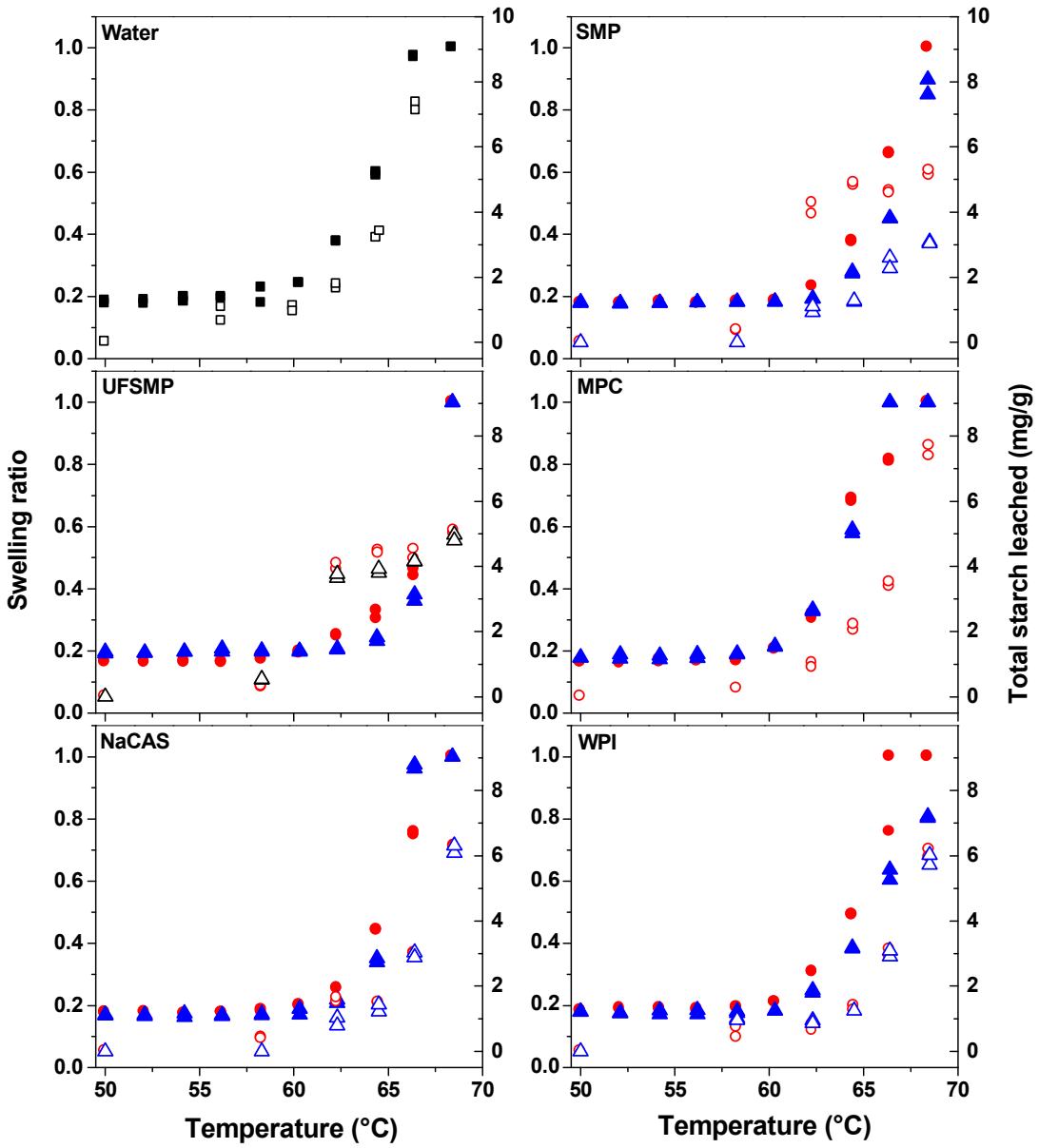

Figure 6.7 Swelling ratio (solid symbols) and total starch leached (open symbols) for waxy rice starch/water mixture and waxy rice starch/milk protein ingredients mixtures as a function of temperature. Concentration of milk protein ingredients: 5% (●,○) and 10% (▲,△).

Table 6.2 Amylose leached (mg/g starch) for waxy starch/water mixture and waxy rice starch/milk protein ingredient mixtures at various temperatures. The values of the duplicate measurements are given, with their mean and standard deviation given inside the brackets.

Dairy ingredient	Conc. (%)	Temperature (°C) [*, **]					
		50ns	56.2	60.3	62.4	64.4	66.5
None	0	0.00±0.00	0.71±0.25b	0.81±0.09bc	0.70±0.01e	0.77±0.12d	1.47±0.03ab
SMP	5	0.00±0.00	0.15±0.01de	1.40±0.12a	1.40±0.14a	1.15±0.15bc	1.39±0.22ab
	10	0.00±0.00	1.31±0.29a	1.28±0.15a	1.15±0.05bcd	1.15±0.06bc	1.31±0.02bc
UFSMP	5	0.00±0.00	0.12±0.02de	1.28±0.09a	1.22±0.29abc	1.16±0.07bc	1.27±0.00bc
	10	0.00±0.00	0.15±0.00de	0.93±0.02b	1.01±0.02d	1.02±0.03c	1.15±0.07cde
MPC	5	0.00±0.00	0.24±0.01de	0.97±0.13b	1.32±0.08ab	1.39±0.11a	1.53±0.28ab
	10	0.00±0.00	0.18±0.04de	0.88±0.21b	1.06±0.06cd	1.25±0.02ab	1.40±0.08ab
NaCAS	5	0.00±0.00	0.36±0.02cd	0.53±0.05d	0.49±0.01f	0.66±0.01d	1.06±0.02de
	10	0.00±0.00	0.00±0.00e	0.59±0.26cd	0.46±0.02f	0.83±0.01d	0.97±0.09e
WPI	5	0.00±0.00	0.32±0.07d	0.18±0.02e	0.46±0.04f	1.26±0.05ab	1.00±0.02e
	10	0.00±0.00	0.77±0.01b	0.78±0.16bc	0.63±0.05e	0.75±0.03d	1.06±0.13e

[*] Mean value ± SD (n ≥ 2).

[**] Different letters within the same column indicate significant difference at $P < 0.05$ different through the Duncan test.

6.3.4 Viscosity determination at the early stage of swelling from the swelling measurements

The swelling ratio ϕ can be used to estimate the viscosity $\eta(T)$ at different temperatures T during the pasting of rice starch/milk protein ingredient mixtures. If we assume that the milk protein/rice starch mixtures are made up of a suspension of starch granules dispersed in an aqueous solution of milk protein ingredient (because the starch granules are much larger than the milk protein ingredient particles), the milk protein ingredient solution can be considered to be a continuous phase. In this case, the Maron-Pierce relationship can be applied:

$$\eta(T) = \frac{\eta_s(T)}{\left(1 - \frac{\phi_v(T)}{\phi_c}\right)^2} \qquad \text{Equation 6.2}$$

where ϕ_v is the volume fraction of the starch granules and η_s is the viscosity of the continuous phase, i.e. that of the milk protein ingredient in water. As both ϕ_v and η_s are temperature dependent, η_s can be measured using the same temperature profile used during the pasting of milk protein/rice starch mixtures. ϕ_c is the maximum packing fraction of the starch granules. The volume fraction ϕ_v can be obtained from the experimentally measured swelling factor ϕ and the packing fraction ϕ_c of the starch granules in the sediment after centrifugation using the equation below:

$$\phi_v = \phi_c \times \phi \qquad \text{Equation 6.3}$$

Assuming that the fraction in the centrifuged pellet is ϕ_c, and combining equation 6.2 and 6.3, yields:

$$\eta(T) = \frac{\eta_s(T)}{(1 - \phi(T))^2} \qquad \text{Equation 6.4}$$

However, the exact value of ϕ_c is not needed to calculate the viscosity of rice starch/milk protein ingredient mixtures; it can be obtained from the swelling

measurements. The initial value of ϕ is approximately 0.16 for all milk protein/rice starch mixtures before swelling (see Figure 6.4 and 6.6 at 50°C). As the density of rice starch is known to be 1.5 g/cm^3 (Willett, 2001), the exact volume fraction occupied by the starch was 10%/1.5 = 6.7%, because the starch was added at a weight fraction of 10%. Thus the packing fraction ϕ_c of rice starch was approximately 0.067/0.16 ≈ 0.42. This value is in very good agreement with the value of 0.45 obtained by Willett (2001) for rice starch, using a different method.

In addition to the determination of the swelling ratio ϕ, determination of the viscosity of the continuous phase η_s for each milk protein ingredient is required in order to use equation 6.4. The viscosities of the milk protein ingredient solutions (10%, w/w) were measured using the same heating profile as that used for the starch dispersions and the results have been reported in Figure 3.2 (section 3.3.1). The viscosities of the NaCAS and SMP solutions decreased with increasing temperature, as was the case with water (open symbols). The viscosity of the WPI solution also decreased up to 72°C, and then started to increase due to the heat denaturation and aggregation of the whey proteins present in WPI (Paulsson and Dejmek, 1990). Note that the values for the viscosity obtained for water were larger than expected. This was due to the complex design of the stirrer and the wide gap between the stirrer blades and the cup used in starch geometry, and to the limitation of rotational viscometers to measure low viscosities.

Using the swelling ratios ϕ of normal rice starch and waxy rice starch in water (Figure 6.2) and the viscosity of water measured separately (Figure 3.2, open symbols); the viscosities of the starch dispersions were calculated using equation 6.4. The calculated and measured viscosities as a function of temperature for normal rice starch and waxy rice starch are reported in Figure 6.8.

The same analysis was performed on the different milk protein/rice starch mixtures, using equation 6.4, the measured swelling ratios ϕ (Figure 6.4 and 6.6) and the viscosities of the different milk protein ingredients (Figure 3.2). The results of the onset temperature at the early stage of swelling for the calculated viscometric onset temperatures (T_{CS}) are reported in Table 6.3, along with the swelling onset temperatures

(T_S) and the measured onset temperatures obtained by viscometry (T_R). It can be clearly seen that, for all the different mixtures studied here (Table 6.3), there was very good agreement between T_{CS} and T_R.

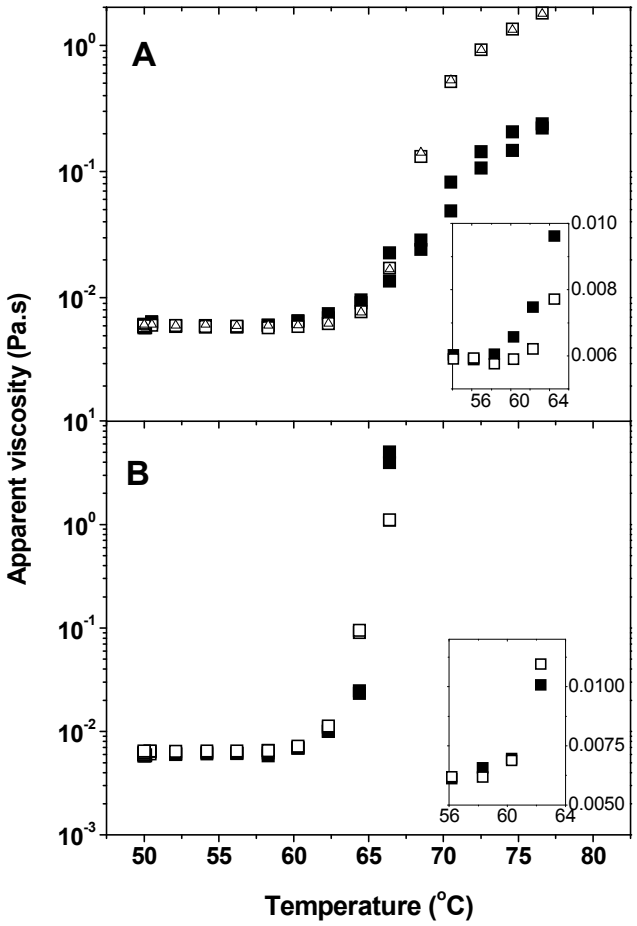

Figure 6.8 Calculated viscosity values at the early stage of the swelling from swelling measurements (solid symbols) and measured viscosity values at the early stage of the swelling (open symbols) for normal rice starch (A) and waxy rice starch (B)

Table 6.3 Onset temperature (°C) at the early stage of rice starch/water mixture and rice starch/10% milk protein ingredients mixtures. T_S is the onset temperature obtained from the swelling measurements, T_{CS} is the onset temperature calculated using equation 6.4 and the swelling measurements, and T_R onset temperature obtained from the rheological measurements.

Dairy ingredient	Normal rice starch			Waxy rice starch		
	T_S	T_{CS}	T_R	T_S	T_{CS}	T_R
None	56.2±0.0	60.3±0.0	60.3±0.0	56.2±1.7	58.3±0.0	58.3±0.0
SMP	60.3±0.0	64.4±0.0	64.4±0.0	60.3±0.0	62.4±0.0	62.4±0.0
UFSMP	60.3±0.0	62.4±0.0	62.4±0.0	59.3±1.2	62.4±0.0	62.4±0.0
MPC	56.2±0.0	60.3±0.0	60.3±0.0	56.2±0.1	58.2±0.1	58.2±0.1
NaCAS	58.3±0.1	60.3±0.0	60.3±0.0	58.2±0.0	59.8±1.0	59.2±1.5
WPI	56.2±0.0	60.3±0.0	60.3±0.0	56.2±0.0	58.3±0.0	58.3±0.1

6.4 Discussion

The swelling ratio (ϕ) versus temperature graphs for all starch/water and starch/milk proteins ingredient mixtures all followed the same basic shape. The granules did not start to swell until a specific temperature that was determined to some extent by the starch used and also the milk ingredient added to the starches. For instance the T_{onset} of swelling for a normal rice starch/water mixture was approximately 1°C higher than that of a waxy rice starch/water mixture. The granules then swelled rapidly with increasing temperature until ϕ reached a plateau value of 1. Waxy rice granules swelled faster than normal rice starch over this critical temperature range. A number of other research groups (Tester and Morrison, 1990a; Tester and Morrison, 1990b; Hermansson and Svegmark, 1996; Vandeputte *et al*., 2003a; Hagenimana and Ding, 2005) had also observed that waxy rice starch granules increased in size more rapidly than normal rice starch. The general swelling results were similar to those of Tester and Morrison (1990a), who reported three phases of starch swelling; an initial phase of slow swelling, a second phase of rapid swelling, and the final phase when maximal swelling was reached. The temperatures range of the three phases of swelling for normal and waxy rice starch/water mixtures is shown in Table 6. 4

It was found that the T_{onset} of swelling for both normal and waxy rice was lower than the T_{onset} of gelatinization (from section 5.3.1, T_{onset} of gelatinization = 62.20°C and 60.26°C for normal and waxy rice starch, respectively). In the initial phase of swelling both starches were observed to swell only slightly. But the rice starch granules swelled rapidly at temperatures above the T_{onset} of gelatinization (the second phase). During gelatinization, the crystalline and molecular order (double helix) of the starch granules are lost concurrently, but the enthalpy of gelatinization results more from the loss of the double helix structure than from loss of crystallinity (Cooke and Gidley, 1992). Therefore, it is possible that the observed rapid increase in swelling in the second phase was mainly due to the loss of the double helical structure of the starch molecules.

Table 6.4 Temperature (°C) for the phase of swelling of normal rice starch/water and waxy rice starch/water mixtures.

Starch	Phase of swelling		
	Initial phase	**Second phase**	**Final phase**
Normal rice starch	59.8°C - 62.3°C	>62.3°C - 82.8°C	>82.8°C
Waxy rice starch	58.6°C - 60.3°C	>60.3°C - 68.4°C	>68.4°C

Although, the swelling of rice starch occurred earlier than the increase in the apparent viscosity a high correlation was found between the swelling and pasting of rice starch, which is discussed in more detail later on.

The swelling behaviour was highly correlated with the leaching behaviour of the two starches. For example a value of R^2 = 0.97, SD = 0.04 (P <0.001), and R^2 = 0.99, SD = 0.04 (P<0.001) was obtained between the two parameters for normal and waxy rice starch, respectively. This result was also in very good agreement with previous studies (Tester and Morrison, 1990a; Tester and Sommerville, 2003). The total starch leached from the starch granules increased with increasing temperature. Both amylose and amylopectin leached out together during the initial phases of swelling, though a greater amount of amylose was found in the leachate. In the case of normal rice starch, amylose accounted for over 85% of the total starch in the leachate during this initial phase, but dropped back to 50% in the second swelling phase. These results are in agreement with the results of Tsia and Lii (2000). For waxy rice starch, which contains a small amount of amylose (only 3.25%), leach out of starch molecules started at 56.2°C, approximately 1^{o}C lower than for normal starch. Moreover, amylopectin accounted for practically most of the leached starch molecules. This trend has also been reported in previous studies on waxy starches (Tester and Morrison, 1990a; Tester and Sommerville, 2003).

The addition of different milk protein ingredients affected the swelling and the leaching of total starch from normal rice starch differently. SMP markedly increased the T_{onset} of

swelling but caused a reduction in the amount of total starch leached from normal starch. To further investigate this effect, the swelling and the leaching of total starch were performed on both rice starches in rice starch/UFSMP mixtures. The results were similar to SMP, UFSMP increased the T_{onset} of swelling and decreased the leaching of total starch from the normal rice starch compared to a normal starch/water mixture. This would suggest that it was the lactose and cations in SMP that were responsible for the fact that SMP increased the T_{onset} of swelling as well as reducing starch molecules leach out compared to rice starch alone in water. However, the various milk proteins cannot be ruled-out from having some effect on the observed influence of SMP on the swelling and the leaching behaviour of the rice starches.

Unlike SMP, MPC had no effect on the T_{onset} of the swelling of normal rice starch. MPC contains all the milk proteins in SMP, but has little lactose in comparison to SMP. The fact that the MPC proteins in greater concentration than in SMP had no noticeable effect would tend to suggest that it was the lactose and cations that influenced the onset swelling behaviour of the starches when SMP was added to the starches. Although MPC did not affect the T_{onset} of swelling it did have a marked affect on the swelling behaviour of normal rice starch. MPC increased the rate of normal rice starch swelling by about 1.7 - 2.7 times compared to normal rice starch in the absence of MPC.

NaCAS increased the T_{onset} of swelling of normal rice starch. In the presence of 5% NaCAS the swelling ratio and the amount of starch that leached from normal rice starch were decreased. Apparent viscosity is dependent on the size and number of the insoluble particles in a system as well as the concentration of soluble macromolecules in the solution, so it was not surprising to see that the apparent viscosity of the normal rice starch/5% NaCAS mixtures being lower than for normal rice/water mixture and that the swelling ratio and apparent viscosity were highly correlated given the above observations. Moreover there was a good relationship between low swelling ability and total starch leached; $R^2 = 0.99$, SD = 0.03 for 5% NaCAS, $R^2 = 0.96$, SD = 0.08 for 10% NaCAS ($P<0.0001$), and low swelling and low apparent viscosity; $R^2 = 0.91$, SD = 0.04 for 5% NaCAS, $R^2 = 0.98$, SD = 0.01 for 10% NaCAS ($P<0.01$), in the initial and the early part of the second phase of swelling.

On the addition of 5% NaCAS to normal rice starch the peak viscosity was lower than in the absence of NaCAS, but 10% NaCAS resulted in a peak viscosity higher than in the absence of NaCAS. A number of studies have reported these pasting anomalies on the addition of NaCAS. For example, Kelly *et al*. (1995) reported that caseinate levels as low as 0.01% dramatically decreased the swelling ratio of potato starch. However, for maize starch in deionized water, casein increased the viscosity of the starch paste and this increase in apparent viscosity increased with increasing caseinate concentration. Doublier *et al*. (1994) reported that the addition of caseinate decreased the swelling and solubility of wheat starch, potato and tapioca starches but only caseinate/tapioca starch mixtures displayed a decrease in apparent viscosity. They also found that NaCAS lowered starch granule deformability and mechanical fragility as the swelling of the granules was reduced and proposed that apart from causing more rigid starch granules, the interaction betweens caseins and starch macromolecules can lead to thermodynamical incompatibility. Thermodynamic incompatibility, results in phase separation between the NaCAS and the starch molecules. However, the CSLM observations made in this study on normal rice starch/5% or 10% NaCAS mixtures, (section 4.3.3), showed no evidence of any thermodynamic incompatibility between the caseins and the starch components. The resulting starch gels showed a homogeneous microstructure.

Note that, in the case of the normal rice starch/10% NaCAS mixture the swelling ratio of the starch granules was decreased up to the early part of the second phase but rose rapidly at the end of the second phase. However, at the end of the second phase of swelling, even though the swelling was sharply increased, there was no simultaneous increase in total starch loss, and in fact the loss of starch was lower than for starch/water mixture during this last phase. The results possibly suggest that the NaCAS had increased the granules' rigidity and resistance to shear (i.e. disintegration) and their loss of amylose and amylopectin from the granules at granule sizes where they would be expected to be quite vulnerable to shear, i.e., near the peak viscosity of the pasting curve. This argument fits in with conclusions by Steeneken (1989), who states that in a concentrated system, the viscosity of a starch paste is governed by the rigidity of the starch granules.

Although the effect of NaCAS on the swelling and total starch loss from granules is not fully understood, it could be the result, according to Tester and Sammerville (2003), of the competition between proteins and starch for available water in the system and it is this competition that limits the mobility of water. Water acts as a plasticiser of the amorphous regions of the starch granules and if there is less water available for plasticisation then gelatinisation of starch will thus be delayed. This effect is well-known in the case of starch and other biopolymer mixtures (Appelqvist and Debet, 1997). However, this mechanism is unlikely to be the cause for the results observed in this study because the extent of the changes, i.e., reduced swelling and starch loss were less for 10% NaCAS/starch mixture than for a 5% NaCAS/starch mixture. One would expect the swelling to be further delayed, and the peak viscosity decreased on increasing the protein concentration if competition for water was the main driving force for the observed changes, but this did not happen in this study.

It is also possible, as shown for other proteins such as bovine serum albumin (BSA) that the proteins may have been adsorbed to the starch granules (Dahle, 1971; Dahle, Montgomery and Brusco, 1975; Lundh, Eliasson and Larsson, 1988; Eliasson and Tjerneld, 1990; Wannerberger, Wahlgren and Eliasson, 1996; Ryan and Brewer, 2005a; Ryan and Brewer, 2006). According to a number of researchers, adsorbed surface lipids and proteins affect the swelling behaviour of starches (Baldwin, 2001; Debet and Gidley, 2006; Eliasson and Gudmundsson, 2006).

In this study NaCAS appeared to stabilise the starch granules in some way and prevent the leaching of starch molecules. This would suggest that NaCAS proteins such as α_s- and/or β-casein were being adsorbed to the surface of the starch granules and stabilising them in some way by either preventing moisture ingress into the granules and/or coating the granules with a layer of protein and thus slowing down their expansion and the leaching out of starch molecules. This adsorbed flexible protein layer could have held the granules together and restricted the expansion of the granules thus delaying the T_{onset} of swelling. However, the layer of adsorbed protein ruptured once the temperature reached some critical value, which then allowed the granules to swell.

The addition of WPI had no affect on the T_{onset} of swelling for normal rice starch. Moreover, there was no starch loss until the mixture reached 58.3°C. Upon further heating the amount of total starch leached increased with temperature. WPI, like NaCAS, tended to decrease the leaching out of total starch from normal rice starch granules except at the end of second phase of swelling (68.5°C). Like MPC, the addition of both 5 and 10% WPI to normal rice starch resulted in a dramatic increase in the swelling rate of normal rice starch compared to starch without any added protein. Unexpectedly, the total starch loss did not increase with increases in swelling of the granules. In fact the reverse happened. In the present study, the swelling ratio is a measure of the swelling volume and in normal rice starch/WPI mixtures there is not only starch, which can absorb water but also whey proteins and also whey protein aggregates that formed through the heat-denaturation of these globular proteins. From section 5.3.1, the T_{onset} for the denaturation temperature of α-la and β-lg; the main whey proteins in bovine milk, measured by DSC were 58.19°C and 72.57°C, respectively. The temperature range for normal rice starch swelling is 59.8°C to 82.8°C. Thus, in normal rice starch/WPI mixtures, the swelling of starch granules occurs at the same time as the thermal denaturation of α-la and β-lg. It is likely that the gelation of the whey proteins, α-la at the T_{onset} of rice starch and β-lg at temperatures of 72^{o}C or above, probably gave exaggerated figures for the rate of swelling increase. Both of the above proteins gel, and the method for determining the degree of swelling relies on the determination of the respective heights of the solid and centrifugate regions of a thin tube. If the proteins gel then this gel will hinder the settling velocities of the starch granules or may even prevent them from settling at all if the viscosity is high enough. The consequence of this is a higher than expected swelling rate at temperatures above the thermal gelling temperature of firstly α-la (58^{o}C) and then again with β-lg (72^{o}C).

Whilst there was conflict in the relationship between swelling ratio and total starch leaching, the relationship between swelling ratio and apparent viscosity of normal rice starch/WPI was in good agreement. Moreover, the temperatures at which the maximum swelling plateau was reached; 74.6°C, and 72.5°C for the addition of 5 and

10% WPI, respectively, correlated well with the denaturation temperature of β-lg in a 10% WPI solution from section 5.3.1.

As described earlier, the swelling and leaching behaviour of waxy rice was different to normal rice starch. However, it was found that the addition of SMP and UFSMP to the waxy rice starch exhibited similar effects to its addition to normal rice starch; the T_{onset} of swelling markedly increased, but the amount of total starch leached decreased. It is believed that lactose and the cations in SMP and UFSMP were responsible for these changes in behaviour.

The addition of NaCAS increased the T_{onset} of swelling and decreased the amount of starch that was leached out of waxy rice starch, as was the case with normal rice starch, suggesting that something in NaCAS, possibly α-casein and/or β-casein; the main caseins in NaCAS, were influencing the mobility, i.e., the ability of the amylose and amylopectin to leach out of the granules in some way. Here again it is feasible that an adsorbed casein on the granule surface, could prevent the starch molecules from leaching out of the granules until sufficient energy has been injected into the system to disrupt this adsorbed casein layer and allow the starch molecules to leach from the granules. Although the swelling rate of waxy rice starch on the addition of both 5% and 10% NaCAS was not increased compared to a starch/water system, the peak and final viscosities were higher than in the absence of NaCAS. This would tend to support the view that the addition of NaCAS might somehow increase the rigidity of waxy rice starch granules and the adsorption of caseins onto the starch granules is possibly the mechanism underlying the observations made in the study.

In NaCAS the caseins exist as either individual proteins or small associations, whilst in SMP and MPC the caseins are mainly associated with micelles. The caseins in NaCAS were able to dramatically alter the physico-chemical properties of rice starch, whilst the caseins in SMP and MPC, either because they existed in the micelle form and/or because of the interference of the whey proteins were not able to influence the physico-chemical properties of the two starches. This suggests that the individual caseins were probably better adsorbed/absorbed to the surfaces/interior of the granules than the

micelle caseins in either SMP or MPC. It would be interesting to see whether the proteins were firstly adsorbed to the granule surfaces, and secondly and more importantly, whether higher amounts of protein was adsorbed from NaCAS than from either SMP or MPC solutions of comparable concentration. If this was indeed the case then the observed differences in the physico-chemical properties of the two starches when NaCAS and MPC were added could be attributed to the types and properties of the adsorbed/absorbed proteins by the respective granules.

MPC and WPI did not dramatically increase the rate of swelling of waxy starch granules, whereas the two proteins dramatically increased the rate of swelling of normal rice starch, particularly over the temperature range 65^{o}C to 72^{o}C. By the time the temperature reached 72^{o}C (the denaturation/gelation temperature of β-lg) the normal starch was fully swollen according to the test procedure used to measure swelling. It could be argued that the increased rate of apparent swelling over the temperature range 65 – 72^{o}C of the normal rice starch granules, as compared with the waxy granules, was brought about by the imbibing of water by the granules and the subsequent increase in concentration of the WPI in the remaining solution. When the concentration of WPI exceeded 15% there was a marked increase in viscosity of the solution (Appendix E) and this had the effect of hindering granule settling and as a consequence a higher rate of swelling than was actually the case. Now the gelation of whey proteins is a concentration dependent process (Aguilera, 1995). Moreover, from Appendix E, a solution of 10% WPI alone showed little increase in viscosity with temperature and as a consequence no apparent viscosity peak at 72 - 74^{o}C due to the gelation of β-lg as was expected, but did so at WPI concentrations ≥15%. If the observed increase in swelling of natural rice starch was the result of a WPI concentration increase with granule swelling then the trend should have been observed for the WPI and MPC/waxy starch mixtures, which was not the case. Secondly the rate of swelling should have decreased with increasing WPI concentration as the WPI would have been competing for the water with the granules as the WPI concentration rose, but no decrease in swelling rate was observed. A more likely explanation for the observed differences was that the adsorbed WPIs, probably α-la, given the fact it denatures at 58°C, removed or altered the native proteins on the natural starch, thus

allowing the granules to swell at a faster rate than when no proteins were added. This argument is based on the results of Debet and Gidley (2006) who found that the removal of the native surface proteins and fat from maize starch made them swell like waxy maize; which contain less surface proteins and fats than waxy rice starch. However, the explanations advanced here for the observed differences for the effects of WPI on the swelling of normal and waxy rice starches should be taken cautiously as there are fundamental differences between these two starches. Firstly, the swelling rate of waxy is faster than that of normal rice starch. In addition, the swelling of normal rice starch spans a wider temperature range than that of waxy rice starch. Secondly the range of temperatures for the swelling of normal starch granules overlaps that over which WPI denatures. Thirdly, it is expected that normal starch granules will retain their integrity better than waxy rice starch granules which would be expected to completely disintegrate once the gelatinization temperature had been reached due to their lack of protein at the surface. Moreover, the natural starch granules should leave more intact ghost starch granules which will affect the viscosity of resulting starch/protein solutions. These three main differences could have a different influence, on the effect of WPI in these systems.

Although, WPI had little or no influence on the observed increase in swelling rate of waxy rice starch compared to normal rice starch, its addition noticeably increased the T_{onset} of swelling and substantially decreased the amount of starch that leached out of the waxy rice. As the surface protein and lipid is a critical factor for the swelling of starch it is hypothesized that the whey proteins were adsorbed onto the waxy starch granules and they prevented water being imbibed into the starch granules and thus prevented the starch molecules from leaching out of the granules.

During the early stage of swelling, the viscosity of the starch/water or starch/milk protein ingredient mixtures can be calculated using the volume fraction of the swollen starch granules (the swelling ratio ϕ) and the Maron-Pierce equation. However, at higher temperatures, > 66°C for normal rice starch (Figure 6.8A) and > 62°C for waxy rice starch (Figure 6.8B), there are discrepancies between the calculated and the measured viscosities. This could be due to the fact that the Maron-Pierce relationship is

established for suspensions of spherical particles, where the volume fraction of the suspensions is very well-known. Unfortunately, starch granules do not swell uniformly as spheres but are twisted and deformed so that not only the diameter but also the form of the granules is affected. This has been shown by microscopic observations performed on unheated and heated rice starch granules (Rani and Bhattacharya, 1995). As a result, it can be expected that the maximum packing of starch can vary significantly with temperature.

In addition, it was assumed that the maximum packing in the sediment ϕ_s was equal to that in the dispersion ϕ_c. These packing fractions could be different as centrifugal forces are expected to increase the value of the maximum packing in the sediment due to possible deformation of the swollen starch granules. Finally, the contribution of the leached starch to the viscosity of the continuous phase was not taken into account in the calculation, where the viscosity of water alone was used. Despite these discrepancies seen at high temperatures, there was very good agreement between the calculated and measured viscosities at low temperatures (see the insets in Figure 6.8, Table 6.3), which thus allowed the determination of the onset temperature of swelling T_C from the calculated viscosity.

6.5 Summary to chapter

The study showed that the swelling ratio and the amount of starch leaching from the starches were decreased on the addition of SMP compared to the starches on their own in water. The T_{onset} of swelling was delayed too by the addition of SMP to the starches. It is likely that these observed changes on the addition of SMP were due to the presence of lactose and ions, as the results for the respective parameters were practically identical on the addition of UFSMP to the rice starches.

The addition of MPC and WPI decreased the loss of total starch from the granules but the reduction was not as great as it was for the addition of either SMP or UFSMP. MPC and WPI affected the swelling rates of normal and waxy rice starch differently. The two protein ingredients instigated a rapid swelling in normal rice starch granules, but had no effect on the swelling rate of waxy rice granules. It would appear as though the whey proteins in both ingredients were interacting with either the starch molecules at the surface or lipids or proteins, but more likely the observed differences in water holding ability over the denaturation/gelation of whey proteins between normal and waxy rice starch played a key role.

The T_{onset} of swelling of both rice starches was slightly increased in the presence of NaCAS. Moreover, NaCAS, like SMP and UFSMP, also decreased the total amount of starch that was leached from the two starches. Hence, it is believed that NaCAS increased the rigidity of both normal and waxy rice starches. Interestingly, the amount of total starch that leached from both types of starch was lower for the addition of NaCAS compared to starch alone.

It was also demonstrated, using the Maron-Pierce equation and the swelling measurements, that it was possible to calculate a viscometric onset temperature. For all the milk protein/rice starch mixtures investigated here. The calculated viscometric onset temperature (T_{CS}) was in very good agreement with the onset temperature measured by viscometry (T_R). The predicted viscosity values in the early stages of

pasting agreed with the calculations based on the phase volume fraction of swollen starch granules.

Based on the results from this chapter and previous chapters, in summary, it was found that the milk protein ingredients affect and affect differently the T_{onset} and T_{peak} of pasting, and gelatinization and swelling of the two starches. Microscopical differences were also observed in the appearance and resistance to shear of the starch molecules on addition of the various milk protein ingredients at various temperatures. The lactose and ions in SMP appeared to overwhelm any differences that might have been observed for the proteins. The probable mechanism for lactose effects was it anti- plasticisation effects and specific lactose-starch interactions. The cations in SMP tend to protect and stabilize the starch granule structure and thus cause a delay in the pasting, gelatinisation and swelling of starch.

However, when MPC, WPI and NaCAS were added the effects on the T_{onset}, T_{peak} and leach-out of starch were different. Their effects could not be attributed to anti-plasticisation effects as most of the protein molecules could not diffuse into the starch molecules and so they probably exerted some effect as a consequence of their interaction on the starch granule surfaces with starch molecules and/or the proteins and lipids. Whilst there are numerous studies that have shown that milk proteins are adsorbed onto oil/water interfaces (Hunt and Dalgleish, 1994a; Hunt and Dalgleish, 1994b; Hunt and Dalgleish, 1996; Sharma *et al*., 1996a; Sharma *et al*., 1996b; Srinivasan *et al*., 1996; Sharma and Singh, 1998; Euston and Hirst, 1999; Segall and Goff, 1999; Srinivasan *et al*., 1999; Euston and Hirst, 2000), reported significant amount of milk proteins adsorbed and they covered the the milk fat globule surface. There are only a few studies describing the adsorption of proteins to granule surfaces (Eliasson and Tjerneld, 1990; Larsson and Eliasson, 1997; Ryan and Brewer, 2005b; Ryan and Brewer, 2006). For starch granules, it is clear therefore, that a study should be carried out to establish whether milk proteins are adsorbed to the surfaces of rice starch granules and to identify the mechanisms of the interactions identified.

Chapter 7

ADSORPTION OF MILK PROTEINS ONTO NORMAL AND WAXY RICE STARCH GRANULES

7.1 Introduction

Several studies have shown that proteins do adsorb onto starch granules (Dahle, 1971; Dahle *et al*., 1975; Lundh *et al*., 1988; Eliasson and Tjerneld, 1990; Wannerberger *et al*., 1996; Ryan and Brewer, 2005a; Ryan and Brewer, 2006) and that this adsorption reduces the ability of the starch granules to absorb water. Eliasson and Tjerneld (1990) studied the adsorption of BSA, low molecular weight wheat protein fraction (WP1), and high molecular weight wheat protein fraction (WP2) onto wheat, maize and potato starch granules. The adsorption was found to be low for BSA and WP1 but higher for WP2. They also found that the adsorption depended not only on the type of protein, but also on the type of starch. Ryan and Brewer (2006) removed the native wheat starch granule surface protein and found that it decreased the binding of added proteins, suggesting that native granule proteins might mediate the binding of exogenous protein. But they did not investigate how this removal of the proteins affected swelling, pasting and gelatinisation of starch. However, the surface protein was reported to have an influence on the physico-chemical properties of starch, such as altering the pasting/gelatinisation of starch (Seguchi, 1986; Eliasson and Tjerneld, 1990; Hamaker and Griffin, 1990; Marshall, Normand and Goynes, 1990; Baldwin, 2001). The results from the previous chapters showed that the swelling and the pasting/gelatinization including the viscoelastic behaviours of rice starches were modified in the presence of milk protein ingredients. Therefore, it is hypothesized that milk proteins could adsorb onto the starch granule surface and in turn affect the behaviour of rice starches.

Although there is currently an increased awareness of the exogenous proteins at the starch granule surface (Ryan and Brewer, 2005a; Ryan and Brewer, 2005b), the actual

underlying mechanism regarding the nature of the protein/starch granule interaction on the starch surface is still not understood. The objective of this chapter was to establish whether milk proteins are adsorbed to the surfaces of rice starch granules and to identify the mechanisms of these interactions and then to finally, link the findings of this chapter to the observed changes in swelling, leaching and pasting/gelatinisation behaviour.

7.2 Materials and Methods

7.2.1 Materials

The details of normal rice starch, waxy rice starch, milk protein ingredients (SMP, MPC, NaCAS, and WPI) are described in section 3.2.1.

All the chemicals used were of analytical grade, unless otherwise specified. They were obtained from either Sigma Chemical Co. (St Louis, MO, USA), or BDH Chemicals (BDH Ltd, Poole, England).

7.2.2 Methods

7.2.2.1 Characterization of normal and waxy rice starch granules.

7.2.2.1.1 Starch granules particle size distribution

A Malvern MasterSizer 2000 (Malvern Instruments Ltd, Malvern, UK) was used to determine the average particle size of the starch granules using the general purpose (spherical) analysis mode. Because the starch granules are relatively large, and not spherical, the approximate particle size was obtained using the Fraunhofer approximation of light scattering theory, which does not require the refractive index of the starch granules (Xu, 2002).

7.2.2.1.2 The absolute density of starch.

The absolute density of the rice starch was determined using a modification of the xylene displacment method of Schoch (1964). The volume of the density bottle used was 10 ml and the xylene density at 20°C was 0.86 g/cm^3 (catalogue number 1330-20-7, Labscan, Dublin, Ireland). Starch was dried by heating it in a hot air oven overnight at 105±5° C. The dried starch (2 g) was mixed well with xylene then transferred to the density bottle, which was filled to its total volume of 10 ml by further addition of xylene. The filled density bottle was equilibrated for 15 min at 20°C and weighed. Absolute density (g/ml) was calculated with the following formula (Schoch, 1964):

$$\text{Absolute density} = \frac{(a \times d)}{(a + b - c)} \qquad \text{Equation 7.1}$$

Where; a = weight of starch (g)

b = weight of density bottle filled with xylene (g)

c = weight of density bottle filled with starch and xylene (g)

d = density of xylene at 20°C (g/ cm^3)

7.2.2.1.3 Morphology and surface properties of starch granules

Light microscopy observations on native normal and waxy rice starch granules

The native normal or waxy rice starch granule morphologies were observed using a light microscope Nikon Eclipse E600POL, Nikon Corporation, Tokyo, Japan).equipped with a Nikon Digital camera (DXM 1200F, Nikon Corporation, Tokyo, Japan). The images were subsequently captured using ACT-2U software version 1.4 (Nikon Corporation, Tokyo, Japan). The suspensions of 10% normal or waxy rice starch in MilliQ water were prepared by mixing 3 g of starch with 27 g of MilliQ water at room temperature for 30 mins using a magnetic stirrer, at medium speed. Each suspension was placed on a glass slide and covered with a cover-slip, the sides of which were sealed with nail polish. The starch granule morphology was observed under 50× magnification using the light microscope.

CSLM observation on SDS treated- starch granules

Sodium dodecyl sulphate (SDS) is a reagent usually used for the extraction of proteins and lipids from hydrophilic substrates like starch granules. It removes starch granule proteins and lipids; particularly surface proteins and lipids, but does not change the visual appearance and birefringence of the starch granules (Debet and Gidley, 2006).

Normal or waxy rice starch samples (3 g) were mixed with 27 g of Milli-Q water or a 2% (w/w) SDS solution using a magnetic stirrer at room temperature for 30 minutes. The rice starch/SDS suspension was centrifuged at 1,000 *g* for 10 min, the supernatant discarded and the pellet made of rice starch granules collected. This pellet was resuspended in Milli-Q water and centrifuged to remove the supernatant once more; this washing procedure was repeated 6 times. The samples were prepared for observation under the CSLM as described in section 7.2.2.3.

The 1% (w/w) normal and waxy rice starch granules before and after extraction of starch proteins with SDS in MilliQ water were mixed with a fluorescent-labelling protein dye Alexa Fluor 488 solution (carboxylic acid, succinimidyl ester, dilithium salt), then observed under CLSM as described in section 7.2.2.3.

Effect of surface protein and fat on the pasting properties of normal and waxy rice starches

The starch granules were extracted with 2% (w/v) sodium dodecyl sulphate (SDS) to remove their surface proteins and fat. This SDS extraction method was modified from Debet and Gidley (2006). Rice starch (3 g) was gently mixed with 27 g of a 2% SDS solution using a magnetic stirrer at room temperature for 30 minutes. The rice starch/SDS mixture was centrifuged at 1,000 *g* for 10 min and the supernatant removed. The pellet was washed six times then resuspended in MilliQ water to give a 10% (w/w) concentration of a starch solution. The 10% (w/w) starch solution was mixed using a vortex mixer to ensure the starch granules were fully redispersed. The pasting behaviour of the starch suspension was determined as previously described (section 3.2.3.2).

7.2.2.2 Determination of milk proteins adsorbed onto the rice starch granules

An adaption of the method used by Hunt and Dalgleish (1994) was used to determine the amount of protein and the proportions of the individual proteins that were adsorbed onto the rice starch granules. The proportions of the individual proteins in milk protein ingredients were also determined by this method, which is based on the quantification of the milk proteins by sodium dodecyl sulphate polyacrylamide gels electrophoresis; SDS-PAGE (Appendix F).

The rice starch/milk protein ingredients mixtures were prepared as described in section 3.2.2. Approximately 30 g of the rice starch/milk protein ingredients mixtures were weighed (to 4 decimal places) directly into 50 ml polypropylene centrifuge tubes (catalogue number 430829, Corning Co., Corning, New York, USA) and centrifuged at 1,000 *g* for 10 min in a Heraeus Megafuge 1.0 (Heraeus, Hanau, Germany). The supernatant was removed and the pellet was resuspended in MilliQ water and centrifuged to remove any non-adsorbed milk proteins. The procedure was repeated six times. Then the final pellet was resuspended in enough MilliQ water to produce a 40% (w/w) starch slurry. The adsorbed milk proteins were desorbed from the rice starch granule with a 2% SDS in buffer (0.5 M Tris, 0.009% bromophenol blue, pH 6.8). The 40% starch slurry was weighed out (~0.6 g) into 1.5 ml. Eppendorf vials and 500 mg of SDS sample buffer was added. The vial was mildly shaken using a Vortex shaker for 30 mins, then centrifuged at 14,000 rpm for 5 min at 20°C using an Eppendorf centrifuge (5417R; Eppendorf AG, Hamburg, Germany). A 20 µl aliquot of the supernatant was loaded onto the sodium dodecyl sulphate-polyacrylamide electrophoresis gel (SDS-PAGE).

7.2.2.3 Confocal scanning microscopy

Dye preparation

Labelled protein and unincorporated dye were prepared following Molecular Probes' Alexa Fluor™ 488 Protein Labeling Kit; A-10235 (Molecular Probes, Eugene, OR, USA). The Alexa Fluor 488 reactive dye (Alexa Fluor 488 carboxylic acid,

succinimidyl ester, dilithium salt, M_W~643) has a succunimidyl ester moiety, which can react efficiently with primary amines in proteins to form stable and highly fluorescent dye-protein conjugates.

Protein preparation

Stock solutions of NaCAS and WPI were prepared by dissolving the NaCAS or WPI powder in Milli-Q water at ambient temperature with gentle stirring for 1 hour and the stock solutions were stored overnight at 4°C. The stock solutions were diluted to a concentration of 2 mg/ml in 0.1 M sodium bicarbonate to meet the recommended dye to protein ratio for adequate protein labelling.

Labelling reaction

One M sodium bicarbonate (50 µl, pH ~8.3) was added to the 2 mg/ml protein solution (0.5 ml) and the protein solution was transferred to a vial containing Alexa Fluor 488 reactive dye, mixed well by inverting the vial a few times to fully dissolve the dye. The reaction mixture was stirred for 1 hour at ambient temperature. Hydroxylamine solution (17 µl) was added to the reaction vial to stop the reaction, and the reaction mixture was further stirred for 30 minutes.

Purification of the labelled protein

The reaction mixture of protein and dye was purified through a purification resin, which can separate free dye from proteins with M_W >15,000. The elution buffer was prepared by diluting a phosphate-buffered saline solution (0.1 M potassium phosphate, 1.5 M NaCl, pH 7.2, with 2 mM sodium azide) 10 times. The purification resin separates the reaction mixture into two coloured bands when viewed under a UV lamp, representing the separation of labelled protein from unincorporated dye. The first coloured band contains the labelled protein. The second (the slower moving band) consists of unincorporated dye. The portion containing the labelled protein was collected. The eluted volume between the labelled protein and the die was discarded to ensure that that there was no labelled protein in the unincorporated dye portion. The eluted portion of unincorporated dye alone was also collected.

Preparation of adsorbed labelled protein or dye alone onto starch granules

Normal or waxy rice starch 1% (w/w) in labelled protein solution or in dye solution alone was prepared at room temperature by mixing for 30 minutes. The starch solutions with labelled protein solution or unincorporated dye in vials were covered by aluminium foil to protect the labelled protein or unincorporated dye from light. The mixtures were incubated overnight at 4°C. The next day the mixtures were centrifuged at 1000 *g* for 10 min and the supernatant discarded, in order to remove any excess of the labelled proteins or dye from the mixture. The pellet was rewashed by resuspending in Milli-Q water and centrifuging at 1000 *g* for 10 min to remove any non-adsorbed labelled proteins or unincorporated dye. This washing procedure was repeated 3 times.

CSLM observation

A portion of each sample was loaded onto a glass slide, covered by a cover slip, transferred to the confocal microscope stage then observed under the 100 mm oil immersion objective lens CSLM (Leica TCS 4D confocal microscope, Leica Lasertechnik GmbH, Heidelberg, Germany) using an Argon laser, for which the absorption and fluorescence emission maxima were 494 and 519 nm, respectively.

7.2.2.4 Determination of milk proteins adsorbed onto the rice starch granules free-surface protein and fat rice starch granules

7.2.2.4.1 Removal of starch surface protein and lipid by 2% SDS

To obtain the rice starch granules free of surface protein and fat, the starch granules were extracted with 2% (w/v) sodium dodecyl sulphate (SDS) to remove their surface protein and fat. This SDS extraction method was a modification of the method of Debet and Gidley (2006). Rice starch (3 g) was gently mixed with 27 g of a 2% SDS solution using a magnetic stirrer at room temperature for 30 minutes. The rice starch/SDS mixture was centrifuged at 1,000 *g* for 10 min and the supernatant removed. The pellet was then washed six times with MilliQ water.

7.2.2.4.2 Determination of milk proteins adsorbed onto free-surface protein and fat rice starch granules

A sub-sample (3 g) of either the normal or waxy rice starches that had their surface-proteins and fat removed according to the procedure outlined in section 7.2.2.4.1 was mixed with 27 g of 10% NaCAS or 10% WPI solution using a magnetic stirrer at room temperature for 30 minutes, and then centrifuged at 1,000 *g* for 10 min in a Heraeus Megafuge 1.0. The supernatant was discarded and the pellet with adsorbed caseins or whey proteins on the surface of the granules was collected. This pellet was resuspended in Milli-Q water and centrifuged to remove any non-adsorbed added protein; this washing procedure was repeated 6 times.

CSLM observation

The granules (normal and waxy starch) with only either the adsorbed caseins or whey proteins on their surface (no native proteins or fat) were prepared for observation under the CSLM as described in section 7.2.2.3. The 1% (w/w) normal and waxy rice starch granules, with caseins or whey proteins adsorbed onto their granules were mixed with a fluorescent-labelling protein dye Alexa Fluor 488 solution (carboxylic acid, succinimidyl ester, dilithium salt), then observed under CLSM as described in section 7.2.2.3.

The SDS-PAGE

The amounts of the various caseins or whey proteins that were adsorbed to normal and waxy rice granules that were free of their native proteins and fat were determined according to the method described in section 7.2.2.2.2. 10 µl aliquot of the supernatant was loaded onto the SDS-PAGE, and the qualitative method for determining the amounts of the various caseins that were adsorbed to the granules is described in Appendix F.

7.2.2.4.3 Pasting behaviour of rice starch with different surface materials

The pasting behaviour of rice starch with different surface materials; rice starch with native proteins present, rice starch free of surface proteins and fat obtained from section 7.2.2.4.1 and the caseins that had been adsorbed onto the surfaces of the starch granules that were free of surface proteins and fat obtained from section 7.2.2.4.2, were investigated.

The starch pellet after the sixth wash with MilliQ water as described in section 7.2.2.4.1 and 7.2.2.4.2 was resuspended in enough water to give a starch concentration of 10% (w/w). This starch suspension was mixed using a vortex mixer to ensure that the starch granules were fully redispersed. The pasting behaviour of the sample was determined using a stress-controlled rheometer (Parr Physica UDS 200; Physica, Stuttgart, Germany) with the starch cell geometry TC 20 as described in section 3.2.3.2. The apparent viscosity of the milk protein/rice starch mixtures during pasting was measured.

7.3 Results

7.3.1 Characterisation of normal and waxy rice starch granules

The granule particle size distribution of normal and waxy rice starch, and their morphology under light microscopy are presented in Figures 7.1 and 7.2, respectively. Normal and waxy rice starches were similar in shape and size, with granule shape ranging from polygonal to irregular shapes, and with an average size diameter of 5.60, and 5.79 μm, for normal and waxy rice starch granules respectively. The densities of the normal and waxy rice starches were 1.51±0.00 and 1.50±0.00 g/cm^3, respectively.

Han and Hamaker (2002) stated that starch granule-associated proteins in their intact state are able to be detected under CLSM using a protein-specific dye. In this study the Alexa Fluor 488 reactive dye was used; Alexa Fluor 488 carboxylic acid, succinimidyl ester, dilithium salt, M_W~643, which has a succunimidyl ester moiety. This succunimidyl ester moiety can react specifically with primary amines in the proteins to form stable and highly fluorescent dye-protein conjugates (Product Information, Alexa Fluor^{TM} 488 Protein Labeling Kit A-10235, Molecular Probes). Under the CLSM images (Figure 7.3), the fluorescent dye-protein conjugates appear as bright light region.

Figure 7.3 shows the CLSM images of normal and waxy rice starch granules stained with fluorescent dye (Alexa Fluor^{TM} 488) before and after removal of proteins and lipids with SDS. For normal rice starch granules and waxy rice starches not treated with SDS, fluorescent regions throughout the starch granules are observed (Figure 7.3A and 7.3B, respectively). Removal of proteins and lipids from the normal and waxy starch granules through extraction by 2% SDS changed the staining pattern of the rice starch granules. All of the outer fluorescent regions of the normal rice starch granules disappeared but a lightly fluorescent area in the inner part of the granules remained (Figure 7.3C). For waxy rice starch granules no fluorescent regions were observed (Figure 7.3D). These results indicated that the surface granules of both normal and waxy rice starches contained native proteins which were largely remove by the SDS treatment.

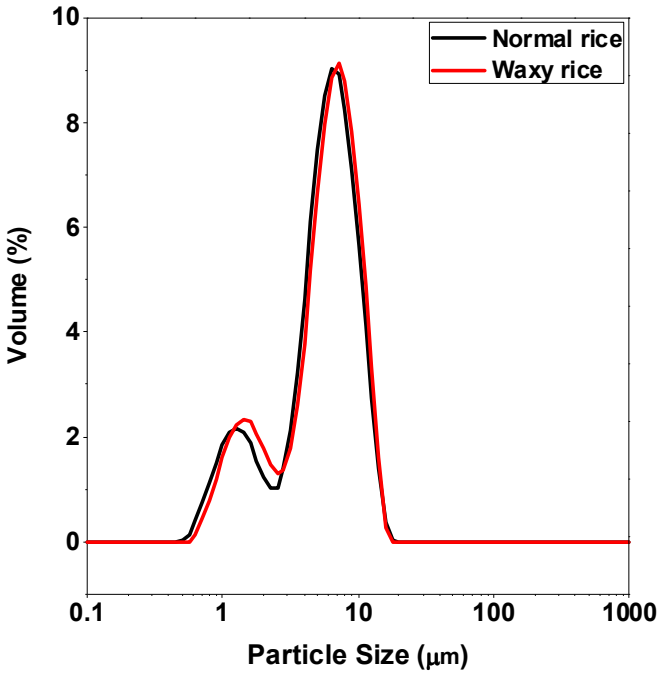

Figure 7.1 Particle size distribution of 10% normal and 10% waxy rice starch granules, (n=3).

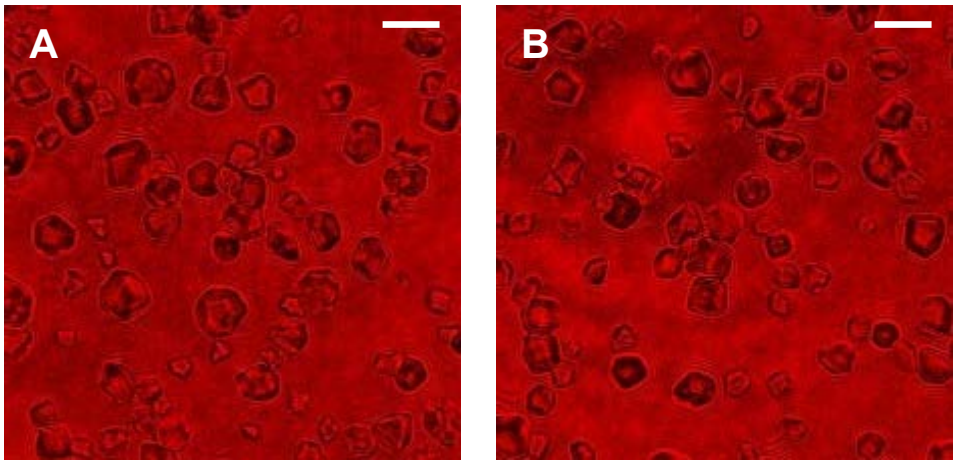

Figure 7.2 Morphology of normal (A) and waxy (B) rice starch granules as observed under light microscopy. Bar scale = 10 μm.

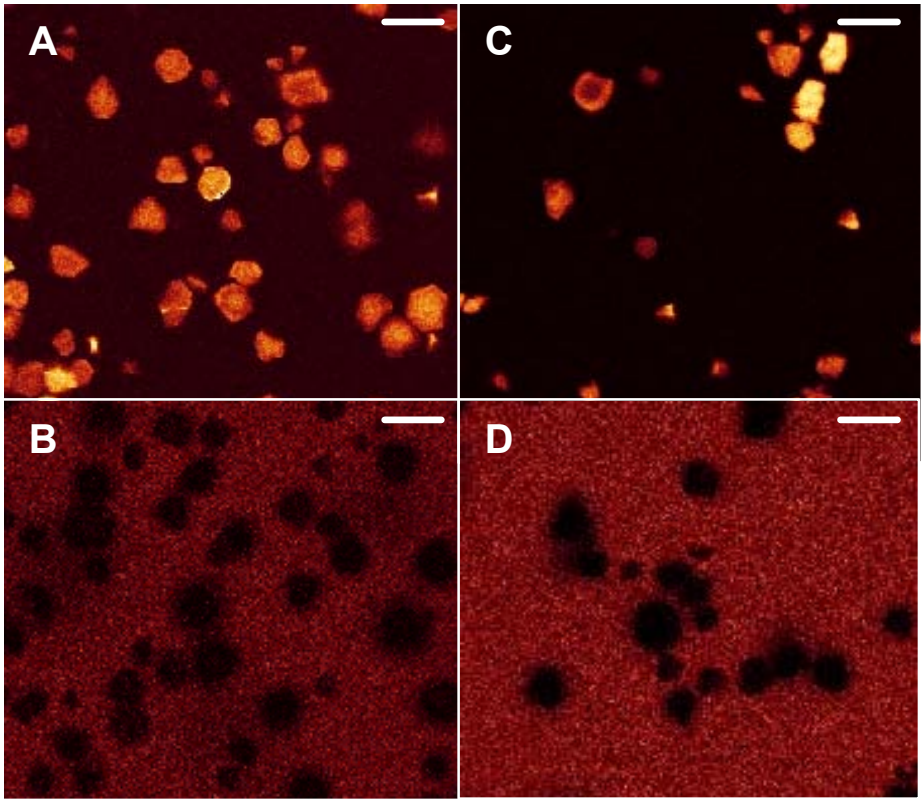

Figure 7.3 Confocal observation of normal rice starch (A), SDS-treated normal rice starch (B), waxy rice starch (C), and SDS-treated waxy rice starch (D). Bar scale = 10 μm.

7.3.2 Adsorption of milk proteins onto rice starch granules

The amount of each of the milk proteins adsorbed onto the normal and waxy rice starch granules (mg of adsorbed milk protein per g of starch) was divided by the surface area of normal or waxy rice starch, by assuming that the starch granules were spherical, to obtain the protein surface coverage.

Experimental data from the adsorption isotherms were fitted to the Langmuir isotherm if they showed monolayer adsorption behaviour (Equation 7.2) (Weber *et al*., 1991), but if they exhibited multilayer adsorption behaviour, the adsorption isotherms were

fitted to the Brunauer, Emmett and Teller (BET) equation (Equation 7.3) (Brunauer *et al*., 1938).

$$\Gamma = \frac{A_{BS}\Gamma_m C}{\left(1 + A_{BS}C\right)} \qquad \text{Equation 7.2}$$

$$\Gamma = \frac{\Gamma_m A_{BS}C}{\left[(C_S - C)(1 + (A_{BS} - 1)(C / C_S)\right]} \qquad \text{Equation 7.3}$$

Where Γ = the surface protein concentration

Γ_{max} = the monolayer coverage value of surface protein concentration

C = solute concentrations in the bulk solution

Cs = solute concentrations in the subsurface area

A_{BS} = the affinity of the adsorbed molecules related to the enthalpy of adsorption

7.3.2.1 Normal rice starch

The amount and proportion of α_s-casein and β-casein from SMP adsorbed onto normal rice starch is shown in Figures 7.4A, and 7.4B, respectively. The amount of adsorbed α_s-casein and β-casein gradually increased with concentration to reach a plateau at 1% SMP. The monolayer values calculated from the Langmuir equation for α_s-casein and β-casein were 0.47, and 0.46 mg/m^2, respectively (Table 7.1). The bulk concentration of SMP affected the proportion of adsorbed α_s-casein and β-casein. At low SMP concentration (≤0.1%), a greater amount of α_s-casein than β-casein was adsorbed onto normal rice starch granules (with a α_s- to β-casein ratio between 1.36 to1.76), however the amount of α_s-casein that was adsorbed decreased with increased SMP concentration. At high concentrations of SMP (>0.1%), equal amounts of α_s-casein and β-casein were adsorbed onto normal rice starch granules (with an α_s- to β-casein ratio between 1.07 to1.12). Note that, the ratio of α_s- to β-casein in the original SMP

solution was 1.35, thus in the high SMP range of concentrations, β-casein was preferentially adsorbed to the normal starch granules, than α_s-casein.

Table 7.1 Saturated surface concentrations (Γ_{max}) of monolayer coverage, the affinity of the adsorbed molecule (A_{BS}), and the solute concentrations in the subsurface area (C_S) for normal rice starch mixed with each milk protein ingredients.

Milk protein	Langmuir		BET		
	Γ_{max} (mg/m^2)	A_{BS}	Γ_{max} (mg/m^2)	A_{BS}	C_S (mg/g)
SMP					
α_s -casein	0.47±0.02	3.94±1.15	-	-	-
β-casein	0.46±0.03	2.19±0.57	-	-	-
MPC					
α_s-casein	0.33±0.02	-2.08±1.15	-	-	-
β-casein	0.38±0.02	1.41±0.38	-	-	-
NaCAS					
α_s-casein	-	-	0.37±0.02	308.57±65.37	87.32±8.40
β-casein	0.26±0.01	4.06±0.86	-	-	-
WPI					
β-lg	-	-	0.10±0.01	147.85±33.55	116.51±12.36
α-la	-	-	0.02±0.00	116.70±63.44	53.62±31.72

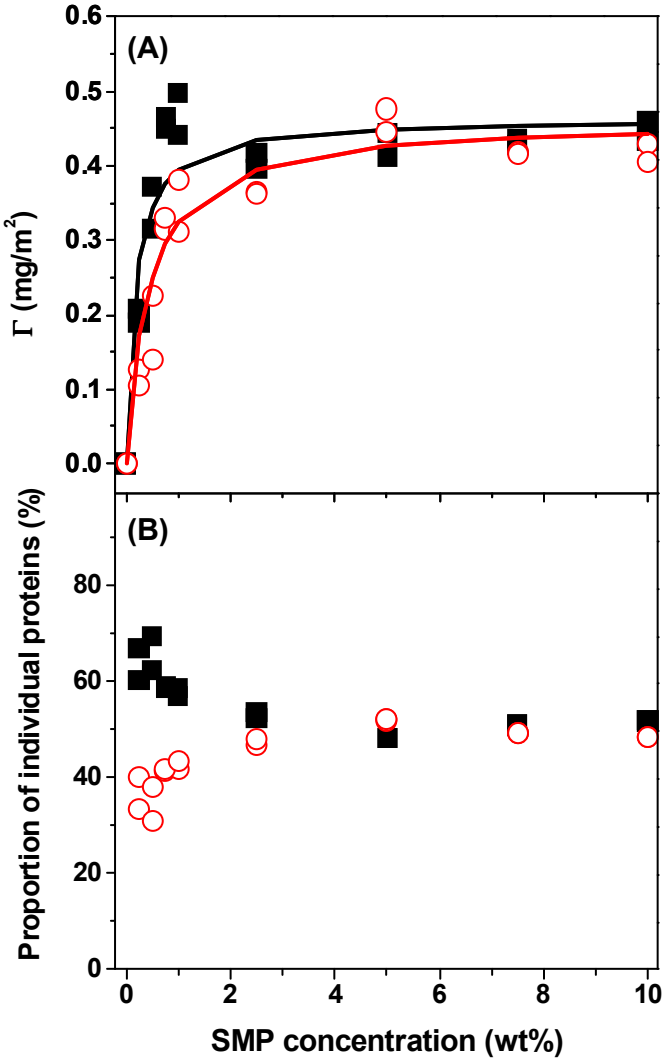

Figure 7.4 Adsorption isotherms for SMP proteins on normal rice starch granules (A) and the proportion of the individual proteins (B). Solid lines represent the fits using the Langmuir equation. SMP proteins are: α_s-casein (■), and β-casein (○). Chi^2 are 0.00266, and 0.00327 for α_s-casein and β-casein, respectively.

The amount and proportion of α_s-casein and β-casein from MPC adsorbed onto normal rice starch is shown in Figures 7.5A, and 7.5B, respectively. The maximum amount of adsorbed α_s-casein occurred at very low MPC (0.5%) concentrations. Further increases in MPC resulted in declining amounts of adsorbed α_s-casein until a plateau isotherm was reached at 2.5% MPC. The adsorbed monolayer value calculated from the Langmuir equation was 0.33 mg/m^2 (Table 7.1). In contrast, the amount of adsorbed β-casein gradually increased with increasing MPC concentration to reach a plateau, similarly to α_s-casein, at 2.5% MPC. The adsorbed monolayer value calculated for β-casein, using the Langmuir equation, was 0.38 mg/m^2.

In the original MPC solution, the ratio of α_s- to β-casein was 1.54. But in the low concentration range of MPC (0.1 to 1%), the ratio of adsorbed α_s- to β-casein was found to be 1.58 to 1.97. This indicates, that at these low MPC concentrations (<1%), α_s-casein was preferentially adsorbed to β-casein. However, at high MPC concentrations (2.5 to 10%) β-casein was adsorbed preferentially to α_s-casein (with a ratio of α_s- to β-casein between 0.63 and 0.91). Note that, the ratio of adsorbed α_s- to β-casein decreased with increasing MPC concentration.

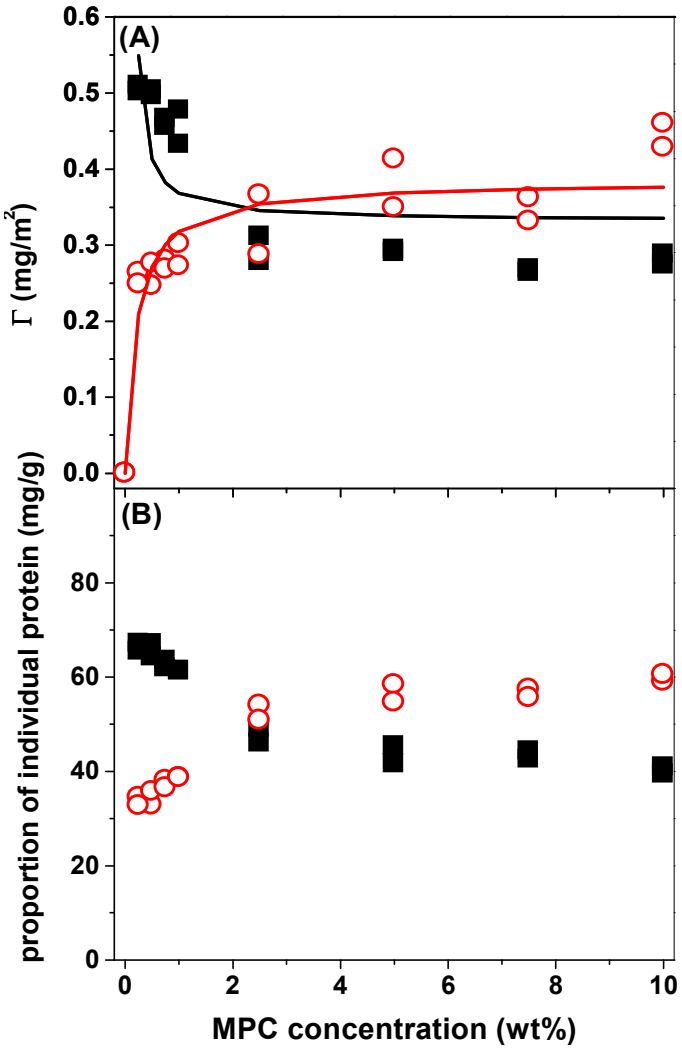

Figure 7.5 Adsorption isotherms for MPC proteins on normal rice starch granules (A) and the proportion of the individual proteins (B). Solid lines represent the fits using the Langmuir equation. MPC proteins are: α_s-casein (■), and β-casein (○). Chi^2 are 0.00207, and 0.00095 for α_s-casein and β-casein, respectively.

The amount and proportion of α_s-casein and β-casein that was adsorbed onto the surface of normal rice starch granules from NaCAS solutions is shown in Figures 7.6A, and 7.6B. The data suggests that the β-casein was adsorbed as a monolayer and the amount adsorbed increased with increasing NaCAS concentration until it reached a plateau at 0.2 % NaCAS. The monolayer value of β-casein calculated using the Langmuir equation was 0.26 mg/m^2. The adsorption isotherms for α_s-casein from the NaCAS/starch mixture showed a multilayer pattern (Figure 7.6A), which was quite different to the adsorption isotherms of β-casein, or α_s-casein in the case of SMP and MPC. At low NaCAS concentration (< 0.2%), the amount of adsorbed α_s-casein gradually increased with NaCAS concentration and reached the plateau for monolayer adsorption at 0.2% NaCAS, with a value of 0.37 mg/m^2 (Table 7.1) obtained using the BET equation. At NaCAS concentrations greater than 2.5%, the amount of adsorbed α_s-casein suddenly increased, which would indicate that a second layer of α_s-casein was adsorbing onto the normal rice starch granules. Note that the amount of adsorbed α_s-casein increased with further increase in NaCAS concentration.

The amount of α_s-casein that was adsorbed to the surface of the starch granules was much higher than the amount of β-casein for all concentrations of NaCAS, and the difference in the amounts adsorbed of the two proteins increased with increasing NaCAS concentration. The ratio of α_s- to β-casein in the original NaCAS solution was 0.83, but the ratio for adsorbed α_s- to β-casein was between 1.08 and 2.25 indicating a preferential adsorption of α_s-casein to β-casein.

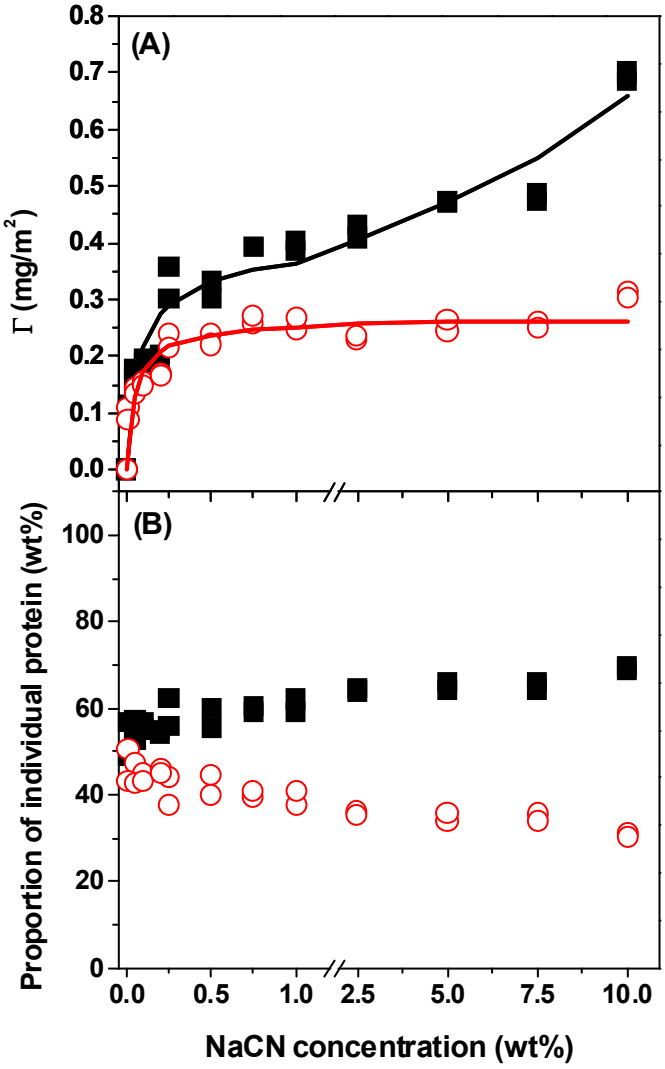

Figure 7.6 Adsorption isotherms for NaCAS proteins on normal rice starch granules (A) and the proportion of the individual proteins (B). Solid lines represent the fits using the BET equation and the Langmuir equation for α_s-casein and β-casein, respectively. NaCAS proteins; (■) α_s-casein, and (○) β-casein. Chi^2 values are for the BET fits and the Langmuir fits are 0.00193, and 0.00064 for α_s-casein and β-casein, respectively.

The amount and proportion of β-lg and α-la, adsorbed onto normal rice starch granules from WPI is shown in Figures 7.7A, and 7.7B, respectively. The amount of adsorbed β-lg and α-la gradually increased with increasing WPI concentration. Compared to other milk protein ingredients, very small amounts of these two proteins were adsorbed onto the normal rice starch granules from the starch/WPI mixture. The monolayer values calculated by the BET equation were 0.1 and 0.02 mg/m^2 for β-lg, and α-la, respectively. However, Figure 7.7A shows that β-lg started to form a second adsorption layer, in much the same way as α_s-casein did from NaCAS, when the WPI concentration exceeded 2.5%. In addition, it was found that the ratio of adsorbed α-la to β-lg was not affected by the WPI concentration. An average of the ratio of adsorbed β-lg to α-la was 5.35, whereas the ratio of β-lg to α-la in the original WPI solution was 2.65, showing the preferential adsorption of β-lg.

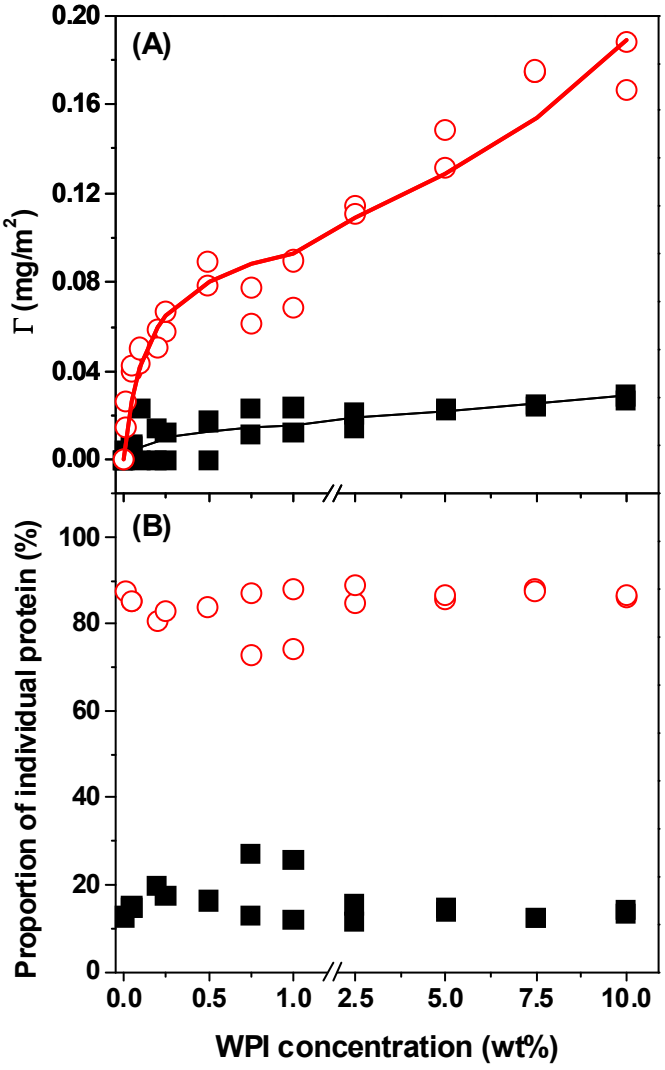

Figure 7.7 Adsorption isotherms for WPI proteins on normal rice starch granules (A) and the proportion of the individual proteins (B). Solid lines represent the fits using the BET equation. WPI proteins are: α-la (■), and β-lg (○). Chi^2 are 0.00019, and 0.00004 for α-la and β-lg, respectively.

7.3.2.2 Waxy rice starch

Figures 7.8A and 7.8B show the amount and proportion of α_s-casein and β-casein adsorbed onto waxy rice starch from SMP, respectively. Similarly to normal rice starch, the amount of adsorbed α_s-casein and β-casein gradually increased with increases in SMP concentration, to reach a plateau at 0.75% SMP. The monolayer values calculated from the Langmuir equation for adsorbed α_s-casein and β-casein onto waxy rice starch granules were 0.42, and 0.40 mg/m^2, respectively (Table 7.2), which is slightly less than the adsorbed α_s-casein and β-casein onto normal rice starch granules. The proportion of adsorbed α_s-casein and β-casein was dependent on the bulk concentration of SMP. At low concentration of SMP (≤0.1%), α_s-casein was preferentially adsorbed, with a ratio of α_s- to β-casein of 2.01 at 0.25% SMP. However, when the SMP concentration exceeded 2.5% the proportion of adsorbed α_s-casein and β-casein was almost constant and provided a ratio of α_s- to β-casein in the range of 1.01 to 1.16.

Table 7.2 Saturated surface concentrations (Γ_{max}) for monolayer coverage, the affinity of the adsorbed molecule (A_{BS}), and the solute concentrations in the subsurface area (C_S) for waxy rice starch mixed with each milk protein ingredient.

Milk protein	Langmuir		BET		
	Γ_{max} (mg/m^2)	A_{BS}	Γ_{max} (mg/m^2)	A_{BS}	C_S (mg/g)
SMP					
α_s-casein	0.42±0.01	2.90±1.46	-	-	-
β-casein	0.40±0.01	2.55±0.30	-	-	-
MPC					
α_s-casein	0.25±0.01	-3.58±0.93	-	-	-
β-casein	0.26±0.01	3.11±1.00	-	-	-
NaCAS					
α_s-casein	-	-	0.29±0.01	1347.82±257.79	146.28±23.0
β-casein	0.21±0.01	18.73±5.10	-	-	-
WPI					
β-lg	-	-	0.10±0.01	557.01±189.23	124.35±15.64
α-la	-	-	0.02±0.00	320.61±221.53	53.32±4.45

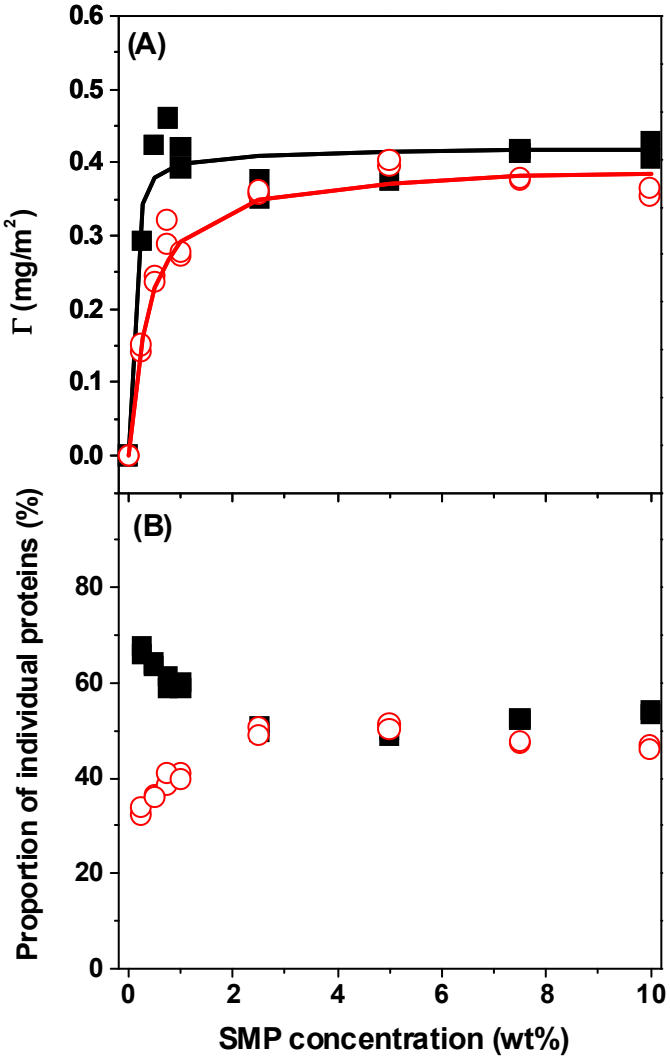

Figure 7.8 Adsorption isotherms for SMP proteins on waxy rice starch granules (A) and the proportion of the individual proteins (B). Solid lines represent the fits using the Langmuir equation. SMP proteins; (■) α_s -casein, and (○) β-casein. Chi^2 values are 0.00167, and 0.00048 for α_s-casein and β-casein, respectively.

Figures 7.9A, and 7.9B show the amount and proportion of α_S-casein and β-casein adsorbed onto waxy rice starch from MPC, respectively. The adsorption of milk proteins from MPC onto waxy rice starch showed similar behaviour to normal rice starch, but the amounts of adsorbed milk proteins were smaller. α_s-casein clearly had a higher affinity for the starch granules at MPC concentrations $\leq$ 0.5% and then decreased with increasing MPC concentrations until it reached a plateau at an MPC concentration of 2.5%. The monolayer value of adsorbed α_s-casein and β-casein onto waxy rice starch granules were 0.25, and 0.26 mg/m^2, respectively. The ratio of adsorbed α_s- to β-casein was higher at low MPC concentration (0.25 to 1%) than at higher MPC concentration (>1%). The ratio of adsorbed α_s- to β-casein for low MPC concentrations was in a range of 1.39 to 1.80, which decreased with increasing concentrations of MPC. At higher concentrations of MPC (>2.5%), the ratio of α_s- to β-casein was almost constant with values in the range of 0.82 to 0.87.

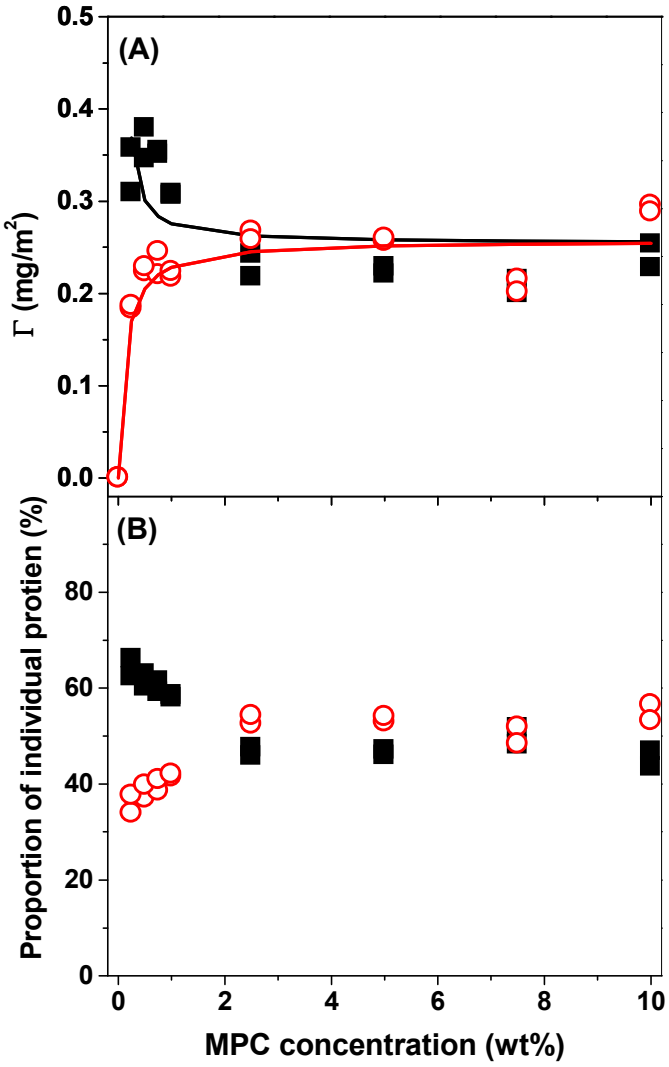

Figure 7.9 Adsorption isotherms for MPC proteins on waxy rice starch granules (A) and the proportion of the individual proteins (B). Solid lines represent the fitted Langmuir equation. MPC proteins; (■) α_s-casein, and (○) β-casein. Chi^2 values are 0.00144, and 0.00057 for α_s-casein and β-casein, respectively.

The amount and proportion of α_s-casein and β-casein from NaCAS adsorbed onto waxy rice starch are shown in Figures 7.10A, and 7.10B, respectively. The adsorption behaviour of α_s-casein and β-casein present in NaCAS onto waxy rice starch is similar to that of normal rice starch, but the amount of adsorbed α_s-casein and β-casein was smaller in the case of waxy rice starch than in the case of normal rice starch. The adsorption of β-casein onto waxy rice starch granules increased gradually with NaCAS concentration and reached a plateau value at 0.2% NaCAS, the monolayer value calculated by Langmuir equation was 0.21 mg/m^2. There is evidence of the adsorption of a multilayer of α_s-casein onto waxy rice starch, as was observed with normal rice starch. After the plateau is reached at 0.2% NaCAS, with a monolayer value of 0.29 mg/m^2, α_s-casein seems to form a second layer when the concentration of NaCAS exceeded 2.5%.

Similarly to normal rice starch granules, the amounts of α_s-casein adsorbed onto waxy rice starch granules was higher than the amount of adsorbed β-casein. At the lowest NaCAS concentration used in this experiment (0.01%), the ratio of adsorbed α_s- to β-casein is 1.05. This ratio increased with increases of NaCAS concentration up to 5%, above which the ratio was not affected.

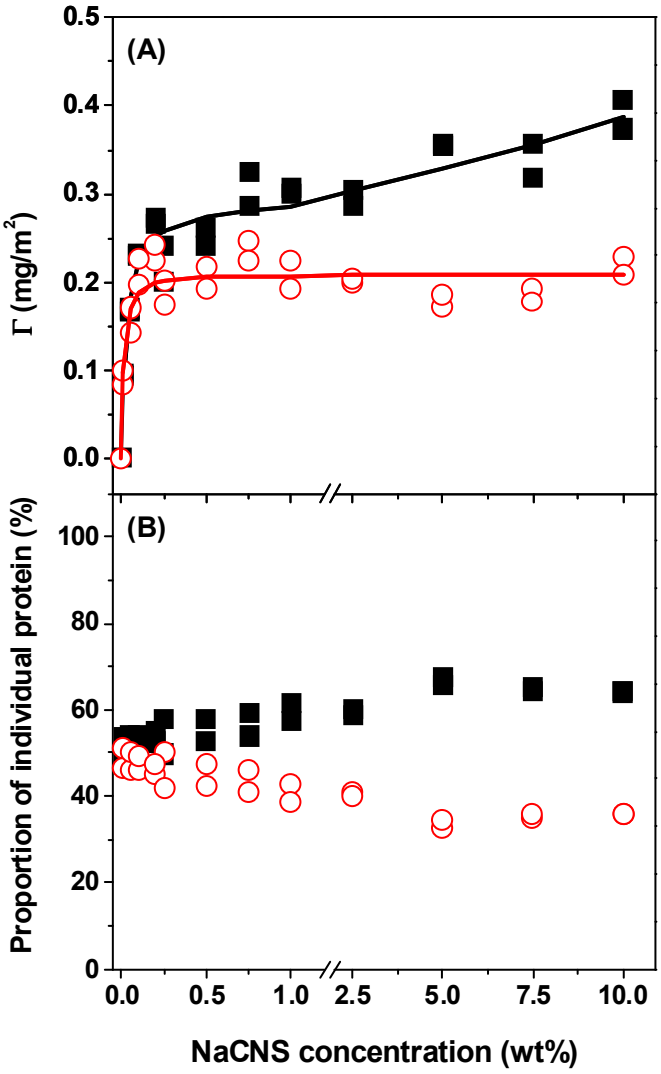

Figure 7.10 Adsorption isotherms for NaCAS proteins on waxy rice starch granules (A) and the proportion of the individual proteins (B). Solid lines represent the fits using the BET equation and the Langmuir equation for α_s-casein and β-casein, respectively. NaCAS proteins; (■) α_s-casein, and (○) β-casein. Chi^2 values for the BET fits and the Langmuir fits are 0.00111, and 0.00053 for α_s-casein and β-casein, respectively.

The amount and proportions of β-lg and α-la that were adsorbed from WPI onto waxy rice starch is shown in Figures 7.11A, and 7.11B, respectively. The adsorption behaviour of β-lg, and α-la onto waxy rice starch is similar to that of their adsorption onto normal rice starch. The amount of both β-lg, and α-la that was adsorbed increased with an increase in the bulk concentration of WPI and reached a plateau at 0.1% WPI. The monolayer values calculated by the BET equation were 0.10 and 0.02 mg/m^2 for β-lg, and α-la, respectively. A second adsorbed layer for both β-lg, and α-la occurred when the WPI concentration exceeded 2.5%. The proportion of adsorbed WPI β-lg, and α-la onto waxy rice starch was not affected by concentration of WPI over the range used in this experiment.

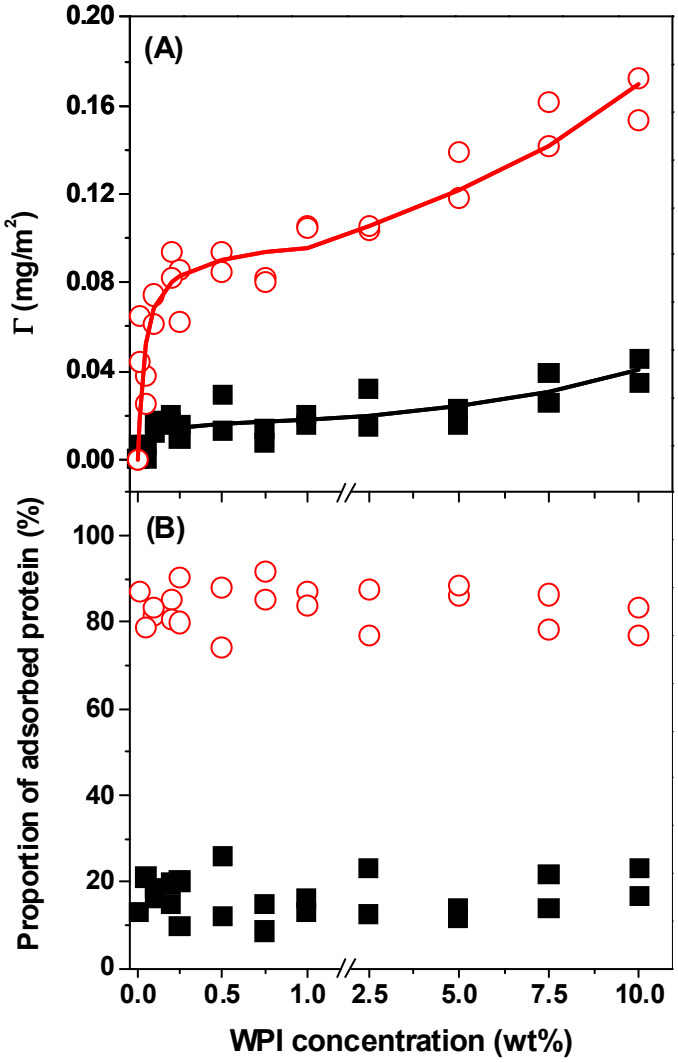

Figure 7.11 Adsorption isotherms for WPI proteins on waxy rice starch granules (A) and the proportion of the individual proteins (B). Solid lines represent the fits using the BET equation. WPI proteins are: α-la (■), and β-lg (○). Chi^2 are 0.00026, and 0.00004 for α-la and β-lg, respectively.

7.3.3 CSLM observation for milk proteins adsorbed onto normal rice starch granules.

CSLM images for normal and waxy rice starch granules after 24 hour incubation with the incorporated fluorescent dye (Alexa Fluor™ 488) are shown in Figures 7.12A and 7.12D, respectively. Brightly fluorescent regions are evident in the slides for both normal and waxy rice starch and are indicative of the presence of protein on the granule surfaces for each type of rice starch.

In Figures 7.12B and 7.12E, CSLM images for rice starch granules after 24 hour incubation with the labeled NaCAS proteins for normal and waxy rice starch are shown. In both starches, only a few of the individual granules showed a fluorescent region on the granule surface or a ring around the granule's periphery, but there was no evidence of proteins on the inside of the granule. This shows that the labeled proteins from NaCAS were unable to penetrate into the starch granules, but were adsorbed either fully to the surface of the starch granules or to parts of the granules' surfaces.

CSLM images for normal and waxy rice starch granules after 24 hour incubation with the labeled proteins from 0.01% WPI are shown in Figures 7.12C and 7.12F, respectively. Similarly to starches incubated with the labeled proteins from NaCAS, not all of starch granules were covered by the WPI labeled proteins. However, some granules appeared to be fully fluorescent, which could be a result of the absorption of the WPI molecules into the granule interior. Overall the CSLM observations were very similar to those for NaCAS.

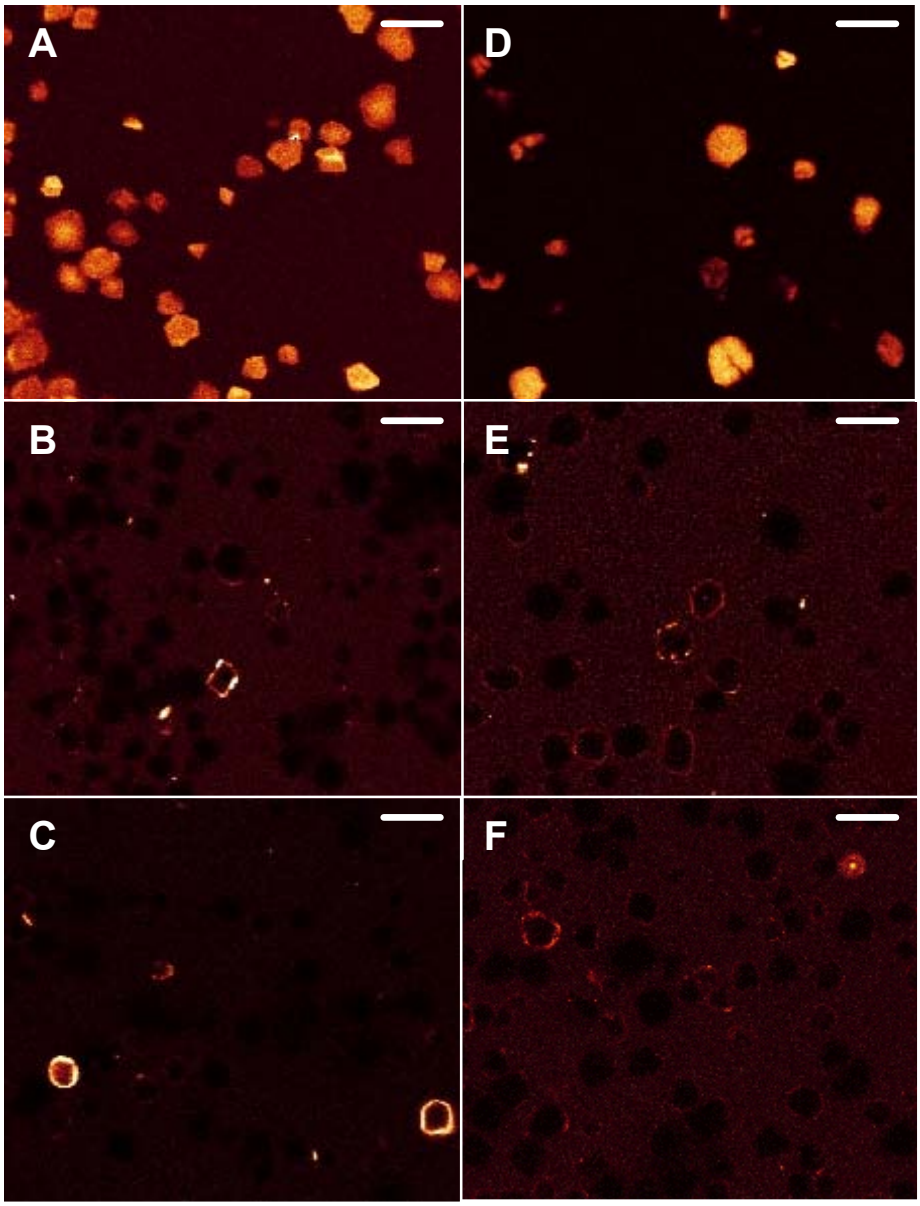

Figure 7.12 Confocal micrographs of normal rice starch (A, B, and C) and waxy rice starch (D, E, and F) after incubation for 24 hr at 4°C with; incorporation of Alexa Fluor^{TM} 488 dye (A, or D), labelled proteins from 0.01% NaCAS (B or E), and labelled proteins from 0.01% WPI (C or F), scale bar is represent 10 μm.

7.3.4 Determination of milk proteins adsorbed onto starch granules free-of native surface proteins and fat

CSLM images of normal and waxy rice starch granules with adsorbed caseins and whey proteins onto starch granule surfaces that had been stripped of all native proteins and fat and incubated for 24 hour with the incorporated fluorescent dye (Alexa Fluor^{TM} 488) are shown in Figure 7.13. There are a few individual granules which showed a fluorescent region on the granule surface or a ring around the granule's periphery, indicating the adsorption of proteins from NaCAS onto both normal and waxy rice starch granule surfaces (Figures 7.13A and B, respectively). Like NaCAS, a few of the individual granules also showed a fluorescent region on the granule surface (Figures 7.13C and D) when treated with whey protein.

The electrophoresis patterns of adsorbed α-casein and β-casein from NaCAS or α-la and β-lg from WPI obtained from normal or waxy rice starch granules with their native proteins and fat either present or denuded are shown in Figure 7.14. For normal and waxy rice starches without adsorbed milk proteins there was a surface protein of M_W~ 15kDa band (a band in Lane Rice). This band almost disappeared (Lane R-SDS) when either type of starch was treated with SDS to remove the surface proteins and fat, indicating that most of the surface protein was removed by the SDS extraction. As with NaCAS, the WPI proteins, α-la and β-lg, adsorbed to the surfaces of normal and waxy rice granules that either had or were free of their native proteins and fat. It seems that the intensity of the α_S-casein and β-casein bands was higher for granules that had their surface proteins and fat removed.

The pasting curves of 10% normal and waxy rice starch with and without their surface proteins and fat are shown in Figure 7.15. The SDS extraction dramatically decreased the T_{onset} and T_{peak} of normal rice. The peak viscosity of normal rice was increased after SDS extraction but the final viscosity was not affected by the SDS extraction. The SDS extraction displayed less effect on waxy rice starch than normal rice starch. The T_{onset} and T_{peak} of waxy rice decreased slightly after SDS extraction. The SDS extraction

increased the peak viscosity of waxy rice, without affecting its final viscosity. Although SDS has been reported to complex with amylose and/or amylopectin (Gudmundsson and Eliasson, 1990; Svensson, Gudmundsson and Eliasson, 1996; Debet and Gidley, 2006), Debet and Gidley (2006) have suggested that starch/SDS complexes have less of an effect on the gelatinisation and swelling of starch than results from the removal of the surface lipids and proteins from the granules' surfaces. In summary, the removal of the starch surface protein completely changed the swelling behaviour of normal rice starch, but had no affect on waxy rice starch. The normal rice changed from a slow-swelling rice to a rapid-swelling rice, a behaviour not unlike waxy rice starch.

Changes in the composition of the surface materials on the rice starch granules significantly altered their pasting behaviour. For granules free of their surface protein and fat T_{onset} and T_{peak} shifted to higher temperatures for both normal and waxy rice starch when caseins were added to the starch (Figure 7.16). The result is similar to what happens when NaCAS is added to waxy rice starch with the native proteins and fat still present (Figure 7.16). The T_{onset} of normal rice starch with the native proteins still present on the surface was not affected by adsorbed caseins, but T_{peak} shifted to a higher temperature. For both normal rice with and without its native protein and fat the final viscosity was increased by the adsorbed caseins.

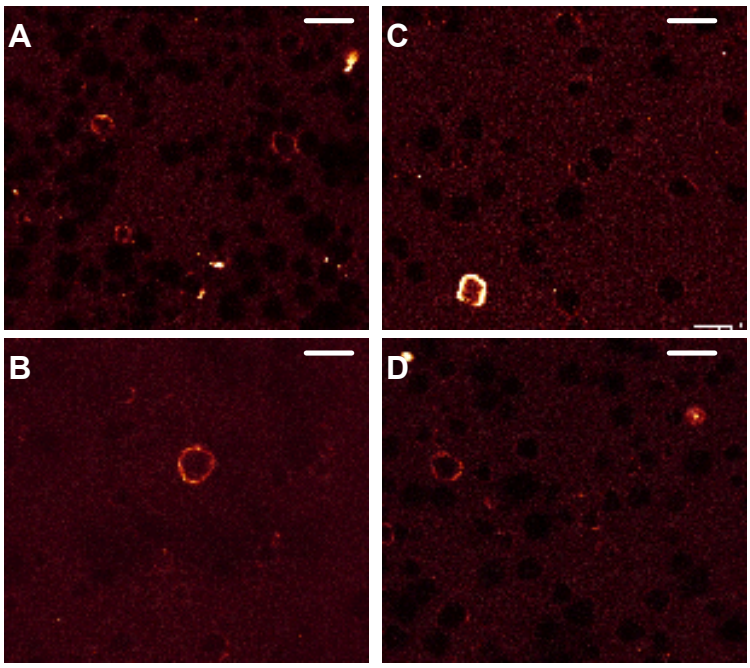

Figure 7.13 Confocal observation for the free surface-protein and fat normal rice starch with caseins (A) or whey proteins (B) adsorbed onto granules and the free surface-protein and fat waxy rice starch with caseins (C) or whey proteins (D) adsorbed onto granules. Bar scale = 10 μm.

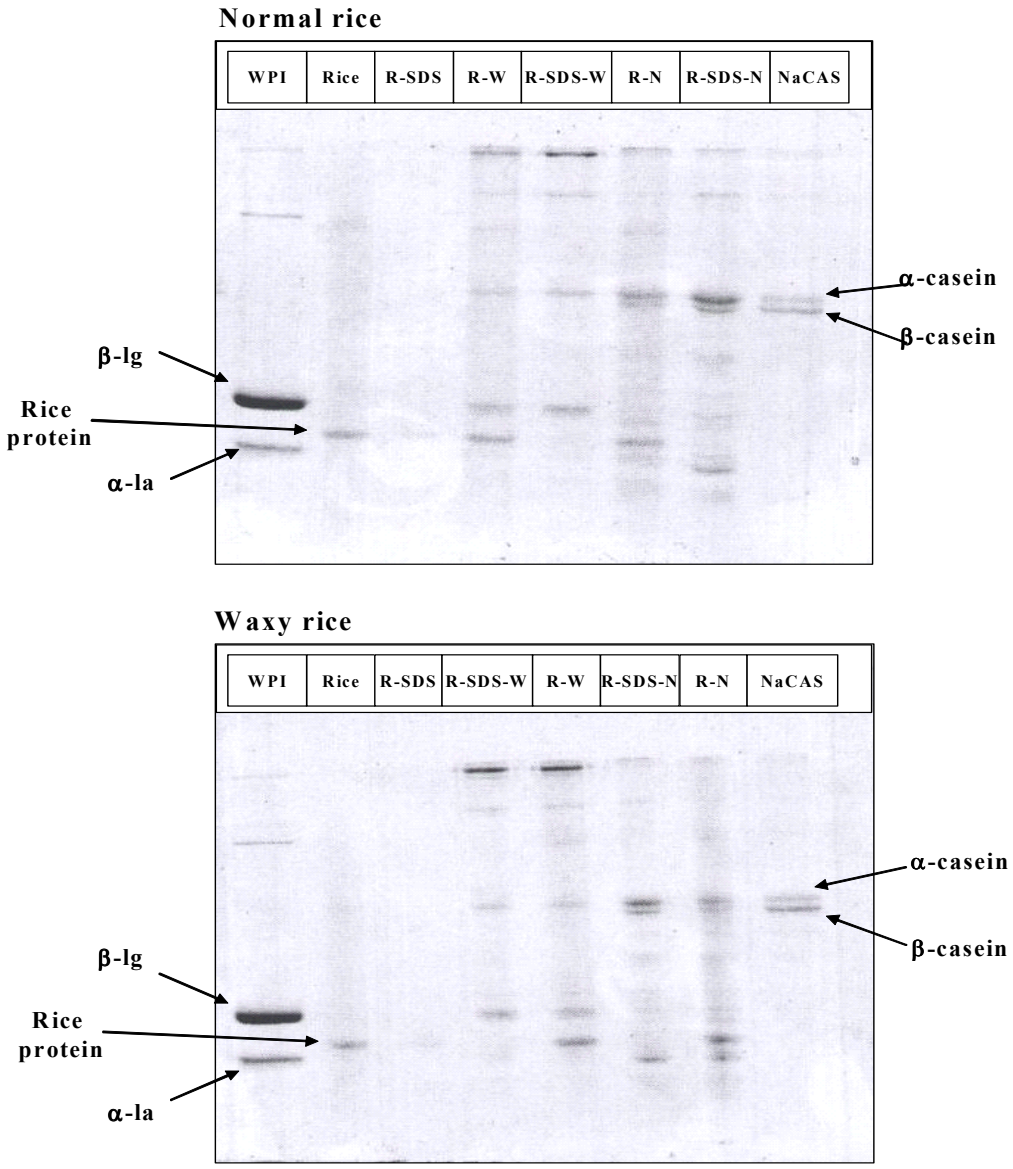

Figure 7.14 The electrophoresis patterns of adsorbed α-casein and β–casein from NaCAS or α-la and β–lg from WPI obtained from normal and waxy rice with and without their native proteins and fat. Standard WPI (WPI), native rice starch without adsorbed milk proteins (Rice), rice starch without surface-protein and fat (R-SDS), rice starch without free surface-protein and fat and with adsorbed whey protein (R-SDS-W), rice starch with adsorbed whey protein (R-W), rice starch free of surface-protein and fat with adsorbed caseins (R-SDS-N), native rice starch with adsorbed caseins (R-N) and standard NaCAS (NaCAS).

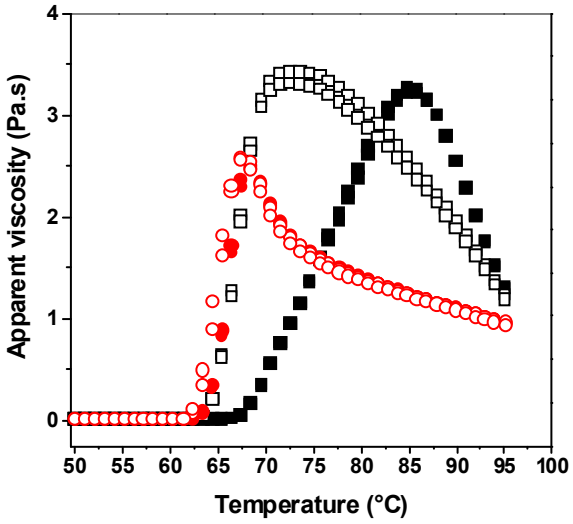

Figure 7.15 Apparent viscosity as a function of temperature for 10% normal rice starch (square) and 10% waxy rice starch (circle) in water, native surface starch (close) or surface-free protein and fat starch (open)

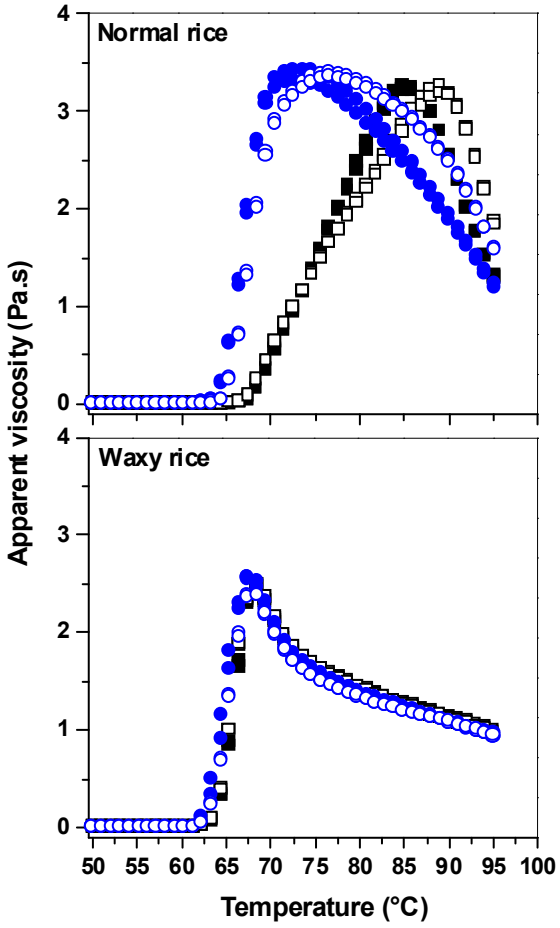

Figure 7.16 Apparent viscosity as a function of temperature for 10% normal rice starch and 10% waxy rice starch. Normal starch with native surface proteins and fat (■), native surface with adsorbed caseins from NaCAS (□), normal starch without surface protein and fat (●), and normal rice without surface protein and fat with adsorbed caseins from NaCAS (○).

7.4 Discussion

7.4.1 The adsorption of milk proteins on to normal and waxy rice starch granules

The normal and waxy rice starch granules used in this project had polyhedral shapes with an average diameter of 5.60 and 5.79 μm, respectively. This is in agreement with several studies from several research groups, which showed that rice starch granules were polyhedral with irregular shape and having a size range of 1.5 to 15 μm (BeMiller and Whistler, 1996; Champagne, 1996; Belitz and Grosch, 1999; Qi *et al*., 2003; Bao and Bergman, 2004). The absolute densities of normal and waxy rice starch were 1.51±0.00, and 1.50±0.00 g/cm^3, respectively. These are similar to values reported by Juliano (1984) of 1.48-1.50, and 1.49-1.51 g/cm^3 for normal and waxy rice starch, respectively.

Confocal scanning laser microscopy (Figure 7.12), suggests that milk proteins do adsorb to or are absorbed into the rice starch granules. Despite not being widely studied, the adsorption of proteins to starch granules, as reported in the introduction to this chapter, has been previously demonstrated (Lund, 1984; Seguchi, 1984; Eliasson and Tjerneld, 1990; Wannerberger *et al*., 1996; Baldwin *et al*., 1997; Baldwin, 2001; Debet and Gidley, 2006).

There are many possible mechanisms for added proteins to be adsorbed onto starch granules; 1) hydrophobic interaction between starch granule surface proteins-and added proteins, 2) hydrophobic interaction between starch granule surface lipids-proteins, 3) electrostatic interaction between charged groups at starch granule surface – and charged domain of added proteins, and 4) hydrogen bonds between the hydroxyl group from terminated glucan molecule that protrude around starch granule surfaces – hydroxyl, amino, or other electron-donating or electron-accepting groups of the added proteins. Despite these complexities, the interaction between starch granules and added milk protein ingredients will be discussed from the milk protein angle. As described in the literature review section, the milk ingredients used in this study could be separated into two groups. The first group, comprised of SMP and MPC, where the majority of the

proteins are organised into casein-micelles. And a second group made of individual proteins, such as individual caseins in NaCAS; and α-la and β-lg in the case of WPI. It is thus expected that the proteins of these different ingredients will adsorb/absorb differently.

In the case of casein-micelle type ingredients, the present study showed that for both SMP and MPC, a Langmuir-type isotherm was obtained for the adsorption of the individual proteins (Figures 7.4, 7.5, 7.8 and 7.9). At low SMP or MPC concentration, the adsorbed α_s- to β-casein ratio is similar to the α_s- casein to β-casein ratio in casein micelles, then at high concentrations of SMP or MPC, this ratio decreases indicating that more β-casein than α_s-casein is adsorbed. This could be explained as follows. At low concentration there is no preferential adsorption of the different milk proteins, then when the concentration of SMP reaches some critical value, β-casein, which is known to be more hydrophobic than α_s-casein (Dickinson, 1997; Dickinson, 1999b; Walstra *et al*., 2006), is adsorbed more readily, probably by displacing the adsorbed α_s-casein. It is however difficult to speculate under which form the SMP or MPC proteins adsorb, since at low concentration the casein micelles in these ingredients would undergo dissociation due to the lack of minerals which are known to contribute to the formation of the casein micelle (Horne, 1998).

In the case, of NaCAS the caseins exist as free casein molecules and self-assembled protein particles; both casein complexes and aggregates (Creamer and Berry, 1975; Srinivasan *et al*., 1999). The main caseins that adsorb onto normal and waxy rice starches are α_s-casein and β-casein with the amount of adsorbed α_s-casein being higher than that of the adsorbed β-casein. The ratio of adsorbed α_s-casein to β-casein increased slightly with increasing NaCAS concentration (Figures 7.6 (B) and 7.10 (B) for normal and waxy rice starch, respectively). Finally in the case of WPI, for both normal and waxy rice starches, the adsorbed amount of β-lg and α-la from WPI continuously increased with increasing WPI concentration. This continuous increase could be due to the ability of β-lg, at high concentration, to adsorb as multilayers to both hydrophobic and hydrophilic surfaces at high concentration of β-lg (Hunt and Dalgleish, 1994a; Addesso and Lund, 1997; Elofsson, Paulsson and Arnebrant, 1997).

Since there are nearly no published studies (except the adsorption of BSA to wheat starch in Eliasson and Tjerneld (1990)) on the interaction between milk proteins and starch granules, the results obtained here will be discussed from a consideration of the observations that have been made on the behaviour of milk proteins at oil-water (emulsions) and solid-water (example polystyrene beads dispersions) interfaces. In the present study, it was found that the amount of adsorbed total protein from milk protein ingredients onto normal and waxy rice starches follows the order; SMP> MPC>NaCAS>WPI (From Table 7.1, and 7.2). This is in agreement with the studies performed on emulsions stabilised by different milk protein ingredients, where the same order in the amounts was reported (Singh, Fox and Cuddigan, 1993; Sharma *et al*., 1996a; Sharma and Singh, 1998; Euston and Hirst, 1999). This was explained by the fact that SMP and MPC tend to adsorb as aggregates (probably in a similar form to the initial casein micelle).

Furthermore Euston and Hirst (1999) reported lower levels of surface coverage of the oil droplets for WPI than NaCAS and explained that this was due to the highly flexible structure of extended casein molecules, compared to the more rigid structure of globular whey proteins. Unfortunately, despite this qualitative agreement, the measured amounts of adsorbed proteins to oil-water interfaces are much higher than the amounts measured in the case of normal and waxy rice starch. For example, Euston *et al*. (1999), reported values for the surface coverage of SMP and MPC >15 mg/m^2, while the measured values of <1 mg/ m^2 were found in the present study for the two starches. The same was observed in the case of WPI and NaCAS, where values of about 3.20 mg/m^2 for both ingredients have been reported for emulsions (Hunt and Dalgleish, 1994a), while values <1 mg/m^2 and 0.12 mg/m^2 were measured for NaCAS and WPI respectively on rice granules in the present study.

The study showed firstly that significantly lower amounts of protein are adsorbed to rice starch granule surfaces than emulsion oil/water interfaces. The study also showed that more protein was adsorbed to normal rice granule surfaces than to waxy rice granule surfaces. These differences suggest that the interaction between milk proteins and starch granules might not be due to hydrophobic interaction as in the case of emulsions. If they are driven by hydrophobic interaction, then the interaction is

influenced by the amounts and types of native protein and fat on the starch granule surfaces. If there is an interaction between milk protein with the starch protein and lipids, then it is expected that more milk protein will be adsorbed to normal rice starch, since it has higher amounts of lipids and protein than waxy rice starch.

The results from Tables 7.1 and 7.2 showed that higher amounts of α_s-casein and β-casein from SMP, MPC and NaCAS were adsorbed onto normal rice starch than waxy rice starch (i.e. Γ_{max} of total milk protein from SMP were 0.93 and 0.82 mg/m^2 for normal and waxy rice starch, respectively). Where there were no differences in the amounts of adsorbed proteins from WPI onto normal and waxy rice starch. Moreover, the differences in the amounts of α_s-casein and β-casein from SMP, MPC and NaCAS adsorbed by normal and waxy rice starch were not high enough to conclude that surface protein and fat mediate the binding of the added milk proteins, even though small differences in native protein and fat content between normal and waxy rice starch significantly influence their pasting behaviour. Removal of surface protein and fat from normal rice starch by 2% SDS converted the initial viscosity behaviour of normal rice from its normal behaviour of developing slowly with increasing temperature to one where it behaved like waxy rice starch (Figure 7.15). In other word, without surface protein and fat, over the initial phase of pasting, normal rice starch behaves as waxy rice starch. Therefore, it is uncertain whether surface protein and fat mediate the binding of the added milk proteins.

The additional experiment as described in section 7.2.2.4, was carried out to investigate the potential mechanisms for explaining the adsorption of added proteins onto starch granules, i.e., whether surface protein and fat play a critical role. The CLSM images (Figure 7.13) give a pictorial view of what happens to the adsorbed proteins and the electrophoresis pattern (Figure 7.14) gives a qualitative view of what happens to individual milk proteins when they are being adsorbed to normal and waxy rice starches with and without their native proteins. These figures clearly indicate that the starch granule surface without protein and fat has the ability to adsorb milk proteins. The surface of the rice starch granule contains the non-reducing end-groups from the amylose and amylopectin molecules that protrude from the surface all around the starch

granule (Baldwin *et al*., 1998). It is possible that the hydroxyl groups on the end of the amylose or amylopectin molecules form hydrogen bonds between the hydroxyl, amino, or other electron-donating or electron-accepting groups and it is these reactions that are primarily involved in the adsorption of the milk proteins to the granule surfaces.

There is some suggestion that both hydrogen bonding between glucan molecules on the surface of the rice starch granules and hydrophobic interactions are responsible for the adsorption of the milk proteins, given the fact that slightly higher amounts of protein are adsorbed from SMP, MPC and NaCAS onto normal starch granules than waxy rice granules. However, the magnitude of the hydrophobic bonding is probably less pronounced than the hydrogen bonding between the glucan molecules and the added milk proteins. As hydrophobic bonds are formed between the added proteins and surface protein and fat (Oda and Schofield, 1997; Baldwin, 2001; Debet and Gidley, 2006), the influence of these bonds was probably not significant given the fact that only small amounts of surface protein and fat are present on normal starch and even less on waxy starch granule surfaces.

Another mechanism which cannot be overruled is the absorption of the milk protein into starch granules in the case of WPI. Whilst the CSLM images showed that the casein proteins from NaCAS were adsorbed only onto the granule surfaces of normal and waxy rice starches (Figures 7.12B and 7.12E, respectively), the whey proteins from WPI were not only adsorbed onto starch granule surfaces but also penetrated into the interior of the starch granules (Figures 7.12C and 7.12F, respectively). In WPI small low molecular weight proteins with a molecular weight range between 4000 to 22000 Da (Walstra *et al*., 2006), such as proteose peptones (PP) are present and it is possible that these migrated to the inside of the starch granules via starch surface pores. The proteoses result from the hydrolysis of β-casein. It has been reported that wet starch is penetrable by proteins with a hydrodynamic radius less than 0.6 nm (Planchot *et al*., 2000). However, the main proteins in WPI, for example β-lg, cannot penetrate into the inner parts of the starch granule due to their large molecular size; the molecular weight and the hydrodynamic radius for the monomer of β-lg are 18,283 Da, and 2.04 nm, respectively (Aymard *et al*., 1996; Walstra *et al*., 2006).

7.4.2 Relation between the adsorption of milk proteins and the physico-chemical behaviours of normal and waxy rice starches

Although very small amounts of α_s-casein and β-casein are adsorbed onto the surface of rice starch granules if they have their native proteins and fat removed, they still have a significant affect on the pasting behaviour of normal rice starch (Figure 7.16). For instance, the pasting T_{onset} value was increased by the adsorption of casein onto waxy rice starch and both normal and waxy rice starch granules that had their native proteins and fat removed. T_{onset} of native normal rice starch was not affected by the adsorption of caseins. This suggests that the native starch proteins and fat on normal rice starch control the pasting behaviour (T_{onset}) of normal rice starch, but once removed by SDS that the pasting behaviour can be significantly influenced by the adsorbed proteins. However, the native proteins and fat on waxy rice starch have little influence on the pasting behaviour (T_{onset}) of the starch and can be greatly influenced by any adsorbed protein (Figure 7.16).

The adsorption of caseins onto native normal rice starch had a more pronounced affect on the T_{peak} of pasting than T_{onset}. This trend was also observed in the case of the pasting of a normal rice starch/NaCAS mixture (Figure 7.16 and Chapter 3) and the thermal behaviour of a normal rice starch/NaCAS mixture (Chaper 5). Note that, the endothermic peak (T_{peak}) of a normal rice starch/NaCAS mixture measured by DSC was not only shifted to a higher temperature but displayed a clearly broader peak than was the case for the starch/water mixture. It is known that the strength of hydrophobic interactions increase with increasing temperature (Chandler, 2005), therefore any increase in temperature (T_{onset} or T_{peak}) during the pasting of a normal rice starch/NaCAS mixture could probably alter the pattern of adsorbed caseins and thus lead to an increase in the hydrophobic interaction between adsorbed caseins and surface protein and/or fat. The shifts of T_{onset} and T_{peak} to higher temperatures may possibly also be due to the fact that the adsorbed casein proteins strengthened the starch granules in some way and prevented then from disrupting until sufficient energy (either heat or shear) has been applied into the mixtures to disrupt this adsorbed casein layer. The result from leaching of starch molecules experiments (Chapter 6) showed that the

amount of amylose and amylopectin that were leached tended to decreased in the presence of NaCAS in both normal and waxy rice starch. This indicates that the adsorbed caseins had increased the granules' rigidity and resistance to disruption/disintegration during heating under shear.

The results from section 7.3.2 showed that the adsorption pattern of caseins was dependent on the concentration of NaCAS. At low NaCAS concentration (≤ 2%), the adsorption of both α_s- and β-casein is likely to be in the monolayer form. At higher concentration of NaCAS, there is evidence for α-casein multilayer formation onto rice starch surface granules. This second adsorbed layer might have been driven by a hydrophobic interaction between hydrophobic regions on the first adsorbed layer and the second adsorbing layer of α-casein. The additional adsorbed layers of α-casein resulted in an increase in the rigidity of the starch granules and thus delayed T_{peak} of the endothermic peak and increased the peak viscosity and the swelling ratio of rice starch/NaCAS mixtures (Chapter 3 and Chapter 6, respectively).

Although it was also found that milk proteins from SMP adsorbed/absorbed to/into rice starch granules their effects on pasting/gelatinization, swelling and starch molecules leaching were clearly hindered by the presence of lactose and the minerals in SMP. Among the four types of milk protein ingredients examined, SMP displayed the greatest effects on the pasting/gelatinization and swelling of both normal and waxy rice starches. All these effects were also observed for UFSMP, which is a solution of salts and lactose present in SMP. For instance, T_{onset} and T_{peak} for the pasting of normal rice starch in the presence of either10% SMP or 10% UFSMP were shifted to higher temperatures to a similar degree; 2.52°C for T_{onset} and 5.15°C for T_{peak}, on the addition of 10% UFSMP and 3.23°C for T_{onset} and 5.10°C for T_{peak}, on the addition of 10% SMP, respectively (Chapter 3).

In the case of MPC and WPI, it was found that the thermal properties of waxy rice starch were altered by the presence of MPC and WPI; T_{onset} and T_{peak} of the endothermic peak was shifted to a higher temperature (Chapter 5). The T_{onset} and T_{peak} values obtained for the pasting experiments also increased in the presence of WPI,

whilst only T_{peak} (pasting) increased on the addition of MPC (Chapter 3). The absorbed α-la and β-lg might have been the cause of this increase in gelatinization/pasting temperature. The leaching of starch molecules was decreased in both normal and waxy rice starch on the addition of MPC and WPI (Chapter 6). From these findings, it is possible that the adsorbed α-la and β-lg layer that covered the waxy starch granules prevented the starch molecules from leaching. However, as with NaCAS, MPC and WPI had no effect on the thermal properties of normal rice.

7.5 Summary to chapter

The normal and waxy rice starches used in this study had a similar polyhedral shape with an average diameter of 5.60 and 5.79 μm, respectively. The densities of the normal and waxy rice starches were 1.51 and 1.50g/cm^3, respectively.

The results from the CSLM images showed that the milk proteins were adsorbed and possibly absorbed (in the case of the WPI proteins) to the granule surface of both normal and waxy rice starch granules. It was found that the milk protein ingredients adsorbed in the order SMP>MPC>NaCAS>WPI. This was explained in terms of the aggregated nature (casein micelle type) of the milk protein in SMP and MPC, and to the individual nature of the proteins in NaCAS and WPI. Unlike the adsorption of milk protein to oil-water interfaces, the hydrophilic interactions; hydrogen bonds between hydroxyl group from terminated glucan molecules that protrude around starch granule surface-hydroxyl; amino, or other electron-donation or electron-accepting groups of the added proteins, could also contribute to the adsorption of milk protein to rice starch granules.

Chapter 8

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDIES

8.1 General conclusions

This thesis had the daunting task of investigating the effects that four milk protein ingredients had on some physico-chemical properties of an aqueous dispersion of rice starch. The milk protein ingredients were chosen as they are readily available commercially, and they cover a large range of dairy systems. For instance, SMP has the same composition as milk, except for fat; MPC is similar to SMP but does not contain large amounts of lactose, NaCAS is made of caseins alone, and WPI of whey proteins alone. The caseins are associated with the micelles in SMP and MPC, but are either associated with other caseins or individual molecules in NaCAS. Both normal and waxy rice starch were considered due to the difference in their amylose contents.

The addition of these milk protein ingredients to starch suspensions resulted in different effects, particularly in the case of the pasting behaviour of the mixtures, as could be seen in Figure 8.1. The main findings of this thesis are highlighted below:

1. The addition of the different milk protein ingredients had a greater effect on the pasting behaviour of normal than waxy rice starch. With the exception of MPC, SMP, NaCAS and WPI tended to increase the peak temperatures of normal starch more than they did for waxy rice starch. The effects of SMP on both starches were probably due to the effects of lactose and the minerals as the effects of UFSMP (lactose and minerals minus the proteins) were comparable to SMP. Normal rice starch has a higher onset temperature and its granules hold their integrity better than those of waxy rice starch. Thus normal rice starch competes for water more efficiently than waxy rice starch. The WPI and particularly the non-micelle associated caseins in NaCAS further increased the rigidity of both normal and waxy rice starch granules compared to the starches

alone and thus increased the peak viscosity of both starches. These effects were greater on normal than waxy rice starch.

2. Despite the differences observed during pasting, the viscoelastic properties of the resulting gels (cooled pastes) are qualitatively the same as for starch alone. This is an indication that the rheological behaviour is dominated by the continuous phase made of starch molecules. In fact, at low milk protein concentrations (≤5%) the milk protein ingredients behaved as inert fillers. Only at high concentration was an effect from these ingredients observed, and this is due either to protein aggregation as in the case of heat-denatured WPI, or to phase separation between the starch molecules and the casein micelles present in SMP and MPC.

3. The onset gelatinisation temperature was also measured using DSC, and a correlation between this temperature and the initial temperature of pasting (measured by rheology) was established.

4. Contrary to common belief, this study showed that it is most probable that the preferential leaching of amylose in the early stage of pasting is overestimated. This study showed that both amylose and amylopectin leach together, and that a majority of the leaching is driven not by diffusion, but through the break-up of the swollen starch granules. The addition of the milk protein ingredients decreased the amount of starch that was leached during the early stages of pasting.

5. An elegant and simple sedimentation-based method was developed to measure accurately the swelling of the starch granules. The measurements of the swelling obtained using this method allowed (i) the calculation of the theoretical viscosity during the early stage of swelling, and (ii) the determination of the exact onset temperature of swelling. This method confirmed unequivocally that SMP increases the onset temperature of swelling and this is due to lactose and the delay in swelling observed when NaCAS was added. It was also clearly demonstrated that the real onset temperature at which the starch granules start to swell is the T_S value obtained from the swelling measurements. Alternatively, the onset temperature of swelling could be

obtained from the rheological measurements, by taking into account the change in the viscosity of the continuous phase with temperature.

6. Finally, since small amounts of NaCAS had large effects on the swelling and pasting behaviour of the starch suspensions, the interactions between the milk proteins and the starch granules was investigated. Confocal microscopy indicated the possibility of heterogeneous adsorption/absorption of the milk proteins to the starch granules. Adsorption isotherms were established, and it was found that the milk protein ingredients adsorbed in the order SMP>MPC>NaCAS>WPI. Similarly, to emulsions the high amounts observed for SMP and MPC are due to the aggregated (casein micelle type) of the protein present in these ingredients.

Figure 8.1 summarises the observed trends in pasting behaviour upon addition of different dairy ingredients to both waxy and normal rice starch.

A possible mechanism to explain the effects of milk proteins on pasting behaviour was postulated. Proteins present on the surface of the granules both compete for moisture and resist swelling. As a result a delay in swelling and consequently viscosity increase results when more proteins are present on the surface. Peak viscosity is also affected due to the increased resistance to shear imparted by the surface coating of protein. This mechanism could explain the differences in early pasting behaviour observed for waxy and normal rice starches in the absence of added milk protein ingredients. This is supported by the similar pasting behaviour of waxy rice starch and normal rice starch after surface proteins had been removed by washing in SDS.

The composition and amount of proteins present on the granule surface is likely to depend on the nature of the surface (e.g. number of hydrophobic and hydrophilic binding sites) and the form of proteins added in the milk protein ingredient (e.g free whey proteins or casein micelles). Understanding of these interactions and how the granule surface can be modified (e.g. by washing out of native proteins) could offer insights on how protein/starch interactions can be exploited in industrial products.

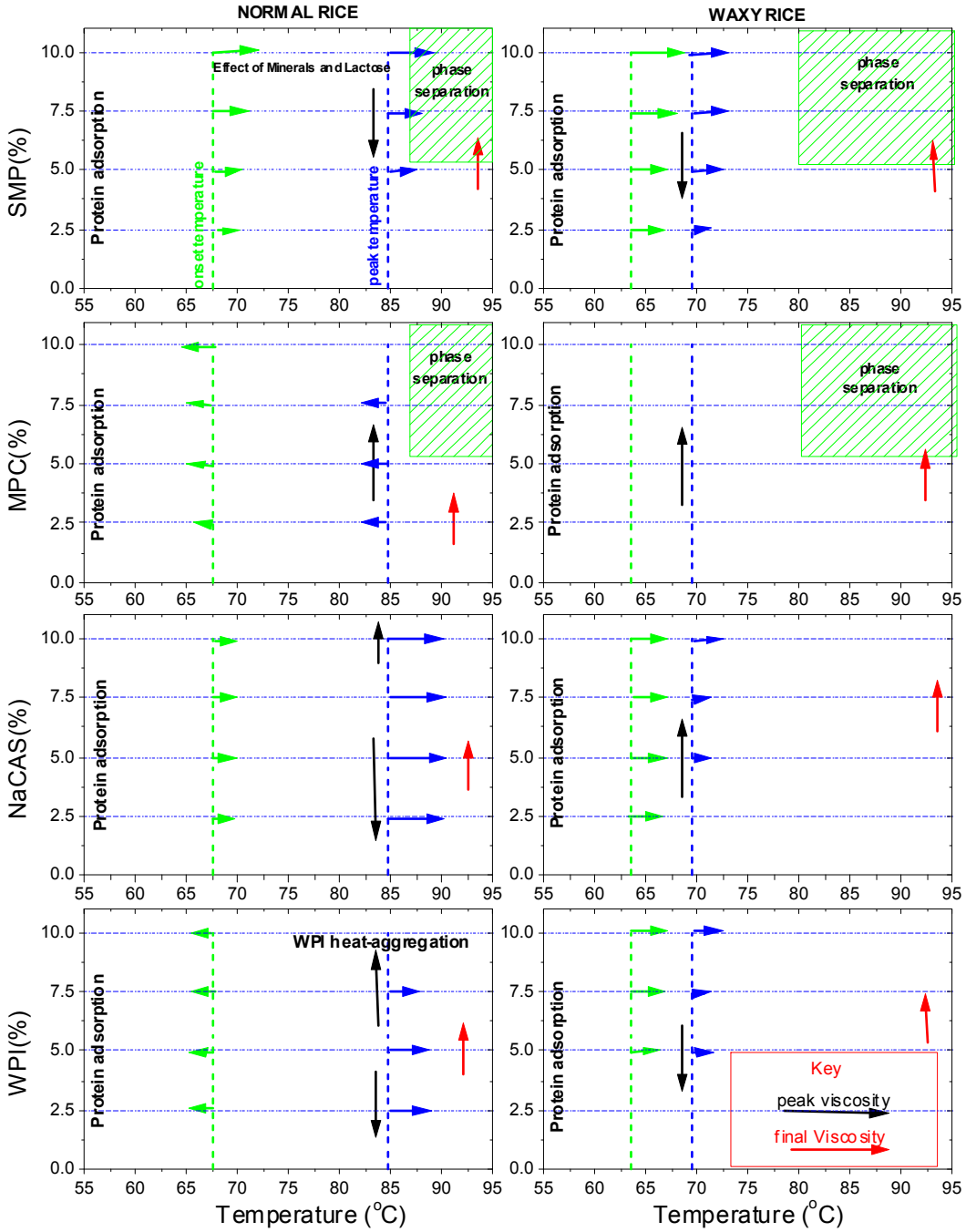

Figure 8.1 Schematic representation of the effects of different milk protein ingredients on the pasting of normal (left hand side) and waxy (right hand side) rice starch.The green dash line (⋮) and blue dash line (⋮) represent the onset and peak temperature of starch without dairy ingredient, which is decreased (⬅, ⬅) or increased (➡ , ➡) depending on type and concentration of dairy ingredients. The peak (➡) and final viscosity (➡), which is also decreased (⬇,⬇) or increased (⬆,⬆) depending on type and concentration of dairy ingredients. The mechanisms influencing the pasting of rice starches on the addition of dairy ingredients at various concentrations could be the adsorption of milk proteins onto rice starch granules, the phase separation, the heat-aggregation of whey proteins and the effects of mineral and lactose present in dairy ingredient.

8.2 Recommendations for further studies

Although the amount of material presented in this systematic study is extensive, it only skimmed the surface of an area where a great deal further research remains to be done. The findings of this thesis pinpoint several venues which need pursuing:

1. The present study was conducted from the milk protein view point. Mixtures where the concentrations of both starch and milk protein are varied should be considered, particularly in the case of SMP and MPC. This should allow the establishment of the concentration conditions (phase diagrams) under which phase-separation and phase inversion are observed.

2. The effect of milk proteins on other starches, of different botanical origins to rice should be also investigated. A good candidate will be potato starch, which could be affected differently from other starches, as it contains phosphorus. Further, the influence of minerals, pH, ionic strength and temperature should be investigated.

3. The adsorption mechanism of the milk proteins to starch granules is not fully elucidated. Further studies should be carried out on purer systems under more controlled conditions. This will include the use of individual milk proteins (α-casein *vs* β-casein) and narrow range granule sizes, which could be obtained by successive partitioning for example. In this way understanding of the equilibrium between proteins bound to the granule surface (either added or native), free in solution and in micelles could be further developed. The effect of micro-peptides and hydrolysed proteins should also be investigated to explore whether these milk proteins are absorbed into the starch granules.

4. For the sake of industrial relevance, gelled milk proteins containing starch and multi-component systems, such as starch-protein-fat mixtures, should be studied. This will allow the investigation of how these mixtures perform in yoghurt and processed-cheese type systems.

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APPENDIX A

Amylose/amylopectin determination

The amylose content of starches was determined by using an amylose/amylopectin assay kit (Megazyme, Ireland), according to the method described by Gibson, Solah, & McCreary (1997). The principle of this method is based on specific binding of non-reducing end-groups of amylopectin with concanavalin A, which is then removed from amylose by centrifugation. The amylose is enzymatically hydrolysed to glucose, and analysed using glucose oxidase/peroxidase reagents.

Equipment

1. Glassware
    - Volumetric flask (25 ml)
    - Glass test tube (10 x 120 mm, 15 ml)
    - Screw capped sample tubes (Kimax$^{@}$) (10 ml)
2. Micropipettors, to dispense 50-1000 µl
3. Positive displacemented pipettor
4. Eppendorf$^{@}$ microfuse tubes (2.0 ml capacity)
5. Boiling water bath
6. Bench centrifuge (speed required to give 2,000g)
7. Vortex mixer
8. Spectrophotometer, set at 510 nm
9. Stop clock
10. Analytical balance
11. Microfuge, capacity of 14,000g
12. Thermostated water bath set to 40°C

Preparation of chemical reagents

1. Con A solution;
   Dissolve the entire contents of the Con A vial (200 mg) enclosed with the kit in 50 ml of Con A solvent (working concentration).

2. GOPOD reagent;
   Dilute the entire contents of the glucose reagent buffer container enclosed with the kit to 1 litre with distilled water and use this to dissolve the glucose determination reagent. Reagents concentrations after dissolution in buffer:
   Glucose oxidase > 12,000 U/litre
   Peroxidase > 650 U/litre
   4-Aminoantipyrine 0.4 mM
   Divide this reagent (GOPOD reagent) into aliquots of desired volume for storage.
   Stability: 2-3 months at 4°C
   12 months at -20°C

3. Glucose standard solution;
   Glucose 100 μg/0.1 ml in 0.2% benzoic acid, which is enclosed with the kit.

4. Sodium acetate buffer (100 mM, pH 4.5);
   Glacial acetic acid (5.9 ml, 1.05 g/ml) is added to 900 ml of distilled water. This solution is adjusted to pH 4.5 by the addition of 1 M (4 g/ 100 ml) sodium hydroxide solution. Sodium azide (0.2 g) is added and the volume is adjusted to 1 litre. The solution is stored at room temperature.

5. Concentrated Con A solvent (pH 6.4, 600 mM in acetate);
   Anhydrous sodium acetate (49.2 g BDH Cat. No: 10236)
   Sodium chloride (175.5 g, BDH Cat. No: 10241)
   $CaCl_2.2H_2O$ (0.5 g, Mallinckrodt Cat. No: 4160)
   $MgCl_2.6H_2O$ (0.7 g BDH, Cat. No: 10149) and

MnCl2.4H2O (0.7 g Ajax, cat. No: 307) are dissolved in 900 ml of distilled water. The pH is adjusted to 6.4 by dropwise addition of glacial acetic acid and the volume is adjusted to 1 litre with distilled water. The solution is stored at 4°C.

6. Con A solvent (working concentration);
   Dilute 30 ml of concentrated Con A solvent to 100 ml with distilled water on the day of use.

7. Dimethyl sulphoxide (DMSO);
   Analytical reagent grade (BDH Analar Cat. No: 10323).

Assay procedure:

A. Starch pre-treatment

1. Accurately weigh starch sample (20-25 mg to the nearest 1.0 mg) into 10 ml screw capped Kimax@ sample tube. Record sample weigh to the nearest 1.0 mg.

2. Add 1.0 ml of DMSO to the tube while gently stirring it at low speed on a vortex mixer. Cap the tube and heat the tube contents in the boiling water bath until the sample is completely dispersed (about 1 min). Ensure that no gelatinous lumps of starch are remaining.

3. Vigorously mix the contents of the sealed tube at high speed on a vortex mixer, place a tube in a boiling water bath and heat it for 15 minutes, with intermittent high speed stirring on a vortex mixer.

4. Store the tube at room temperature for approximately 5 minutes and add 2 ml 95% ethanol with continuous stirring on vortex mixer. Add a further 4 ml of ethanol, cap the tube and invert to mix. A starch precipitate will formed. Allow tube to stand for 15 minutes (or over night if desired).

5. Centrifuge (2,000 *g*, 5 minutes), discard the supernatant and drain the tubes on tissue paper for 10 minutes. Ensure that all of the ethanol has drained. The pellet is used for subsequent amylose and starch determination.

6. Add 2 ml of DMSO (with gentle vortex mix) to the starch pellet. Place a tube in a boiling water bath for 15 minutes and mix occasionally. Ensure that there are no gelatinous lumps.

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7. Add, with mixing, 4 ml of Con A solvent and qualitatively transfer the tube contents (by repeat washing with Con A solvent) to a 25 ml volumetric flask. Dilute to volume with Con A Solvent (this is solution 1).

B. Con A precipitation of amylopectin and determination of amylose

1. Transfer 1.0 ml of solution 1 to a 2.0 ml Eppendorf@ microfuge tube. Add 0.50 ml of Con A solution (4 mg/ml), cap the tube and gently mix by repeated inversion. Avoid frothing of the sample.

2. Allow the tube to stand for 1 hour at room temperature. Centrifuge at 14,000 *g* for 10 minutes in a microfuge at 20°C.

3. Transfer 1 ml of the supernatant to a 15 ml centrifuge tube. Add 3 ml of 100 mM sodium acetate buffer, pH 4.5. This reduces the pH to 5. Mix the contents, lightly stopper (wit a marble) and heat in a boling water bath for 5 minutes to denature the Con A.

4. Place the tube in a water bath at 40°C and allow to equilibrate for 5 minutes. Add 0.1 ml of amyloglucosidase/α-amylase enzyme mixture and incubate at 40°C for 30 minutes. Centrifuge the tube at 2,000 *g* for 5 minutes.

5. To 1.0 ml aliquots of the supernatant add 4 ml GOPOD reagent. Incubate at 40°C for 20 minutes. Incubate the reagent blank and the glucose controls concurrently.

- The reagent blank is prepared by adding 1.0 ml of sodium acetate buffer to 4.0 ml of GOPOD reagent and incubating at 40°C for 20 minutes.

- Glucose controls consist of 0.1 ml of glucose standard solution (1 mg/ml), 0.9 ml of sodium acetate buffer and 4.0 ml of GOPOD reagent.

6. The absorbance at 510 nm for each sample, and the glucose controls are read against the blank reagent.

C. Determination of total starch

1. Mix 0.5 ml of Solution 1 with 4 ml of 100 mM sodium acetate buffer, pH 4.5.

2. Add 0.1 ml of amyloglucosidase/α-amylase solution and incubate the mixture at 40°C for 10 minutes.

3. Transfer aliquots 10 ml (in duplicate) of this solution to glass test tubes and add 4 ml of GOPOD reagent. Incubate at 40°C for 20 minutes. This incubation should be performed concurrently with the samples and standards from section B.

D. Calculation of amylose content (%)

$$Amylose(\%) = \frac{Absorbance\ Con\ A}{Absorbance\ Total\ Starch} \times \frac{6.15}{9.2} \times \frac{100}{1}$$

Where 6.15 and 9.2 are dilution factors for the Con A and total starch extracts respectively.

APPENDIX B

The viscoelastic parameters for rice starch with and without milk protein ingredient from small and large-deformation rheological measurement

Table B.1 The viscoelastic parameters for 10% normal rice starch/water and normal rice starch/milk protein ingredient mixtures. Values of G'(Pa), G''(Pa) and G^*(Pa) at 1Hz obtained from the frequency sweep measurement (10^{-2} to 10 Hz and 1% strain at 25°C). γ_{max} and σ_{max} are the critical values of the strain and the stress obtained giving $G \fallingdotseq G''$ in the strain sweep measurement (10^{-2} to 10^4 % strain and 1 Hz at 25°C).

Dairly ingredient	Conc. (%)	Small-deformation			Large-deformation	
		G'(Pa)	G''(Pa)	G^*(Pa)	γ_{max} (%)	σ_{max} (Pa)
None	0	52.95± 0.38	10.85± 0.07	53.79± 0.85	763± 11.7	208± 5.9
SMP	2.5	43.52± 0.68	11.14± 0.08	49.19± 5.30	764± 4.7	196± 0.8
	5	51.02± 0.60	12.43± 0.04	51.28± 1.17	758± 9.5	217± 0.8
	7.5	69.35± 0.35	15.10± 0.00	70.98±0.29	776± 12.0	264± 0.8
	10	109.75± 0.49	19.95± 0.03	111.55± 0.49	839± 0.0	413± 1.5
MPC	2.5	38.37± 0.26	12.38± 0.01	40.33± 0.26	620± 45.3	161± 0.9
	5	41.07± 0.08	14.40± 0.03	43.53± 0.09	576± 12.0	161± 0.8
	7.5	50.91± 1.46	18.40± 0.39	54.15± 1.51	521± 0.0	177± 0.0
	10	81.68± 0.90	28.41± 0.50	86.48± 1.02	500± 4.7	267± 3.8
NaCAS	2.5	134.15± 2.76	19.22± 0.48	135.50± 4.10	510± 9.5	253± 0.7
	5	146.65± 4.03	23.13±0.40	148.45± 2.76	492± 16.8	273± 16.1
	7.5	146.70± 1.56	26.35± 0.58	149.05± 1.63	507± 9.5	297± 0.0
	10	148.25± 1.34	31.40± 0.17	151.55±1.34	503± 14.3	332± 6.9

Continued-

Table 5.1 Continued-

Dairly ingredient	Conc. (%)	Small-deformation			Large-deformation	
		G'(Pa)	G''(Pa)	G^*(Pa)	γ_{max} (%)	σ_{max} (Pa)
WPI	2.5	90.74± 1.37	15.51± 0.08	92.05± 1.37	666± 23.8	262± 9.2
	5	77.24± 0.15	18.41± 0.01	79.41± 0.14	763± 16.5	305± 3.0
	7.5	86.72± 0.42	29.29± 0.02	91.53± 0.40	896± 15.1	487± 3.1
	10	334.9± 4.38	92.53± 1.17	347.45± 4.60	835± 14.3	889± 19.1
UFSMP	2.5	49.39± 0.31	10.93± 0.03	50.59± 0.32	752± 2.3	202± 0.0
	5	58.68± 0.92	11.20± 0.06	59.74± 0.92	770± 2.6	224± 1.5
	7.5	93.34± 1.98	13.10± 0.20	94.26± 1.99	676± 2.6	245± 3.6
	10	118.95± 2.62	15.21± 0.12	119.90± 2.55	541± 2.6	236± 5.0
Lactose	2.5	58.60± 0.57	11.30± 0.00	59.68± 0.56	770± 2.6	232± 4.6
	5	65.40± 1.13	11.90± 0.00	66.47± 1.11	756± 2.6	235± 3.9
	7.5	73.90± 0.14	12.75± 0.07	74.99± 0.15	721± 12.3	277± 5.4
	10	89.20± 0.71	14.10± 0.14	90.31± 0.72	702± 4.9	266± 2.4

Table B.2 The viscoelastic parameters for 10% waxy rice starch/water and waxy rice starch/milk protein ingredient mixtures. Values of G'(Pa), G''(Pa) and G^*(Pa) at 1Hz obtained from the frequency sweep measurement (10-2 to 10 Hz and 1% strain at 25°C). γ_{max} and σ_{max} are the critical values of the strain and the stress obtained giving $G' \fallingdotseq G''$ in the strain sweep measurement (10^{-2} to 10^4 % strain and 1 Hz at 25°C).

Dairy ingredient	Conc. (%)	Small-deformation			Large-deformation	
		G'(Pa)	G''(Pa)	G^*(Pa)	γ_{max} (%)	σ_{max} (Pa)
None	0	17.61± 0.02	7.67± 0.04	19.20± 0.04	628± 24.7	92± 4.1
SMP	2.5	16.35± 0.11	7.94± 0.02	18.18± 0.11	453± 2.3	63± 2.3
	5	16.53± 0.01	8.53± 0.02	18.60± 0.02	392± 5.9	59± 0.0
	7.5	18.15± 0.13	9.43± 0.07	20.46± 0.15	368± 31.9	61± 5.9
	10	27.28± 0.28	12.38± 0.13	29.96± 0.30	504± 3.6	122± 0.0
MPC	2.5	17.95± 0.46	8.98± 0.26	20.08± 0.53	422± 12.8	68± 0.2
	5	19.29± 0.16	10.31± 0.09	21.88± 0.18	341± 5.4	59± 0.9
	7.5	22.25± 0.11	12.06±0.09	25.32± 0.13	278± 2.4	54± 0.7
	10	26.61± 0.83	14.25± 0.05	30.19± 0.76	236± 10.1	52± 1.0
NaCAS	2.5	19.00± 0.08	8.91± 0.01	20.98± 0.07	461± 0.0	74± 0.8
	5	21.83± 0.13	10.98± 0.13	24.44± 0.18	364± 2.3	68± 4.1
	7.5	26.00± 0.09	13.97± 0.14	29.53± 0.15	295± 1.1	71± 0.8
	10	30.99± 0.30	17.68±0.32	35.68± 0.42	245± 2.3	73± 1.3
WPI	2.5	18.01± 1.61	9.06± 1.12	18.74± 0.07	469± 12.8	70± 4.6
	5	17.74± 0.00	9.59± 0.00	20.17± 0.01	423± 2.2	71± 2.2
	7.5	22.27± 0.03	13.50± 0.02	26.05± 0.04	350± 2.7	78± 2.1
	10	102.10± 0.85	56.97± 0.32	116.95± 0.92	530± 8.2	381± 7.9

Continued-

Table 5.2 Continued-

Dairly ingredient	Conc. (%)	Small-deformation			Large-deformation	
		G'(Pa)	G''(Pa)	G^* (Pa)	γ_{max} (%)	σ_{max} (Pa)
UFSMP	2.5	18.15± 0.01	7.58± 0.01	19.67± 0.01	658± 9.9	99± 1.4
	5	17.41± 0.01	7.60± 0.03	19.00± 0.01	572± 0.0	82± 2.5
	7.5	17.03± 0.08	7.69± 0.02	18.69± 0.06	533± 7.0	77± 1.7
	10	16.95± 0.04	7.82± 0.02	18.67± 0.05	477± 4.8	66± 1.6
Lactose	2.5	18.84± 0.14	7.89± 0.04	20.02± 0.14	640± 39.3	101± 3.9
	5	18.45± 0.07	7.96± 0.01	20.09± 0.06	671± 39.3	106± 7.0
	7.5	18.80± 0.14	8.13± 0.02	20.48± 0.12	673± 26.9	109± 7.0
	10	18.95± 0.07	8.33± 0.01	20.70± 0.06	644± 39.3	106± 6.2

APPENDIX C

The amount of total starch leaching at various temperatures

Table C.1 Total starch leached (mg/g starch) for normal rice starch/water (without protein), normal rice starch/ milk protein ingredient mixtures at different temperatures.

Dairy ingredient	Conc. (%)	Temperature (°C)					
		50ns	**58.3**	**62.3**	**64.4**	**66.5**	**68.5**
None	0	00±0.00	0.56±0.06ab	3.13±0.42b	4.30±0.36b	6.86±0.43a	8.15±0.03b
SMP	5	0.00±0.00	0.32±0.02d	3.10±0.24b	3.60±0.04c	4.85±0.06ef	6.69±0.31d
	10	0.00±0.00	0.00±0.00e	0.83±0.05e	3.21±0.04d	3.67±0.03h	5.82±0.12f
MPC	5	0.00±0.00	0.44±0.00bcd	3.82±0.39a	4.55±0.17ab	5.71±0.16c	6.87±0.32d
	10	0.00±0.00	0.00±0.00e	0.78±0.09d	3.07±0.11d	4.52±0.20fg	6.19±0.21e
NaCNS	5	0.00±0.00	0.33±0.01cd	4.10±0.24a	4.85±0.06a	5.00±0.10d	6.31±0.16e
	10	0.00±0.00	0.32±0.16d	2.17±1.00cd	2.13±0.24e	4.30±0.14gh	5.19±0.11g
WPI	5	0.00±0.00	0.45±0.06bc	2.62±0.11c	3.59±0.41c	6.42±0.08b	6.31±0.16e
	10	0.00±0.00	0.00±0.00e	1.02±0.27e	3.02±0.16d	5.98±0.37c	11.19±0.28a
UFSMP	5	0.00±0.00	0.68±0.20a	3.16±0.17b	4.49±0.10ab	4.90±0.08e	5.32±0.06g
	10	0.00±0.00	0.00±0.00e	0.68±0.11e	3.18±0.13d	3.95±0.15h	5.66±0.11f

Table C.2 Total starch leached (mg/g starch) for waxy rice starch/water (without protein), waxy rice starch/ milk protein ingredient mixtures at different temperatures.

Dairy ingredient	Conc. (%)	Temperature (°C)					
		50ns	**56.2**	**60.3**	**62.4**	**64.4**	**66.5**
None	0	.00±0.00	0.85±0.30b	1.01±0.12d	1.70±0.10e	3.29±0.14c	7.22±0.18b
SMP	5	0.00±0.00	0.36±0.01cde	4.10±0.24a	4.85±0.06a	4.60±0.04a	5.19±0.11f
	10	0.00±0.00	0.00±0.00f	1.01±0.19d	1.26±0.02gh	2.44±0.20e	3.06±0.02h
MPC	5	0.00±0.00	0.25±0.00def	0.96±0.10d	2.11±0.12d	3.44±0.10c	7.54±0.24a
	10	0.00±0.00	0.22±0.08ef	0.53±0.11f	1.12±0.18h	2.65±0.18e	5.65±0.34e
NaCNS	5	0.00±0.00	0.39±0.03cde	1.56±0.09c	1.48±0.02f	2.99±0.03d	6.28±0.03c
	10	0.00±0.00	0.00±0.00f	0.91±0.17de	1.33±0.15fg	2.96±0.10d	6.20±0.16c
WPI	5	0.00±0.00	0.57±0.22bc	0.71±0.13ef	1.32±0.10fg	3.01±0.15d	6.06±0.16cd
	10	0.00±0.00	0.99±0.05a	0.91±0.06de	1.25±0.13gh	2.30±0.03d	5.87±0.03de
UFSMP	5	0.00±0.00	0.30±0.01cde	3.98±0.13a	4.44±0.06b	4.36±0.21ab	5.05±0.07fg
	10	0.00±0.00	0.54±0.00cd	3.71±0.08b	3.87±0.09c	4.16±0.01b	4.89±0.13g

APPENDIX D

The onset temperature (T_{onset}) of rice starch in the presence of milk protein ingredients obtained by swelling, viscosity and thermal (DSC) measurements.

Table D.1 Onset temperature (T_{onset}) of 10% normal rice starch/milk protein ingredient mixtures.

Dairly ingredient	Conc. (%)	Swelling[a]	Viscosity (Rheometer)[a]	Gelatinization (DSC)[b]
None	0	59.84±0.30	67.01±0.50	62.62±0.18
SMP	5	61.20±0.19	69.87±0.06	64.72±0.22
	10	62.03±0.40	70.59±0.12	66.11±0.02
MPC	5	59.39±0.18	66.49±0.12	62.43±0.36
	10	60.03±0.08	65.04±0.27	62.36±0.27
NACAS	5	60.61±0.15	68.35±0.18	62.48±0.1
	10	61.11±0.16	68.72±0.11	62.62±0.39
WPI	5	59.92±0.13	66.46±0.19	62.58±0.25
	10	59.77±0.24	66.83±0.10	62.12±0.25
UFSMP	5	61.28±0.15	69.22±0.23	65.54±0.37
	10	62.07±0.11	70.25±0.18	67.46±0.15

[a] Data from section 6.3

[b] Data from section 5.3

Table D.2 Onset temperature (T_{onset}) of 10% waxy rice starch/milk protein ingredient mixtures.

Dairly ingredient	Conc. (%)	Swelling [a]	Viscosity (Rheometer)[a]	Gelatinization (DSC)[b]
None	0	58.61±0.15	63.62±0.13	60.26±0.33
SMP	5	60.13±0.15	66.27±0.02	61.47±0.52
	10	61.88±0.11	67.91±0.33	64.68±0.12
MPC	5	58.34±0.06	63.37±0.05	60.45±0.78
	10	58.62±0.17	63.21±0.13	62.17±0.23
NACNS	5	59.49±0.25	64.42±0.13	61.94±0.56
	10	59.92±0.53	64.94±0.21	62.32±0.08
WPI	5	59.71±0.16	64.57±0.09	60.49±0.23
	10	60.09±0.01	65.39±0.19	61.26±0.89
UFSMP	5	60.48±0.49	65.16±0.12	63.86±0.68
	10	61.83±0.24	66.90±0.40	65.13±1.29

[a] Data from section 6.3

[b] Data from section 5.3

APPENDIX E

Apparent viscosity of WPI solutions as a function of temperature

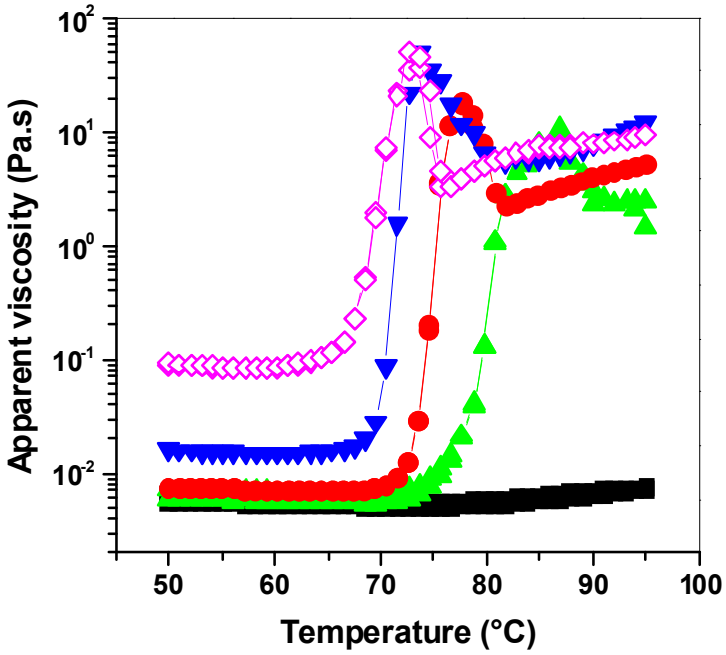

Figure E.1 Apparent viscosity of WPI solutions as a function of temperature. Concentration of WPI: 10% (■), 15% (▲), 20% (●), 30% (▼) and 40% (◇).

APPENDIX F

Sodium dodecyl sulphate polyacrylamide gels electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to the method of Singh and Creamer (1991).

Preparation of stock solutions

30% Acrylamide/Bis solution 37.5:1 (2.67%C)

Acrylamide/Bis mixture 37.5:1 (2.6% C) (BioRad, Richmond, CA, USA) was dissolved in approximately 60 ml of Milli-Q water. Bring to a final volume of 100 ml and stored in an amber bottle at 4°C.

10% Ammonium persulphate (APS)

Dissolve 100 mg of APS (BioRad Richmond, CA, USA; Electrophoresis purity reagent) in 1.0 ml of Milli-Q water, made up fresh each day.

Bromophenol Blue 0.4% (w/v)

Dissolve 1.6 g of Bromophenol Blue in approximately 7 ml of 0.1 M NaOH. Make up to 400 ml in a measuring cylinder with Milli-Q water.

1.5 M Tris-HCl buffer, pH 8.8

Tris base (18.15 g) was dissolved in about 60 ml of Milli-Q water, mix, and then adjusts the pH to 8.8 with 6 M HCl and the volume made up to 100 ml with Milli-Q water. The buffer was store at 4°C.

0.5 M Tris-HCl buffer, pH 6.8

Tris base (6.0 g) was dissolved in about 60 ml of Milli-Q water, mix, then adjusts the pH to 6.8 with 6 M HCl and the volume made up to 100 ml with Milli-Q water. The buffer was stored at 4°C.

10% SDS

Dissolve 10 g of SDS in Milli-Q water with gentle stirring and the volume was made up to 100 ml. The 10% SDS solution was stored at room temperature.

SDS Sample buffer

To 500 ml of Milli-Q water, 125 ml 0.5 M Tris-HCl buffer (pH 6.8), 100 ml Glycerol, 200 ml 10% (w/v) SDS, 25 ml 0.4 (w/v) Bromophenol Blue solution were added and mixed well. The total volume of this SDS sample buffer is 950 ml.

5 X electrode buffer

Tris(hydroxymethyl) methylamine (15 g), glycine (72 g), and SDS (5 g) were dissolved in Milli-Q water and the volume was made up to 1 litre with Milli-Q water.
Check the pH of 5 X electrode buffer, which is 8.6 (±0.2), store at 4°C. For each electrophoresis run, dilute 80 ml (5 X) stock to 400 ml with Milli-Q water for one electrophoresis run.

Coomassive Brilliant Blue R-250 staining solution

Coomassive Brilliant Blue R-250(BioRad Richmond, CA, USA; Electrophoresis purity reagent) 2.5 g were dissolved in 1250 ml of isopropanol, glacial acetic acid 500 ml was added and the volume was made up to 5 litre with Milli-Q water. The mixture was stirred overnight and then filter under vacuum through Whatman No.1 filter paper.

Coomassive destaining solution

Isopropanol (500 ml) was added to 500 ml of glacial acetic acid. The volume was made up to 5 litres with Milli-Q water then mixed well.

Preparation of gel

Resolving gel

To prepare 2 gels, Milli-Q water (2.00 ml), 1.5 M Tris-HCl buffer (2.50 ml), and acrylamide/Bis (30% T) (5.30 ml) were mixed in a 100 ml Buchner flask and degased under stirring for 15 min. The 10%SDS solution (100 µl), 5 µl of the TEMED (N, N, N′, N′-tetramethylethylenediamine, BioRad Richmond, CA, USA; Electrophoresis purity reagent) and then the 10% APS (50 µl) were added, with gently mixing the mixture after each addition. 3.30 ml of the mixture was quickly transferred in between the electrophoresis casting plates. Note that, it is important to keep the flow of gel buffer constant while pipetting so as not to introduce air bubbles into the gel. A small quantity of Milli-Q water (~200 µl) was added to form an upper layer over the gel solution. The acrylamide mixture was allowed to polymerize at room temperature for about 15-30 min, decant off the layer of water and remove the last traces of water using a piece of filter paper placed carefully between the two glass plates just above the gel line before pouring the staking gel.

Stacking gel

To prepare 2 gels, Milli-Q water (3.05 ml), 0.5 M Tris-HCl buffer (1.25 ml), and acrylamide/Bis (30% T) (0.65 ml) were mixed in a 100 ml Buchner flask and degased under stirring for 15 min. The 10%SDS solution (50 µl), 5 µl of the TEMED and then freshly prepared of the 10% APS (25 µl) were added. This mixture was made by gentle mixing after each addition. 10% SDS, the TEMED and APS, were added by swirling the flask gently to achieve mixing after each addition. This mixture (2.20 ml) was quickly poured on top of the resolving gel. Immediately the slotted comb was placed at the top of stacking gel (between the two plates) to form appropriate slots for samples in the gel. Care was taken to ensure that there are no bubbles trapped around the slots. Keep the gel stand for at least 1 hour at room temperature and store the gels in the cold room (4°C) sealed in plastic bags with a small amount of water to prevent them from drying out. The comb was removed before the samples were loaded into gel slots.

Running of gels

The gels were run on a Mini-PROTEAN II dual slab cell (Bio-Rad, Richmond, CA, USA) at constant voltage 210 V, current, power, and time were set at 70 mA, 6.5 W using a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Richmond, CA, USA) until the tracking dye front reaches the bottom of the gel. The approximate running time was about 0.9 hour for one gel and 1.1 hour for two gels. The gels were removed from the gel glass plate's sandwich and gently transfer into containers.

Staining

Dispense 50 ml of Coomassive brilliant Blue R-250 staining solution into each gel container. Set the container on the platform rocking table in such a way that the gels were uniformly stained with the staining solution, stain the gels for exactly 1 hour.

Destaining

The staining solution was drained the gel container carefully, replace with 100 ml destaining solution then the gel container was returned to the rocker for 1 hour. The detsaining solution was replaced by draining off the used destain solution then adding 100 ml of a fresh destaining solution. The gels were left to destain on the rocker for a further 19 hours.

Quantitative determination of individual proteins

The gel was scanned on a Molecular Dynamics Scanner (Molecular Dynamics, Sunnyvale, CA) and quantitative determination of the volume intensity of proteins in each band of the samples, separated by SDS-PAGE, was performed by using ImageQuant 5.0 software. The standards were prepared and loaded in exactly the same way as the samples. In each gel, the standard protein solution was run in conjunction with samples to indicate the position of known proteins on the gel. The amount of protein in each band for each milk protein ingredient was determined from the respective protein concentration/volume band densitometry standard curve. For standard curve, a dilution series of a known standard made and run, at least five triplicates points on a standard curve were achieved. The standard curves were linear in the range of the concentrations used.

APPENDIX G

The electrophoresis patterns of adsorbed α-casein and β-casein obtained from rice starch as function of NaCAS concentration

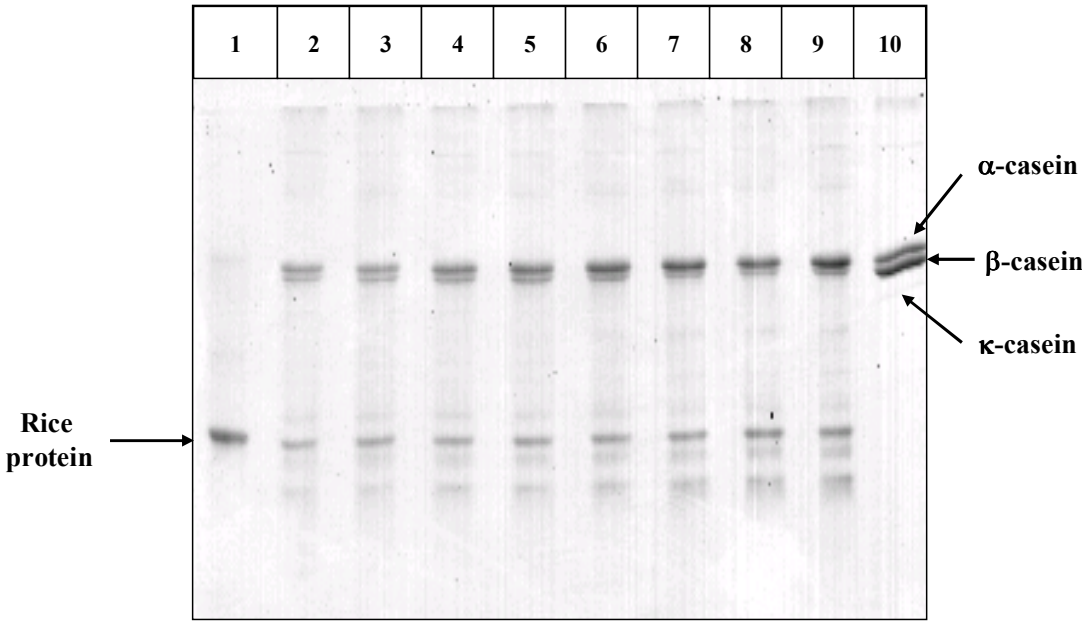

Figure G.1 The electrophoresis patterns of adsorbed α-casein and β-casein obtained from normal rice as function of NaCAS concentration from 0.25 to 10% (w/w). Lane1: starch in MilliQ water, lane2: starch in 0.25% NaCAS, lane3: starch in 0.50% NaCAS, lane4: starch in 0.75% NaCAS, lane5: starch in 1% NaCAS, lane6: starch in 2.5% NaCAS, lane7: starch in 5% NaCAS, lane8: starch in 7.5% NaCAS, lane9: starch in 10% NaCAS, lane10: 0.0075% NaCAS.

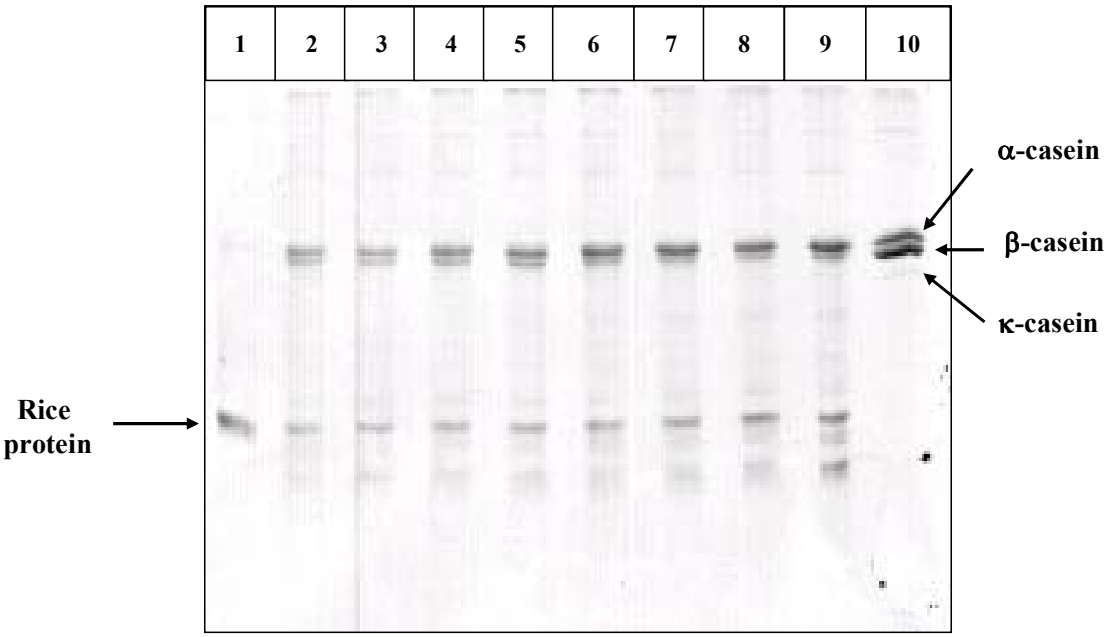

Figure G.2 The electrophoresis patterns of adsorbed α-casein and β-casein obtained from waxy rice as function of NaCAS concentration from 0.25 to 10% (w/w). Lane1: starch in MilliQ water, lane2: starch in 0.25% NaCAS, lane3: starch in 0.50% NaCAS, lane4: starch in 0.75% NaCAS, lane5: starch in 1% NaCAS, lane6: starch in 2.5% NaCAS, lane7: starch in 5% NaCAS, lane8: starch in 7.5% NaCAS, lane9: starch in 10% NaCAS, lane10: 0.0075% NaCAS.

APPENDIX H

Kinetics of individual milk proteins adsorbed onto rice starch granules.

Kinetics of individual milk proteins adsorbed onto normal and waxy rice starch were studied by using 5% NaCAS or 5% WPI. Rice starch (3 g) was mixed with Milli Q water or a 5% solution of NaCAS or WPI using a magnetic stirrer. The following adsorption times were used for the study; 15 mins, 30 mins, 1 hr, 4 hrs and 24 hrs. Sample preparation and the method to determine the amount of protein adsorbed onto the starch granules by quantitative SDS-PAGE were as described in section 7.2.2.3 and appendix F, respectively.

The adsorption of α_S-casein, β-casein and total caseins present in NaCAS, and β-lg**,** α-la and total proteins present in WPI onto normal rice starch granules as a function of time are reported in Figure H.1, and H.3, respectively.

The adsorption of caseins onto rice starch granule was slow, which the amount of adsorbed proteins not reaching adsorption saturation values even after 4 hours. This might be due to packing and an extensive molecular rearrangement of caseins at the starch granule interface.

There is a different in the adsorption behavior of α_S-casein and β-casein from NaCAS. It was found a preference adsorption of α_S-casein onto rice starch granules throughout the adsorption time used in this experiment. At the first 30 minutes of adsorption, not only higher the amount of adsorbed α_S-casein but the adsorption rate is also faster than β-casein. However, although the adsorption rate of β-casein decrease with adsorption time the amount of adsorbed β-casein slightly increased. This indicates that β-casein was not displaced by α_S-casein. Thus co-adsorption of the two proteins, with a greater

adsorption ability of α_S-casein over β-casein was clearly seen in the initial stage of adsorption.

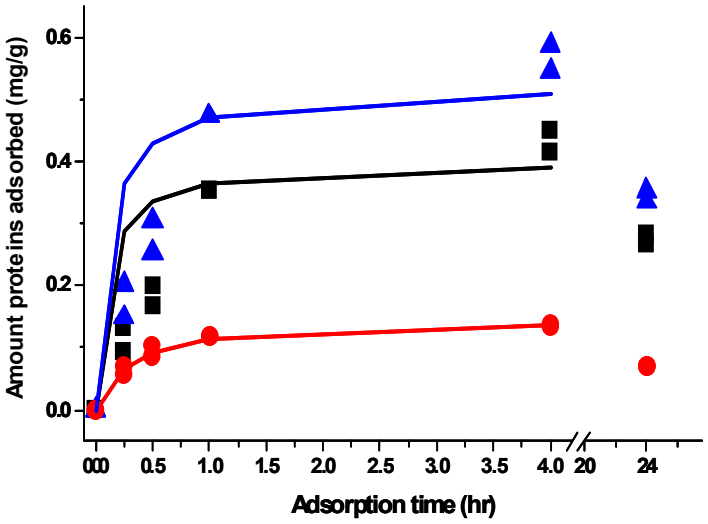

Figure H.1 Adsorption isotherms for NaCAS caseins on normal rice starch granules. Solid lines represented fitted Langmuir isotherm. NaCAS caseins; (■)α_S-casein, (●) β-casein, and (▲) total caseins. Chi^2 are 0.00193, and 0.00064 for α-casein and β-casein, respectively.

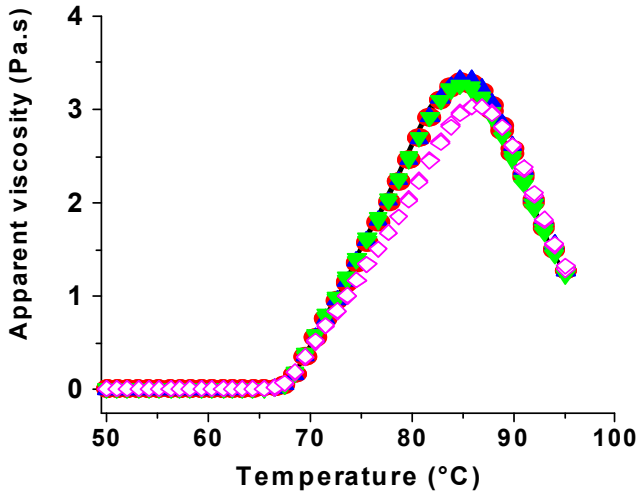

Figure H.2 Apparent viscosity as a function of temperature for 10% normal rice starch in MilliQ water with different mixing times; — 15 min, ● 1 hour, ▲ 4 hours, and ◇ 24 hours.

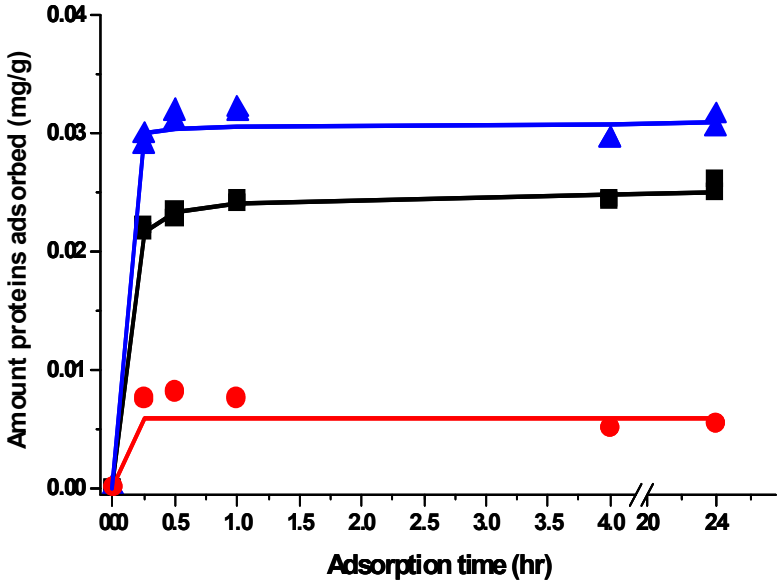

Figure H.3 Adsorption isotherms for WPI proteins on normal rice starch granules. Solid lines represented fitted Langmuir isotherm. WPI proteins; (■) β-lg, (●) α-la, and (▲) total whey proteins. Chi^2 are 0.00193, and 0.00064 for α-casein and β-casein, respectively.

After 24 hours adsorption time, the amount of adsorbed both α_S-casein and β-casein were decreased; ~37% and 50% for α_S-casein and β-casein, respectively. This is an unexpected result, however it is possible that desorption could have occurred. However, there are another two possibilities to explain the drop off of the adsorbed amount of α_S-casein and β-casein. Firstly, applied constant stirring to suspension of starch in both MilliQ and 5% NaCAS for 24 hours caused a significant decrease in the peak viscosity of suspensions compare to shorter stirring time (Figure H.2) due to starch granules damage through shear. The damage of starch granules is an opportunity for amylose and short chain of amylopectin to leach out from the granule into the suspension. Hence, the solution composition is altered by these amylose and amylopectin molecules. Secondly, it is possible that there is a loss of starch granules during the washing process. Since, the original mass of starch was used in the calculation, it is possible that the amounts of protein is under-estimated.

Appendix

The adsorption of α-la and β-lg from 5% WPI solution onto normal rice starch granules shows a higher adsorption affinity compared to the adsorption of caseins from NaCAS (Figure H.3). Both adsorbed α-la and β-lg rapidly reached a plateau at 15 minutes. After 30 minutes of adsorption, the amount of adsorbed α-la decreased with time. In contrast, there is a slightly increase of adsorbed amount of β-lg. β-lg has higher molecular weight than α-la; 18,283, and 14,176, respectively. Moreover, it is well-known that β-lg, at pH similar to that of the present experiment, is likely present as dimers of two non-covalently linked monomeric subunits, where as α-la exists as free globular molecules. Hence, the displacement of smaller size of α-la by the larger size of dimers β-lg via the orogenic displacement mechanism could have taken place and resulted in the reduction in the amount of adsorbed α-la.
