High-Pressure-Induced Starch Gelatinisation and

Its Application in a Dairy System

A thesis presented in partial fulfilment of the requirements for the Doctor of Philosophy in Food Science at Massey University, Auckland, New Zealand.

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Abstract

This study investigated pressure-induced starch gelatinisation in water and milk suspensions. A rheological method, termed 'pasting curves', provided an objective and analytical means to determine the degree of pressure-induced starch gelatinisation. In addition, a polarised light microscope was used to observe birefringence of the starch granules and the degree of starch swelling was measured. The preliminary investigation into pressure-induced gelatinisation of six different starches showed that potato starch was the most pressure resistant and was not gelatinised after a pressure treatment of 600 MPa for 30 min at 20 °C. Waxy rice, waxy corn and tapioca starches showed complete gelatinisation after the same treatment while normal rice and normal corn starches were only partially gelatinised despite the disappearance of birefringence.

Based on the preliminary study, two starches (normal and waxy rice starches) were selected for more detailed studies. The effects of treatment conditions (pressure, temperature and duration) on the gelatinisation were investigated with these selected starches. The degree of gelatinisation was dependent on the type of starch and the treatment conditions. The results also indicated that different combinations of the treatment conditions (e.g. high treatment pressure for a short time and low treatment pressure for a longer time) could result in the same degree of gelatinisation. Both starch types exhibited sigmoidal-shaped pressure-induced gelatinisation curves and there was a linear correlation between the degree of swelling and the apparent viscosity of the starch suspension. After treatments at ≥500 MPa for 30 min at 20 °C, both starches lost all birefringence although the apparent viscosity and the degree of swelling of normal rice starch did not increase to the same extent as observed in waxy rice starch.

Pressure-induced gelatinisation of starch was retarded when starch was suspended in skim milk. This was attributed to the effect of soluble milk minerals and lactose present in the milk whereas milk proteins (casein and whey) did not affect the degree of gelatinisation at the levels present in 10% total solids skim milk. The presence of soluble milk and/or lactose may lead to less effective plasticising of starch chains by the suspension medium. Interactions between milk components and starch molecules may also play a role in retarding gelatinisation by reducing the mobility of starch chains.

The functionality of starch in a dairy application was tested using acid milk gels as a model system. Skim milk with added starch (waxy rice or potato starch) was either pressure treated (500 MPa, 20°C, 30 min) or heat treated (80°C, 30 min) and subsequently acidified to form acid milk gels. The addition of waxy rice starch resulted in firmer acid milk gels, and increasing the amount of starch caused an increase in the firmness of both pressure-treated and heat-treated samples. However, pressure-treated samples with added potato starch did not show significant changes in the firmness whereas the heat-treated counterparts showed a marked increase in the firmness as the level of potato starch increased. The difference between the effects of the two different starches can be explained by the extent of starch gelatinisation in skim milk. Starch granules absorb water during gelatinisation whether induced by pressure or heat which effectively increases milk protein concentration in the aqueous phase to form a denser protein gel network on acidification. The firmness of acid milk gels can be increased by adjusting the pH at pressure or heat treatment to higher than the natural pH of milk. The effect of pH at pressure or heat treatment and addition of starch on the acid milk gel firmness was additive and independent of each other up to a starch addition level of 1%.

This study provided an insight into pressure-induced gelatinisation of starch by showing gelatinisation properties of starches of different botanical origins and the effects of the treatment conditions (treatment pressure, treatment temperature and duration) on the degree of gelatinisation. Furthermore, the results from the pressure treatments of starch in dairy-based suspensions showed that pressure-induced gelatinisation was affected by other components in the system. These results demonstrate the importance of understanding the gelatinisation properties of starch in complicated food systems in which a number of other components are present. In terms of the application of starch in dairy systems, when starch was added to milk and gelatinised by pressure treatment, the acid milk gel produced by subsequent acidification was firmer than the acid milk gel made from skim milk alone.

Acknowledgements

List of Contents

List of Figures

In publication, Oh, H. E., D. N. Pinder, Y. Hemar, S. G. Anema and M. Wong (2008a). "Effect of high-pressure treatment on various starch-in-water suspensions." *Food Hydrocolloids* **22**(1): 150-155:

Fig. 1: Viscosity of starch suspensions as a function of temperature (pasting curve) after no pressure treatment (●), pressure treatment at 400 MPa (▽) and pressure treatment at 600 MPa (■): A, normal rice starch; B, waxy rice starch; C, normal corn starch; D, waxy corn starch; E, tapioca starch; F, potato starch....4-7

Fig. 2: Polarized light micrographs of starches after no pressure treatment (1), pressure treatment at 400 MPa (2) and pressure treatment at 600 MPa (3): normal rice starch (A-1, A-2, A-3); waxy rice starch (B-1, B-2, B-3); normal corn starch (C-1, C-2, C-3); waxy corn starch (D-1, D-2, D-3); tapioca starch (E-1, E-2, E-3); potato starch (F-1, F-2, F-3)……………………………………………….4-8

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List of Tables

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List of Symbols

$\eta_{initial}$	Apparent viscosity at 20 °C before pasting begins
η_{peak}	Maximum apparent viscosity attained during pasting
T_{onset}	Onset temperature of gelatinization at which the apparent viscosity starts to increase
T_{peak}	Temperature at η_{peak}
G'	Storage modulus
G''	Loss modulus

List of Abbreviations

CCP	Colloidal calcium phosphate
GDL	Glucono-δ-lactone

CHAPTER 1

Introduction

High pressure processing (HPP) technology has been attracting much interest in recent years as a non-thermal technology to process food products, along with other emerging technologies such as pulsed electric fields, ultrasound, and ultraviolet light radiation (Tewari et al., 1999; Rastogi et al., 2007). The ultimate goal of these technologies is to offer alternatives to the traditional thermal processing so that foods undergo minimal change in sensory and nutritional properties during processing, and the HPP technology has shown good potential. The treatment pressure range of 100–1000 MPa is typically used in HPP of food products (**Figure 1-1**). HPP is used to inactivate pathogenic and spoilage microorganisms in food, for example, fresh oysters can be pressure-treated to extend the shelf life and ensure food safety (He et al., 2002). Deteriorative enzyme reactions that result in colour changes in avocados can also be inactivated by the HPP (Guerrero-Beltran et al., 2005).
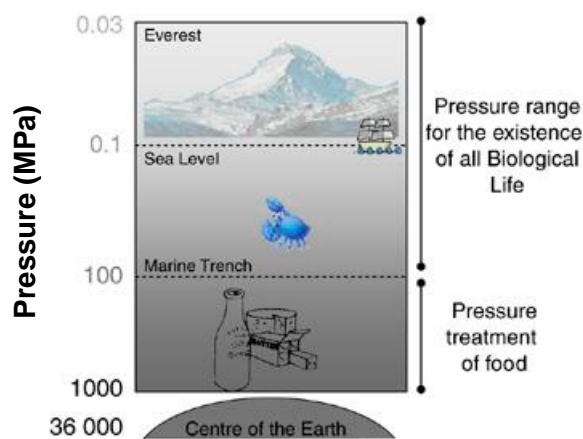
Figure 1-1: Schematic representation of the pressures used in food processing. Source: Considine et al. (2008).

The development of the high pressure technology for non-food applications such as ceramics has led to the wider availability of high pressure equipment (Farr, 1990). This has enabled studies of food systems with pressure as a variable, which has provided opportunities to gain fresh insights into changes in various physico-chemical

phenomena. Aside from commercial applications in food, the HPP technology itself is a research tool in food science. Pressure is an important factor that may affect various physico-chemical phenomena in food, for example, denaturation of proteins and gelatinisation of starch (Balny et al., 2002). However, the effects of pressure on these phenomena have not been studied as extensively as have the effects of temperature. The knowledge and understanding gained from such studies can then be applied in manufacturing of food products.

Gelatinisation of starch is conventionally induced by heating starch in aqueous suspension and is an important reaction in terms of the functionality and nutritional value of the starch. It is now known that starch gelatinisation can be induced at room temperature without prior heating by pressure treatment (Stute et al., 1996; Knorr et al., 2006). However, there is limited information available regarding rheological changes due to pressure-induced starch gelatinisation and the application of pressure-induced gelatinisation in food systems where starch is commonly used as an ingredient.

The aim of this study was to investigate the effect of high pressure treatment on starch in water and milk based systems. The objectives of this study were:

- to study the effect of pressure treatment on starch-in-water suspensions (Chapter 4 & 5)

- to study the effect of pressure treatment on starch-in-skim milk suspensions and investigate the effect of different skim milk components (Chapter 6)

- to investigate starch addition to skim milk prior to pressure or heat treatment and compare the effect of the different treatments on the

acidifications of the milk and the properties of acid milk gels that were formed (Chapter 7).

The preliminary experiments in this study investigated the differences in pressure-induced gelatinisation behaviour of six different starches. Two types of starch were then selected for further investigation into the effects of treatment pressures, temperatures and duration on the pressure-induced gelatinisation. To characterise pressure-induced gelatinisation of starch in a dairy system, the effect of skim milk components was examined by using suspension media with different compositions. Functionality of starch in a dairy application was tested using acid milk gel as a model system and the effects of pressure treatment and heat treatment on the system were compared.

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CHAPTER 2

Literature Review

2.1   Introduction

Consumer demand for fresh-like food products with an extended shelf life has been increasing in recent years. With this trend, high pressure processing technology has been gaining popularity as a non-thermal technique for the manufacture of food products. One of the major advantages of high pressure processing over more traditional thermal processing is that the process allows for the retention of nutritional and sensory characteristics of food while inactivating pathogens and food spoilage microorganisms (Tewari et al., 1999). Many pressure-treated food products are therefore considered 'fresh-like' food with extended shelf life.

High pressure processing is not a new technology. The first study on the effect of high pressure processing on food was published by Hite (1899) over a century ago. Hite (1899) reported that the souring of milk could be delayed by pressure treatment. He also noted physical changes in milk after pressure treatment such as the translucent appearance. However, his system did not prove to be sufficiently reliable for repeated use. **Figure 2-1A** shows the high pressure processing device used by Hite (1899).

With advances in the engineering aspects of high pressure processing, it is now used in the commercial manufacture of a variety of food products, for example, guacamole (USA), jams (Japan) and for the improved shucking of oysters as well as inactivation of pathogens (USA). Studies have shown that high-pressure processing can be used for microbial inactivation, denaturation of enzymes and alteration of functional properties of food (Hoover et al., 1989; Cheftel, 1995; Johnston et al., 2005). High pressure processing systems with automated product loading and removal are now available for commercial plants, an example is shown in **Figure 2-1B**. There are two types of industrial high pressure processing systems which do not involve heating; direct compression and indirect compression systems (Balci and Wilbey,

1999). Most industrial pressure systems use the indirect compression method in which the food sample is first packaged and then immersed in a liquid medium that transmits the applied pressure (pressure transmitting fluid) to it (Balci and Wilbey, 1999). In the direct systems, a liquid food product is pressurised directly.
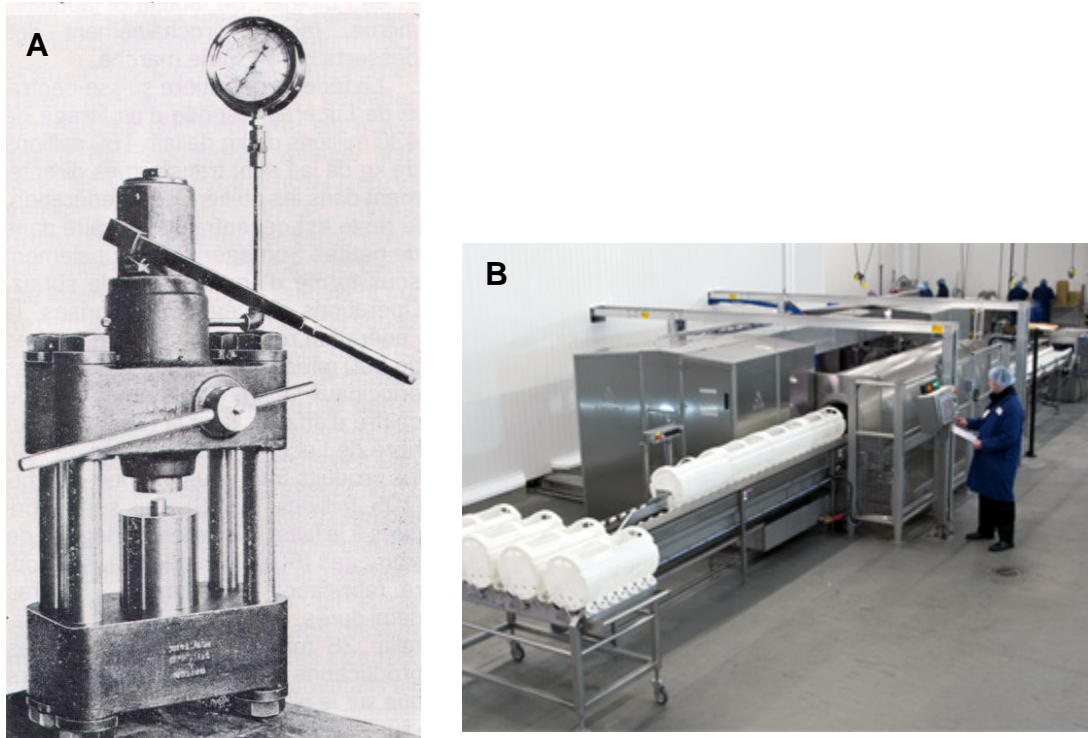
Figure 2-1: (A) High pressure processing device used by Hite (1899), Source: Hite, 1899. (B) Modern high pressure processing plant (Avure 350L-600, Avure technologies). Source: Avure Technologies (2008).

There are a number of published articles and reports on the effect of high pressure processing on milk (Balci and Wilbey, 1999; Huppertz et al., 2002) and fewer on high pressure processing of starch suspensions (Hibi et al., 1993; Katopo et al., 2002). This chapter will provide a general background on the high pressure process, a review of the literature on pressure-induced starch gelatinisation with comparison to thermal gelatinisation and a brief review of the literature on the effects of pressure treatment on bovine milk with comparison to heat treatment where relevant to this study.

2.2　High pressure process

2.2.1　Principle

Hydrostatic pressure can be applied to a system by reducing the volume mechanically or by increasing the temperature while keeping the volume constant. The work reported in this thesis is only concerned with the former, reducing the volume at a constant temperature. The behaviour of molecules and chemical reactions under high pressure follows the Le Chatelier's principle: application of pressure shifts an equilibrium towards the state which occupies a smaller volume (Tewari et al., 1999). This means that reactions that result in volume reduction are favoured when pressure is applied whereas reactions that result in a volume increase are retarded. The van't Hoff equation (**Equation 2-1**) relates the pressure dependence of the equilibrium constant (k) with the volume difference (ΔV) of products and reactants. ΔV is useful for predicting the effect of pressure on the equilibrium (Knorr et al., 2006). A negative ΔV indicates that the equilibrium shifts towards forming products and the reaction is favoured whereas a positive ΔV indicates that the equilibrium does not shift towards product formation, thus the reaction is not favoured (Knorr et al., 2006).

$$\frac{\partial \ln k}{\partial p} = -\frac{\Delta V}{RT}$$　.................　**Equation 2-1**

$R = 8.314\ Jmol^{-1}K^{-1}$, the universal gas constant

$T = temperature\ (K)$

$p = pressure\ (Pa)$

$k = equilibrium\ constant$

2.2.2 Adiabatic heating during pressure treatment

Pressurisation results in a temperature increase due to the work of compression (adiabatic heating) whereas depressurisation results in a temperature decrease due to the work of expansion (adiabatic cooling) in both the product being treated and in the pressure transmitting fluid (Otero and Sanz, 2003). Therefore, in a pressure treatment cycle, the temperature increases on pressurisation, re-equilibrates during the holding phase, which is then followed by a corresponding adiabatic cooling effect on depressurisation. The extent of adiabatic temperature change is dependent on the nature of pressure transmitting fluid. **Figure 2-2** shows the pressure and temperature profiles of water, ethylene glycol and ethanol during pressure treatment.
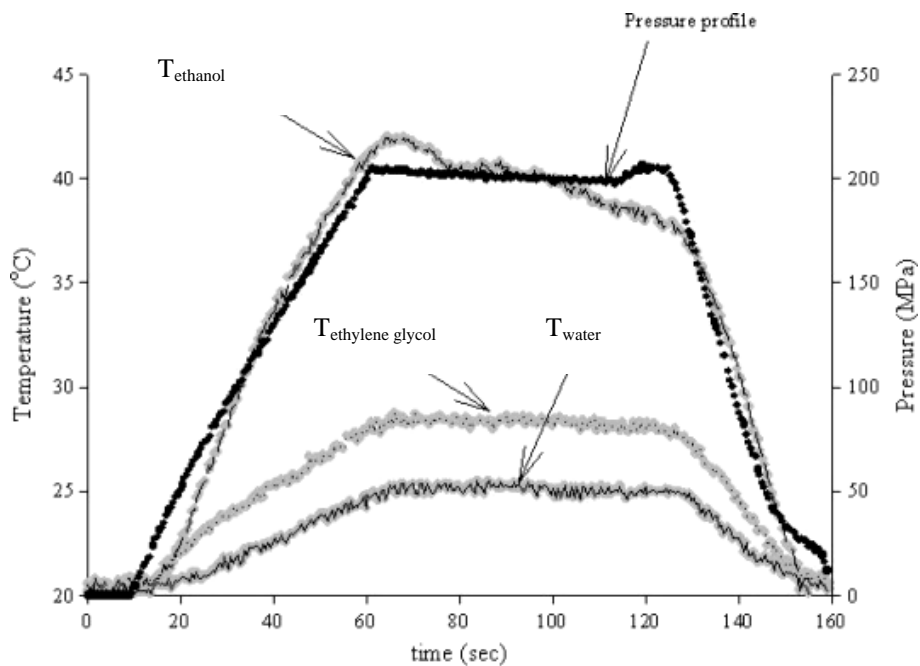
Figure 2-2: Pressure and temperature profiles of water, ethylene glycol and ethanol during pressurisation to 200 MPa, holding for ~60 seconds and depressurisation. Initial temperature of the substances was 20 °C and compression rate was set to 200 MPa/min. Source: Buzrul et al. (2008).

Food samples may be subjected to unintended thermal effects during pressurisation if the pressure transmitting fluid exhibits high adiabatic heating. For example, many oils have an adiabatic temperature increase three times that of water.

Water is often used as the pressure transmitting fluid in the food industry as it is the major component in most raw foods including milk (Otero and Sanz, 2003) and also for food safety and economic reasons (Ting et al., 2002).

2.2.3 Effects of pressure on food molecules

Table 2-1 summarises the effect of pressure and temperature on different chemical bonds. Covalent bonds do not undergo changes under pressure, and therefore the application of pressure does not affect some of the low molecular weight food components such as flavours, vitamins and amino acids (Tewari et al., 1999). However, non-covalent bonds (hydrophobic, ionic, and hydrogen bonds) can be affected by pressure (**Table 2-1**). As a result, the structure of the food components with non-covalent bonds may undergo changes when pressure is applied. Using methane molecules as model systems, Hummer et al. (1998) showed that pressure destabilises the contact configuration of non-polar molecular groups, thereby destabilising hydrophobic aggregates. Breaking of ionic bonds leads to a volume decrease due to charges on water molecules in the proximity of ions and is therefore promoted under high pressure (Balci and Wilbey, 1999). On the contrary, the formation of hydrogen bonds are favoured under pressure as their formation results in a volume decrease (Balci and Wilbey, 1999).

Table 2-1: Effect of pressure and temperature on weak linkages in proteins. Source: Balci and Wilbey (1999).

	Pressure	Temperature
Electrostatic bonds	Very labile	Little effect
Hydrophobic interactions	Complex but usually easily disrupted	Stronger $\leq 60°C$ then weaker at the higher temperatures
Hydrogen bonds	Slightly stronger	Very labile
Oxidation of –SH	Favoured	Favoured

Pressure-induced changes in lipids and polysaccharides (except for starch) can be considered as a pressure-induced ordering of the structure, similar to the effect of lowering the temperature (Balny et al., 2002). In contrast, proteins and starch show a pressure-induced disordering in their structures that is similar, but not identical to that induced by heating (Balny et al., 2002). Proteins undergo pressure-induced unfolding of the structures that may be largely attributed to penetration of water into the structure (Hummer et al., 1998). As the pressure increases, the protein-water system may be packed more efficiently and have a lower total volume when water molecules are incorporated into the structure, swelling the protein (Hummer et al., 1998). Penetration of water into the starch structure due to increasing pressure is also the first phase in the induction of starch gelatinisation by pressure (Rubens et al., 1999). The amorphous parts of the starch granules are hydrated first and as the granules continue swelling under pressure, the crystalline structures eventually collapse.

2.3   Starch

2.3.1 Properties of starch

Starch is the predominant food reserve substance in plants and provides 70-80% of the calories consumed by humans worldwide (BeMiller and Whistler, 1996). Starch occurs naturally as discrete particles (granules) which are relatively dense and insoluble, hydrating only slightly in cold water (**Figure 2-3**; BeMiller and Whistler, 1996). Most starch granules consist of two biopolymers: an essentially linear polysaccharide called amylose and a highly branched polysaccharide called amylopectin (**Figure 2-4**; Tester et al., 2004).
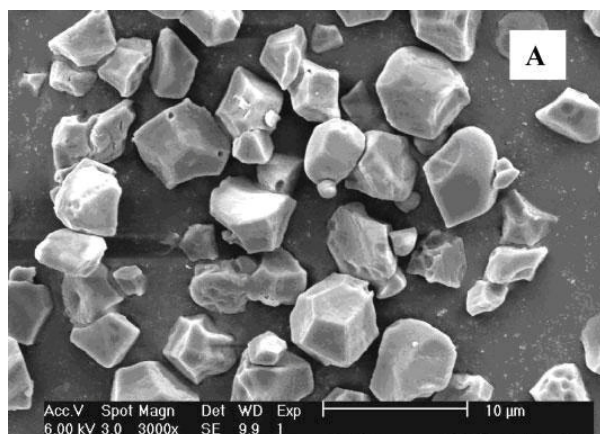
Figure 2-3: Scanning electron micrographs of native rice starch granules. Source: Wang et al. (2007).

Amylose has a helical structure as it mainly consists of α–1→4 linked glucose units with a few α–1→6 branches. Amylose molecules typically consist of 200–2000 glucose units. The molecular weight of amylose has been estimated to be $5.1 - 6.9 \times 10^5$ g mol^{-1} (Chen and Bergman, 2007). The interior of amylose helices is hydrophobic in nature and is able to form complexes with linear hydrophobic portions of molecules that can fit into the hydrophobic cavity (BeMiller and Whistler, 1996). Linear regions of amylose form a dark blue complex with polyiodide ions in aqueous solution at room temperature. This iodine binding property of amylose does not occur with amylopectin and this allows a distinction to be made between amylose and amylopectin. Therefore, it can be used as a basis for determining the amylose content of native starch (Parker and Ring, 2001).

Amylopectin is one of the largest biopolymers known with typical molecular weights being approximately 10^8 g mol^{-1} and a hydrodynamic radius of 21–75nm (Parker and Ring, 2001). Unlike amylose, amylopectin has a highly branched structure. The α–1→6 links between glucose units constitutes the major branching linkages in amylopectin and these α–1→6 branch links comprise about 4 to 5 % of the

total number of linkages in amylopectin (French, 1973). The α–1$\rightarrow$6 branch links occur every 20–30 glucose units and each branch can consist of up to 30 glucose units.

(A)

(B)
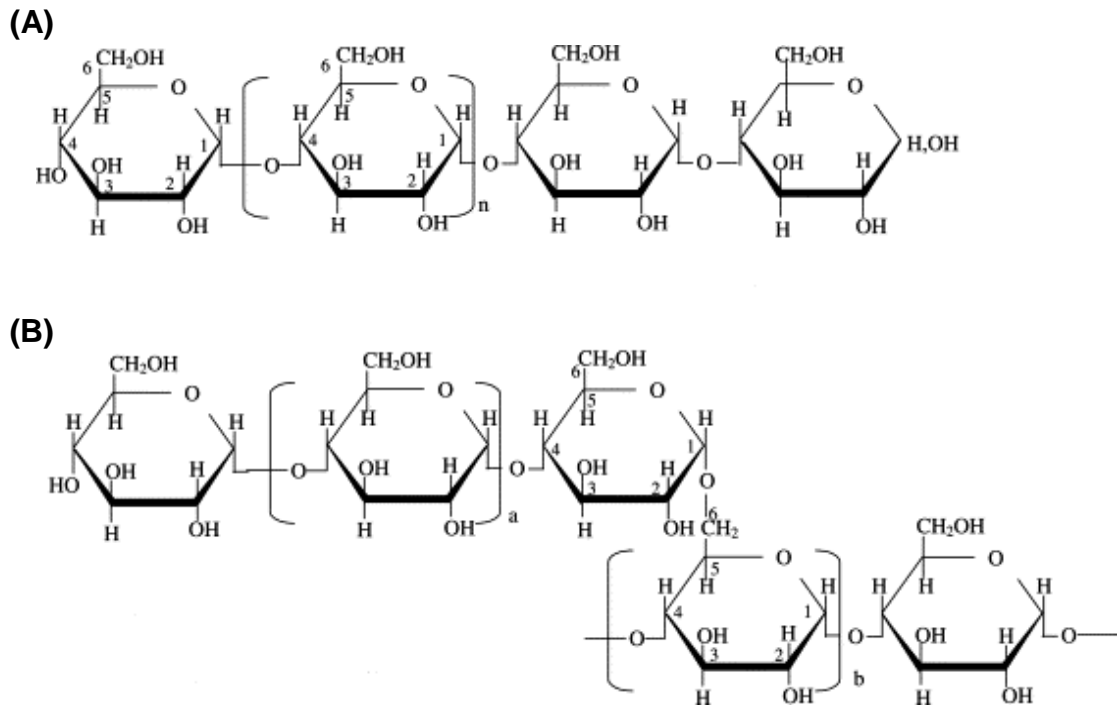
Figure 2-4: Structure of amylose (A) and amylopectin (B). Source: Tester et al. (2004).

Table 2-2 shows selected properties of common starches. Starches are categorised as normal or waxy starch. Normal starches typically contain about 25% amylose and 75% amylopectin. Starches that contain little or no amylose are called waxy starches. Waxy corn was the first grain recognised as one in which the starch consists almost exclusively of amylopectin. It is termed 'waxy' corn because when the kernel is cut the new surface appears vitreous or waxy (BeMiller and Whistler, 1996).

Table 2-2: Some properties of common starch granules. Source: BeMiller and Whistler (1996) and Singh et al. (2003).

	Granule size (µm)	Amylose (%)	Gelatinisation temperature (°C)[a]
Normal corn	2 – 30	28	62 – 80
Waxy corn	2 – 30	< 2	63 – 72
Potato	5 – 100	21	58 – 65
Tapioca	4 – 35	17	52 – 65
Wheat	2 – 56	28	52 – 85
Normal rice	1.5 – 9	5 – 28	66 – 78
Waxy rice	1.5 – 9	< 2	67 – 79

[a] From the initial temperature of gelatinisation to complete pasting.

2.3.2 Structure of starch granules

Starch granules are partially crystalline with alternating layers of semi-crystalline growth rings and amorphous growth rings (**Figure 2-5A**). The semi crystalline growth rings contain stacks of crystalline and non-crystalline (amorphous) lamellae (**Figure 2-5B**). Amylopectin is the basis of the crystalline structure of the granules, whereas amylose is present in the non-ordered (non-crystalline) state within the granule (Hermansson and Svegmark, 1996). The crystallinities of the starch granules are estimated to be approximately 30 per cent of the starch granules (Parker and Ring, 2001).
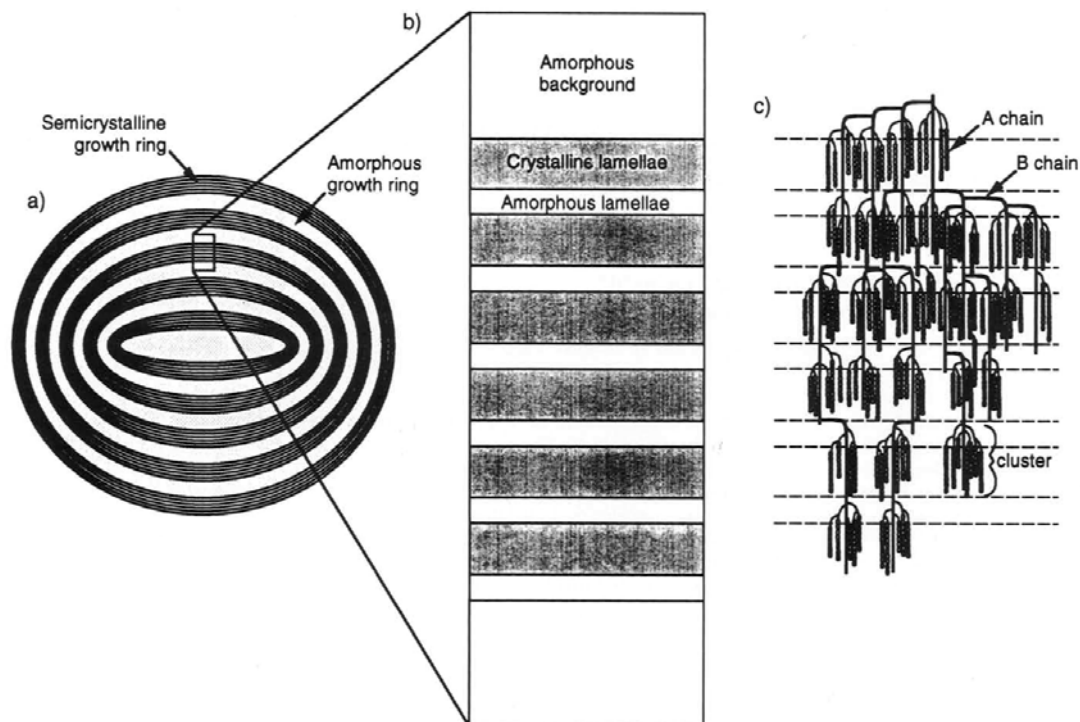
Figure 2-5: Schematic diagram of starch granule structure. (a) A single granule, comprising concentric rings of alternating amorphous and semi-crystalline composition. (b) Expanded view of the internal structure. (c) The currently accepted cluster structure for amylopectin within the semi-crystalline growth ring. Source: Jenkins and Donald (1995).

Amylopectin has two types of chains, *A-chains* and *B-chains*. The *A-chains* are linked only through their reducing ends to carbon 6 of a glucose unit (**Figure 2-5C**; Thompson, 2000). The *B-chains* are linked in the same way as *A-chains* except that it has at least one other additional chain attached at carbon 6 of one of its glucose units (**Figure 2-5C**; Thompson, 2000). This other chain could be either another *A* or *B-chain*. *A-chain* sections of amylopectin (**Figure 2-5C**) form double helices, that are regularly packed into crystalline lamellae. *B-chains* provide inter-cluster connections (**Figure 2-5C**). Branching points for both *A* and *B chains* are predominantly located within the amorphous lamellae (Jenkins and Donald, 1995). Due to the radial orientation of crystalline layers, ungelatinised native starch granules show a characteristic birefringence (white cross on a dark background) when starch granules

2-10

are viewed using a polarised light microscope (**Figure 2-6**). The centre of the cross is

at the hilum, the origin of growth of the granule (BeMiller and Whistler, 1996).
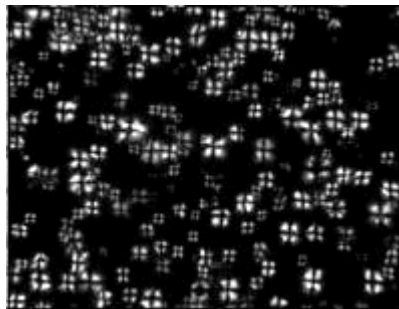
Figure 2-6: Example of birefringence: Corn starch granules observed under polarised light microscope. Source: Chen et al. (2006).

Starch can form different crystalline forms which can be observed in the X-ray

diffraction patterns of hydrated starch samples. These crystalline forms have been

categorised into A-, B- and C-types. The main distinguishing characteristic between

the different crystalline forms is the packing density of the double helices in the unit

cell (**Figure 2-7**). In the A-type structure, there is a close packed arrangement of

double helices, whereas in the B-type, the structure is more open, with a greater

amount of inter-helical water (Appelqvist and Debet, 1997).
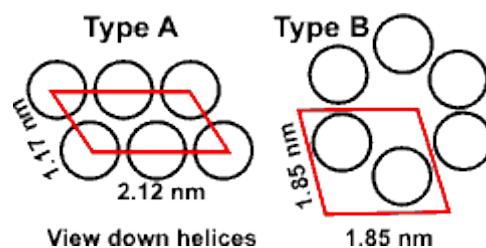
Figure 2-7: Double-helix packing arrangement in A- and B-type starches. Source: Parker and Ring (2001).

The X-ray diffraction pattern of C-type starch is considered to be a

combination of A- and B-type diffraction patterns (Buleon et al., 1998). For example,

in pea starch the A-type pattern is located essentially in the outer part of the granules

whereas the B-type pattern is found mostly at the centres (Buleon et al., 1998). A- and B-type diffraction patterns are shown in (**Figure 2-8**). In general, cereal starches such as rice, wheat or maize are of the A-type crystalline form, whereas tuber and root starches such as potato starch are of the B-type crystalline form. The rare C-type crystalline form is found in some legume starches such as pea or tapioca (Gidley, 1992).
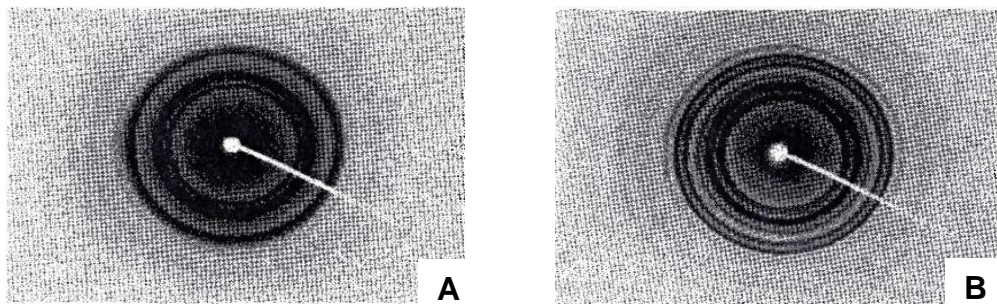
Figure 2-8: (A) A-type starch X-ray diffraction pattern, (B) B-type starch X-ray diffraction pattern. Source: French (1973).

2.3.3 Thermal gelatinisation of starch

2.3.3.1 Physico-chemical changes during starch gelatinisation

Starch is a commonly used ingredient in food products and the gelatinisation of starch is a critical process for both functional and nutritional purposes. **Figure 2-9** shows such changes in starch granules during gelatinisation as observed using a light microscope. Starch gelatinisation is defined as the disruption of the molecular order within the starch granules and this is manifested as irreversible changes in properties such as granular swelling (**Figure 2-9D** compared to **2-9B**), native crystallite melting, loss of birefringence (**Figure 2-9H, J & L** compared to **2-9B, D & F**) and starch solubilisation (Atwell et al., 1988). When starch is gelatinised, the polysaccharide chains within the starch granules are exposed, making the starch more readily available for modifying the functional properties in the food system as well as being available for hydrolysis by digestive enzymes (Oates, 1997). For example, gelatinised

starch can increase the viscosity and the intensity of 'creaminess' in yoghurt as well as reducing syneresis of stirred yoghurt (Ares et al., 2007). Dessert sauces can also be thickened by gelatinised starch when used in combination with another polysaccharide such as xanthan (Sikora et al., 2007).
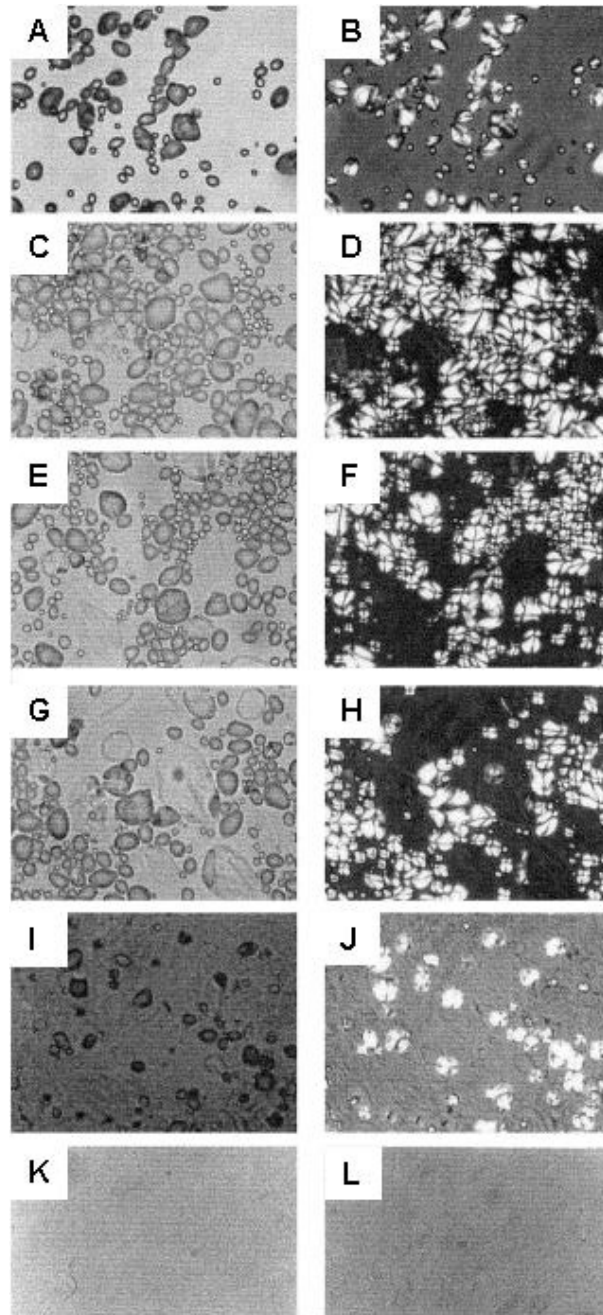
Figure 2-9: Light microscopy with (right) and without (left) polarised light for a potato starch-water system (18% starch content) upon heating at temperatures of 30°C (C, D), 50 °C (E, F), 58°C (G, H), 65°C (I, J) and 75°C (K, L). (A, B) starch granules without water at 30°C (Magnification ×200). Source: Liu et al. (2002).

Glicksman (1969) has given a theoretical rationale for the gelatinisation of starch. Starch polymers in the starch granules are held together by hydrogen bonds. When an aqueous suspension of starch is heated to a temperature at which the hydrogen-bonding forces are sufficiently weakened, water can enter into the granules. This critical temperature is called the initial gelatinisation temperature from which point the starch granules start to swell and lose their characteristic birefringence. Gelatinisation begins at the botanical centre (hilum) of the granule and spreads rapidly to the periphery of the granule. Amorphous regions of the granule where the hydrogen bonding is weakest are affected first. The gelatinisation temperature depends on the botanical source, genetic variety and growing conditions of the starch source (**Table 2-2**). The degree of association (hydrogen bonding strength) in the amorphous regions differs in individual granules of each starch type (Jane, 2004).

As the swelling of the granules continues, amylose (and some short chain amylopectin) that has become fully hydrated separates from the intricate network and diffuses into the surrounding aqueous medium (Glicksman, 1969). Such swelling and disruption of the starch granules produces a viscous mass consisting of a continuous phase of solubilised amylose and amylopectin and a discontinuous phase of granule remnants (BeMiller and Whistler, 1996). Gelatinised starch suspensions convert to a turbid visco-elastic paste, or an opaque elastic gel at sufficiently high starch concentrations ($\geq$ 6% w/w; Morris, 1990). The rheological properties of such pasted and gelled starch materials are affected by the volume fraction of swollen starch granules and the network formation of solubilised amylose. Starch gels are, therefore, considered composites consisting of swollen granules filling an amylose gel network. The granules increase the stiffness of the amylose gel (Morris, 1990).

2.3.3.2 Factors affecting thermal gelatinisation of starch

Starch gelatinisation is influenced by temperature, time, moisture and other solutes in suspension (Lund, 1984). Due to the complex nature of starch gelatinisation, it is difficult to model the entire gelatinisation process from the initial state (i.e. no gelatinisation) to maximum extent of gelatinisation (Lund, 1984). Nevertheless, attempts at modelling have been made by some researchers (Okechukwu and Rao, 1996; Spigno and De Faveri, 2004; Lagarrigue et al., 2008). For example, Okechukwu and Rao (1996) have shown that the gelatinisation of starch followed pseudo first-order kinetics with respect to temperature after an initial lag time. Also, the gelatinisation rate constant was shown to increase with temperature and could be described by the Arrhenius model.

Critical levels of water are required for the initiation of gelatinisation. For example, to start gelatinisation of rice starch, more than 3 mol of water/glucose unit are required (0.25 water/starch ratio) and to obtain complete gelatinisation, approximately 13.5 mol of water are required per mole of glucose units (Lund, 1984). From this aspect starch gelatinisation has been considered as a solvent-assisted, polymer melting phenomena (Lund, 1984).

Ionic and non-ionic soluble constituents influence starch gelatinisation. Generally, non-ionic constituents result in a decreased extent of gelatinisation and an increased temperature of gelatinisation (Lund, 1984). For example, increasing the concentration of sugar (non-ionic solute) in a starch suspension increases the gelatinisation temperature of starch (Spies and Hoseney, 1982). The effect of ionic substances depends on the type of ion. Oosten (1990) showed that anions are capable of rupturing hydrogen bonds, acting as gelatinisation-promoting agents, while cations have the opposite effect and protect starch granules from gelatinisation.

2.3.3.3 Techniques used in characterisation of thermal gelatinisation of starch

Starch gelatinisation results in numerous changes in starch granules and in the starch suspension. These changes can be monitored by a number of different techniques. Estimation of the gelatinisation can be based on changes in viscosity during gelatinisation (Lund, 1984). The viscosity of a starch suspension increases until the integrity of the starch granules is lost. Once all the starch granules have lost integrity, the viscosity decreases (**Figure 2-10**; BeMiller and Whistler, 1996). Historically, microscopy methods were also widely used to study starch gelatinisation (Di Paola et al., 2003). Since ungelatinised starch granules show birefringence under polarised light (**Figure 2-6 & 2-9**), the degree of gelatinisation can be determined by calculating the percentage loss of birefringence. In essence, the microscopy method can quantify the loss of crystallinity. However, this method is limited to dilute granule suspensions (Zobel, 1984) and it can be time consuming to monitor a large sample of granules.
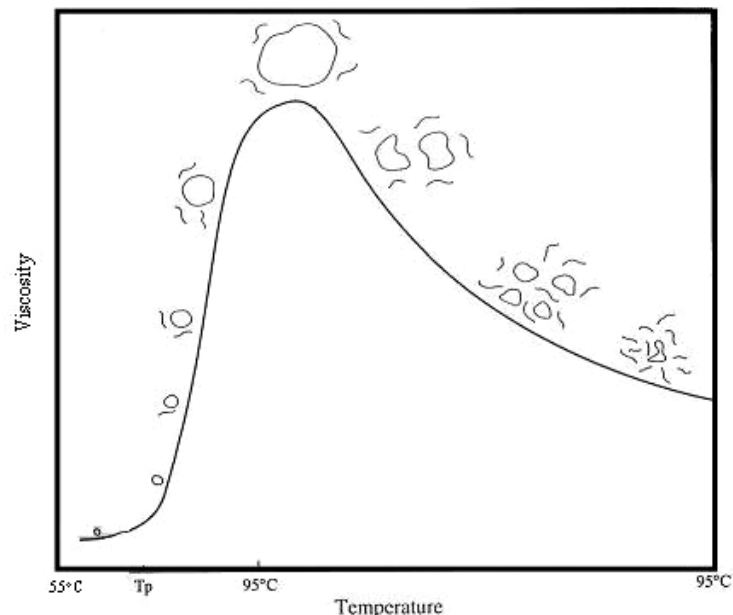
Figure 2-10: Representative Brabender curve (viscosity versus temperature) showing viscosity changes related to typical starch granule swelling and disintegration as a suspension of granules is heated to 95° C and held at the temperature. T_p is the pasting temperature which is the temperature at which a viscosity increase is recorded. Source: BeMiller and Whistler (1996).

Gelatinisation is an endothermic process, therefore can be monitored by thermal methods such as differential scanning calorimetry (DSC). DSC is a thermo analytical technique for monitoring changes in the physical or chemical properties of materials as a function of temperature by detecting the heat changes associated with such processes (**Figure 2-11**). The measuring principle of DSC is to compare the rate of heat flow to the sample and to an inert material, which are heated or cooled at the same rate. Changes in the sample that are associated with absorption or evolution of heat cause a change in the differential heat flow which is then recorded as a peak.
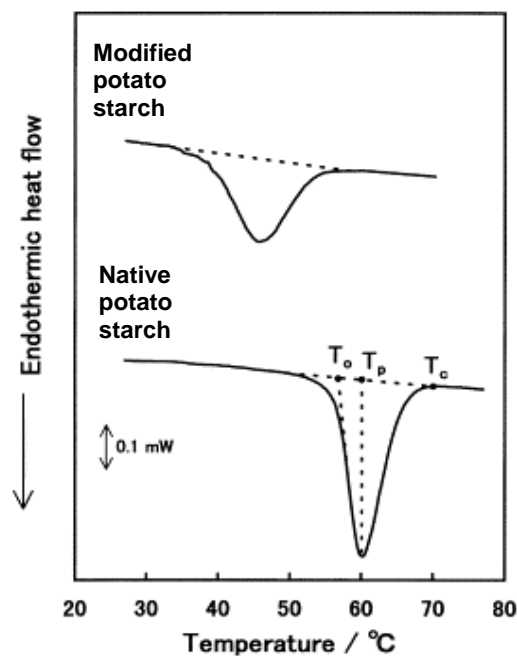
Figure 2-11: The heating DSC curves of native potato starch and chemically modified potato starch (heating rate: 0.2°C/min). Adapted from Morikawa and Nishinari (2000).

The area of the peak is directly proportional to the enthalpy change and its direction indicates whether the thermal event is endothermic or exothermic (**Figure 2-11**; Biliaderis, 1983). For starch, other parameters such as T_o (onset temperature of gelatinisation), T_p (peak temperature) and T_c (completion temperature of gelatinisation) can be extracted from the DSC curves, and these parameters are useful in the study of starch gelatinisation (**Figure 2-11**). DSC provides insights into the

order-to-disorder phase transition phenomena of starch granules as this is an endothermic process.

Changes in the crystallinity of starch granules and the transition of crystal structures can be monitored by X-ray diffraction (XRD) methods (**Figure 2-12**; Ratnayake and Jackson, 2007). Percent relative crystallinity can be calculated according to the method by using quartz as 100% crystalline material and an 85 °C treated sample of each starch as 100% amorphous material (**Equation 2-2** ; Ratnayake and Jackson, 2007).
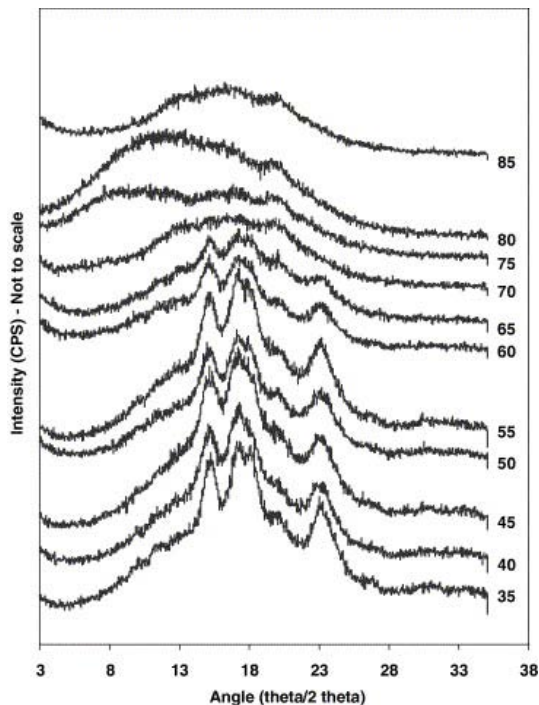
Figure 2-12: X-ray diffraction patterns of normal corn starch. Numbers to the right of each profile represent the treatment temperature (°C).
Source: Ratnayake and Jackson (2007).

$$\% \, Relative \, crystallinity = \frac{\left(\sum|\boldsymbol{I_s}-\boldsymbol{I_a}|\right)}{\left(\sum|\boldsymbol{I_c}-\boldsymbol{I_a}|\right)} \times 100 \quad \textbf{.................... Equation 2-2}$$

$|I_s - I_a|$ = absolute difference between the sample [I_s] and amorphous [I_a] intensities.

$|I_c - I_a|$ = absolute difference between the crystalline (quartz) [I_c] and amorphous [I_a] intensities. Source: Ratnayake and Jackson (2007).

Other techniques such as nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FTIR) have been used to study thermal gelatinisation of starch. The NMR technique measures water mobility and interactions at the molecular level (Gonera and Cornillon, 2002) whereas the FTIR technique detects absorption by different bond vibrations in starch molecules during gelatinisation (Liu et al., 2002).

2.4 Pressure-induced starch gelatinisation

There have been numerous studies on heat-induced starch gelatinisation over the decades, but there have been fewer studies on pressure-induced starch gelatinisation. The studies on pressure-induced starch gelatinisation have been published mainly in the last 12 years. Many of these have been published in the last three years, since the initiation of this study.

2.4.1 Mechanism

A number of studies have demonstrated that starch gelatinisation can be induced by high hydrostatic pressure (Douzals et al., 1996; Rubens et al., 1999; Kawai et al., 2007a). **Figure 2-13** shows the changes in the starch granules and the suspension volume during the different stages of pressure treatment (Douzals et al., 1996). While temperature effects involve energy and volume aspects due to thermal expansivity, pressure effects are mainly on the volume aspects via the compressibility of the system (Balny et al., 2002). Knorr (1999) has shown that at 20°C water was compressed by approximately 4% at 100 MPa and up to 15% at 600 MPa. The compressibility (relative volume change as a response to a pressure change) of a wheat starch suspension was close to that of pure water at pressures below 300 MPa (Douzals et al., 1996). Above 300 MPa, the reduction in the volume was higher for the wheat starch suspension than for pure water at the same pressure. This indicates

that the water molecules linked with starch to occupy a smaller volume than the molecules in pure water (**Figure 2-13**). Hence, gelatinisation of starch is induced by sufficiently high pressure which satisfies the principle of Le Chatelier.
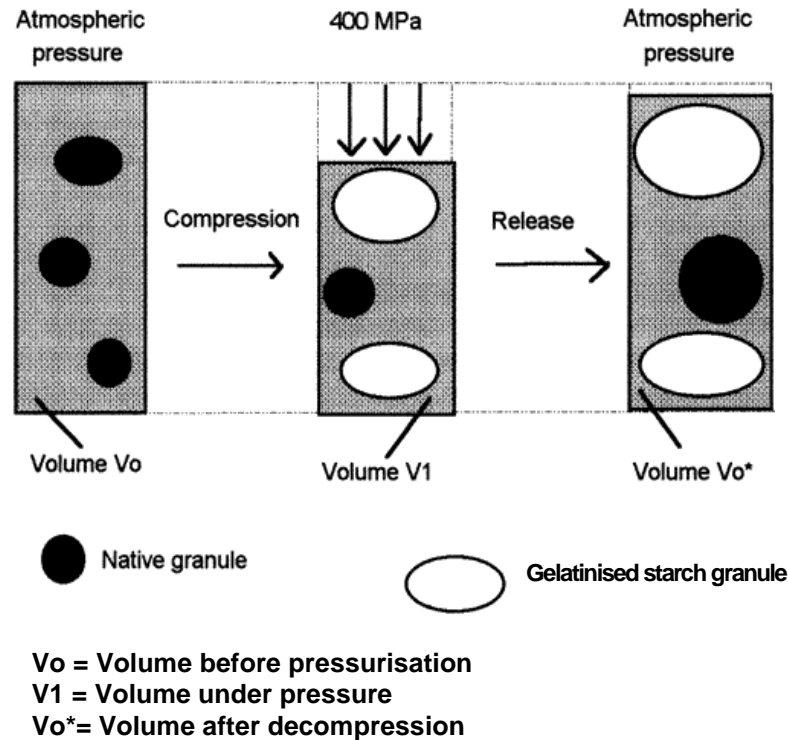
Figure 2-13: Schematic representation of wheat starch granule behaviour in suspension with water during a 400 MPa treatment. Source: Douzals et al. (1996).

The in-situ FTIR study by Rubens et al. (1999) showed that during gelatinisation under high pressure, the hydration of starch occurred before changes in crystallinity. They proposed a two-step mechanism for pressure-induced gelatinisation of starch. The amorphous regions of the starch granule are hydrated first, similarly to thermal gelatinisation. This hydration induces a swelling of the granule leading to a distortion of the crystalline regions. In the second step, the crystalline regions become more accessible to water. The two steps are not necessarily well separated and are dependent on the type of starch (Rubens et al., 1999).

2.4.2 Experimental methods to estimate the degree of gelatinisation in pressure-treated starch

As with heat-induced gelatinisation, loss of birefringence is often used to indicate the degree of gelatinisation of starch in pressure-treated samples, despite some drawbacks (Thevelein et al., 1981; Stute et al., 1996; Bauer and Knorr, 2005; Rumpold and Knorr, 2005). Stute et al. (1996) stated that the differentiation between gelatinised and ungelatinised starch granules after pressure treatment was more difficult to assess than after heat treatment because a large number of the granules showed partial loss or fading of the birefringence. The subjective nature of the method was also recognised by Kawai et al. (2007a). Another method commonly used to determine the degree of gelatinisation of pressure-induced starch gelatinisation is to use DSC (Douzals et al., 2001; Ahromrit et al., 2007; Kawai et al., 2007a). However, the results obtained from the microscopic method (birefringence) and the calorimetric method (DSC) showed discrepancies. The microscopic method has been reported to overestimate the number of gelatinised granules (Douzals et al., 2001).

2.4.3 Factors affecting pressure-induced starch gelatinisation

Thermal gelatinisation of starch occurs over a temperature range, rather than at a specific temperature because the degree of association between starch polymers in the amorphous regions differs in individual granules (Glicksman, 1969). The gelatinisation temperature range is different depending on the type of starch (**Table 2-2**). This also seems to apply with pressure in that the starch gelatinisation is observed over a range of pressures and the pressure range over which gelatinisation occurs is dependent on the starch type (**Table 2-3**). For example, normal corn starch gelatinises at pressures between 400 and 550 MPa (Stute et al., 1996) whereas potato starch gelatinises in the pressure range of 700-1200 MPa (**Table 2-3**; Kawai et al. 2007a).

Table 2-3: Minimum gelatinisation pressure reported for different types of starches

Starch type	Minimum gelatinisation pressure	Source
Wheat	300 MPa	Douzals et al. (1996)
Normal corn	420 MPa	Stute et al. (1996)
Normal rice	300 MPa	Rubens et al. (1999)
Potato	700 MPa	Kawai et al. (2007a)

Within the gelatinisation pressure range, the degree of gelatinisation is dependent on the applied pressure at a given temperature, so that increasing pressure at temperatures below the thermal gelatinisation temperatures of starch leads to a higher degree of gelatinisation (Bauer and Knorr, 2005; Kawai et al., 2007b). Pressure-induced gelatinisation is also affected by the duration of treatment as long as the pressure is above the gelatinisation pressure (Stolt et al., 2000; Bauer and Knorr, 2004; Bauer and Knorr, 2005). Blaszczak et al. (2005b) demonstrated that more destruction of the microstructure of starch granules occurred as the treatment duration increased from time zero to three minutes at 600 MPa. Bauer and Knorr (2005) found that the degree of gelatinisation was strongly dependent on the treatment time during the first hour of treatment for wheat, tapioca and potato starch suspensions. However, prolonged pressurisation (>1h) was found to lead to little further changes in the starch (Bauer and Knorr, 2005; Kawai et al., 2007b). Kawai et al. (2007b) showed that starch gelatinisation was affected by treatment pressure and temperature when the treatment duration was between one and 66 hours.

Most studies have shown that the lower the treatment pressure, the slower the gelatinisation occurs and as the treatment pressure increases, the gelatinisation occurs at a greater rate (Stolt et al., 2000; Bauer and Knorr, 2004; Bauer and Knorr, 2005). In addition, the effect of pressure can be combined with the temperature. Bauer and

Knorr (2005) showed that at constant pressure, the degree of gelatinisation increases with increasing treatment temperature (below the thermal gelatinisation temperature). In fact, the extent of pressure-induced starch gelatinisation at pressures above the gelatinisation pressure has been shown to follow the first-order kinetic model with the rate constant (k) increasing progressively as the pressure increased (Ahromrit et al., 2007).

Rumpold and Knorr (2005) showed that solutes such as sugars and salts in the starch suspension could influence the gelatinisation in a similar way to that reported for thermal gelatinisation. When sugars (e.g. fructose, glucose, sucrose and trehalose) were added to starch suspensions, the degree of pressure-induced gelatinisation was lowered, and the sugars with higher molecular weights and more hydroxyl groups showed more pronounced effects. Salts (e.g. NaCl, $CaCl_2$, KCl) showed gelatinisation suppressing or promoting effects depending on the concentration. The extent of the effect on the gelatinisation pressure depended on the type of salt and the type of starch.

2.4.4 Structural changes of starch granules due to pressure treatment

Although water can be channelled into the crystalline regions, Knorr et al. (2006) suggested that disintegration of the crystalline regions is incomplete in pressure-induced starch gelatinisation. In thermal gelatinisation, the crystalline structure of the starch granule is disintegrated through helix-helix dissociation of amylopectin chains followed by helix-coil transitions when the gelatinisation temperature is exceeded (**Figure 2-14**). However, under pressure, the helix-helix dissociation and helix unwinding of amylopectin chains might be restricted as the stability of van der Waals forces and hydrogen bonds are maintained, therefore, the helix structure is promoted. Consequently, starch gelatinisation does not proceed further as the crystalline regions are not completely disintegrated (**Figure 2-14**).
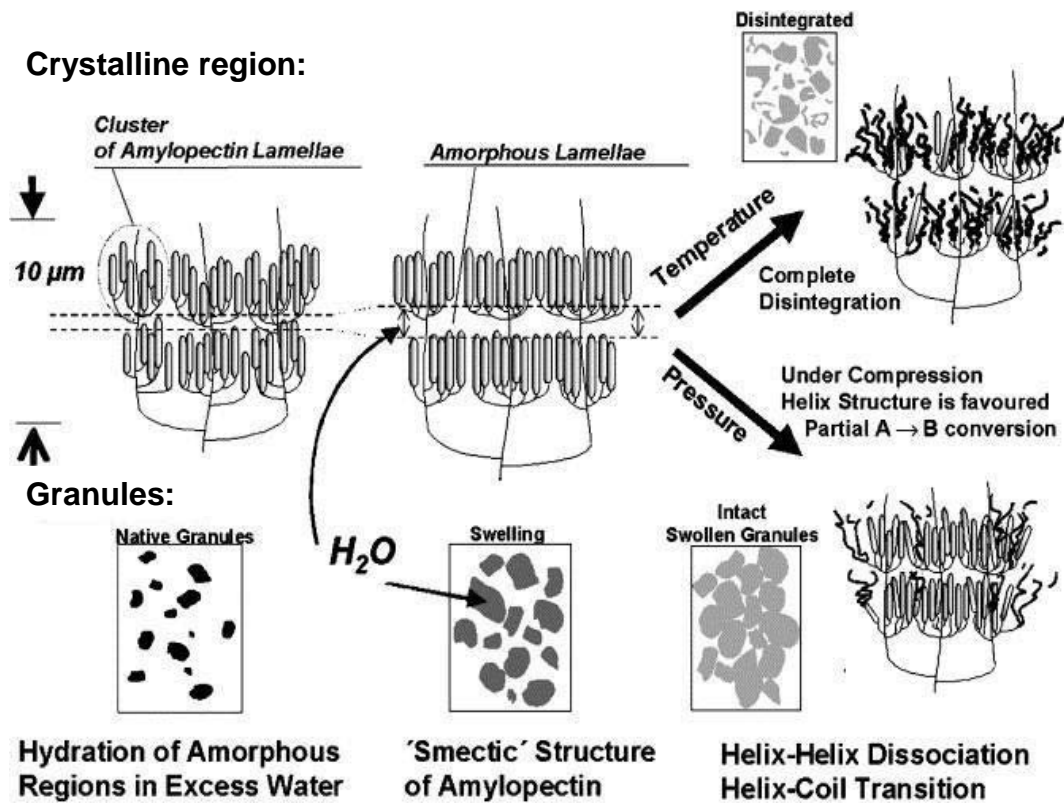
Figure 2-14: Comparison of structural changes of starch granules during heat- or pressure-induced starch gelatinisation. Source: Knorr et al. (2006).

Using X-ray diffraction techniques, it has been shown that the crystalline structure of amylopectin changed after high pressure treatment (Hibi et al., 1993; Katopo et al., 2002). Starches with an A-type pattern were converted into a pattern similar to B-type starches whereas B-type starches kept their original B-type pattern. For example, the crystalline structure of normal corn starch (A-type) showed a faint B-type X-ray diffraction pattern after pressure treatment at 500 MPa for 20 min whereas potato starch (B-type) retained the native granular features and increased the B-type X-ray diffraction pattern, even after application of pressures up to 500 MPa for 60 min (Hibi et al., 1993).

Gel-like inner part

Unchanged outer part
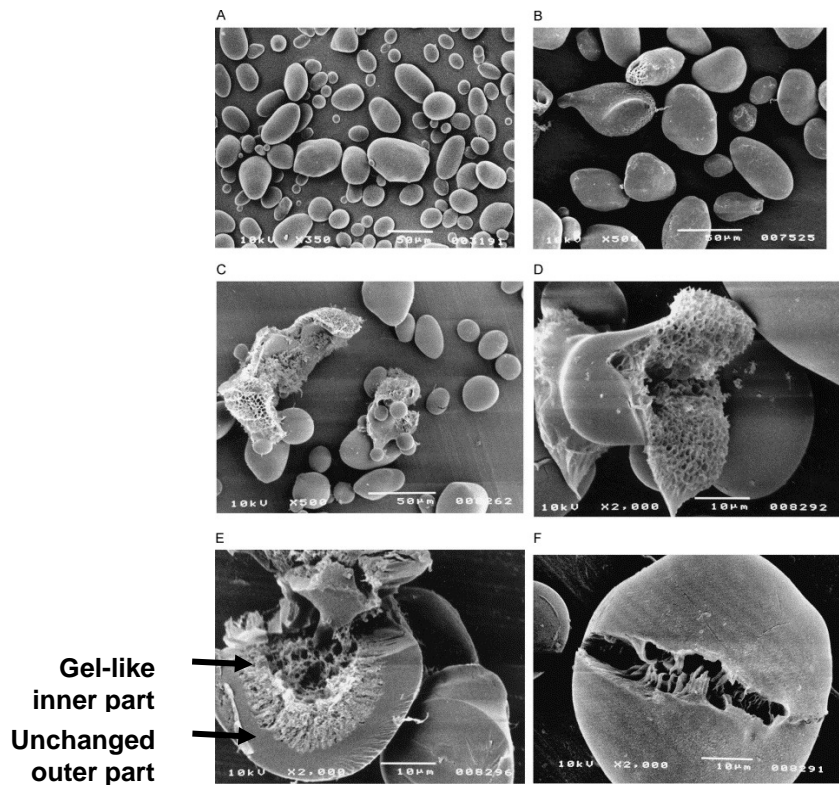
Figure 2-15: Microstructure of potato starch shown by scanning electron microscopy (SEM): native (A); treated with high pressure at 600 MPa for 2 min (B) and 3 min (C); (D)–(F) details of starch structure treated for 3 min. Source: Blaszczak et al. (2005b).

There are a number of factors that may contribute to the stability of the starch granule structure under pressure such as the thickness of the crystalline region and extent of interaction of the starch chains within the amorphous and crystalline regions. Potato starch and high amylose corn starch exhibit a far greater level of ordered structure in the outer part of the granule than other starches such as normal corn starch and waxy corn starch (Sevenou et al., 2002). For potato starch, Blaszczak et al. (2005b) observed that the outer part of pressure-treated and freeze-dried granules had unchanged structure whereas the inner part was completely filled with a gel-like network which had larger voids towards the centre of the granule (**Figure 2-15**). This indicates that the outer part of the starch granule was more pressure-resistant, which may have contributed to the overall stability of the granular structure of potato starch under pressure.

2.4.5 Swelling of starch granules

As with thermal gelatinisation, water is a critical component in the pressure-induced gelatinisation of starch. When water was replaced with hexane, the crystalline structure of starches, as indicated by the X-ray diffraction pattern, did not change after pressure treatment (Hibi et al., 1993). According to Stute et al. (1996), at least 50% (w/w) moisture level was necessary to gelatinise wheat starch when the starch was treated at 600 MPa for 15 min, as indicated by changes in the DSC-curves. Furthermore a higher water content in the starch suspension can promote more effective gelatinisation as indicated by more gelatinisation-related changes in the DSC-curves (Stute et al., 1996). Katopo et al. (2002) found that the starches that were pressurised in 2/1 (v/w) water/starch suspensions showed a higher degree of gelatinisation compared with those pressurised in 1/1 (v/w) water/starch suspensions. In addition, Ahromrit et al. (2007) showed that the degree of gelatinisation of rice starch in rice grains, measured by gelatinisation enthalpy change (ΔH) at any given pressure, temperature and time, was highly correlated with the moisture content of the grain.

Entry of water into starch granules occurs predominantly through the hilum and the subsequent swelling of granules follows a first order reaction (Snauwaert and Heremans, 1999). Swelling of starch granules was shown to occur using microscopic examination (Hibi et al., 1993; Stolt et al., 2000; Bauer et al., 2004), swelling index measurements (Douzals et al., 1998) or particle size analysis (Stute et al., 1996). The swelling index of starch increased with treatment pressure within the gelatinisation range (Douzals et al., 1998).

The swelling capacity of starch granules was found to vary among different types of starches. Pressure treatment of normal corn, arrowroot and wheat starches led

to a lower degree of swelling than waxy corn starch or tapioca starch (Stute et al., 1996). Starch from potato and pea also did not swell as extensively as waxy corn starch or tapioca starch (Rubens et al., 1999). Starches do not necessarily swell to the same extent under pressure as in thermal gelatinisation. The microscopic analysis by Blaszczak et al. (2005a) showed that pressure treatment of waxy corn starch resulted in a complete breakdown of the granules, similarly to that expected after heat treatment, whereas high-amylose (68%) corn starch maintained its granular structure after a pressure treatment at 650 MPa for 9 min. Wheat starch was also reported to show limited swelling in pressure-induced gelatinisation when compared with thermal gelatinisation (Douzals et al., 1998).

The reason for restricted swelling and preservation of granule structure observed in some starches under pressure is not fully understood. It is possible that the complex packing of amylose and amylopectin chains in the starch granules influences gelatinisation behaviour of starches (Rubens and Heremans, 2000). Stolt et al. (2000) suggested that amylose somehow stabilises the starch granule structure under pressure since starch with a higher amylose content (e.g. normal corn starch) showed restricted swelling while starch with very low levels of amylose (e.g. waxy corn starch) could swell and disintegrate completely.

2.4.6 Leaching of amylose from starch granules

Bauer and Knorr (2004) found that the electrical conductivity of the tapioca starch suspension increased as the degree of pressure-induced gelatinisation of starch increased, which indicates a release of ions and/or amylose from the starch granule during pressurisation or after pressure release. The extent of amylose leaching seems to depend on the type of starch. Bauer et al. (2004) observed a complete decolouration of iodine stain in wheat and tapioca starch granules after 1 hour at 300 MPa using a

light microscope with a high pressure cell with windows (**Figure 2-16**). The authors also reported darkening of the surrounding liquid phase and brownish precipitation. The decolouration was considered to be due to the release of amylose from the amorphous regions of the starch granules as a result of pressure-induced starch gelatinisation. The released amylose formed complexes and this caused the darkening of the surrounding liquid.
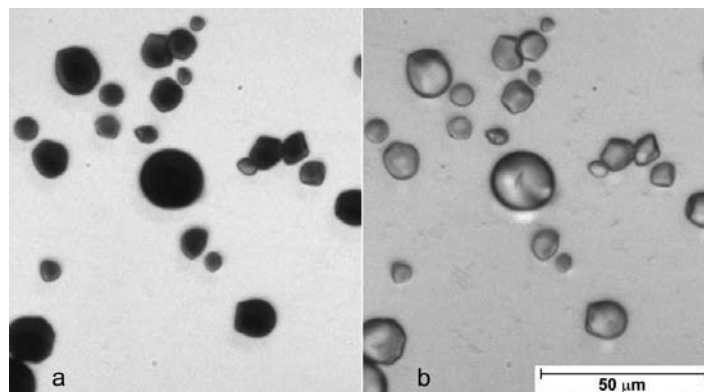
Figure 2-16: Iodine stained tapioca starch suspensions before a pressure treatment (a) and after 1 h at 300 MPa (b). Source: Bauer et al. (2004).

Potato starch granules, on the other hand, were only slightly decoloured with a minor darkening of the surrounding liquid phase after the same treatment. In a barley starch suspension, no leaching of amylose was observed when suspensions were pressure-treated at 550 MPa for 45 min (Stolt et al., 2000). In comparison to thermal gelatinisation, Douzals et al. (1998) found that pressure-induced gelatinisation of wheat starch involved much less leaching of amylose. This could be due to a lower water binding (swelling) in pressure-induced gelatinisation than in thermal gelatinisation.

2.4.7 Properties of pressure-induced starch gels

Starch can form a gel-like network when pressure-treated (Stute et al., 1996; Stolt et al., 1999; Blaszczak et al., 2007). Stolt et al. (1999) studied the effect of pressure treatment time on the storage modulus (G') of pressure-induced 10% waxy

corn starch gel and found that excessive pressurisation could weaken the gel structure. Douzals et al. (1998) carried out specific gravity measurements and showed that wheat starch suspensions gelatinised by pressure treatment were denser than the untreated suspensions, whereas the suspensions gelatinised by heating were less condensed than the untreated suspensions. This difference in suspension volumes between pressure-treated and heat-treated suspensions suggests that the conformation of hydrogen bonds between the starch polymers and water in the network may be different from each other.

2.5   Bovine Milk

Milk is an opaque white fluid secreted by female mammals for the nourishment of their young (Pearsall and Trumble, 1995). It provides both energy and the building materials necessary for growth (Bylund, 1995). Although milk is chemically very stable at its natural pH, it is perishable in nature as it is designed to be fed directly to young mammals soon after production (Muir, 1998). Bovine milk is the major milk processed in the world for human consumption. The processing technologies for milk have been developing to adapt to the needs of the consumers beyond the simple preservation requirements. Bovine milk has both nutritional values in the human diet and commercial significance and is also an extremely versatile raw material for a number of food products consumed by humans. As this study intends to examine the pressure-induced gelatinisation of starch in milk as well as in water and to evaluate potential applications of starch in dairy food systems, relevant aspects of milk are reviewed in this section.

2.5.1 Basic Physical-Chemical Properties of Bovine Milk

Milk is a complex mixture of a variety of components. Bovine milk consists of about 86% water and 14% dry matter. The dry matter is suspended or dissolved in the water (Swaisgood, 1996). **Table 2-4** displays the typical composition of bovine milk and the state of each group of components in the milk. Milk has components that are in true solution, in molecular solution, in colloidal suspension or in an emulsion form depending on which component is considered (**Table 2-4**). The quantities of the various components in milk can vary between cows of different breeds and between individual cows of the same breed (Swaisgood, 1996). The composition of milk also changes slightly depending on the stage of the lactation cycle (Bylund, 1995).

Table 2-4: Size, state and typical percentage of components in bovine milk. Sources: Walstra and Jenness (1984); Swaisgood (1986); Jenness (1988); Bylund (1995).

Component	Size (diameter, nm)	State in milk	Sub-component	Typical (% w/w)
Water	-	-	-	**86**
Total Solids	-	-	-	**14**
Fat	2000-6000	Oil in water emulsion	-	**5**
Lactose	0.5	True solution		**4.7**
Protein			**Total proteins**	**3.5**
Casein micelles	50-300	Colloidal suspension	**Total casein**	**2.8**
			α_{S1}-casein	1.1
			α_{S2}-casein	0.3
			β-casein	1.0
			κ-casein	0.4
Whey proteins	4-6	Molecular solution	**Total whey protein**	**0.7**
			β-lactoglobulin	0.35
			α-lactalbumin	0.14
			others	0.21
Minerals	-		**Total minerals**	**0.75**
		True solution/ Colloidal suspension	calcium	0.17
			phosphate	0.23
		True solution	potassium	0.19
		Mainly true solution	other minerals	0.16

2.5.2 Milk Proteins

Understanding the effects of various processing conditions on the milk proteins is essential for dairy processing as their behaviour largely determines the behaviour of milk during processing. Milk proteins are also responsible for the functional properties of milk such as rennet gelling and acid gelling properties which are the basis for the manufacture of cheese and yoghurt, respectively (Muir, 1998). The nitrogen content of milk is distributed among caseins, whey proteins, and the non-protein nitrogen, as well as some minor proteins that are associated with the fat globule membrane (Goff and Hill, 1993). The principal protein component of milk is casein, which accounts for 80% of the protein content of skim milk. The whey proteins make up the remaining 20% of the proteins in milk (**Table 2-4**).

2.5.2.1 Whey Proteins

The proteins remaining soluble when milk is adjusted to pH 4.6 are collectively referred to as 'whey proteins' (Goff and Hill, 1993). The whey proteins are a complex mixture of proteins of which β-lactoglobulin (~50%) and α-lactalbumin (~20%) are the major components (**Table 2-4**). Lactoferrin, bovine serum albumin and immunoglobulin are also present at significant levels (Wong et al., 1996).
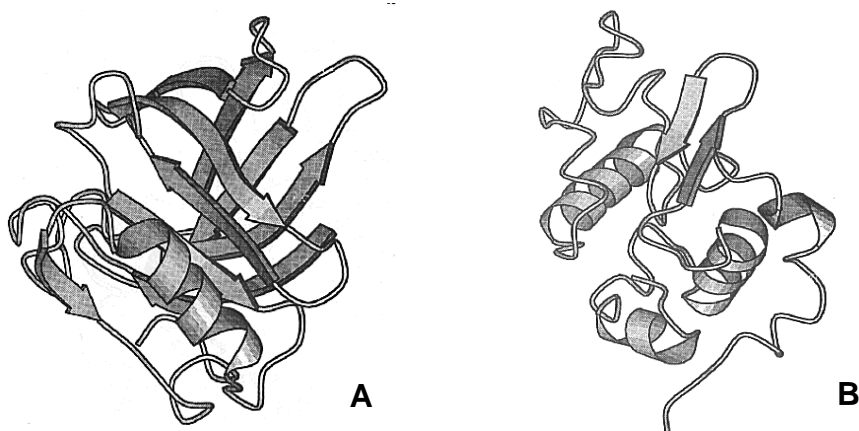
Figure 2-17: (A) Structure of β-lactoglobulin, (B) Structure of α-lactalbumin. Source: Swaisgood (1996).

The structure of the two main whey proteins, β-lactoglobulin and α-lactalbumin (**Figure 2-17**) are typical of globular proteins (Swaisgood, 1996), and therefore have a considerable native internal architecture, consisting of several different non-covalent bonds occurring within or between protein molecules or between protein groups and solvent molecules (Relkin, 1996). As whey proteins are typical globular proteins, they can be denatured by processes such as heat treatment, as is discussed in detail in section 2.5.3.

β-lactoglobulin contains two disulfide bonds and a partially buried sulphydryl group. β-lactoglobulin consists of eight strands of anti-parallel β-sheets wrapped around to form a β-barrel with the shape of a flattened cone and a single α-helix lying on its surface (**Figure 2-17**; Wong et al. 1996). There are six known genetic variants of β-lactoglobulin and each genetic variant exists as a stable dimer at pH values near its isoelectric point up to and including the pH of milk and at room temperature (Swaisgood, 1986). At pH values below 3.5 the dimers reversibly dissociates due to the strong electrostatic repulsive forces. It is also known that genetic variants A and B associate to form octamers at pH between 3.5 and 5.2 while dissociation of the dimers occurs at alkaline pH values (Swaisgood, 1986).

α-lactalbumin is ellipsoid in shape with a deep cleft dividing the molecule into two lobes, four α-helices form one lobe and two β-strands together with a loop-like chain make up the other lobe (**Figure 2-17B**; Wong et al. 1996). Four disulphide groups form linkages with helical segments on at least one side of the bond (Wong et al., 1996). There are two known genetic variations of α-lactalbumin (Swaisgood, 1986).

2.5.2.2 Caseins

Caseins are phosphoproteins which are precipitated from raw milk at pH4.6 at 20°C (Wong et al., 1996). There are four types of caseins: α_{S1}-, α_{S2}-, β- and κ-casein, and the approximate percentage of each casein in milk is listed in **Table 2-4**. Caseins in milk are associated together in the form of large macromolecular assemblies called casein micelles. Native casein micelles in milk can be considered as association colloids (i.e. the proteins and calcium phosphate are not covalently bound), and are generally stable during technological treatments such as heating, freezing and even drying (de Kruif, 1999). Unlike whey proteins, heat treatment of casein micelles does not lead to denaturation (Singh and Fox, 1985).

The exact structure of the casein micelle has not been unequivocally established. Nevertheless, several models of casein micelle structure have been proposed over the years. The model of the casein micelle as a roughly spherical, fairly swollen particle with average diameters of 100-200 nm, with a hairy outer layer consisting of κ-casein, is now generally accepted. The classical sub-micelle model of the casein micelle is shown in **Figure 2-18** (Schmidt, 1982; Walstra, 1990; Walstra, 1999). In this model, it was proposed that the casein micelle is built of tightly aggregated sub-micelles which are roughly spherical units of about 14 nm diameter. The sub-micelles can be divided into two types, those rich in κ-casein and those with little κ-casein. The sub-micelles are held together by hydrophobic bonds and salt bridges. Regions of amorphous calcium phosphate link the sub-micelles to each other with the ester phosphate groups of the caseins forming part of this colloidal phosphate. κ-casein is located predominantly at the micelle surface with the C-terminal part of the κ-casein present as flexible 'hair' extending from the surface of micelle. It has been suggested that colloidal calcium phosphate (CCP) binds to the α_{S1}-, α_{S2}- and β-

casein via their phosphate side chains but it does not bind to phosphoserine-poor κ-casein. Hence, sub-micelles with a low κ-casein content or with no κ-casein are located in the interior of the micelles and those with high κ-casein content are located at the surface of the micelles. Micellar growth would stop when the entire surface is covered by κ-casein (Schmidt, 1982; Walstra, 1990; Walstra, 1999).
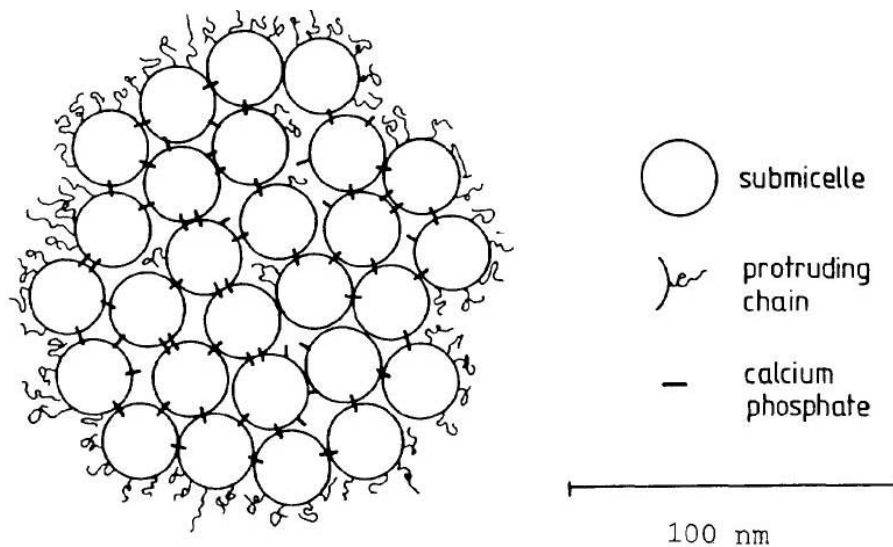
Figure 2-18: Sub-micelle model (cross section) of a casein micelle. Source: Walstra (1990).

Recently, an alternative model of the casein micelle that does not have discrete sub-micelles was proposed by Horne (1998) and is shown in **Figure 2-19**. In this new model, two types of linkages between protein molecules were suggested. The first type is a hydrophobic linkage which is formed between two or more hydrophobic regions from different casein molecules. Electrostatic repulsion of the protein charged residues limits extension of these polymers. The second type of linkage is formed as CCP forms bridges between phosphoserine clusters on casein proteins. The CCP also neutralises high charges associated with phosphoserine clusters. Although the κ-casein can interact via their hydrophobic domains with the hydrophobic regions of the other caseins, further growth beyond the κ-casein is not possible because it does not

have a phosphoserine cluster for linkage via CCP, nor another hydrophobic anchor point to extend the chain. Hence, κ-casein acts as a terminator for both types of growth and becomes part of the surface structure of the micelle (Horne 1998; **Figure 2-19**).
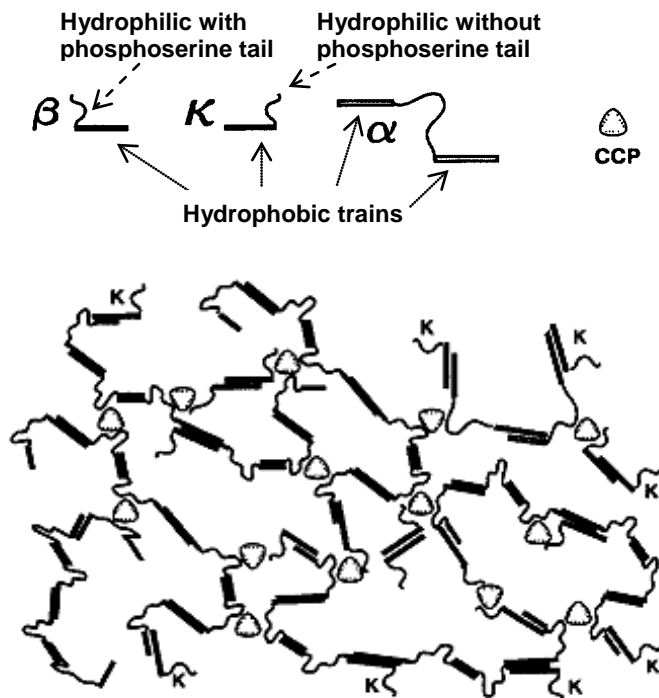
Figure 2-19: Dual binding model of a casein micelle with α-, β- and κ-casein. Bonding occurs between the hydrophobic regions, shown as rectangular bars, and by linkage of hydrophilic regions containing phosphoserine clusters to colloidal calcium phosphate (CCP) clusters. Source: Horne (1998).

The colloidal suspension of casein micelles in milk serum is a stable protein system. Although the surface charge on the casein micelle contributes to the stability of casein micelle via electrostatic repulsion, it is now considered that steric stabilisation due to the protruding glycomacropeptide hairs of κ-casein is largely responsible for the high stability of casein micelles (Holt and Horne, 1996; Horne, 1998). When the hairy surfaces of two micelles interpenetrate on close approach the local osmotic pressure increases so that the proximity of the two micelles is restricted. This inter-penetration also restricts the freedom of motion of the flexible hairs,

leading to an entropic repulsion at close approach (Holt and Horne, 1996; Horne, 1998).

2.5.3 Effect of thermal processes on the proteins in skim milk

Processing of virtually all milk products involves heating at some stage for food safety and product functionality reasons. Therefore, it is vital to understand the effect of thermal processes on milk. There are a variety of different thermal processes including pasteurisation (about 72°C for 15 seconds) and ultra-high temperature treatment (UHT, 138-142°C for several seconds). Heating results in a number of different changes to the proteins in milk. This section will discuss the effect of heating on milk proteins, focusing on the denaturation of whey proteins and association of the casein micelles with the denatured whey proteins.

2.5.3.1 Effect of heating on whey proteins

Heat treatment is one of the major processes that causes the denaturation of whey proteins which leads to substantial alterations in their structural arrangements and consequently their functional properties whether in milk, whey or in model protein solutions (de Wit, 1984; Dannenberg and Kessler, 1988a; Dannenberg and Kessler, 1988b; Dannenberg and Kessler, 1988c; Anema, 2001; Singh and Havea, 2003). The whey proteins are known to denature when milk is subjected to heat treatment above about 70°C (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996; Oldfield et al., 1998a; Oldfield et al., 1998b; Anema, 2000; Anema, 2001). The extent of denaturation for the two main whey proteins, β-lactoglobulin and α-lactalbumin, increases with an increase in heating temperature and time as shown in Arrhenius plots in **Figure 2-20** (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996; Oldfield et al., 1998a; Oldfield et al., 1998b; Galani and Apenten, 1999; Anema 2000; Anema 2001).
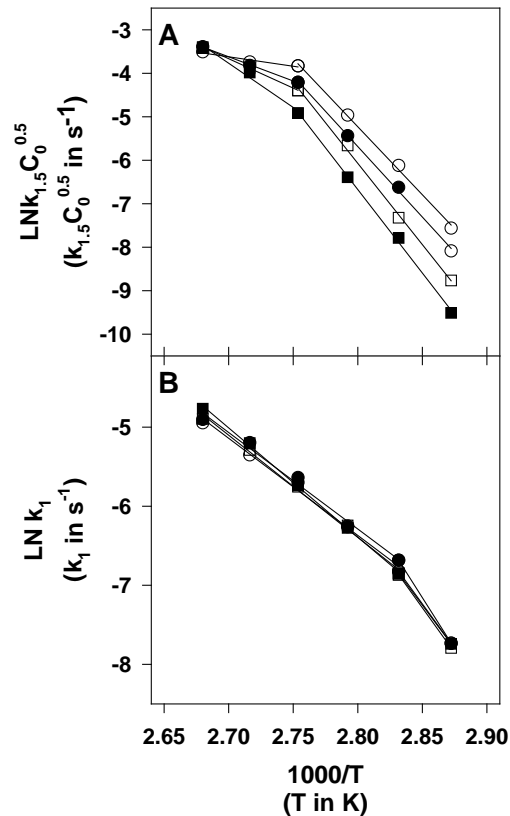
Figure 2-20: The Arrhenius plots for the thermal denaturation of β-lactoglobulin (A) and α-lactalbumin (B) over a 75–100°C temperature range, at various milk concentrations. ○ : 9.6% total solids milk; ● : 19.2% total solids milk; □ : 28.8% total solids milk; ■ : 38.4% total solids milk. Sources: Anema (2000) and Anema (2001).

However, the irreversible denaturation of whey proteins is not a simple process. As shown in **Figure 2-20**, the temperature dependence of β-lactoglobulin denaturation changes at about 90°C (363K) and that of α-lactalbumin changes at about 80°C (353K). It has been suggested that this change in the temperature dependence of the rate constants for denaturation (**Figure 2-20**) could be due to the change in rate-determining steps occurring at these critical temperatures (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996). The enthalpy of activation, entropy of activation and the activation energy suggest that the rate-determining step is the reversible unfolding of the protein tertiary structure at temperatures below the critical temperatures, whereas irreversible aggregation processes involving the unfolded

protein become rate-determining at temperatures above the critical temperatures (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996).

The denaturation of whey proteins also depends considerably on the pH of the milk at heating. Law and Leaver (2000) found that the rate of denaturation of β-lactoglobulin and α-lactalbumin increased as the pH of the milk increased from the natural pH and decreased as the pH of the milk was decreased. In addition, the composition and concentration of milk affects the whey protein denaturation. The rate of denaturation of β-lactoglobulin decreased with an increasing milk concentration (Anema, 2000) while that of α-lactalbumin was unaffected by the milk concentration (**Figure 2-20**; Anema, 2001). These effects of milk concentration has been related to various components in milk (Anema, 2000; Anema et al., 2006). In particular, Anema et al. (2006) showed that increasing the concentration of non-protein soluble components in milk, especially lactose, stabilised the whey proteins against denaturation.

Whey proteins become reactive when denatured, as the change in the conformation exposes reactive side chain groups that are normally buried within the native structure (Anema, 2000; Anema, 2001). In β-lactoglobulin, the exposure of the free thiol group instigates thiol-disulfide exchange reactions involving the denatured β-lactoglobulin, denatured α-lactalbumin, κ-casein and possibly, α_{S2}-casein (Anema, 2000; Anema, 2001). Denatured β-lactoglobulin and α-lactalbumin interact both with other denatured whey proteins and with casein micelles to form aggregates via non-covalent bonds as well as thiol-disulphide exchange reactions (Anema, 2000; Anema, 2001).

2.5.3.2 Heat induced association of casein micelles and whey proteins

Heat treatment of milk leads to the association between denatured whey proteins and κ-casein at the casein micelle surface (Smits and van Brouwershaven, 1980). When denatured, whey proteins can react with each other and with the casein micelles (Elfagm and Wheelock, 1978; Smits and van Brouwershaven, 1980; Mohammad and Fox, 1987). The interactions predominantly involve thiol-disulphide interchange reactions, as well as hydrophobic interactions (Smits and van Brouwershaven, 1980). Mohammad and Fox (1987) reported that the ragged appendages on the surface of casein micelle observed under electron micrographs after heating appeared to be the β-lactoglobulin and κ-casein complexes. On the other hand, α-lactalbumin can interact with κ-casein only in the presence of β-lactoglobulin as it does not contain free sulphydryl groups to initiate the interaction by itself (Calvo et al., 1993). It was suggested that β-lactoglobulin and α-lactalbumin interacted first and then this product formed a complex with κ-casein (Elfagm and Wheelock, 1978).

Oldfield et al. (1998a) reported that the association behaviour of the whey proteins and casein micelles in skim milk was affected by heating temperature (**Figure 2-21**). During the initial stages of heating in the range 80-130°C, mainly β-lactoglobulin appeared to associate with the casein micelles (Oldfield et al., 1998a). After prolonged heating, α-lactalbumin started to associate with the casein micelles (Oldfield et al., 1998a). However, both β-lactoglobulin and α-lactalbumin associated simultaneously with the casein micelles when the heating temperature was below 80°C (Oldfield et al., 1998a).
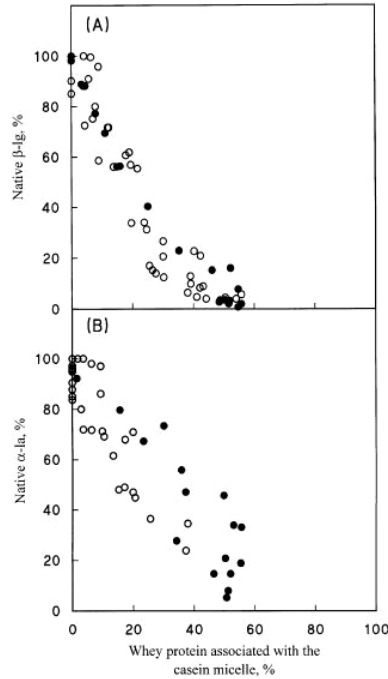
Figure 2-21: Relationship between the quantities of native β-lactoglobulin (A) and α-lactalbumin (B) and the β-lactoglobulin and α-lactalbumin that were associated with the casein micelles. Heating temperature; 95-130°C (○) and 75-90°C (●). Source: Oldfield et al. (1998a).

Anema and Li (2003a) reported that when skim milk at pH 6.55 was heated, the rate of association of the denatured whey proteins with the casein micelles was considerably slower than the rate of denaturation of the whey proteins. At the lower temperatures (75-85°C), the level of whey protein associated with the micelles increased relatively slowly throughout the heating time. At higher temperatures (90-100°C), the level of association increased rapidly during the initial period of heating and tended to plateau on prolonged heating. Also, not all the denatured whey proteins were associating with the casein micelles. They found that the association level was time and temperature dependent. The size of casein micelles was suggested to be related to the association of denatured whey proteins with the casein micelles after heating (Anema and Li, 2003a; Anema et al., 2004). At any particular temperature (75-100°C), the casein micelle size increased with longer holding time (up to 60 min),

and at any particular holding time, the casein micelle size increased with increasing temperature. The maximum increase in casein micelle size was reported to be about 30-35nm and a maximum of about 70-80% of the denatured whey proteins associated with the micelles at any given heating temperature.

In general, at the pH of milk between 6.4 and 6.8 approximately, it was reported that the level of association of denatured whey proteins with the casein micelles increased as the pH was decreased (Corredig and Dalgleish, 1996; Oldfield et al., 2000; Anema and Li, 2003b). For example, the level whey proteins associated with the casein micelles in heated milk increased from approximately 32% when skim milk was at pH 6.7 to approximately 70% when skim milk was at pH 6.5 (Anema and Li, 2003a). The higher level of whey proteins associated with casein micelles at the lower pH is not due to higher levels of denatured whey proteins available for association (Anema and Li, 2003a). Anema and Li (2003b) demonstrated that denaturation of α-lactalbumin and β-lactoglobulin was not affected by pH range studied (pH 6.5-6.7) at 80-100°C. It has been suggested that this change in association behaviour of whey proteins and casein micelles is related to dissociation of κ-casein from the casein micelles which was found to increase as the pH of the milk was increased from pH 6.5 to 7.1 (Anema and Li, 2003a; Anema, 2007). The denatured whey proteins preferentially interacted with the dissociated κ-casein. Hence, less whey proteins associated with the casein micelles at higher pH where higher levels of κ-casein were dissociated (Anema, 2007).

2.5.4 Effects of high pressure processing on skim milk

When milk is subjected to a high pressure process, a number of changes can occur. High pressure can affect protein conformation and can lead to denaturation and/or aggregation of the whey proteins in milk (Huppertz et al., 2004b; Anema et al.,

2005b; Anema et al., 2005c). The mineral equilibrium in milk may also be affected as the CCP of casein micelles is solubilised by pressure (López-Fandiño, 2006). The effects of pressure on whey protein denaturation and on casein micelles structures are discussed in this section as milk proteins (whey proteins and casein micelles) are one of the major components in skim milk.

2.5.4.1 Effect of pressure on whey proteins

Once the treatment pressure reaches a critical level, whey protein denaturation occurs and the degree of pressure-induced whey protein denaturation increases as the treatment pressure increases or treatment duration at a given pressure increases (Lopez-Fandino et al., 1996; Huppertz et al., 2004b). Pressure-induced effects in protein may be attributed to the penetration of water into the protein structure and this results in unfolding of protein structures and disruption of hydrophobic bonds that maintain the protein conformations (Balny et al., 2002). Different whey proteins have different degrees of pressure resistance to denaturation. Denaturation of β-lactoglobulin starts at pressure treatments above about 100 MPa (Lopez-Fandino et al., 1996; Lopez-Fandino and Olano, 1998; Huppertz et al., 2004b), while α-lactalbumin is resistant to pressures up to 400 MPa (Lopez-Fandino et al., 1996; Garcia-Risco et al., 2000). Denaturation of β-lactoglobulin reached almost 100% after treatment at 600 MPa for 30 min while that of α-lactalbumin reached ~72% denaturation after 30 min at 800 MPa (Huppertz et al., 2004b). Bovine serum albumin is resistant to pressure treatment up to 400 MPa (Lopez-Fandino et al., 1996).

The degree of pressure-induced denaturation of whey proteins depends on the pressure, temperature at pressurisation and treatment duration (Huppertz et al., 2004b; Anema et al., 2005c; Hinrichs and Rademacher, 2005). **Figure 2-22** shows the effect of these treatment conditions on two genetic types (A and B) of β-lactoglobulin.

Above the threshold pressure (>100MPa), the level of native β-lactoglobulin decreased, indicating the denaturation of β-lactoglobulin. More β-lactoglobulin was denatured as the treatment pressure, temperature at pressurisation or the treatment duration was increased (**Figure 2-22**). The two varieties of β-lactoglobulin behaves similarly. Also, Lopez-Fandino and Olano (1998) showed that the denaturation level of α-lactalbumin increased as the temperature at treatment increased.
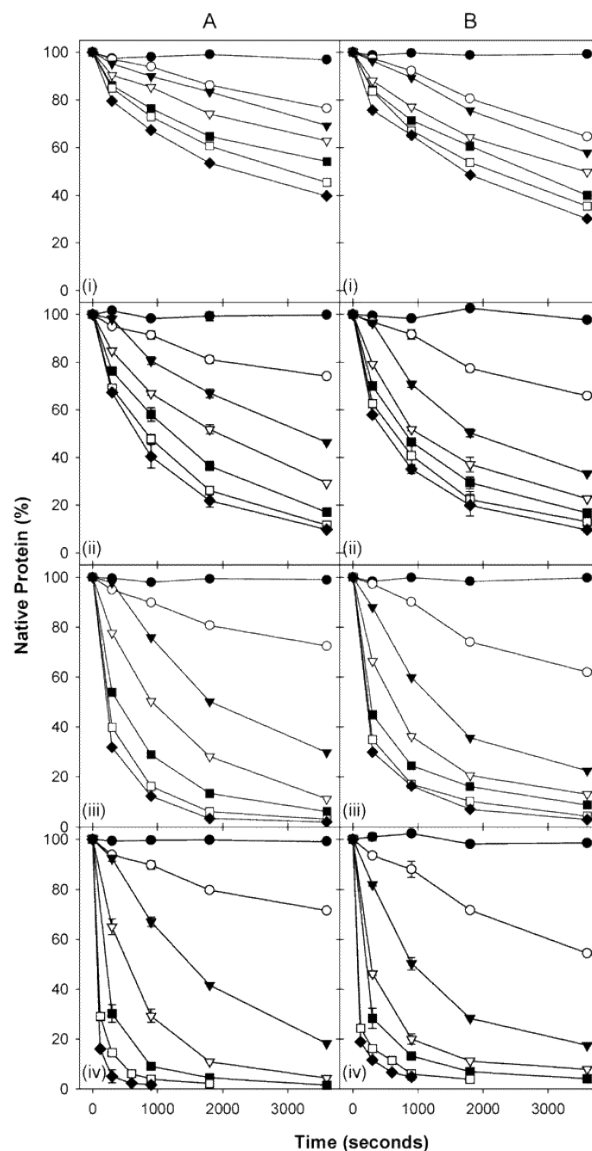
Figure 2-22: (A) Denaturation of β-lactoglobulin A and (B) Denaturation of β-lactoglobulin B in skim milk. (i): 10° C; (ii): 20° C; (iii) 30° C; and (iv): 40° C. ●: 100 MPa; ○: 200 MPa; ▼: 250 MPa; ▽: 300 MPa; ■: 400 MPa; □ : 500 MPa; and ◆ : 600 MPa. The level of native β- lactoglobulin in samples was determined using native polyacrylamide gel electrophoresis. Source: Anema et al. (2005c).

In addition, the pH of milk affects the degree of denaturation of whey proteins by pressure. Huppertz et al. (2004b) reported that adjusting the pH of milk to 6.2 before pressure treatment at 250-600 MPa reduced the extent of denaturation of α-lactalbumin and β-lactoglobulin compared to milk at pH 6.7. However, the extent of denaturation of both whey proteins increased when the pH of milk was adjusted to 7.0 compared to milk at pH 6.7 (Huppertz et al., 2004b). This is similar to the effect of pH on the thermal denaturation of whey proteins (Law and Leaver, 2000).

2.5.4.2 Casein micelles

The effects of pressure treatment on the size of casein micelles in skim milk is dependent on the treatment pressure, temperature at pressurisation and duration of the pressure treatment (**Figure 2-23**). The size of casein micelles is not affected by treatment at pressures below about 200 MPa when the temperature at treatment was at or below 20°C (**Figure 2-23**). At pressures of 200 MPa or above, a number of studies showed that casein micelles start to disintegrate into smaller particles. Needs et al. (2000) showed that pressure treatment of raw milk at 200 MPa for 15 min caused partial disintegration of casein micelles. Similarly, Huppertz et al. (2004b) showed that pressure treatment at 250 MPa for 5 min and at 20°C reduced casein micelle size and Schrader and Buchheim (1998) showed that casein micelles are disintegrated into smaller aggregates in pasteurised skim milk pressurised at 300 MPa for 5 min at room temperature.

However, at 200-300 MPa, the size of casein micelles increases with prolonged pressure treatments (**Figure 2-23**; Anema et al., 2005b; Considine et al., 2007, Huppertz et al., 2004b). Schrader and Buchheim (1998) also observed a aggregation of casein micelles and sub-micelles occurring in skim milk pressurised at 300 MPa, 20°C for 20 min. The aggregation was found to be dependent on the

treatment pressure, temperature at pressurisation and duration of the pressure treatment (**Figure 2-23**; Anema et al. 2005b; Huppertz et al., 2004b). Huppertz et al. (2004b) also found that treatment for 10 to 60 min at 250 MPa, 20°C, progressively increased micelle size by up to a maximum of approximately 30% compared to in untreated milk after 40 min.
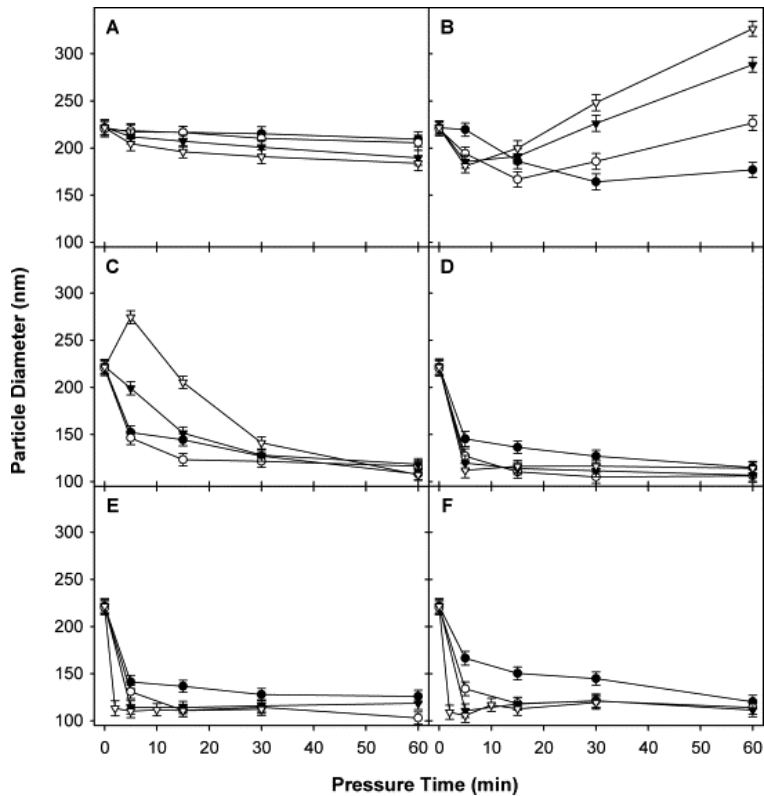
Figure 2-23: Casein micelle size following pressure treatment at 100-600 MPa from 0 to 60 min at 10-40 °C. (A) 100 MPa. (B) 200 MPa. (C) 300 MPa. (D) 400 MPa. (E) 500 MPa. (F) 600 MPa. ●, 10°C; ○, 20°C; ▼, 30°C; ▽, 40°C. Error bars represent the standard deviation of repeated measurements. Source: Anema et al. (2005b).

It has been shown that whey proteins are not involved in this aggregation of casein micelles occurring at pressure treatment between 200-300 MPa (**Figure 2-23**). The particle size variation of casein micelles in skim milk was similar to that in whey-protein-depleted skim milk (Anema et al., 2005b) or native phosphocaseinate suspensions (Gaucheron et al., 1997) where whey proteins were absent. Also, heating

of milk (90°C for 10 min) prior to pressure treatment had no effect on the changes of average casein micelle size on pressure treatment indicating that heat-induced interactions with β-lactoglobulin did not influence casein micelle size after pressure treatment (Huppertz et al., 2004c). The caseins in milk exist as large colloidal aggregates called casein micelles and CCP and hydrophobic interactions have been known to play an important role in maintaining micelle integrity (Walstra, 1990; Horne, 1998). Disruption of casein micelles due to pressure treatment (≥200 MPa) may involve dissolution of the CCP (Schrader et al., 1997). It has been suggested that the degree of hydrophobic interactions may not change at pressure treatments between 200-300 MPa (Anema et al., 2005b). Under these pressures, hydrophobic bonds between disrupted casein micelles may be involved in the aggregation of casein micelles during prolonged pressure treatment or at pressure treatment at higher temperatures (Huppertz et al., 2004a; Anema et al., 2005b).

Pressure treatments between 400 and 600 MPa results in the disintegration of casein micelles with the size of the micelles decreasing markedly (**Figure 2-23**; Needs et al. 2000; Anema et al. 2005b; Huppertz et al., 2004b; Gaucheron et al., 1997). Needs et al. (2000) suggested that disruption of hydrophobic interactions and solubilisation of CCP contributed to the disintegration of the micelles. This change in micelle structure after pressure treatment is presumably related to the concomitant increase in the translucence of skim milk that has been reported by a number of researchers (Johnston et al., 1992; Gaucheron et al., 1997; Huppertz et al., 2004c; Orlien et al., 2006). In contrast to pressure-treated milk, heat-treated skim milk samples appear whiter than the untreated sample (**Figure 2-24**). Pressure-treated skim milk maintains the translucent appearance for several days when stored at about 5°C.

However, the appearance of pressure-treated skim milk becomes gradually more opaque when stored at room temperature (Considine et al., 2007).
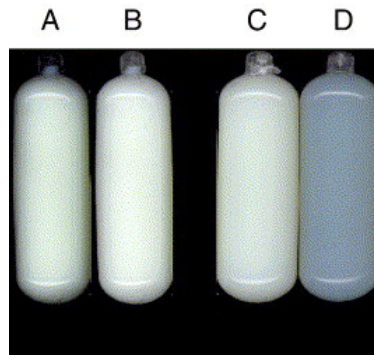
Figure 2-24: Changes in the appearance of milk on heat treatment and pressure treatment. A: untreated milk; B: heated milk (100 °C/3 min); C: untreated milk; D: high-pressure-treated milk (600 MPa/30 min). Source: Considine et al. (2007).

2.5.4.3 Pressure-induced association of casein micelles and whey proteins

It has been suggested that denatured whey proteins associate with casein micelles during pressure treatment of milk (Huppertz et al., 2004b; Patel et al., 2006). However, there is only limited information available in the literature regarding the pressure-induced association of casein micelles and whey proteins. Individual whey proteins have different denaturation sensitivity towards heat treatment and pressure treatment and this may result in different protein aggregates (Considine et al., 2007). Patel et al. (2006) reported that protein aggregates formed in milk by pressure treatment were smaller than those formed by heat treatment. Associations via disulphide bonds such as those between β-lactoglobulin and κ-casein occur at relatively mild pressure treatment (200 MPa, for 30 min; Patel et al., 2006). Huppertz et al. (2004b) showed that after pressure treatment at 250 MPa for 5 min, the level of sedimentable denatured β-lactoglobulin (presumably associated with casein micelles) was about 15% of the native β-lactoglobulin existed in untreated milk and the level increased to approximately 68% when the treatment duration increased to 60 min. At

the higher pressures (>400 MPa), β-lactoglobulin can also be associated with α_{S1}-casein (Patel et al., 2006).

2.5.5 Acid gelation of milk

2.5.5.1 Effect of heat or pressure treatment on the properties of acid milk gel systems

Acid milk gels are particle gels that are formed by aggregation of milk proteins when milk is acidified, as the negative charge on the casein micelles are neutralised and steric repulsion between casein micelles is diminished (Lucey and Singh, 1998; Horne, 1999). This gel-forming characteristic of milk proteins is the fundamental basis for making dairy products such as yoghurt and some types of cheeses. This study intends to use the acid milk gel as a model dairy application to examine the effects of pressure treatment on skim milk and starch mixed systems and compare the results with those from conventional heat treatment.

The acid milk gels made from heat-treated milk has been reported to be firmer than that made from untreated milk (**Figure 2-25**; Lucey and Singh 1998; Anema et al. 2004; Anema et al. 2005a). When milk is heated, whey proteins denature and interact with casein micelles to form milk protein aggregates (Corredig and Dalgleish, 1999; Anema and Li, 2003a). It has been shown that as the level of denatured whey proteins in milk increases, the firmness of the acid milk gel increases (Dannenberg and Kessler, 1988b; Anema et al., 2004). The incorporation of whey proteins in the acid milk gels is assumed to produce a more inter-connected structure with a higher protein concentration (Lucey and Singh, 1998; Anema et al., 2004) and lowers the syneresis of the acid milk gels (Dannenberg and Kessler, 1988c). In addition, denatured whey proteins associated with casein micelles contribute to the increased pH at gelation found in acid gelation of heated milk compared to that of unheated milk (Lucey and

Singh, 1998). The isoelectric pHs of whey proteins are higher than that of casein (pH 4.6) and there are increased interactions between whey proteins near their isoelectric pHs due to the exposed hydrophobic groups after heating (Zhu and Damodaran, 1994; Lucey and Singh, 1998). For example, the isoelectric pH of β-lactoglobulin is ~5.3 (Kinsella and Whitehead, 1989). **Figure 2-26** shows the protein networks of acid milk gels made from unheated and heated milk as observed by confocal microscopy. The protein network in the acid milk gel made from heated milk appears to be denser and more cross-linked than that made from unheated milk (**Figure 2-26**).
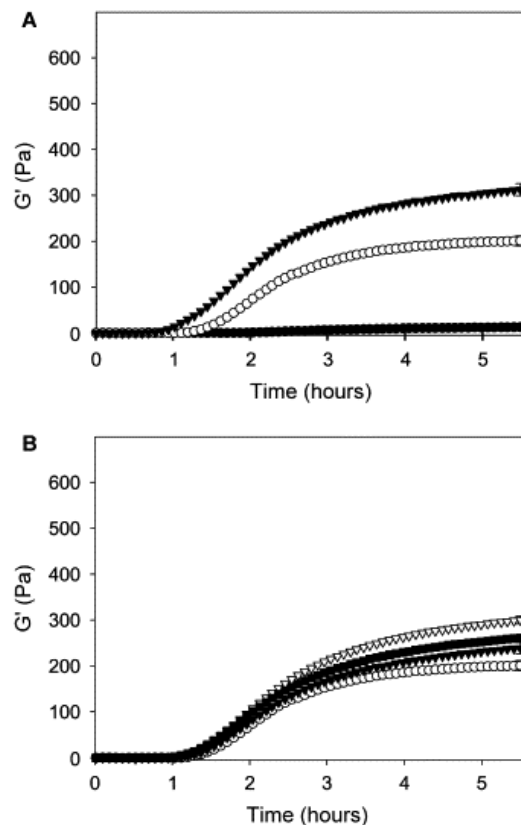
Figure 2-25: (A) Changes in storage modulus, G', with time after glucono-δ-lactone (GDL) addition for unheated and heated skim milk samples: ●, untreated skim milk; ○, skim milk heated at 80 °C for 2 min; ▼, skim milk heated at 90 °C for 15 min. (B) Changes in storage modulus, G', with time after pressure treatment: ○, skim milk heated at 80 °C for 2 min; ▼, skim milk heated at 80 °C for 2 min and pressurised to 400 MPa with 0 min holding time; ▽, skim milk pressurised to 400 MPa with 60 min holding time; ■, skim milk pressurised to 400 MPa with 120 min holding time. Source: Anema et al. (2005a).
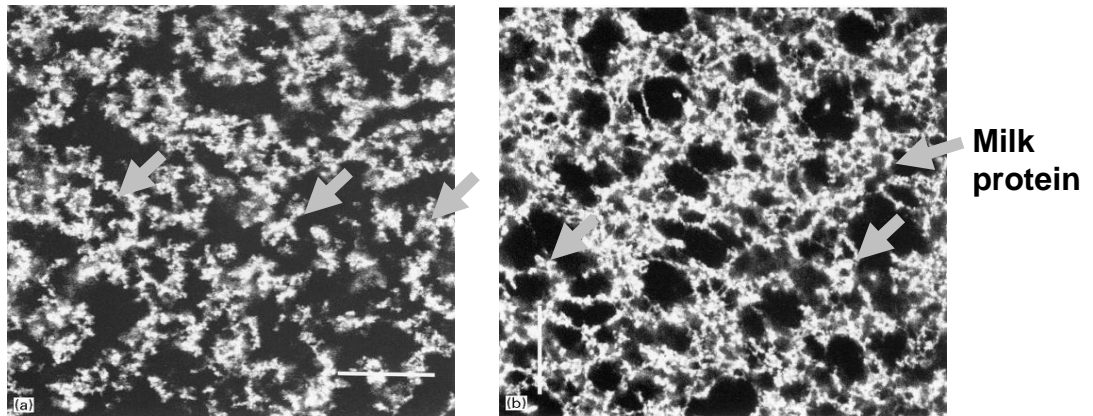
Figure 2-26: Confocal scanning laser micrographs of acid milk gels made at 30°C by acidification with 1.3% (GDL) from unheated milk (a) and heated milk (b). Heat treatment was 80°C for 30 min. Source: Lucey et al. (1999).

The acid milk gels made from pressure treated milk were firmer than that made from untreated milk (Anema et al., 2005a) and more resistant to syneresis (Johnston et al., 1993). However, the firmness of acid milk gels made from pressure-treated milk was lower than those made from heat-treated milk (**Figure 2-25**). Whey proteins can be denatured in milk by pressure treatment (Huppertz et al., 2004b). It has been suggested that the increased gel firmness of the acid milk gels made from pressure-treated milk is due to the effect of denatured whey proteins on the subsequent acid gelation of milk via a similar mechanism to that revealed in the acid gelation of heat-treated milk (Considine et al., 2007).

2.6   Conclusions

Thermal gelatinisation of starch has been reported extensively in the literature; however, there is still limited information available for pressure-induced starch gelatinisation. The application of high pressure technology in starch-containing food products requires more detailed information regarding physical and rheological

properties of starch after pressure treatment. Moreover, the differences in pressure-induced gelatinisation characteristics of different types of starch require further understanding, which may also provide a basis for choosing starches in different product applications. Since starch can be used in a number of different food products it is also vital to understand starch gelatinisation in different environments as well as in a simple starch-in-water suspension. For dairy systems, there have been a number of studies showing the effects of pressure treatment on milk components. However, there is lack of knowledge concerning the effect of pressure treatment on mixed systems such as starch and milk. Therefore, as well as a need for more information on the pressure-induced starch gelatinisation, there is a need for understanding the effect of dairy components on the pressure-induced starch gelatinisation.

2.7   References

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CHAPTER 3

Materials and Methods

3.1   Materials

Normal corn, waxy corn and potato starches were obtained from Penford New Zealand Limited (Auckland, New Zealand). Unmodified normal rice starch and waxy rice starch were obtained from Remy Industries (Leuven-Wijgmaal, Belgium). Tapioca starch was obtained from National Starch Food Innovation (Auckland, New Zealand). All starches were used as supplied and stored in air-tight containers. 'Low heat' skim milk powder was obtained from the Edendale site, Fonterra Co-operative Group Limited, New Zealand. The skim milk powder was composed of 33% protein, 54% lactose, 3.8% moisture, 0.8% fat and 8.4% ash.

Table 3-1: Typical composition of starches. Source: Penford New Zealand Limited, Remy Industries and National Starch Food Innovation.

Starch type	Moisture	Protein	Fat	Ash
Normal corn	11-14%	0.5% max.	0.8%	0.3%
Waxy corn	11-14%	0.5% max.	0%	0.1%
Potato	20% max.	0.05%	0%	0.3%
Normal rice	12%	0.13%	0.09%	0.06%
Waxy rice	11%	0.07%	0.06%	0.08%
Tapioca	15% max.	0.25% max.	0.15% max.	0.15% max.

3.2   Sample preparation

3.2.1 Reconstituted skim milk

Reconstituted skim milk samples were prepared by adding low heat skim milk powder to purified water (reverse osmosis followed by filtration through a Milli-Q apparatus) to a final concentration of 5–15% (w/w) total solids. Skim milk with 10% (w/w) total solids was considered the standard reconstituted skim milk with composition and concentration similar to fresh skim milk. The reconstituted skim milk samples were stirred with a magnetic bar at ambient temperature (approximately

20 °C) for at least 10 h before use to ensure complete equilibration (Anema and Li, 2003). A small amount of sodium azide (0.02% (w/v)) was added to all the milk samples as a preservative.

3.2.2 Simulated milk ultra-filtrate (SMUF)

Simulated milk ultrafiltrate (SMUF) was prepared based on the method of Jenness and Koops (1962) with some modifications. SMUF is a solution which simulates the composition of the soluble minerals in milk. To prevent precipitation of minerals, two separate aqueous stock solutions were prepared which contained the ingredients listed in **Table 3-1**. The ingredients used were obtained from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions were stored at 4 °C for up to 2 weeks until they were needed.

Table 3-2: Compositions of stock solutions 1 and 2 used for SMUF.

Stock solution 1 (500 mL)	
KH_2PO_4	7.90g
K_3citrate.H_2O	6.00g
Na_3citrate.$2H_2O$	8.96g
KCl	3.00g
Milli-Q water	to 500 mL
Stock solution 2 (500 mL)	
$CaCl_2.2H_2O$	6.60g
$MgCl_2.6H_2O$	3.25g
Milli-Q water	to 500 mL

To make 1 L of SMUF, 100 mL of stock solution 1 was added to 750 mL of Milli-Q water and 100 mL stock solution 2 was then added slowly with stirring. After thorough mixing of the stock solutions and water, 1M KOH was added slowly to adjust the pH to 6.6 and Milli-Q water was added to bring the total volume to 1 L. When required, lactose (Sigma-Aldrich, St. Louis, MO, USA) was added to

appropriate SMUF samples at 5% w/w which is a similar concentration to that found in 10% total solids skim milk.

3.2.3 Whey protein- and lactose- depleted (WPLD) skim milk

Whey protein- and lactose-depleted (WPLD) skim milk was prepared by filtering reconstituted 10% total solids skim milk through a polyethersulfone microfiltration membrane (Vivaflow 200, 0.2 μm pore size, Sartorius Stedim Biotech, Aubagne, France) connected to a peristaltic pump and flexible plastic tubes. The active membrane area was 200 cm^2 and the operating pressure was 2 bar. The whey proteins and lactose were removed in the permeate and the retentate was recirculated back to the feed. SMUF was constantly added back to the skim milk, replenishing the feed volume as the permeate was removed. The volume of SMUF used was approximately six-times that of skim milk. The average flowrate with this regime was 400 mL/h.

The WPLD milk was used as a suspension medium that contained casein micelles and soluble milk minerals equivalent to their concentrations in 10% total solids skim milk. The WPLD milk contained < 10% of α-lactalbumin and < 20% of β-lactoglobulin that were present in the initial 10% TS skim milk while the casein content remained unchanged. Lactose was added to appropriate WPLD milk samples at 5% w/w, which is a similar concentration to that found in 10% total solids skim milk. Polyacrylamide gel electrophoresis (PAGE) as described in Anema and McKenna (1996) was used to determine the residual whey protein content in the WPLD milk.

3.2.4 Preparation of starch suspensions

Starch suspensions were prepared by adding the required amount of starch to the appropriate suspension medium (water, reconstituted skim milk, SMUF, SMUF

with lactose, WPLD milk or WPLD milk with lactose) at the required concentration (w/w). The suspensions were stirred with a magnetic bar at ambient temperature until the starch was completely dispersed. For pressure or static heat treatments, the samples were transferred to polycarbonate ultracentrifuge tubes (13.5 or 5.1 mL capacity, Denville Scientific Inc., Metuchen, NJ, USA) and the tubes were heat sealed. For heat treatment using a Rapid-viscoanalyser (RVA), 30 mL of sample was transferred to an aluminium RVA cup.

3.2.5 pH adjustment for acid gelation samples

The pH of the milk samples was adjusted from the natural condition (pH 6.64) to a pH value in the range from 6.5 to 7.1 by the slow addition of hydrochloric acid (1 M) or sodium hydroxide (1 M) while stirring the milk. Starch was added to the pH-adjusted skim milk at the required level. After pressure treatment and decompression, or heat treatment and cooling of the sample, hydrochloric acid (1 M) or sodium hydroxide (1M) was slowly added at room temperature (20 °C) with stirring, to readjust the pH back to the natural pH (~pH 6.64) of the milk prior to acidification. The pH of samples was measured by a pH meter (Radiometer PHM92 LAB pH meter, Radiometer, Copenhagen, Demark) with an electrode (Schott-Gerate N61 electrode, Schott-Gerate GmbH, Hofheim, Germany).

3.3   Pressure treatment of samples

Pressure treatments of samples prepared as per section 3.2.4 were carried out using a laboratory-scale high-pressure unit (Food-Lab, model S-FL-065-200-9-W for Chapter 4 or, model S-FL-850-9-W for Chapter 5, 6 and 7, Stansted Fluid Power Ltd., Stansted, Essex, UK) as shown in **Figure 3-1A.** The unit contains a cylindrical high-pressure chamber (65 mm $\times$ 220 mm) which holds a sample canister of 30 mL capacity (**Figure 3-1B**). In both types of high-pressure units, the chamber is filled

with a pressure-transmitting fluid which is an emulsion of 10% vegetable oil in water with 0.18% of Tween 80, 0.013% of Span 60, and 0.0065% of potassium sorbate added (Sigma-Aldrich, St. Louis, MO, USA). Pressure is generated by displacement of the high pressure plunger into the chamber and the plunger is retracted to release pressure (**Figure 3-1B**). The temperature in the high-pressure chamber is controlled by circulating water through a water jacket which encloses the shell part of the chamber. However, this temperature control does not instantaneously offset the adiabatic heating or cooling during a pressure treatment. The pressure inside the chamber and the temperature of the pressure-transmitting fluid in the chamber during pressure treatments were recorded. These are assumed to be the pressure and temperature of the samples in the chamber during pressure treatments.

(A)   **(B)**
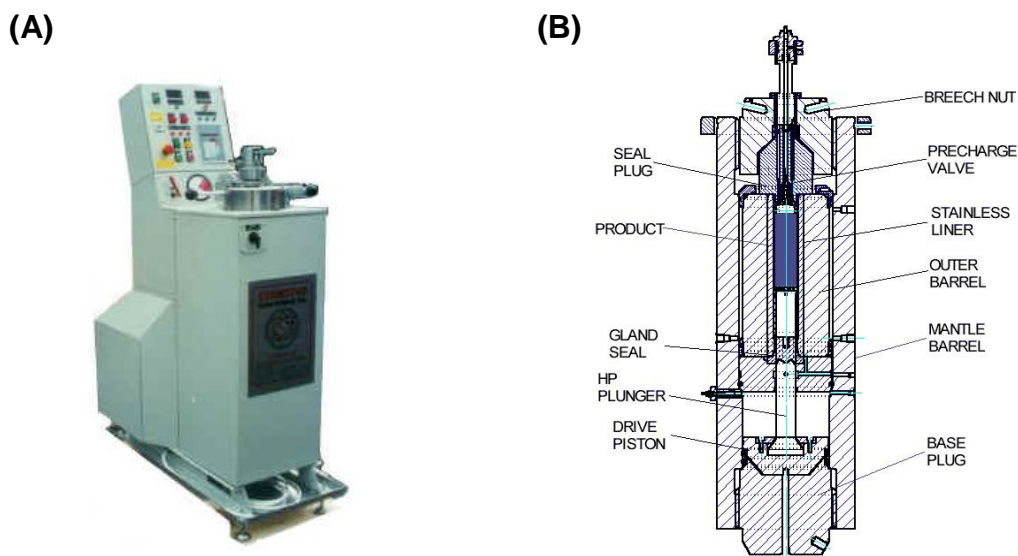
Figure 3-1: (A) High pressure unit (Stansted Fluid Power Ltd.), (B) Inside the high pressure unit.

To carry out a pressure treatment, sample tubes were loaded into the canister and the interlock was closed. The pressure cycle was started. In the first step of a cycle the pressure chamber was pre-charged with the pressure transmitting fluid. In the pressurisation step, the pressure in the pressure chamber is increased to the set

pressure at the rate of 4.4 MPa/s. Compression during the pressure treatment generates heat as it works against repulsive intermolecular forces and the temperature inside the pressure vessel increases. The average rate of adiabatic heating during pressurisation was 1.9-2.0 °C/100 MPa. In the second step, the unit holds the pressure for a set duration. To maintain the set pressure the unit automatically checks the pressure every 45 seconds and if the pressure in the chamber is below the set pressure, the pressure level is restored by more compression. The pressure in the chamber was maintained within ±20 MPa from the set pressure. The last step is the depressurisation during which pressure is released at the rate of 9.2 MPa/s. The vessel temperature decreases in this step at 1.7-2.2 °C/100 MPa due to adiabatic cooling. A typical temperature and pressure profile of pressure treatment is shown in **Figure 3-2**.
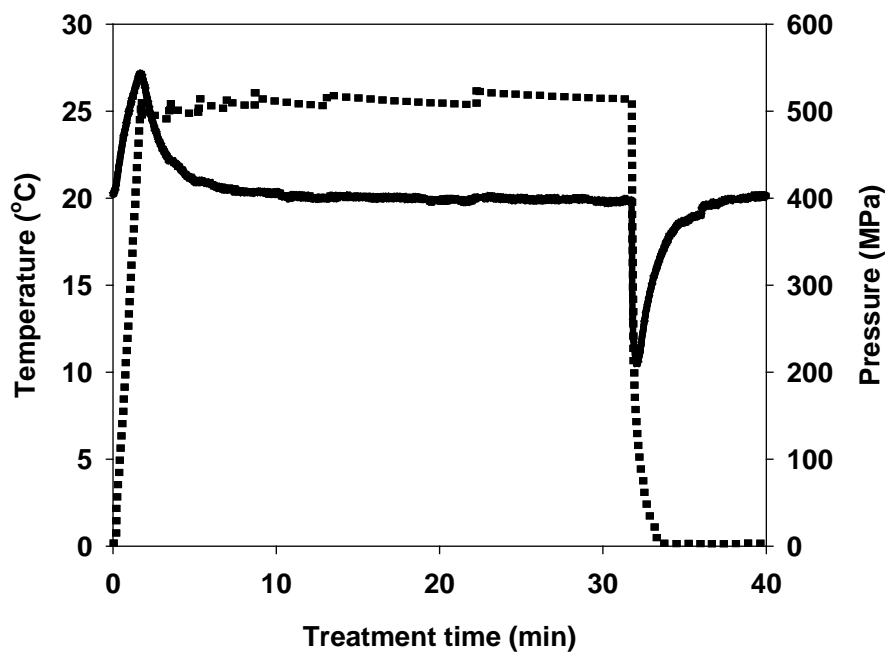
Figure 3-2: Temperature (solid line) and pressure (dotted line) profile during a typical pressure treatment at 500 MPa for 30 min at 20 °C.

Samples were treated at pressures up to 700 MPa combined with temperatures between 10 and 60 °C for up to 30 min. Water was circulated in the water-jacket

around the high-pressure chamber to achieve the desired temperature at treatment. Samples were equilibrated to the temperature at treatment in a water-bath for 20 min before the treatment. To minimise sedimentation of starch during treatment, sample tubes were shaken before loading them into the high pressure unit. After depressurisation, sample tubes were removed from the high pressure unit. The contents of sample tubes were transferred into a 50 mL plastic container with lid for storage at 20 °C for approximately 10 h before analyses. Any sediment found in the sample was mixed thoroughly by hand using a metal spatula to ensure sample homogeneity.

3.4 Heat treatment of samples

3.4.1 Samples for characterising gelatinisation of starch under static heating conditions

Heat treatment of the samples prepared as per section 3.2.4 was conducted at temperatures between 55 and 90 ± 1 °C. Samples were placed in a water-bath set to the required treatment temperature. Once the temperature of the sample reached the treatment temperature, the sample was held in the water-bath for 30 min. The maximum time to reach the required temperature was 4 min. Samples were not mixed or sheared during the treatment to be consistent with the static conditions used during the pressure treated starch counterparts. After the treatment, the samples were transferred into a different water-bath at 10 °C to cool down to 20 °C in less than 6 min. The contents of sample tubes were transferred into a 50 mL plastic container with lid for storage at 20 °C for at least 10 hours before analyses. Any sediment found in the sample was mixed thoroughly by hand using a metal spatula to ensure sample homogeneity.

3.4.2 Samples for acid gelation under stirred heating conditions

A Rapid Visco Analyser (RVA, Newport Scientific, Warriewood, New South Wales, Australia) was used to heat the samples for acid gelation that were prepared as per section 3.2.4 (**Figure 3-3A**). An aluminium RVA cup was filled with 30 mL of sample. The cup and a stirring paddle were installed to the RVA and the sample was heated with continuous stirring at 200 rev/min. The temperature of the sample was increased to 80±1 °C at a rate of 20 °C/min, held at 80 °C for 30 min and then cooled to 20 °C at a rate of 13 °C/min (**Figure 3-3B**).
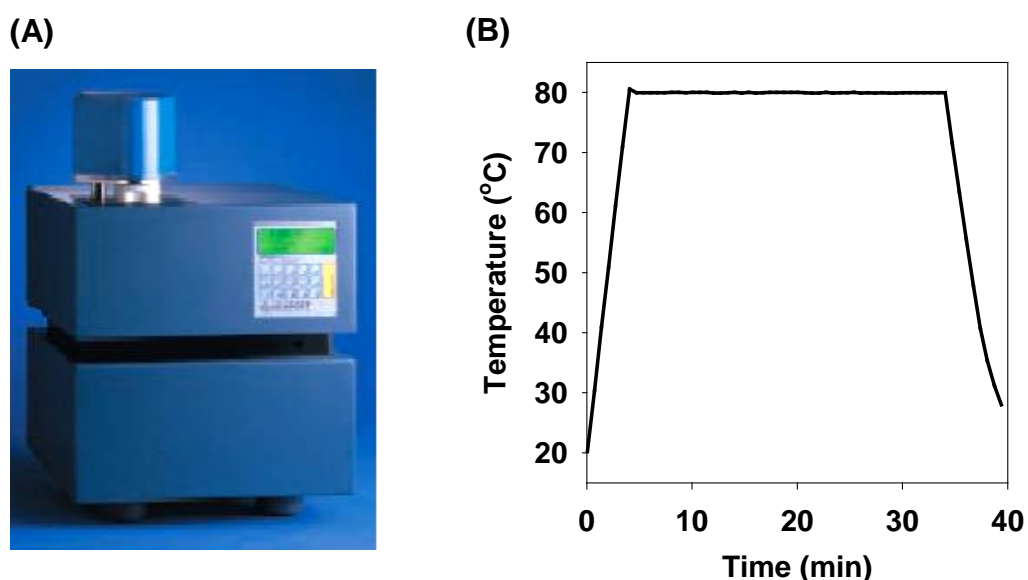
Figure 3-3: (A) Rapid visco-analyser (RVA), New Port Scientific, (B) RVA heating profile.

3.5   Rheological analyses of samples

3.5.1 Initial apparent viscosity and pasting curves of starch suspensions

A stress-controlled rheometer, the Physica UDS200 (Anton Paar GmbH, Graz, Austria) equipped with a starch cell and stirrer arrangement (C-ETD 160/ST) was used to measure initial apparent viscosity and construct pasting curves (**Figure 3-4A**). Several parameters, which provide information about gelatinization characteristics,

can be extracted from a pasting curve. The initial apparent viscosity ($\eta_{initial}$) was defined as the viscosity at 20 °C before pasting begins. The onset temperature, 'T_{onset}', is the temperature at which the viscosity starts to increase, the peak viscosity, 'η_{peak}', is the maximum viscosity attained and 'T_{peak}' is the temperature at the peak viscosity.

The starch cell was filled with 22 mL of sample and the stirrer was lowered into the operating position in the sample. The contents were stirred at 100 rev/min for 1 min at 20 °C to thoroughly mix the sample and the $\eta_{initial}$ was measured. For pasting of the sample, the stirring continued at 100 rev/min and the temperature was increased from 20 to 95 °C at a constant rate of 2 °C/min while measuring the apparent viscosity at 30 seconds intervals. The procedure is illustrated in **Figure 3-4B**. The apparent viscosity was plotted against temperature as a pasting curve.
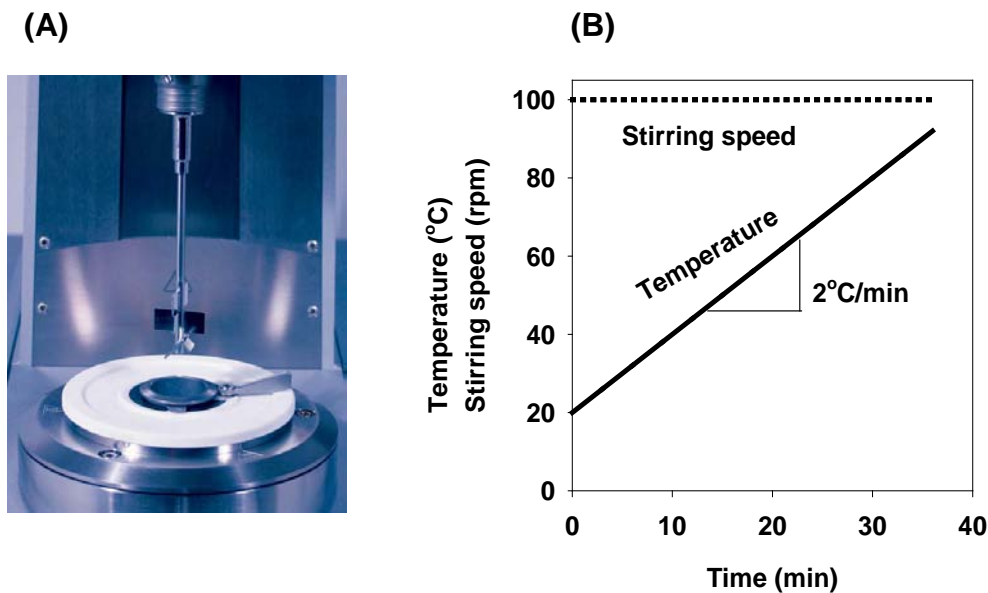
Figure 3-4: (A) Starch cell and stirrer arrangement (C-ETD 160/ST) with the Physica UDS200 (Anton Paar GmbH, Graz, Austria) used for pasting, (B) Pasting conditions used.

3.5.2 Flow properties of milk samples with added starch

The shear stress of heated milk samples was measured at a shear rate 0.1–100 s^{-1} using an AR2000 rheometer (TA Instruments, New Castle, DE, USA) and a cone and plate geometry (4 cm, 4° and 100 μm truncation). The apparent viscosity was determined at a shear rate of 100 s^{-1}.

3.5.3 Acid gelation of skim milk with added starch

The majority of food materials can be considered to exhibit a mixture of solid and liquid-like properties, i.e. viscoelasticity (Ross-Murphy, 1984). To separate these two components, a small amplitude oscillatory test is often employed using a rheometer with a set of geometry arrangement such as a cone and plate, parallel plates or, a cup and bob arrangement (Bourne, 2002). The geometry is made to oscillate about a central point with a sinusoidal angular velocity at low amplitude while the shear stress is measured (Bourne, 2002). **Figure 3-5** shows typical stress responses of various material types after an applied strain. This test is non-destructive when the amplitude of the strain is small.

An AR2000 rheometer (TA Instruments, New Castle, DE, USA) and a cone and plate geometry (4 cm, 4° and 100 μm truncation) were used to monitor rheological changes of skim milk with different levels of added starch during acid gelation. The heat-treated or pressure-treated samples of skim milk (10% total solids) with different levels of added starch were acidified using glucono-δ-lactone (GDL; Sigma-Aldrich, St. Louis, MO, USA) at a level of 2% (w/w) and at 30 °C. The GDL was added to the sample, which was stirred for 30 seconds. An aliquot of 1.2 mL was transferred to the rheometer plate, the cone was lowered into position and a cover and water trap arrangement was placed over the sample to prevent evaporation. For selected samples, 40 mL sub-samples were transferred to 50 mL plastic containers and

the pH change with time was monitored for 6 hours in a $30 \pm 1°C$ water bath. The same pH meter and electrode arrangement mentioned in section 3.2.5 was used to measured the pH of the samples.

The rheological measurements were performed at a frequency of 0.1 Hz, a constant strain of 0.5% and a constant temperature of $30 \pm 1°C$. Measurements were taken every 5 minutes for 3 hours or 6 hours of the acid gelation process. After the acid gelation, the final gel was subjected to a frequency sweep from 0.01 to 10 Hz. Once the frequency sweep had been completed, the sample was then subjected to a temperature sweep. The temperature of the sample was decreased from 30 to 5 °C at a rate of $0.9 °C \, min^{-1}$ and the rheological properties at a frequency of 0.1 Hz were monitored.

The storage modulus (G'), loss modulus (G'') and tan δ were derived from the shear stress and shear strain. The stress component in phase with the shear strain is defined as the storage modulus (G') which is the ratio of the stress in phase with the strain, to the strain (**Equation 3-1**; Bourne, 2002). The stress component 90° out of phase with the shear strain is defined as the loss modulus (G'') which is the ratio of the shear stress out of phase with the strain, to the strain (**Equation 3-2**; Bourne, 2002). Tan δ is the ratio of G' to G'' (**Equation 3-3**; Bourne, 2002). If an oscillation strain is applied to a perfectly elastic material, the resulting stress will be exactly in phase with the strain (**Figure 3-5**; Ross-Murphy 1984). For a purely viscous liquid the stress will be exactly 90° out of phase with the applied strain because it will have its maximum when the rate of change of strain with time is at its maximum value (**Figure 3-5**; Ross-Murphy 1984).
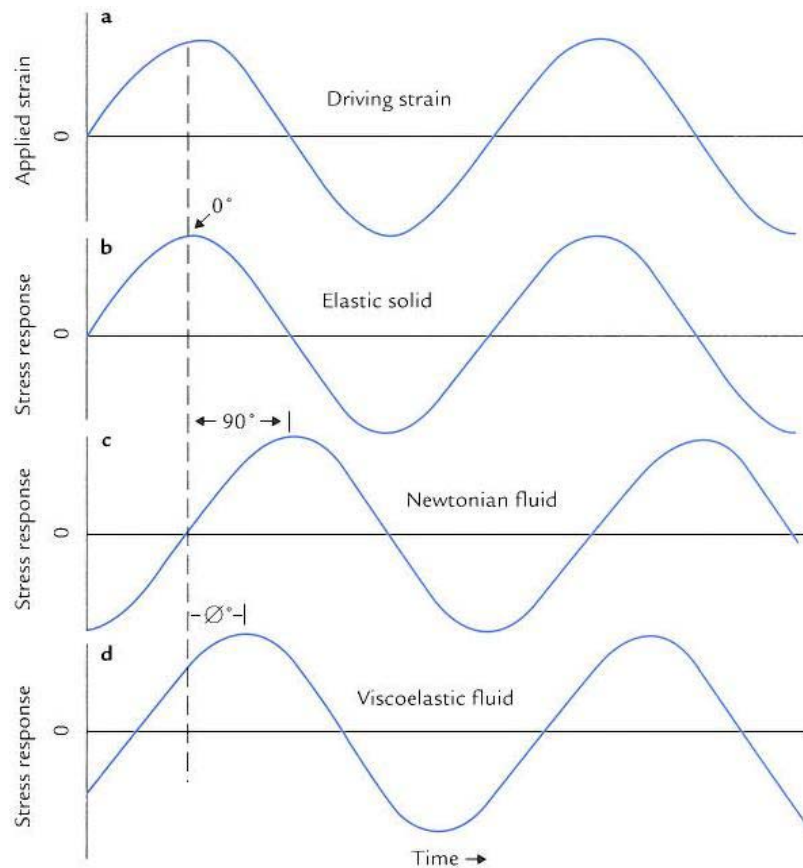
Figure 3-5: The principle of oscillation rheology. Applied strain versus time on various types of materials. $\varnothing°$ indicates the degree out of phase. Source: Bourne (2002).

$$G' = \frac{\sigma'}{\gamma} \qquad \text{………..} \quad \textbf{Equation 3-1}$$

σ' = shear stress in phase

$$G'' = \frac{\sigma''}{\gamma} \qquad \text{………..} \quad \textbf{Equation 3-2} \quad \sigma'' = \text{shear stress } 90° \text{ out of phase}$$

γ = strain

$$\tan \delta = \frac{G''}{G'} \qquad \text{………..} \quad \textbf{Equation 3-3}$$

Separate samples were prepared for a strain sweep. After 3 hours of gelation at 30 °C, the temperature was dropped to 5 °C and a strain sweep was performed by increasing the strain from 0.5 to 300% to measure the breaking strain and breaking stress of the acid gel. A typical strain sweep curve is shown in **Figure 3-6**. As the

strain increases, the stress increases to a maximum and then decreases as the gel breaks. The maximum stress in the curve represents the breaking stress of the gel and the corresponding strain at this point is the breaking strain.
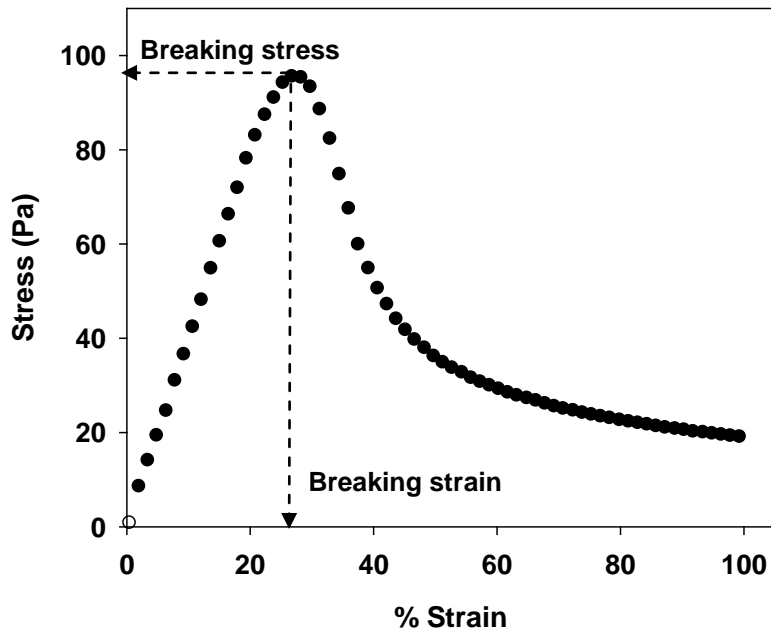
Figure 3-6: Typical strain sweep curve of acid milk gels.

3.6 Degree of swelling of starch suspensions

The degree of swelling of the starch granules was measured using a simple centrifugation technique, modified from that developed by Hemar and Horne (1998). A sample of a starch suspension was transferred into 75 mm long glass capillary tubes, leaving about 10 mm of the tube void so that the sample was not overheated when sealing one end of the tube with a bunsen flame. The sealed tubes were loaded into a Haemofuge centrifuge (Heraeus-Christ, Hanau, Germany) with the sealed ends to the outer rim, and centrifuged at 12 000 rev/min for 10 min at ambient temperature. The centrifuged tubes were scanned to obtain magnified images using a scanner (hp Scanjet 5590, Hewlett-Packard Development Company, USA). **Figure 3-7** shows the reference points to determine the height of centrifuged sediment and total sample.
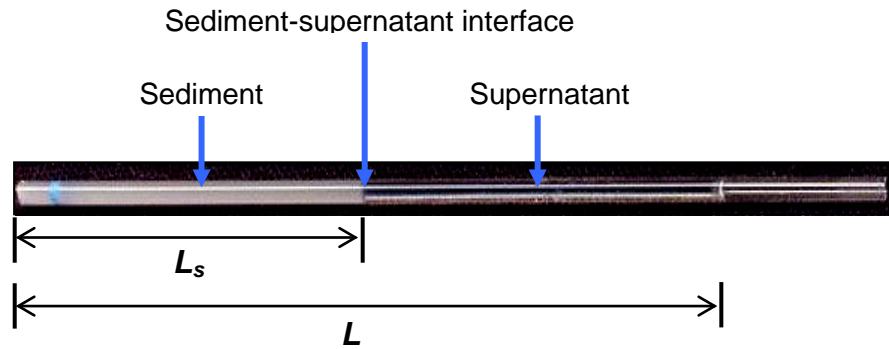
Figure 3-7: Reference points used for degree of swelling.

The degree of swelling was essentially a volume ratio of centrifuged sediment over the total volume of sample. **Equation 3-4** was used to calculate the degree of swelling:

$$Degree\ of\ swelling\ (\%) = \frac{L_s}{L} \times 100 \qquad \textbf{Equation 3-4}$$

3.7 Microscopy of samples

3.7.1 Light microscopy of starch suspensions

An aliquot of a starch suspension sample was put onto a glass slide and a cover slip was placed on top of the sample. The perimeter of the cover slip was sealed with nail polish to prevent dehydration of the sample during microscopic examination. A polarising light microscope (Nikon Eclipse E600 Pol, Nikon Corporation, Tokyo, Japan) with a 50× or 20× objective was used to observe birefringence of the starch granules. The microscope was also used without the polarising filter to observe the appearance of starch granules in the sample.

3.7.2 Confocal laser scanning microscopy (CLSM) of acid milk gels

The heat- or pressure-treated milk samples were mixed with 2% (w/w) GDL, and Fast Green CFC dye (Merck, Darmstadt, Germany) was added to stain the protein

in the sample. A small aliquot of the sample was then transferred to a concave glass slide and a cover slip was placed on top. The perimeter of the cover slip was sealed with nail polish and the prepared slide was placed on water-saturated tissue papers in a plastic container with lid to prevent dehydration of sample until the sample was ready for microscopic examination. The container was kept at 30 °C for 6 h which allows the pH of the sample to decrease to approximately pH 4.2, thus forming an acid gel.

Confocal scanning laser microscopy (CSLM) was performed on a Leica TCS 4D confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a 100 mm oil immersion objective. The microscope was used in a fluorescent mode. The excitation using an air-cooled Ar/Kr laser was performed at 488 nm.

3.8   Assay of leached amylose and starch

3.8.1 Separation of solution phase

The solution phase was separated from the starch suspension by centrifugation. A sub-sample of the starch suspension sample (8 g) was transferred into a 10 mL centrifuge tube and centrifuged at ~ 4000 rev/min (2000 g) for 10 min in a Mistral 2000 centrifuge (MSE (UK) Ltd., London, UK). The supernatant was weighed and freeze-dried.

3.8.2 Determination of amylose and total starch

The assay procedure developed by Gibson et al. (1997) was followed to measure the amounts of amylose and total starch leached from starch granules. A Megazyme amylose/amylopectin assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) was used to measure the amount of amylose and total starch in the freeze-dried supernatant. The kit contains freeze-dried concanavalin A (Con A),

amyloglucosidase/α-amylase suspension, glucose determination reagent, glucose reagent buffer, glucose standard solution and a starch reference sample.

3.8.2.1 Preparation of reagents, buffers, solvents and enzymes

Reagents, buffers, solvents and enzymes used in the analysis were prepared following the instructions enclosed in the Megazyme amylose/amylopectin assay kit. All other materials (chemicals) were obtained from Sigma-Aldrich (St. Louis, MO, USA)

Sodium acetate buffer. Glacial acetic acid (5.9 mL) was added to 900 mL of Milli-Q water and the pH of the solution was adjusted to pH 4.5 by adding 1M sodium hydroxide solution. A small amount of sodium azide (0.2 g) was added as a preservative and the volume was adjusted to 1L Milli-Q water. The sodium acetate buffer was stored at room temperature.

Con A solvent. Anhydrous sodium acetate (49.2 g), sodium chloride (175.5 g), $CaCl_2.2H_2O$ (0.5 g), $MgCl_2.6H_2O$ (0.7 g), $MnCl_2.4H_2O$ (0.7 g) were dissolved in 900 mL of Milli-Q water. The pH of the solution was adjusted to pH 6.4 by drop-wise addition of glacial acetic acid and the volume was adjusted to 1L with Milli-Q water. This solution was a concentrated form and stored at 4°C. For a working concentration of Con A solvent, 30 mL of the solution was diluted to 100 mL with Milli-Q water on the day of use.

Con A solution. The freeze-dried Con A was dissolved in 50 mL of Con A solvent (working concentration) and stored at -20°C until needed.

GOPOD (Glucose Oxidase/Peroxidase) reagent. Glucose reagent buffer was diluted to 1 L with Milli-Q water and this was used to dissolve the glucose determination reagent. The GOPOD reagent was stored at 4°C.

3.8.2.2 Pre-treatment

The freeze-dried starch supernatant was weighed into a 10 mL screw capped test tube. An aliquot (1 mL) of dimethyl sulphoxide (DMSO) was added to the tube and mixed on a vortex mixer. The tube was then capped and heated in a boiling water bath for 1 min to completely disperse the dried material. The contents of the tube were mixed using a vortex mixer and returned to the boiling water bath and heated for further 15 min, with intermittent mixing with a vortex mixer.

The tube was allowed to stand at 20°C for 5 min and then 2 mL of 95% ethanol was added. The contents were mixed with a vortex mixer and a further 4 mL of 95% ethanol was added. The cap was replaced and the contents were mixed by repeated inversions. After allowing the tube to stand at 20°C for 15 min, the tube was centrifuged at 2000g for 5 min in a Mistral 2000 centrifuge (MSE (UK) Ltd., London, UK) and the supernatant was discarded. To ensure all the ethanol was drained and removed, the tube was placed under a fume hood for at least 20 min. An aliquot (1 mL) of DMSO was added to the drained pellet and the tube was mixed on a vortex mixer. The sample tube was then heated in a boiling water bath for 15 min, with intermittent mixing on a vortex mixer. The contents were diluted to 25 mL by adding Con A solvent. The contents were repeatedly washed with Con A solvent and transferred into a 25 mL volumetric flask. This solution is termed '*Solution 1*'.

3.8.2.3 Determination of amylose

A centrifuge tube was filled with 1 mL of *Solution 1* and mixed with 0.5 mL of Con A solution by repeated inversion. The mixture was stored at 20°C for 1 hour and then centrifuged at 20,000g for 10 min in an Eppendorf centrifuge (5417R, Eppendorf, Hamburg, Germany) at 20°C. A screw capped test tube was filled with 1 mL of the supernatant and 3 mL of sodium acetate buffer was added to reduce the pH

to pH 5 ± 0.05. The tube was then heated in a boiling water bath for 5 min to denature the Con A. The tube was then placed in a water bath at 40°C for 5 min to equilibrate before adding 0.1 mL of amyloglucosidase/α-amylase enzyme mixture. After 30 min of incubation at 40°C, the sample was centrifuged at 2,000g for 5 min in a Mistral 2000 centrifuge (MSE (UK) Ltd., London, UK).

After centrifugation, 1 mL of the supernatant was mixed with 4 mL of glucose determination reagent in a test tube and the mixture was incubated at 40°C for 20 min. A reagent blank was prepared by mixing 1 mL of sodium acetate buffer and 4 mL of glucose determination reagent. A glucose control was prepared by mixing 0.1 mL glucose standard (1mg/mL), 0.9 mL sodium acetate buffer and 4 mL of GOPOD reagent. The reagent blank and the glucose control were incubated concurrently with the sample at 40°C for 20 min. The absorbance at 510 nm for the Con A supernatant (sample) and glucose control was read against the reagent blank using a spectrophotometer (Jasco V-560 UV-Vis spectrophotometer, Jasco Corporation, Tokyo, Japan; 10mm path length). The amylose was calculated as described in section 3.8.2.5.

3.8.2.4 Determination of total starch

In a test tube, 0.5 mL of *Solution 1* was mixed with 4 mL of sodium acetate buffer and 0.1 mL of amyloglucosidase/α-amylase enzyme mixture was then added. The test tube was incubated at 40°C for 10 min. After incubation, 1 mL of the test tube contents were mixed with 4 mL of GOPOD reagent in a test tube and the mixture was incubated at 40 °C for 20 min concurrently with the other test tubes prepared in section 3.8.2.3. The absorbance at 510 nm for the sample and glucose control was read against the reagent blank using a spectrophotometer after the incubation period.

3.8.2.5 Calculations

The total starch (%) and amylose (%) were calculated using **Equation 3-5** and

3-6, respectively which were modified from Gibson et al. (1997).

$$Total\ starch\ (\%) = A_{510} \times F \times 230 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$ **Equation 3-5**

A_{510} = absorbance of reaction solutions read against reagent blank at 510nm

F = factor to convert absorbance values to µg glucose = 100µg glucose/ A_{510} 100µg

glucose

230 = volume correction

1/1000 = conversion from µg to mg

100/W = conversion to %, where W = sample weight in mg

162/180 = factor to convert from free glucose (as determined), to anhydrous glucose

(as occurs in starch).

$$Amylose(\%) = \frac{A_{510}\ ConA\ supernatant}{A_{510}\ total\ starch\ aliquot} \times \frac{6.15}{9.2} \times 100$$ **Equation 3-6**

6.15/9.2 = dilution factor

3.9 Sampling and data analysis

All experiments (pressure treatment, heat treatment and acid gelation) were

carried out in at least duplicate from sample preparation to sample analyses. Standard

deviations and pooled standard deviations were used where appropriate to indicate the

variability between repeated experiments or measurements. Analysis of variance

(ANOVA, $p<0.05$) using MINITAB Statistical Software was conducted to examine

the significance of observed differences where appropriate.

3.10 References

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CHAPTER 4

Effect of High-Pressure Treatment on Various Starch-in-Water Suspensions

This chapter presents the results from preliminary experiments to investigate the gelatinisation behaviour of a variety of starches after high-pressure treatments. The results from this chapter have been published in the following article: [1]Oh, H. E., D. N. Pinder, Y. Hemar, S. G. Anema and M. Wong (2008a). "Effect of high-pressure treatment on various starch-in-water suspensions." *Food Hydrocolloids* **22**(1): 150-155. This article can be found on page 4-4 of this chapter

Aim. To investigate the effect of high pressure treatment on the gelatinisation of a range of starches.

Relevance. Starches from different botanical sources have been found to show different susceptibility to both heat- and pressure-induced gelatinisation (Hibi et al., 1993; BeMiller and Whistler, 1996; Stute et al., 1996). In previous studies, microscopic and calorimetric techniques were commonly used to characterise the gelatinisation behaviour of the starches. In this study, rheological techniques are investigated for their use in characterising the gelatinisation behaviour of starches. Such techniques have not previously been used to characterise pressure-induced gelatinisation of starch. The hypothesis tested in this chapter was that pressure-induced behaviour of starches can be related to one or more of the following factors - botanical origins, amylose content and crystalline forms. This chapter reports the results on the pressure-induced gelatinisation of normal corn, waxy corn, normal rice, waxy rice, tapioca and potato starch-in-water suspensions. The results from this study were used to develop and test the methodology for sample analyses and also provided a basis for the selection of starches for more detailed investigations.

Approach. Starch-in-water suspensions (10% w/w starch concentration) were prepared and pressure treated using common treatment conditions (400 and 600MPa,

[1]- Oh et al. (2008a) contains the original work of the author H. E. Oh and has been written by the author H. E. Oh.

20°C, 30 min). The pressure-treated samples were analysed for initial apparent viscosity ($\eta_{initial}$), pasting curves of the starch suspensions, degree of swelling and changes in the birefringence of starch granules.

Summary of results. It was found that different starches can have very different susceptibility to pressure-induced gelatinisation. Furthermore, susceptibility of starch to heat-induced gelatinisation cannot be used to predict the susceptibility to pressure-induced gelatinisation. Potato starch was the most pressure-resistant starch compared with the other starches used in this study. Waxy rice, waxy corn and tapioca starches showed 100% degree of swelling and were completely gelatinised after the pressure treatment at 600 MPa, which resulted in a maximum $\eta_{initial}$ increase. Although normal rice and normal corn starches showed a complete loss of birefringence, the degree of swelling did not reach 100% and the $\eta_{initial}$ did not increase to the maximum.

From a sample handling point of view, the rice starches (normal rice and waxy rice starches) were preferred over the corn starches (normal corn and waxy corn starches) or tapioca starch. The starch suspension sample cannot be stirred during pressure treatment in the equipment used in this study therefore, some sedimentation of starch was inevitable despite thorough mixing of the sample immediately before the pressure treatment. The sedimentation layer often formed a gel. Since rice starch granules are, on average smaller than corn starch granules, they stayed suspended in the suspension medium for longer and produced less sedimentation during pressure treatment. Also, the resulting sedimentation layers in the rice starch suspensions were easier to mix than other starch suspensions where sediments were found. Reduced sedimentation in rice starch samples therefore minimised any potential sample handling errors. The sedimentation layer found in tapioca starch suspension was the

4-2

most difficult to incorporate into the rest of the sample, hence required more attention in handling to achieve the same degree of reproducibility of the analysis results.

Conclusions.

- Starches that can be readily gelatinised by heat are not necessarily gelatinised readily by pressure. Potato starch, which can be gelatinised at relatively low temperature (58-65°C), was the most pressure-resistant starch when compared with other starches examined in this study.

- The $\eta_{initial}$ and degree of swelling of normal rice and normal corn starches were not increased to the same extent as that of waxy rice, waxy corn and tapioca starches despite the disappearance of birefringence in the granules.

- The rheological techniques used in this study ($\eta_{initial}$ measurements and pasting curves) were reliable methods to characterise the pressure-induced gelatinisation of starch.

The two rice starches, normal rice starch and waxy rice starch, were selected to be used in the subsequent studies (Chapters 5 & 6). Waxy rice starch was fully gelatinised within the experimental pressure range and normal rice starch could show an interesting contrast to this.

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Effect of high-pressure treatment on various starch-in-water suspensions

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Abstract

This study investigated the high-pressure-induced gelatinization of different starches, namely normal rice, waxy rice, normal corn, waxy corn, tapioca and potato starches. The high-pressure-treated starch solutions were characterized by pasting behaviour, degree of swelling and changes in birefringence. Potato starch was found to be less affected by pressure treatment than the other starches, as it retained birefringence after a pressure treatment of 600 MPa for 30 min. Waxy and tapioca starches showed complete gelatinization after the same treatment, whereas normal starches were only partially gelatinized. The pasting curves of the normal starches showed an increase in the initial viscosity after pressure treatment, whereas the initial viscosities of the waxy starches after the 600 MPa for 30 min treatment were already equal to their respective peak viscosities.
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Keywords: High pressure; Starch; Gelatinization; Pasting; Viscosity

1. Introduction

Starch occurs naturally as discrete particles (granules), which are relatively dense and insoluble and hydrate only slightly in cold water (BeMiller & Whistler, 1996). When starch is heated in an aqueous medium, swelling of the granules becomes irreversible and the structure of the granules is altered significantly. This process is called 'gelatinization' (Lund, 1984). During gelatinization, swelling and disruption of the starch granules produces a viscous mass consisting of a continuous phase of solubilized amylose and amylopectin and a discontinuous phase of granule remnants (BeMiller & Whistler, 1996). Heat-induced gelatinization depends on the botanical source, genetic variety and growing conditions of the starch (Jane, 2004).

Starch can also be gelatinized by high pressure (Douzals, Marechal, Coquille, & Gervais, 1996; Katopo, Song, & Jane, 2002; Stute, Klingler, Boguslawski, Eshtiaghi, & Knorr, 1996). Rubens, Snauwaert, Heremans, and Stute

(1999) proposed a two-step mechanism for pressure-induced gelatinization, similar to that for heat-induced gelatinization. In the first step, hydration of amorphous parts of the starch granules occurs, which leads to swelling of the granules and distortion of the crystalline regions. In the second step, the crystalline regions become more accessible for water. In heat-induced gelatinization, different starches have different gelatinization temperatures and the extent of gelatinization can change depending on the time and temperature of heating (Lund, 1984). Similarly, in pressure-induced gelatinization, different types of starch gelatinize over different ranges of pressure and the extent of gelatinization is dependent on the treatment pressure, the treatment time and the temperature of pressurization (Bauer & Knorr, 2005).

Previous studies have shown that B-type starches such as potato starch are more resistant to pressure than A- or C-type starches (Katopo et al., 2002; Rubens et al., 1999; Stute et al., 1996). It has also been shown by X-ray diffraction that high-pressure treatment converts starches that display the A-type pattern into B-type-like starches, whereas B-type starches keep their original B-type pattern (Hibi, Matsumoto, & Hagiwara, 1993; Katopo

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et al., 2002). Katopo et al. (2002) attributed this difference between A- and B-type starches to their amylopectin structures. They suggested that, for B-type starches, water fills up the channel in the cell unit of the crystallite and stabilizes the structure. In contrast, the A-type crystallite has a more scattered amylopectin branching structure, which is more flexible, and therefore allows rearrangement of double helices to generate a channel in which water molecules are included under pressure. Consequently, the crystalline structure goes through a transformation from the A-type crystallite to the B-type crystallite. The importance of sufficient water in high-pressure-induced gelatinization has been highlighted in a number of studies (Hibi et al., 1993; Rubens et al., 1999).

In this study, we examined the effect of high-pressure treatments on different starches by comparing the degree of swelling, the pasting behaviour of the starch suspensions and the birefringence of the granules. In particular, this study investigated the changes in the viscosity of pressure-treated starch suspensions, which have not been extensively reported in the literature.

2. Materials and methods

2.1. Materials

Normal rice starch (16% amylose) and waxy rice starch (3% amylose) were obtained from Remy Industries (Leuven-Wijgmaal, Belgium). Normal corn, waxy corn and potato starches were obtained from Penford New Zealand Limited (Auckland, New Zealand). Tapioca starch was obtained from National Starch Food Innovation (Auckland, New Zealand). All starches were used as supplied.

2.2. Sample preparation

Starch suspensions were prepared by adding starch to purified (reverse osmosis followed by filtration through a Milli-Q apparatus) water to a final concentration of 10% (w/w). The suspensions were stirred at ambient temperature (approximately 20 °C) until the starch was completely dispersed. A small amount of sodium azide (0.02%, w/v) was added to all samples as a preservative. The samples were then transferred into Beckman Polyallomer centrifuge tubes (13 mm internal diameter × 15 mm high or 16 mm × 76 mm, Beckman Instruments Inc., Spinco Division, Palo Alto, CA) and the tubes were heat sealed.

2.3. High-pressure treatment

The samples were treated in a high-pressure unit ('Food-Lab' food processor, model S-FL-065-200-9-W, Stansted Fluid Power Ltd., Stansted, Essex, UK), at pressures of 400 or 600 MPa, for 30 min at 20 °C. An emulsion of 10%

vegetable oil in water with surfactant and preservative was used as a pressure-transmitting fluid in the 65 mm × 220 mm cylindrical high-pressure chamber.

The pressurization and depressurization rates were 6 and 27 MPa s^{-1}, respectively. The average adiabatic heating during pressurization was ~2.0 °C/100 MPa. The cooling rate during depressurization was ~1.7 °C/100 MPa.

Once the high-pressure treatment was complete, the sample tubes were opened and the samples were transferred into beakers. Whenever sediment was found, it was mixed thoroughly by hand with the rest of the sample from the same tube to ensure sample homogeneity. The samples were stored in closed containers at room temperature for approximately 10 h before analyses.

2.4. Methods

A stress-controlled rheometer, the Physica UDS200 rheometer (Anton Paar GmbH, Graz, Austria) equipped with a starch cell and stirrer arrangement (C-ETD 160/ST), was used to measure the rheological properties of the samples. A sub-sample (22 ml) was transferred into the starch cell and was stirred at 100 rev min^{-1} for 1 min at 20 °C. Due to the characteristics of the starch cell geometry, the exact shear rate is difficult to calculate; however, a stirring speed of 100 rev min^{-1} corresponds to a shear rate of approximately 44 s^{-1}. The viscosity of the sample was then measured as the temperature was increased from 20 to 95 °C, at a constant rate of 2 °C min^{-1} with constant stirring at 100 rev min^{-1}. The viscosity was recorded at 30 s intervals. All measurements were performed in duplicate.

The swelling test was performed using a simple centrifugation method based on that developed by Hemar and Horne (1998). The samples were transferred to 75 mm glass capillary tubes and one end of each capillary tube was sealed by flame. At least 10 mm of the capillary tube was left void to ensure that the sample was not subjected to excessive heat during flame sealing. For each sample, three tubes were prepared and analyzed.

The capillary tubes were loaded into a Haemofuge centrifuge (Heraeus Christ, Hanau, Germany), sealed ends to the outer rim and centrifuged at 12 000 rev min^{-1} for 10 min at ambient temperature. The tubes were scanned to obtain magnified images, which were used to calculate the degree of swelling using Eq. (1):

$$\text{Swelling}(\%) = \frac{\text{Height of centrifuge sediment}}{\text{Height of sample}} \times 100 \qquad (1)$$

For the birefringence observations, an aliquot of starch suspension was transferred on to a glass slide and a cover slip was placed on top of the slide. A polarized light microscope (Nikon Eclipse E600 Pol, Nikon Corporation, Tokyo, Japan) with a 20× or 50× objective was used to observe birefringence of the starch granules.

3. Results

3.1. Degree of swelling

The degree of swelling for each starch type after the different pressure treatments is summarized in Table 1. As expected, different types of starch were affected differently by the pressure treatments. Normal rice starch showed an increase in swelling with an increase in treatment pressure such that it reached 51.8% swelling after pressure treatment at 600 MPa. Normal corn starch also showed an increase in swelling with pressure but the swelling at 600 MPa was still less than 50%. Stute et al. (1996) reported similar observations regarding the limited swelling of normal corn starch and have stated that restricted swelling is typical of the high-pressure-induced gelatinization of some starches. Waxy rice starch was most affected by high pressure, showing 40.3% swelling after pressure treatment at 400 MPa. Waxy corn starch did not swell considerably after pressure treatment at 400 MPa but reached 100% swelling after treatment at 600 MPa. For both rice starch and corn starch, the waxy type was more susceptible to pressure than the normal type. Similar to waxy rice starch and waxy corn starch, tapioca starch was also fully swollen (100%) after pressure treatment at 600 MPa. Potato starch was least affected by pressure treatment. Even after treatment at 600 MPa, the degree of swelling was only 20.4%.

3.2. Initial viscosity and pasting curves

The initial viscosity measurements, taken at 20 °C before the pasting of each starch type, are summarized in Table 1. As expected, the initial viscosity values corresponded well with the swelling results. Waxy rice, waxy corn and tapioca starches displayed considerable increases in viscosity after pressure treatment at 600 MPa, when the swelling reached 100%. For example, the initial viscosity for waxy corn starch increased from $5.7\,\text{mPa s}^{-1}$ before pressure treatment to $3530\,\text{mPa s}^{-1}$ after pressure treatment at 600 MPa. Normal rice starch and normal corn starch showed an increase in the initial viscosity with pressure whereas

potato starch was hardly affected by the pressure treatment.

Pasting curves from 20 to 95 °C for each starch type are shown in Figs. 1A–F. Three viscometric parameters can be extracted from the pasting curve. The onset temperature, 'T_{onset}', is the temperature at which the viscosity starts to increase, the peak viscosity, 'η_{peak}', is the maximum viscosity attained and 'T_{peak}' is the temperature at the peak viscosity. Untreated starch suspensions showed a rapid increase in viscosity at temperatures beyond T_{onset}, which indicates the start of starch gelatinization. The pasting curves for waxy rice, waxy corn and tapioca starches that were pressure treated at 600 MPa did not show a η_{peak} because the initial viscosity was already equal to the η_{peak}; this is a clear indication of complete gelatinization (Figs. 1B, D and E). The viscosity of these starch suspensions decreased slightly with temperature during pasting. As they were already gelatinized by the pressure treatment, the decrease in viscosity was due to the increase in temperature during pasting.

Partial gelatinization is observed when the initial viscosity of the treated starch suspension is higher than that of the untreated suspension, but the suspension still exhibits an increase in viscosity on an increase in temperature above T_{onset}. Normal rice starch and normal corn starch (Figs. 1A and C) pressure treated at either 400 or 600 MPa and waxy rice starch pressure treated at 400 MPa (Fig. 1B) showed this behaviour. For normal rice starch, η_{peak} and T_{peak} were unaffected by the pressure treatment. Normal corn starch was slightly different from normal rice starch. After pressure treatment at 600 MPa, both T_{onset} and T_{peak} of the normal corn starch suspension increased compared to the untreated sample. Especially T_{peak} increased from 89 (untreated) to 93 °C (600 MPa). Katopo et al. (2002) also reported an increase in pasting temperature and related this to the development of an amylose–lipid complex under high pressure, which then intertwines with amylopectin molecules to restrict the swelling and dispersion of the starch granules.

For the starches that were fully gelatinized on pressure treatment (waxy rice, waxy corn and tapioca starches), η_{peak} was not notably different from that of the untreated starch. This is contrary to the findings by Katopo et al.

Table 1
Initial viscosity and swelling of starches after different pressure treatments

Starch	No treatment	400 (MPa)		600 (MPa)	
	Initial viscosity (mPa s^{-1})	Swelling (%)	Initial viscosity (mPa s^{-1})	Swelling (%)	Initial viscosity (mPa s^{-1})
Normal rice	6.9 (0.09)	20.1 (0.80)	6.0 (0.5)	51.8 (0.16)	58.3 (2.5)
Waxy rice	6.9 (0.05)	40.3 (0.51)	44.1 (3.2)	100.0 (0.00)	4100 (79.9)
Normal corn	5.9 (0.04)	18.7 (0.21)	7.1 (1.5)	42.9 (0.46)	22.6 (3.3)
Waxy corn	5.7 (0.03)	18.1 (0.58)	6.7 (1.1)	100.0 (0.00)	3530 (95.5)
Tapioca	5.7 (0.30)	17.1 (0.68)	5.8 (0.2)	100.0 (0.00)	8254 (99.7)
Potato	5.5 (0.06)	15.5 (0.30)	6.0 (1.0)	20.4 (0.44)	7.6 (1.1)

Standard deviations of repeated measurements are given in parentheses.

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* Misprint correction: The unit for all initial viscosity data in Table 1 on this page should be "mPa.s".
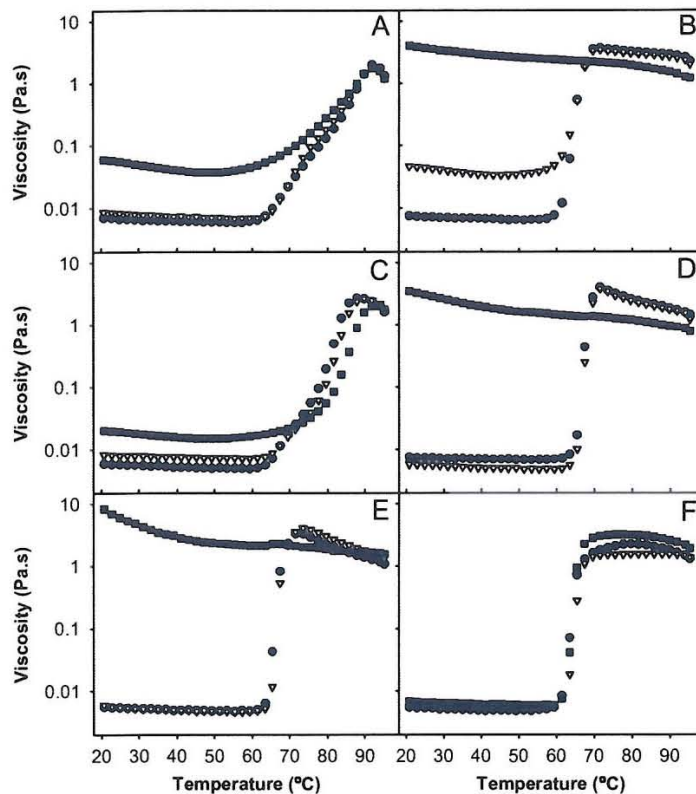
Fig. 1. Viscosity of starch suspensions as a function of temperature (pasting curve) after no pressure treatment (●), pressure treatment at 400 MPa (▽) and pressure treatment at 600 MPa (■). A, normal rice starch; B, waxy rice starch; C, normal corn starch; D, waxy corn starch; E, tapioca starch; F, potato starch.

(2002) who reported that the peak viscosity of pressure-treated waxy corn starch was lower than that of the untreated sample. The disagreement with Katopo et al. (2002) could be due to the different handling of the pressure-treated samples before pasting and the heating profiles during pasting. Furthermore, in this study, pressure-treated starch suspensions were used in pasting without further processing whereas the method used by Katopo et al. (2002) involved drying and milling of the samples after pressure treatment.

In contrast to the other starches, the pasting behaviour of potato starch was relatively unaffected by pressure treatment up to 600 MPa, especially at temperatures less than T_{onset}. However, there appeared to be a small difference in η_{peak} after the different pressure treatments (Fig. 1F).

3.3. Polarized light microscopy

Micrographs of starch suspensions treated at different pressures are shown in Fig. 2. As expected, the starch granules in the untreated suspensions displayed birefringence. Some granules lost birefringence after treatment at 400 MPa, especially granules of normal rice starch and

waxy rice starch. After pressure treatment at 600 MPa, birefringence was still observed in the granules of potato starch; however, there was no birefringence in the other types of starch. This is in agreement with Stute et al. (1996). However, the granules of normal rice starch and normal corn starch still retained some degree of integrity despite the loss of birefringence after pressure treatment at 600 MPa (Figs. 2A-3 and C-3). In Katopo et al. (2002), scanning electron micrographs showed that normal corn starch retained its granular integrity after pressure treatment at 690 MPa whereas the granules of waxy corn starch partially lost integrity. It has been suggested by Douzals, Cornet, Gervais, and Coquille (1998) that a lower release of amylose due to less water binding could be related to the preservation of the integrity of the granules.

4. Discussion

The different starch suspensions investigated in this work can be qualitatively divided into three classes: the waxy starches, which can fully gelatinize at sufficiently high pressure (> 400 MPa); the normal starches, which partially gelatinize under high pressure (up to the pressure of 600 MPa applied here), probably as a result of the partial
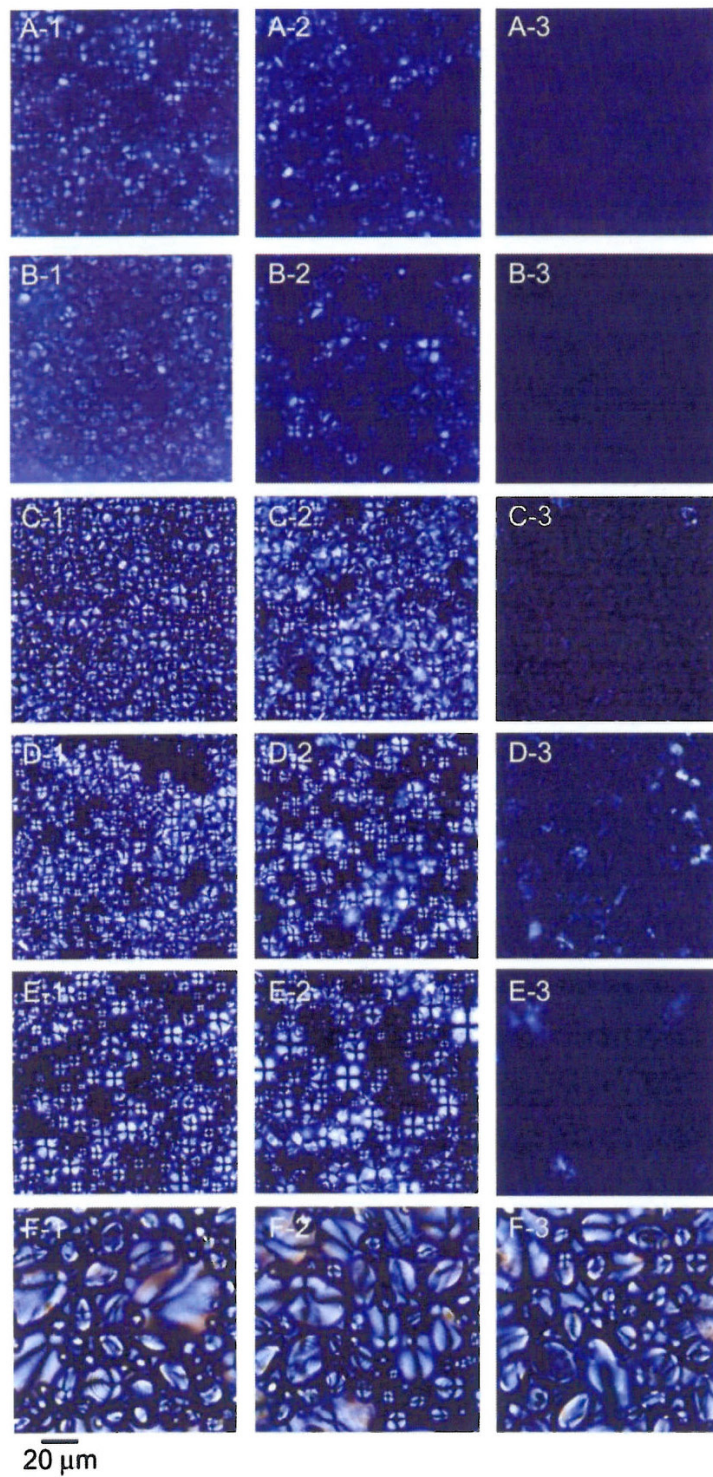
Fig. 2. Polarized light micrographs of starches after no pressure treatment (1), pressure treatment at 400 MPa (2) and pressure treatment at 600 MPa (3): normal rice starch (A-1–A-3); waxy rice starch (B-1–B-3); normal corn starch (C-1–C-3); waxy corn starch (D-1–D-3); tapioca starch (E-1–E-3); potato starch (F-1–F-3).

swelling of the starch granules and pressure-resistant starches such as potato starch that are not affected by high-pressure treatment up to 600 MPa.

The differences in the behaviour of these starches under high pressure could be due to the differences in their physico-chemical properties. First, differences in the composition of the starches may lead to different interactions under pressure. Swelling of starch granules relates to the magnitude of the interaction between starch chains within the amorphous and crystalline domains. The extent of this interaction is influenced by the amylose to amylopectin ratio and by the characteristics of amylose and amylopectin in terms of molecular weight, distribution, degree and length of branching, and conformation (Hoover, 2001).

Second, normal starches contain considerably more lipids and proteins than waxy starches and the amylose to amylopectin ratio is higher than that of waxy starches (BeMiller & Whistler, 1996). It has been suggested that amylose–lipid complexes could be formed in normal starches under pressure and that these complexes may restrict the swelling of the starch granules (Katopo et al., 2002).

Third, potato starch was more resistant to pressure than the other starches examined in this study, in agreement with the literature (Hibi et al., 1993; Katopo et al., 2002; Stute et al., 1996). This could be due to differences in the structure of the external region of the starch granules. Sevenou, Hill, Farhat, and Mitchell (2002) suggested that potato starch exhibits a far greater level of ordered structure in the external region of the granule than other starches such as normal corn starch and waxy corn starch. Using scanning electron micrographs, Blaszczak, Valverde and Fornal (2005) showed that potato starch granules have a very compact condensed layer that seems to be more pressure resistant than the inner part of the granule and remains unchanged by high-pressure treatment.

The marked difference between potato starch and other starches in their resistance to high-pressure treatment can also be related to the difference in their crystalline properties. Using X-ray diffraction, starches can be classified as A-type starch (normal rice, waxy rice, normal corn and waxy corn starches), B-type starch (potato) and C-type starch (tapioca). B-type starches are known to be more resistant to pressure than A- or C-type starches (Katopo et al., 2002; Rubens et al., 1999; Stute et al., 1996). Katopo et al. (2002) suggested that A-type crystallites have more scattered branching amylopectin structures than B-type crystallites. These structures in the A-type crystallite can be rearranged under pressure to generate a channel in which water molecules can be included whereas the B-type crystallite stays stable under pressure. This may mean that

the B-type crystalline structure has a lower compressibility than the A-type crystalline structure, as mentioned in Hibi et al. (1993).

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4-9

CHAPTER 5

Effect of High-Pressure Treatment on Normal Rice and Waxy Rice Starch-in-Water Suspensions

This chapter presents a detailed characterisation study of the pressure-induced gelatinisation behaviour of normal and waxy rice starches. The results from this chapter have been published in the following article: [1]Oh, H. E., Y. Hemar, S. G. Anema, M. Wong and D. N. Pinder (2008b). "Effect of high-pressure treatment on normal rice and waxy rice starch-in-water suspensions." *Carbohydrate Polymers* **73**(2): 332-343. This article can be found on page 5-4 of this chapter. This chapter also contains some unpublished results that show the effect of static heating on the starch-in-water suspensions as a comparison which can be found on page 5-16.

Aim. To conduct an in-depth study of the pressure-induced gelatinisation of starch-in-water suspensions using normal rice starch and waxy rice starch. These were the starches selected from the preliminary study (Chapter 4).

Relevance. Understanding the pressure-induced gelatinisation of starch and the effects of various processing conditions (pressure, temperature and duration) is essential for the applications of high-pressure treatment in starch-containing products in order to achieve the desired product functionality. The hypothesis tested in this chapter was that for any starch different combinations of treatment pressure, temperature and duration may result in the same degree of gelatinisation but two different starches may show different gelatinisation behaviours under the same processing conditions.

Approach. Rheological methods ($\eta_{initial}$ measurements and pasting curves), were employed to determine the degree of starch gelatinisation after pressure treatments, instead of the qualitative microscopic methods (birefringence counts) or the thermal method (DSC) commonly used in the field. The rheological methods provided a more objective and analytical means to determine the degree of gelatinisation compared to the other methods. In addition, rheological changes are directly related to the

[1]- Oh et al. (2008b) contains the original work of the author H. E. Oh and has been written by the author H. E. Oh.

functional properties of the starch that are crucial in product application. A method of quantifying the leached amylose after pressure treatment was adapted from published methodologies and used for the pressure-treated starch suspensions.

Summary of results. Both normal and waxy rice starches exhibited sigmoidal-shaped pressure-induced gelatinisation curves ($\eta_{initial}$ as a function of treatment pressure). The gelatinisation curves can be divided into three regions. In the first region, the $\eta_{initial}$ value was low and did not change markedly until a critical level of pressure was applied, which was approximately 350 MPa for normal rice starch and 300 MPa for waxy rice starch. The second phase starts from these critical pressures, in which the $\eta_{initial}$ increased sharply as the treatment pressure was increased. The third phase starts from about 500 MPa at which both starch types showed no further increase in $\eta_{initial}$.

The degree of gelatinisation was dependent on the type of starch, the pressure, the temperature, and the duration of treatment. Different combinations of these factors could result in the same degree of gelatinisation of a particular starch. There was a linear correlation between the degree of swelling and $\eta_{initial}$. After pressure treatments at 500 MPa, both starches lost all birefringence although they experienced different extents of change in $\eta_{initial}$ and the degree of swelling. Normal rice starch retained the granular structure and did not swell to 100% after the pressure treatment ($\leq$700 MPa) whereas waxy rice starch reached 100% swelling and the granules were completely disrupted. This can be related to the smaller $\eta_{initial}$ increase compared with that found in waxy rice starch. A maximum of ~2% leaching of starch material including amylose was detected for normal rice starch.

Conclusions.

-       The relationship between the degree of starch gelatinisation and the treatment pressure was found to produce a sigmoidal-shaped curve.

- The degree of starch gelatinisation increased as the treatment duration or temperature increased.

- Pressure-induced gelatinisation of starch is dependent on the swelling of starch granules.

- Although both normal rice starch and waxy rice starch can be gelatinised by pressure, the difference in the degree of swelling between the two starches after pressure treatment led to differences in the rheological properties of the pressure-treated suspensions.

Effect of high-pressure treatment on normal rice and waxy rice starch-in-water suspensions

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Abstract

The effects of treatment pressure ($\leqslant 700$ MPa), temperature at treatment (10–60 °C), and treatment duration (0–30 min) on the gelatinization of normal and waxy rice starches were investigated. Pressure-treated starch suspensions were examined for pasting behaviour, initial apparent viscosity ($\eta_{initial}$), degree of swelling, birefringence changes, and leaching of starch and amylose. The $\eta_{initial}$ measurements provided an objective and analytical means of determining the degree of pressure-induced gelatinization of starch. Both normal and waxy rice starches exhibited sigmoidal-shaped pressure-induced gelatinization curves. The degree of gelatinization was dependent on the type of starch, the pressure, the temperature, and the duration of treatment. Different combinations of these factors could result in the same degree of gelatinization. There was a linear correlation between the degree of swelling and $\eta_{initial}$. After treatments at $\geqslant 500$ MPa, both starches lost all birefringence although they experienced different extents of change in $\eta_{initial}$ and the degree of swelling.
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Keywords: High-pressure; Starch; Gelatinization; Pasting; Viscosity; Swelling

1. Introduction

Starch is a major food reserve substance in plants, and occurs in discrete granules. Starch consists of two biopolymers: an essentially linear polysaccharide called amylose and a highly branched polysaccharide called amylopectin (Parker & Ring, 2001). Amylose and crystalline amylopectin are organized into alternating radial layers to form the mechanical structure of starch granules (Parker & Ring, 2001). Applications of starch in food products often involve its gelatinization for functional and nutritional properties. Starch gelatinization is defined as the disruption of molecular orders within the starch granule, manifested in irreversible changes in properties such as granular swelling, native crystallite melting, loss of birefringence, and starch solubilization (Atwell, Hood, Lineback, Varriano- marston, & Zobel, 1988). Although heating starch in the presence of water is a common method of inducing gelatinization, high-pressure treatment of starch can also induce its gelatinization (Katopo, Song, & Jane, 2002; Stute, Klingler, Boguslawski, Eshtiaghi, & Knorr, 1996).

High-pressure treatment, among other non-thermal technologies, is gaining interest in the food industry. For example, high-pressure treatment may provide a preservative technique that can satisfy the consumer demands for 'fresh-like' products while maintaining shelf life. High-pressure treatment causes disordering of biopolymers, including proteins and starch, as it modifies non-covalent intermolecular interactions (Balny, 2002). The pressure-induced disordering is similar to heat-induced disordering, but not identical (Balny, 2002). For starches, this disordering results in pressure-induced gelatinization. Understanding pressure-induced gelatinization of starch is, therefore, vital for applications of high-pressure treatment in starch-containing products in order to understand and achieve the desired product functionality.

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High-pressure-induced starch gelatinization has been investigated in a number of studies in recent years (Katopo et al., 2002; Stute et al., 1996). The pressure range in which the gelatinization occurs depends on the type of starch. For instance, gelatinization of wheat starch begins below 300 MPa and is achieved completely at 600 MPa (Douzals, Marechal, Coquille, & Gervais, 1996). In contrast, the treatment pressure needs to be at least 600 MPa for potato starch to start to gelatinize (Bauer & Knorr, 2005). The findings of Stute et al. (1996) suggest that, for all starches, the characteristics of pressure-induced gelatinization can be different from those of heat-induced gelatinization. The authors showed that some starches, including normal corn starch, did not swell under pressure as much as they did during thermal gelatinization. In addition, Douzals, Cornet, Gervais, and Coquille (1998) reported that pressure-induced starch gelatinization resulted in a lower release of amylose compared with that from heat-induced gelatinization.

In this study, the effects of different treatment pressures, temperatures at pressurization, and treatment durations on normal and waxy rice starch suspensions were investigated. Pressure-treated starch suspensions were analyzed for pasting profile, initial apparent viscosity, degree of swelling, birefringence changes, and leached starch and amylose to explore different aspects of high-pressure-induced gelatinization. Pasting profiles and initial apparent viscosity provided information on physical changes that indicate the degree of gelatinization. The information gathered from the rheological measurements was related to the other analyses results to characterize the high-pressure-induced gelatinization of each starch. The observed differences and similarities in the behaviour of normal and waxy rice starches are compared and discussed.

2. Materials and methods

2.1. Materials

Unmodified normal rice starch (12% moisture, 0.09% fat, 0.13% protein, 0.06% ash) and waxy rice starch (11% moisture, 0.07% fat, 0.06% protein, 0.08% ash) were supplied by Remy Industries (Leuven-Wijgmaal, Belgium) and were used as supplied. The starches were stored in air-tight containers. A Megazyme amylose/amylopectin assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) was used for the analysis of leached starch and amylose.

2.2. Preparation of starch suspensions

Starch was dispersed in purified water (reverse osmosis followed by filtration through a Milli-Q apparatus) by stirring at room temperature (~20 °C) to produce starch suspensions with a final concentration of 10% (w/w). Sodium azide (0.02%, w/v) was added to all samples as a preservative. Beckman Polyallomer centrifuge tubes

(13 mm internal diameter × 51 mm high, or 16 × 76 mm, Beckman Instruments, Inc., Spinco Division, Palo Alto, CA, USA) were used to hold the samples for high-pressure treatment. Once the centrifuge tubes had been filled with sample, the tubes were heat sealed.

2.3. High-pressure treatment

Pressure treatments of samples were conducted using a laboratory-scale high-pressure unit (Food-Lab, model S-FL-850-9-W, Stansted Fluid Power Ltd., Stansted, Essex, UK). Various treatment conditions were used: pressures ranged between 100 and 700 MPa, treatment durations ranged from 0 to 30 min, and temperatures at pressurization were between 10 and 60 °C. The samples were equilibrated to the pressure treatment temperature in a water bath for 20 min before treatment commenced. The 65 × 220 mm cylindrical high-pressure chamber was filled with a pressure-transmitting fluid consisting of an emulsion of 10% vegetable oil in water with small amounts of Tween 80, Span 60, and potassium sorbate. Control samples were prepared and kept in a water bath at the set pressurization temperature for the duration of the relevant pressure treatment.

The pressurization rate was 4.4 MPa/s and the depressurization rate was 9.2 MPa/s. The average adiabatic heating during pressurization was ~1.9 °C/100 MPa. The cooling rate during depressurization was ~2.2 °C/100 MPa. Samples from three separate runs with identical set conditions were collected to produce enough volume for analyses. The samples were transferred into storage containers after depressurization. Any sediment was mixed carefully by hand with the rest of the sample to ensure sample homogeneity. Lids were placed on the sample containers and the samples were held at ambient temperature (20 °C) overnight (~10 h) before analysis.

2.4. Rheological properties

The rheological properties of the samples were analyzed using a stress-controlled rheometer, the Physica UDS200 rheometer (Anton Paar GmbH, Graz, Austria) equipped with a starch cell and stirrer arrangement (C-ETD 160/ST). The starch cell was filled with 22 mL of sample and the contents were stirred at 100 rev/min for 1 min at 20 °C before pasting. The pasting procedure entailed measuring the viscosity of the sample while increasing the temperature from 20 to 95 °C at a constant rate of 2 °C/min with a constant rotational speed of 100 rev/min. The viscosity was measured at 30 s intervals. This experiment was carried out in duplicate for all samples.

2.5. Degree of swelling

A simple centrifugation technique, modified from that developed by Hemar and Horne (1998), was used to examine the degree of swelling of the starch granules. Glass capillary tubes (75 mm long) were filled with sample, leaving

about 10 mm of the tube void so that the sample was not overheated when sealing the end of the tube with a bunsen flame. After sealing, the tubes were placed into a Haemofuge centrifuge (Heraeus-Christ, Hanau, Germany), sealed ends to the outer rim, and centrifuged at 12,000 rev/min for 10 min at ambient temperature. Magnified images of the centrifuged tubes were obtained by scanning the tubes using a scanner (hp Scanjet 5590, Hewlett-Packard Development Company, USA). The degree of swelling was calculated using Eq. (1):

$$\text{Degree of swelling (\%)} = \frac{\text{Height of centrifuged sediment}}{\text{Height of sample}} \times 100 \tag{1}$$

Three tubes were analyzed for each sample.

2.6. Light microscopy

An aliquot of each sample was put on to a glass slide and a cover slip was placed on top of the sample for microscopic examination. A polarizing light microscope (Nikon Eclipse E600 Pol, Nikon Corporation, Tokyo, Japan) with a 50× objective was used to observe birefringence of the starch granules. The microscope was also used without the polarizing filter to observe the appearance of the sample.

2.7. Total starch and amylose assay

The assay procedure developed by Gibson, Solah, and McCleary (1997) was followed to measure the amounts of amylose and total starch leached from starch granules. The solution phase of the sample was first separated by centrifugation. A sub-sample of the aqueous phase (8 g) was transferred into a 10 mL centrifuge tube and centrifuged at ~4000 rev/min (2000g) for 10 min in a Mistral 2000 centrifuge (MSE (UK) Ltd., London, UK). The supernatant was weighed and freeze dried. The freeze-dried samples were dispersed by heating in dimethyl sulfoxide (DMSO). Lipids were removed by successive ethanol washing and the precipitated starch was recovered. The precipitated starch was then dissolved in an acetate/salt solution and a sub-sample was taken. Concanavalin A was added to precipitate amylopectin, which was then removed by centrifugation. A sub-sample of the supernatant was taken after the centrifugation. The total starch in a sub-sample of the acetate/salt solution with dissolved starch and the amylose in a sub-sample of the supernatant were enzymically hydrolyzed to glucose. Glucose oxidase/peroxidase reagent was then added to each sub-sample and the absorbances at 510 nm of these mixtures were measured. The relative concentration of amylose in the starch sample was estimated as the ratio of the absorbance of the supernatant to that of the total starch sample. The total starch in the sample (%) was calculated using the total starch content equation in McCleary, Gibson, and Mugford (1997). This assay was carried out in duplicate.

Analysis of variance (ANOVA, $p < 0.05$) using MINITAB Statistical Software was conducted to examine the significance of observed differences.

3. Results

3.1. Pasting behaviour

In this study, "pasting" was defined as the heating of the starch suspension from 20 to 95 °C at 2 °C/min while stirring at 100 rev/min. Changes in apparent viscosity were recorded during pasting while stirring the sample, to construct a pasting curve. Pasting curves for normal and waxy rice starch suspensions that had received no pressure or heat treatment are shown in Fig. 1. Several parameters, which provide information about gelatinization characteristics, can be extracted from a pasting curve; these are marked in Fig. 1. The initial viscosity, "$\eta_{initial}$", is the apparent viscosity at 20 °C before pasting begins. The onset temperature of gelatinization, "T_{onset}", is the temperature at which the apparent viscosity starts to increase. The peak viscosity, "η_{peak}", is the maximum apparent viscosity attained during pasting and "T_{peak}" is the temperature at η_{peak}. The two types of rice starch had similar $\eta_{initial}$ values (approximately 0.007 Pa.s) but showed different pasting patterns (Fig. 1). T_{onset} was 64.5 °C for normal rice starch and 60.1 °C for waxy rice starch. Untreated waxy rice starch suspensions showed a rapid increase in viscosity over a narrow temperature range, so that T_{peak} was 72 °C. The viscosity increase for normal rice starch after T_{onset} was more gradual and over a wider temperature range, so that T_{peak} was 92 °C. The η_{peak} value was 2.1 Pa.s for normal rice starch and 3.5 Pa.s for waxy rice starch.

Selected pasting curves for normal and waxy rice starches after pressure treatment are shown in Fig. 2. The pressure treatments were carried out for 30 min at 40 °C
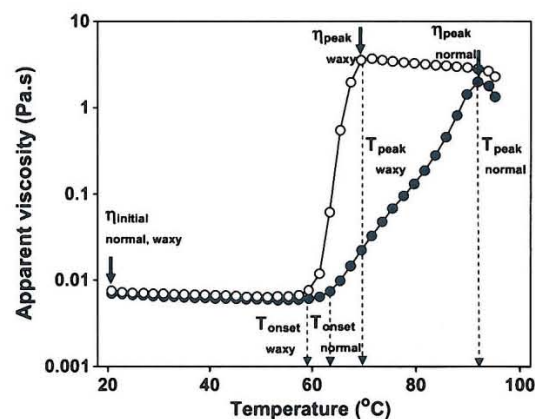
Fig. 1. Pasting curve. Apparent viscosity of starch suspensions (10% w/w) as a function of temperature for untreated normal rice starch (●) and untreated waxy rice starch (○).
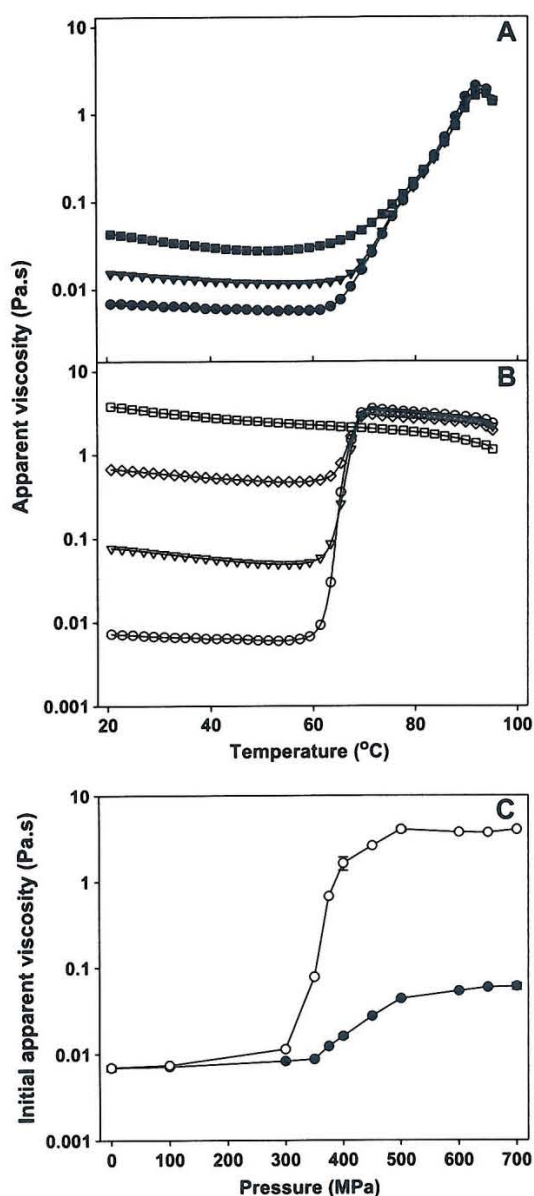
Fig. 2. (A) Pasting curves for normal rice starch after no pressure treatment (control) (●), pressure treatment at 400 MPa (▼), and pressure treatment at 500 MPa (■). (B) Pasting curves for waxy rice starch after no pressure treatment (control) (○), pressure treatment at 350 MPa (▽), pressure treatment at 375 MPa (◇), and pressure treatment at 500 MPa (□). (C) Initial apparent viscosity as a function of treatment pressure for normal rice starch (●) and waxy rice starch (○). The temperature at treatment was 40 °C and the treatment duration was 30 min.

at different pressures. The adiabatic heating was ∼1.9 °C/100 MPa, which increased the temperature of the pressurizing unit during treatment. For example, the temperature increased to ∼49.5 °C and cooled back to 40 °C over ∼4 min during the 500 MPa treatment. Starch gelatiniza-

tion involves granule swelling and the release of starch material and results in an increase in viscosity (BeMiller & Whistler, 1996). The increased $\eta_{initial}$ after pressure treatment indicates the degree of gelatinization of starch as a consequence of the pressure treatments. The viscosity of the starch suspensions subsequently increased with temperature during pasting when the starch had not been completely gelatinized by the pressure treatment.

The $\eta_{initial}$ value of normal rice starch suspensions was 0.007 Pa.s when untreated and increased to 0.043 Pa.s after treatment at 500 MPa. However, even after treatment at ⩽500 MPa, the initial viscosity of normal rice starch did not increase to the η_{peak} that could be attained on pasting. The η_{peak} value for the pressure-treated normal rice starch was approximately 2.1 Pa.s, was not notably different between suspensions that received different pressure treatments, and was very close to the value achieved in the untreated sample (Fig. 2A).

Waxy rice starch showed more noticeable changes in $\eta_{initial}$ after pressure treatments, compared with normal rice starch (Fig. 2B). Pressure treatment at 350 MPa was enough to increase the $\eta_{initial}$ value of the waxy rice starch suspension approximately tenfold, from 0.007 (untreated) to 0.078 Pa.s, followed by a further approximately tenfold increase after the 375 MPa treatment. On pasting, the viscosities of these samples increased to η_{peak} values that were similar to the values achieved for untreated waxy rice starch. After the 500 MPa pressure-treatment, the waxy rice starch suspension showed a $\eta_{initial}$ value that was slightly higher than the η_{peak} value of the untreated suspension and the viscosity did not increase further during pasting. Instead, the viscosity of the suspension decreased as the temperature increased. This decrease may have been a consequence of the stirring, which may have broken down the swollen granules and remnants, therefore decreasing the viscosity of the suspension (BeMiller & Whistler, 1996).

3.2. Initial viscosity ($\eta_{initial}$)

Fig. 2C shows the change in $\eta_{initial}$ after different pressure treatments at 40 °C. For both normal rice starch and waxy rice starch, the plot of $\eta_{initial}$ against pressure exhibited a sigmoidal-shaped curve. The $\eta_{initial}$ value did not change markedly until a critical level of pressure was applied, which was approximately 350 MPa for normal rice starch and 300 MPa for waxy rice starch. Above these critical pressures, there was a phase where $\eta_{initial}$ increased sharply as the treatment pressure was increased. This phase was between 350 and 500 MPa for normal rice starch and between 300 and 500 MPa for waxy rice starch. Above 500 MPa, both starch types showed no further increase in $\eta_{initial}$.

The effect of the duration of pressure treatment at 40 °C on $\eta_{initial}$ of the starch suspensions was also examined (Fig. 3). At 300 MPa, a small but significant increase in $\eta_{initial}$ of normal rice starch was observed with increased duration of pressure treatment (Fig. 3A). When the treat-
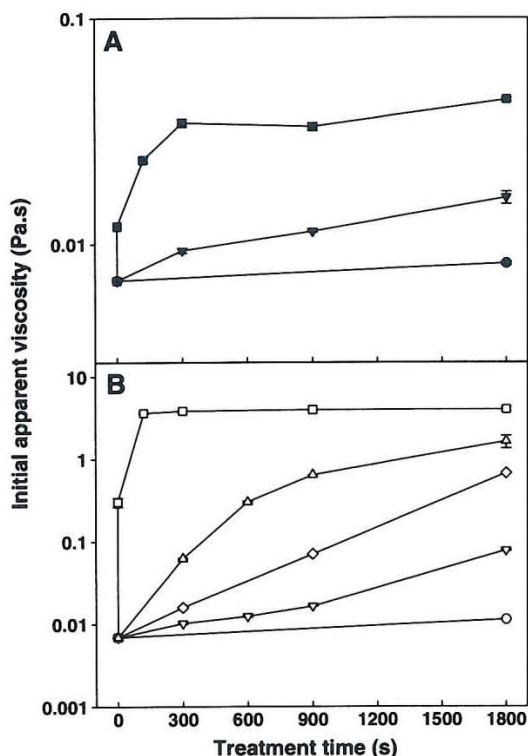
Fig. 3. Initial apparent viscosity as a function of treatment duration. (A) Normal rice starch after pressure treatment at 300 MPa (●), 400 MPa (▼), and 500 MPa (■). (B) Waxy rice starch after pressure treatment at 300 MPa (○), 350 MPa (▽), 375 MPa (◇), 400 MPa (△), and 500 MPa (□). The temperature at treatment was 40 °C.

Fig. 4. Initial apparent viscosity as a function of temperature at treatment. (A) Normal rice starch after no pressure treatment (control) (●), pressure treatment at 400 MPa (▼), and pressure treatment at 500 MPa (■). (B) Waxy rice starch after no pressure treatment (control) (○), pressure treatment at 350 MPa (▽), and pressure treatment at 500 MPa (□). The treatment duration was 30 min.

ment pressure was increased to 400 MPa, $\eta_{initial}$ increased gradually with treatment time. At 500 MPa, there was a sharp increase in $\eta_{initial}$ over the first 300 s of pressure treatment but prolonged treatment did not result in a significant further increase in $\eta_{initial}$.

Waxy rice starch showed a similar behaviour to that observed for normal rice starch (Fig. 3B). The treatment pressure of 300 MPa led to a slight increase in $\eta_{initial}$ with increased duration of pressure treatment. At 350 MPa, $\eta_{initial}$ increased steadily with treatment time, whereas, at 375 MPa, the increase in $\eta_{initial}$ was linear ($R^2 = 0.98$) with treatment duration. When the treatment pressure was increased to 400 MPa, the increase in $\eta_{initial}$ was no longer proportional to the treatment duration. At 400 MPa, $\eta_{initial}$ increased considerably after 300 s of pressure treatment and the increase in $\eta_{initial}$ was more gradual with longer treatment. At 500 MPa, $\eta_{initial}$ increased abruptly after the first 120 s of pressure treatment and did not change further as the treatment duration was extended.

The effect of temperature at pressure treatment on $\eta_{initial}$ of the starch suspensions is shown in Fig. 4. The set temperatures at pressurization were 10, 20, 40, and 60 °C. As a result of the adiabatic heating (~1.9 °C/100 MPa), the

temperature increased by up to 9.5 °C at 500 MPa and then decreased back to the set temperature within 5 min of the set holding time of 30 min. As T_{onset} was 64.5 °C for normal rice starch and 60.1 °C for waxy rice starch when the set temperature at treatment was 10, 20, or 40 °C, the temperature of the unit stayed below T_{onset} for both starch types even though adiabatic heating occurred. However, when the set temperature at treatment was 60 °C, the adiabatic heating increased the temperature of the unit above T_{onset} for both starch types, especially at 500 MPa when the temperature increased to ~69.5 °C.

The $\eta_{initial}$ value of untreated normal rice starch was essentially constant when it was held at temperatures between 10 and 40 °C, which were well below T_{onset} (64.5 °C) (Fig. 4A). There was a small but significant increase in $\eta_{initial}$ of the untreated control sample at 60 °C as it was held near T_{onset}. At 400 MPa, $\eta_{initial}$ increased gradually as the temperature was increased from 10 to 60 °C. At 500 MPa, normal rice starch increased in $\eta_{initial}$ when the temperature was increased from 10 to 60 °C. For untreated waxy rice starch, $\eta_{initial}$ increased only when the temperature was increased to 60 °C, which was T_{onset} (Fig. 4B). At 350 MPa, $\eta_{initial}$ increased with an increase

in temperature at treatment from 20 to 60 °C. However, $\eta_{initial}$ of waxy rice starch was not affected by the temperature at treatment when the treatment pressure was increased to 500 MPa, with a high $\eta_{initial}$ observed at all temperatures. At 500 MPa, waxy rice starch was completely gelatinized even at the lowest temperature at pressurization (10 °C).

3.3. Degree of swelling

Degree of swelling was defined here as the volume fraction of the centrifuged sediment relative to the volume of total sample, calculated using Eq. (1). During thermal gelatinization, water molecules form hydrogen bonds with the exposed hydroxyl groups of amylose and amylopectin in starch, causing swelling of the starch granules (Ratnayake, Hoover, & Warkentin, 2002). Similarly, when pressure is applied to starch-in-water suspension, water molecules enter into starch granules and form hydrogen bonds with starch polymers. At the individual granule level, this means an increase in granule size (swelling). However, when considering the whole system at the suspension level, such linkages between starch polymers and water reduce the bulk suspension volume (Douzals et al., 1996). Since phenomena that result in volume reduction is favoured under pressure, hydration of starch granules (swelling) can be induced by pressure instead of heating. The degree of swelling after different pressure treatments is shown in Fig. 5A.

In normal rice starch, the degree of swelling did not change until the treatment pressure was greater than 300 MPa and then increased rapidly as the treatment pressure increased up to 500 MPa. The maximum degree of swelling was approximately 50%. Waxy rice starch showed a minor increase in the degree of swelling at treatment pressures below 300 MPa. The degree of swelling then increased very sharply between 300 and 400 MPa and reached 100% at 400 MPa.

Although waxy rice starch showed 100% swelling and normal rice starch could reach only 50% swelling, the degree of swelling curves of both starches had similar sigmoidal shapes and were also similar to the $\eta_{initial}$ curves in Fig. 2C. In fact, there was a linear relationship between the degree of swelling and $\eta_{initial}$ of starch suspensions after all pressure treatments and a single regression line represented the results from both normal rice starch and waxy rice starch (Fig. 5B). It was clear that the swelling of starch granules was correlated with the increase in $\eta_{initial}$.

3.4. Light microscopy

The radial orientation of crystallites in native starch granules causes the characteristic birefringence (Maltese cross-pattern) under a polarized light microscope (Yuryev, Wasserman, Andreev, & Tolstoguzov, 2002). As starch undergoes a phase transition from the ordered state to a disordered state during gelatinization, it loses crystallinity which leads to loss of this birefringence (Ratnayake
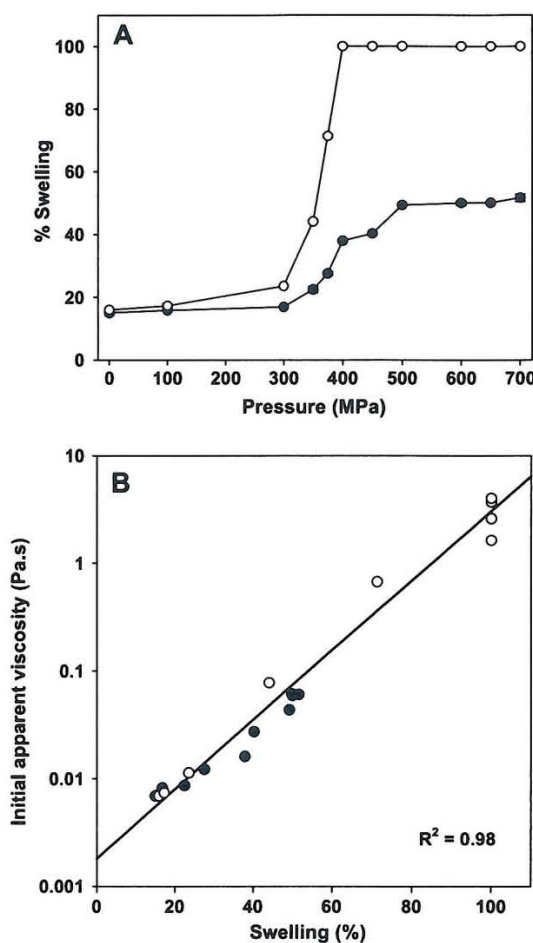
Fig. 5. (A) Degree of swelling (%) as a function of treatment pressure. (B) Plot of swelling (%) versus initial apparent viscosity for normal rice starch (●) and waxy rice starch (○). The temperature at treatment was 40 °C and the treatment duration was 30 min.

et al., 2002). Fig. 6 shows the change in birefringence of starch granules at different stages of starch gelatinization. Untreated starch granules of both starch types had characteristic birefringence patterns (Fig. 6A1 and B1). The starch samples shown in Fig. 6A2 and B2 were those that corresponded to the midpoints (approximately) of the rapid increasing phase on the $\eta_{initial}$ and degree of swelling curves for normal and waxy rice starch, respectively (Fig. 2C and Fig. 5A). The treatment pressures at the midpoints were 400 MPa for normal rice starch and 350 MPa for waxy rice starch. A number of normal rice starch granules lost birefringence after treatment at 400 MPa (Fig. 6A2). Similarly, some loss of birefringence was observed in waxy rice starch after treatment at 350 MPa (Fig. 6B2). After treatment at 500 MPa, at which point the $\eta_{initial}$ and degree of swelling curves for both starches had plateaued (Fig. 2C and Fig. 5A), no birefringence
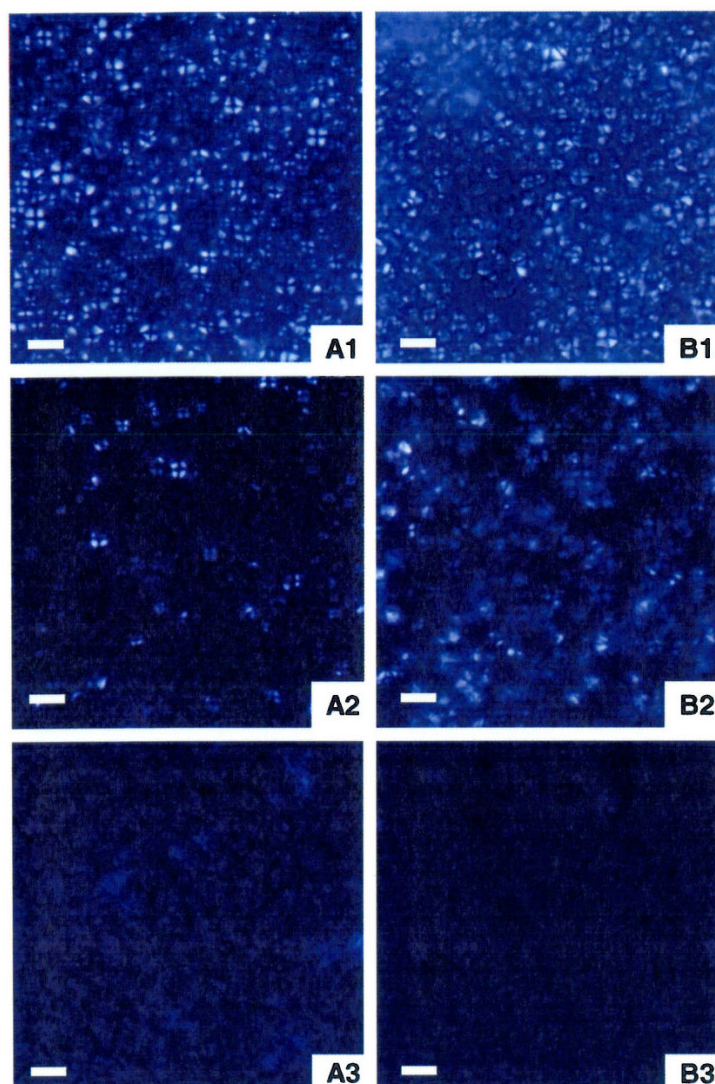
Fig. 6. Polarized light micrographs. (A) Normal rice starch suspension after [A1] no pressure treatment, [A2] pressure treatment at 400 MPa, and [A3] pressure treatment at 500 MPa. (B) Waxy rice starch suspension after [B1] no pressure treatment, [B2] pressure treatment at 350 MPa, and [B3] pressure treatment at 500 MPa. The bar is 20 μm. The temperature at treatment was 40 °C and the treatment duration was 30 min.

was observed in either of the starches (Fig. 6A3 and B3) despite the different extents of change in $\eta_{initial}$ and the degree of swelling between the two starches (Fig. 2C and Fig. 5A).

The micrographs in Fig. 7 were taken without the polarizing filter to observe the granular structure of the starches. Untreated samples of both normal rice starch and waxy rice starch showed intact granular structures (Fig. 7A1 and B1). After the treatment at 500 MPa, normal rice starch appeared to be swollen but still retained the granular structure (Fig. 7A2). In contrast, after the same treatment, waxy rice starch lost most of its granular structure and only a few swollen granules and

fragments were observed after the same treatment (Fig. 7B2). This suggests that, for normal rice starch, swelling of starch granules during the pressure treatment was sufficient to distort the crystalline region of starch, as indicated by the loss of birefringence, but not enough to disrupt the granular structure. The observation also confirms the incomplete swelling (to only 50%) of normal rice starch shown in Fig. 5A, which in turn resulted in the smaller increase in $\eta_{initial}$ compared with waxy rice starch (Fig. 2C). For waxy rice starch, the almost complete disruption of the granule structure led to 100% swelling (Fig. 5A) and a much higher $\eta_{initial}$ than measured for normal rice starch (Fig. 2C).
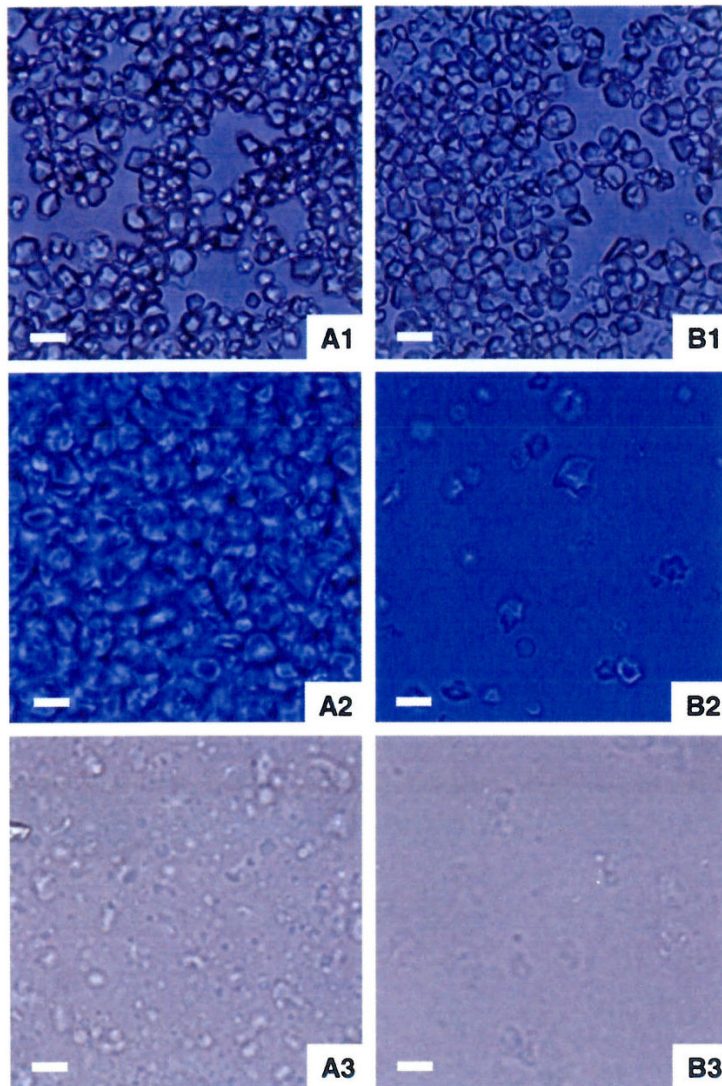
Fig. 7. Light micrographs without polarizing filter. (A) Normal rice starch suspension; (B) Waxy rice starch suspension. After no pressure treatment [A1 and B1], after pressure treatment at 500 MPa [A2 and B2], and after pressure treatment at 500 MPa and subsequent pasting [A3 and B3]. The bar is 20 μm. The temperature at treatment was 40 °C and the treatment duration was 30 min.

The granular structure of normal rice starch, which was still observed after the pressure treatment, was destroyed after the subsequent pasting (Fig. 7A3). This breakdown of granules accounts for the apparent viscosity increase of the pressure-treated sample during pasting (Fig. 2A). The fragments of waxy rice starch observed in Fig. 7B2 was also further broken down after pasting (Fig. 7B3).

3.5. Leaching of starch and amylose

Table 1 summarizes the leached starch and amylose analyses. "Leached starch" was defined as the amount of starch in the supernatant relative to the total starch in the suspension. "Amylose in leached starch" was defined as the percentage of amylose in the leached starch. "Leached amylose" was defined as the amount of amylose in the supernatant relative to the total amylose in the starch suspension. The amounts of leached starch and leached amylose after pressure treatment were very low in both starch types but some trends in the results were found.

For normal rice starch, the leached starch in the sample increased as the treatment pressure increased up to 400 MPa. However, the amount of leached starch did not change significantly when treatment pressure increased

Table 1
Average amount of starch leached from granules, amount of leached amylose and percentage of amylose in the leached starch from granules after pressure treatments ($n = 2$)

	Leached starch (% w/w)	Leached amylose (% w/w)	Amylose in leached starch (% w/w)
Normal rice			
No treatment	1.70	1.10	10.50
350 MPa	1.98	1.71	14.30
400 MPa	2.07	2.22	16.57
500 MPa	2.16	2.22	16.09
600 MPa	2.48	2.39	15.41
700 MPa	2.38	2.07	14.60
Pooled SD[a]	0.07	0.33	2.19
Waxy rice			
No treatment	1.53	2.70	4.90
100 MPa	1.62	2.80	4.87
300 MPa	2.43	5.72	6.63
350 MPa	2.81	6.20	6.17
Pooled SD[a]	0.17	1.04	0.69

The temperature at treatment was 40 °C and the treatment duration was 30 min.

[a] Pooled standard deviation of values in the same column.

above 400 MPa. For waxy rice starch, the leached starch increased from 1.53% (w/w) when untreated to 2.81% (w/w) after the treatment at 350 MPa.

In untreated normal rice starch suspensions, approximately 1.1% (w/w) of amylose leached from starch granules into the aqueous phase and this figure increased slightly to 1.7% (w/w) after treatment at 350 MPa. The amount of leached amylose did not change significantly from 400 to 700 MPa and was around 2% (w/w). The percentage of amylose in leached starch was slightly higher after pressure treatment than in the untreated sample but the results for the samples that received pressure treatments (350–700 MPa) were not significantly different.

In waxy rice starch suspensions, the leached amylose increased steadily with treatment pressure, from 2.7% (w/w) when untreated to 6.2% (w/w) after treatment at 350 MPa, which was higher than for normal rice starch after treatment at all pressures, i.e., a maximum of ~2% (w/w). Above 350 MPa, the waxy rice starch suspensions were too viscous to separate the aqueous phase by centrifugation. However, given that the majority of the waxy rice starch granules had been disintegrated after the 500 MPa treatment (Fig. 7B2), it can be assumed that most starch material in the granules, including amylose, would have leached into the aqueous phase eventually as the treatment pressure increased. On a dry basis, waxy rice starch contains approximately 3% amylose and normal rice starch contains a higher amount, ~16% amylose.

4. Discussion

The effects of increasing temperature are essentially energy and volume effects due to thermal expansivity (Balny, Masson, & Heremans, 2002). In contrast, the effects of pressure are mainly volume effects through compressibility of the system (Balny et al., 2002). According to Douzals et al. (1996), at pressures over 300 MPa, the reduction in volume is greater for a wheat starch suspension than for pure water at the same pressure. This indicates that the water molecules linked with starch occupy a smaller volume than the molecules in pure water. Consequently, uptake of water by starch granules occurs under pressure in order to reduce the suspension volume; hence gelatinization of starch is induced. The in-situ FTIR study by Rubens, Snauwaert, Heremans, and Stute (1999) showed that the amorphous regions of the starch granule are hydrated first, similar to heat-induced gelatinization. This hydration induces swelling of the granules, leading to distortion of the crystalline regions which then become more accessible for water.

The present study explored aspects of the pressure-induced gelatinization of normal and waxy rice starches. By using $\eta_{initial}$ as an indicator for the degree of gelatinization, we showed that pressure treatments gelatinized normal rice starch and waxy rice starch to different extents depending on the treatment pressure, the duration, and the temperature at treatment. Although loss of birefringence is often used as an indicator of starch gelatinization, whether induced by heat or pressure, its limitation in determining the degree of pressure-induced gelatinization objectively and quantitatively has been acknowledged in the literature (Kawai, Fukami, & Yamamoto, 2007; Stute et al., 1996). For example, Stute et al. (1996) reported that many corn starch granules showed a partial loss or "fading out" of birefringence so that distinguishing between gelatinized and non-gelatinized granules was difficult.

In contrast, the $\eta_{initial}$ method used in this study provides an objective and analytical means of determining the degree of pressure-induced gelatinization of starch. The viscosity measurement encompasses the swelling and the leaching of starch material that would occur during starch gelatinization. This study established that the increase in $\eta_{initial}$ of starch suspensions after pressure treatment is directly correlated with the degree of swelling (Fig. 5B). Likewise, in thermal gelatinization of starch, Bagley, Christianson, and Beckwith (1983) showed that the viscosity of starch suspensions correlated directly with the volume fractions of the swollen granules when leaching of soluble material was insignificant.

The relationship between $\eta_{initial}$ and treatment pressure followed a sigmoidal-shaped curve in both starches (Fig. 2C). Such sigmoidal curve shapes, which also represented the relationship between the degree of swelling and the treatment pressure (Fig. 5A), seem to be typical of pressure-induced starch gelatinization. Bauer and Knorr (2005) used the ratio of starch granules having lost birefringence as an indicator for the degree of gelatinization and reported similar sigmoidal curves for pressure-induced gelatinization of wheat and tapioca starches. The swelling index curve for wheat starch presented by Douzals et al. (1998) also exhibited a sigmoidal shape.

5-12

The sigmoidal-shaped gelatinization curve means that pressure-induced gelatinization occurred over a pressure range and that the treatment pressure had to be above a critical level for gelatinization to occur effectively. Individual granules of starch in the population of normal rice starch or waxy rice starch have different degrees of association between starch polymers in the amorphous regions (Glicksman, 1969), which suggests that they will pose different resistances to water uptake. Therefore, it can be assumed that the critical pressure is the pressure at which granules with, overall, the weakest associations between starch polymers start breaking and that granules with stronger associations will subsequently swell over a pressure range. Likewise, the effect of treatment duration on the degree of gelatinization ($\eta_{initial}$) observed in Fig. 3 can be explained. Granules with weaker associations between starch polymers will gelatinize early and those with stronger associations will gelatinize later during a pressure treatment (Glicksman, 1969).

However, treatment duration and $\eta_{initial}$ displayed different types of relationship depending on the treatment pressure (Fig. 3). Although different treatment pressure and duration combinations can result in the same $\eta_{initial}$ value of a starch suspension, the channelling of water into the starch granules during pressurization may not necessarily occur in the same way under different treatment pressures. It is also possible that the observed effects of treatment pressure, temperature at pressurization, and treatment duration on starch gelatinization are kinetic effects (Figs. 3 and 4).

The effect of temperature at treatment on the gelatinization of normal and waxy rice starches (Fig. 4) can be related to the pressure–temperature (P–T) diagram of wheat starch suspensions, shown by Douzals, Perrier-Cornet, Coquille, and Gervais (2001). The P–T diagram of wheat starch suspension was divided into three zones. "Zone A" corresponded to high temperatures (about 40–76 °C) and low pressures (<300 MPa) where the degree of gelatinization could be increased by increasing either the pressure or the temperature. "Zone B" corresponded to higher pressures (>300 MPa) and temperatures of 0–40 °C where there was almost no influence of temperature on gelatinization. "Zone C" corresponded to subzero temperatures, where an increase in pressure resulted in an increase in gelatinization temperature. Because of the differences in starch type and the method for determining gelatinization, the P–T diagrams of the normal and waxy rice starches used in this study would have a slightly different shape from that in Douzals et al. (2001). Nevertheless, assuming similar trends, examples for Zone A in our study include normal rice starch suspensions that received pressure treatment at 400 MPa at temperatures of 20–60 °C and waxy rice starch suspensions that received pressure treatment at 350 MPa at 20–60 °C (Fig. 4). The degree of gelatinization ($\eta_{initial}$) of these samples increased at a constant pressure as the treatment temperature was increased. At 500 MPa, the gelatinization of the waxy rice starch

suspension was unaffected by the temperature at treatment, which can be related to Zone B type behaviour (Fig. 4B).

Although the two different starches (normal rice starch and waxy rice starch) both lost all birefringence after pressure treatment at 500 MPa (Fig. 6), their pressure-induced gelatinization characteristics were different. This was demonstrated by examining the pasting curves (Fig. 2A and B) and the degree of swelling (Fig. 5A). Normal rice starch maintained the granular entity more effectively than waxy rice starch (Fig. 7). It can be assumed that the granular entities seen in the normal rice starch (Fig. 7A2) were swollen starch granules rather than starch "ghosts", as the leaching of starch was minimal (Table 1) and the viscosity of the sample increased considerably during subsequent pasting (Fig. 2A). In contrast, the waxy rice starch sample can be considered to contain starch ghosts only (Fig. 7B2) as no further gelatinization-related changes occurred upon further heating. Blaszczak, Fornal, Valverde, and Garrido (2005) showed a similar trend when waxy corn starch and high-amylose corn starch suspensions were compared. Pressure treatment at 650 MPa for 6 min resulted in a complete breakdown of the granules in waxy corn starch, whereas the high-amylose corn starch retained a granular structure (Blaszczak et al., 2005).

Buckow, Heinz, and Knorr (2007) suggested that disintegration of the crystalline region was not completed by pressure because the side-by-side dissociation and helix unwinding of amylopectin units might be suppressed as van der Waals' forces and hydrogen bonds are stabilized, which should favor the helix structure. Although this proposal may explain the pressure behaviour of normal type starches such as the maize starch used by Buckow et al. (2007) or the normal rice starch in this study, it does not explain how the crystalline structure of waxy type starches can be disintegrated completely by pressure.

Starch is composed primarily of a mixture of two polymers, amylopectin and amylose (Parker & Ring, 2001). Normal rice starch contains 16% (w/w of total starch) amylose and waxy rice starch contains considerably less amylose, 3% (w/w of total starch). Collapse of the crystalline structure of waxy rice starch, which contains only a small amount of amylose (3%), indicates that the amylopectin crystalline structure can be destroyed by pressure. Although crystallinity of starch granules is formed by the ordering of amylopectin chains (BeMiller & Whistler, 1996), the extent of interaction of the starch chains within the amorphous and crystalline regions can be affected by a number of other factors such as the amylose/amylopectin ratio and the characteristics of amylose and amylopectin in terms of molecular weight distribution, degree and length of branching, and conformation (Hoover, 2001). It seems possible that amylose, which occurs among the amylopectin molecules in starch, contributes to the different susceptibilities of normal and waxy rice starches to pressure in terms of preserving the granular entity.

It can be speculated that, when starch contains more amylose, such as normal rice starch, amylose and displaced

amylopectin in starch may develop a more pressure-stable arrangement. In other words, amylose may form thermodynamically favorable complexes with displaced amylopectin molecules instead of leaching into the aqueous phase. Similarly, in thermal gelatinisation, Debet and Gidley (2007) proposed that slower swelling starches (non-waxy or tuber starches) give sufficient time and polymer concentration for glucan cross-linking to take place. Also, the pressure treatments in this study were carried out at 40 °C, if the amylose in the normal rice starch was solubilized during pressure treatment, it may have formed a gel structure within the starch granules, incorporating displaced amylopectin units, similar to the amylose gel network that can be formed on cooling after thermal gelatinization (Morris, 1990).

Debet and Gidley (2006) stated that lipid, protein and amylose are all necessary to restrict swelling in wheat and maize starches (non-waxy). In particular, Kuakpetoon and Wang (2007) suggested that more amylose was present in the form of an amylose–lipid complex in the outer 10% layer of the starch granules than in the core. This could restrict swelling of granules and leaching of amylose. For amylose-containing corn starches, more long chain amylopectin molecules were found in this region which could also contribute to restricted swelling and leaching. Normal rice starch used in this study not only contained more amylose than waxy rice starch but also slightly more fat and protein. These small compositional differences between the two starch types may, in part, contribute to the different pressure-induced gelatinization characteristics. However, further studies are required to determine the significance of such compositional differences.

It remains unknown if further changes can occur in normal rice starch when the treatment pressure is increased beyond the levels used in this study. Bowler, Williams, and Angold (1980) described the swelling behaviour of cereal starches during heating as a two-stage phenomenon. In the first stage, starch granules swell radially. The rate of amylose leaching is relatively slow during the first stage of swelling (Hermansson & Svegmark, 1996). Amylose–lipid complexes are believed to restrict swelling in the first stage as they do not dissociate unless heated above 94–98 °C (Hermansson & Svegmark, 1996; Zeleznak & Hoseney, 1987). Once the temperature is sufficiently high, the amylose–lipid complexes dissolve and amylose leaches out, and the granules swell tangentially, deform, and lose their original shape in the second stage (Hermansson and Svegmark, 1996). If the swelling behaviour of starch under pressure follows the same stages as that described for thermal gelatinization, it can be speculated that the treatment pressures used in this study (up to 700 MPa) did not solubilize the amylose complexes that may have existed.

5. Conclusions

Both normal rice starch and waxy rice starch followed sigmoidal-shaped pressure-induced gelatinization curves.

The degree of starch gelatinization ($\eta_{initial}$) was dependent on the treatment pressure, the temperature at pressure treatment, and the treatment duration, and different combinations of these factors could result in the same degree of gelatinization. There was a linear relationship between the degree of swelling and $\eta_{initial}$, indicating contribution of swelling to $\eta_{initial}$ increases. Disappearance of the characteristic birefringence in the starch granules was observed with an increase in treatment pressure for both starches. After treatments at $\geqslant 500$ MPa, both starches lost all birefringence despite the different extents of change in $\eta_{initial}$ and the degree of swelling. In terms of industrial application, the $\eta_{initial}$ results show product viscosity changes due to pressure treatment and the pasting profiles show the further changes in viscosity that might occur in the subsequent heating processes.

Acknowledgements

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5.1 Effect of static heating on the starch-in-water suspensions

5.1.1 Introduction

Pressure treatments are always conducted under static conditions, as it is not possible to stir the starch suspension samples during the pressure treatment. Therefore, it was necessary to test whether the static conditions could affect the degree of gelatinisation and the viscosity of the suspension. In particular, normal rice starch (16% amylose) was only partially gelatinised based on the $\eta_{initial}$ measurements, by the treatment pressures used in this study unlike waxy rice starch (3% amylose) which was completely gelatinised. However, both starch types showed complete loss of birefringence after pressure treatment at $\geq$500 MPa. It is important to examine if static conditions during pressure treatment affected the two starch types differently as they have different compositions or, the observed differences between the two starches were solely due to the different susceptibility to pressure treatment

5.1.2 Materials and methods

Normal rice starch and waxy rice starch-in-water suspensions were prepared as described in section 3.2.4. Static heating (55-90°C) of suspensions was carried out in a water bath as described in section 3.4.1. The samples were examined for pasting curves as described in section 3.5.1.

5.1.3 Results and Discussion

Figures 5-1A and 1B show the pasting curves for normal rice starch and waxy rice starch suspensions after different heat treatments. The T_{onset} was 64.5 °C for normal rice starch and 60.1 °C for waxy rice starch. Untreated waxy rice starch suspensions showed a rapid increase in apparent viscosity over a narrow temperature range, so that T_{peak} was 72 °C. The apparent viscosity increase for normal rice starch after T_{onset} was more gradual and over a wider temperature range, so that T_{peak} was 92

°C. This can be attributed to restrained swelling of amylose containing starches such as normal rice starch caused by the amylose-lipid complexes that dissolve at temperatures higher than the gelatinisation temperature (Hermansson and Svegmark, 1996).

Figure 5-1C shows the $\eta_{initial}$ (degree of gelatinisation) of normal rice and waxy rice starch suspensions against the treatment temperature. The onset of gelatinisation was observed at 60°C for normal rice starch. The degree of gelatinisation of normal rice starch increased gradually as the treatment temperature increased so that after the treatment at 90°C, the starch was completely gelatinised (**Figure 5-1A & 1C**). In comparison, normal rice starch was not completely gelatinised by pressure treatment up to 700 MPa (Fig. 2, page 5-5).

Waxy rice starch required lower treatment temperature to be gelatinised completely. Waxy rice starch was partially gelatinised after heat treatment at 60°C (**Figure 5-1C**). Further increase in the treatment temperature by 5 degrees resulted in an almost complete gelatinisation of waxy rice starch as indicated by the $\eta_{initial}$. This is consistent with the gelatinisation behaviour of waxy-type starches which contain minimal or no amylose to restrain the swelling of starch granules (Hermansson and Svegmark, 1996). The heat-induced gelatinisation curve of waxy rice starch shown in **Figure 5-1C** resembles the pressure-induced gelatinisation curve shown in Fig. 2C (page 5-5).

The results indicate that stirring of starch suspension is not a requirement for a complete heat-induced gelatinisation of normal rice starch or waxy rice starch. Starch can be gelatinised under static conditions when the treatment temperature (driving force) was sufficiently high regardless of the amylose content in starches. Hence, it is suggested that the observed differences in the pressure-induced gelatinisation

behaviour between the two starches shown in Fig. 2 (page 5-8) were not due to the effect of static conditions during pressure treatments. Static conditions during pressure treatment was not the reason why the $\eta_{initial}$ of normal rice starch did not increase to the same extent as that of waxy rice starch.
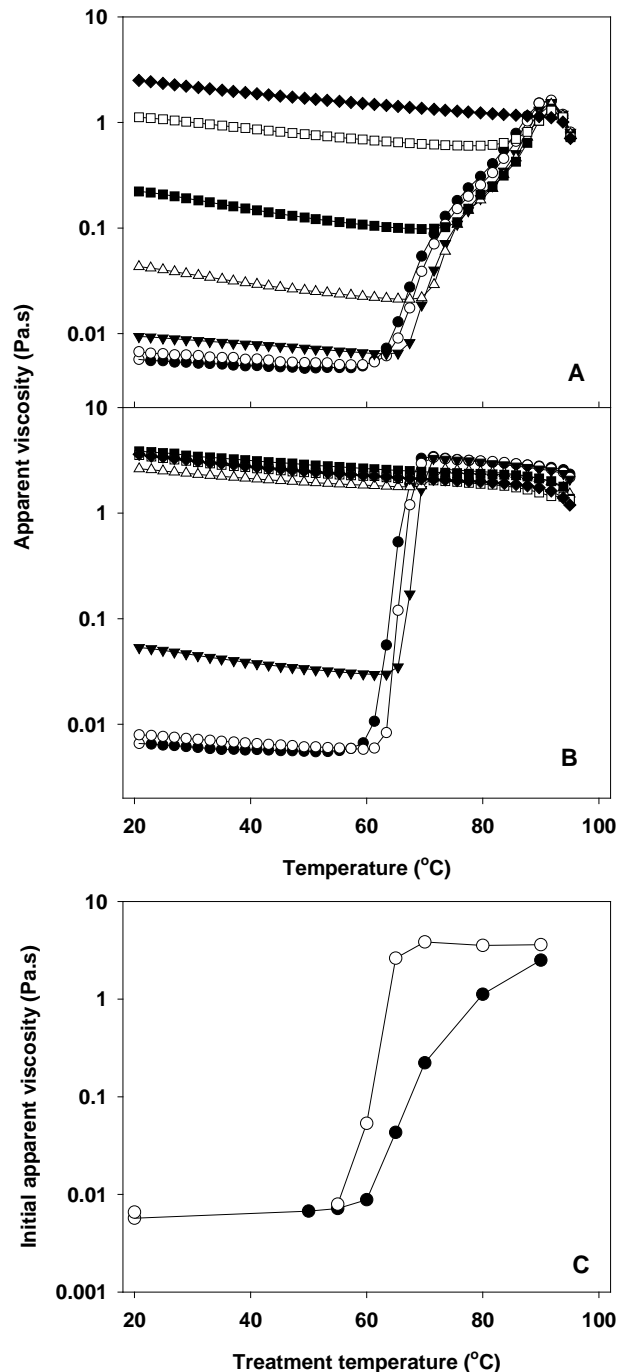
Figure 5-1: Pasting curves for normal rice starch (A) and waxy rice starch (B) after no heat treatment (control) (●), 55°C (○), 60°C (▼), 65°C (△), 70°C (■), 80°C (□) and 90°C (◆); (C) Initial apparent viscosity ($\eta_{initial}$) of normal rice starch (●) and waxy rice starch (○) as a function of heating temperature. The treatment duration was 30 min for all samples.

5.1.4 Conclusions

Static conditions of heat treatment did not prevent starches from being completely gelatinised. Both normal rice starch and waxy rice starch can be completely gelatinised under static conditions provided that the treatment temperature is sufficiently high.

5.1.5 References

Hermansson, A. M. and K. Svegmark (1996). "Developments in the understanding of starch functionality." *Trends in Food Science & Technology* **7**(11): 345-353.

CHAPTER 6

Effect of Different Components in Skim Milk on High-Pressure-Induced Gelatinisation of Waxy Rice Starch and Normal Rice Starch

This chapter presents a study of the pressure-induced gelatinisation behaviour of normal and waxy rice starches in skim milk and the effects of skim milk components on the pressure-induced gelatinisation of the starches. The results from this chapter have been published in the following article: [1]Oh, H. E., S. G. Anema, D. N. Pinder and M. Wong (2009). "Effects of different components in skim milk on high-pressure-induced gelatinisation of waxy rice starch and normal rice starch." *Food Chemistry* 113(1): 1-8. This article can be found on page 6-3 of this chapter

Aim. To study the pressure-induced gelatinisation of starch in skim milk.

Relevance. Starch is often used as an ingredient in a variety of food systems and may undergo gelatinisation in an aqueous environment in the presence of other food components. However, the gelatinisation properties of starch may be affected by the other components such as sugars, minerals and proteins in food systems. This study investigates the pressure-induced gelatinisation properties of normal and waxy rice starches when the starches were suspended in skim milk and other aqueous solutions/suspensions containing dairy components, and compares this with suspending them in water. The hypothesis in this chapter was that pressure-induced gelatinisation properties of starches can be affected by the presence of dairy components in the suspension medium. The results from this study will contribute towards the understanding of the interactions between starch molecules and components in the suspension medium under pressure which have not been reported extensively in the literature.

Approach. The effect of varying the skim milk concentration on starch gelatinisation was examined first to test the effect of skim milk solids on the pressure-induced gelatinisation behaviour of waxy and normal rice starches. The effect of individual

[1]- Oh et al. (2009) contains the original work of the author H. E. Oh and has been written by the author H. E. Oh.

skim milk components in the suspension medium: lactose, soluble milk minerals and milk proteins (casein and whey proteins); on starch gelatinisation was then examined. The rheological methods ($\eta_{initial}$ measurements and pasting curves), degree of swelling and light microscopy techniques as used in the experiments described in Chapter 5 were employed to study the degree of starch gelatinisation in dairy suspensions.

Summary of results. Normal rice starch and waxy rice starch suspended skim milk were found to be gelatinised by pressure. Similarly to the pressure-induced gelatinisation in water, the relationship between the degree of gelatinisation of both starches and the treatment pressure showed a sigmoidal-shaped curve. However, the pressure-induced gelatinisation of starch was retarded in skim milk (i.e. a higher pressure was required to achieve the same degree of gelatinisation). This was attributed to the effect of soluble milk minerals and lactose. Milk proteins (casein and whey protein) did not affect the degree of pressure-induced gelatinisation at the concentrations of these components in skim milk at 10% (w/w) total solids.

Conclusions.

- Starch in skim milk can be gelatinised by pressure treatment. However, a higher treatment pressure is required to induce gelatinisation of starch when it was suspended in skim milk compared with that in water.

- Lactose or soluble milk minerals were responsible for retarding starch gelatinisation under pressure.

- The gelatinisation retarding effects of lactose and soluble milk minerals were additive to each other.

- Milk proteins at the concentration used in this study did not affect starch gelatinisation.
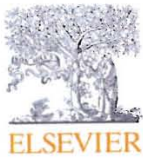
Rapid Communication

Effects of different components in skim milk on high-pressure-induced gelatinisation of waxy rice starch and normal rice starch

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ABSTRACT

The gelatinisation of starch in skim milk required higher pressures than did the gelatinisation of starch in water. This study examined the effects of various milk components on the pressure-induced gelatinisation of waxy rice starch and normal rice starch, in order to understand the differences between the gelatinisation characteristics of starch in skim milk and starch in water. Gelatinisation was retarded in skim milk, which was attributed to the effects of soluble milk minerals and lactose. The presence of these components may reduce the plasticising ability of the suspension medium. Direct interactions between the milk components and starch molecules may also contribute to retarded gelatinisation. Milk proteins (casein and whey protein) did not affect the degree of pressure-induced gelatinisation at the concentrations of these components in skim milk, at 10% total solids.

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1. Introduction

Starch is used as an ingredient in a range of food systems, and controlling its gelatinisation is often critical in the manufacture of starch-containing foods. Although gelatinisation is commonly induced by heating of an aqueous starch suspension, it is known that starch can also be gelatinised by high pressure. Douzals, Marechal, Coquille, and Gervais (1996) showed that water molecules linked with starch occupy a smaller volume than do pure water molecules. Because events that result in volume reduction are favoured under pressure, the hydration of starch granules can be induced by pressure instead of by heating. Whether induced by heat or pressure, starch gelatinisation is a swelling phenomenon, which starts as water from the suspension enters starch granules (Rubens, Snauwaert, Heremans, & Stute, 1999).

Skim milk contains various components, such as water, proteins, lactose and minerals. Some of the components in skim milk are known to affect the thermal gelatinisation of starch. Noisuwan, Bronlund, Wilkinson, and Hemar (2008) showed that different milk protein products affect the pasting behaviour of starch differently. For example, the peak viscosity temperature of normal rice starch was increased by skim milk powder and sodium caseinate but de-creased by whey protein isolate. Bertolini, Creamer, Eppink, and Boland (2005) indicated a possible interaction between starch and sodium caseinate. Gels formed from a mixture of starch (cassava, waxy corn, corn, wheat or rice) and sodium caseinate resulted in higher storage modulus and viscosity than did gels formed from starch alone. Also, the onset temperature, the peak temperature and the end temperature of starch gelatinisation were delayed when sodium caseinate was added (Bertolini et al., 2005). Matser and Steeneken (1997) showed that the addition of lactose caused highly cross-linked waxy maize starch to gelatinise at a higher temperature than that when no lactose was present. Lactose addition also increased the storage modulus of the gelatinised starch system.

In dairy product applications, starch can be used as a thickener, a fat replacer or a filler. In order to achieve the desired product functionality in starch-containing foods, it is important to understand the effects of other components in the environment on the gelatinisation characteristics of the starch. Although pressure-induced gelatinisation of starch-in-water suspensions has been studied by a number of researchers, the effect of changing the suspension medium from water to milk (or the effect of the various milk components) on starch gelatinisation has not been investigated.

In this study, pressure-induced gelatinisation, of waxy rice starch and normal rice starch in skim milk media, was investigated

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and compared with that in water. The effect of varying the skim milk concentration and the effects of adding different milk components to the suspension medium on the degree of gelatinisation were examined. The results of this study will be useful in predicting the functional properties of starch in dairy applications that involve high pressure processing.

2. Materials and methods

2.1. Materials

Unmodified normal rice starch (12% moisture, 0.09% fat, 0.13% protein and 0.06% ash) and waxy rice starch (11% moisture, 0.07% fat, 0.06% protein and 0.08% ash) were obtained from Remy Industries (Leuven–Wijgmaal, Belgium) and were used as supplied. The starches were stored in air-tight containers at ambient temperature. Low heat skim milk powder was obtained from the Edendale site, Fonterra Co-operative Group Limited, New Zealand, and was stored in multilayered foil bags at ambient temperature. The skim milk powder was composed of 33% protein, 54% lactose, 3.8% moisture, 0.8% fat and 8.4% ash. Lactose was obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of suspension media

Reconstituted skim milk samples were prepared by adding low heat skim milk powder to purified water (reverse osmosis, followed by filtration through a Milli-Q apparatus) to final concentrations of 5–15% (w/w) total solids (TS). The reconstituted skim milk was stirred for at least 1 h and stored overnight at ambient temperature (approximately 20 °C) before use.

Simulated milk ultrafiltrate (SMUF) was prepared using the reagents and methods described by Jenness and Koops (1962). SMUF contains soluble milk minerals equivalent to their concentrations in 10% TS skim milk. Lactose was added to appropriate samples at 5% w/w, which is similar to its concentration in 10% TS skim milk.

Whey-protein- and lactose-depleted (WPLD) milk was prepared by filtering reconstituted 10% TS skim milk through a microfiltration membrane (Vivaflow 200, 0.2 µm pore size, Sartorius Stedim Biotech, Aubagne, France). The whey proteins and lactose were removed in the permeate. SMUF was constantly added back to the skim milk, replenishing the feed volume as the permeate was removed. The WPLD milk was used as a suspension medium that contained casein micelles and soluble milk minerals equivalent to their concentrations in 10% TS skim milk. Lactose was added to appropriate WPLD milk samples at 5% w/w, which is a similar concentration to that found in 10% TS skim milk. Polyacrylamide gel electrophoresis (PAGE), as described in Anema and McKenna (1996), was used to determine the residual whey protein content in the WPLD milk. The WPLD milk contained <10% of α-lactalbumin and <20% of β-lactoglobulin, that were present in the initial 10% TS skim milk, whilst the casein content remained unchanged. A small amount of sodium azide (0.02% (w/v)) was added to all the suspension media as a preservative.

2.3. Preparation of starch suspensions

Starch suspensions were prepared by adding starch to the appropriate suspension medium at 10% w/w. The suspensions were stirred at ambient temperature until the starch was completely dispersed. The samples were transferred to ultracentrifuge tubes (13.5 or 5.1 ml capacity, Denville Scientific Inc., Metuchen, NJ, USA) for high pressure treatment and the tubes were heat-sealed (Oh, Hemar, Anema, Wong, & Pinder, 2008).

2.4. High pressure treatment

The various samples were pressure-treated at 100–700 MPa and at 20 °C for 30 min, using the equipment and methods described previously (Oh et al., 2008).

2.5. Analysis of samples

The pressure-treated samples were examined for their rheological properties, degree of swelling and birefringence, as described previously (Oh et al., 2008).

3. Results and discussion

3.1. General

In this paper, "pasting" is defined as the heating of a starch suspension from 20 to 95 °C at 2 °C/min whilst stirring at 100 rev/min in a rheometer. To construct a "pasting curve", changes in the apparent viscosity of the sample were recorded over time during pasting. The initial apparent viscosity ($\eta_{initial}$) is defined as the measured viscosity at 20 °C at the start of pasting and is used as an indicator of the degree of gelatinisation. A higher $\eta_{initial}$ indicates a higher degree of gelatinisation of the starch caused by the pressure treatment prior to pasting. The peak viscosity (η_{peak}) is defined as the maximum viscosity attained during pasting. The apparent viscosity of a sample increased during pasting when its $\eta_{initial}$ value was lower than the η_{peak} value of the untreated control (Oh et al., 2008).

3.2. Gelatinisation of starch-in-skim-milk suspensions

Pasting curves for waxy rice starch and normal rice starch in skim milk samples of different concentrations after pressure treatment at 450 MPa for 30 min are shown in Fig. 1 and the $\eta_{initial}$ values are displayed in Table 1. Complete gelatinisation of waxy rice starch in water after pressure treatment was observed, as indicated by the pasting curve, which showed no η_{peak} (Fig. 1A). However, there was only partial gelatinisation in skim milk suspensions, as indicated by the apparent viscosity increase that occurred during pasting. Normal rice starch was partially gelatinised in both water and skim milk; however, there was less gelatinisation at higher skim milk concentrations (Fig. 1B).

Table 1 summarises the $\eta_{initial}$ values of suspensions of waxy rice starch and normal rice starch in skim milk after different pressure treatments. The $\eta_{initial}$ values of the untreated samples showed a slight but significant increase as the TS concentration of the skim milk increased. This increase in $\eta_{initial}$ is related to the contribution of the milk solids to the viscosity of the suspension. After pressure treatment at 450 MPa, the $\eta_{initial}$ values of both types of starch suspension decreased significantly as the concentration of the skim milk increased. For example, the $\eta_{initial}$ value of the waxy rice starch suspension decreased, by approximately 50%, from 4.067 Pa s in water to 2.026 Pa s in 5% TS skim milk and decreased further as the skim milk concentration increased further (Table 1).

Fig. 1C shows the gelatinisation curves for normal rice starch and waxy rice starch suspended in water or 10% TS skim milk, based on the $\eta_{initial}$ values after treatment at the different pressures. Regardless of the suspension medium, there was a sigmoidal-shaped relationship between the $\eta_{initial}$ values and the treatment pressures, with a low essentially constant $\eta_{initial}$ at low pressure, a transition zone where $\eta_{initial}$ increased sharply over a narrow pressure range, followed by a high essentially constant $\eta_{initial}$ at high pressure. For both starch types, the treatment
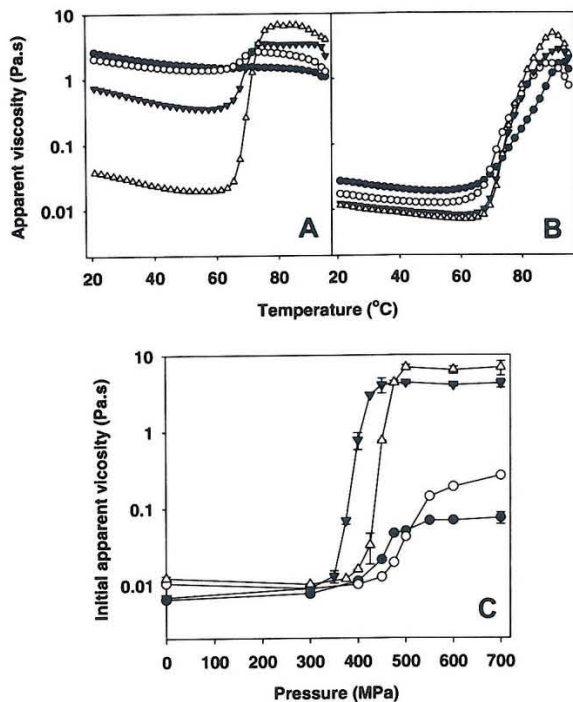
Fig. 1. Pasting curves of (A) waxy rice starch and (B) normal rice starch (10% w/w) after pressure treatment at 450 MPa in: (●) water; (○) 5% TS skim milk; (▼) 10% TS skim milk; (△) 15% TS skim milk. (C) Initial apparent viscosity ($\eta_{initial}$) as a function of treatment pressure (gelatinisation curves) for: waxy rice starch in (▼) water and (△) 10% TS skim milk; normal rice starch in (●) water and (○) 10% TS skim milk. All pressure treatments were carried out at 20 °C for 30 min.

Table 1
Initial apparent viscosity ($\eta_{initial}$) of waxy rice starch and normal rice starch (10% w/w) in different skim milk suspensions with different skim milk concentrations after no treatment or pressure treatment at 450 MPa and 20 °C for 30 min

Skim milk concentration (% w/w)	Initial apparent viscosity (Pa s)			
	Waxy rice starch		Normal rice starch	
	Untreated	450 MPa	Untreated	450 MPa
0	0.007^a	4.067^a	0.006^a	0.021^a
5	0.008^b	2.026^b	0.008^b	0.016^b
10	0.012^c	0.755^c	0.010^c	0.012^c
15	0.015^d	0.037^d	0.013^d	0.013^c

Means with the same letter within a column are not significantly different ($P < 0.05$) as determined by analysis of variance (ANOVA).

pressure at which $\eta_{initial}$ started to increase was higher in skim milk than in water; thus, the gelatinisation curves for skim milk were shifted to the right, to higher pressure ranges. For suspensions of normal rice starch in water, $\eta_{initial}$ increased markedly at pressures above ∼300 MPa, whereas, for suspensions of normal rice starch in skim milk, gelatinisation did not start until the pressure was above ∼400 MPa. Likewise, for suspensions of waxy rice starch, $\eta_{initial}$ started to increase from ∼300 MPa in water and from ∼375 MPa in skim milk.

This indicates that gelatinisation of starch in skim milk was retarded compared with that in water and this effect was dependent on the concentration of skim milk (Table 1). To examine reasons for this change in gelatinisation behaviour between starch-in-water suspensions and starch-in-skim-milk suspensions, starch suspensions containing different milk components were prepared to test the effect of different groups of components in skim milk on the pressure-induced gelatinisation of starch.

3.3. Effect of different skim milk components on the gelatinisation of starch

3.3.1. Gelatinisation curves and degree of swelling curves

Fig. 2A and B shows the gelatinisation curves for starch suspended in solutions containing mixtures of the various milk components (water, soluble milk minerals, lactose, casein micelles and skim milk). These curves illustrate which components of skim milk were responsible for the shift in the gelatinisation curves to higher pressure when the suspension medium was changed from water to skim milk, as seen in Fig. 1C.

For waxy rice starch, the gelatinisation curve was shifted to higher pressure with the addition of soluble milk minerals and then to even higher pressure with the addition of lactose (Fig. 2A). When the waxy rice starch suspension contained both soluble milk minerals and lactose, gelatinisation occurred over a pressure range similar to that observed for skim milk, even though no casein micelles were present (Fig. 2A). The gelatinisation curve of normal rice starch was also shifted to higher pressure when both soluble milk minerals and lactose were added (Fig. 2B). Although casein micelles did not affect the pressure at which the gelatinisation of normal rice starch started, the gelatinisation curve for suspensions containing casein micelles plateaued at a higher $\eta_{initial}$, similar to that when starch was suspended in skim milk (Fig. 2B).

Fig. 2C shows the swelling curves for waxy rice starch. When the suspension medium was water, the treatment pressure required for 100% swelling was 425 MPa, which was lower than that for all other media. The swelling curve of waxy rice starch was shifted to higher pressure when soluble milk minerals were added to the suspension and then to even higher pressure with the addition of lactose. When the suspension contained casein micelles, the degree of swelling was slightly lower at some treatment pressures compared with the suspension containing soluble milk minerals and lactose only. However, this was not reflected in the $\eta_{initial}$ results shown in Fig. 2A. The swelling curve of the waxy rice starch suspension with soluble milk minerals, lactose and casein micelles was very similar to that of the starch-in-skim-milk suspension.

Fig. 2D shows the swelling curves of normal rice starch. Normal rice starch did not reach 100% swelling within the pressure range used in this study, regardless of the suspension medium. The swelling curve of normal rice starch was also shifted to higher pressure when soluble milk minerals were added to the suspension. This indicates that the degree of swelling was restrained by the presence of soluble milk minerals; however, this was not reflected in the $\eta_{initial}$ results shown in Fig. 2B. The swelling curve was again shifted to even higher pressure with the addition of lactose. When the normal rice starch suspension contained soluble milk minerals and lactose, with or without casein micelles, the swelling curve was very similar to that of normal rice starch suspended in skim milk.

There was a positive correlation between the degree of swelling and $\eta_{initial}$ for both waxy rice starch and normal rice starch suspensions after pressure treatments in all the suspension media and this relationship could be represented by a single regression line (Fig. 2E). This relationship between $\eta_{initial}$ and the degree of swelling is consistent with our previous finding for starch-in-water suspensions (Oh et al., 2008).

3.3.2. Pasting curves and initial apparent viscosity ($\eta_{initial}$)
3.3.2.1. Waxy rice starch. Fig. 3A–E shows the pasting behaviour of waxy rice starch in different suspension media after various
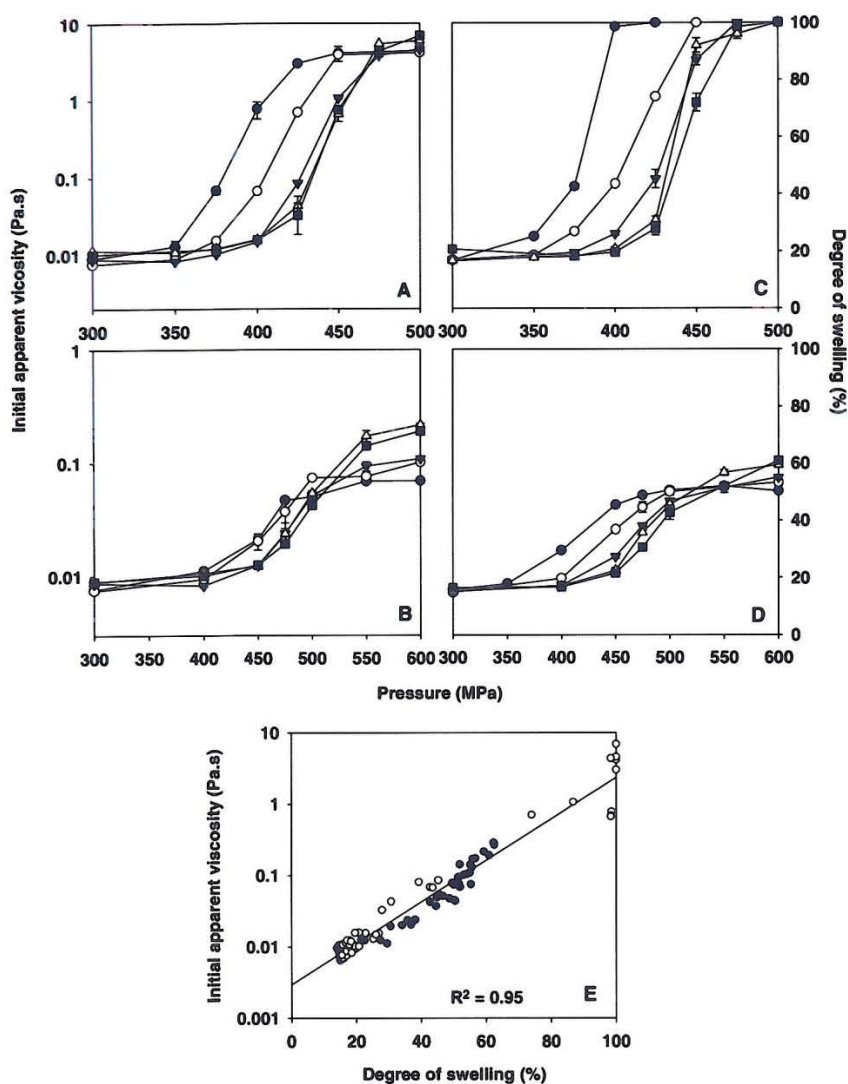
Fig. 2. Initial apparent viscosity ($\eta_{initial}$) as a function of treatment pressure within the gelatinisation pressure range for (A) waxy rice starch and (B) normal rice starch (10% w/w), and degree of swelling as a function of treatment pressure within the gelatinisation pressure range for (C) waxy rice starch and (D) normal rice starch in various suspension media containing: (●) water only; (○) soluble milk minerals; (▼) soluble milk minerals and lactose; (△) soluble milk minerals, lactose and casein micelles; (■) 10% TS skim milk. (E) Plot of initial apparent viscosity ($\eta_{initial}$) versus degree of swelling (%) for (○) waxy rice starch and (●) normal rice starch, all suspension media. All pressure treatments were carried out at 20 °C for 30 min.

pressure treatments. The 600 MPa treatment was enough to gelatinise waxy rice starch in all the suspension media examined in this study, which was indicated by the high $\eta_{initial}$ and the disappearance of η_{peak} in the pasting curves (Fig. 3A–E). At the lower pressure (400 MPa), where waxy rice starch was not completely gelatinised by the pressure treatment, the degree of gelatinisation varied, depending on the suspension medium. A moderate treatment pressure of 400 MPa was selected to highlight the differences in the $\eta_{initial}$ values of waxy rice starch in various suspensions, as displayed in Table 2. Sufficient water has been shown to be a prerequisite for high-pressure-induced gelatinisation in starch suspensions (Hibi, Matsumoto, & Hagiwara, 1993; Rumpold & Knorr, 2005); the starch-to-water ratios used in this study were well below the limiting ratio for gelatinisation. The gelatinisation-retarding effect of the milk components examined in this study could not

be explained by the reduced water-to-starch ratio as a result of the addition of milk components to the suspension medium. The effective concentration of starch in water was highest (at 11% (w/w)) when the suspension medium was 10% TS skim milk. At this concentration of starch in water, the $\eta_{initial}$ (the degree of gelatinisation after a pressure treatment) of waxy rice starch was significantly higher than that of all other suspensions examined in this study, 1.477 Pa s (Table 2). The high $\eta_{initial}$ indicates that gelatinisation was not retarded at this concentration and at this pressure. Therefore, it is unlikely that competition between starch granules and skim milk components for hydration played a part in retarding the gelatinisation process. The pressure-induced gelatinisation observed in this study was more probably affected by the properties of the suspension medium than by the absolute quantity of water in the suspension.
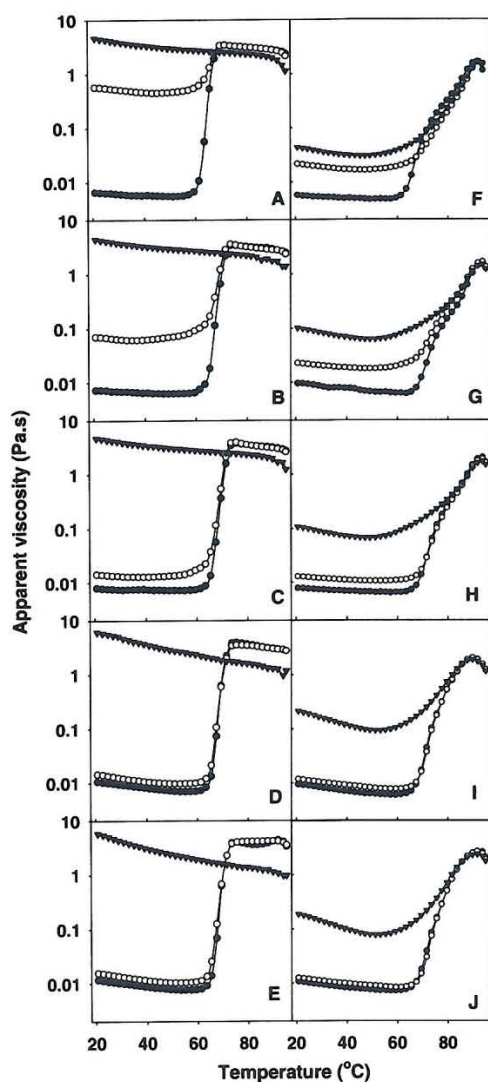
Fig. 3. Pasting curves of (A–E) waxy rice starch (10% w/w) after (●) no pressure treatment, (○) pressure treatment at 400 MPa and (▼) pressure treatment at 600 MPa and (F–J) normal rice starch (10% w/w) after (●) no pressure treatment, (○) pressure treatment at 450 MPa and (▼) pressure treatment at 600 MPa in various suspension media containing: (A,F) water only; (B,G) soluble milk minerals; (C,H) soluble milk minerals and lactose; (D,I) soluble milk minerals, lactose and casein micelles; (E,J) 10% TS skim milk. All pressure treatments were carried out at 20 °C for 30 min.

As reported previously, pressure treatment affected the pasting behaviour of suspensions of waxy rice starch in water (Oh et al., 2008). The $\eta_{initial}$ of the suspension increased over 100-fold, from 0.007 Pa s (untreated) to 0.773 Pa s (after treatment at 400 MPa) (Fig. 3A). The $\eta_{initial}$ increased only 7-fold to 10-fold when soluble milk minerals or lactose was added to the suspension (Fig. 3A and B, Table 2), i.e. to 0.067 Pa s when soluble milk minerals were added alone and to 0.047 Pa s when lactose was added alone. Both $\eta_{initial}$ values were significantly lower than that of the suspension in pure water, which suggests that soluble milk minerals and lactose had a gelatinisation-retarding effect on waxy rice starch.

Table 2
Initial apparent viscosity ($\eta_{initial}$) of aqueous suspensions of waxy rice starch and normal rice starch (10% w/w) after pressure treatment at 400 MPa (waxy) or 450 MPa (normal) and 20 °C for 30 min

Suspension medium	Initial apparent viscosity (Pa s)	
	Waxy rice starch (400 MPa)	Normal rice starch (450 MPa)
Water	0.773[a]	0.021[a]
Soluble milk minerals	0.067[b]	0.020[a]
Lactose	0.047[c]	0.023[a]
Soluble milk minerals + lactose	0.015[d]	0.012[b]
Soluble milk minerals + casein micelles	0.080[b]	0.020[a]
Soluble milk minerals + casein micelles + lactose	0.016[d]	0.012[b]
Skim milk	0.016[d]	0.012[b]
Increased starch concentration (11% w/w)	1.477[e]	0.088[c]
Pooled standard deviation of the data within the column	0.005	0.001

Means with the same letter within a column are not significantly different ($P < 0.05$) as determined by ANOVA.

Soluble milk minerals and lactose showed an additive effect when they were present together in the waxy rice starch suspension (Fig. 3B and C, Table 2). The $\eta_{initial}$ was 0.015 Pa s after the 400 MPa treatment, which was significantly lower than that of the suspension containing lactose or soluble milk minerals only.

Casein micelles at the level present in 10% TS skim milk did not affect the $\eta_{initial}$ value significantly. Soluble milk minerals and lactose still showed the gelatinisation-retarding effect when casein micelles were included in the suspensions (Fig. 3C and D, Table 2). In fact, the degree of gelatinisation in skim milk after the 400 MPa treatment, as indicated by $\eta_{initial}$, was not significantly different from that in the medium containing soluble milk minerals and lactose, which suggests that neither whey proteins nor casein micelles had a significant effect on the gelatinisation of waxy rice starch (Table 2). Providing the suspension medium contained soluble milk minerals and lactose, the pasting curves of waxy rice starch after the 400 MPa treatment were not notably different from those of their untreated counterparts (Fig. 3C and E).

3.3.2.2. Normal rice starch. Pressure treatment at 450 MPa was selected to highlight the differences in $\eta_{initial}$ for normal rice starch suspended in different media (Table 2). Increasing the starch concentration to 11%, which was equivalent to the starch-to-water ratio in the starch-in-skim-milk suspension, resulted in a significant increase in $\eta_{initial}$ of normal rice starch, indicating that the slight reduction in water in the suspensions as a result of the added components was not the reason for the retarded gelatinisation (Table 2). This is in keeping with the findings for waxy rice starch.

Unlike waxy rice starch, normal rice starch was not completely gelatinised by pressure within the range used in this study, regardless of the suspension medium, as the degree of swelling did not reach 100% (Fig. 2C and D). Fig. 3F–J shows the pasting behaviour of normal rice starch in different suspension media after various pressure treatments. The pasting curves after the 600 MPa pressure treatment were included to indicate the approximate maximum degree of gelatinisation as the $\eta_{initial}$ of all the suspensions reached almost the maximum after this treatment.

Compared with the untreated control sample, the $\eta_{initial}$ of suspensions of normal rice starch in water increased approximately fourfold after the 450 MPa treatment and increased further to 0.045 Pa s after the 600 MPa treatment (Fig. 3F). The $\eta_{initial}$ and the pasting curve of normal rice starch were not significantly affected by the addition of either soluble milk minerals or lactose alone (Table 2). However, when both soluble milk minerals and

lactose were present in the suspension together, the $\eta_{initial}$ of the normal rice starch suspension after the 450 MPa treatment was 0.012 Pa s, which was significantly lower, by about 50%, than that of the pure water suspension (0.021 Pa s; Fig. 3H, Table 2). Casein micelles did not affect the degree of gelatinisation after the pressure treatment (Fig. 3H and I). The $\eta_{initial}$ after the 450 MPa pressure treatment did not change significantly when the suspension medium contained casein micelles as well as soluble milk minerals and lactose (Table 2).

The $\eta_{initial}$ of the suspension of normal rice starch in skim milk after the 450 MPa treatment was not significantly different from that of the suspension that contained soluble milk minerals and lactose (Table 2). In both types of medium, pressure treatment at 450 MPa did not alter the pasting curve of normal rice starch from that of the untreated sample. However, after the higher pressure treatment at 600 MPa, the $\eta_{initial}$ of these suspensions increased markedly (Fig. 3I and J).

In the presence of soluble milk minerals and lactose, the $\eta_{initial}$ (degree of gelatinisation after a pressure treatment) of waxy rice starch or normal rice starch did not change significantly when casein micelles were included or both casein micelles and whey proteins were included (i.e. skim milk). However, it was observed that, when casein micelles were introduced into the suspension medium, the general shape of the pasting curve of normal rice starch was changed such that the viscosity peak was broader than that in suspension media without casein micelles (Fig. 3I and J). Aggregation of casein micelles may have been responsible for this change, and could have occurred as the effective concentration of micelles increased due to the exclusion of these molecules with the swelling of the starch granules during pasting.

The starch granule is composed of semi-crystalline growth rings and amorphous rings in alternating layers. As milk proteins are macromolecules suspended in skim milk, they cannot penetrate the intricate structure of starch granules at the start of gelatinisation, whereas small dissolved molecules, such as lactose and mineral ions, can penetrate this structure. Casein micelles can be as large as 300 nm and, even though they can be disintegrated by pressure ($\geqslant$400 MPa), the average particle size will still be above 100 nm (Anema, Lowe, & Stockmann, 2005). Hence, considering that the thickness of each semi-crystalline growth ring or amorphous ring in starch granules is around 120–400 nm (Jenkins & Donald, 1995), it is unlikely that casein micelles can readily penetrate into the layered ring structure of starch granules.

However, at higher protein concentrations, there may be interactions between proteins and starch molecules on the surface of starch granules that may restrict swelling. Debet and Gidley (2006) identified starch protein on the surface of starch granules as one of the factors involved in the restricted swelling of starch. At high concentrations, milk proteins that are in contact with starch granules may have a similar effect, which could effectively shield starch granules from water uptake.

3.4. Light microscopy

The disappearance of the characteristic birefringence is one of the changes that indicates the gelatinisation of starch (BeMiller & Whistler, 1996). Polarised light micrographs of waxy rice starch suspensions after pressure treatment at 400 MPa and normal rice starch suspensions after pressure treatment at 450 MPa are displayed in Fig. 4. The birefringence observations mostly agree with the rheological results. The majority of the waxy rice starch granules lost birefringence in the water suspension after pressure treatment at 400 MPa (Fig. 4A). In the presence of soluble milk minerals, more waxy rice starch granules retained birefringence (Fig. 4B). When soluble milk minerals and lactose were present, together, in suspensions, with or without casein micelles, most of the starch

granules retained birefringence (Fig. 4C and D), and this was very similar to the observation for the suspension of waxy rice starch in skim milk (Fig. 4E). Although the degree of swelling of waxy rice starch was slightly decreased in the presence of casein micelles (Fig. 2C), this effect was not manifested in the $\eta_{initial}$ values or in the display of birefringence.

Normal rice starch displayed a trend similar to that of waxy rice starch with the addition of different milk components. In comparison with water, when no granules displayed birefringence, a few granules displayed birefringence when soluble milk minerals were added (Fig. 4F and G). When the suspension medium contained both soluble milk minerals and lactose, with or without casein micelles, most of the starch granules retained birefringence (Fig. 4H and I) and this was similar to the observation for the suspension of normal rice starch in skim milk (Fig. 4J).

One of the important factors in starch gelatinisation is the structure of water and its modification when solutes are added (Jane, 1993). Milk minerals contain a number of highly charged ions, such as Ca^{2+}, Mg^{2+}, PO_4^{3-} and citrate (Jenness & Koops, 1962). Assuming that water is a mixture of hydrogen-bonded clusters and unbound free water molecules, ions with low charge density act as structure breakers and break or weaken hydrogen bonds between water molecules, thereby increasing the fraction of free water (Chiotelli, Pilosio, & Le Meste, 2002). However, ions of high charge density have strong electrostatic interactions with water molecules (structure makers), thereby reducing the fraction of free water (Chiotelli et al., 2002). It is possible that the highly charged ions in soluble milk minerals reduced the amount of free water available for starch gelatinisation, thereby contributing to the observed retardation of gelatinisation.

Ionic interactions between starch molecules and mineral ions in skim milk may also need to be considered. Oosten (1990) suggested that anions and cations have different interactions with starch molecules. It was explained that starch acts as a weak acid ion-exchanger and that cations tend to protect and stabilise the granule structure. In contrast, anions promote gelatinisation by rupturing hydrogen bonds between starch molecules. It is possible that the shielding effect of free cations, such as Ca^{2+}, Mg^{2+}, Na^+ and K^+, around starch granules in skim milk, was dominant compared with the effect of anions. This led to reduced swelling of the starch granules as a net effect and therefore a retardation of the gelatinisation in skim milk compared with that in water.

Lactose is known to affect thermal gelatinisation. Matser and Steeneken (1997) showed that higher temperatures were required for the gelatinisation of highly cross-linked waxy corn starch when 5.6% or 11.2% lactose was added to the suspension prior to heating. In pressure-induced gelatinisation Rumpold and Knorr (2005) showed that the gelatinisation of starch occurred at higher pressure when sugars such as fructose, glucose, sucrose and trehalose were added. In aqueous starch suspensions, water acts as a plasticiser that lowers the glass transition temperature of the amorphous regions and increases the mobility of starch chains (Biliaderis, 1991). Chiotelli, Rolée, and Le Meste (2000) showed that sugar solutions increased the glass transition temperature of wheat starch compared with a sample without sugar at the same starch-to-water ratio. The authors suggested that the sugar solution acted as an anti-plasticiser that reduced the flexibility of the amorphous regions and contributed to the delay in the gelatinisation process.

In addition, the possibility of direct interaction between lactose and starch molecules may need to be considered. Kohyama and Nishinari (1991) suggested that the crystalline region in starch could be stabilised by sugar molecules. Spies and Hoseney (1982) stated that sugar molecules can stabilise the amorphous regions of the granule by linking starch chains together (sugar-bridge effect). As the flexibility of the starch chains in the amorphous regions directly influences the gelatinisation temperature,
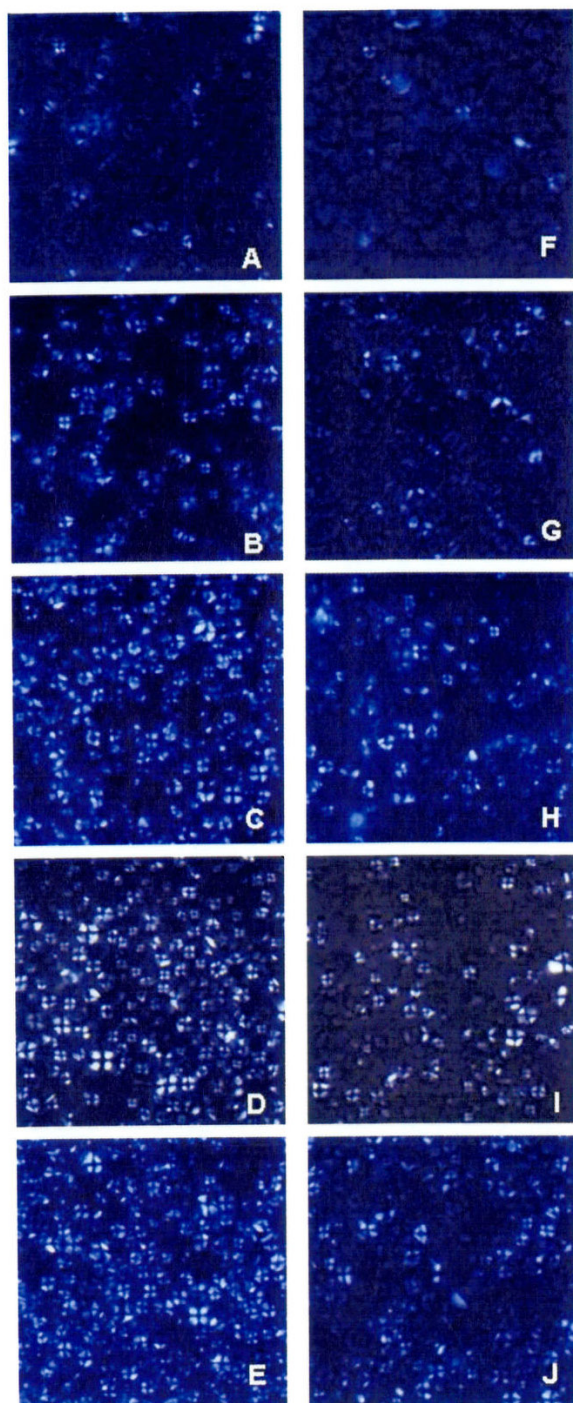
cross-linking of the starch chains would increase the gelatinisation temperature. Hoover and Senanayake (1996) showed that leaching of amylose was decreased when sugar was added, which indicated that sugars interacted with amylose chains within the amorphous regions of the starch granule. Interaction of lactose with starch molecules in the amorphous or crystalline regions, to stabilise the structure, would slow down the progress of gelatinisation.

The presence of small solutes, such as lactose and milk minerals, could lower the water activity (a_w), which results in lowering of the chemical potential and consequently reactions involving water require more energy input (Spies & Hoseney, 1982). To a lesser degree, the small increase in viscosity that is caused by the presence of solutes reduces the ability of water molecules to diffuse into starch granules.

Small solutes can also change the mobility of water, thereby altering its plasticising effectiveness. Chinachoti, Kim-Shin, Mari, and Lo (1991) conducted water mobility measurements during starch gelatinisation using oxygen-17 nuclear magnetic resonance and showed that the mobility of water decreased in the presence of sucrose or sodium chloride compared with a solute-free starch suspension. If lactose and milk minerals played roles similar to those of these solutes in starch gelatinisation and reduced the water mobility, this could in turn lower the plasticising ability of water and restrain gelatinisation.

In summary, the observed reduction of starch gelatinisation in skim milk (Fig. 1, Table 1) was attributed to the combined effect of soluble milk minerals and lactose (Figs. 2–4 and Table 2). Gelatinisation is viewed as a phase transition phenomenon that eventually leads to melting of crystalline regions of the starch granule. However, the amorphous growth ring regions of the starch granule are where gelatinisation starts, when the degree of molecular mobility within these regions reaches a critical level. Molecular mobility is, in turn, controlled by the total degree of plasticisation of starch chains (Perry & Donald, 2002). Soluble milk minerals and lactose, which were identified as the main gelatinisation-retarding components in skim milk, potentially affect various aspects of the starch gelatinisation process. The degree of molecular mobility of starch chains may be influenced by the direct interactions of starch chains with soluble milk minerals and lactose. As small solutes, soluble milk minerals and lactose can also decrease the chemical potential of water and the fraction of free water, making water less available for the gelatinisation process. Moreover, the mobility of water can be reduced by the presence of these components, which may directly affect its plasticising effectiveness.

4. Conclusions

Pressure-induced gelatinisation of starch was found to be a swelling phenomenon in skim milk as it is in water. Gelatinisation occurred at a higher pressure in skim milk than in water. It is concluded that the retarded gelatinisation in skim milk was caused by the presence of soluble milk minerals and lactose. Milk proteins did not affect the degree of pressure-induced gelatinisation at a concentration equivalent to that found in 10% TS skim milk. However, it is possible that, at higher concentration, casein micelles or whey proteins may interact with starch granules on the surface, which may lead to restricted swelling of the granules.

Fig. 4. Polarised light micrographs of (A–E) waxy rice starch after pressure treatment at 400 MPa and (F–J) normal rice starch after pressure treatment at 450 MPa in various suspension media containing: (A,F) water only; (B,G) soluble milk minerals; (C,H) soluble milk minerals and lactose; (D,I) soluble milk minerals, lactose and casein micelles; (E,J) 10% TS skim milk. All pressure treatments were carried out at 20 °C for 30 min.

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CHAPTER 7

Application of Starch Gelatinisation in a Dairy System (Acid Milk Gel)

This chapter contains three studies investigating the application of starch in a model dairy system (acid milk gel). The results from the first study were published in the article: [1]Oh, H. E., S. G. Anema, M. Wong, D. N. Pinder and Y. Hemar (2007a). "Effect of potato starch addition on the acid gelation of milk." *International Dairy Journal* **17**(7): 808-815. This article can be found on page 7-6 of this chapter. The results from the second study were published in the article: [2]Oh, H. E., M. Wong, D. N. Pinder, Y. Hemar and S. G. Anema (2007b). "Effect of pH adjustment at heating on the rheological properties of acid skim milk gels with added potato starch." *International Dairy Journal* **17**(12): 1384-1392. This article can be found on page 7-14 of this chapter. The results from the third study have been written as a paper ([3]Oh, H. E., M. Wong, D. N. Pinder, and S. G. Anema. "Comparison of pressure treatment and heat treatment of skim milk with added starch on subsequent acid gelation of milk") which is to be submitted for publication. This study can be found on page 7-23 of this chapter.

Aim. To study and compare the properties of acid milk gels prepared from pressure- or heat-treated skim milk containing starch which was added prior to the pressure or heat treatment.

Relevance. Acid milk gels are particle gels formed by aggregation of milk proteins when milk is slowly acidified to ~pH 4.6 (Horne, 1999). The manufacture of dairy products such as yoghurt and some types of cheese is based on the acid gelation of milk. In this study, the incorporation of starch into acid milk gels was first tested in the heat-treated milk systems as heat treatment is the method traditionally used in acid milk gel applications of dairy products. Also, more information is available to understand the behaviour of starch after heat treatment than after pressure treatment.

[1]- Oh et al. (2007a) contains the original work of the author H. E. Oh and has been written by the author H. E. Oh.

[2]- Oh et al. (2007b) contains the original work of the author H. E. Oh and has been written by the author H. E. Oh.

[3]- Oh et al. (to be submitted) contains the original work of the author H. E. Oh and has been written by the author H. E. Oh.

The effect of adding starch into pressure-treated milk systems was investigated subsequently and compared with the results from the heat-treated counterparts.
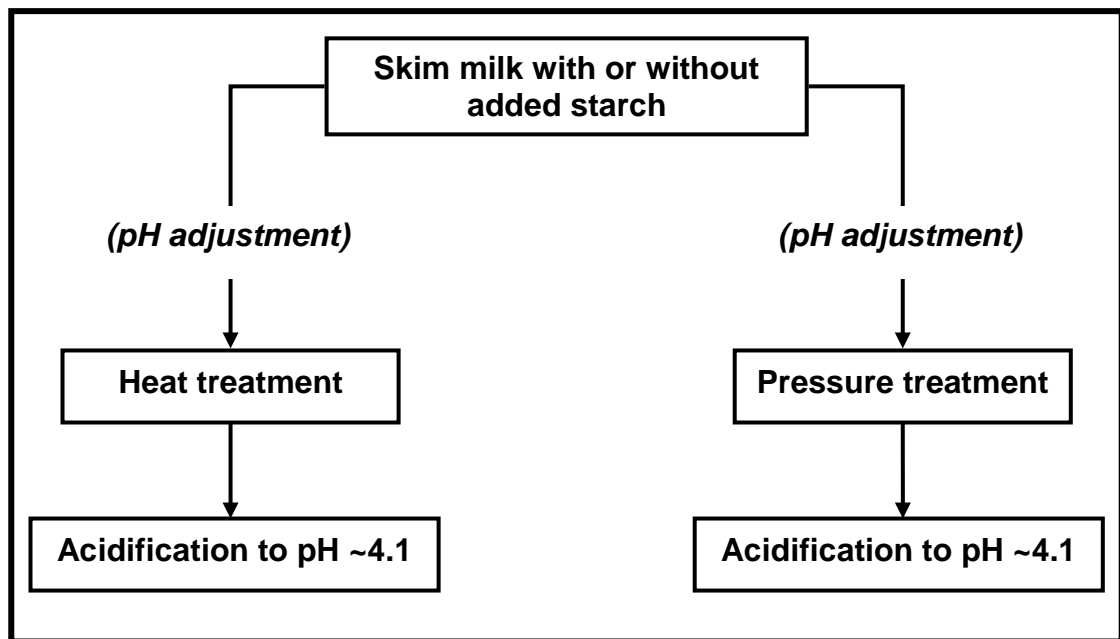
Figure 7-1: Block diagram of acid gelation experiments

Approach. The studies presented in this chapter are outlined in **Figure 7-1**. In the first study, potato starch (0–1.5% w/w) was added to skim milk and the skim milk samples were heat-treated at the natural milk pH. The aim of the first study was to examine the properties of acid milk gels made from the heated skim milk samples with different levels of potato starch added prior to the heat treatment. Potato starch was selected as a representative starch as it is widely used in food applications and gelatinises readily when heated in an aqueous environment. It was hypothesised that when starch is added to skim milk prior to heat treatment, gelatinisation of starch and heat-induced changes in milk components such as protein denaturation would occur simultaneously during heat treatment.

Once the properties of acid milk gels prepared from skim milk at its natural pH with the chosen starch (potato starch) added prior to heat treatment had been understood, the effect of potato starch addition (0–2% w/w) to pH-adjusted milk (pH

6.5–7.1) prior to heat treatment on properties of acid gels was examined in the second study. Anema et al. (2004) showed that the firmness of acid milk gel is influenced by the pH at heating as the pH affects the interaction behaviours of casein micelles and whey proteins. Hence, in the second study, milk samples were pH-adjusted prior to starch addition, heat treatment and acidification (**Figure 7-1**). It was hypothesised that the effect of pH adjustment and the effect of starch addition may not be additional to each other in terms of increasing the firmness of acid milk gels. The combined effects of adjusted pHs of milk sample at heat treatment and starch addition on the acid gelation and properties of the acid skim milk gels were examined.

In the last study presented in this chapter, the traditional heat treatment was replaced by pressure treatment (**Figure 7-1**). It was hypothesised that pressure treatment of milk with added starch may produce firmer acid milk gels than milk with no added starch, similar to the effect of starch addition with heat treatment. Two different starches, waxy rice starch and potato starch were used and their effects on acid milk gels after different treatments were compared. Waxy rice starch and potato starch have very different pressure-induced gelatinisation properties, therefore selected for contrast. Combined effects of adjusted pHs of milk samples at pressure treatment and starch addition on the acid gelation and properties of the acid skim milk gels were also examined. Although the pressure-induced gelatinisation of normal rice starch was also investigated in the studies from the previous chapters, it was not used in the detailed acid milk gels application study. Preliminary results showed that when normal rice starch was added to milk and pressure-treated, the resultant milk sample could not produce acid gels with consistent properties and no meaningful results can be obtained. It appeared that the swollen normal rice starch granules interferes with the formation of protein networks during acidification of milk.

Summary of results. The addition of potato starch prior to heating and subsequent acidification resulted in a higher storage modulus, *G'*, in the final acid gels. Increasing the level of starch caused a linear increase in the final *G'* of the acid gels. In addition, the gelation time was reduced and the gelation pH was increased when potato starch was added, compared with acid gels prepared with no starch. Confocal microscopy showed that the acid milk gels contained swollen starch granules embedded in a protein network, and that the protein network appeared denser as the level of starch added increased.

The *G'* of the final acid gels was increased by heating the milk at higher pH prior to acidification and further increased by adding starch prior to heat treatment and acidification. The effect of pH at heating and addition of starch appeared to be additive and independent of each other up to a starch addition level of 1%. Above this starch level, the pH at heating had a lesser effect. This may be attributed to the changes in the aggregation behaviour of proteins due to the increased viscosity of the aqueous phase as a result of starch gelatinisation or a possible phase separation into amylose-rich and protein-rich regions. Confocal microscopy showed that milk proteins developed fewer but broader protein clusters at higher pH than at lower pH. Starch addition resulted in an increased density of the protein network which can be explained by an increase in the effective concentration of milk protein as the added starch gelatinises during heating and absorbs water.

The last study compared the effects of heat treatment and pressure treatment of milk samples on the subsequent acidification. The *G'* of the final acid milk gels increased as more waxy rice starch was added to milk before pressure or heat treatment and acidification. However, acid gels made from pressure-treated milk with added potato starch did not show significant changes in the *G'* in the final acid gels

whereas those made from the heat-treated counterparts showed a marked increase in the final *G'* as the potato starch level increased. These results can be explained by the extent of starch gelatinisation in milk during pressure or heat treatment. Waxy rice starch was gelatinised in milk by both pressure treatment and heat treatment whereas potato starch was gelatinised by heat treatment only. Increasing the pH of milk (with no added starch) before pressure or heat treatment increased the final *G'* of the acid milk gel produced on subsequent acidification and the final *G'* was increased further by addition of waxy rice starch before the pressure or heat treatment. The effect of starch was unaffected by the pH of milk as the range of adjusted pH used in this study does not influence the gelatinisation of starch.

Conclusions.

- The firmness of acid milk gel was higher when starch was added to milk compared with that of acid milk gel made from skim milk alone, provided that starch was gelatinised during pressure or heat treatment prior to acidification.

- The firmness of acid milk gel increased as the pH of milk at pressure or heat treatment increased.

- The effect of pH at pressure or heat treatment and the effect of starch addition appeared to be additive and independent of each other up to a starch addition level of 1%.

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Effect of potato starch addition on the acid gelation of milk

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Abstract

Potato starch was added to skim milk at levels of 0–1.5%. The milks were heated and then acidified to form acid milk gels. The properties of the milks during acidification and the final properties of the acid gels were studied. The addition of starch resulted in a higher storage modulus, G', in the final acid gels, and increasing the level of starch caused a linear increase in the final G'. Compared with acid gels prepared with no starch, the gelation time was reduced and the gelation pH was increased. However, the temperature and frequency dependences of the acid gels were not affected by the addition of starch. Furthermore, the breaking strain of the acid gels was not markedly affected by the addition of starch, whereas the breaking stress was dependent on the level of starch added. Confocal microscopy showed that the acid gels contained swollen starch granules embedded in a protein network, and that the protein network increased in density as the level of starch added increased.
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1. Introduction

Milk can be gradually acidified to form acid gels using bacterial cultures, which ferment lactose to lactic acid, or using glucono-δ-lactone (GDL), where the hydrolysis of GDL to gluconic acid results in a reduction in the pH (Lucey & Singh, 1997). The properties of these acid gels can be altered by a number of different methods. For example, different properties of acid gels, such as the pH of gelation, the gelation time and the storage modulus, can be manipulated using heat treatment of the milk before acidification (Lucey, 2004; Lucey & Singh, 1997). The rheological properties of acid gels or yoghurt can be further modified by fortifying the milk with dairy-based ingredients, non-dairy ingredients or a combination of both prior to heat treatment and acidification.

The addition of whey-protein-based solids prior to heating and acidification has been investigated in a number of studies (Graveland-Bikker & Anema, 2003; Lucey,

Munro, & Singh, 1999). Lucey et al. (1999) reported that the addition of whey protein concentrates to milk followed by heat treatment at 80 °C caused a further increase in the pH of gelation, a reduction in the gelation time and an increase in the storage modulus of the skim milk acid gel. The effects of the whey proteins were found to be dependent on the type of whey proteins and the addition levels (Bikker, Anema, Li, & Hill, 2000; Graveland-Bikker & Anema, 2003).

Non-dairy ingredients, especially polysaccharides such as locust bean gum, xanthan gum, guar gum, pectin and starches, can also be used in yoghurt in conjunction with dairy ingredients or on their own to modify the rheological properties (Decourcelle, Lubbers, Vallet, Rondeau, & Guichard, 2004; Keogh & O'Kennedy, 1998; Williams, Glagovskaia, & Augustin, 2003; Williams, Glagovskaia, & Augustin, 2004). The viscosity of stirred yoghurt increased when 1% (w/w) modified waxy corn starch was added to yoghurt milk but the yoghurt developed a grainy texture (Williams et al., 2003, 2004). Keogh and O'Kennedy (1998) used an array of different polysaccharides in stirred yoghurt and showed that the yoghurts made

with wheat starch had the highest shear consistency. Sandoval-Castilla, Lobato-Calleros, Aguirre-Mandujano, and Vernon-Carter (2004) examined the use of tapioca starch as a fat replacer along with other whey-protein-based fat replacers. They reported that yoghurt with tapioca starch showed higher firmness than full-fat yoghurt. The microstructure of the yoghurt with tapioca starch showed some solubilized starch molecules integrated into the casein micelle network as well as starch gel fragments forming independent structures.

In addition to modifications to the rheological properties, added polysaccharides were also found to cause a decrease in the concentration of aroma compounds in the headspace of yoghurt (Decourcelle et al., 2004). In the case of starch, it was suggested that molecular interactions between the helical chains of starch and the aroma compounds could cause the decrease in the concentration of aroma compounds in the headspace of the yoghurt. Heinemann, Zinsli, Renggli, Escher, and Conde-Petit (2005) also found that linear amylose in particular was able to form inclusion complexes with a wide variety of flavour compounds.

Changes in the native structures of both milk proteins and starch occur upon heat treatment above their relevant critical temperatures. The heat treatment of milk at temperatures above about 70 °C results in denaturation of the whey proteins (Anema & McKenna, 1996; Dannenberg & Kessler, 1988). These denatured whey proteins can undergo a complex series of aggregation reactions with other denatured whey proteins and with the casein micelles (Anema & Li 2003a; Corredig & Dalgleish, 1996; Mulvihill & Donovan, 1987). In contrast, starches 'gelatinize' when heated in the presence of water, with the critical temperature dependent on the starch type. Starch gelatinization encompasses disruption of the granular structure, swelling and hydration, and solubilization of starch molecules (Appelqvist & Debet, 1997). Such developments in a mixed system of milk and starch during heat treatment may lead to different characteristics in the final acid gel compared with acid gels made from milk alone.

The current study was conducted to examine the effect of the addition of potato starch to skim milk at its natural pH on the rheological properties of acid gels. The starch was added prior to heat treatment and acidification. As well as the rheological and physical properties of final acid milk gels with added starch, which previous studies have shown (Keogh & O'Kennedy, 1998; Sandoval-Castilla et al., 2004; Williams et al., 2003, 2004), this study provides insight into the rheological changes during the acid gelation of skim milk samples with added potato starch to provide more comprehensive understanding of the acid gelation of milk in the presence of starch. The acid gelation processes and the properties of the final gels with different levels of added starch were compared and their microstructures were studied.

2. Materials and methods

2.1. Materials

Native potato starch (amylose:amylopectin, 24:76) was obtained from Penford New Zealand Limited (Auckland, New Zealand) and was used as supplied. The potato starch contains 0.05% protein, 0.3% ash and a maximum of 20% moisture. Low-heat skim milk powder was obtained from the Edendale site, Fonterra Co-operative Group Limited, New Zealand. GDL was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

Reconstituted skim milk samples were prepared by adding low-heat skim milk powder to purified water (reverse osmosis followed by filtration through a Milli-Q apparatus) to a final concentration of 10% (w/w) total solids. The reconstituted skim milk samples were stirred at ambient temperature (approximately 20 °C) for at least 10 h before further treatment to ensure complete equilibration (Anema & Li, 2003b). A small amount of sodium azide (0.02% (w/v)) was added to all the milk samples as a preservative. Potato starch was added to the reconstituted skim milk before heating. The starch was added at levels of 0, 0.25, 0.5, 1 or 1.5% (w/w).

2.3. Heat treatment of samples

A Rapid Visco Analyser (RVA, Newport Scientific, Warriewood, New South Wales, Australia) was used for heating the samples. The samples (30 mL) were transferred to aluminium RVA cups and heated in the RVA with continuous stirring at 200 rev min^{-1}. The samples were heated to 80 °C at a rate of 20 °C min^{-1}, held at 80 °C for 30 min and then cooled to 20 °C at a rate of 13 °C min^{-1}.

2.4. Acid gelation and rheological measurements

The heat-treated samples were acidified using GDL at a level of 2% (w/w) and at 30 °C. The change in pH with time was monitored using a glass electrode and a standard pH meter.

For the rheological measurements, an AR2000 rheometer (TA Instruments, New Castle, DE, USA) and a cone (4 cm, 4° and 100 μm truncation) and plate geometry were used for all experiments. Before the rheological measurements, the GDL was added to the sample, which was stirred for 30 s. An aliquot of 1.2 mL was transferred to the rheometer plate, and a cover was placed over the sample to prevent evaporation.

To monitor the gelation process as the milk was acidified, the rheological measurements were performed at a frequency of 0.1 Hz, a constant strain of 0.5% and a constant temperature of 30 °C. Measurements were taken every 5 min for 6 h. After 6 h, the final gel was subjected to

a frequency sweep from 0.01 to 10 Hz. Once the frequency sweep had been completed, the sample was then subjected to a temperature sweep. The temperature of the sample was dropped from 30 to 5 °C at a rate of 0.9 °C min^{-1} and a frequency of 0.1 Hz was used.

Separate samples were prepared for a strain sweep. After 3 h of gelation at 30 °C, the temperature was dropped to 5 °C and a strain sweep was performed by increasing the strain from 0.5% to 300% to measure the breaking strain and breaking stress of the acid gel.

2.5. Confocal scanning laser microscopy (CSLM)

The heat-treated sample was mixed with 2% (w/w) GDL, and Fast Green CFC dye (Merck, Darmstadt, Germany) was added to stain the proteins in the sample. A small aliquot of the sample was then transferred to a concave glass slide, a cover slip was placed on top and the sample was kept at 30 °C for 6 h for acid gelation.

CSLM was performed on a Leica TCS 4D confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a 100 mm oil immersion objective. The microscope was used in a fluorescent mode. The excitation using an air-cooled Ar/Kr laser was performed at 488 nm.

All experiments were repeated in duplicate. Analysis of variance (ANOVA) using MINITAB Statistical Software was conducted for statistical analyses where appropriate.

3. Results and discussion

3.1. Change in pH with time after GDL addition

The addition of starch to milk had only a small effect on the change in pH with time after GDL addition. As shown in Fig. 1a, at a starch concentration of 1.5%, which was the highest level used in this work, there was a slightly faster rate of pH decrease than for the samples with no starch. In both cases, the initial pH of the sample was 6.5 and dropped to approximately pH 4.1 after 360 min of acidification with 2% GDL. As the differences in pH change were small, no attempts to correct for this were made. A similar observation was reported by Williams et al. (2003) upon the addition of starch to milk before acidifying to make yoghurt. These authors showed that the addition of 1% (w/w) starch had little effect on the fermentation time, which was the time required for the pH of the milk inoculated with starter culture to fall to 4.6.

3.2. Acid gelation curves

Fig. 1b shows the storage modulus, G', as a function of time after the addition of GDL, for samples containing different starch levels; the result for the unheated milk sample is included for comparison. The G' values displayed a similar behaviour, irrespective of the starch concentration.
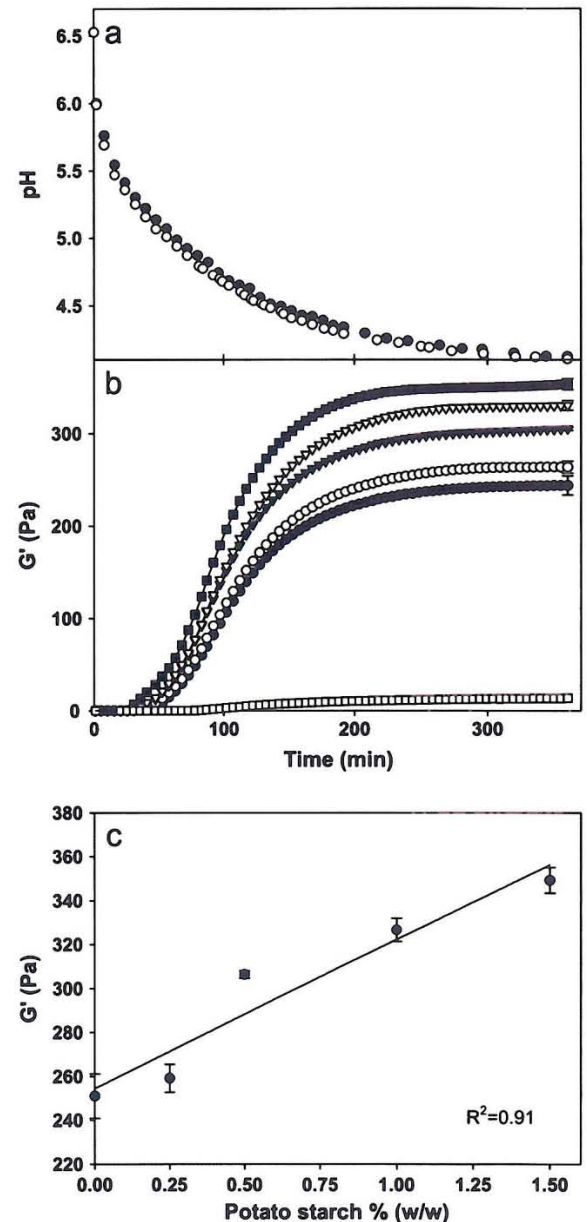
Fig. 1. (a) Change in pH with time after GDL addition for (●) heated milk with 0% potato starch and (○) heated milk with 1.5% potato starch. (b) Change in storage modulus, G', at 30 °C, with time after GDL addition: (□) unheated milk; (●) heated milk with 0% potato starch; (○) heated milk with 0.25% potato starch; (▼) heated milk with 0.5% potato starch; (▽) heated milk with 1% potato starch; (■) heated milk with 1.5% potato starch. (c) Final value of the storage modulus, G', at 30 °C as a function of the starch addition level. Error bars represent standard deviations.

In the early stages of acidification, low values of G' were measured. When G' had reached a value of 0.5 Pa, it increased rapidly, indicating rapid progress of the

gelation process. This period of rapid increase in G' was followed by a period in which the curve plateaued (Fig. 1b). The 'gelation time' is arbitrarily referred to as the time at which G' reached 0.5 Pa and the 'gelation pH' is referred to as the pH at the gelation time. The G' value of the acid gels after 6 h of acid gelation is referred to as the final G'. The final G', gelation time and pH values for all samples are reported in Table 1.

The acid gels prepared from unheated skim milk had very low final G' values (13 Pa at 360 min) and a long gelation time of 83 min. Heat treatment of the skim milk at 80 °C for 30 min resulted in an acid gel with a markedly higher final G' value (244 Pa at 360 min) and the gelation time was also markedly reduced (Table 1). These observations are in agreement with the published literature (Graveland-Bikker & Anema, 2003; Lucey et al., 1999). The gelation occurred at pH 4.82 for the unheated milk sample and at pH 5.23 for the heated milk sample (Table 1). These pH values are in agreement with results reported in the literature (Anema, Lauber, Lee, Henle, & Klostermeyer, 2005; Bikker et al., 2000).

The addition of starch had a clear effect on the final G' value of the acid milk gels. As the level of starch addition increased, the final G' value increased linearly ($R^2 = 0.91$, $P < 0.001$) (Fig. 1c, Table 1). The difference in final G' values between the acid milk gel made from milk alone and that made from the milk with the highest starch concentration, 1.5%, was 110 Pa at 30 °C, which was an approximately 45% increase in firmness (Table 1). The addition of starch did not have a significant effect on the gelation time or the gelation pH up to a starch addition level of 1%. At a starch addition level of 1.5%, the gelation time was reduced and the gelation pH was increased significantly (Fig. 1b, Table 1); the gelation time was 10 min shorter and the gelation pH was 0.11 units higher than for the sample with no added starch (Table 1). Similar effects of starch addition on the properties of stirred yoghurts

have been reported by Williams et al. (2003); these workers reported that the viscosity of the stirred yoghurt increased when a modified starch was added to the milk before heating and acidification.

Adding ungelatinized starch to heated milk did not have any effect on the final G' value, the gelation time or the gelation pH (results not shown). This indicates that gelatinization of starch in milk is a key mechanism for the observed changes in acid gelation properties. As potato starch is known to gelatinize between 58 and 65 °C (BeMiller & Whistler, 1996), the heat treatment at 80 °C for 30 min prior to acid gelation was sufficient for starch gelatinization to take place in milk. During gelatinization, swelling and disruption of the starch granules produces a viscous mass consisting of a continuous phase of solubilized amylose and amylopectin and a discontinuous phase of granule remnants (BeMiller & Whistler, 1996). When a drop of iodine was added to milk with added starch after heating, the continuous phase turned blue, indicating that amylose and amylopectin leached into the continuous phase during heating. However, no colour change was observed in milk containing unheated starch granules. Therefore, the viscosity of the continuous phase of the acid milk gel increased, as a result of both solubilized amylose and solubilized amylopectin. Furthermore, the uptake of water by the starch granules during swelling would result in an increase in the protein concentration in the continuous phase, leading to a stronger gel network.

3.3. Viscoelastic properties of acid milk gels

Fig. 2 shows the frequency dependence of the storage modulus, G', and the loss modulus, G'', for acid milk gels containing different amounts of starch. It was observed that the frequency dependences of G' and G'' were not

Table 1
Storage modulus, G', of final skim milk acid gels with various potato starch concentrations[a]

Acid gel sample[b]	n	Gelation time (min)	Gelation pH	Final G' at 30 °C (Pa)	Final G' at 5 °C (Pa)
UH–0% PS	1	83	4.82	13	—
H–0% PS	2	37[a]	5.23[a]	244[a]	601[a]
H–0.25% PS	2	35[a]	5.25[a]	264[a]	604[a]
H–0.5% PS	2	31[ab]	5.30[ab]	305[b]	723[b]
H–1% PS	2	31[ab]	5.30[ab]	330[bc]	811[c]
H–1.5% PS	2	27[b]	5.34[b]	353[c]	857[c]
Pooled standard deviation		2.7	0.033	6.5	11.7

[a]Means with the same superscript letter within a column are not significantly different ($P < 0.05$) as determined by ANOVA.
[b]UH, unheated; H, heated; PS, potato starch.
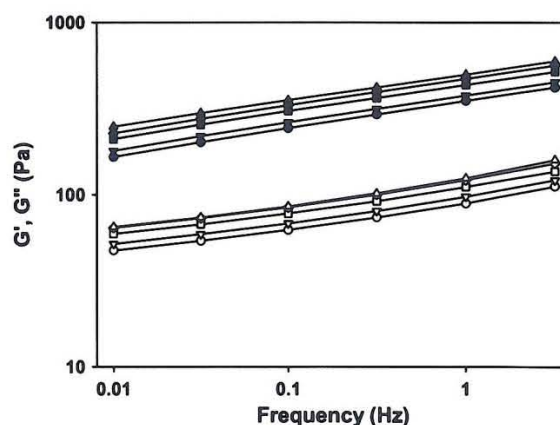
Fig. 2. Storage modulus, G', and loss modulus, G'' of final acid gels at 5 °C as a function of frequency for acid milk gels containing (●) 0%, (▼) 0.25%, (■) 0.5%, (◆) 1% and (▲) 1.5% potato starch. Open symbols are for G'' and solid symbols are for G'.

different for the acid gels regardless of the starch concentration. For all the acid gels, the plots of G' and G'' were straight lines with a slope of 0.153 on a logarithmic scale and G' was greater than G'', which indicates that the gels were elastic. In addition, the difference between G' and G'' was less than a log, indicating that these systems could be classified as weak gels (Lapasin & Pricl, 1995).

To study the effect of temperature, a rheological temperature sweep measurement was performed on the set acid gel (Fig. 3a). As the temperature of the set acid gels was decreased from 30 to 5 °C, the G' for all gels increased approximately twofold regardless of the level of starch addition (Fig. 3a). Similar observations have been reported in the literature for acid milk gels (Bikker et al., 2000; Lucey, Teo, Munro, & Singh, 1997) and for gels prepared from pressure-treated milks and transglutaminase-treated skim milk (Anema et al., 2005). The lowering of the

Fig. 3. (a) Storage modulus, G', as a function of temperature for acid milk gels containing (●) 0%, (○) 0.25%, (▼) 0.5%, (▽) 1% and (■) 1.5% potato starch. Inset: plot of G'/G' at 5 °C versus $1/T$ for the same acid milk gels. (b) Loss modulus, G'', as a function of storage modulus, G', for acid milk gels at (●) 30 °C and (○) 5 °C.

temperature increases the viscosity of the continuous phase and provides more rigidity to the gel network and therefore an increase in G'. The results suggest that the addition of starch did not influence this effect of temperature on the acid gels, as the Arrhenius plot of the ratio of G' at any particular temperature to the G' at 5 °C showed a quasi-linear relationship regardless of the starch addition (Fig. 3a inset). This viscosity effect was also confirmed by the linear relationship between G' at any particular temperature to the G'' that was obtained for all samples measured in this study (Fig. 3b). This indicates that the $\tan \delta$ was similar for all samples.

3.4. Large deformation rheology

The final gels were also subjected to a strain sweep at 30 °C and the shear stress was measured (Fig. 4a). The stress increased with increasing strain to a maximum, and then decreased markedly. The maximum stress was considered to be the stress at which the gel broke, and the corresponding strain was defined as the breaking strain. As shown in Fig. 4b, the breaking strain was approximately 25% on average and did not change significantly with the starch addition level. However, the breaking stress was affected by the level of starch added ($P < 0.05$). The breaking stress did not change significantly up to a starch addition level of 0.25% but increased markedly at a starch addition level of 0.5%. At a starch addition level of 1%, it was between the breaking stresses at the 0% and 0.5% starch addition levels but increased again with the addition of 1.5% starch to a value equivalent to that observed at the 0.5% starch addition level. These changes in the direction of the effect on the stress between starch addition levels of 0.5% and 1.5% were observed consistently in all the replicate samples and were therefore considered to be real effects.

This behaviour of the breaking stress as a function of the level of starch added is not yet fully understood. However, this behaviour could be due to two reasons. Firstly, it is possible that the amylose and amylopectin that leached from the starch granules during the heating affected the structure and connectivity of the protein network. This was previously proposed by Olsson, Langton, and Hermansson (2002), who observed a similar fracture stress behaviour in a β-lactoglobulin and amylopectin potato starch system.

Secondly, it can be speculated that breaking of the acid milk gels can occur through fracture caused by two effects. On the one hand, it is expected that the stress would increase with the increase in the viscosity of the continuous phase and with the increase in the protein concentration due to the intake of water by the starch granules, as previously discussed. On the other hand, the stress at which the gel would fracture could decrease, as the increase in the level of starch added would result in an increase in the number of voids and defects, caused by the remnant starch granules. However, it is worth noting that at high strains (100%), the value of the stress increased with an increase in the starch concentration (see Fig. 4a). This is an indication
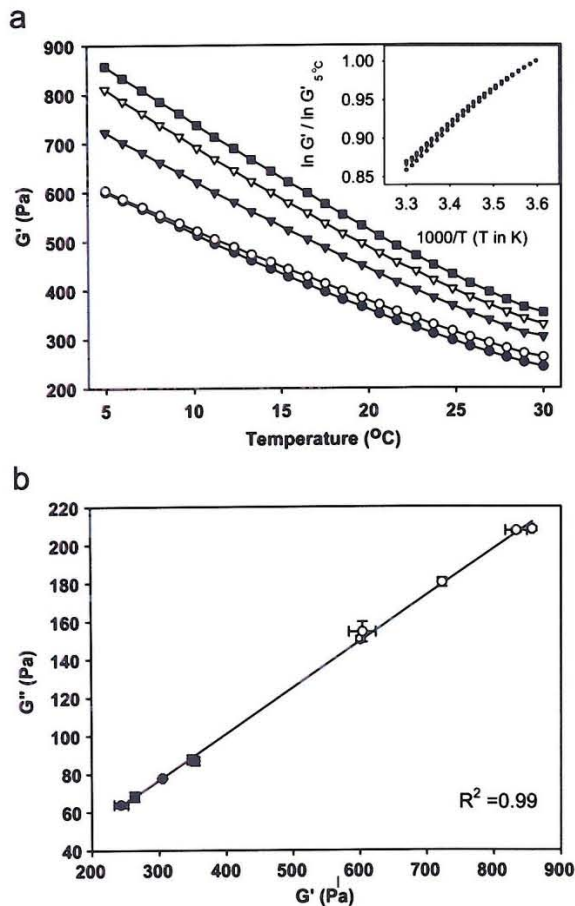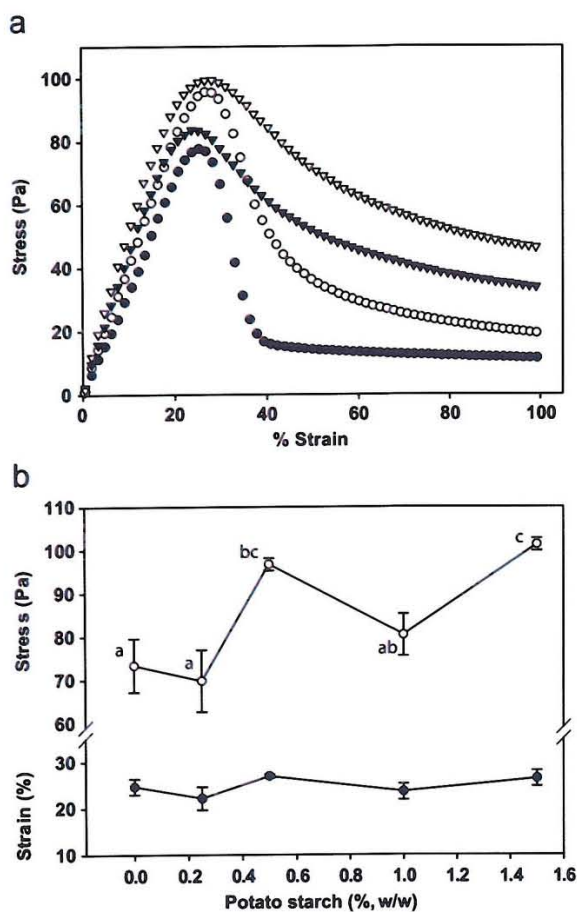
Fig. 4. (a) Stress as a function of strain for acid milk gels containing (●) 0%, (○) 0.5%, (▼) 1% and (▽) 1.5% potato starch. (b) Breaking stress (○) and breaking strain (●) of acid milk gels as a function of potato starch concentration. Error bars represent standard deviations. Data points with the same letter are not significantly different ($P < 0.05$) as determined by ANOVA.

that if these gels were homogenized, which usually occurs in the manufacture of stirred yoghurt, their viscosity would increase, as expected with the addition of starch.

3.5. CSLM

CSLM micrographs of acid milk gels with different levels of added starch are shown in Fig. 5. The addition of Fast Green CFC dye had no effect on the rheological properties during acidification (results not shown). The acid gel from milk with no starch had a relatively open and regular protein network (Fig. 5a), similar to those reported by Lucey et al. (1999). In samples to which starch had been added, swollen granules appeared as dark globules embedded in a protein network; as expected, the number of these globules increased with an increase in the starch

concentration (Fig. 5b–d). Furthermore, at all levels of starch addition, the protein network remained as a dominant continuous phase, with the density of the protein network increasing with an increase in the starch level (Fig. 5b–d). These microstructural observations are in agreement with the rheological measurements, which indicated that the behaviour of the acid milk gels containing starch was dominated mainly by the behaviour of the protein network.

Starch gelatinization involves swelling of granules and leaching of soluble components, primarily amylose, which contribute to an increase in viscosity (BeMiller & Whistler, 1996). Raw potato starch granules are approximately 20 μm in diameter. The granules of potato starch shown in the confocal micrographs (Fig. 5b–d) are up to 50 μm in diameter, which clearly indicates that the granules were swollen and the potato starch added to the milk was, therefore, gelatinized. This was expected, as the heat treatment (80 °C for 30 min) given to the samples upon the addition of starch to the milk provided a sufficiently high temperature for gelatinization of potato starch.

To summarize the findings, although different studies have reported that interactions between starch and milk protein do occur (Bertolini, Creamer, Eppink, & Boland, 2005; Goel, Singhal, & Kulkarni, 1999; Lelievre & Husbands, 1989) and starch components such as amylopectin and amylose may affect the protein network in a similar way, as suggested by Olsson et al. (2002), the effect of starch addition to milk on the firmness of acid milk gels shown in the current study can be explained largely in terms of starch gelatinization in the presence of milk.

Firstly, during heating, the added starch absorbs water, which leads to an increase in the effective concentration of milk protein, which forms the continuous phase. This is clearly observed in the confocal micrographs (Fig. 5a–d), which show the increasing density of the protein network as the starch addition level was increased. Secondly, the leached amylose during starch gelatinization will also increase the viscosity of the aqueous phase, which will result in strengthening of the protein network. Thirdly, the swollen starch granules themselves, which appeared to be embedded in the dominant protein network, will contribute to the gel strength of the acid milk gels. As shown in Fig. 1b and c, the G' of the final acid gels increased almost linearly as the starch addition level increased. This linear increase was also observed by Bikker et al. (2000), when whey protein mixtures containing β-lactoglobulin were added to milk prior to heating and acidification. However, unlike the starch granules, which reinforced the gel network by the protein concentrating effect and the embedding of the granules, the whey proteins provided increased protein concentration and larger aggregate structures that, on acidification, can provide a higher level of cross-linking and a firmer gel structure.

Whereas the final storage modulus, G', of the acid gels increased with an increase in the level of starch addition, the breaking strain of the gels remained relatively constant

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* Refer to Figure A2 in Appendix 2 for 'results not shown' in line 8.

7-11
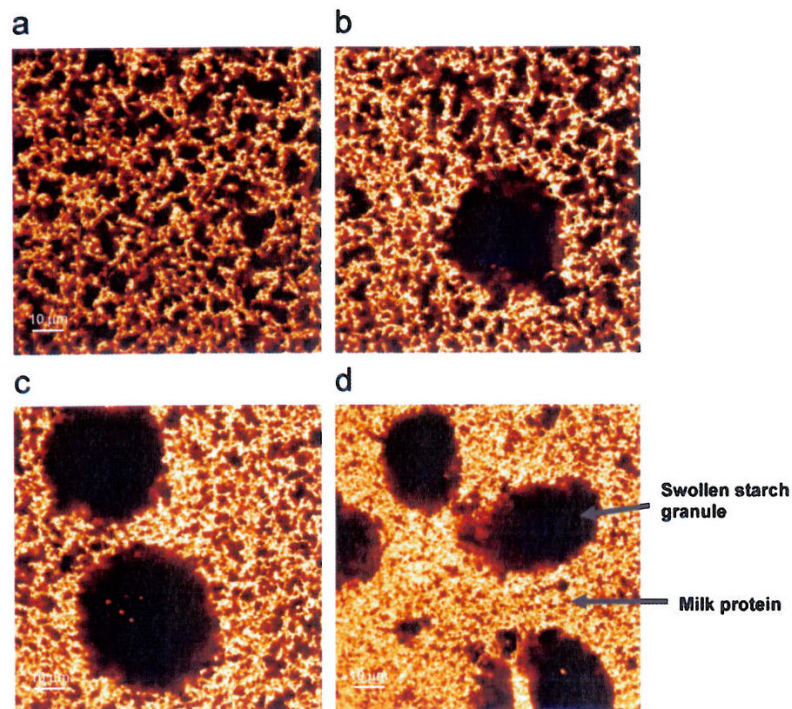
Fig. 5. CSLM micrographs of final acid milk gels containing (a) 0%, (b) 0.5%, (c) 1% and (d) 1.5% potato starch. The bar corresponds to 20 μm.

across the different starch addition levels (Fig. 4b). This indicates again that the rheological behaviour of these systems is dominated mainly by the behaviour of the milk protein network, which depends on the protein–protein bonds. In contrast, the changes in breaking stress could be due to the introduction of defects to the protein network. Although similar complexity in fracture properties of mixed gels was shown in other studies, including that by DeMars and Ziegler (2001), and explained by a phase inversion behaviour, the results in this study cannot be explained in the same way. As shown in the confocal micrographs (Fig. 5), milk protein always formed the continuous phase regardless of the starch addition; therefore, phase inversion did not take place.

4. Conclusions

This study demonstrated the changes in acid gelation curves with the addition of potato starch prior to heat treatment, which have not been shown previously. It was found that starch addition resulted in a reduction in the gelation time and an increase in the gelation pH. The addition of starch increased the final G' values of acid milk gels and the magnitude of the increase was dependent on the level of starch added to the milk. The CSLM micrographs revealed that the starch granules appeared to be embedded in the dominant protein network, which was in agreement with other observations made in this

study. Potato starch increased the firmness of the acid gels but the gels still showed typical acid gel characteristics in terms of viscoelastic properties. The breaking strain remained relatively unchanged regardless of the starch addition level whereas the effect of different starch addition levels on the breaking stress was complex.

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Effect of pH adjustment at heating on the rheological properties of acid skim milk gels with added potato starch

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Abstract

The rheological properties of acid skim milk gels, prepared from milk with added potato starch and pH adjusted (pH 6.5–7.1) prior to heat treatment and acidification, were investigated. The storage modulus, G', of the final acid gels was increased by heating the milk at higher pH and further increased by adding starch. The effect of pH at heating and addition of starch appeared to be additive and independent of each other up to a starch addition level of 1%. Above this starch level, the pH at heating had a lesser effect. This may have been due to the increased viscosity of the aqueous phase as a result of starch gelatinization or to direct contributions of the starch to the gel network structure. Confocal microscopy showed that milk proteins developed fewer but broader protein clusters at higher pH than at lower pH. Starch addition resulted in an increased density of the protein network.
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Keywords: Skim milk; Acid gelation; pH adjustment; Starch; Rheology

1. Introduction

Recent studies have shown that the pH of the milk at heating can modify the properties of acid gels prepared from this heated milk. Compared with samples heated at the natural pH, increasing the pH at heating to about pH 7.1 increased the firmness of the acid gels, whereas decreasing the pH at heating to about pH 6.5 markedly decreased the firmness of the acid gels (Anema, Lee, Lowe, & Klostermeyer, 2004; del Angel & Dalgleish, 2006; Lakemond & van Vliet, 2005; van Vliet, Lakemond, & Visschers, 2004).

These results were explained by changes in the interaction behaviour of the denatured whey proteins with the casein micelles during heating, since the levels of denatured whey protein associated with the casein micelles decreased as the pH at heating was increased (Anema et al., 2004; Anema & Li, 2003; del Angel & Dalgleish, 2006; Vasbinder & de Kruif, 2003). On heating at pH 6.5, about 80% of the

denatured whey proteins are associated with the casein micelles, whereas on heating at pH 7.1 only about 30% of the denatured whey proteins are associated with the micelles.

The structure of the protein network that forms on subsequent acidification is affected by these changes in interactions (Anema et al., 2004; del Angel & Dalgleish, 2006). Lakemond and van Vliet (2005) showed that the permeability of acid gels prepared from milk heated at a higher pH was higher than the permeability of acid gels prepared from milk heated at a lower pH, which in turn indicates that the pore sizes of the gels were changed by the pH at heating.

The addition of starch to milk prior to heat treatment and acidification can be used to modify the properties of acid gels (Decourcelle, Lubbers, Vallet, Rondeau, & Guichard, 2004; Keogh & O'Kennedy, 1998; Williams, Glagovskaia, & Augustin, 2003, 2004). For example, the addition of 1% modified waxy corn starch to milk before heating and acidification increased the viscosity of the resultant stirred yoghurt (Williams et al., 2003, 2004). Similarly, yoghurt containing tapioca starch had a higher

firmness than full-fat yoghurt (Sandoval-Castilla, Lobato-Calleros, Aguirre-Mandujano, & Vernon-Carter, 2004).

We examined the effect of adding potato starch (up to 1.5%, w/w) to skim milk prior to heating and acidification on the rheological properties of acid gels (Oh, Anema, Wong, Pinder, & Hemar, 2007). The firmness (G') of the acid gels increased proportionally with the level of starch added, whereas the breaking stress and breaking strain showed a complex behaviour with increases and decreases depending on the level of starch added. In this previous study, the milk/starch systems were heated at the natural pH of the milk before acidification to form acid gels. As the pH at heating is known to influence the rheological properties of acid skim milk gels prepared from the heated milk, this current study examined whether starch addition modified this effect of the pH at heating. Milk samples were pH adjusted (pH 6.5–7.1) prior to starch addition (0–2%, w/w), heat treatment and acidification. The rheological and microstructural properties of the acid skim milk gels were examined.

2. Materials and methods

The materials and methods used in this study were the same as described in our previous paper (Oh et al., 2007), apart from the pH adjustment of the milk before heating, the readjustment of the pH of the milk after heating and the determination of viscosity of the heated milk samples. Prior to potato starch addition and heating (80 °C for 30 min), the pH of the milk samples was adjusted from the natural condition (pH ≈ 6.64) to a pH value in the range from 6.5 to 7.1 by the slow addition of hydrochloric acid (1 M) or sodium hydroxide (1 M) while stirring the milk. Potato starch was added to the pH-adjusted skim milk at levels from 0% to 2% (w/w). After heat treatment and cooling, hydrochloric acid (1 M) or sodium hydroxide (1 M) was slowly added to the milk at room temperature (20 °C) with stirring, to readjust the pH back to the natural pH of the milk prior to acidification. The shear stress of each heated milk sample was measured at shear rate 0.1–100 s^{-1} using an AR2000 rheometer (TA Instruments, New Castle, DE, USA) and a cone (4 cm, 4° and 100 μm truncation) and plate geometry after readjustment of pH. The apparent viscosity was determined at a shear rate of 100 s^{-1}.

All experiments were repeated in duplicate. Analysis of variance (ANOVA) using MINITAB Statistical Software was conducted for statistical analyses where appropriate.

3. Results

3.1. Flow properties of heated milk samples

The term 'pH at heating' is used to describe the pH of the milk sample at the time of heating, in order to differentiate this from the pH changes observed during the acid gelation process. Fig. 1A shows the shear stress versus shear rate curves for milk samples heated at its natural pH
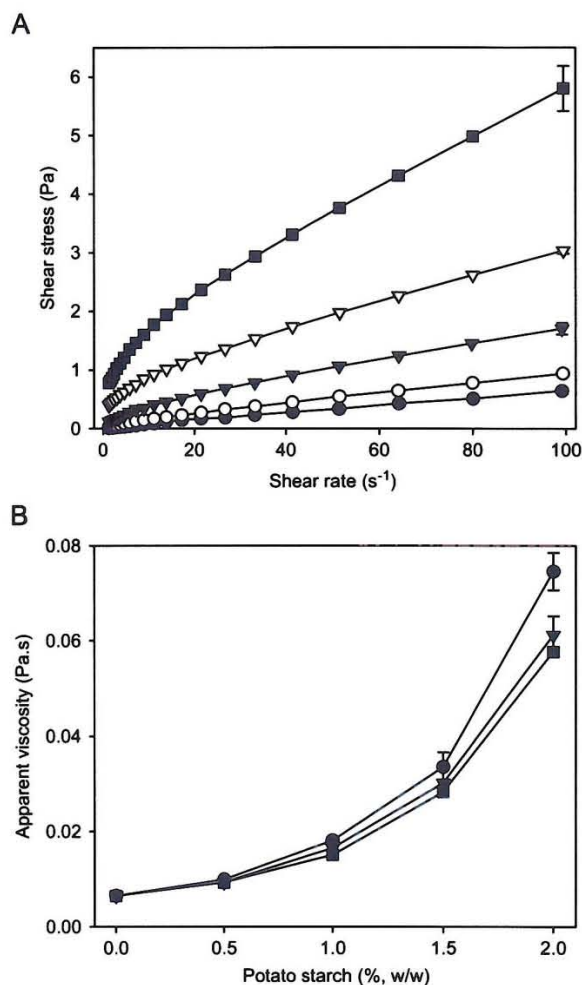
Fig. 1. (A) Shear stress as a function of shear rate for milk heated at pH 6.64 with (●) 0%, (○) 0.5%, (▼) 1%, (▽) 1.5% and (■) 2% potato starch. (B) Apparent viscosity as a function of starch addition level at the shear rate of 100 s^{-1} for milk heated at: (●) pH 6.5; (▼) pH 6.64; (■) pH 6.9.

(pH ≈ 6.64) with different levels of added starch. Shear stress increased with shear rate in all samples and the gradient of the curve increased as the level of added starch increased. Fig. 1B shows the apparent viscosity of milk samples heated at pH 6.5, 6.64 (natural) and 6.9 at shear rate 100 s^{-1} as a function of starch addition levels. The apparent viscosity of all three samples increased progressively as the starch addition level increased and the three curves showed similar trends. The apparent viscosity increase was greater at higher starch addition levels. For example, when the milk samples were heated at pH 6.64, increasing the starch addition level from 0% to 0.5% resulted in an approximately 46% increase in the apparent viscosity. The rate of apparent viscosity rise then increased so that increasing the starch addition level from 1.5% to

2% resulted in an approximately 100% increase in the apparent viscosity.

Compared with milk samples heated at pH 6.64, samples heated at pH 6.5 displayed consistently higher apparent viscosity and those heated at 6.9 displayed consistently lower apparent viscosity over the range of starch addition levels (Fig. 1B). However, statistically significant differences ($P < 0.05$) in apparent viscosity were only found in samples with 2% starch added starch. At the starch addition level of 2%, the milk heated at pH 6.5 had significantly higher apparent viscosity than that heated at pH 6.9 ($P < 0.05$), yet there was no significant difference in apparent viscosity values between the samples heated at pH 6.5 and 6.64 or between those heated at pH 6.64 and 6.9.

3.2. Acid gelation curves

The pH during acid gelation decreased to approximately pH 4.1 after 6 h of acidification with 2% glucono-delta-lactone (GDL). Because each milk was readjusted back to the natural pH after heating, the acidification process always started from the same pH. Therefore, the reduction in pH during acidification was not affected by the pH at heating, which is in agreement with literature reports (Anema et al., 2004; del Angel & Dalgleish, 2006). The addition of starch to the samples did not affect the change in pH on acidification (results not shown), as also found by Williams et al. (2003) and Oh et al. (2007).

Fig. 2 shows the storage modulus, G', as a function of time after the addition of GDL for milk samples with 0% (Fig. 2A) and 1% (Fig. 2B) added starch and heated at different pH values. The shapes of these curves are typical for the acid gelation of milk and are consistent with earlier reports (Anema et al., 2004; Lucey, Teo, Munro, & Singh, 1997; Oh et al., 2007). The 'gelation time' and the 'gelation pH' are defined as the time and the pH at which G' reached 0.5 Pa. The G' and loss modulus (G'') values of the acid gels after 6 h are referred to as the 'final G'' and the 'final G''' respectively.

For the samples without starch (Fig. 2A), the curves showed that gelation occurred after about 30–40 min of acidification in all samples. After gelation, as the pH at heating was increased, the G' value increased at any given acidification time so that the sample heated at pH 7.1 had a final G' value approximately double that of the sample heated at pH 6.5. This is in agreement with other studies (Anema et al., 2004; del Angel & Dalgleish, 2006; Lakemond & van Vliet, 2005; van Vliet et al., 2004). The acid gelation curves for the samples with 1% added potato starch (Fig. 2B) showed that gelation occurred after about 30–35 min. The pH at heating had a similar general effect on the G' of the samples to that observed for the samples without added starch. Comparison of Figs. 2A and B shows that, for samples heated at any given pH, starch addition shifted the acid gelation curve to higher G' when compared with the equivalent samples without added starch.
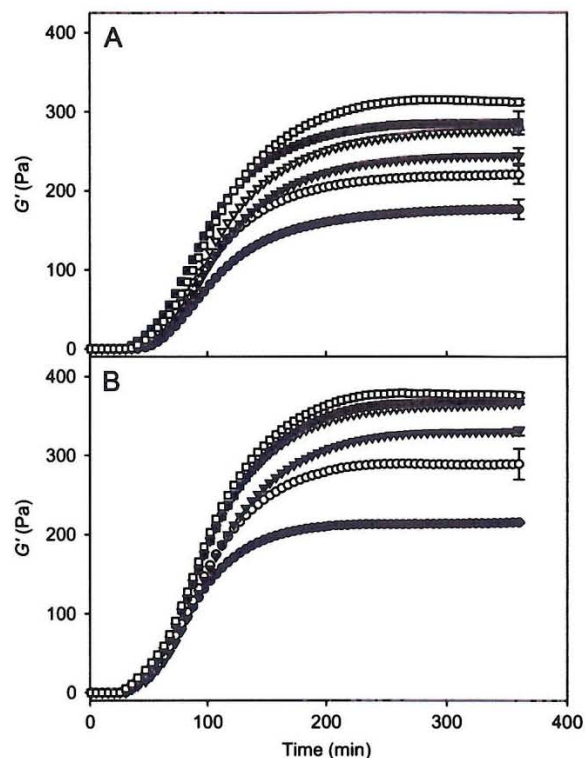
Fig. 2. Changes in storage modulus, G', after GDL addition as a function of time at 30 °C for milk with 0% (A) and 1% (B) potato starch heated at: (●) pH 6.5; (○) pH 6.6; (▼) pH 6.64; (▽) pH 6.75; (■) pH 6.9; (□) pH 7.1. Error bars represent standard deviations of repeated measurements.

For the samples with no added starch, the gelation time decreased and the gelation pH increased as the pH at heating increased (Table 1), which is in agreement with Anema et al. (2004), Lakemond and van Vliet (2005), and del Angel and Dalgleish (2006). At any given starch addition level, a decrease in gelation time and an increase in gelation pH with increasing pH at heating were also observed; however, a statistical analysis indicated that only the differences in gelation times and gelation pH for the samples with 0% and 0.5% added starch were statistically significant. In addition, at any given pH at heating, the gelation time decreased and the gelation pH increased as the level of added starch increased, which is in agreement with studies on milk heated at the natural pH (Oh et al., 2007; Williams et al., 2004). This may be due to the increased effective protein concentration in the aqueous phase due to uptake of water by starch granules during swelling.

Fig. 3A shows the final G' plotted against the pH at heating for the samples with 0–2% added starch. The addition of starch to milk prior to heating resulted in an increase in the final G' of the acid gels at all pHs at heating. For the acid gels with 0–1% potato starch, the most noticeable increase in the final G' value was for milk heated

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* Refer to Fig. 1(a) on page 7-8 for 'results not shown' in line 25.

Table 1
Gelation time and gelation pH of acid skim milk gels with different potato starch levels heated at pH 6.5–7.1[a]

	0% Potato starch		0.5% Potato starch		1% Potato starch		1.5% Potato starch		2% Potato starch	
	Gel time (min)	Gel pH	Gel time (min)	Gel pH	Gel time (min)	Gel pH	Gel time (min)	Gel pH	Gel time (min)	Gel pH
pH 6.5	41[a,A]	5.24[a,E]	35[a,B]	5.32[a,F]	31[a,BC]	5.36[a,FG]	26[a,CD]	5.41[a,GH]	24[a,D]	5.45[a,H]
pH 6.6	36[ab,A]	5.31[bd,E]	34[abc,B]	5.33[ab,EF]	30[a,C]	5.37[a,F]	26[a,D]	5.41[a,G]	–	–
pH 6.64	37[ac,A]	5.29[b,E]	31[bd,AB]	5.36[b,EF]	29[a,AB]	5.38[a,EF]	24[a,BC]	5.44[a,FG]	19[a,C]	5.52[a,G]
pH 6.75	36[ad,A]	5.31[bc,E]	31[bd,AB]	5.36[b,EF]	28[a,B]	5.40[a,F]	24[a,B]	5.44[a,F]	–	–
pH 6.9	33[bd,A]	5.35[cd,E]	30[d,A]	5.37[b,E]	24[a,BC]	5.44[a,EF]	21[a,CD]	5.47[a,FG]	18[a,D]	5.54[a,G]
pH 7.1	34[bc,A]	5.33[cd,E]	30[cd,A]	5.37[b,E]	24[a,B]	5.44[a,EF]	21[a,BC]	5.47[a,FG]	18[a,C]	5.54[a,G]
Pooled S.D.	1.5	0.01	0.8	0.01	1.9	0.03	1.9	0.02	2.0	0.04

[a]Pooled standard deviations (S.D.) of each column are given. Values with the same lower case superscript letter within a column are not significantly different and values with the same upper case superscript letter within a row are not significantly different ($p < 0.05$) as determined by analysis of variance (ANOVA).
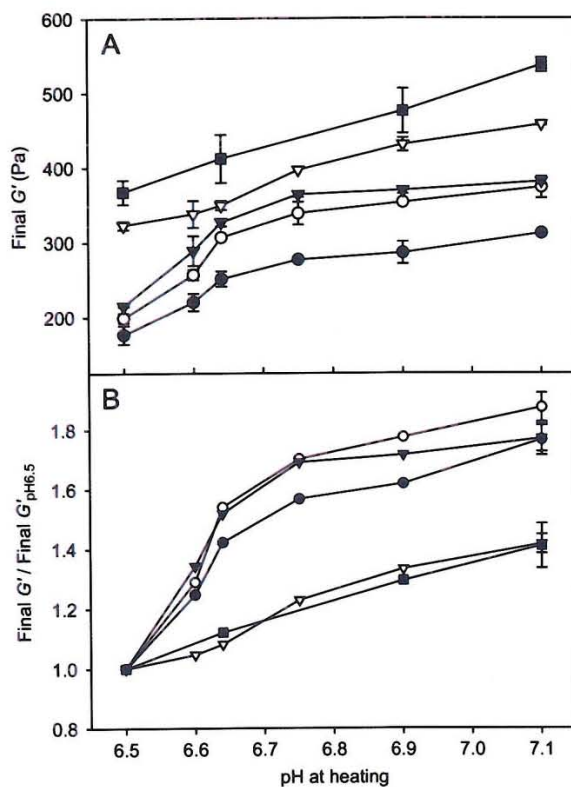
Fig. 3. (A) Final value of the storage modulus, G', and (B) ratio of final G' value at a given pH to final G' value at pH 6.5 for milk with (●) 0%, (○) 0.5%, (▼) 1%, (▽) 1.5% and (■) 2% potato starch at 30 °C as a function of the pH at heating. Error bars in Fig. 3A represent standard deviations of repeated measurements. Error bars in Fig. 3B represent pooled standard deviations.

between pH 6.5 and pH 6.75, whereas the changes in the final G' value were much smaller for milk heated at pH between 6.75 and 7.1. However, at the 1.5% and 2% starch addition levels, the pH at heating had a different effect on the increase in final G', especially for milk heated between

pH 6.5 and 6.75 where the increase with increasing pH was smaller compared with lower starch addition levels (Fig. 3A). This can be seen more clearly in Fig. 3B, in which the changes in the final G' at any given pH at heating relative to the final G' of the gels prepared from the samples heated at pH 6.5 are plotted against the pH at heating. The changes in the final G' for the samples with 0–1% added starch were very similar in shape, with marked changes in the ratio of the final G'/final $G'_{pH\ 6.5}$ with increasing pH. However, the changes in the final G'/final $G'_{pH\ 6.5}$ for the samples with 1.5% and 2% added starch were substantially different as there were much smaller increases in final G' as the pH at heating was increased (Fig. 3B).

3.3. Viscoelastic properties of acid milk gels

For each sample, a temperature sweep showed that the final G' and the final G'' at 5 °C were 2.4 times (± 0.1) greater than those observed at 30 °C (results not shown). Regardless of the temperature of the gels, the level of starch addition or the pH at heating, the relationship between final G' and final G'' was linear ($R^2 = 0.997$, $P < 0.001$), and tan δ was 0.25 ± 0.01 (results not shown). Other studies have also reported relatively constant tan δ value despite different treatment history of the milk prior to preparing the acid gels (Anema, Lauber, Lee, Henle, & Klostermeyer, 2005; Lucey et al., 1997).

It is not fully understood why G' and G'' increase markedly, and tan δ remains essentially constant when the temperature of the set acid gel is decreased. However, it is known that hydrophobic interactions are strongly temperature dependent and play an important role in the assembly of casein micelles, and in the structure of acid casein gels. van Vliet, Roefs, Zoon, and Walstra (1989) have suggested that decreased hydrophobic interactions with decreasing temperature may lead to less compact conformation of casein molecules, thereby increasing the size of casein particles within acid gel networks. Such changes in size and interactions would alter the balance between inter-particle and intra-particle bonds so that

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* Refer to Figure A3 in Appendix 3 for 'results now shown' in line 17.
** Refer to Figure A4 in Appendix 4 for 'results not shown' in line 21.

there were more inter-particle bonds between casein particles as the temperature were reduced, and as a consequence, this could result in increases in G' and G'' (van Vliet et al., 1989). Other factors may also contribute to the changes in the G' and G'' with temperature. For instance, increased viscosity of the aqueous phase (water, lactose, milk salts and soluble starch components) with decreasing temperature may account for some changes in the final G'', although the effect is likely to be smaller and seems to be compensated by other factors so that tan δ is not affected.

3.4. Large deformation rheology

After 3 h of acidification, some of the final set gels were subjected to a strain sweep at 5 °C and the shear stress was measured (Fig. 4). The stress increased to a maximum as the strain was increased and then decreased, indicating that the gel structure had been destroyed (Figs. 4A and B). The stress and the corresponding strain at this maximum were considered to be the breaking stress and the breaking strain, respectively, and these are plotted against the level of added starch in Fig. 4C.

The breaking strain value of the final acid gels was between 20% and 35% for all samples (Fig. 4). The breaking strain was not significantly affected by the pH at heating for the samples with no added starch (Figs. 4A and C). However, for the samples with added starch, the breaking strain appeared to decrease as the pH at heating was increased (Figs. 4B and C). Although this effect of pH was consistently observed for all samples, only the breaking strains for the acid gels from milk with 1% added starch and heated at pH 6.9 were significantly lower than those for the acid gels from milk heated at pH 6.5 ($P < 0.05$).

Increasing the pH of the milk before heating resulted in acid gels that required higher stress to break the gel structure at all starch addition levels (Fig. 4), which is consistent with the report by Lakemond and van Vliet (2005) for acid gels from milk samples without added starch. The effect of starch on the breaking stress was not consistent across the starch addition levels (Fig. 4C). Compared with samples with no added starch, the addition of 0.5% starch significantly increased the breaking stress in all the acid gels at any given pH at heating. The breaking stress decreased when the starch addition level was increased to 1%, although the effect was less pronounced as the pH at heating was decreased. The breaking stress increased again with the addition of 1.5% and 2% starch. These changes in the direction of the effect on the stress between starch addition levels of 0.5% and 2% were reproducible in all samples.

3.5. Confocal scanning laser microscopy (CSLM)

CSLM micrographs of final acid milk gels prepared from milk samples heated at pH 6.5 or 6.9 and with 0% or 1%
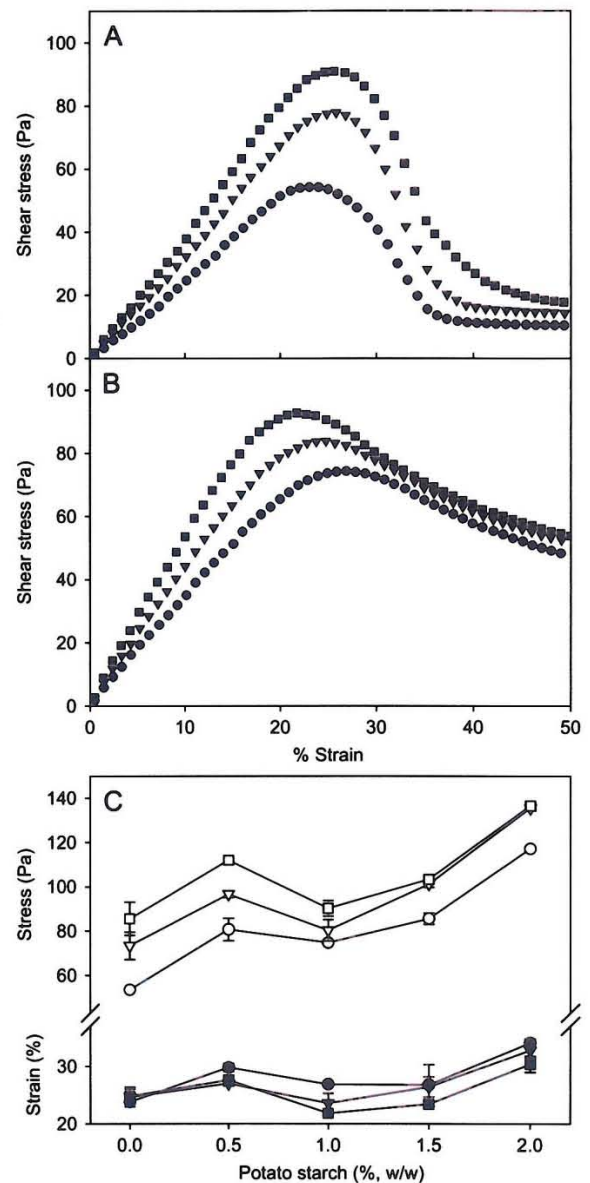
Fig. 4. Stress as a function of strain for acid milk gels containing 0% (A) and 1% (B) potato starch. Milk heated at: (●) pH 6.5; (▼) pH 6.64; (■) pH 6.9. (C) Breaking stress and breaking strain of acid milk gels as a function of starch addition level. Milk heated at: (●, ○) pH 6.5; (▼, ▽) pH 6.64; (■, □) pH 6.9. Filled symbols for strain and open symbols for stress. Error bars in Fig. 4C represent standard deviations of repeated measurements.

added starch are shown in Fig. 5. For the samples with no added starch (Figs. 5A and C), it appears that the different pHs at heating resulted in different arrangements of the protein clusters and pores that formed the acid gel network. In the acid gel sample prepared from the milk heated at pH 6.5, the protein clusters appeared to be
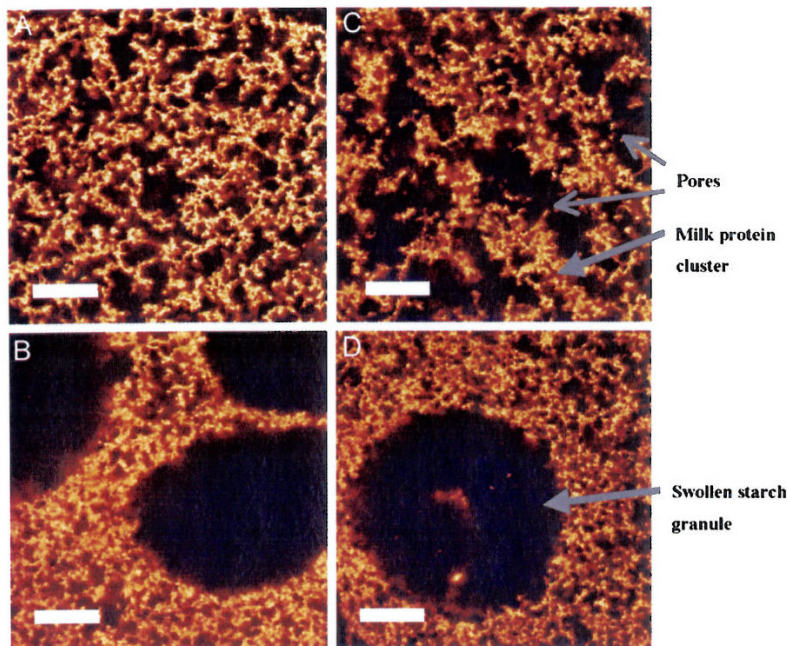
Fig. 5. CSLM micrographs of final acid milk gels: heated at pH 6.5 and containing 0% (A) and 1% (B) potato starch; heated at pH 6.9 and containing 0% (C) and 1% (D) potato starch. The bar corresponds to 20 μm.

relatively fine and evenly spread throughout the gel, with small pores regularly inter-dispersed in this protein network (Fig. 5A). In contrast, in the sample prepared from the milk heated at pH 6.9, there were fewer but broader protein clusters, and many of the pores within the protein network were considerably larger and irregular (Fig. 5C).

Lakemond and van Vliet (2005) reported that the permeability of acid gels made from milk heated at pH 6.9 was higher than that of acid gels made from milk heated at pH 6.2. The increased pore size observed in the samples produced from milk heated at higher pH (Fig. 5C) would account for the increased permeability of these acid gels when compared with those produced from milk heated at lower pH (Fig. 5A).

In the acid gel samples with 1% added starch (Figs. 5B and D), the micrographs displayed swollen starch granules, which appeared as dark globules embedded in the protein network. The addition of starch increased the density of the protein network (Figs. 5B and D). Although the protein network was denser in the presence of starch, it appeared that the addition of 1% starch did not alter the effect of the pH at heating on the protein network in the acid gel samples. There were fewer protein clusters and larger irregularly spaced pores for the acid gels prepared from the milk heated at pH 6.9 (Fig. 5D) than for the acid gels prepared from the milk heated at pH 6.5 (Fig. 5B). At starch addition levels above 1%, the protein network became very dense and no obvious effect of the pH at heating could be observed (results not shown).

4. Discussion

The pH at heating has a pronounced effect on the association behaviour of the denatured whey proteins with the casein micelles in the heated milk. As the pH at heating was increased, the level of denatured whey protein associated with the casein micelles decreased and the level of soluble denatured whey proteins increased (Anema & Li, 2003; del Angel & Dalgleish, 2006; Vasbinder & de Kruif, 2003). The distribution of denatured whey proteins between serum and colloidal phases as a consequence of heating do not change on the subsequent re-adjustment of milk back to the natural pH (unpublished results). These differences influence the aggregation behaviour of the casein and whey proteins on subsequent acidification to form the acid gels, which alters the firmness of the resultant acid gels (Anema et al., 2004; del Angel & Dalgleish, 2006; Lakemond & van Vliet, 2005). The confocal micrographs showed that the pH at heating affected the protein network in the acid gel by influencing the arrangement of the protein clusters and the size of the pores within the gel network on acidification of the heated milks (Figs. 5A and B), and that these differences in protein and pore structures in the gel network influenced the final firmness of the acid gels (Figs. 2 and 3).

Potato starch in an aqueous environment is known to start gelatinizing when heated over a temperature range from 58 to 65 °C (BeMiller & Whistler, 1996), and therefore would be completely gelatinized under the heating

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* Refer to Figure A5 in Appendix 5 for 'results not shown' in the left column line 29.
** Refer to Figure A6 in Appendix 6 for 'unpublished data' in the right column line 13.

conditions used in this study. This indicates that gelatinization of starch would occur concurrently with whey protein denaturation. The confocal micrographs showed the swollen starch granules were over 50 μm (Fig. 5), which is significantly larger than the native starch granules (about 20 μm). The apparent viscosity increase with starch addition also indicates that starch gelatinization had occurred in milk during the heating (Fig. 1). As starch gelatinization is not known to be affected by the range of pH values to which the milk was adjusted (Garcia-Alonso, Jimenez-Escrig, Martin-Carron, Bravo, & Saura-Calixto, 1999), the addition of starch showed a similar effect on apparent viscosity at all the different pH values of the milk at heating (Fig. 1).

Starch gelatinization involves swelling of granules and leaching of soluble components, primarily amylose (BeMiller & Whistler, 1996). On swelling, the starch absorbs water and milk serum, which effectively concentrates the milk protein in the aqueous phase. On acidification to form gels, the presence of swollen starch increases the density of the protein network, which is clearly evident in the confocal micrographs (Figs. 5B and D). Therefore, the presence of the geltainized starch would increase the firmness of the acid gels in a similar fashion to increasing the concentration of the milk solids. The leaching of amylose into the milk serum would also increase the viscosity of the aqueous phase, and this in turn would further increase the firmness of the acid gels.

At low levels of starch (up to 1%), the effect of the pH at heating and the effect of added starch on the properties of the acid gels appeared to be additive and essentially independent of each other (Figs. 2–4). For example, in Fig. 3B, the shape of the curves indicates that the pH at heating had a similar effect, with the added starch shifting the curve to higher G' values by a similar amount at each pH at heating. In addition, the confocal micrographs (Fig. 5) revealed that the microstructure of the acid gel protein network in the samples with 0% or 1% added starch was affected similarly by the pH of the milk at heating, with the starch increasing the density of the protein network. This suggests that the effect of pH at heating on the interaction of whey proteins and casein micelles was similar in milks with up to 1% added starch.

At higher levels of added starch (1.5% or 2%), the effects of the pH at heating and starch addition could no longer be considered to be additive (Fig. 3). The pH at heating had a diminished effect on the final G' of the acid gels and this can be most clearly seen in Fig. 3B. This appeared to be largely a consequence of a markedly increased final G' when the pH at heating was below 6.75 when compared with the samples with lower levels of starch (Fig. 3A). As the effect of the pH at heating on the subsequent acid gel firmness is considered to be due to changes in the interaction of denatured whey proteins with the casein micelles (Anema et al., 2004; del Angel & Dalgleish, 2006), it is possible that, at high starch concentrations (1.5% or above), the increased viscosity of

the aqueous phase (Fig. 1) may affect the diffusion of the protein components during heating and, consequently, may reduce the association of the denatured whey proteins with the casein micelles, particularly at the lower pH at heating. Unfortunately, it was not possible to analyse the distribution of whey proteins between the colloidal and serum phases in the heated milks with added starch. The swollen starch granules interfered with the centrifugation process and this centrifugation is necessary to separate milk protein components between colloidal and serum phases.

The high viscosity of the aqueous phase may also modify the interactions of the protein components on the subsequent acidification of the milk during the preparation of the acid gels. For example, it is possible that the aggregation of the casein micelles in skim milk during the acidification to form acid gels is a predominantly reaction-limited process. However, the increased viscosity on starch addition to the milk (Fig. 1) could influence the aggregation process so that at high starch levels, aggregation becomes a predominantly diffusion-limited process. A change in aggregation from reaction-limited to diffusion limited could account for the unusual effect of high starch levels on the pH-dependence of the final G' (Fig. 3). However, the extent of the viscosity effect on protein aggregation is difficult to determine as respective contributions of swollen granules and leached starch materials to the changes in the viscosity of the aqueous phase cannot be easily measured.

Modelling the aggregation of casein micelles in skim milk based on fractal structures has the potential to differentiate reaction and diffusion limited processes through the inherent differences in fractal dimensions from these extremes in reaction probability (Horne, 1999; Vetier, Desobry-Banon, Eleya, & Hardy, 1997). However, application of fractal modelling to the acid-induced aggregation of casein micelles were inconclusive in determining whether the process was reaction or diffusion limited (Chardot, Banon, Misiuwianiec, & Hardy, 2002; Vetier et al., 1997). The unexpectedly high fractal dimensions in biological systems, such as aggregated casein micelles, may be due to considerable cluster restructuring during aggregation (Horne, 1999; Vetier et al., 1997), which leads to marked differences in fractal dimensionality that are unrelated to the primary aggregation phenomenon.

Although this has not been studied in acid gel systems, the heat-induced aggregation/gelation of β-lactoglobulin in mixed potato amylopectin/β-lactoglobulin systems has been shown to be affected by the concentration of potato amylopectin, a major component of potato starch (Olsson, Langton, & Hermansson, 2002). The temperature of aggregation/gelation of β-lactoglobulin was reduced with increasing concentrations of potato amylopectin. In addition, an increased concentration of potato amylopectin resulted in a more rapid aggregation of β-lactoglobulin, and markedly different connectivity within the gel structures. This also affected the fracture properties of the gels. The modified aggregation behaviour was attributed to the

high viscosity of the aqueous phase due to increased concentration of potato amylopectin. The high viscosity was also found to restrict protein clusters from forming a connected protein network. Although the system used in this study contains different components, the high viscosity of the aqueous phase may have affected the protein aggregation behaviours in a similar manner during acidification.

An alternative explanation for the diminished effect of pH at heating on the rheological properties of the acid gels at high starch concentrations (1.5% or above; Fig. 3) is phase separation into amylose-rich and protein-rich regions. At starch addition levels below 1.5%, the acid gel network is dominated by the milk protein, whereas, at higher levels, the gelatinized starch may have a greater contribution to the gel network. It has been shown that amylose leached into the aqueous phase during heating of milk with added starch (Oh et al., 2007). If the concentration of leached amylose in the aqueous phase was sufficiently high after heating, it could form a separate phase of a weak gel network that excludes proteins, therefore phase separation occurs. Such changes could alter the aggregation behaviour of proteins during acidification, therefore lead to a structure in which both protein and starch have a major contribution to the gel network, replacing the protein-dominant system obtained at lower starch concentrations. Therefore, at high starch levels, changes in the protein component of the network may not have the same impact on the final firmness of the acid gels as observed at lower starch concentrations, and therefore the effect of pH at heating on the acid gels may be diminished (Fig. 3B).

de Bont, van Kempen, and Vreeker (2002) studied phase separation behaviour in a milk protein and amylopectin system prepared by mixing reconstituted skim milk and amylopectin dissolved in milk permeate through heating. The authors found that non-phase separating behaviour was observed in a system containing 2.1% casein and 1.5% amylopectin. When the concentration of amylopectin was increased above 1.5%, phase separation with protein-rich and amylopectin-rich areas occurred. However, there are differences in the methodologies and materials between de Bont et al. (2002) and the current study. de Bont et al. (2002) used dissolved amylopectin, whereas in this study discrete potato starch granules were added to milk. Since materials leaching from starch granules during heating are mainly amylose, especially when the granules are mostly undamaged (BeMiller & Whistler, 1996), even with the highest concentration of added starch (2%) the concentration of leached amylopectin in the aqueous phase is unlikely to reach the phase separation level mentioned in de Bont et al. (2002).

Leached amylose in the aqueous phase was observed using a light microscopy and iodine staining method (Oh et al., 2007). However, it is difficult to conclude whether leached amylose in milk led to phase separation as it was not possible to observe separate phase formation based on this technique. The CSLM micrographs (Fig. 5) revealed the continuous network of protein but did not show any evidence of separate amylose-rich regions. Protein was labelled with a fluorescent dye (Fast green) in the micrographs, therefore appeared bright and all other materials appeared dark in the field (Fig. 5). While the relatively large dark spheres could be readily identified as swollen starch granules, possible presence of amylose-rich regions could not be distinguished from the other non-protein materials which also appeared dark in the micrographs.

The current observations are not sufficient to confirm the possibility of phase separation or the effect of high viscosity on protein aggregation process. Therefore, further studies are required to examine both scenarios and how they influence the properties of acid gels with added starch. Moreover, investigations into the microstructure of acid gels using CSLM with a starch-specific fluorescent dye would show whether amylose-rich regions exist in the gel structure. This could confirm if phase separation had occurred in the acid gels with higher starch addition levels. Likewise, fractal modelling of acid induced aggregation of casein micelles in skim milk would determine whether the process is reaction or diffusion limited. The effect of high viscosity of the aqueous phase on the aggregation process of proteins could then be examined.

5. Conclusions

This study showed that the properties of acid milk gels could be modified by altering the types of protein associations with pH adjustment at heating or by starch gelatinization, which leads to denser protein networks and an aqueous phase of high viscosity. Both methods increased the final acid gel firmness and their effects appeared to be additive and independent of each other up to a starch addition level of 1%. Above this starch level, the pH at heating had a lesser effect, and this may have been due to the increased viscosity of the aqueous phase as a result of starch gelatinization or to direct contributions of the starch to the gel network structure.

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Comparison of pressure treatment and heat treatment of skim milk with added starch on subsequent acid gelation of milk

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Abstract

Skim milk with added starch (waxy rice starch or potato starch at levels of 0 to 1.5%) was either pressure-treated (500 MPa, 20 °C, 30 min) or heat-treated (80 °C, 30 min) and subsequently acidified (using glucono-δ-lactone) to form acid milk gels. The rheological properties of the samples during acidification and of the final acid gels were studied. The storage modulus, *G'*, of the final acid milk gels increased as more waxy rice starch was added to milk before pressure or heat treatment. However, acid milk gels made from pressure-treated milk with added potato starch did not show significant changes in the *G'* of the final acid gels whereas those made from the heat-treated counterparts showed a marked increase in the final *G'* as the potato starch level increased. These results can be explained by the extent of starch gelatinisation in milk during pressure or heat treatment. Waxy rice starch was gelatinised in milk by both pressure treatment and heat treatment whereas potato starch was gelatinised by heat treatment only. Increasing the pH of milk before pressure or heat treatment increased the final *G'* of the acid milk gel produced on subsequent acidification of the milk and the final *G'* was increased further by the addition of waxy rice starch before the pressure or heat treatment.

Key words:
Acid gelation, Milk, Starch, Gelatinisation, Pressure treatment, Heat treatment, pH adjustment

7.1 Introduction

Acid milk gels are particle gels that are formed by aggregation of milk proteins when milk is acidified (Horne, 1999). This gel-forming characteristic of milk proteins is the fundamental basis for making dairy products such as yoghurt and some types of cheeses (Lucey and Singh, 1998; van Vliet et al., 2004). Starch is commonly used as an ingredient in food products and in dairy products, starch can be used to modify the product texture (Keogh and O'Kennedy, 1998; Williams et al., 2003; Sandoval-Castilla et al., 2004; Williams et al., 2004). Our previous studies have shown that when potato starch was added to skim milk prior to heat treatment, the firmness of milk gels made on subsequent acidification of heated milk increased due to the gelatinisation of the starch in skim milk during the heat treatment (Oh et al., 2007a; Oh et al., 2007b). Similar effects of starch addition on yoghurt gels have been reported in other studies (Keogh and O'Kennedy, 1998; Williams et al., 2003; Sandoval-Castilla et al., 2004; Williams et al., 2004). Uptake of water by starch during gelatinisation is proposed to be the primary reason for the increased firmness of acid milk gels prepared from heated milk with added starch as the effective concentration of proteins increases in the aqueous phase which then resulted in a denser protein network on acidification (Oh et al., 2007a).

High pressure processing technology has been gaining popularity as a non-thermal method for the manufacture of food products. As well as inactivating pathogenic and spoilage microorganisms and deteriorative enzymes (Tewari et al., 1999), pressure treatment can lead to changes in functional properties of food by affecting the food constituents. Pressure treatment can affect both milk proteins and starch (Balny et al., 2002). In some respects, the effects of pressure on starch and globular proteins such as whey proteins are similar to, although not identical to, that

of heat (Knorr et al., 2006; Considine et al., 2007). Globular proteins undergo pressure-induced unfolding of the structures, therefore denaturation, which may be largely attributed to penetration of water into the structure (Balny et al., 2002). Water also penetrates into starch granules, which causes swelling of the granules and induces gelatinisation (Rubens et al., 1999).

When heat treatment has been used, milk proteins show different aggregation behaviours on acidification depending on the pH of the milk at heating, and this has resulted in different acid milk gel firmness when the heated milks were subsequently acidified (Anema et al., 2004; Lakemond and van Vliet, 2005; del Angel and Dalgleish, 2006). Compared with samples heated at the natural pH, increasing the pH at heating to about pH 7.1 increased the firmness of the acid milk gels, whereas decreasing the pH at heating to about pH 6.5 decreased the firmness of the acid milk gels (Anema et al., 2004; van Vliet et al., 2004; del Angel and Dalgleish, 2006). These results were attributed to the changes in the interaction behaviour of the denatured whey proteins with the casein micelles during heating, since the levels of denatured whey protein associated with the casein micelles decreased as the pH at heating was increased (Anema and Li, 2003a; Vasbinder and de Kruif, 2003; Anema et al., 2004; del Angel and Dalgleish, 2006). Oh et al. (2007b) showed that the effects of pH at heating and addition of starch was additive and independent of each other up to a starch addition level of 1%.

The properties of acid milk gels produced on acidification may be different when the milk with added starch is pressure-treated instead of heat treated. Therefore, this study examines the acid gels prepared from pressure-treated skim milk with added waxy rice starch or potato starch. The results are compared to those obtained from acid gels prepared from heat treated skim milk with added waxy rice starch and

potato starch. In the first part of this study, skim milk was used at its natural pH. In the second part of this study, the pH of the skim milk was adjusted to pH values between 6.5 and 6.9 to examine the effect of pH at pressure treatment compared to the effect of pH at heat treatment.

7.2 Materials and Methods

7.2.1 Materials

Waxy rice starch was supplied by Remy Industries (Leuven-Wijgmaal, Belgium). Potato starch was supplied by Penford New Zealand Limited (Auckland, New Zealand). All starches were used as supplied. Low heat skim milk powder was obtained from the Edendale site, Fonterra Co-operative Group, New Zealand. Glucono-δ-lactone (GDL) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Skim milk samples were prepared by reconstituting low heat skim milk powder in purified water (reverse osmosis followed by filtration through a Milli-Q apparatus) to a final concentration of 10% (w/w) total solids. The reconstituted skim milk was stirred for at least one hour and stored overnight at ambient temperature (approximately 20 °C) before use.

7.2.2 Sample preparation and treatment

In the first part of the study, waxy rice starch or potato starch (0, 0.5, 1 or 1.5% w/w) was added to skim milk at its natural pH prior to pressure or heat treatment. In the second part of the study, the pH of the skim milk samples was adjusted from the natural condition (pH 6.64) to pH 6.5, 6.6 or 6.9 by the slow addition of hydrochloric acid (1M) or sodium hydroxide (1M) while stirring the milk. Waxy rice starch was then added to the pH-adjusted skim milk at concentrations of 0 or 1% w/w. All samples were either heat-treated at 80°C for 30 min as described by Oh et al. (2007a) or pressure-treated at 500 MPa and 20°C for 30 min as described by

Oh et al. (2008b). For the pH-adjusted milk samples, the pH was re-adjusted to the natural pH after pressure treatment and depressurisation or heat treatment and cooling by the slow addition of hydrochloric acid (1M) or sodium hydroxide (1M) at room temperature (20°C) with stirring.

7.2.3 Acidification and rheology

The samples were acidified by adding GDL at a level of 2%. Changes in the rheological properties of the samples during acid gelation were monitored using an AR2000 rheometer (TA Instruments, New Castle, DE, USA) and a cone (4 cm, 4° and 100 µm truncation) and plate geometry as described in (Oh et al., 2007a). To monitor the acid gelation process of milk, the rheological measurements were performed at a frequency of 0.1 Hz, a constant strain of 0.5% and a constant temperature of 30 °C. Once the acid gelation had been completed, the sample was then subjected to a temperature sweep. The temperature of the sample was decreased from 30 to 5 °C at a rate of 0.9 °C min^{-1} and the rheological properties were monitored as the temperature was decreased using a frequency of 0.1 Hz and a strain of 0.5%.

7.2.4 Microscopy

Birefringence of the starch granules in untreated, pressure-treated or heat-treated skim milk was observed prior to acidification, using a polarizing light microscope (Nikon Eclipse E600 Pol, Nikon Corporation, Tokyo, Japan) with a 50× or 20× objective. The microstructure of the final acid milk gels were observed using a confocal scanning laser microscope (CSLM) by methods described by Oh et al. (2007a).

7.3 Results and Discussion

7.3.1 Acid gelation of skim milk with added starch after pressure or heat treatment at the natural pH

7.3.1.1 Acid gelation curves

Milk samples with various levels of added waxy rice starch or potato starch were either pressure-treated at 500 MPa and 20°C for 30 min or heat-treated at 80°C for 30 min. GDL was added to the milk samples to slowly lower the pH from the natural pH to ~pH 4.2 over a 180 min period. The storage modulus, G', was monitored during acidification and was used to indicate the firmness of the samples during acidification (**Figure 7-2A &B**). Regardless of the treatment the milk samples received, the shapes of the acid gelations curves are typical for the acid gelation of milk as has been previously shown (Lucey et al., 1997; Anema et al., 2004; Oh et al., 2007b). The first phase of acid gelation is a lag phase where the G' is low as the milk remains liquid. The length of the lag phase is termed the 'gelation time' in this study. The second phase is a rapid gelation phase in which the increase in G' is almost directly proportional to time. In this study, the term '$G'_{30°C}$' denotes the G' of milk sample during acidification at 30°C. The term 'final $G'_{30°C}$' denotes the G' of acid milk gel at 30°C after 180 min of acidification by GDL and 'final $G'_{5°C}$' denotes the G' of acid milk gel when the temperature of acid milk gel formed after 180 min of gelation at 30°C was subsequently decreased to 5°C.

The gelation times were different between the pressure-treated milk with no added starch and the heat-treated counterpart (**Table 7-1, Figure 7-2**). The gelation time (lag phase) for pressure-treated milk was 61 min which was markedly longer than that for heat-treated milk (43 min; **Table 7-1**). The longer gelation time of pressure-treated milk indicates that the pH at which the protein aggregates start

forming gel networks was lower than that for heat-treated milk (**Figure 7-2**). The heated milk sample with no added starch showed a higher final $G'_{30\,°C}$ value than the pressure-treated counterpart. The final $G'_{30\,°C}$ value was 115 Pa for the acid milk gel made from pressure-treated milk and 165 Pa for the acid milk gel made from heat-treated milk.

The gelation time was shortened and the final $G'_{30\,°C}$ value of acid gel increased as the amount of added waxy rice starch increased regardless of whether the milk samples were pressure-treated or heat-treated (**Table 7-1, Figure 7-2**). The effect of waxy rice starch addition on the final $G'_{30\,°C}$ was more pronounced for pressure-treated samples than for heat-treated samples (**Figure 7-2A inset**). The final $G'_{30\,°C}$ of acid gel made from pressure-treated milk with 1.5% added waxy rice starch was 186 Pa which was a 62% increase compared with that made from pressure-treated milk with no added starch (115 Pa; **Table 7-1**). In contrast, for the heated counterparts, the final $G'_{30\,°C}$ increased from 165 Pa to 204 Pa which was only a 24% increase (**Table 7-1**).

Table 7-1: Mean gelation time of milk containing different levels of added starch after pressure or heat treatment, final $G'_{30°C}$ and final $G'_{5°C}$ of the acid milk gels.

Starch (%)	Gelation time (min)				Final $G'_{30°C}$ (Pa)				Final $G'_{5°C}$ (Pa)			
	Waxy rice		Potato		Waxy rice		Potato		Waxy rice		Potato	
	P^1	H^2	P	H	P	H	P	H	P	H	P	H
0	61^a	43^a	61^a	43^a	115^a	165^a	115^a	165^a	252^a	355^a	252^a	355^a
0.5	57^b	41^{ab}	56^a	34^{ab}	127^{ab}	168^{ab}	113^a	254^b	267^a	360^a	269^a	541^b
1	56^b	38^{bc}	58^a	32^b	151^b	192^b	105^a	278^b	313^a	394^a	240^a	606^b
1.5	52^c	35^c	55^a	25^b	$186^{c,A}$	$204^{b,A}$	120^a	351^c	$392^{b,B}$	$413^{a,B}$	276^a	771^c

Means with the same lower case superscript letter within a column are not significantly different ($P<0.05$) as determined by ANOVA.

Mean values of gelation time, final $G'_{30°C}$ or final $G'_{5°C}$ in the column 'P' and column 'H' for the same starch type are significantly different $P<0.05$) as determined by ANOVA, except for the pairs indicated with the upper case superscript letters (A and B).

Keys:

1 - Pressure treatment

2 - Heat treatment
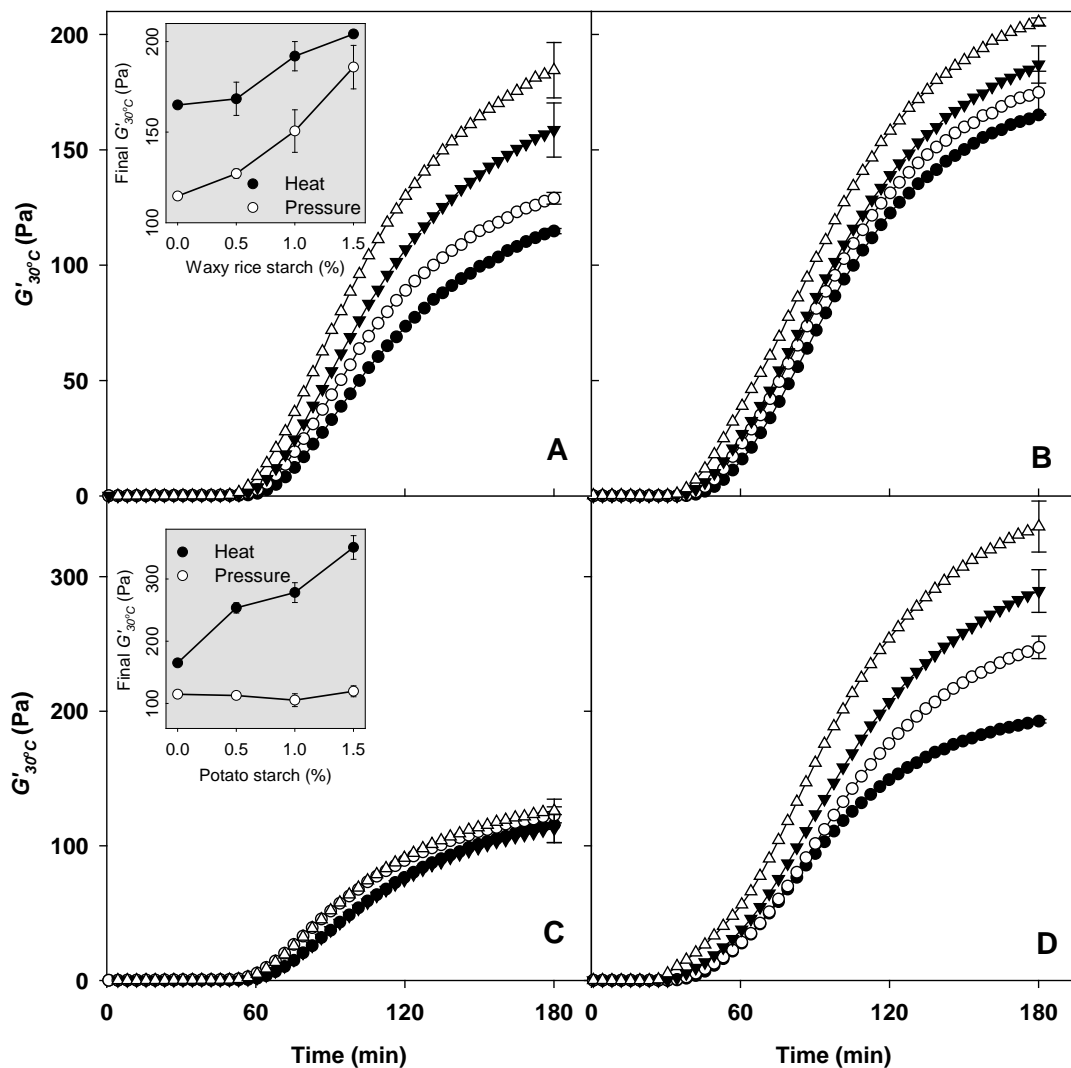
Figure 7-2: Acid gelation curves of (A) milk samples with waxy rice starch after pressure treatment (500MPa / 20°C / 30min), Inset: Final $G'_{30°C}$ as a function of waxy rice starch addition level, (B) milk samples with waxy rice starch after heat treatment, (C) milk samples with potato starch after pressure treatment, Inset: Final $G'_{30°C}$ as a function of potato starch addition level and (D) milk with potato starch after heat treatment (80°C / 30min) at addition levels (●) 0%, (○) 0.5%, (▼) 1% and (△) 1.5% (w/w). The pH at treatment was pH 6.64 (natural). Error bars represent standard deviations of repeated measurements.

Figure 7-2C & 2D show the acid gelation curves for milk samples with added

potato starch. For pressure-treated samples, addition of potato starch to milk prior to

pressure treatment did not result in any significant change in the acid gelation curves,

gelation times or the final $G'_{30°C}$ of the acid gels (**Figure 7-2C & 2C inset, Table 7-**

1). In contrast, milk samples that received heat treatment showed shortened gelation times and notably higher final $G'_{30°C}$ values as more potato starch was added to the milk prior to heat treatment (**Figure 7-2D, Table 7-1**). The final $G'_{30°C}$ of the acid gel made from heat-treated milk with 1.5% potato starch added prior to heat treatment was 351 Pa, which was a 113% increase from the final $G'_{30°C}$ of acid milk gel made from milk with no added starch (**Figure 7-2C inset, Table 7-1**). Regardless of the type of added starch (waxy rice starch or potato starch), the starch addition level or the type of treatment (pressure or heat treatment), the final $G'_{5°C}$ of acid milk gels was approximately twice the value of final $G'_{30°C}$ (**Table 7-1**). Similar effects of decreasing the temperature on the firmness (G') of the acid milk gel has been reported in previous studies (Lucey et al., 1997; Bikker et al., 2000; Oh et al., 2007a).

7.3.1.2 Confocal scanning laser microscopy (CSLM) of acid milk gels

The acid milk gel network is composed of milk proteins that have aggregated when the pH of the milk was gradually lowered (Lucey and Singh, 1998). Fast Green CFC dye was added to the samples prior to acidification to label proteins. The milk samples were then acidified with GDL at 30°C for 180 min and the acid milk gels were examined for their microstructures using the CSLM (**Figure 7-3**). The microstructure of acid milk gels made from pressure-treated milk and heat-treated milk can be compared, as can the effect of adding different starches (waxy rice starch and potato starch) to the milk prior to each treatment.

The acid milk gel made from pressure-treated milk with no added starch appeared to be composed of intricately connected thin protein strands whereas those made from the heat-treated counterpart were composed of broader but fewer protein

strands in the gel network (**Figure 7-3A & 3B**). Also, the pores in the acid milk gel network appeared smaller and more numerous for the acid milk gel made from pressure-treated milk compared to that made from heat-treated milk (**Figure 7-3A & 3B**). Scanning electron micrographs shown by Penna et al. (2007) revealed that the microstructure of yoghurt made from pressure treated milk consisted of smaller and more interconnected particles compared to the heat treated counterpart which is in line with the results found in this study.

Addition of 1% waxy rice starch prior to pressure or heat treatment of milk produced denser protein gel networks on acidification compared to the corresponding samples with no added starch (**Figure 7-3A & 3C, 7-3B & 3D**). The increase in the final $G'_{30°C}$ of acid milk gels with added waxy rice starch made from either pressure- or heat-treated milk samples (**Figure 7-2A & 2B**) can be related to this increased density of the protein network. The acid milk gel made from pressure-treated milk with 1% waxy rice starch appeared to have broader protein strands than the sample with no added starch (**Figure 7-3A** compared to **7-3C**) which, in fact, resembled the appearance of the acid milk gel made from heated milk with no added starch although with a markedly denser network (**Figure 7-3B & 3C**). As shown in **Figure 7-2A**, the final $G'_{30°C}$ value of the acid gel increased from 115 Pa with no added starch to 152 Pa with 1% waxy rice starch. The result again suggests that the gel firmness increases when the gel network is made of broader protein strands. The protein network in the acid gel made from heat-treated milk with 1% waxy rice starch appeared to be denser than that made from heat-treated milk with no added starch or that made from pressure-treated milk with 1% waxy rice starch (**Figure 7-3B** compared to **7-3D** & **7-3C**).
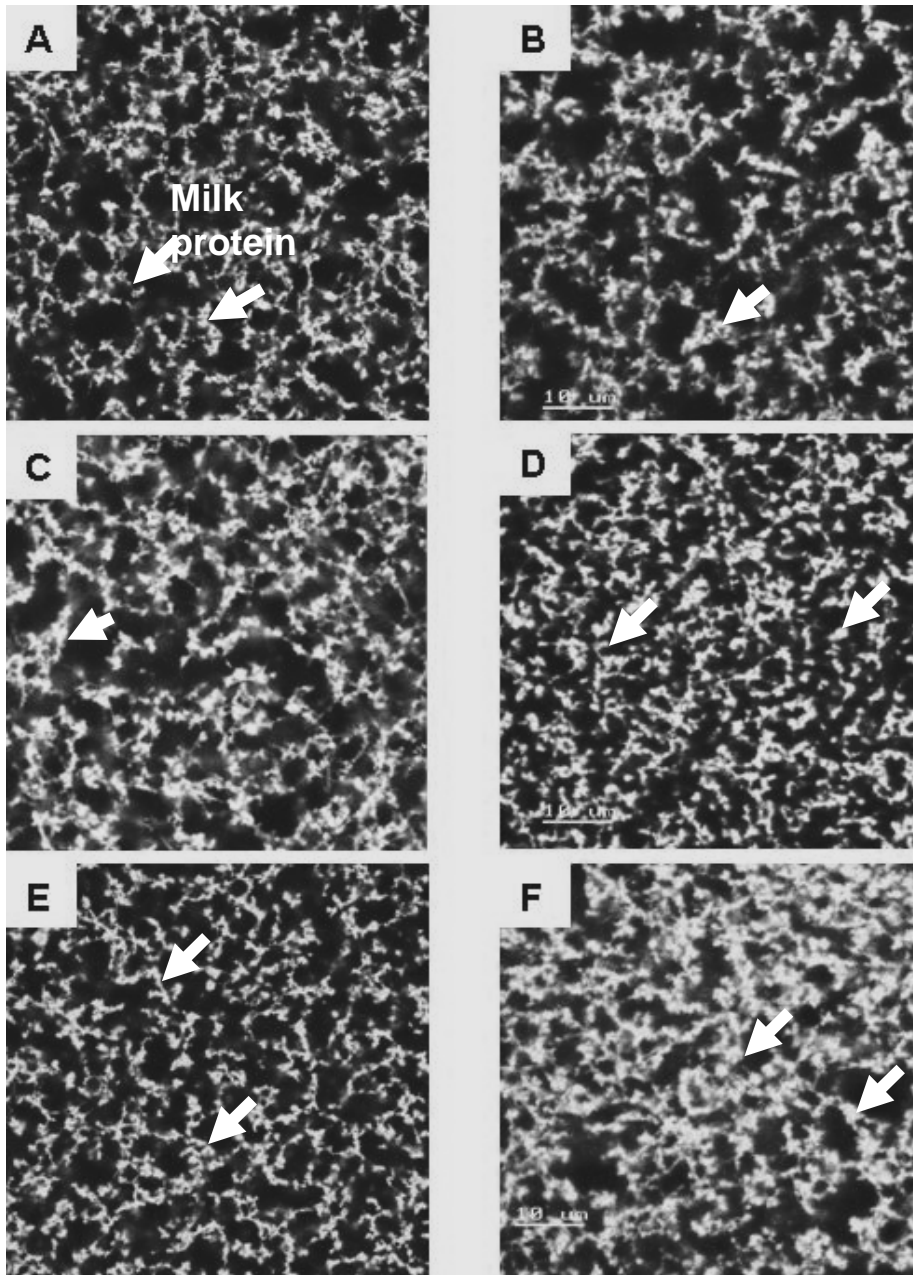
Figure 7-3: CSLM micrographs of final acid milk gels made from pressure-treated milk (500MPa / 20°C / 30 min; A, C, E) and heat-treated milk (80°C / 30 min; B, D, F). A, B: with no added starch; C, D: with 1% waxy rice starch; E, F: with 1% potato starch added prior to treatment.

The confocal micrographs from the acid milk gel network made from pressure-treated milk with 1% potato starch did not show noticeable differences compared with the sample with no added starch (**Figure 7-3A & 3E**). However, the heat-treated counterpart appeared to be more densely packed protein gel networks

than the sample with no added starch (**Figure 7-3B** compared to **7-3F**) and also seemed somewhat denser than the heat-treated sample with 1% waxy rice starch (**Figure 7-3D** compared to **7-3F**).

7.3.1.3 Polarised light microscopy

Figure 7-4 shows the appearance of starch granules in untreated, pressure- or heat-treated milk samples with 1% waxy rice starch or 1% potato starch prior to acidification as viewed using a polarised light microscope. Ungelatinised starch granules have an intact crystalline structure which is birefringent and display a characteristic cross (Yuryev et al., 2002). Untreated samples containing either waxy rice starch or potato starch showed granules with birefringence as expected (**Figure 7-4A & 4B**). Potato starch granules (~40 μm) were larger than waxy rice starch granules (~10 μm; **Figure 7-4A & 4B**) which is consistent with literature reports (BeMiller and Whistler, 1996; Singh et al., 2003).

After the pressure or heat treatment, waxy rice starch granules no longer showed any birefringence which indicated that waxy rice starch was completely gelatinised by both treatment regimes. Potato starch showed a different behaviour compared to waxy rice starch. The same level of birefringence was still observed in potato starch granules after the high pressure treatment, indicating that the starch was not gelatinised (**Figure 7-4D**), which explains why the acid gelation curve was not changed when potato starch was added to the milk prior to pressure treatment and acidification (**Figure 7-2C**). However, the heat treatment resulted in a complete gelatinisation of potato starch as indicated by the loss of birefringence (**Figure 7-4F**) which resulted in the observed increase in the final $G'_{30°C}$ value when potato starch was added to the milk prior to heating and acidification (**Figure 7-2D**).
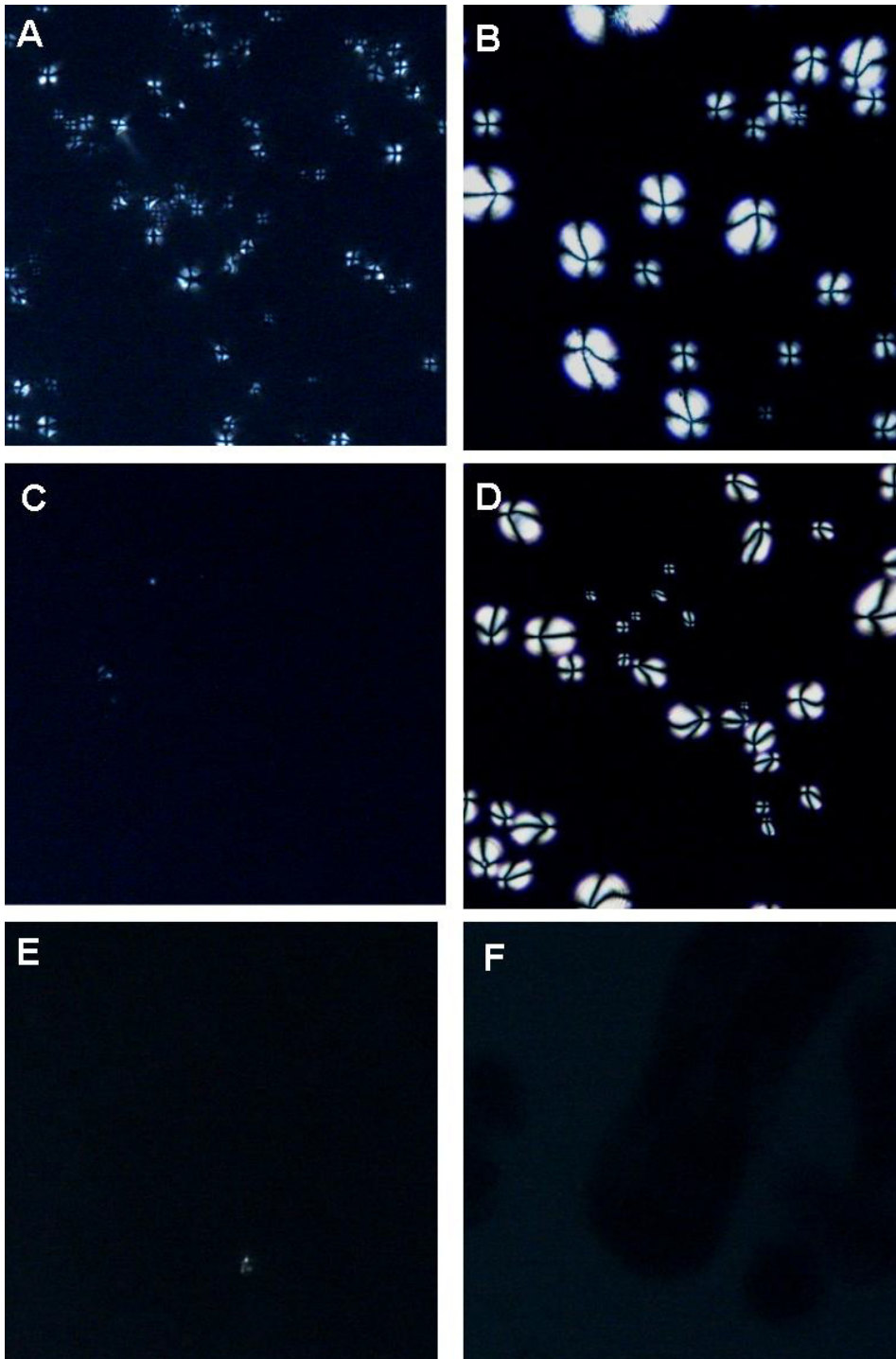
Figure 7-4: Polarised light micrographs of milk samples before acidification with 1% waxy rice starch (50×) (A, C, E), and 1% potato starch (20×) (B, D, F) added prior to pressure or heat treatment. (A, B) untreated, (C, D) pressure-treated at 500MPa and 20°C for 30 min and (E, F) heat-treated at 80°C for 30 min.

7.3.2 Effect of pH at pressure or heat treatment of skim milk with added waxy rice starch on the acid gelation properties of the milk

7.3.2.1 Acid gelation curves

Studies have shown that the pH of milk at heating can change the properties of acid gels produced on the subsequent acidification of the milk. Increasing the pH of milk at heating to about pH 7.1 increased the firmness of the acid gels when compared with samples heated at the natural pH (~pH 6.64), whereas decreasing the pH at heating to about pH 6.5 markedly decreased the firmness of the acid gels (Anema et al., 2004; van Vliet et al., 2004; Lakemond and van Vliet, 2005; del Angel and Dalgleish, 2006; Oh et al., 2007b). The pH of milk has been shown to influence the interaction behaviour of the denatured whey proteins with the casein micelles during heating. Anema and Li (2003b) showed that the levels of denatured whey protein that associated with the casein micelles decreased from 80 to 30% as the pH at heating was increased from pH 6.5 to 6.7. The pH of milk also affects pressure-induced denaturation of β-lactoglobulin so that the level of denaturation is higher when the pH of milk increased (Arias et al., 2000).

In this study, the effect of adjusting the pH of milk with added waxy rice starch prior to pressure or heat treatment and subsequent acidification was investigated. The properties of acid gels made from milk that was pressure-treated at adjusted pHs with added starch has not been studied previously. **Figure 7-5** shows the acid gelation curves of milk samples with no added starch or with 1% waxy rice starch which were adjusted to different pHs prior to pressure or heat treatment. Milk samples were pressure-treated at 500 MPa and 20°C for 30 min or heat-treated at 80°C for 30 min. The term "pH at pressure treatment (pH$_{pressure}$)" or "pH at heat

treatment (pH_{heat})" is used to describe the pH of the milk samples at the time of pressure or heat treatment, respectively, in order to distinguish this from the pH changes observed during acidification of milk samples.
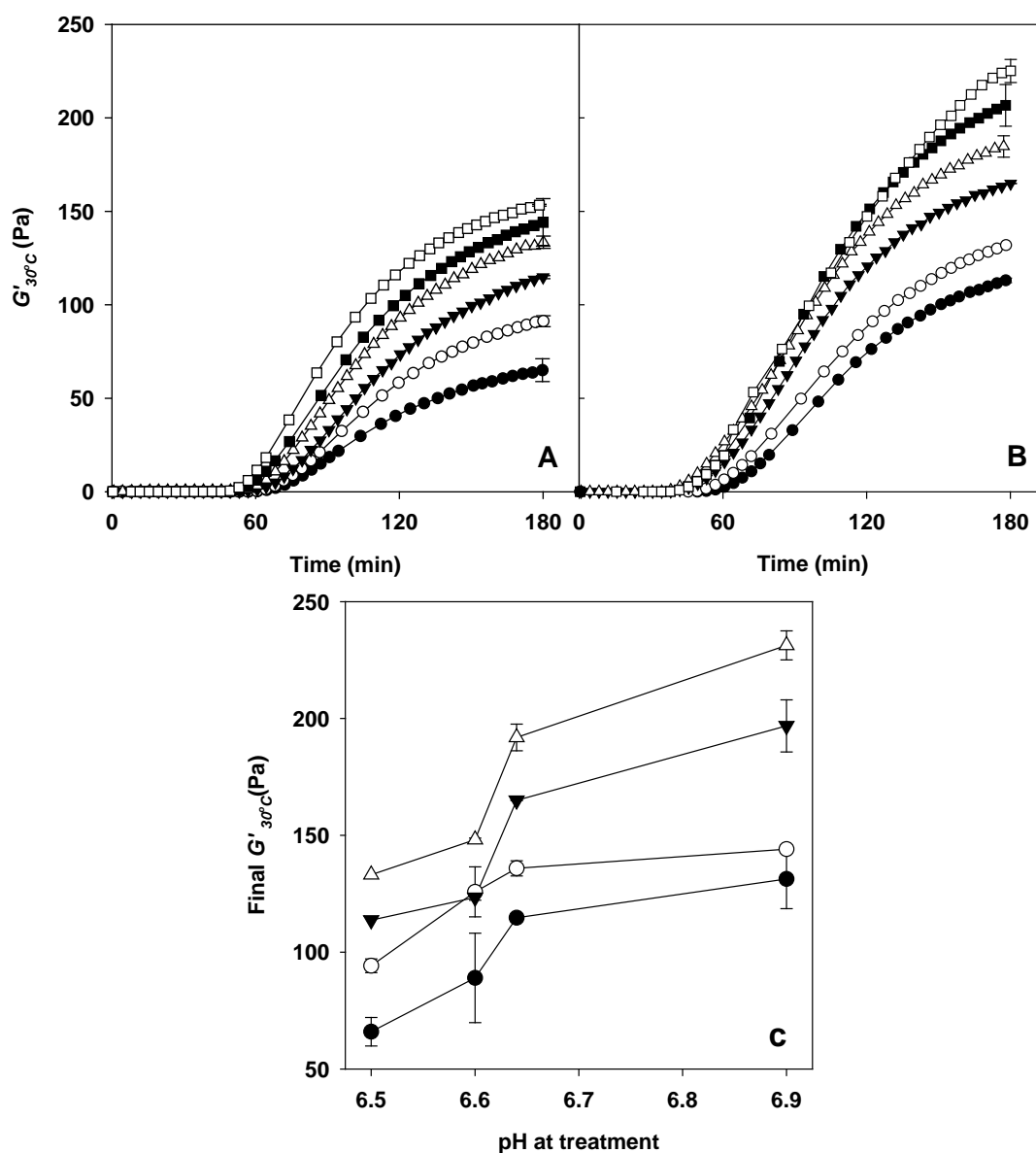
Figure 7-5: Acid gelation curves for (A) pressure-treated (500MPa / 20°C / 30min) milk samples (B) heat-treated (80°C / 30min) milk samples at pH 6.5 (●) with no added starch and (○) with 1% waxy rice starch; at pH 6.64 (natural) (▼) with no added starch and (△) with 1% waxy rice starch; at 6.9 (■) with no added starch and (□) with 1% waxy rice starch. (C) Final $G'_{30°C}$ as a function of pH at treatment. (●) no added starch, pressure-treated, (○) 1% waxy rice starch, pressure-treated, (▼) no added starch, heat-treated and (△) 1% waxy rice starch, heat-treated. Error bars represent standard deviations of repeated measurements.

For acid milk gels prepared from milk with no added starch, adjusting the pH$_{pressure}$ or pH$_{heat}$ to below the natural milk pH (6.64) resulted in acid milk gels with lower $G'_{30\,°C}$ values at a given pH during acidification and consequently lower final $G'_{30\,°C}$ values (**Figure 7-5**). Conversely, adjusting the pH$_{pressure}$ or pH$_{heat}$ to above the natural milk pH resulted in acid milk gels with higher $G'_{30\,°C}$ values at a given pH during acidification and consequently higher final $G'_{30\,°C}$ values (**Figure 7-5**). The effect of increasing the pH of milk at treatment on the final $G'_{30\,°C}$ was similar to that of increasing the addition level of waxy rice starch at the natural milk pH as seen in **Figure 7-2**. For both pressure-treated and heat-treated samples, addition of 1% waxy rice starch prior to the respective treatment resulted in acid milk gels with higher $G'_{30\,°C}$ values than the samples with no added starch with a similar level of increase at all pH$_{pressure}$ or pH$_{heat}$.

7.3.2.2 Confocal scanning laser microscopy (CSLM) of acid milk gels

Figure 7-6 shows the structures of final acid milk gels made from pressure treated samples with different pH$_{pressure}$ as observed using CSLM. The appearances of the protein gel networks may provide some explanations for the rheological results shown in **Figure 7-5**. At pH$_{pressure}$ 6.5 (below the natural milk pH) with no added starch, the protein clusters seemed smaller and the gel network was somewhat disjointed in areas, which may provide relatively weak support towards the overall firmness of the acid milk gel (**Figure 7-6A**). The final $G'_{30\,°C}$ of this sample was, in fact, the lowest of all the acid milk gels samples examined (**Figure 7-5C**). In comparison, at pH$_{pressure}$ 6.9 the gel network contained protein clusters that seemed

larger and more intricately connected (**Figure 7-6B**), which is in line with the observed higher final $G'_{30°C}$ for this sample (**Figure 7-5B & 5C**).

Addition of 1% waxy rice starch to milk prior to pressure treatment resulted in acid milk gels with denser and more connected protein networks at both levels of $pH_{pressure}$ compared to the respective counterparts with no added starch (**Figure 7-6A & 6C, 7-6B & 6D**). The acid milk gel made from milk with 1% waxy rice starch pressure-treated at $pH_{pressure}$ 6.9 appeared to have denser protein networks than that pressure-treated at $pH_{pressure}$ 6.5 (**Figure 7-6C & 6D**).
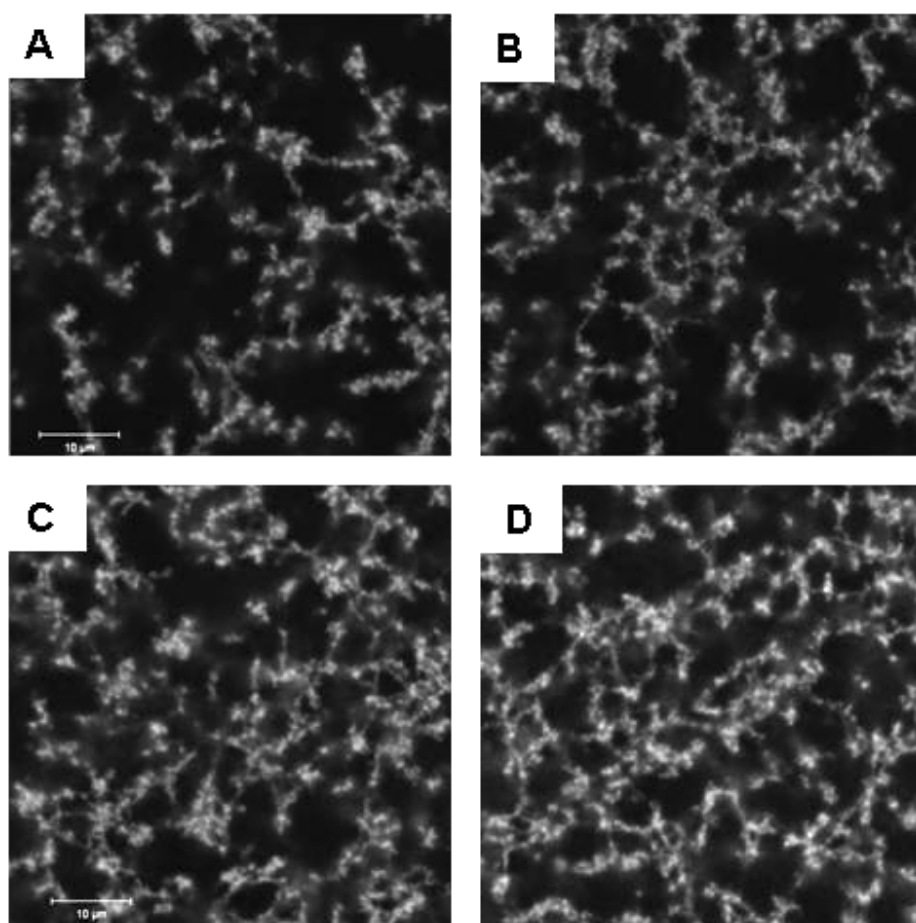
Figure 7-6: CSLM micrographs of final acid milk gels made from milk samples pressure-treated (A) at $pH_{pressure}$ 6.5 with no added starch and (B) at $pH_{pressure}$ 6.9 with no added starch, (C) at $pH_{pressure}$ 6.5 with 1% waxy rice starch and (D) at $pH_{pressure}$ 6.9 with 1% waxy rice starch added prior to pressure treatment (500MPa / 20°C / 30min).

7.4 Discussion

When milk is heated above 70°C, denaturation of whey proteins occurs, with the level of denaturation dependent on the temperature and duration of heating as well as other factors such as the composition and pH of the milk (Dannenberg and Kessler, 1988a; Anema and McKenna, 1996; Corredig and Dalgleish, 1996b; Oldfield et al., 1998). The denatured whey proteins can interact with each other and with the casein micelles, forming protein aggregates (Mulvihill and Donovan, 1987; Corredig and Dalgleish, 1996a). These heat-induced interactions between milk proteins involve non-covalent interactions and disulfide bonds, with the latter being important and involves free sulphydryl groups of β-lactoglobulin (Lowe et al., 2004; Considine et al., 2007). When the whey proteins in milk are denatured by heat treatment, the texture and consistency of the acid milks gels produced after a subsequent acidification can be modified and the level of denatured whey proteins is positively correlated to the gel firmness (Dannenberg and Kessler, 1988b; McKenna and Anema, 1993; Lucey and Singh, 1998).

Whey proteins can also be denatured by pressure and pressure-denatured whey proteins have been reported to be associated with casein micelles in pressure-treated milk (Lopez-Fandino et al., 1996; Huppertz et al., 2004; Patel et al., 2006). Anema et al. (2005a) showed that pressure treatment of milk resulted in an increase in the firmness of acid milk gels when compared with that of acid milk gels prepared from untreated milk. This is similar to the effect observed on heat treatment of milk prior to acidification. It has been suggested that the increase in the firmness of acid milk gels prepared from pressure-treated milk is related to whey protein denaturation by a mechanism similar to that for heat-treated milk (Anema et al., 2005a).

However, there are some differences in the composition and size of the aggregated whey proteins as the individual whey proteins have different susceptibility to denaturation by pressure and by heat (Lopez-Fandino et al., 1996; Garcia-Risco et al., 2000; Huppertz et al., 2004; Anema et al., 2005a). Denaturation of β-lactoglobulin starts at pressure treatments above about 100 MPa while α-lactalbumin and bovine serum albumin are not denatured by pressures up to 400-500 MPa (Lopez-Fandino et al., 1996; Lopez-Fandino and Olano, 1998; Huppertz et al., 2004; Anema et al., 2005b; Hinrichs and Rademacher, 2005). In the heat treatment of milk, minor proteins such as bovine serum albumin are more labile than the major whey proteins, β-lactoglobulin and α-lactalbumin (Considine et al., 2007). Patel et al. (2006) reported that the sizes of protein aggregates in pressure-treated samples are smaller than those in the heat-treated counterpart. This suggests that the basic units (protein aggregates) for the acid milk gel network are smaller in the pressure-treated milk than in the heat-treated milk and may be related to the thinner and shorter protein strands formed on acidification of the pressure-treated milk compared with that formed from the heat-treated milk when treated at the natural pH of milk (**Figure 7-3A & 3B**). Despite the more interconnected gel network (**Figure 7-3A & 3B**), the firmness (final $G'_{30°C}$) of the acid milk gel made from pressure-treated milk was lower than the heat-treated counterpart (**Figure 7-2A & 2B**).

The effects of starch addition on the microstructure of the acid milk gel from milks treated at their natural pH's are in line with the rheological results. Regardless of the type of treatment, the addition of waxy rice starch resulted in a denser protein network, hence firmer gels as indicated by the higher $G'_{30°C}$ values compared to that of the acid milk gel with no added starch (**Figure 7-2A & 2B, Figure 7-3A–D**). On

the other hand, potato starch contributed to the gel network density only in the case of heat-treatment, which was also indicated by the gel firmness ($G'_{30°C}$) results (**Figure 7-2C & 2D, Figure 7-3A, 3B, 7-3E & 3F**).

The effect of starch during heating of milk at the natural pH on the physical properties of acid milk gels prepared from the heated milks is primarily due to starch gelatinisation in milk as discussed by Oh et al. (2007a). It can be concluded that the firmness of acid milk gels can be increased by the addition of starch to skim milk when the starch was gelatinised in milk by the pressure or heat treatment received prior to acidification (**Figure 7-2 & Figure 7-4**). Waxy rice starch was completely gelatinised by both treatments whereas potato starch was gelatinised by the heat treatment only (**Figure 7-4**). As potato starch was not gelatinised after the pressure treatment, the presence of potato starch granules did not affect the acid gelation of skim milk (**Figure 7-4D & Figure 7-2C**).

It is important to note that susceptibility of a particular type of starch to pressure-induced gelatinisation cannot be predicted from its susceptibility towards heat-induced gelatinisation. For example, the onset temperature of heat-induced gelatinisation for waxy rice starch and potato starch are both approximately 60°C (BeMiller and Whistler, 1996; Singh et al., 2003; Oh et al., 2008b). Yet, waxy rice starch can be gelatinised by pressure treatment from 300 MPa at room temperature whereas potato starch is resistant to pressure-induced gelatinisation and does not gelatinise even at pressures up to 600 MPa (Hibi et al., 1993; Stute et al., 1996; Katopo et al., 2002; Oh et al., 2008a; Oh et al., 2008b).

The pressure resistance of potato starch has been attributed to the compact and condensed external layer of potato starch granule which remains unchanged by high

pressure treatment (Blaszczak et al., 2005). The amylopectin crystalline structure in starch granules are commonly divided into three types based on their X-ray diffraction patterns. Cereal starches including waxy rice starch display A-type crystallinity while potato starch displays B-type crystallinity. The B-type starch granules have a more open-structure than A-type granules which may suggest that the B-type structure can better accommodate water molecules migrating into the starch granules under pressure and channel the water through without disrupting the crystalline order under pressure. Therefore, this B-type crystallinity of potato starch granules may also contribute to the pressure resistance (Katopo et al., 2002).

The heat treatment used in this study was enough to gelatinise both waxy rice starch and potato starch completely (Oh et al., 2008b). Yet, when potato starch was added to milk prior to heat treatment the acid milk gels produced on acidification were firmer than the acid milk gels made from the milk with added waxy rice starch at the same addition level prior to heat treatment (**Figure 7-2B & 2D**). Starch gelatinisation contributes to the strength of the dominant protein network primarily by increasing the effective concentration of milk proteins in the aqueous phase as water is absorbed by the starch granules resulting in a denser protein gel network on acidification (Oh et al., 2007a). Potato starch has higher swelling power than waxy rice starch when heated in water (Singh et al., 2003), i.e. it absorbs more water than waxy rice starch. This higher swelling power may lead to an increased effective concentration of milk protein in the aqueous phase for milk heated in the presence of potato starch than that heated in the presence of waxy rice starch. Consequently, the heated milk with potato starch formed a denser protein network than that with waxy

rice starch on acidification as shown in the CSLM micrographs (**Figure 7-3D & 3F**), therefore the firmer acid milk gel (**Figure 7-2B & 2D**).

The amylose content of starch may also contribute to the observed difference between the effect of waxy rice starch and potato starch on the acid milk gel firmness after heat treatment. Waxy rice starch does not contain amylose, unlike potato starch which contains approximately 19% amylose. Amylose is known to leach into the suspension during thermal gelatinisation of starch (Morris, 1990; BeMiller and Whistler, 1996). When released from starch granules amylose contributes to the viscosity of the suspension and also forms a starch gel network upon cooling whereas solubilised amylopectin contributes to the viscosity but does not form a gel network on its own (Morris, 1990). The amylose network may contribute to the overall firmness of the acid milk gel containing heat-induced gelatinised potato starch.

The protein strands found in the acid milk gel made from pressure-treated milk with 1% waxy rice starch appeared broader and there were less interconnections than those found in the acid milk gels made from pressure-treated milk with no added starch (**Figure 7-3A** compared to **7-3C**). This indicates that the aggregation behaviour of milk proteins was affected by the starch addition (**Figure 7-2A & 2C**). It is possible that a phase separation into protein-rich and starch-rich regions occurred in the sample with added waxy rice starch after pressure treatment. The uptake of water by starch granules during gelatinisation increases the effective concentration of protein in the aqueous phase which in itself may contribute to the formation of larger protein aggregates under pressure, especially if starch gelatinisation occurred at a faster rate or at the lower pressure than changes in milk proteins such as dissociation of casein micelles, whey protein denaturation and aggregation of milk proteins. The

phase separation may further promote the formation of larger protein aggregates during pressure treatment by effectively keeping groups of proteins in close proximity in the protein-rich regions.

It is possible that phase separation into a protein-rich phase and a starch-rich phase occurred when milk with added starch was heated. Assuming this phase separation was occurring, the diffusion rate of proteins during acidification from one protein-rich region to another in the sample with added waxy rice starch would be slower than in the sample with no added starch as starch gelatinisation caused an increase in the viscosity. A phase separation coupled with the slow diffusion rate of proteins may reduce the interconnections of proteins during acidification. Consequently, proteins developed more interconnections within each protein-rich region on acidification to form broader protein strands and fewer interconnections with proteins in the other protein-rich regions (**Figure 7-3C***).*

On heating of milk, the pH_{heat} affects the association behaviour of the denatured whey proteins with the casein micelles in milk. The amount of denatured whey protein associated with the casein micelles decreases and the level of soluble denatured whey proteins increases as the pH_{heat} was increased (Anema and Li, 2003a; Vasbinder and de Kruif, 2003; del Angel and Dalgleish, 2006). Anema (2007) reported that the level of serum κ-casein increased as the pH of milk increased from pH_{heat} 6.5 to 7.1. This pH dependence of the levels of serum phase κ-casein has been suggested to be responsible for the change in distribution of the whey proteins between the colloidal and serum phases (Anema, 2007). The aggregation behaviour of the casein and whey proteins on acidification is consequently affected by the association of proteins after heating, which alters the firmness of the resultant acid

gels (Anema et al., 2004; Lakemond and van Vliet, 2005; del Angel and Dalgleish, 2006). It can be said that as the pH_{heat} increases, the level of soluble denatured whey proteins in milk increases after the heat treatment and upon acidification of milk the aggregation of proteins occur in such a way to establish stronger gel networks (**Figure 7-5**).

Huppertz et al. (2004) showed that when the $pH_{pressure}$ is adjusted to lower than the natural pH of milk, to $pH_{pressure}$ 6.2, the degree of whey proteins denaturation (α-lactalbumin and β-lactoglobulin) decreases compared to the milk pressure-treated at its natural pH. The effect of pH_{heat} on the degree of whey proteins denaturation was small in comparison to pressure treatment (Anema et al., 2004). Increasing $pH_{pressure}$ also increases the level of sedimentable denatured β-lactoglobulin (Huppertz et al., 2004), indicating increased associations with casein micelles. For example, Huppertz et al. (2004) showed that the level of sedimentable denatured β-lactoglobulin increased from about 49% to about 74% after pressure treatment at 400 MPa and at 20°C for 30 min as the $pH_{pressure}$ increased from 6.2 to 6.7. These pH-dependent changes in whey protein denaturation and association behaviours may contribute to the effect of $pH_{pressure}$ on the acid gel firmness. As the samples at $pH_{pressure}$ 6.9 may contain more denatured whey proteins to incorporate, they may develop larger and more interconnected protein aggregates on acidification compared to the samples at the lower levels of $pH_{pressure}$.

The effect of pressure treatment on casein micelles at the different pHs may also contribute to the observed differences between the acid milk gel networks at $pH_{pressure}$ 6.5 and 6.9. Huppertz et al.(2004) showed that the average size of casein micelles after pressure treatments up to 800 MPa was higher when the $pH_{pressure}$ was

7.0 compared to that of milk pressure treated at the natural pH. The larger protein clusters observed in the $pH_{pressure}$ 6.9 sample compared to those of $pH_{pressure}$ 6.5 may be attributed to the larger casein micelles which in turn contribute to firmer gel network (**Figure 7-6A & 6B**).

When waxy rice starch was added to milk prior to pressure treatment, the final $G'_{30°C}$ of acid milk gels produced on subsequent acidification increased by ~25 Pa to higher G' compared with those made from pressure-treated milk with no added starch regardless of the $pH_{pressure}$. Likewise, the final $G'_{30°C}$ of acid gels made from heat-treated milk with starch added prior to heat treatment also increased by ~25 Pa regardless of the pH_{heat}. Therefore, in both pressure treatment and heat treatment cases, the whole $G'_{30°C}$ versus $pH_{pressure}$ or pH_{heat} plot was displaced by ~25 Pa (**Figure 7-5C**). Starch contributes to the firmness of acid milk gels as a consequence of gelatinisation and the range of milk pH used in this study does not affect the starch gelatinisation behaviours (Bao and Corke, 2002; Hirashima et al., 2005).

7.5 Conclusions

The addition of waxy rice starch to milk prior to pressure or heat treatment followed by acidification produced acid milk gels with the higher firmness (final $G'_{30°C}$) than the acid milk gels made from milk with no added starch. Increasing the waxy rice starch addition caused an increase in the final $G'_{30°C}$ in both pressure-treated and heat-treated samples. However, acid milk gels made from pressure-treated milk with added potato starch did not show significant changes in the $G'_{30°C}$ whereas the heat-treated counterparts showed a marked increase in the final $G'_{30°C}$ as more potato starch was added to milk. Increasing the $pH_{pressure}$ or pH_{heat} increased the final

$G'_{30^\circ C}$ of the acid milk gel made from the pressure- or heat- treated samples. Adding waxy rice starch (1% w/w) prior to pressure or heat treatment resulted in further increase in the final $G'_{30^\circ C}$ of acid milk gels.

Acknowledgements

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CHAPTER 8

Discussion: Contribution Towards the Understanding of High-Pressure-Induced Starch Gelatinisation

Increasing pressure favours the state of lower volume. 'Swelling' of starch granules occurs under high pressure in accordance to this thermodynamic principle. Starch molecules linked to water molecules can occupy a smaller volume than unassociated starch molecules and water molecules (Douzals et al., 1996). Therefore, starch suspended in aqueous medium can be gelatinised by pressure without prior heating as the reaction will result in a total volume reduction. Pressure is recognised as an important dimension to starch gelatinisation and has been attracting more interest in recent years (Douzals et al., 1996; Katopo et al., 2002; Knorr et al., 2006). This study investigated pressure-induced gelatinisation of starch using rheological methods as the main tools to assess the degree of gelatinisation together with the degree of swelling measurement, amylose/amylopectin assay and light microscopy (birefringence).

One of the earlier findings of this study was that pressure-induced gelatinisation of starch was dependent on the starch type. Starches can be divided into three general groups based on their pressure-induced gelatinisation behaviours (**Table 8-1**). Group I and II starches can be gelatinised by moderate to high pressures at temperatures below their T_{onset}. The differences between the two groups are the maximum degree of swelling and the initial apparent viscosity ($\eta_{initial}$) that can be attained by pressure treatment. For example, the $\eta_{initial}$ of waxy corn starch in Group I after a pressure treatment at 600 MPa was as high as the maximum viscosity that can be attained by thermal gelatinisation. In comparison, the $\eta_{initial}$ of normal corn starch in Group II after a pressure treatment at 600 MPa was 0.023 Pa.s which was lower than the maximum viscosity that can be attained by thermal gelatinisation (2.7 Pa.s). Group III is represented by potato starch which is the most pressure-resistant starch examined in this study. No gelatinisation of potato starch was observed at pressures

up to 600 MPa, despite the relatively low thermal gelatinisation temperature (58–65°C) for this starch. This finding suggests that the pressure-induced gelatinisation behaviour of starch cannot be predicted based on the thermal gelatinisation behaviours.

Table 8-1: Starch groups and their pressure-induced gelatinisation characteristics

Group	Starch examples	Gelatinisation pressure range at 20 °C	Degree of swelling and change in $\eta_{initial}$	Birefringence
I	Waxy rice Waxy corn Tapioca	300–600 MPa	100% swelling and complete gelatinisation which led to $\eta_{initial}$ similar to that achieved in thermal gelatinisation	Disappears
II	Normal rice Normal corn	300–600 MPa	Swelling increases but does not reach 100%. $\eta_{initial}$ increases but not to the level achieved in thermal gelatinisation	Disappears
III	Potato	>600 MPa	No change up to 600 MPa	Remains

The pressure resistance of potato starch (Group III) may suggest that potato starch has a more ordered crystalline structure than other starches and so withstands higher pressures and maintains the granular structure. Scanning electron micrographs taken by Blaszczak et al. (2005) showed that potato starch granules have a very compact and condensed layer which remains unchanged by high pressure treatment and is, therefore, more pressure resistant than the inner part of the granule. The pressure resistance of potato starch may also be attributed to the B-type crystallinity of potato starch granules. The amylopectin crystalline structure in starch granules are

commonly divided into three types based on their X-ray diffraction patterns. Cereal starches display A-type crystallinity while potato starch displays B-type crystallinity. Tapioca starch shows C-type crystallinity which is a mixture of A-and B-type crystallinity. The B-type starch granules have a more open-structure than A-type granules which may suggest that the B-type structure can better accommodate water molecules migrating into the starch granules under pressure and channel them through without disrupting the crystalline order.

An in-depth study of normal rice starch (Group I) and waxy rice starch (Group II) revealed that gelatinisation of starch occurs over a pressure range for both starch types. The relationship between the degree of gelatinisation and treatment pressure follows a sigmoidal-shaped curve. It was noted that there is a minimum critical pressure required to initiate starch gelatinisation. The minimum critical pressure was 350 MPa for normal rice starch and 300 MPa for waxy rice starch. Once the treatment pressure is increased to this level, gelatinisation is induced. Above the minimum critical pressure, the degree of gelatinisation depends on the treatment pressure. This suggests that pressure treatment can be used as a technique to gelatinise starch to a desired degree of gelatinisation in a more controlled way than the conventional thermal gelatinisation. Transmittance of pressure through a sample is known to be uniform and instantaneous and independent of size and geometry of the sample (Tewari et al., 1999), whereas heat transfer depends on time and position in the sample, hence homogeneous gelatinisation and the precise degree of gelatinisation may be more difficult to achieve with heat treatment when compared with pressure treatment.

The results from this study suggest that different combinations of treatment pressure, treatment temperature and duration can result in the same $\eta_{initial}$ value for a

starch suspension. Pressure-induced gelatinisation occurs at a faster rate as the treatment pressure is increased above the minimum critical pressure. For example, $\eta_{initial}$ of waxy rice starch was increased from 0.007 Pa.s (untreated) to 0.3 Pa.s after a pressure treatment at 400 MPa for ten minute whereas at 500 MPa, the same level of $\eta_{initial}$ was attained after the treatment duration of only one second. In fact, waxy rice starch was completely gelatinised after two minutes at 500 MPa. Elevated treatment temperatures (but below the thermal gelatinisation onset temperature of starch) can increase the degree of pressure-induced gelatinisation. It is possible that a small amount of thermal energy can enhance the mobility of amylose and amylopectin molecules in starch which, in turn, accelerates the plasticising process of these molecules by water.

Pressure-induced starch gelatinisation is a swelling phenomenon and the degree of swelling is directly correlated to the $\eta_{initial}$. Unlike waxy rice starch which showed 100% swelling, the degree of swelling against treatment pressure curves for normal rice starch plateaued at 50% although the birefringence of the normal rice starch granules had completely disappeared at this point. Consequently, the $\eta_{initial}$ curve also plateaued at a lower level than that of waxy rice starch. Normal rice starch contains 16% amylose and the rest is mainly amylopectin, whereas waxy rice starch contains amylopectin only.

This difference in the starch amylose/amylopectin compositions may explain the different pressure-induced gelatinisation behaviours. The leaching of amylose from normal rice starch granules was only about 2% at the most (Table 1, Page 5-12). It is possible that leaching of solubilised amylose molecules into the aqueous suspension medium does not involve a volume reduction hence, it is not promoted under pressure. In thermal gelatinisation, an amylose gel network can be formed on

cooling (Morris, 1990). However, as pressure treatment does not involve the heating of the starch suspension, amylose may form a gel network as soon as solubilised. The solubilised amylose molecules may be trapped inside the starch granules and form a gel network within the starch granules comprising amylopectin molecules which come loose as the disruption of the crystalline structure continues due to migration of water into the granules. If the resultant network is thermodynamically stable under pressure, the starch granules do not undergo any further structural changes. Alternatively, the solubilised amylose molecules may migrate to the surface of starch granules and form a gel layer onto the surface instead of leaching into the aqueous phase. This gel layer can potentially block the further entry of water into the starch granules and pressure-induced gelatinisation does not progress further from this point. The light micrographs of normal rice starch suspensions after pressure treatments showed starch granules that were swollen yet, maintaining the granular entity whereas waxy rice starch granules were ruptured and had lost the structure completely when the treatment pressure was sufficiently high.

Pressure-induced gelatinisation is driven by the compressibility of the starch suspension and thermal gelatinisation is driven by thermal expansion of the structures in starch granules. In both cases, gelatinisation of starch under pressure starts in the amorphous regions (Rubens et al., 1999). When pressure is applied water molecules migrate into the amorphous regions of starch granules and the interaction between the water molecules and starch chains begin. Any factors impeding this interaction therefore could result in retarding of starch gelatinisation. Pressure-induced gelatinisation of waxy rice starch and normal rice starch suspended in skim milk were studied and compared to those suspended in water. Skim milk contains a number of different components (proteins, minerals and lactose) which may influence starch

gelatinisation. It was found that when starch was suspended in skim milk instead of water more pressure was required to achieve a given degree of gelatinisation. This indicates that components in skim milk impeded the interactions between water molecules and starch chains.

Examining pressure-induced gelatinisation of starch in suspension media with different milk components showed that soluble milk minerals and lactose were the gelatinisation-retarding components in the skim milk. Similar effects have been reported in thermal gelatinisation. If water has any dissolved solutes, the plasticising ability of water can be compromised (Perry and Donald, 2002). In addition, solutes may interact with starch chains so that the mobility of the starch chains are restricted (Spies and Hoseney, 1982). These similarities of the effects of solutes on starch gelatinisation induced by pressure and by heat indicate that, although the process is initiated for different reasons, the effectiveness of the process still depends on the plasticisation of the starch chains by the solvent (water).

Gelatinisation of starch is important for the functional properties of food containing the starch as it leads to rheological changes in food applications. Acid milk gels are particle gels that are formed by aggregation of milk proteins when milk is acidified (Horne, 1999). Starch is often used as an ingredient in acid gel manufacture. When starch is added to milk prior to pressure or heat treatment, the uptake of water by starch due to gelatinisation increases the effective concentration of proteins in the aqueous phase which then leads to a denser protein network on acidification. In addition, gelatinisation of starch increased the viscosity of the aqueous phase which provides support for the protein gel network and the swollen starch granules themselves may also provide structural support for the network. Hence, the addition of starch results in firmer acid milk gels if the added starch is gelatinised in milk

whether by pressure or heat. Added waxy rice starch was gelatinised in milk by either pressure or heat treatment so that on acidification, firmer acid milk gels were produced than the gels made from milk with no added starch. On the other hand, potato starch was only gelatinised by heat treatment, not by pressure treatment, therefore the addition of potato starch to milk did not contribute to the acid milk gel firmness (final G') when the pressure treatment was used.

Anema et al. (2004) showed that the firmness of acid milk gel is influenced by the pH of milk at heating as the pH affects the interaction behaviours of casein micelles and denatured whey proteins. In this study, the combined effects of starch addition and pH adjustment (pH 6.5–7.1) prior to heat or pressure treatment were examined. It was found that increasing the pH of milk at pressure or heat treatments increased the final G' of the acid milk gel with or without added starch. Addition of starch can further increase the final G' when added starch was gelatinised in milk by the treatment received.

The effect of pH at heat treatment was reduced when more than 1% potato starch was added to milk. The increased viscosity of the aqueous phase as a result of starch gelatinisation may affect the diffusion of the protein components during heating and, consequently, reduce the association of the denatured whey proteins with the casein micelles, particularly at the lower pH at heating. In addition, the high viscosity of the aqueous phase may alter the interactions of the protein components on the subsequent acidification of the milk during the preparation of the acid gels. It is also possible that starch contributes to the gel network directly as the addition level increases.

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CHAPTER 9

Conclusions and Recommendations

9.1   Conclusions

- Starch can be gelatinised by pressure treatment without prior heating. The relationship between the degree of gelatinisation and treatment pressure follows a sigmoidal-shaped curve.

- Starches can be divided into three general groups based on their pressure-induced gelatinisation behaviours:

  - Starches in Group I (waxy rice, waxy corn starches and tapioca starch) and Group II (normal rice and normal corn starches) can be gelatinised by moderate to high pressures at temperatures below their T_{onset}:

    o Group I starches showed 100% swelling and complete gelatinisation which led to $\eta_{initial}$ similar to that achieved in thermal gelatinisation.

    o Group II starches did not show 100% swelling by the treatment pressure range used in this study although the degree of swelling increased as the treatment pressure increased. Hence, the $\eta_{initial}$ increased as the treatment pressure increased but not to the level achieved in thermal gelatinisation.

  - Group III is represented by potato starch which is the most pressure-resistant starch examined in this study.

- Pressure-induced gelatinisation characteristics of starch are dependent on the starch type as shown by the pasting curves, initial apparent viscosity ($\eta_{initial}$), degree of swelling and birefringence.

- The $\eta_{initial}$ of pressure-treated starch suspension and the degree of swelling have a linear correlation.

- Different treatment pressure, duration and treatment temperature can result in the same degree of starch gelatinisation.

- Pressure-induced starch gelatinisation is retarded in skim milk compared with that in water.

- Soluble milk minerals and lactose retarded pressure-induced starch gelatinisation in skim milk.

- When starch was added to skim milk and gelatinised during pressure or heat treatment, the acid milk gel produced on subsequent acidification was firmer than the acid milk gel made from skim milk alone.

- Increasing the pH of milk at pressure or heat treatment increased the final G' of the acid milk gel produced on subsequent acidification.

- The final firmness of an acid milk gel increased further by adding starch to milk that can be gelatinised by the treatment employed (pressure or heat treatment).

- For potato starch, the effect of pH at heating and addition of starch appeared to be additive and independent of each other up to a starch addition level of 1%. Above this starch level, the pH at heating had a lesser effect.

- For waxy rice starch, the effect of pH at pressure treatment or heat treatment and addition of starch appeared to be additive and independent of each other up to a waxy rice starch addition level of 1%.

9.2 Recommendations

- Pressure treatment comprises three steps – compression to the set pressure, holding at the set pressure, and decompression to atmospheric pressure. The current study examined the effect of pressure treatments on starch while keeping the compression rate and decompression rate (MPa/min) constant. Further study into the effect of altering these rates of compression and/or decompression on starch granules on pressure-induced starch gelatinisation is recommended to extend the understanding of the pressure treatment on starch.

- Milk proteins at the levels present in 10% total solids skim milk did not affect the degree of pressure-induced gelatinisation. However, at higher milk protein concentration there may be interactions between starch and milk proteins under pressure which could have applications in the food industry. Further study to higher milk protein concentration is recommended.

- Based on the birefringence observations from this study, it can be said that individual starch granules have different susceptibility to high pressure treatment. It is possible that difference size starch granules in a population have different pressure susceptibility. In thermal gelatinization, it has been shown that the degree of gelatinisation was different depending on the size of starch granule (Vermeylen et al., 2005). Sahlström et al. (2003) reported that large wheat starch granules showed the lower gelatinisation onset and peak temperature than small wheat starch granules. Further study of the effect of starch granule size on the kinetics of pressure-induced gelatinisation of starch is recommended.

- It has been suggested that the external structure of potato starch contributes to the reported pressure-resistance of potato starch (Blaszczak et al., 2005). A

further study is recommended to investigate the role of the external structure of potato starch by chemically removing the external (surface) layer of starch granules before pressure treatment and examining the pressure-induced gelatinisation of the starch.

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APPENDICES

Appendix 1: Effect of storage time after pressure treatment on rheological analyses results of starch suspensions

The standard experimental protocol for pressure-treated starch suspension samples involved storing the samples at 20°C for approximately. It was found that there was no significant difference ($p<0.05$) between pasting curves of samples stored for 10h before analysis and samples that were analysed immediately after pressure treatments as shown in **Figure A1**. 10 hours before the rheological analyses (Page 3-7, section 3.3).
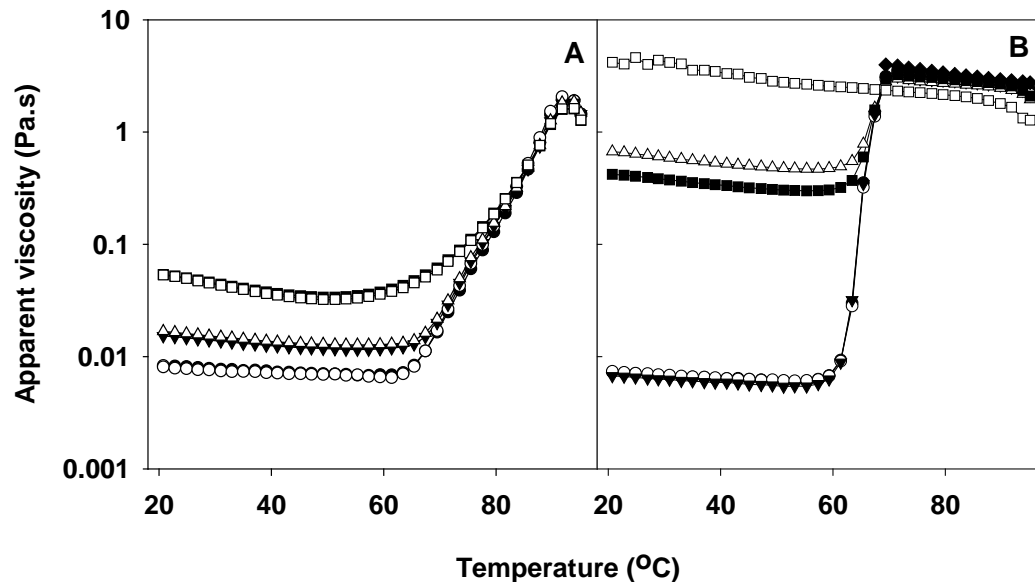
Figure A1: Pasting curves of (A) normal rice starch suspensions after pressure treatment at (●) 300 MPa and storage for 10h, (○) 300 MPa and no storage time, (▼) 400 MPa and storage for 10h, (△) 400 MPa and no storage time, (■) 600 MPa and storage for 10h, (□) 600 MPa and no storage time and (B) waxy rice starch suspensions after (●) 100 MPa and storage for 10h, (○) 100 MPa and no storage time, (▼) 375 MPa and storage for 10h, (△) 375 MPa and no storage time, (■) 500 MPa and storage for 10h, (□) 500 MPa and no storage time.

Appendix 2: Effect of adding Fast Green CFC on acid gelation of milk

Results that were mentioned but not shown on page 7-11, section 3.5 in Chapter 7 is presented in this appendix.
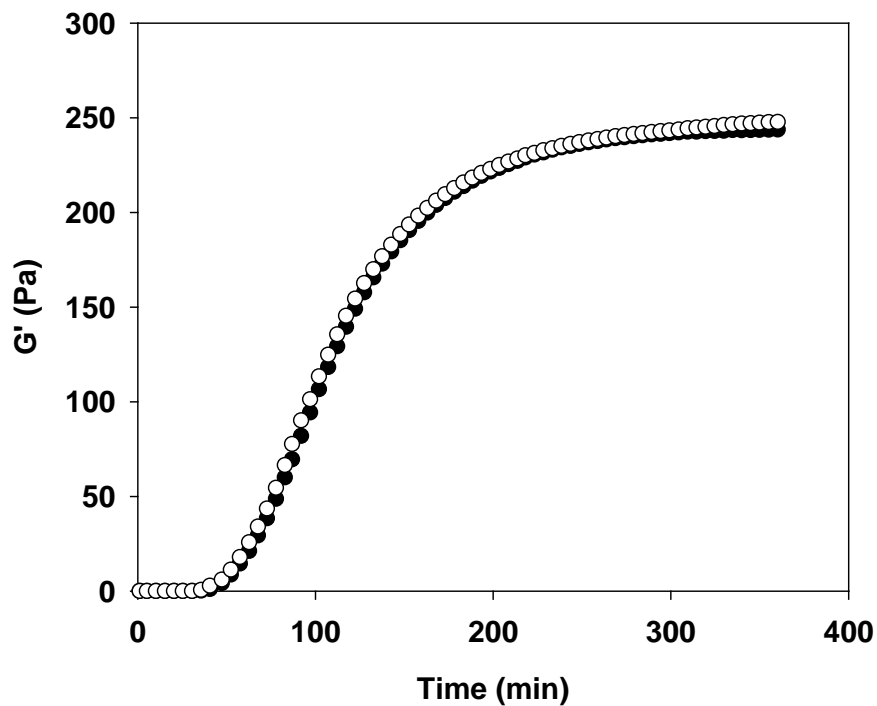

Figure A2: Change in storage modulus, G', at 30 °C, with time after GDL addition (●) heated milk and (○) heated milk with added Fast Green CFC dye.

Figure A2 shows the typical acid gelation curve of heated milk with or without added Fast Green CFC dye. The addition of the dye did not alter the acid gelation curve, indicating that the dye did not affect the formation of protein gel network during acidification of milk.

Appendix 3: Effect of temperature on *G'* and *G"* of acid milk gels

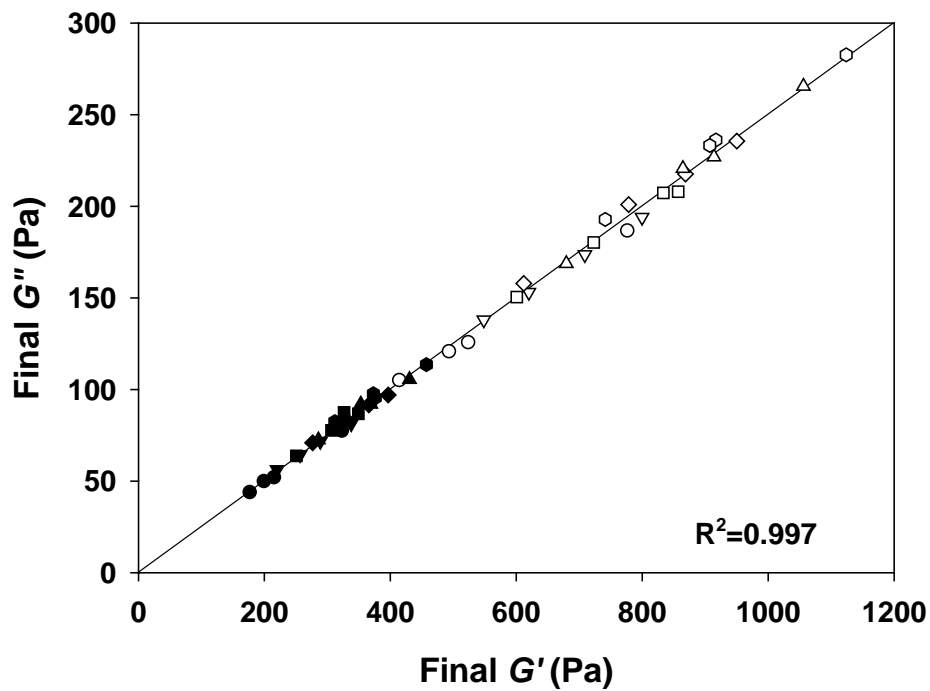Results that were mentioned but not shown on page 7-17, section 3.3 in Chapter 7 is presented in this appendix.
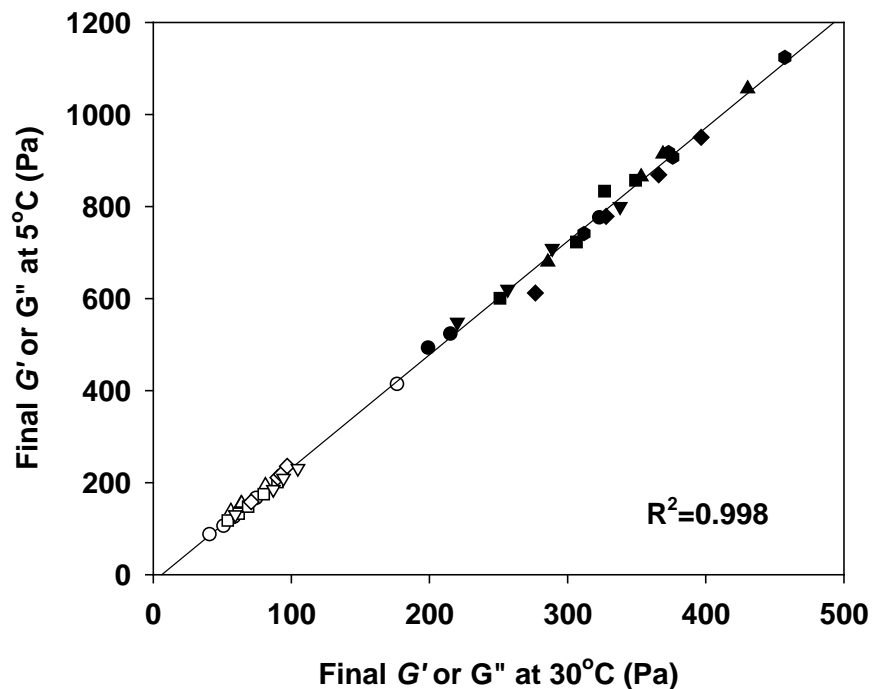
Figure A3: Final G' of acid milk gels at 30°C as a function of final G' at 5°C (filled symbols) and final G" of acid milk gels at 30°C as a function of final G" at 5°C (empty symbols). The acid milk gels were made from milk containing different levels of added potato starch (0-1.5% w/w) and heated at different adjusted pH values (pH 6.5-7.1).

Figure A3 shows the changes in *G'* and *G"* when the temperature of acid milk gels was decreased from 30°C to 5°C. The relationship between the *G'* and *G"* at 30°C and those at 5°C was linear (R^2 =0.998) with the gradient of 2.4. The results indicate that regardless of the starch addition level in milk or the pH of milk at heating, *G'* and *G"* of the acid milk gels increased by 2.4 times when the temperature was decreased from 30°C to 5°C.

Appendix 4: Final G" versus final G' (tan δ) of acid milk gels

Results that were mentioned but not shown on page 7-17, section 3.3 in Chapter 7 is

presented in this appendix.

Figure A4: Loss modulus, *G"*, as a function of storage modulus, *G'*, for acid milk gels heated at: (●, ○) pH 6.5; (▼, ▽) pH 6.6; (■, □) pH 6.64; (◆, ◇) pH 6.75; (▲, △) pH 6.9; (⬟, ○) pH 7.1. Filled symbols for final acid gels at 30 °C and open symbols for final acid gels at 5 °C.

The gradient of the best fit line on Figure A4 is approximately 0.25 (=tan δ).

Results that were mentioned but not shown on page 7-19, section 3.5 in Chapter 7 is presented in this appendix.
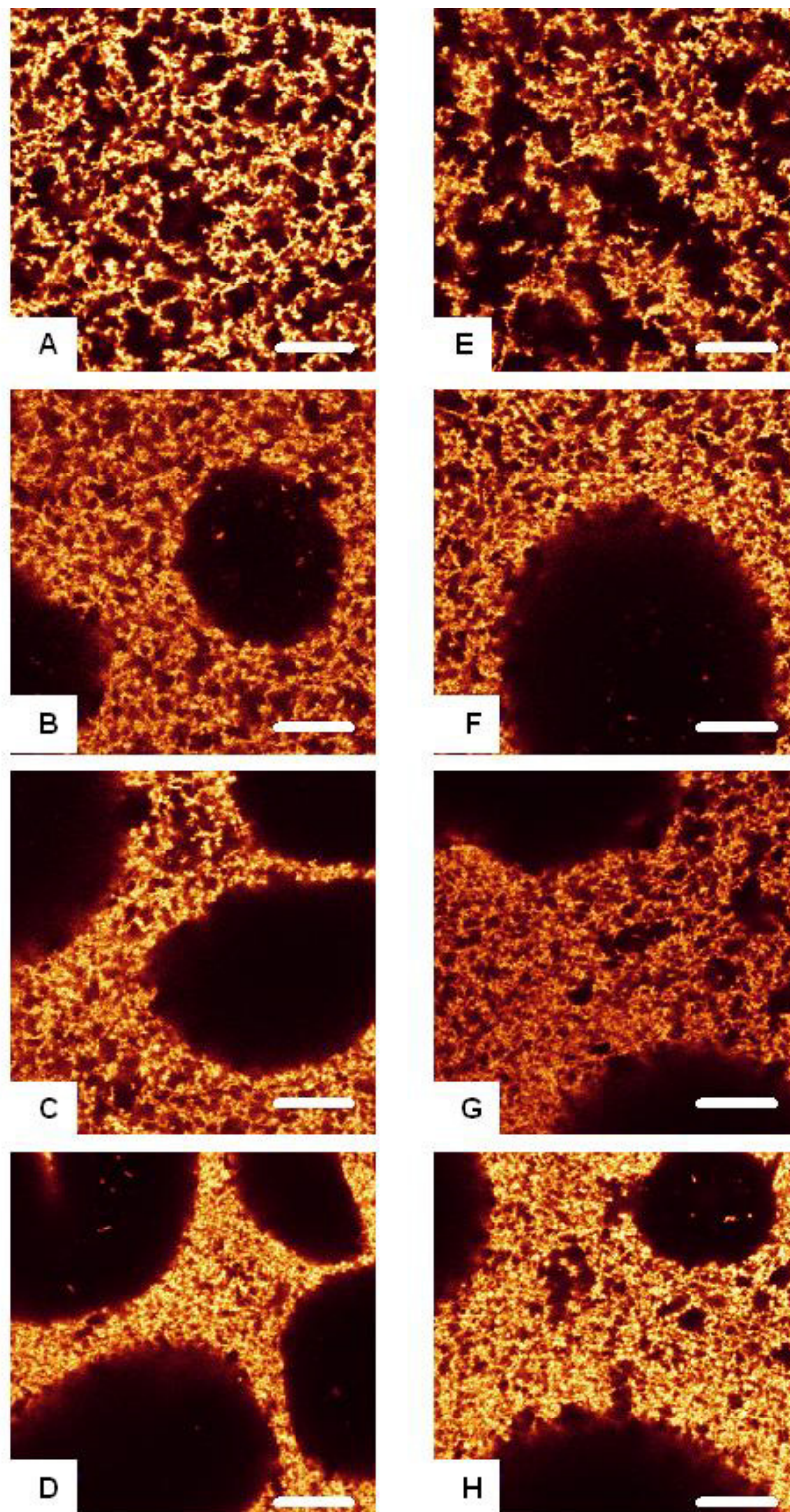
Figure A5: CSLM micrographs of final acid milk gels: heated at pH 6.5 containing (A) 0%, (B) 0.5%, (C) 1% and (D) 1.5% potato starch; heated at pH 6.9 containing (E) 0%, (F) 0.5%, (G) 1% and (H) 1.5% potato starch. The bar corresponds to 20 μm.

Appendix 6: Effect of pH re-adjustment on the distribution of denatured whey proteins between serum and colloidal phases

Results that were mentioned but not shown on page 7-19, section 4 in Chapter 7 is presented in this appendix.
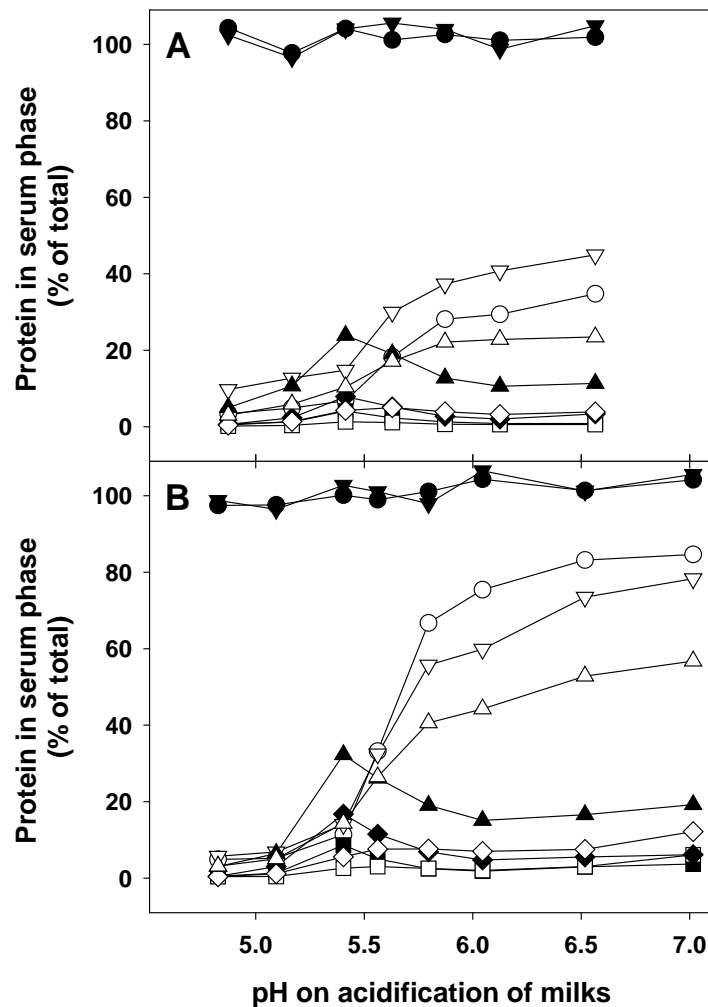
Figure A6: Serum phase protein in heated milks that were acidified using GDL to various pH. (A) skim milk at an initial pH of 6.5 before treatment. (B) Skim milk at an initial pH of 7.1 before treatment. ●, ○: β-lactoglobulin, ▼, ▽: α-lactalbumin, ■, □: α_s-casein, ◆, ◇: β-casein, ▲, △: κ-casein. Filled symbols: unheated milks; open symbols: milks heated at 100°C for 6 minutes. Source: Anema, unpublished.

Skim milk was adjusted to pH 6.5 or 7.1. The milk was either unheated (20°C) or

heated at 100°C for 6 min. The milks were then slowly pH adjusted to various pH

between the initial pH (6.5 or 7.1) and pH 4.6 by adding different levels of GDL to the milks and holding for 24 hours at 30°C (i.e. the lower the pH when the more GDL was added, the GDL level ranged from 0 to about 1.5%). The milks were then centrifuged (25000×g 1 hour) and the supernatants were analysed for protein, and compared to the original milks by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (Anema, unpublished). As shown in **Figure A6** the whey proteins and κ-casein that were in the supernatant did not re-associate with the casein micelles until the pH was below about 5.75, or even lower, at this point the milk was starting to gel (Anema, unpublished).