Interactions between wheat starch and *Mesona chinensis* polysaccharide

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Abstract

This thesis studies the interaction between wheat starch and *Mesona chinensis* polysaccharide (MCP) and its impact on the properties of the composite gel. Composite MCP and wheat starch gels were studied using solid-state NMR, whereby close proximity (5 Å) between MCP and glucan polymers was established, indicating that both polymers interacted at molecular level. MCP was found to increase the molecular mobility of the carbon 6 fraction found in starch glucan polymers, suggesting that it is likely to be the site of interaction.

The granular swelling, amylose leaching, and gelatinisation properties of 2 % w/w wheat starch were studied in the presence of increasing concentrations of MCP. At room temperature, the presence of MCP (0.1 – 5 % w/w) induced shear-reversible starch aggregation. Heating of starch-MCP suspension resulted in an earlier onset of viscosity increase that was characterised by a peak "M". This was also accompanied by delayed granular swelling and a 40 % reduction in amylose leaching when 7 % w/w MCP suspension was present. Interaction was thought to occur when amylose leaches out of wheat starch granules forming an MCP-amylose barrier, increasing the apparent size of the granules. The final *G'* of starch gel (1.1 Pa) increased during cooling in the presence of increasing concentrations of MCP up to 20 Pa when 5 % w/w MCP was present, indicative of a 3D network comprising of amylose, MCP and starch granules. The final gel properties were dependent on the concentration of MCP present whereby an extensive MCP-amylose network was required to stabilise the starch granule aggregates despite a reduction in the amount of amylose leached. The elastic modulus (*G'*) and hardness of gels containing high starch concentration (≥ 8 % w/w) was decreased at low (< 3 % w/w) MCP level and subsequently increased by more than 35 % at 5 % w/w MCP. In contrast, when starch concentration was lowered (5 % w/w), the viscoelasticity gradually increased in the presence of MCP. Microscopy, DSC and syneresis data showed that a heterogeneous gel network microstructure was formed when there was insufficient MCP and/or amylose, which reduced the final *G'* and hardness of the gels, and accelerated their retrogradation.

Factors such as starch type, starch to MCP ratio, pH and salt were found to affect the rheological properties of wheat starch-MCP gels. All of the starches (wheat, maize, potato and waxy maize) studied were found to gel at high MCP concentrations (3 – 5 % w/w). At low MCP concentration (1 % w/w), starch gelation was only observed for non-waxy starches, with the largest increase (1800 %) in gel strength being observed for 5 % w/w wheat starch. This was reduced when the ratio of wheat starch to MCP was decreased and this was thought to be due to a reduction in the total amylose present to form a 3D network and insufficient granules to reinforce the network. The pH of the system was found to influence the strength of the composite gel, whereby the *G'* of the gel was decreased at

pH 4 and increased when the pH was higher than 5. This is likely to be due to a change in the conformation of MCP (compact at low pH and open random coil at high pH), which allows more/less sites for interaction with amylose. The addition of monovalent salts was found to increase the strength of the gel, while the opposite was found for divalent salts.

The digestibility of wheat starch gels in the presence of MCP was also investigated. MCP was found to be the most effective polysaccharide in reducing wheat starch digestion in comparison to starch gels of similar hardness containing xanthan, guar, locust bean gum (LBG) or agar. A 33 % reduction in the digestibility of intact starch gel containing 5 % w/w MCP (after 120 minutes of digestion) was observed and this was attributed to the strengthening of the gels in the presence of high concentration of the polysaccharide. In contrast, despite a reduction in the firmness of the gel when 2 % w/w MCP was present, there was a 7 % reduction in starch digestibility and hence, firmness was deduced to be not solely responsible for the digestibility of the gels. When these gels were macerated, starch digestibility was reduced regardless of the MCP concentration. Starch digestion in the macerated samples seemed to cease after 10 minutes with about 30 % more starch remaining when 5 % w/w MCP was present. This suggests that the amount of starch available for digestion was reduced when MCP was present. The reduced availability of starch for digestion was hypothesised to be due to starch-MCP interaction, which formed amylose-MCP complexes that would be resistant to enzymatic digestion.

Additionally, MCP itself was found to inhibit α-glucosidase uncompetitively. Upon calcium removal from the extract, the extent of this inhibition was reduced but MCP exhibited an additional inhibition towards α-amylase in the same manner (uncompetitive inhibition). The removal of calcium increased the zeta potential of MCP, indicating that the conformation of MCP may be less compact due to the presence of negatively charged groups on the polysaccharide. This was thought to expose sites that could bind onto α-amylase, hence inhibiting the enzyme. Due to its enzyme inhibitory activities, MCP may be used as an ingredient to reduce postprandial glycaemia by inhibiting α-amylase and α-glucosidase.

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1. Yuris, A., Matia-Merino, L., Hardacre, A. K., & Goh, K. K. T. (2015). *Mesona Chinensis* extract as a potential food ingredient to reduce postprandial hyperglycaemia in starchy foods. Presented at Plants for the Future 2015. Massey University, Palmerston North.
2. Matia-Merino, L., Kumar, R., Yuris, A., Hardacre, A. K., & Goh, K. K. T. (2016). Starch and plant extracts interactions. Poster session presented at the meeting of 18th World Congress of Food Science and Technology, Dublin.
3. Yuris, A., Goh, K. K. T., Hardacre, A. K., & Matia-Merino, L. (2017). Understanding the interaction between wheat starch and *Mesona chinensis* polysaccharide. *LWT - Food Science and Technology*, 84, 212 - 221.
4. Yuris, A., Matia-Merino, L., Goh, K. K. T., Hardacre, A. K. & Hindmarsh, J. (2017). Wheat starch digestibility in the presence of *Mesona chinensis* polysaccharide. Presented at 7th International Symposium on Delivery of Functionality in Complex Food Systems. Auckland, New Zealand.
5. Yuris, A., Matia-Merino, L., Hardacre, A. K., Hindmarsh, J., & Goh, K. K. T. (2018). Molecular interactions in composite wheat starch-*Mesona chinensis* polysaccharide gels: Rheological, textural, microstructural and retrogradation properties. *Food Hydrocolloids*, 79, 1 - 12.
6. Yuris, A., Matia-Merino, L., Hardacre, A. K., & Goh, K. K. T. (In submission). The effect of gel structure on the *in vitro* digestibility of wheat starch-Mesona chinensis polysaccharide gels. *Food & Function*.
7. Yuris, A., Hardacre, A. K., Goh, K. K. T. & Matia-Merino, L., (In submission). *In vitro* digestibility of wheat starch-MCP gels – Enzyme inhibition and role of calcium. *LWT - Food Science and Technology*.
8. Yuris, A., Hindmarsh, J., Hardacre, A. K., Goh, K. K. T. & Matia-Merino, L. (In preparation). The interactions between wheat starch and *Mesona chinensis* polysaccharide: A study using solid-state NMR. *Food Chemistry*.

Chapter 1 Introduction

While most of us may not have heard of the herb *Mesona chinensis*, the name "grass jelly" may ring a bell, especially if you have visited an Asian country. "Grass jelly" is a black gel dessert that is consumed in many parts of the world for its unique or, rather, acquired taste. The making of the gel requires the concurrent heating of starch and the herbal extract, which is followed by their gelation during cooling. While starch itself is known to have the ability to form a paste or gel, *Mesona chinensis* extract by itself is liquid and does not form a gel when heated and cooled. Hence, its unique property lies in the fact that when cooked together with starch, it results in a synergistic increase in the brittleness and strength of the gel. How this occurs is one of the many unknowns that triggered this thesis.

In fact, the research questions addressed in this thesis came about from a preliminary research project, which looked at the effect of adding *Mesona chinensis* extract into different starches. While it is definite that the extract can increase the viscosity of certain starch pastes, it is not known whether it is the result of the interaction between both polymers or, just simply, the occurrence of phase separation resulting in starch granules and polymer segregation. If interactions do occur (as it will be demonstrated in this thesis), there are questions such as what is interacting, how the interaction takes place, what affects the interaction and how does this change the properties of the gel, that need to be answered. It is essential to note that the interest of this study is the polysaccharide from MC extract known as the *Mesona chinensis* polysaccharide (MCP), which is established very early on (Chapter 4) to be the component in the extract interacting with the glucan polymers in starch. A mechanism of associative interaction involving amylose and MCP has previously been proposed by Lai and Chao (2000b) but without any evidence to prove their interaction at the molecular level. One of the aims of this thesis will be to prove the mechanism of interaction (Chapter 5).

Regardless of whether the interaction between the two is segregative or associative, the changes in the viscosity of the paste have been shown in the literature to be influenced by the starch type, concentration of starch and MCP and the addition of salt. But yet, there is very little knowledge on the textural and viscoelastic properties of the mixed MCP-starch gel because most studies employ a continuous rotational shear, which does not allow the gel to set during cooling. There is also an absence of a systematic study on the effect of polymer concentration—both starch and MCP—on the rheological properties of the gel. Hence, there is a lack of understanding of how low and high MCP concentrations affect starch gel properties progressively from a non-gelling system of low starch concentration—where viscosity is solely governed by the starch granule volume fraction—all the way to a gelling system containing high starch concentration—where viscosity is governed by both starch volume fraction and MCP polymer-starch granule interactions. Additionally, there is only a single study

on the microstructure of MCP-starch gels and the interpretation of these micrographs is debatable. Therefore, the importance of starch and MCP concentration, as well as the microstructure of the gels will be explored across Chapter 5 and Chapter 6 of this thesis. Furthermore, the effect of pH on their interaction and, subsequently, on the rheological properties has never been studied despite the fact that MCP is a charged polysaccharide. Therefore, the various factors influencing the rheological properties of the composite gel will be discussed in Chapter 7 and these include the starch type, starch to MCP ratio, addition of salt (type and concentration) and pH. Apart from the increase in the viscosity or strength of the gel, for most polysaccharides, their ability to reduce starch retrogradation and prevent syneresis is one of the key reasons of why they are incorporated in starch-based food products. The ability of MCP to reduce starch retrogradation is not known and, hence, it is one of the objectives of this thesis to measure the retrogradation endotherms and the syneresis of starch gels in the presence of the polysaccharide (Chapter 6).

The proportion of polysaccharide in the extract could be increased following purification (Chapter 9) but this was also accompanied by an increase in its calcium content, suggesting that the mineral may be naturally bound onto the MCP. Interestingly, despite the high mineral content of MC extract, there is no study carried out on the role of these salts in the gelling mechanism of starch and MCP. It is widely known that calcium is usually involved in the gelation of many polysaccharides such as low methoxy pectin, alginate and iota carrageenan. Thus, it is also the interest of this study to look at the role of calcium in the gelation of starch containing MCP, which will be explored in Chapter 9.

Apart from the fundamental mechanisms of interaction and the factors that influence the properties of the gels, there is also the question of how these gels are digested in the human gastrointestinal tract. A previous study has quantified the effectiveness of the extract in reducing the postprandial rise in blood glucose following a high carbohydrate meal (Chusak, Thilavech, & Adisakwattana, 2014). “Grass jelly” is made out of two ingredients, mainly starch and the extract of *Mesona chinensis* (and glucose); while these jellies are commonly consumed yet we do not know how they are digested in the body. The idea is that if the combination can lead to a reduction in starch digestibility, there is a possibility to formulate starchy food products where the digestibility can be manipulated by the presence of MCP. Therefore, the digestibility of starch-MCP gels will be measured in Chapter 8 and the mechanisms—such as the gel strength, amylose-MCP interaction, reduction in granular swelling and enzyme inhibition—associated with the changes in the digestibility of these gels will be elucidated across Chapter 8 and Chapter 9.

Hence, this thesis follows a logical flow from identifying the components interacting in the composite gel to how MCP affects the properties of the gels and the factors that influence their interactions, to finally investigating how the digestibility of starch gels is affected by MCP and how calcium influences

starch-MCP interactions and digestibility. The objectives of the thesis together with the chapter structure are summarised in Figure 1.1.

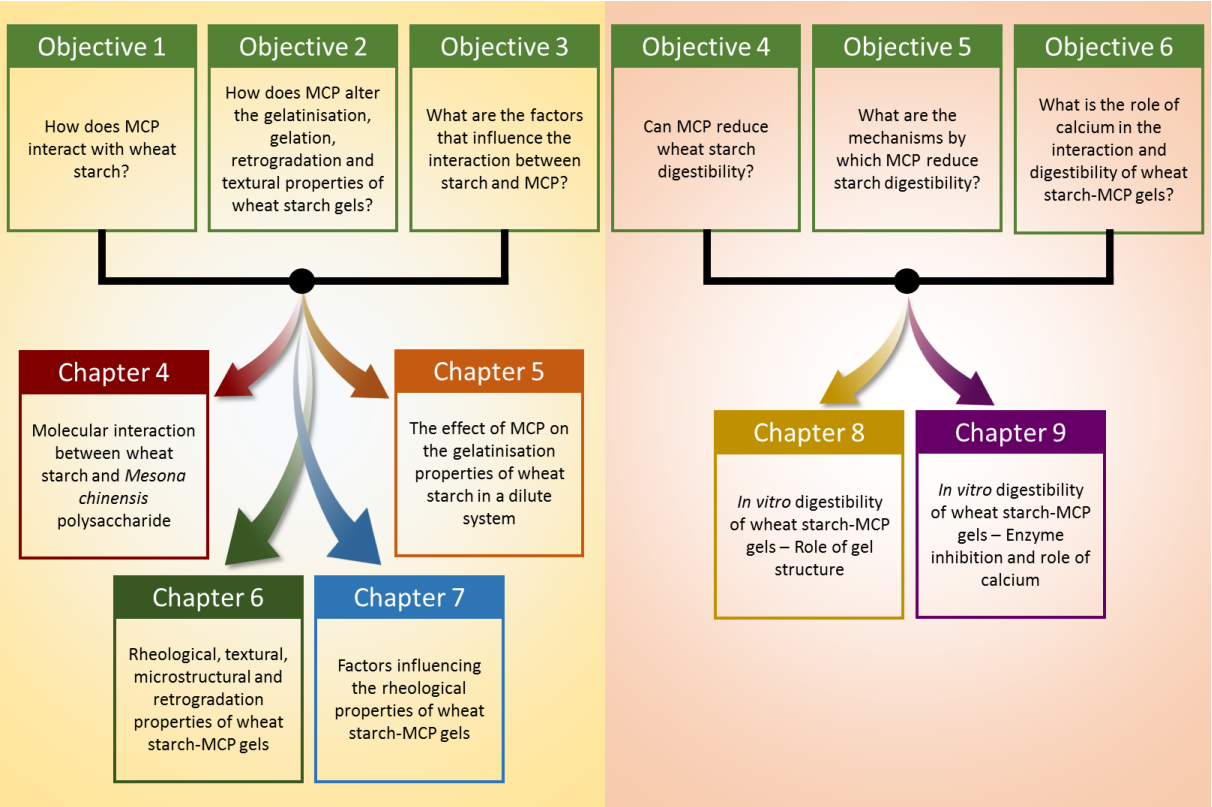

Figure 1.1 The objectives of the thesis and the chapters where they are addressed.

Chapter 2 Literature review

2.1 Starch

Starch is one of the main source of carbohydrates in the human diet and is obtained from a variety of sources, the most important of which are; seeds, roots, tubers, stems, leaves and fruits of plants. Starch is naturally resistant to enzymes in its native state but becomes readily digestible upon gelatinisation (described at a later section). Starch is stored in plants as insoluble granules and are hydrolysed into sugar to support growth (establishment of seedlings following germination) and facilitate processes such as nectar production (Zeeman, Kossmann, & Smith, 2010). Starch granules are synthesised at the hilum and grow by apposition (Pérez & Bertoft, 2010). This gives rise to a variety of shapes, sizes, and distributions of the granules (Table 2-1).

Table 2-1 The shape and size of starches of different origin (Tester, Karkalas, & Qi, 2004).

Starch origin	Type	Shape	Distribution	Size (µm)
Barley	Cereal	Lenticular (A-type) Spherical (B-type)	Bimodal	15 – 25 2 – 5
Maize (Waxy & Normal)	Cereal	Spherical/polyhedral	Unimodal	2 – 30
Amylomaize	Cereal	Irregular	Unimodal	2 – 30
Millet	Cereal	Polyhedral	Unimodal	4 – 12
Oat	Cereal	Polyhedral	Unimodal	3 – 10 (single) 80 (compound)
Pea	Legume	Reniform (single)	Unimodal	5 – 10
Potato	Tuber	Lenticular	Unimodal	5 – 100
Rice	Cereal	Polyhedral	Unimodal	3 – 8 (single) 150 (compound)
Rye	Cereal	Lenticular (A-type) Spherical (B-type)	Bimodal	10 – 40 5 – 10
Sorghum	Cereal	Spherical	Unimodal	5 – 20
Tapioca	Root	Spherical/lenticular	Unimodal	5 – 45
Triticale	Cereal	Spherical	Unimodal	1 – 30
Sago	Cereal	Oval	Unimodal	20 – 40
Wheat	Cereal	Lenticular (A-type) Spherical (B-type)	Bimodal	15 – 35 2 – 10

Starch granules are composed of two glucose chain units, namely the amylopectin (70 – 80 %) and amylose (20 – 30 %), which are made up of α-D-glucopyranosyl units. The proportion of amylose and amylopectin found in a starch granule varies depending on its source as shown in Table 2-2. The organisation of these polymers in the granule contributes to the variation in starch properties including water absorption, swelling, pasting, gelling, and susceptibility to enzyme attack (Wang & Copeland, 2013b).

Table 2-2 Amylose and amylopectin composition of various starches.

Starch origin	Amylose (%)	Amylopectin (%)
Maize	21	79
Potato	21	79
Rice	15 – 25	85 – 75
Tapioca	16.7	83.3
Wheat	21.7	78.3

2.1.1 Amylopectin

Amylopectin is a highly branched polymer that is linked by α-1,4-linkages with about 5 – 6 % α-1,6-linkages, making it a highly branched molecule. The backbone of an amylopectin molecule is commonly termed as C-chain and it carries the reducing end of the molecule. Attached to the C-chain are branching B-chains which may be linked to another B-chain or A-chains (Figure 2-1) (Manners, 1989). The chains within the amylopectin molecules are tightly packed and this influences the behaviour of starch gelatinisation. The exact positioning of the chains is not clear; however, it is predicted that clusters of A- and B-chains form the superhelical structure of amylopectin.

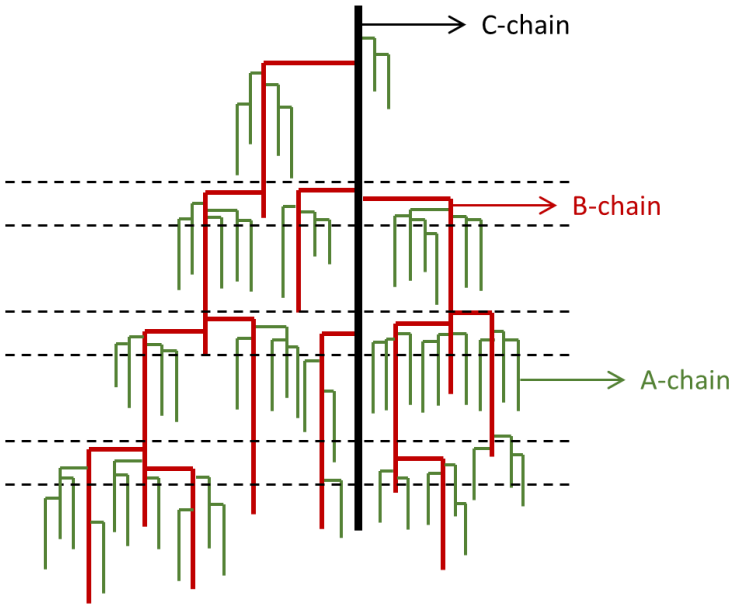

Figure 2-1 Structure of amylopectin molecule.

While most normal (or non-waxy) starches contain roughly 70 – 80 % amylopectin and 20 – 30 % amylose, starches containing high proportions of amylopectin (> 95 %) exist and they are known as waxy starches. The gelatinisation and gelation properties of these starches differ from their normal counterpart. Waxy starches exhibit a high swelling power, meaning that during gelatinisation, the volume of their granules increases more than their normal counterpart. Gelatinisation of waxy starches results in a rapid and large increase in their viscosity, which is typically followed by a large breakdown due to the susceptibility of their granules to shear at high temperatures (Abdel-Aal, Hucl, Chibbar, Han, & Demeke, 2002). During cooling, the paste of waxy starches does not form a gel due to the lack/absence of amylose to create a 3D structure. As a result, waxy starches are typically used as thickeners, binders and stabilisers in food products (Šárka & Dvořáček, 2017).

2.1.2 Amylose

In contrast to the branched amylopectin, the glucan units in amylose are linked mostly by (1→4)-α-linkages, forming a fairly linear polymer. While amylose is generally regarded as a linear, chiral molecule, studies have shown branching in its molecular structure (Hizukuri, Shirasaka, & Juliano, 1983; Hizukuri, Takeda, & Yasuda, 1981). In aqueous solution, amylose adopts a random coil conformation that is unstable. Hence, there is a tendency for amylose to form single helical structures with suitable complexing agents (also known as V-complexes) or double helices among themselves (Takeo, Tokumura, & Kuge, 1973). In the presence of a complexing agent such as alcohol, ketones, and fatty acids, single helical structures form instantaneously. The hydrophobic region of the amylose molecule is buried within the helix, interacting with the non-polar moiety of the complexing agent.

When no complexing agent is available, two amylose molecules align themselves to form a double helix with their hydrophobic regions folded within the helix (Jane, 2009).

Starches containing high proportions of amylose (> 50 %) are termed as high amylose starches. They typically swell to a lesser extent than normal starches—due to a lower level of amylopectin—and require higher temperatures (> 90°C) to achieve full gelatinisation (Richardson, Jeffcoat, & Shi, 2011). High amylose starches are less susceptible to enzymatic digestion as they contain high proportions of starch that is resistant to enzymatic attacks (resistant starch). This proportion of starch is indigestible in the small intestine but fermentable in the colon, a property that is similar to dietary fibre (Van Hung, Maeda, & Morita, 2006). Hence, high amylose starches have found applications as additives to increase the level of dietary fibre and resistant starch in food products.

2.1.3 Molecular organisation

Under polarised light, intact starch granules display a characteristic maltese cross (Figure 2-2) due to optical polarisation caused by the radial orientation of the amylopectin molecules (Perez, Baldwin, & Gallant, 2009). These crosses are irreversibly lost following starch gelatinisation.

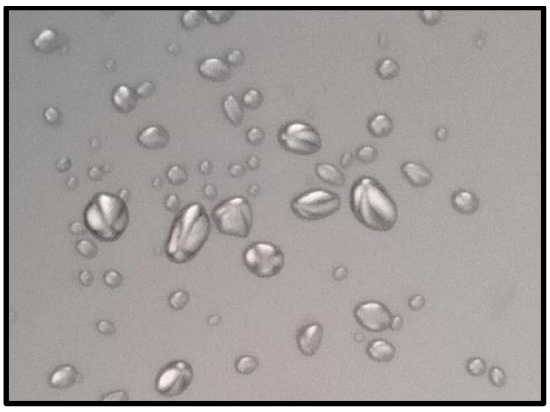

Figure 2-2 Maltese cross of starch granules.

X-ray diffraction patterns reveal a periodicity of approximately 9 – 10 nm within the granule which is interpreted as growth rings (Pérez & Bertoft, 2010). These rings are composed of alternating amorphous and semi-crystalline shells which can be viewed under SEM (Figure 2-3a). Depending on the origin of the starch granule, the growth ring may range from 120 to 400 nm thick. Presently, the accepted model of the granule structure depicts the crystalline lamellae as being formed by double helices of amylopectin side chains, and the amorphous lamellae as where the amylose and branch points of amylopectin are located (Wang & Copeland, 2013a). The core of a non-waxy starch (starches that lack amylose) granule is thought to be amorphous and contain mostly amylose and the reducing ends of amylopectin (Wang & Copeland, 2013b).

Figure 2-3 Starch granule and its hierarchical structure (Wang, Blazek, Gilbert, and Copeland (2012) modified by Wang and Copeland (2013b)).

With mild acid hydrolysis, X-ray diffraction patterns of starch granules provide information on the allomorph of the starch crystals based on the peaks that appear at different diffraction angles (2Θ) (Figure 2-4). The diffraction pattern of A-type starches typically consists of a single peak occurring at 2Θ = 15° and an unresolved doublet at 2Θ = 17° and 18°. On the other hand, B-type starches produce a diffraction pattern which consists of a single peak at 2Θ = 17°, several small peaks at 2Θ = 20°, 22° and 23° and an additional small peak at 2Θ = 5°(Cheetham & Tao, 1998). In general, A-type patterns are derived from cereal starches while B-type and C-type patterns are derived from tubers and legumes respectively (Pérez & Bertoft, 2010). The distinction between A- and B-type granules lies in the packing of the crystals and their water content (Figure 2-4). In A-type starches, crystallites are composed of glucosyl double helices arranged in a monoclinic lattice with four water molecules between helices; while in B-type starches, the glucosyl double helices are arranged in a hexagonal

lattice with 36 water molecules bound within the structure (Perez *et al.*, 2009). C-type crystals are a mixture of A- and B-type crystals and the diffraction pattern of these starches is in between those of the A- and B-type starches. The formation of A- or B-type crystals in starch is dependent on the conditions at which the crystals are generated: A-type crystals are formed under warm, dry conditions while B-type crystals are formed under cool, wet conditions (Pérez & Bertoft, 2010). The crystal structure within a starch granule influences its susceptibility to enzymatic hydrolysis: Ungelatinised B-type starches are resistant to digestion by pancreatic amylase (Englyst, Veenstra, & Hudson, 1996; Sun, Lærke, Jørgensen, & Knudsen, 2006) while ungelatinised C-type starches are resistant to hydrolysis by α-amylase (Englyst *et al.*, 1996). However, all starches, regardless of their type, become susceptible to enzymatic hydrolysis following gelatinisation due to hydration of their semicrystalline structure (Zhang, Venkatachalam, & Hamaker, 2006) (see the later section on gelatinisation for a detailed description of this process). This suggests that the semicrystalline structure is highly important in determining the rate of digestion of starches.

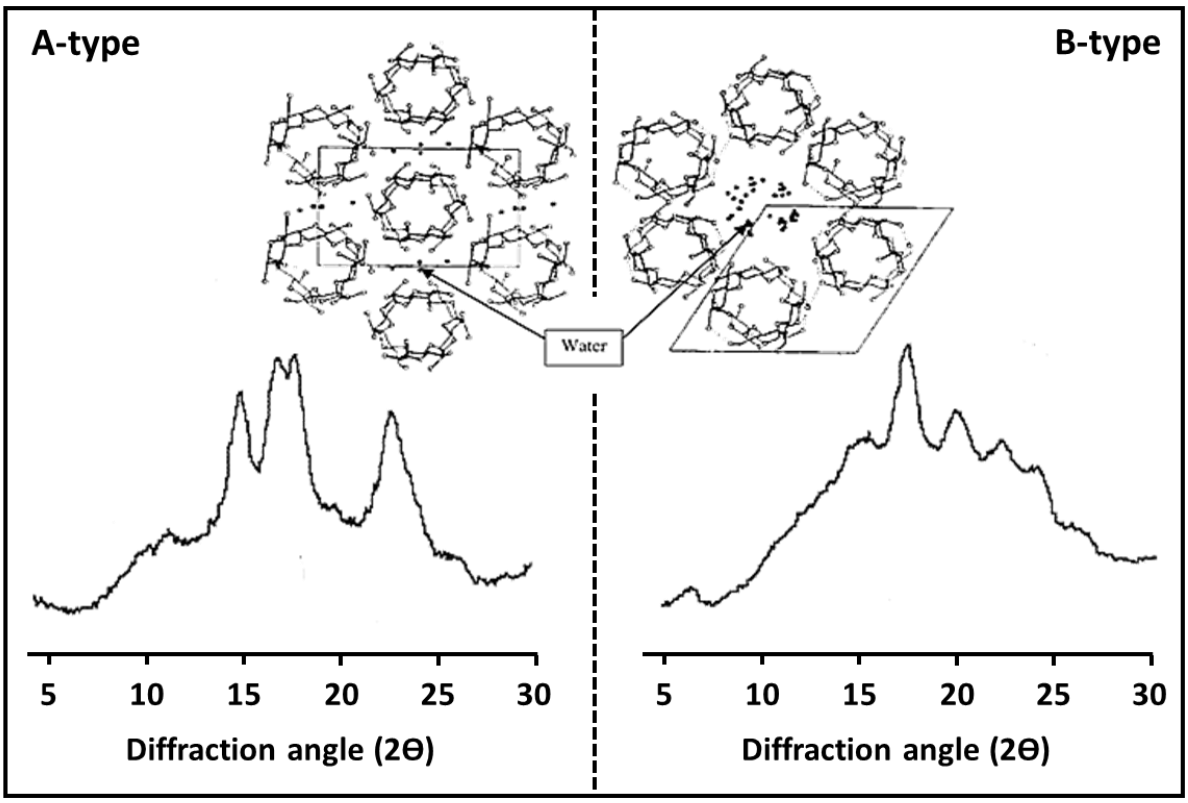

Figure 2-4 Wide-angle X-ray powder diffraction spectra for A-type and B-type starches (Cheetham & Tao, 1998).

2.1.4 Porous surface

The surface of certain starch granules such as maize and sorghum possess pores, which are openings to channels within the granule. Most of these channels span through the external surface of the

molecule to the granule core or hilum (Huber & BeMiller, 2000). The channels have a diameter of approximately 100 nm, and contain structural proteins and membrane proteins such as granule-bound starch synthase (Benmoussa *et al.*, 2010; Han & Hamaker, 2002b). The presence of pores facilitates enzymatic hydrolysis and hence, starches that do not have pores or have very small pores (ungelatinized potato starch) experience a lower degree of hydrolysis as degradation begins on the surface rather than moving rapidly to the interior of the granule (Sarikaya, Higasa, Adachi, & Mikami, 2000; Uthumporn, Zaidul, & Karim, 2010).

2.1.5 Physical properties

2.1.5.1 Gelatinisation

When starch is heated (above 55°C) in the presence of water and/or shear (extrusion), it undergoes a process known as gelatinisation, whereby the molecular organisation of the starch granules is disrupted. This phase transition is irreversible and it follows a sequence of water uptake, leaching of macromolecules, unwinding of double helices, loss of birefringence and finally increase in paste viscosity as a result of the formation of hydrogen bonds between starch and water, which increase the volume of the granules (Biliaderis, 2009; Buleon & Colonna, 2007). At low starch concentration, the viscosity of a starch suspension is largely influenced by the solid volume fraction of the starch alone, which is dependent on the rigidity and the volume fraction of the granules. As the concentration of starch is increased above the maximum packing volume, the viscosity of the suspension becomes more dependent on the interactions between starch granules and leached polymers (Okechukwu & Rao, 1995).

The gelatinisation process is often illustrated using the pasting curves derived from a Rapid Visco Analyser (RVA) where parameters such as pasting temperature, peak viscosity, peak temperature, breakdown, setback, and final viscosity can be obtained (Figure 2-5). The first phase of gelatinisation involves a reversible process of starch granule hydration, which consists of the absorption of water by the starch granules. There is limited swelling at this stage and the molecular structure of the granules remains intact. The temperature at which gelatinisation begins can be defined as the temperature at which viscosity begins to rapidly increase as a starch suspension is heated in water. The initial increase in the viscosity of the starch suspension is associated with the increase in the volume fraction of starch granules as a result of water uptake. This point is commonly termed as “onset of rapid viscosity increase” and the temperature corresponding to it is termed as “temperature of onset of rapid viscosity increase”. As the temperature rises in the presence of excess water, glucan polymer double helices become disordered due to associations of water with the hydroxyl groups on the surface of starch molecules. During this phase, the granules begin to swell, which increases the solid volume

fraction in suspension and, consequently, the viscosity of the suspension. This increase in viscosity leads to the "peak viscosity" in Figure 2-5. The gelatinisation process is accompanied by the leaching of amylose out of the granules, producing a starch paste that contains swollen granules (ghost granules), leached amylose and water (Buleon & Colonna, 2007). The existence of an envelope around ghost granules has been shown to have a role in maintaining the rigidity of the starch granules as they swell (Atkin, Abeysekera, & Robards, 1998; Han & Hamaker, 2002a). This envelope is thought to have an elastic property, which allows granules to swell during gelatinisation without losing their structural integrity. With prolonged swelling and shearing, and especially at high starch concentration, the granular envelope may collapse due to a build-up of internal pressure. This causes the ghost granules to contract resulting in the curling of the granular envelope towards the interior, reaching a point where granules soften and no longer behave as rigid spheres, and the viscosity of the paste then decreases (Atkin *et al.*, 1998). This is observed as the "breakdown" in the pasting curve. Despite the collapse in the envelope structure, most of amylopectin chains remain within the ghost granules. Factors such as amylose content have been shown to affect the integrity of ghost granules due to the cross linking of amylose on the outer region of ghost granules that stabilises them (Debet & Gidley, 2007). Upon cooling of the paste, starch glucan polymer retrogrades and this increases the viscosity of the paste. The retrogradation process is further described in a later section.

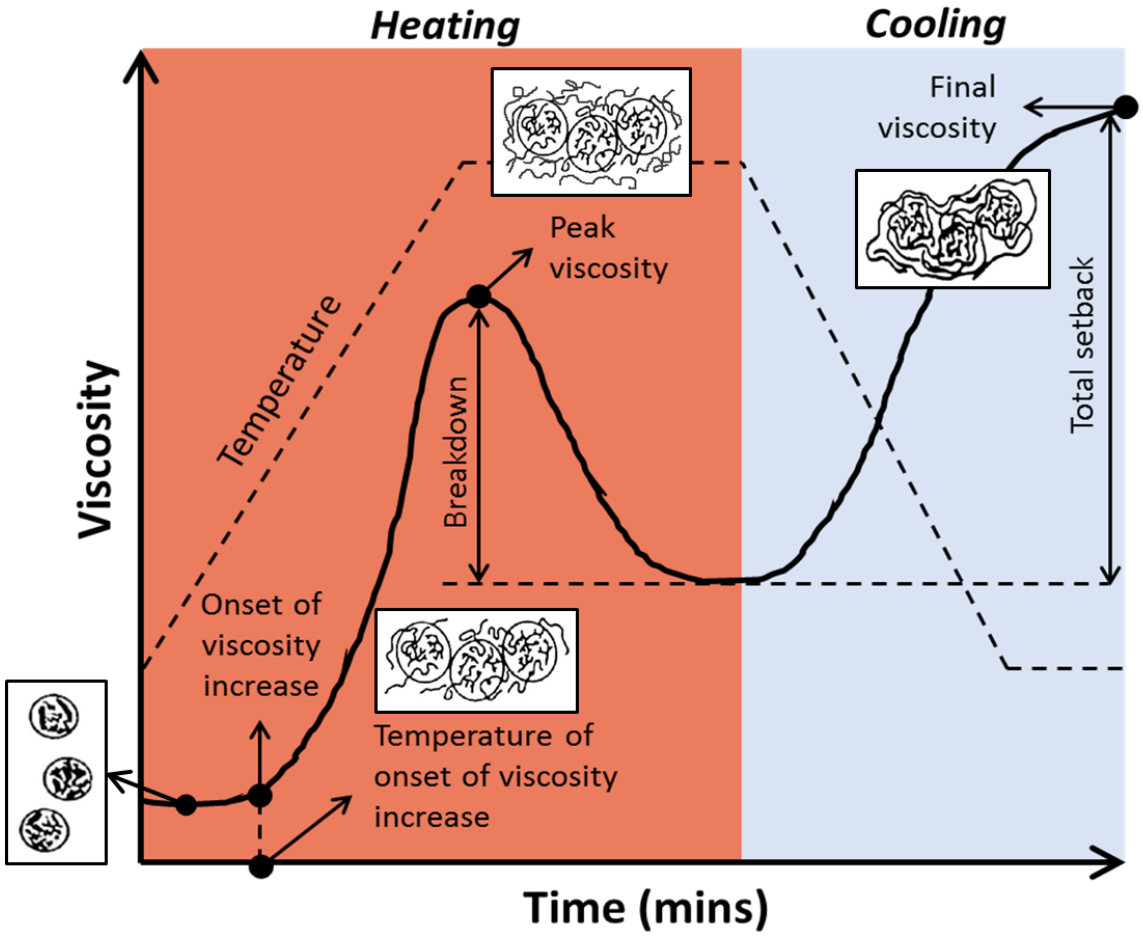

Figure 2-5 Typical pasting curve of a starch.

The early gelatinisation processes associated with the breaking of hydrogen bonds between starch polymers are endothermic and can be detected using differential scanning calorimetry (DSC) as two endotherms (peaks G and M in Figure 2-6) (Singh, Singh, Kaur, Singh Sodhi, & Singh Gill, 2003). The DSC endotherm peaks G and M (Figure 2-6) were first attributed to the disruption of crystalline structure (~60 - 75°C) and melting (>75°C) of crystallites respectively as proposed by Donovan (1979). In excess water, swelling of the granules unfolds and hydrates amylopectin helices completely, leaving none available for melting at higher temperature; thus, the only endotherm peak G was observed at high water content. As the proportion of water is decreased, some helices remain in their native conformation, which melts with increasing temperature (between 75 - 110°C). Thus, both endotherm peaks G and M are observed. At low water content, swelling is limited and thus, helices are not hydrated and remained undisrupted. This results in the helices melting at high temperature, creating a single endotherm peak M.

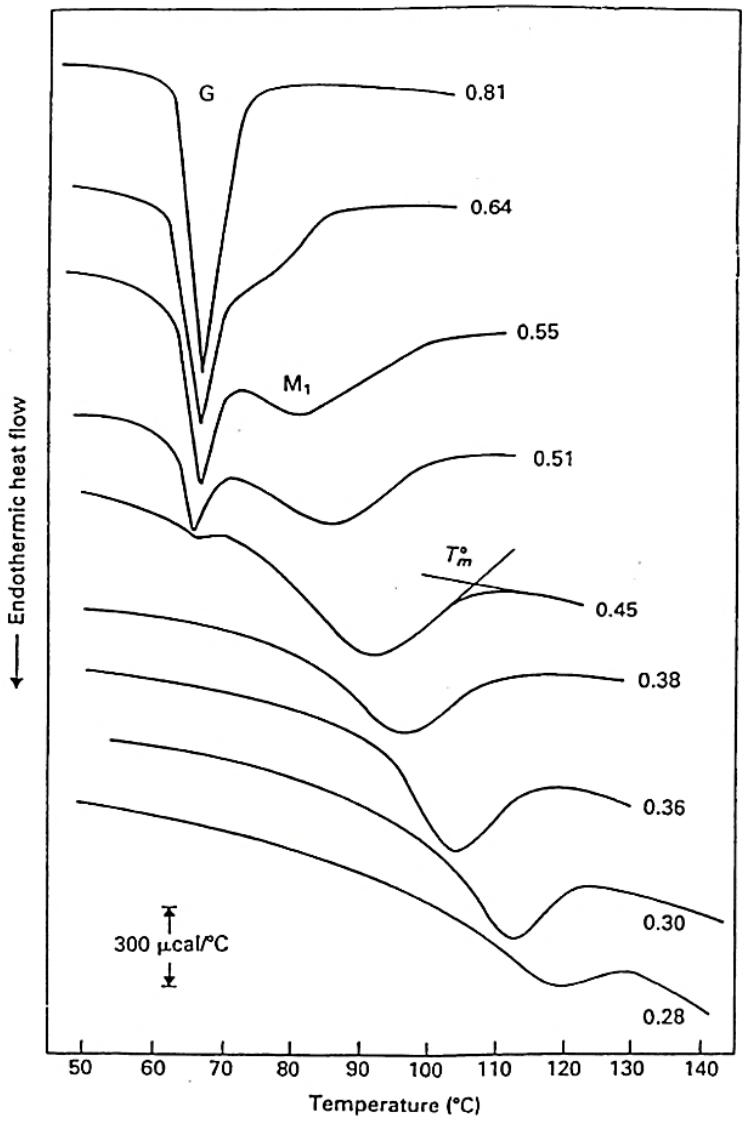

Figure 2-6 DSC profiles of potato starch at various water fractions. Numbers on each DSC profile denotes the fraction of water in the system (Donovan, 1979).

More recently, a model proposed by Waigh, Gidley, Komanshek, and Donald (2000) compares the disordering of starch granules during gelatinisation to the behaviour of liquid crystals. Using data from DSC analysis, small angle X-ray scattering (SAXS), wide angle X-ray diffraction (WAXD), dynamic mechanical analysis, and nuclear magnetic resonance spectrophotometry (NMR), the authors suggest that hydration of amylopectin chains result in smectic-nematic/isotropic transition followed by helix-coil transition (Figure 2-7). In the smectic phase, helices of amylopectin are oriented in a parallel manner on a defined plane, whereas in the nematic state, these helices are not arranged in a defined plane despite being parallel to each other. In the isotropic state, the helices are neither parallel nor arranged on a defined plane. These transitions during the gelatinisation process are water content dependent. It is suggested that at low water content, the single peak M observed in DSC thermographs correspond to the unwinding of the amylopectin helices into individual coils. At

intermediate water content, the first endotherm G is thought to be due to the dislocation of double helices resulting in smectic – nematic transition. This is then followed by unwinding of helices to coils, which is observed as the second endotherm M in the DSC thermograph. In excess water, the dislocation of double helices is thought to happen concurrently with the unwinding of helices to coils, resulting in only endotherm G being observed in the DSC (Waigh *et al.*, 2000).

Figure 2-7 Gelatinisation of starch granules in excess water (a) and in limited water (b) for B-type starch (i) and A-type starch (ii) (Waigh *et al.*, 2000).

2.1.5.2 Retrogradation

As non-waxy starch pastes are cooled, their storage moduli increase during a process termed as retrogradation. This occurs as a result of the formation of a three dimensional network as polymer molecules within the starch suspension reassociate and form junction zones via hydrogen bonds upon cooling (Biliaderis, 2009). Retrogradation results in increased gel turbidity, exudation of water (syneresis) from the gel and increased structural organisation (Wang, Li, Copeland, Niu, & Wang, 2015). The properties of retrograded starches are determined by the proportion of amylose and amylopectin that it contains. Starches containing amylose generally form more extensive networks that create strong elastic gels, whereas those that contain mostly amylopectin form pastes that are sticky, adhesive and soft (Miles, Morris, Orford, & Ring, 1985; Mua & Jackson, 1997). The formation of strong starch gels is associated with amylose-amylose interaction via hydrogen bonds between hydroxyl groups on the starch molecules and water. At low amylose concentration, there is a reduction in the intermolecular bonding between the relatively rigid amylopectin molecules and, therefore, is less conducive to associations between molecules. Hence, pastes are formed instead of gels (Ishiguro, Noda, Kitahara, & Yamakawa, 2000). When amylose is present, 3D networks are formed as mobile amylose chains bond with water and other amylose and amylopectin molecules (Biliaderis, 2009), and this network is reinforced by the presence of ghost starch granules (Miles *et al.*, 1985).

Starch gels are unstable because of the thermodynamic incompatibility between amylose and amylopectin. Prolonged storage results in the separation of the two polymers as they individually reorganise and preferentially associate with themselves, resulting in the formation of polymer-rich and polymer deficient phases. The reorganisation of amylose during cooling is regarded as the beginning of retrogradation and this occurs over minutes to hours. A minimum amylose concentration of 0.8 – 1.1 % is required for gelation (Hayashi *et al.* (1983) as cited in Biliaderis (2009)). This concentration is below the coil overlap concentration (C* ~ 1.45 %) suggesting that molecular entanglements do not determine the critical gelling concentration. Gidley (1989) proposed that the mechanism of gelation is the result of amylose interchain associations forming double helices and aggregation of helices which, in turn, form junction zones.

Prolonged storage of starch gels results in the retrogradation of amylopectins, which takes place over hours to days. Unlike amylose, the retrogradation of amylopectin takes weeks and requires higher polysaccharide concentrations than their coil overlap concentration (C* ~ 0.9 %). The rigidity of an amylopectin gel is dependent on storage temperature to a high degree and less on concentration. The first stage of gel formation involves the reordering of amylopectin chains from coils to double helices, which then aggregates and form crystals following the nucleation kinetics (Biliaderis, 2009).

Amylopectin gels are thermoreversible and the melting of the gel structure occurs between 45 – 60°C, which can be measured using DSC (Ring *et al.*, 1987).

2.2 Non-starch polysaccharides (NSPs)

Polysaccharides are long chain sugar polymers that can be classified into starches and non-starch polysaccharides (NSPs). NSPs are of interest in this work due to their (1.) functionality to act as a stabiliser, thickener and/or gelling agent (Stephen & Churms, 2006) and (2.) their limited digestibility in the human gut. The different functionality of various NSPs is largely attributed to their molecular structure and conformation. However, there is no single mechanism that explains the ability of some NSPs to reduce starch digestion, which will be discussed further at a later section. Two of the most common functionalities of NSPs when added into foods (thickening and gelation) are described below.

2.2.1 Thickening

The hydrodynamic volume of an individual polysaccharide molecule can be quantified by its intrinsic viscosity, [η], which is dependent on its polymer structure, which in turn is affected by changes in solvent conditions including pH, salt and temperature. The bulk viscosity of a polysaccharide solution is dependent on the polymer concentration and its volume fraction. Most polysaccharides behave as random coils in water as the solvent. At low shear rates, the disruption of existing entanglements and the formation of new entanglements achieve equilibrium resulting in constant viscosity, known as the zero-shear viscosity (η_0). At a higher shear rate, the rate of disentanglement exceeds re-entanglement, which results in the depletion of networks and a fall in viscosity (Figure 2-8) (Morris, 1990).

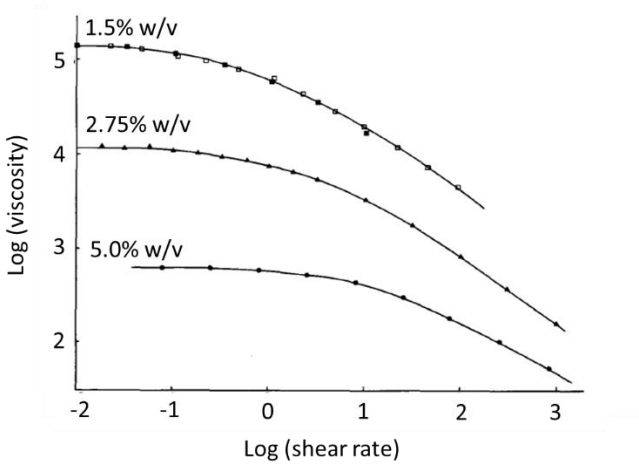

Figure 2-8 Shear thinning of concentrated polysaccharide solutions for lambda carrageenan at various concentrations (Adapted from Morris, Rees, and Welsh (1980)).

The zero shear viscosity of a polymer increases with concentration (slope = 1.4) up to a point known as the critical concentration, C*. The critical concentration marks the point at which coils begin to overlap and the concentration dependency of η_0 changes. Above C*, the concentration dependency of η_{sp} increases as indicated by an increase in the slope of the line—the magnitude of which is dependent on the type of polysaccharide (Figure 2-9) (Morris, Cutler, Ross-Murphy, & Rees, 1981). At this point, entanglement occurs and polysaccharides can only move in a limited manner through the entangled neighbouring chains (Figure 2-9c inserts) (Morris, 1990). This gives hydrocolloids their functionality as a thickener at low concentration.

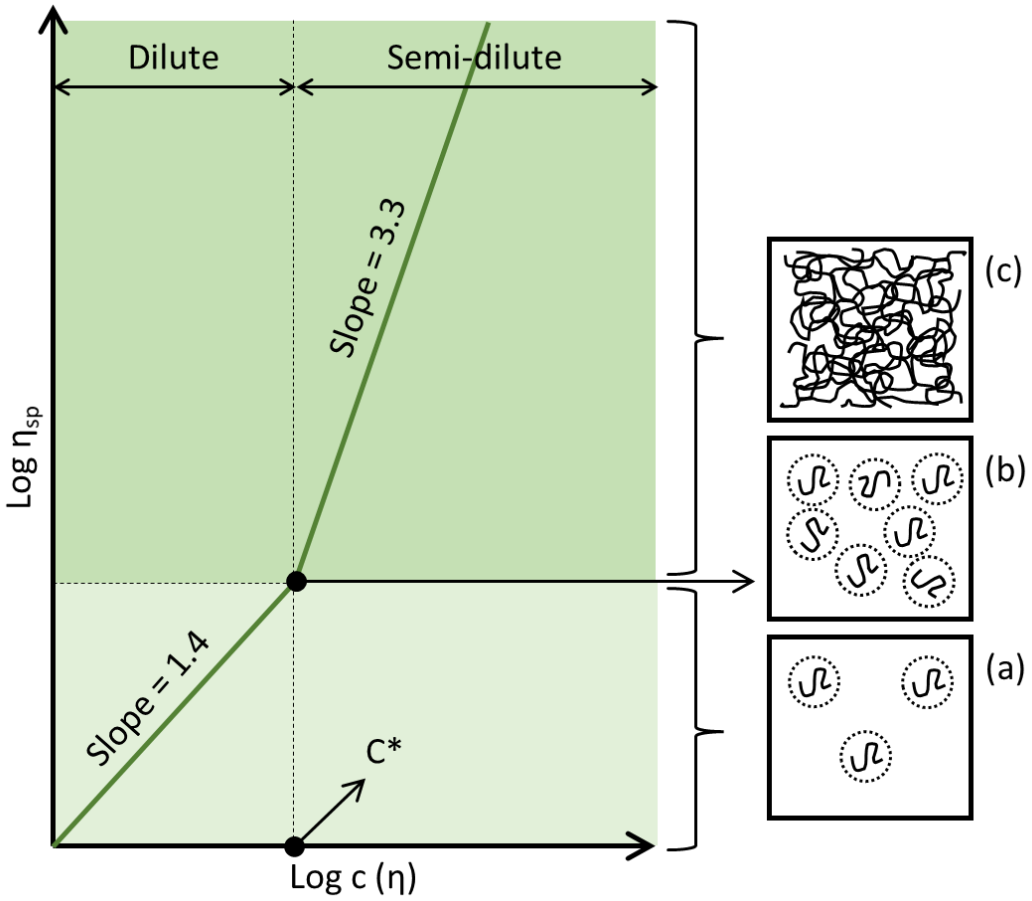

Figure 2-9 Variations in the zero-shear specific viscosity of polysaccharide solutions with the degree of space occupancy by the polymer coils: independent polymer coils at low concentration (c < c*) (a), onset of coil overlap (c = c*) (b) and entangled polymer network (c > c*) (c).

2.2.2 Gelation

Gelation of hydrocolloids occurs when polymers associate to form a network structure. In general, according to the method of gelation, hydrocolloids can be classified into cold-setting gels, hot-setting gels, and gels that set on cooling, after heating the solution. The properties of the gel such as the

strength and whether it is thermal reversible/irreversible, are dependent on the nature of the linkages formed between the polymers (Morris, 2007). Three types of linkages are common among NSPs: (i) point cross-links, (ii) extended cross-links, and (iii) complex cross-links (Morris, 2007).

2.2.2.1 Point cross-links

Point cross-links occur at points between polysaccharide chains. An example of this structure is the diferulic acid linkages generated by sugar beet pectin (Saulnier & Thibault, 1999). Gels with point cross-links can be set at room temperature, and are irreversible and strong (Morris, 2007). The strength of these gels is determined by the number of cross-links per unit volume.

2.2.2.2 Extended cross-links

Extended cross-links occur when blocks of structure on different polymers associate to form a network. Extended cross links can be induced by altering the conditions of the solution in which the hydrocolloids are dispersed. Several examples of these are summarised by Morris (2007) as follow:

a. Konjac mannan gels induced by alkali: Alkali treatment is thought to generate blocks of mannan regions that are insoluble. These regions associate through hydrogen bonds, forming a network.
b. Methylcellulose gels induced by heating: Although poorly understood, gelling is thought to happen due to precipitation of polysaccharides from the solution as a result of hydrophobic associations (Figure 2-10).

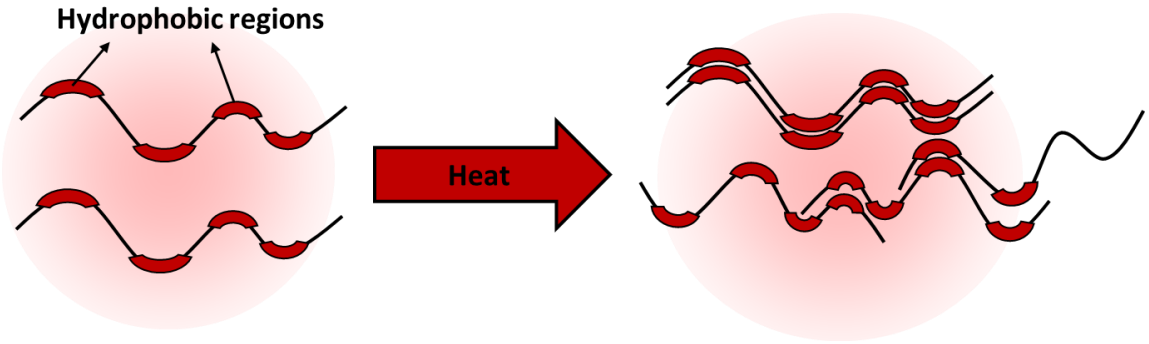

Figure 2-10 Gelation of methylcellulose induced by heating (adopted from Jyoti and Baek (2014)).

c. Alginate gels induced by cooperative binding of cations: Blocks of polyglucuronic acid binds to cations (calcium is preferred) resulting in the neutralisation of charges, and leading to gelation (Figure 2-11).

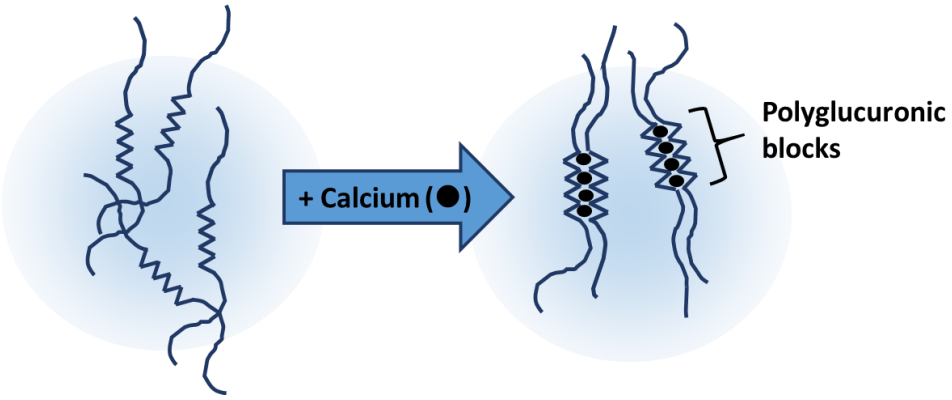

Figure 2-11 Gelation of alginate in the presence of calcium.

d. Low methoxy pectin gels induced by lowering pH or cooperative binding of cations: Gelation occurs in a similar manner to alginate gels. In this case, polygalacturonic acid associates with calcium ions. The charge on polygalacturonic acid can be neutralised by lowering the pH. Gels formed at low pH are thermally reversible (due to the presence of two modes of associations: ionic and non-ionic) while gels formed at neutral pH are thermally irreversible.

2.2.2.3 Complex cross-links

Several gel forming polysaccharides such as gellan, agar and carrageenan form higher order helical structures, which is important for gelation. Helices may vary between single helical structures, three-fold double helices, or five-fold double helices. At high temperature, the thermal energy provided by its environment overcomes the rotational energy barrier, allowing the polysaccharide to be flexible and adopt a random coil formation (Ebewele, 2000). The transition between coils to helices occurs on cooling the heated polysaccharide solution. The formation of hydrogen bonds between the polysaccharide chains drives the formation of the helices (Piculell, 2006). Upon contact, they become sites of nucleation, propagating the chains to create filamentous structures, which aggregates to form a gel (Morris, 2007).

The formation of these gels is also influenced by the ionic strength of the media, whereby the addition of cations to the solution or increasing its ionic strength results in the formation of stronger gels (Morris, 2007). This is attributed to two effects. The first is that increasing the ionic strength of the media results in charge screening, thereby inducing helix formation due to the absence of repulsive forces between the polysaccharide chains. The second is that the presence of cations results in the formation of junction zones between polysaccharide chains, which promote further aggregation of the helices to form thicker branched fibrous structure (Figure 2-12) (Morris, 2007).

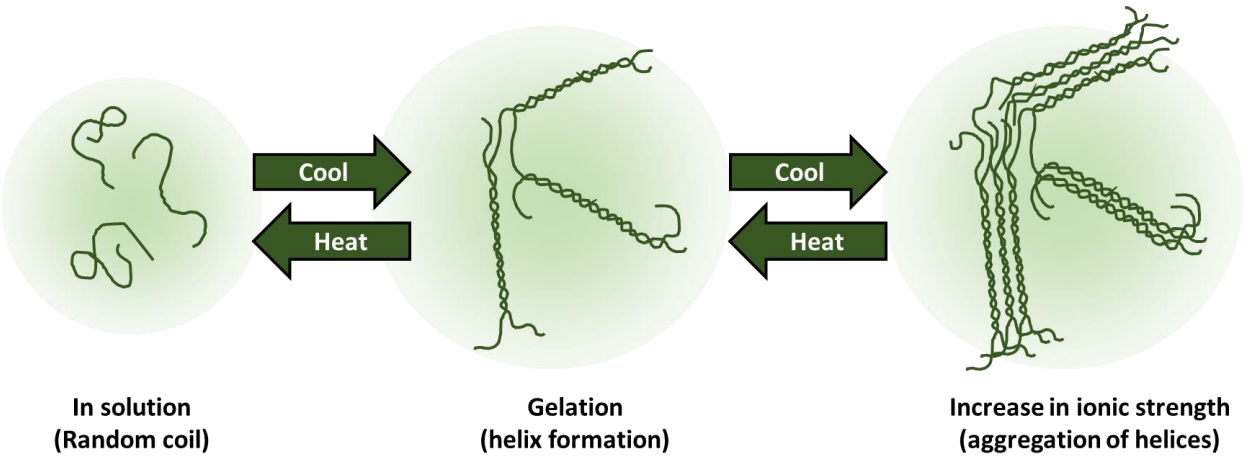

Figure 2-12 Complex cross linking of polysaccharides such as carrageenan.

2.2.3 *Mesona chinensis* polysaccharide (MCP)

2.2.3.1 Composition and structure

Mesona chinensis (MC) is a herb of the mint family which contains neutral and ionic polysaccharides, and is traditionally used as a remedy for heatstroke, hypertension, diabetes, and muscle joint pain (Feng, Ye, Zhuang, Fang, & Chen, 2012). In many Asian countries, the herb's extract is used in combination with starch to make a black gel type dessert known as "grass jelly". The crude MC extract is sold for food applications and typically contains a high proportion (20 – 30 %) of impurities, including protein, fibre, and ash (Table 2-3).

Table 2-3 Composition of MC extract powder.

Component	Composition			
	(Lai & Chiang, 2002)	(Feng, Gu, Jin, & Zhuang, 2010b)	(Lai, Tung, & Lin, 2000)	(Feng *et al.*, 2014)
Crude protein (%)	6.45	9.74	4.56	9.74
Crude fibre (%)	6.75	2.98	1.07	2.98
Crude fat (%)	0.37	-	0.86	0.83
Total sugars (%)	-	42.2	-	-
Moisture (%)	-	14.18	-	5.12
Total Ash (%)	21.44	30.9	26.97	30.9
Minerals (mg/g)				
Sodium	54.84			
Potassium	2.27			
Calcium	7.86	-	-	-
Magnesium	0.50			
Iron	0.63			
Zinc	0.13			

MC extract is known to contain a relatively high proportion of uronic acid that varies from 13.8 % to 48.6 %, depending on the extent of purification (Feng *et al.*, 2010b; Feng, Gu, Jin, & Zhuang, 2008). The extract of MC contains two types of polysaccharides: neutral (0.5 %) and anionic (90 %) and their sugar compositions are shown in Table 2-4 (Feng *et al.*, 2008).

Table 2-4 Monosaccharide compositions of neutral and anionic polysaccharides of MC (Feng *et al.*, 2008).

Monosaccharide composition	Neutral polysaccharide	Anionic polysaccharide
Galactose (%)	22.75	5.51
Glucose (%)	34.99	2.07
Mannose (%)	9.88	0.77
Xylose (%)	3.39	4.75
Arabinose (%)	26.69	25.88
Galacturonic acid (%)	-	48.62
Rhamnose (%)	2.29	12.4

In this thesis, the NSP of MC is termed as the *Mesona chinensis* polysaccharide (MCP) and its structure has been reported to contain an α-(1→4)-galacturonan backbone with insertions of α-1,2-L-Rha*p* residues on which arabinogalactan, arabinan, galactan, and xylan are attached to (Figure 2-13) (Feng *et al.*, 2008). This polysaccharide is of interest due to its ability to synergistically increase the viscosity of starch paste and form a stronger gel—aqueous solutions of MCP on their own do not form gels when heated and cooled (Lai & Liao, 2002a).

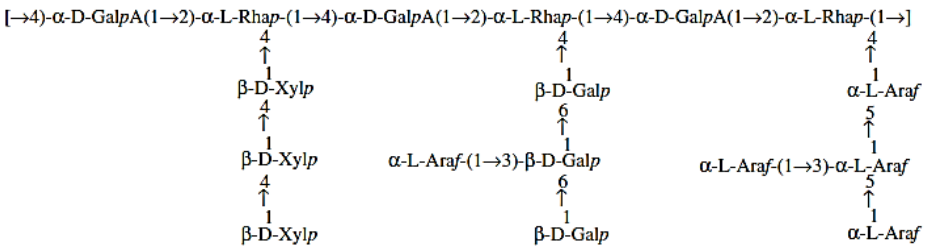

Figure 2-13 Structure of anionic MCP (Feng *et al.*, 2008).

2.2.3.2 Rheological behaviour

Double logarithmic plots of the zero-shear rate specific viscosity vs coil overlap parameter of MCP suggest that the polysaccharides' conformation was more rod-like than random coil. The behaviour of MCP in dilute solution was similar to that of random coil polysaccharides (slope = 1.28) (Figure 2-14). However, in the semi diluted region ($C > C^*$) where coil overlaps and entanglement begins, the slope

of the plot was lower (slope = 2.29) than most random coil polysaccharides (Lai *et al.*, 2000). The viscosity of MCP in the semi diluted region showed a second power dependence on concentration which is similar to the behaviour of rod-like amylose in the semi diluted region (Ellis & Ring, 1985). MCP has a low viscosity and is reported to be Newtonian at a concentration below 1 % w/w (Lin *et al.*, 2017). When the concentration of MCP is increased further, It exhibits shear-thinning properties as a result of polymer entanglement at high polysaccharide concentrations (Lai *et al.*, 2000).

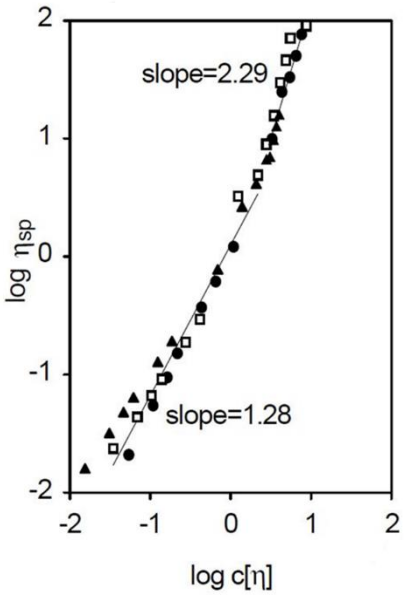

Figure 2-14 Double logarithmic plot of specific viscosity vs. concentration for fresh (●), frozen (□) and dehydrated (▲) MCP.

The properties of MCP solutions are affected by changes in salt and pH. A decrease in MCP solubility was reported when 0.01 – 1 M sodium chloride, calcium chloride and zinc chloride were added (Lin *et al.*, 2017). The viscosity of 10 % w/w MCP solution is also reduced by the addition of < 1 % w/w calcium chloride, more so than with sodium chloride at similar concentration. However, at a concentration greater than 2 % w/w calcium chloride, the viscosity of the polysaccharide increased, presumably due to the cation promoting interchain associations. On the other hand, reports regarding changes in the viscosity of MCP with pH are inconsistent. While some authors reported a decrease in the viscosity of MCP in both acidic and alkaline conditions as a result of polysaccharide hydrolysis (Lin *et al.*, 2017), others have reported that the highest viscosity of MCP was obtained at pH 10 due to charge repulsion that causes MCP to expand in solution (Feng, Gu, & Jin, 2007).

2.3 Interactions between starch and NSPs

Starches are used in food formulations mainly for its nutritive and textural values. The high viscosity of gelatinised starches is a desirable trait that imparts body and mouthfeel to food products. However, with the increase in obesity and associated diabetes worldwide, there is a shift in consumers' perspective regarding desirable attributes in foods. For example, there is a clear rising demand for foods with a lower glycaemic load. Hence, there is a drive to partially substitute starches in food products to reduce their digestibility and hence glycaemic load, but at the same time, retain all desirable textural properties. The ability of many NSPs to cause large increases in viscosity in aqueous solution at relatively low concentrations makes them useful as substitutes for starches in many of these food products. For this reason, the interaction between starch and NSP is widely studied in order to find suitable NSPs for use in the formulation of food products.

2.3.1 Mechanisms of interaction

The interaction between starch and NSP is very specific to the type of starch and NSP and the conditions in which the interactions occur. The nature of their interaction determines the final properties of the composite and are normally characterised by a synergistic increase in the viscosity of the starch paste in the presence of low levels of the NSP. These have been widely explored and several mechanisms of these interactions are explained as follow.

2.3.1.1 Thermodynamic incompatibility

Due to the various conformations and structures of polymers, incompatibilities are likely to occur when starches and NSPs are mixed. This can lead to phase separation, which results in areas of high NSP, amylopectin or amylose concentrations, increasing the overall viscosity of the paste. This has been reported for galactomannans (locust bean and guar gums) when mixed with sago starch (Ahmad & Williams, 1999) or maize starch (Alloncle & Doublier, 1991). In these two instances, amylose leached during gelatinisation and the polysaccharide gum were thought to be present in two separate phases. The concentrations of starch and galactomannan were found to be important to induce phase separation, whereby for a 6 % w/w sago starch dispersion, a galactomannan concentration greater than 0.25 % w/w was required to induce phase separation (Ahmad & Williams, 1999). Thermodynamic incompatibility is very common, and it occurs as starch gelatinises and retrogrades. The release of amylose from the starch granules during gelatinisation results in the formation of a phase that is rich in amylose, while amylopectin is retained within the starch granules as they swell. During retrogradation, amylose and amylopectin chains reassociate with themselves to increase the rigidity of starch gels, resulting in polymer-rich and polymer-deficient areas, thus causing phase separation. However, at a relatively high starch concentration, phase separation of the polymers can be slowed

due to the formation of a uniform amylose network (Morris, 2007), which plays a large role in increasing the viscosity of the system (Alloncle & Doublier, 1991). Hence, macroscopic phase separation is not always detected and has to be determined using other techniques such as rheology and microscopy.

2.3.1.2 Polymer association

Polymer association between non-waxy starches and the *Mesona chinensis* polysaccharide, which is of interest in this study, have previously been hypothesised. Based on rheological data and thermal analysis, the interaction between the two is thought to be due to amylose and MCP interactions (Lai & Chao, 2000b) via hydrogen bonds (Feng *et al.*, 2012; Kumar, 2012). However, the exact nature of their molecular interaction has never been proven. Similarly, interaction between the glucan polymers of starch and the polysaccharide of yellow mustard have been suggested, but without further evidence of the nature of the interaction (Liu, Eskin, & Cui, 2003).

2.3.1.3 Surface association

Adsorption of NSP onto the surface of starch granules have been observed for κ-carrageenan (Espinosa-Dzib, Ramírez-Gilly, & Tecante, 2012), xanthan (Gonera & Cornillon, 2002) and soybean-soluble polysaccharide (Funami *et al.*, 2008) using confocal microscopy. While the mechanism by which NSPs adsorb onto the surface of starch granules is not known, it has been shown that the phenomenon is accompanied by a decrease in the proportion of amylose leached and peak viscosity of the paste (Funami *et al.*, 2008). The formation of a barrier around starch granules by NSPs is likely to result in two things; the first is that water absorption into starch granules during gelatinisation is hindered and, therefore, granules are less gelatinised leading to less amylose being leached. The second is that the presence of a barrier prevents amylose from exiting the granules during gelatinisation and, hence, led to a reduction in the proportion of amylose that can be leached from the granules. The combination of the two factors causes the viscosity of the paste to decrease as a result of granules being less swollen and a lack of amylose associations in the continuous phase.

2.3.1.4 Depletion flocculation

Depletion flocculation (Figure 2-15) is a phenomenon that occurs when polymers are excluded from a gap (depletion zone) between two particles due to osmotic potential difference, resulting in the aggregation of the particles (Abdulmola, Hember, Richardson, & Morris, 1996; McClements, 2000). For depletion flocculation to occur, a critical concentration of non-adsorbed biopolymer has to be exceeded and this is dependent on the size of the biopolymer relative to the size of the particle (Chanamai & McClements, 2001).

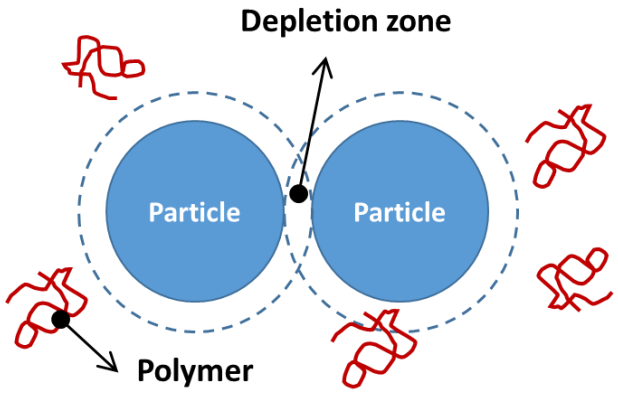

Figure 2-15 Depletion flocculation.

Depletion flocculation has previously been reported to occur in beverage emulsions when 0.4 % w/w gum arabic or 1.7 % w/w modified starch was added to 5 % oil in water emulsions made with hexadecane stabilised with a non-ionic surfactant, Tween 20 (Chanamai & McClements, 2001). While relatively uncommon in a starch system, depletion flocculation as a result of excess NSPs in a starch system has been proposed for xanthan gum (Abdulmola *et al.*, 1996; Achayuthakan & Suphantharika, 2008).

2.3.2 Starch and MCP interaction

2.3.2.1 Effect of interactions on gel/paste properties

Gelatinisation of some non-waxy starches such as wheat, maize and rice in the presence of MCP has been shown to result in an increase in the peak viscosity of the paste, which forms a thermo-irreversible gel upon cooling (Feng, Gu, Jin, & Zhuang, 2010a; Feng *et al.*, 2010b). For many starches, the formation of a transient peak at the early stages of starch gelatinisation in the presence of MCP is observed as shown in Figure 2-16. However, this has never been pointed out or discussed by any authors despite it being a distinct character of starch being gelatinised in the presence of MCP.

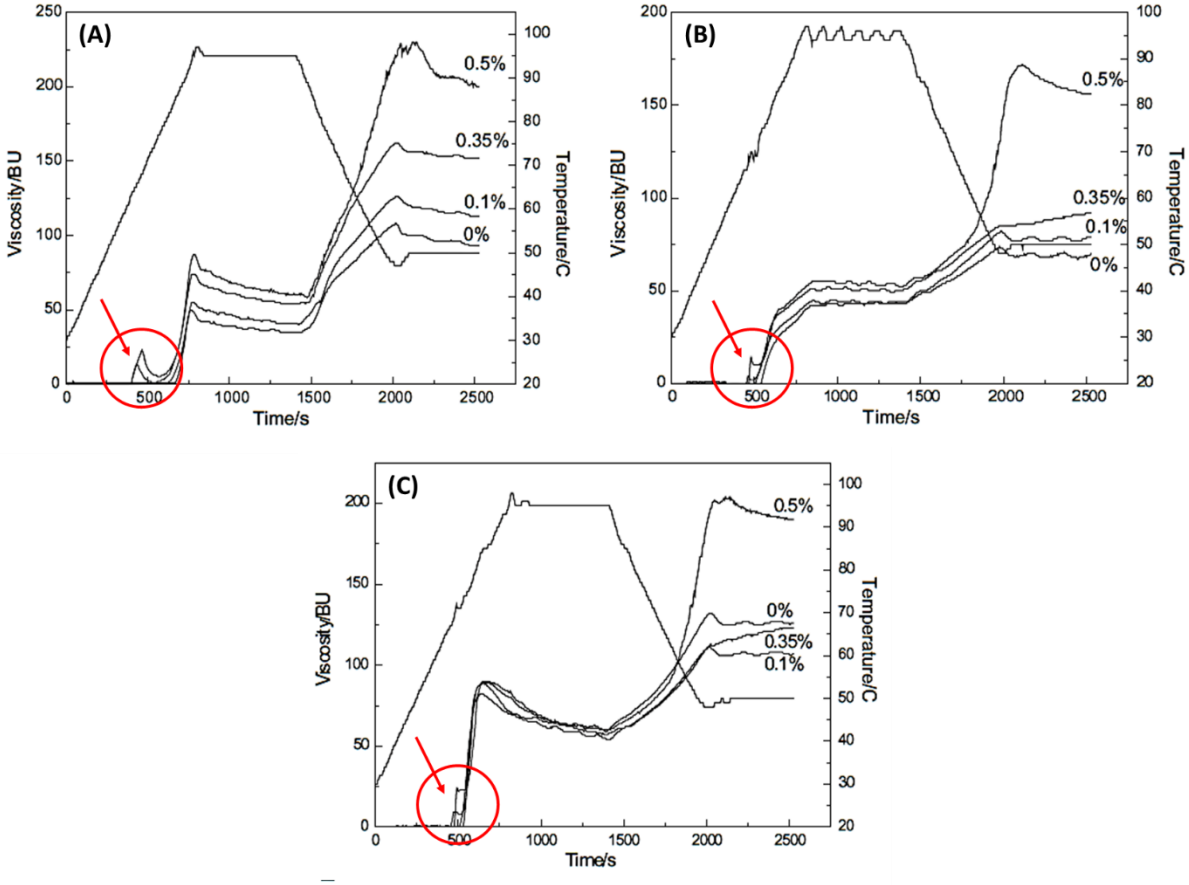

Figure 2-16 Pasting curve of rice (A), pea (B) and mung bean (C) starches containing increasing concentrations of MCP. Red arrow and circle indicates the characteristic transient peaks in the presence of MCP (Feng *et al.*, 2014).

Thermodynamic incompatibility between polymers resulting in segregation of starch granules and polysaccharide in the continuous phase has been proposed as an explanation for the increase in the viscosity of starch pastes containing MCP (Lai, Liu, & Lin, 2003). However, this does not eliminate the possibility of associative interaction between the two. The microstructure of rice starch gels containing MC extract (Figure 2-17) has been reported and it was proposed that the presence of MCP resulted in the formation of thicker membranes, indicative of interaction between amylose and MCP (Feng *et al.*, 2010b).

Figure 2-17 SEM photographs of Rice starch-MNSP mixtures with 0 % (a), 0.1 % (b), 0.35 % (c), 0.5 % (d), and 0.7 % (e) w/w MNSP concentration (Feng *et al.*, 2010b).

While the interpretation of the micrographs is debatable, other studies have demonstrated that gel formation and increases in paste viscosity were only observed when MCP is gelatinised with non-waxy starches, supporting the notion that interaction was primarily between amylose and MCP (Figure 2-18) (Lai & Chao, 2000b). Further studies suggest the involvement of hydrogen bonds in their interaction and the importance of the early stage of gelatinisation in the development of starch-MCP gels (Feng *et al.*, 2012; Kumar, 2012). The proposed mechanism of starch and MCP interaction was then expanded by Kumar (2012) to include the involvement of not only amylose, but also the starch granules, whereby MCP interacts with the granule surface to limit swelling, making them more rigid. However, there is no further understanding or definitive evidence on the mechanism of interaction.

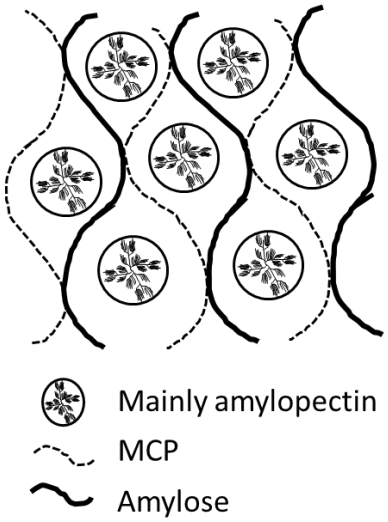

Figure 2-18 Proposed mechanism of starch-MCP interaction (adapted from Lai and Chao (2000b)).

2.3.2.2 Factors influencing the properties of starch-MCP paste/gel

2.3.2.2.1 Starch type

The effect of adding MCP (0.1 – 0.5 % w/w) into different starches (6 % w/w) have previously been reported by Feng *et al.* (2014), whereby interaction resulting in an increase in the viscosity of the cooled paste (measured using RVA) was reported for rice, wheat, pea and sweet potato starches. The opposite was observed for potato and tapioca starches, whereby the addition of MCP generally resulted in a decrease in their cooled paste viscosity. The mechanism by which this occurred is not known, however, it has been hypothesised that electrostatic repulsion between the phosphate groups in potato starch and the negatively charged groups in the NSP (MCP in this case) caused the decrease in the viscosity of the composite paste (Shi & BeMiller, 2002).

2.3.2.2.2 Starch and MCP concentrations

In a dilute system, the increase in the viscosity of starch pastes by the addition of MCP has been shown to be starch and MCP concentration dependent. Lai *et al.* (2003) reported that critical MCP concentrations to obtain maximum viscosities were specific for the individual starches that were studied (tapioca, wheat and corn) at concentrations ranging from 2 – 4 % w/w starch. With increasing starch concentrations, the amount of MCP needed to achieve a maximum viscosity was found to increase (for example 1.2 % and 1.8 % w/w MCP were required to obtain the maximum viscosity for 2 % and 4 % w/w wheat starch respectively). At higher starch concentration (6 % w/w), the addition of increasing concentrations of MCP to rice starch resulted in a decrease and for wheat starch an increase in zero shear viscosities respectively (Feng *et al.*, 2010a). For other starches such as corn and mung bean, the addition of low concentrations of MCP (0.1 % w/w for corn and < 0.35 % w/w for mung

bean) resulted in a decrease in the viscosity of the paste. However, further addition of MCP up to 0.5 % w/w resulted in an increase in the viscosity of the paste (Feng *et al.*, 2014). From the given reports, it is likely that the interaction between MCP and starch to increase the paste viscosity is not only dependent on the presence of amylose. Other factors such as the starch swelling properties are likely to be involved in determining the outcome of the interaction since the viscosity of a starch paste is not only dependent on the interaction between polymers in the continuous phase but also on the starch granule volume fraction.

2.3.2.2.3 Salt

The effect of salt on the viscoelastic properties of 2 % w/w wheat starch + 1 % w/w MCP (Lai & Chao, 2000a) and 6 % w/w rice starch + 0.5 % w/w MCP (Feng *et al.*, 2016) has been studied. The authors reported an increase in the gelling temperature, gelling rate and viscosity of the system with increasing concentrations of monovalent cations from 5 to 75 mM (NaCl and KCl). Viscosity increases were also observed for divalent cations ($CaCl_2$ and $MgCl_2$), but these increases were dependent on the salt concentration— viscosity increase was in the order of 6.8 mM > 5.1 mM > 3.4 mM > 8.5 mM divalent salts. At higher calcium chloride concentration (20 – 75 mM), a decrease in the viscosity of rice starch paste was observed (Feng *et al.*, 2016). The increase in the viscosity of wheat starch-MCP pastes in the presence of divalent salts at a concentration between 6.8 and 8.5 mM was attributed to the formation of junction zones within the structure through ionic interactions with the uronic acids found in MCP (Feng *et al.*, 2007; Lai & Chao, 2000a). At a higher concentration (25 – 75 mM), the decrease in rice starch paste viscosity was attributed to charge screening effect of excess calcium ions, resulting in MCP adopting a more compact conformation, hence disrupting network formation.

2.4 Digestion of starch and polysaccharide gels

2.4.1 Digestion of starch

Carbohydrate digestion begins in the mouth where salivary amylase is secreted. Salivary α-amylase hydrolyses α-1,4-glycosidic linkages in amylose and amylopectin to produce glucose, maltose, maltotriose, and α-limit dextrins. This enzyme is inactive at low pH, and therefore salivary digestion ceases as food enters the stomach (pH 2.6). However, some hydrolysis by salivary amylase may still occur as gastric secretions do not immediately penetrate into the food bolus to inactivate the enzyme. Digestion of starches is continued in the small intestine where bicarbonate in the pancreatic juice along with bile neutralises gastric acidity (Berdanier, 2000). Pancreatic juice contains amylase (1,4-α-D-glucanohydrolase, EC 3.2.1.1) which hydrolyses carbohydrate in the same manner as salivary α-

amylase (Lehmann & Robin, 2007). The hydrolysis of carbohydrate polymers is specific. The catalytic subsite of α-amylase is specific to the five glucose residues adjacent to the terminal reducing glucose unit on the polymer (amylose and amylopectin) chain. Once bound, the enzyme cleaves the second and third α-1,4-linked glucosyl residue (Singh, Dartois, & Kaur, 2010). α-amylase is unable to hydrolyse α-1,4 linkages adjacent to the α-1,6 linkages branching point due to steric hindrance (Singh *et al.*, 2010). Therefore, the product of α-amylase digestion is further hydrolysed by the brushborder enzymes of the small intestine. A group of enzymes known as α-glucosidases located in the brushborder membrane of the small intestine hydrolyses the α-limit dextrins to produce glucose. Maltase, lactase, and sucrase hydrolyse maltose, lactose, and sucrose respectively. The remnants of the carbohydrates that are not hydrolysed are passed into the colon whereby intestinal flora metabolises them to organic acids and gases (Berdanier, 2000; Champ, 2004).

2.4.2 Measuring glycaemic response and starch digestibility

2.4.2.1 Glycaemic index, glycaemic load, and glycaemic glucose equivalent

The concept of glycaemic index (GI) was developed from the dietary fibre hypothesis which suggests that metabolic benefits relating to diabetes and reduction of coronary heart disease can be achieved from foods that are slowly absorbed from the human intestinal tract (Jenkins *et al.*, 2002). The GI value for a food describes the postprandial blood glucose response over a 2h period in comparison with the rise in blood sugar following the consumption of a standard starchy or sugary meal, often being a standard quantity of white bread or glucose. It can be calculated using the following equation (Brennan, 2005):

$$\text{GI} = \frac{Incremental\ area\ under\ blood\ glucose\ response\ curve}{Corresponding\ area\ of\ blood\ glucose\ curve\ from\ consumption\ of\ a\ glucose\ standard} \times 100$$

The concept of GI has brought about other concepts such as glycaemic loading (GL) which takes into account the carbohydrate content of the food consumed and, therefore, represents the impact of the total food on glucose accumulation in the blood (Brennan, 2005):

$$\text{GL} = \text{GI of food product} \times \text{carbohydrate content}$$

As the GL concept takes into account the quality and quantity of carbohydrates ingested, it may be used as an indicator of the dietary insulin demand of an individual (Salmeron *et al.*, 1997). However, this concept was developed as “a measure of cumulative exposure to glycaemia as a risk factor in disease” and, therefore, may not be a good measuring tool for the glycaemic effect of a single meal (Monro, 2002). From this, Monro (2002) developed a concept known as glycaemic glucose equivalent

(GGE) which allows for the quantification of the glycaemic effect of a meal. GGE is calculated as follows:

$$GGE = \Sigma \text{ (servings of food} \times \text{Food weight/serving} \times (\%\ CHO \times GI)/10\ 000)$$

All of these concepts possess shortcomings such as their failure to take into consideration the influence of the foods' matrix, microstructure, and other physical properties that may affect the observed glycaemic response (Brennan, 2005). They also fail to take into account the variation in the digestive process and nutritional status of individuals, which deem the estimation of the glycaemic response inaccurate. Hence, the quantification of glycaemic response using these methods is somewhat debatable.

2.4.2.2 Readily digestible starch, slowly digestible starch, and resistant starch

A different concept of measuring starch digestibility is by classifying them into readily digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Englyst, Kingman, & Cummings, 1992). This classification is based on the amount of glucose released at times during the course of digestion. In simple terms, the amount of RDS starch corresponds to the proportion of glucose released after 20 minutes of digestion in simulated gastric conditions, SDS is the proportion released between 20 and 120 minutes, and RS the remaining starch which is not digested in that time frame (total starch minus the amount of glucose released after 120 minutes) (Englyst *et al.*, 1992). This classification method was developed for usage with *in-vitro* digestions involving gastric and intestinal phases. Hence, it is a good predictor of starch digestibility.

Interpreting data from a digestion curve to obtain qualitative data for comparisons between different systems can be straight forward. The amount of glucose released/starch digested after a certain period of time gives a good indication of the amount of RDS, SDS and RS present. However, to obtain a more in depth understanding of the digestion kinetics can be difficult for reasons that the digestion process involves an initial zero order kinetic where excess substrate is present upon the addition of enzymes. As the digestion progresses, it follows a first order reaction and the substrate is gradually depleted. There is also the added complications that the mixing rate will determine the rate at which enzymes bind onto the substrate and the products released from the enzyme. To add to this, the presence of inhibitors and the rate at which they bind onto the enzymes will further alter the kinetics of the digestion. For these reasons, fitting a model to the digestion curve to understand the enzyme kinetic is not always possible.

2.4.3 Factors influencing starch digestion

The digestibility of starch is influenced by many factors and these are summarised in Table 2-5.

Table 2-5 Factors influencing starch digestibility.

Factors	Observations	Explanation	References
Structure of starch granules	Starches with porous surface have higher digestibility.	Pores present on the surface of starches facilitate the penetration of amylase into the molecule, resulting in a faster rate of hydrolysis (endocorrosion). Starches that have smooth surfaces experience surface hydrolysis (exocorrosion).	(Li, Gao, Wang, Jiang, & Huang, 2010; Singh *et al.*, 2010)
	Gelatinising starch increases its digestibility.	Disruption of structure during gelatinisation leads to loosening of amylopectin packing, which increases enzyme accessibility to the polymer.	(Lentle & Janssen, 2011)
	Retrograded starch are less digestible.	Association of polymer chains to form helices during retrogradation reduces enzyme accessibility.	(Lentle & Janssen, 2011)
Size of starch granules	Starch digestibility decreases with increasing granule size.	Small granules have larger surface area which increases the chance of amylase hydrolysis.	(Kaur, Singh, McCarthy, & Singh, 2007; Noda *et al.*, 2008)
Amylose/ amylopectin ratio	Starches with high amylose content have lower digestibility.	Amylose is present as helices, held together by hydrogen bonds, making them less available for amylolytic attack.	(Hu, Zhao, Duan, Linlin, & Wu, 2004; Singh *et al.*, 2010)
Food matrix	Viscous matrix results in reduced starch digestibility.	High viscosity inhibits peristalsis propulsion and mixing. This reduces substrate accessibility to the enzyme.	(Singh *et al.*, 2010)
Presence of other materials	The presence of certain hydrocolloid reduces starch digestion.	Various mechanisms (discussed in section 2.4.4)	(Sasaki & Kohyama, 2011, 2012; Singh *et al.*, 2010; Slaughter, Ellis, Jackson, & Butterworth, 2002)

2.4.4 Digestion of starch in the presence of NSP

The effectiveness of NSP in altering starch digestibility varies depending on the type of NSP and starches. While some NSPs such as gum arabic (Brennan, Suter, Luethi, Matia-Merino, & Qvortrup, 2008) can increase the rate of starch digestion, most have been shown to reduce it. These polysaccharides include guar gum (Brennan, Blake, Ellis, & Schofield, 1996; Brennan *et al.*, 2008; Brennan & Tudorica, 2008; Dartois, Singh, Kaur, & Singh, 2010; Slaughter *et al.*, 2002), fenugreek gum (Hannan *et al.*, 2007; Madar & Shomer, 1990), locust bean gum (Brennan *et al.*, 2008; Brennan & Tudorica, 2008), xanthan gum (Brennan & Tudorica, 2008; Sasaki & Kohyama, 2012) and agar (Sasaki & Kohyama, 2011). The mechanisms by which these NSPs reduce starch digestibility is unclear. However, several mechanisms have been proposed to explain this:

1. NSPs increase digesta viscosity, affecting the mass transfer of sugar and enzyme molecules (Bordoloi, Singh, & Kaur, 2012; Dartois *et al.*, 2010).
2. NSPs increase the viscosity and/or strength of starch gel, consequently decreasing the ability of the enzymes to penetrate into the gel and products to be released from the gel (Brennan *et al.*, 2008; Singh *et al.*, 2010).
3. NSPs form physical barriers by associating with granule surfaces, protecting them from hydrolysis (Brennan, Kuri, & Tudorica, 2004; Dartois *et al.*, 2010; Gonera & Cornillon, 2002).
4. NSPs act as inhibitors by binding onto enzymes to prevent them from hydrolysing starch (Slaughter *et al.*, 2002; Wang, Yang, & Wei, 2010).

The increase in the viscosity of the system is one of the most common mechanism that has been used to explain the decrease in starch digestibility in the presence of an NSP. Since many NSPs are viscous, it is common that this effect cannot be discerned from other effects such as the encapsulation of starch granules by NSP or the ability of an NSP to inhibit amylolytic enzyme. Unlike most NSPs, the polysaccharide of *Mesona chinensis* is not viscous and, therefore, digesta viscosity would have minimal effect on the digestibility of starch in the presence of MCP. The digestibility of starch-MCP gels has never been studied. However, it has been shown that the consumption of 1 g of MC extract following a high carbohydrate meal resulted in a reduction in the postprandial rise in blood glucose: Δ plasma glucose after 30 minutes = ~+30 mg/dL vs ~+20 mg/dL in the absence and presence of MC extract respectively (Chusak, Thilavech, & Adisakwattana, 2014). This provides a strong basis to study the digestibility of starch gels containing MCP as it could potentially be a novel ingredient to formulate starchy food product with low glycaemic loads.

2.5 Gaps in the literature

Two major research groups from China and Taiwan have studied the interaction between starches and MCP. The outcome of the studies provided good insights into the impact that MCP has on starch gelatinisation properties. However, the gaps in the present literature are clear; and these are listed as follow:

1. While a mechanism of interaction involving MCP and amylose has been proposed (Lai & Chao, 2000b), the existence of molecular interaction has never been proven. Furthermore, SEM images of the gel microstructures to prove the interaction between amylose and MCP are somewhat debatable and, therefore, there is no direct evidence of amylose involvement in the interaction.
2. There is a lack of in depth discussion of the early stages of starch gelatinisation in the presence of MCP, which show consistent appearance of a transient peak in the pasting curve of starches containing MCP at the onset of gelatinisation—this has never been pointed out or discussed. While there is no mention of this transient peak in the literature, the fact that only MCP is known to cause the appearance of this peak when starch is gelatinised would suggest its significance and importance in the gelatinisation process.
3. Several studies on the effect of MCP on starch paste viscosities have been reported. However, both the starch and MCP concentrations used were relatively low (forming paste), with no systematic study done at higher starch concentrations, where stronger gels can be formed.
4. There is a lack of understanding on the textural properties of the gels since most of the studies focused on the viscosity of the paste. Given the fact that MCP is typically used to make a gel dessert that is set on cooling, it is logical to put more emphasis on the textural properties of the set gels rather than the paste. Therefore, there is a need to analyse the formation of these gels on cooling as the rheological properties of starch-MCP gels are important for understanding the interactions in the mixed systems.
5. Despite being an anionic polysaccharide, the effect of pH on the properties of starch-MCP gels has never been studied.
6. The effect of starch type on the gel properties is not completely understood due to the differences in the concentrations of starch and MCP used in different publications. Hence, there is a need to develop a systematic study where starch and MCP quantities can be varied to observe the impact on the properties of starch-MCP gels.
7. Whereas, the effect of additional salt on the viscosity of starch and MCP gels has previously been reported, there is a lack of impetus on the salt that is present naturally in the MC extract and in particular, calcium. It is known that calcium is involved in the gelation of many

polysaccharides such as low methoxy pectin and alginate. Therefore, it is probable that the calcium ions naturally present in MC extract have a role in the gelation of starch-MCP gels.

8. In terms of the starch-MCP gels, there has been no study carried out on their retrogradation properties, which is one of the most important factors in determining the acceptability of starch based products.
9. The digestibility of starch-MCP gels is unknown, despite the gels being widely consumed. The extract itself has been shown to reduce postprandial rise in blood glucose after a high carbohydrate meal (Chusak *et al.*, 2014). Therefore, it is likely that starch gels containing MCP may have reduced digestibility. Whether this would be due to changes in the physical properties of the gel (increased strength), the interaction between amylose and MCP or the ability of the extract to inhibit enzymes, the mechanisms associated with decreasing/increasing the digestibility of the gels need to be explored.

With all of the above, it is clear from the literature review that a systematic study on the effect of starch type, starch and MCP concentrations, salt and pH on the rheological and textural properties of the mixed gel, as well as the retrogradation, microstructure and digestibility of these gels is needed to further understand the polymer interactions.

Chapter 3 Experimental techniques

3.1 Gel properties

Techniques such as rheometry, texture analysis, microscopy, DSC and nuclear magnetic resonance spectroscopy (NMR) are commonly used to study starch-NSP interactions because they provide useful data on the gelatinisation process and properties of the resultant starch-NSP gels. Individually, these techniques do not directly show the presence of hydrogen bonds, electrostatic bonds or hydrophobic interactions, which makes interpreting starch-NSP interactions rather difficult. However, a combination of these techniques can be used to provide sufficient evidence to infer starch polymer and NSP association. Hence, reports on starch-NSP associations are normally inexplicit and deduced based on results from several experimental techniques that are described in this chapter.

3.1.1 Rheology

The viscosity of a substance can be defined as its resistance to flow. In a dilute starch suspension, this is determined largely by the volume fraction of the starch granules. As the concentration of starch is increased, a critical concentration C* is reached, whereby the viscosity of the solution becomes dependent on the rigidity of the granules rather than its volume fraction (Steeneken, 1989). The changes in the viscosity of a starch suspension during gelatinisation and gelation are typically measured using the Rapid-Visco Analyser (RVA), whereby the viscosity of the paste during heating and cooling is measured under constant rotational shear. The viscosity (η) of a fluid is defined as its resistance to flow and is directly related to the shear stress and shear rate. The two parameters are typically explained using the two-plates-model (Figure 3-1). Shear stress (τ) is defined using the following equation:

$$\tau = \frac{F}{A}$$

Where F = force and A = area. The movement of fluid in between the two plates occurs when the upper plate is set in motion with the bottom plate remaining stationary. This generates a resulting velocity (V) over the distance between the two plates (h) and this is defined as the shear rate ($\dot{\gamma}$) with a dimension of reciprocal time (s^{-1}). Therefore, the viscosity (Pa.s) of a solution is defined as the ratio of shear stress to shear rate using the following equation:

$$\eta = \frac{\tau}{\dot{\gamma}}$$

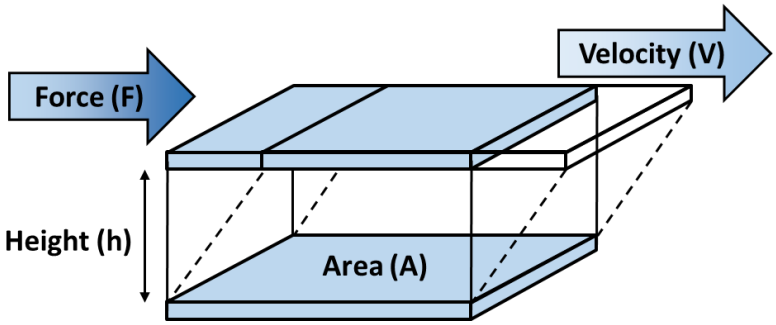

Figure 3-1 Two-plates-model showing the flow velocity of a fluid as a result of applied force (Mezger, 2011).

While the viscosity of a starch paste can be measured during gelatinisation, constant shearing of the paste during cooling disrupts network formation and, hence, viscosity measurements during this period do not accurately represent the gelation process. The gelation process is best characterised under minimum disruption, which can be achieved under oscillation within the linear viscoelastic region of a gel (Mandala, 2012). Viscoelastic materials, like starch gels, possess both viscous and elastic behaviour, with the latter characterising its ability to recover its original shape upon the removal of stress. An ideal solid material behaves in accordance with Hooke's law, whereby the strain is directly proportional to shear stress. On the other hand, an ideal fluid obeys the Newton's law, whereby shear rate is directly proportional to shear stress.

Viscoelasticity is typically measured using oscillatory techniques, whereby small sinusoidal small-amplitude deformations are applied to the material to measure their storage (*G'*) and loss (*G''*) moduli. The storage modulus represents the elastic component of the material and it indicates the amount of stored energy. On the other hand, the loss modulus represents the viscous component and it indicates the energy that is being dissipated. The larger value between the two moduli determines the predominant property of the material, for e.g. if *G'* is larger than *G''*, the material has predominantly elastic properties (Miri, 2011). Using the two-plates-model in an oscillatory measurement, a sinusoidal stress at an angular frequency (ω) is applied to the upper plate while the bottom plate remains stationary. This generates a sinusoidal shear strain (γ) that is defined by the following equation:

$$\gamma = \gamma_0 \sin \omega t$$

Where γ_0 = amplitude strain and t = time. Within the linear viscoelastic region, this strain produces an out-of-phase sinusoidal stress response (σ) that is defined by the following equation:

$$\sigma = \sigma_0 \sin(\omega t + \delta)$$

Where σ_0 = shear stress amplitude and δ = phase lag. The application of a sinusoidal strain to an elastic solid and viscous fluid results in a stress response that is 90° in phase and out of phase with the strain respectively (Figure 3-2).

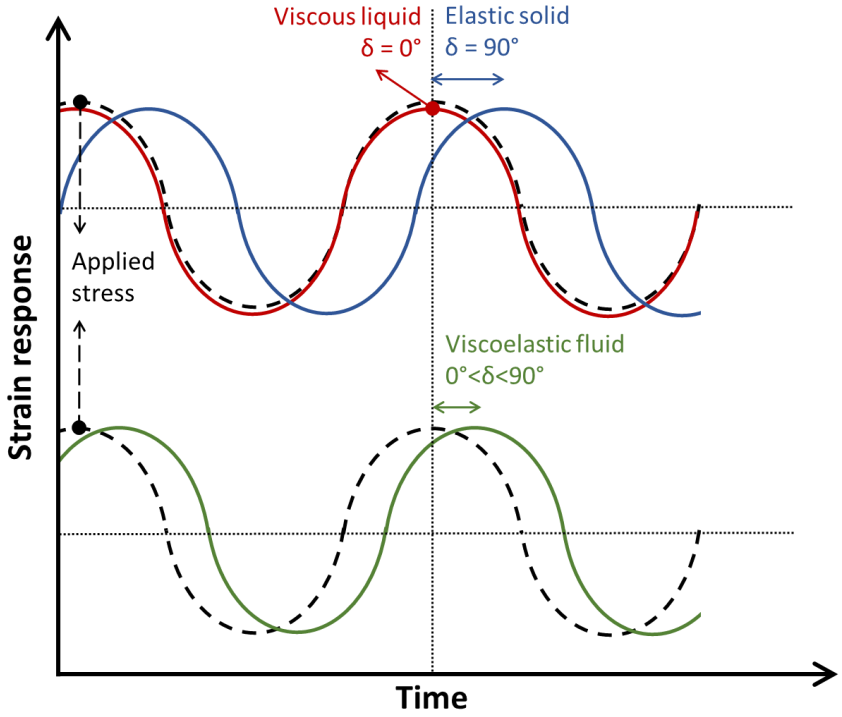

Figure 3-2 Strain response of viscous liquid, elastic solid and viscoelastic material under applied stress (Bourne, 2002).

Hence, when applied to a viscoelastic material, depending on their elastic and viscous behaviour, the phase lag between stress and strain would lie between 0° and 90°. The storage and loss moduli can, therefore, be quantified using the following equations:

$$G' = \frac{\tau_0 \cos \delta}{\gamma_0}$$

$$G'' = \frac{\tau_0 \sin \delta}{\gamma_0}$$

For these moduli to be determined accurately, it is important that measurements are carried out within the linear viscoelastic region (LVR) (Figure 3-3), whereby the properties of the material are not influenced by the magnitude of stress and deforming strain, or the rate of strain application. Hence, within this region, the strain response is proportional to the applied stress (Miri, 2011). In order to determine the LVR of a sample, a large deformation amplitude sweep test can be carried out, whereby an increasing deformation is applied to a sample (by increasing the shear stress) and the range of strain at which the moduli remains constant is regarded as the LVR. Once the deformation goes

beyond this range, the response is no longer linear and the shear moduli decrease, normally indicating a breakage in the structure of the sample. Other oscillatory tests such as the frequency sweep test are also useful in characterising the properties of a starch gel. In a frequency sweep test, a sample is subjected to increasing frequencies under a constant amplitude strain while its dynamic moduli are measured. The *G'* of a solid material is typically independent of frequency, while for a liquid like material, changes in the frequency influences its *G'* (TA Instruments, 2015).

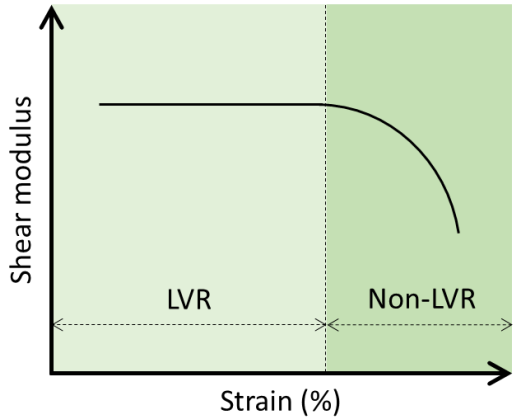

Figure 3-3 Linear and non-linear viscoelastic regions of a material with increasing strain.

However, since starch sediments in water under oscillation, there is a need to still apply constant rotational shear during gelatinisation to ensure that all starch granules are in suspension. A combination of rotational and oscillatory shearing (Figure 3-4) was adopted in this study in order to best characterise the properties of starch during gelatinisation and gelation. This was done by using a controlled-stress rheometer (MCR302, Anton Paar Physica, Graz, Austria) fitted with a starch cell (C-ETD160/ST) and spindle (ST24-2D/2V/2V-30) to apply rotational shear to starch suspension during heating, ensuring that all granules do not sediment during gelatinisation. Once the granules have swollen, the viscosity of the suspension was increased, and this prevented granules from sedimenting. The system was then switched to oscillatory mode in the LVR during gelation to allow the gels to set with minimal disruption. The viscosity of the paste is measured during gelatinisation under rotational shearing and the storage and loss moduli measured during gelation under oscillation (Figure 3-4). Several parameters were investigated to identify the ideal conditions for measurements of starch + MCP gels. These include the rotational shear rate during viscosity measurement, the temperature at which the system is switched to oscillation and the holding time of the starch hot paste at 95°C.

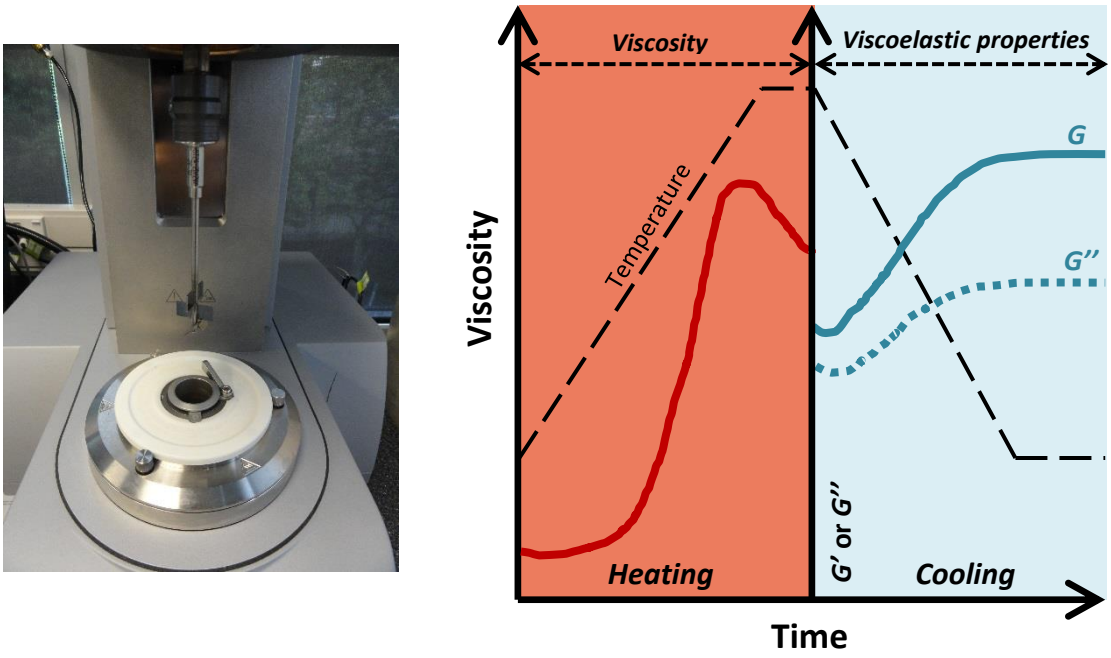

Figure 3-4 Starch cell and spindle in the MCR 302 (left) and typical viscosity and viscoelasticity profile of starch during gelatinisation under rotational shear and gelation under oscillatory shear (right).

3.1.1.1 Rotational shear rate

The rotational shear rate of the rheometer spindle for the initial viscosity measurements were determined by heating 10 % w/w maize starch from 55°C to 85°C at different rotational shear rates before switching it to oscillation (1 % strain and 1 Hz) to check for starch sedimentation. Within the range tested (100/s – 200/s), all shear rates kept the starch granules in suspension during gelatinisation as indicated by results that were superimposed (Figure 3-5). Typically, sedimentation can be visually observed and would result in an increase in the strength of the gel as seen in Figure 3-7 in the next section.

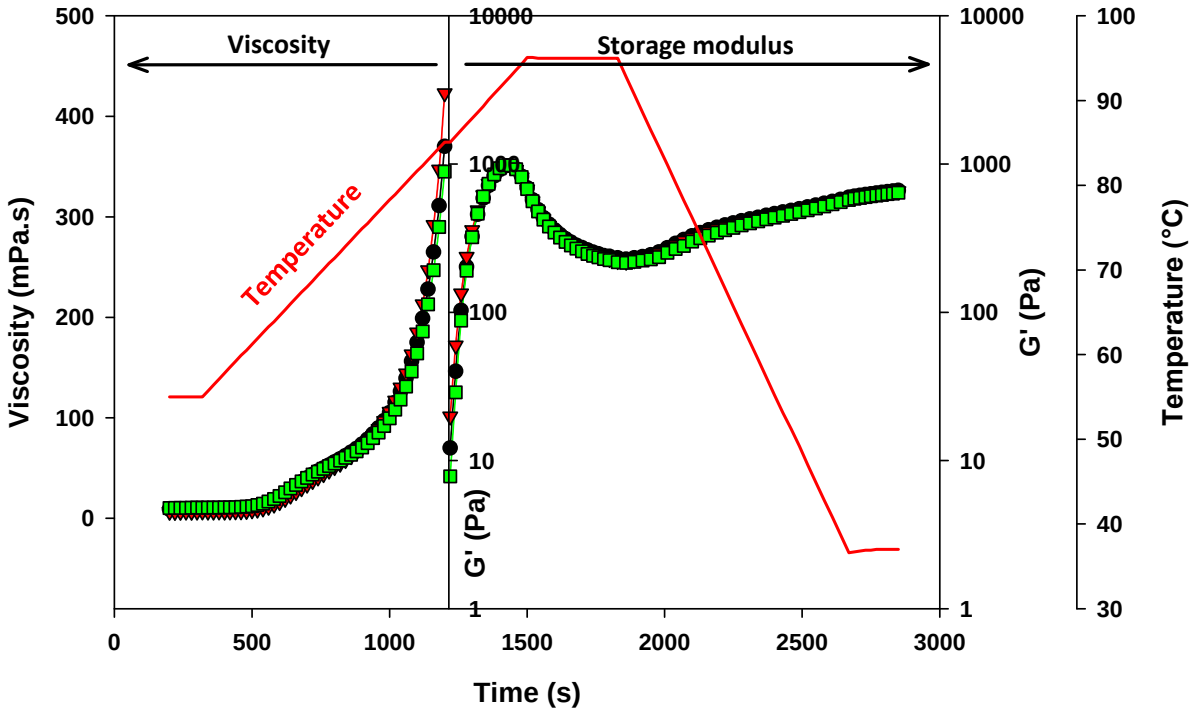

Figure 3-5 Viscosities (left side) of 10 % w/w maize starch gelatinised up to 85°C at a shear rate of 100/s (▼), 160/s (●) and 200/s (■) and the resulting gel strength with further heating and cooling under oscillation (right side).

The effect of shear rate on the pasting properties of 10 % w/w starch was then further explored by carrying out gelatinisation and gelation under complete rotational shear. It was observed that with increasing shear rate, the final viscosity of the paste was decreased (Figure 3-6). At higher temperatures (> 85°C), it is likely that more starch granules and amylose network are being broken up at higher shear rate. Hence, while all of the shear rates tested resulted in no starch granules sedimentation, the lowest shear rate of 100/s was chosen for this study to minimise any damage to the starch granules and any network formation that may occur before the gelation process.

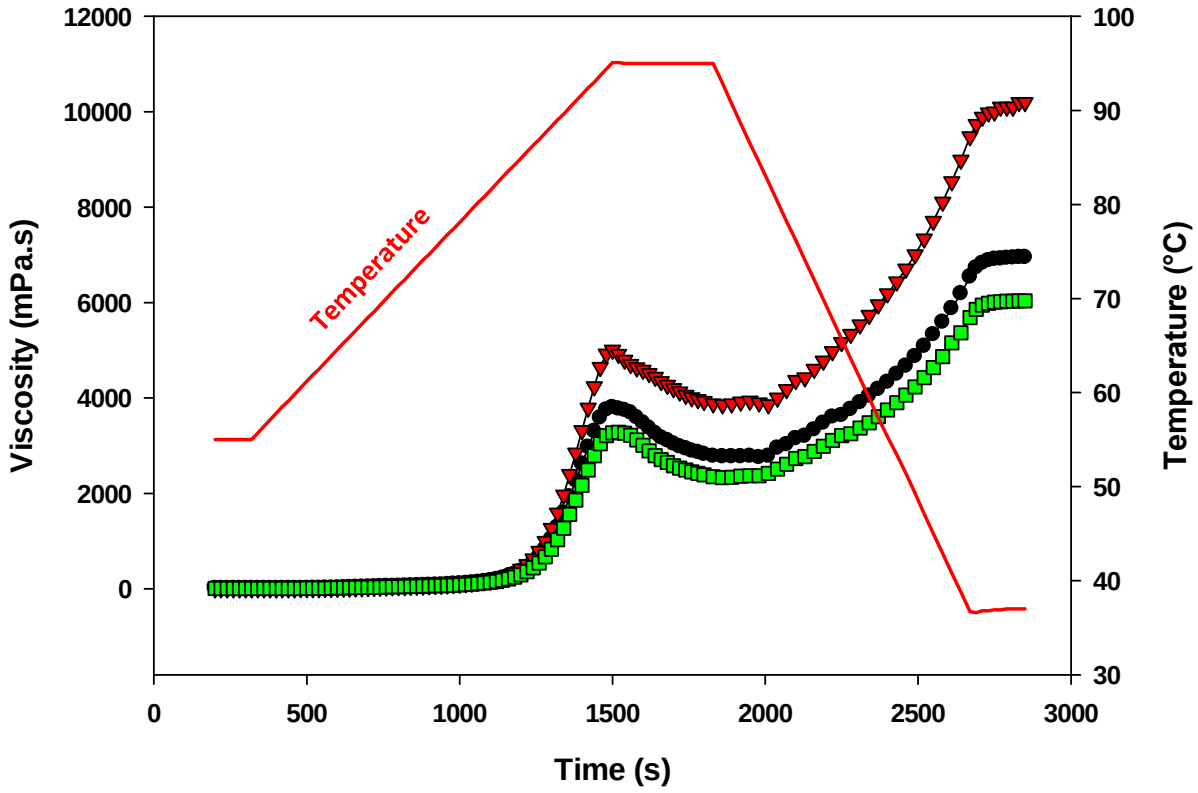

Figure 3-6 Pasting properties of 10 % w/w maize starch at a shear rate of 100/s (▼), 160/s (●) and 200/s (■).

3.1.1.2 Temperature at which the system is switched to oscillation

Following the determination of the ideal shear rate, the temperature at which the system should be switched into oscillation was investigated. The ideal switching temperature was deemed to be one where starch granule sedimentation did not occur, as well as minimal disruption in the network formation resulting from shear. This was done by heating 10 % w/w maize starch suspension under rotational shear from 55°C up to 95°C, holding at 95°C for 5 minutes and then cooling to 37°C. The switch from rotational shear to oscillatory shear took place at different temperatures: 55°C (at the start), 65°C (during gelatinisation), 85°C (just before onset of viscosity increase), 95°C (at peak viscosity) and after 5 minutes at 95°C (end of heating phase). The development of *G'* was then monitored throughout the oscillatory phase (Figure 3-7).

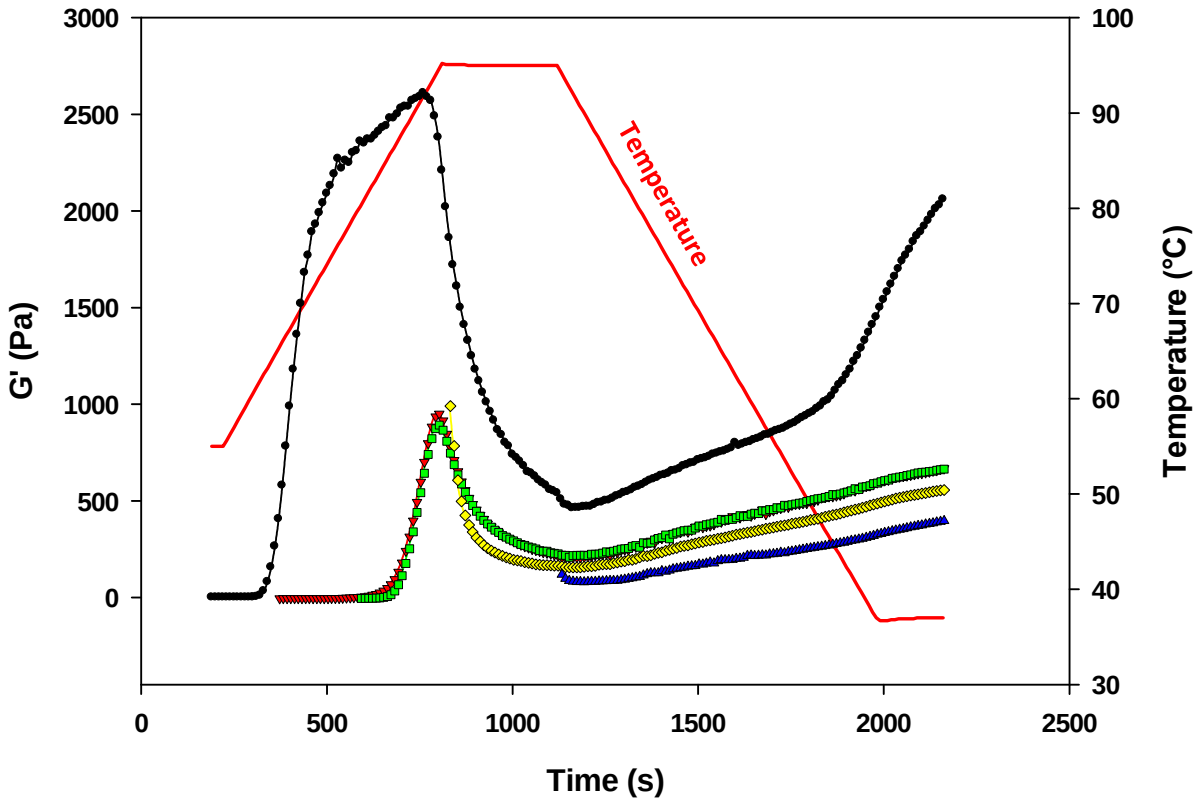

Figure 3-7 Development of storage modulus (*G'*) of 10 % w/w maize starch when switched to oscillation at 55°C (●), 65°C (▼), 80°C (■), 95°C (◆) and after 5 minutes at 95°C (▲).

Starch granules sedimentation was found when rotational shear was switched immediately to oscillatory shear at the beginning of the heating stage (55°C). Apart from visual observation of the gel, this was characterised by a rapid increase in the *G'* of the gel at the early stages of heating as a result of the formation of a layer of concentrated starch granules that gelatinised at the bottom of the cell. Sedimentation was not observed when the system was switched to oscillation at temperatures higher than 65°C. Switching to oscillation after the peak viscosity was reached (95°C) resulted in gels of lower strength. However, switching the system to oscillation before the heating phase ends (after 5 minutes at 95°C) resulted in weaker starch gels. This is demonstrated by subjecting the gels to a compression test. To illustrate the fracture observed after the compression, images of starch gels containing MCP after a single compression is shown in Figure 3-8. The fracturability of these gels was thought to be due to amylose being unevenly distributed in the continuous phase due to a premature switch to oscillation while the polymer is still being leached out. Hence, it was concluded that the best temperature to switch the system to oscillation was after the heating phase has lapsed.

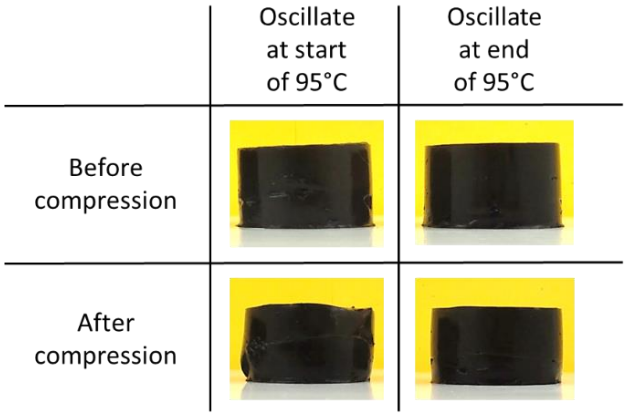

Figure 3-8 The effect of single compression on 8 % w/w maize starch + 2 % w/w MCP gels produced by switching the system to oscillation at 95°C or the end of the heating phase (after 5 minutes at 95°C).

3.1.1.3 Holding time at 95°C

The ideal holding time at 95°C for the starch paste to form a strong gel upon cooling was investigated. This is important because it is hypothesised that amylose plays an important role in the interaction between starch and MCP. Hence, there is a need to hold the paste at a high temperature for a duration that is long enough to allow maximum leaching of amylose. At the same time, prolonged shearing at a high temperature can result in the starch granules breaking, causing the viscosity of the paste to be reduced. Therefore, the optimum holding time at 95°C needs to be determined. A rotational shear rate of 100/s was used (as determined in section 3.1.1.1) to keep the granules in suspension during gelatinisation up to 95°C. The hot paste was then held at the temperature for 1, 5 or 10 minutes under constant rotational shear before the system was switched to oscillation (1 % strain, 1 Hz) at the end of the heating phase (as determined in section 3.1.1.2). The gels produced after cooling were then subjected to a single compression and these are shown in Figure 3-9. It was found that holding the hot paste at 95°C for 1 minute resulted in a gel that fractures upon compression. Prolonged heating at 95°C strengthened these gels and no fractures were identified after 5 and 10 minutes of holding time. However, it was found that increasing the holding time resulted in a significant decrease in the gel hardness—18.1 ± 0.4 N after 5 minutes vs 16.2 ± 0.8 N after 10 minutes ($p < 0.05$). Therefore, 5 minutes was selected as an adequate holding time at 95°C to produce starch + MCP gels of greatest strength.

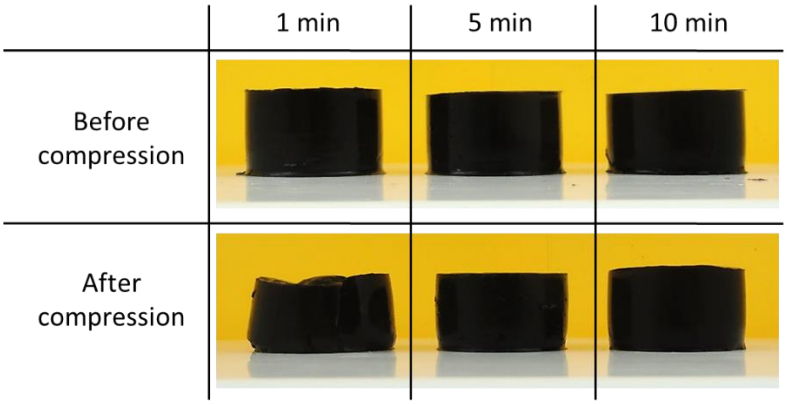

Figure 3-9 The effect of single compression on 8 % w/w maize starch + 2 % w/w MCP gels produced by switching the system to oscillation after holding the hot paste at 95°C for 1, 5 or 10 mins.

3.1.2 Texture

The textural properties of a food product are related to the deformation, disintegration, and flow of the food product under stress. These give rise to a group of physical characteristics that are influenced by the structure of the food (Bourne, 2002). Uniaxial compression is one of the most common method that is used to measure the hardness of a solid food product. This is done by applying force using a plate that has larger diameter than the food specimen. The compression test is the basis of the Texture Profile Analysis (TPA) test, whereby the food material is compressed twice using a destructive force to simulate biting. Several parameters are then derived from the TPA curve (Figure 3-10) generated from a universal testing machine such as the TA.XT2 Texture analyser and the Instron. These can be used to define properties such as hardness, springiness, stringiness, fracturability, cohesiveness (Area 2/Area 1), guminess (hardness × cohesiveness) and chewiness (guminess × springiness).

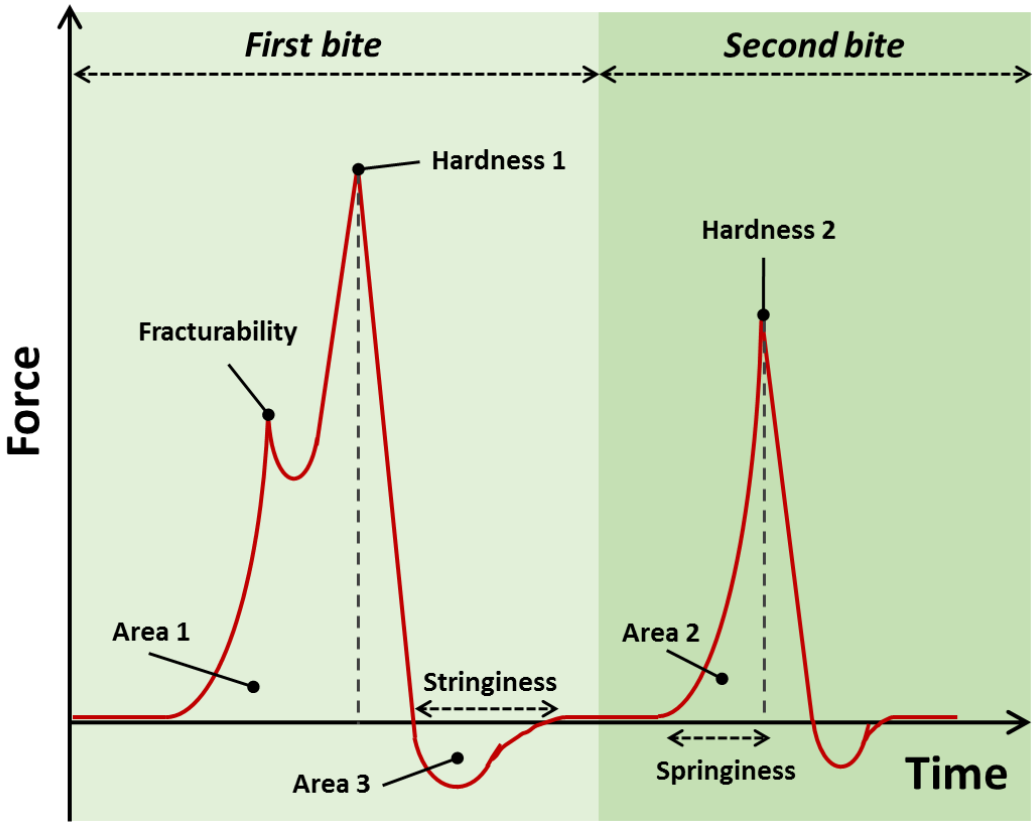

Figure 3-10 A typical Texture Profile Analysis curve.

In contrast to measurements done using small deformation oscillatory shear in the rheometer, the compressions in TPA tests are destructive (90 % deformation) and, hence, parameters obtained from each technique, such as "gel strength" from oscillation or "gel hardness" from TPA do not always necessarily correlate. While the rheological measurements (*G'* and *G''*) allows us to study the formation of bonds during starch gelation, compression tests give results that allow a better understanding of consumer's perception of the textural properties of a gel. The textural parameters obtained from a TPA curve have been highly correlated with sensory evaluations (Szczesniak, Brandt, & Friedman, 1963), making it a reliable method to predict a perceived product's textural properties when consumed. TPA tests were initially performed in this study. However, the results showed that the hardness was the only parameter that differed among gels and, hence, a single uniaxial compression test was adopted to measure the hardness of these gels.

3.1.3 Differential Scanning Calorimeter (DSC)

The DSC measures the heat flow of a material as it is being heated or cooled, which is indicative of transitions occurring in the sample such as starch gelatinisation and polymer melting. The principle of the method is based on the energy released/absorbed by a material during an endothermic/exothermic reaction as a result of bond breaking or formation, which changes the

temperature of the sample. The temperatures of the sample and reference material are maintained at the same level by the instrument and any difference in the amount of power required to maintain this temperature is recorded (Brown, 2001). The DSC is widely used in the characterisation of starch gelatinisation and retrogradation. In terms of gelatinisation properties, the parameters of interest that are derived using this method include the onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) and the heat enthalpy of gelatinisation ($\triangle H_1$) (Figure 3-11).

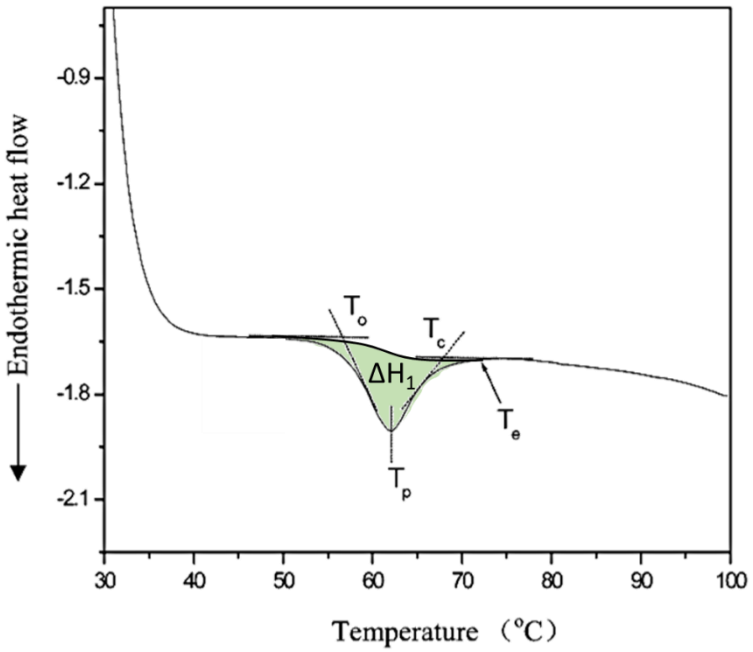

Figure 3-11 A typical DSC thermogram of wheat starch in water (water to starch ratio = 2:1) (Adopted from Wang and Copeland (2013b)).

The enthalpy of a material corresponds to the energy required to heat the material up to a specified temperature (Gabbott, 2008). During gelatinisation, this corresponds to the amount of energy required to disorder the amylopectin crystalline structure in the starch granules (Waigh *et al.*, 2000). During retrogradation, the amylopectin molecules reorder themselves and, hence, reheating of the retrograded starch in the DSC results in the formation of an endotherm (at about 40°C) that corresponds to the melting of the reordered amylopectin ($\triangle H_2$). The ratio of $\triangle H_2$ to $\triangle H_1$ gives a good indication of the extent of starch retrogradation.

3.1.4 Gel syneresis

Together with thermal analysis, the retrogradation properties of starch are typically characterised by the amount of water that is exuded from the gel following storage for a period of time. Starch gels have high water holding capacity that is progressively loss during retrogradation. The reordering of amylopectin and amylose results in the gel contracting, forcing water to be expelled from the network

(Vaclavik & Christian, 2008). The loss of water from starch gel following storage is known as syneresis and is a good indicator of the extent of starch retrogradation and the loss of its water holding capacity (Eliasson, 2017). Syneresis can be accelerated by repeated freeze thawing of the sample to induce phase separation of the starch polymers and water (in the form of ice). Upon thawing, the water is easily separated from the network and is quantified as gel syneresis. Repeated freeze thawing of starch gels induces phase separation promptly and, therefore, is the chosen technique in this thesis (in combination with DSC) to study starch gel retrogradation in the presence of MCP. The syneresis setup is shown in Figure 3-12 (Charoenrein, Tatirat, & Muadklay, 2008).

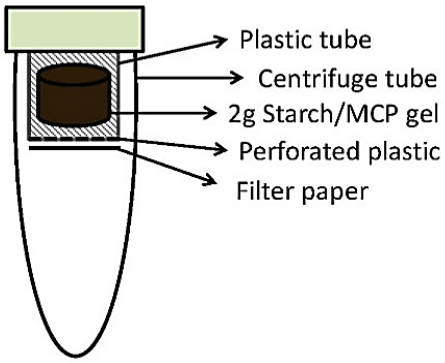

Figure 3-12 Setup for gel syneresis (adapted from Charoenrein *et al.* (2008)).

3.2 Microscopy

3.2.1 Hot stage microscopy

In starch analysis, hot stage microscopes are mainly used to study the loss of starch granule birefringence during gelatinisation to identify the gelatinisation temperature of the starch (Yeh & Li, 1996). The instrumentation of the hot stage microscope is composed of a light source, objective lens (and/or polarised lens) and a heating stage. Dilute starch suspensions are typically placed within a ring of high viscosity oil on a microscope slide and a cover slip is placed on top of the sample. The oil acts as a barrier to prevent steam from fogging up the glass plate (Sablani, 2009). The hot stage microscope has also been used to monitor the size of starch granule during gelatinisation in order to determine its swelling rate (Bean & Yamazaki, 1978). This is the primary use of the instrument in this thesis. The swelling of starch granules in the absence and presence of MCP was recorded and images were extracted out of these recordings at different temperatures, from which granule size was manually measured.

3.2.2 Scanning Electron Microscopy (SEM)

The microstructure of gels in this thesis was studied using SEM. The instrumentation of a typical SEM is composed of an electron gun, two condenser lenses, an objective lens, an electron detection system and deflectors (Figure 3-13). These components are packed in a column and operates in a vacuum. Electrons are accelerated by the electron gun, which is demagnified by the three lenses to obtain a narrow electron probe diameter of 1 – 10 nm on the sample's surface. Upon contact with the sample, the electrons are scattered based on depth to produce secondary electrons (close to the surface) and backscattered electrons (from deeper levels), which are used to generate an image (Khursheed, 2011).

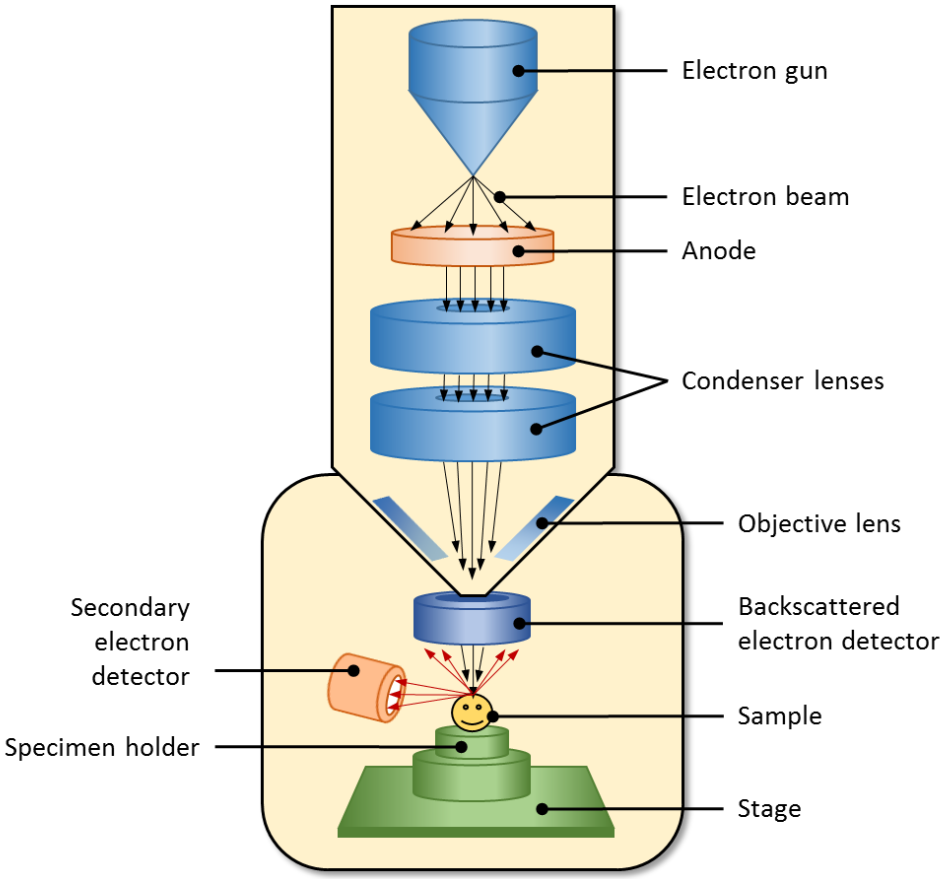

Figure 3-13 Components of a typical SEM.

SEM is a powerful imaging tool that produces high resolution images in a short time. However, it suffers from disadvantages that include high cost and the need for experienced user as sample preparations may lead to the formation of artefacts. The technique has been widely used to characterise the structure of starch granules before and after gelatinisation (Jane, Kasemsuwan, Leas, Zobel, & Robyt, 1994; Liu & Zhao, 1990). In recent years, with more research being done on starch and hydrocolloids mixed system, SEM is being increasingly used to characterise the network that is formed by the mixture following gelatinisation and retrogradation (Chaisawang & Suphantharika, 2006;

Charoenrein, Tatirat, Rengsutthi, & Thongngam, 2011; Lee, Baek, Cha, Park, & Lim, 2002). Pre-treatment of starch gels for imaging under the SEM typically involves a freeze-drying step, whereby water is removed from the sample rapidly to retain the integrity of the gel structure. This process leaves holes within the gel matrix in places where the water was located prior to freeze drying. SEM micrographs of starch gels in the absence and presence of guar and xanthan gum are shown in Figure 3-14.

Figure 3-14 SEM images of sweet potato starch gels in the absence (left) and presence of 0.6 % w/w guar gum (middle) or 0.6 % w/w xanthan gum (right) after three freeze-thaw cycles (Lee et al., 2002).

3.2.3 Confocal scanning laser microscopy (CSLM)

The principle of the confocal scanning laser microscope (CSLM) is based on the elimination of reflected or fluorescent light that is emitted from planes out of focus. This is achieved by positioning pinholes to illuminate a sample at one specific point and eliminate out of focus light, preventing it from reaching the detector. A key feature of confocal microscopy is its ability to image a single focal plane in a sample with an arbitrary thickness. The objective lens collects fluorescent light from the sample and focuses it into a small pinhole, eliminating the out of focus light (Figure 3-15). This ability to focus light into a small pinhole gives an excellent resolution within the focal plane. A 2D image of a sample is obtained through a point by point scanning of the sample in the x- and y-direction of the focal plane. Moving stepwise through the sample gives a 3D image. CSLM relies on the specific fluorescence of dyes/stains used. Therefore, selection of suitable dyes to bind onto specific components in a sample is important. CSLM usually has up to three photomultipliers operating at different wavelengths to detect light emitted from different fluorescent probes. A set of lasers at different wavelengths is used to excite the fluorescent dyes and images of a sample at different wavelengths can be captured separately. These images can then be superimposed to produce one image in which different components are differentiated by dyes excited at different wavelengths (van de Velde, Weinbreck, Edelman, van der Linden, & Tromp, 2003).

Figure 3-15 Schematic diagram of how images are produced in a CSLM (van de Velde *et al.*, 2003).

CSLM was explored initially in this thesis as a technique to identify the location of MCP relative to the starch granules following gelatinisation. FITC, rhodamine B and safranin (van de Velde *et al.*, 2003) were all individually used to stain starch granules following gelatinisation in the presence and absence of MCP. Good fluorescence was obtained using rhodamine B and safranin, whereas granules stained with FITC had poor fluorescence. However, it was found that the use of safranin or rhodamine B resulted in MCP aggregating (Figure 3-16). In order to obtain a good visualisation of these aggregates, both confocal microscopy and differential interference contrast (DIC) microscopy were used to capture images of the aggregates and the starch granules. The black and white images from DIC and the red and black images from CSLM were then superimposed to obtain black, white and red images that represent MCP aggregates, water and starch granules respectively (Figure 3-16 right). These aggregates were found to surround the starch granules but it could not be concluded whether MCP was coating the granules following gelatinisation or there were interactions between the granules and the aggregates. While the desired outcome of the technique was not achieved, the CSLM provided a validation of the findings in the light microscope and SEM—a delay in granular swelling and starch granule aggregation in the presence of MCP. In order to fully utilise the CSLM, a dye that binds specifically onto MCP needs to be identified and this could be part of an integral work for the future.

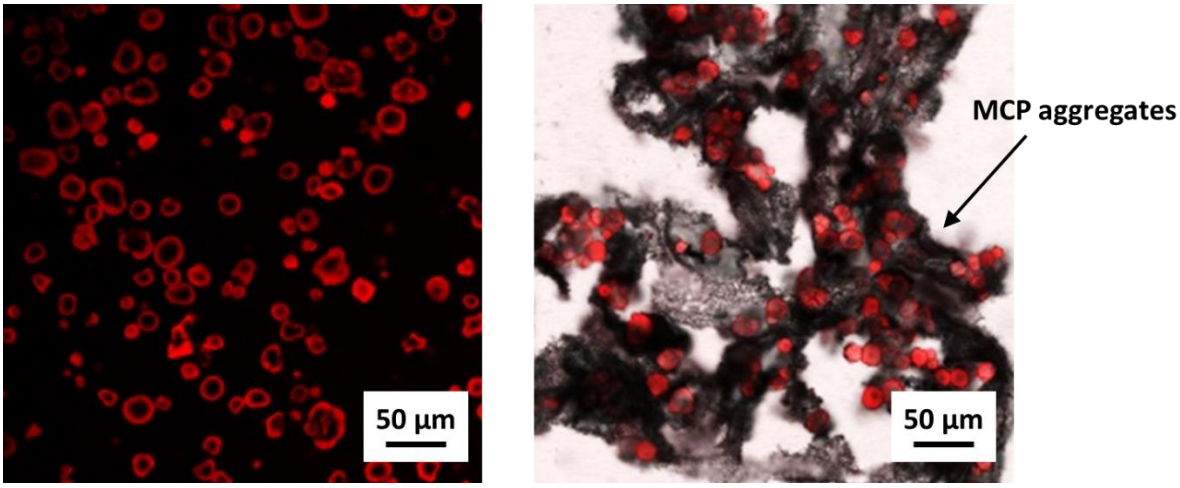

Figure 3-16 Confocal images of maize starch gelatinised to 67°C stained with safranin in the absence (left) and presence of MCP (right), with MCP aggregates.

3.3 Light scattering

Particle size measurement of starch granules was carried out using a Malvern Mastersizer Hydro2000MU. The instrumentation of the Mastersizer is composed of a laser beam light source, a measurement cell attached to a dispersion unit and fixed-angle detectors. The technique is based on the principles of light scattering, whereby a beam of light passing through the sample is scattered by particles in a certain pattern, which is then measured by the detectors. This pattern is then used to define the granule particle size in terms of equivalent spheres, based on the particle volume.

The size of the particles are typically expressed as the volume mean diameter or the surface area mean diameter. The volume mean diameter D (4,3) monitors changes in the droplet size distribution diversity, whereas the surface area mean diameter D (3,2) quantifies the surface area of the particles in equivalent spheres. The volume mean diameter is defined using the following equation:

$$D(4,3) = \left[\frac{\sum_i V_i d_i^4}{\sum_i V_i d_i^3}\right]$$

Where V_i = droplets number of diameter d_i (Malvern Instrument, 2007). In this thesis, the volume mean diameter is the parameter of interest due to its sensitivity to changes in the size distribution with the presence of bigger particles.

In the Mastersizer, the particle size distribution is calculated using the Mie scattering theory, based on the scattering patterns of the particle. The Mie theory assumes two things. The first is that only a single particle is scattering the light and, therefore, dilution of sample is always necessary. The second is that all particles have a varying degree of opacity and, hence, they scatter light with unequal

efficiencies. This opposes the assumptions of earlier diffraction techniques based on the Fraunhofer theory, which assumes that particles are significantly larger than the wavelength of the laser and that all particles are opaque and, hence do not scatter light. Therefore, in Mie theory, the scattering of light by a particle is influenced not only by its size but also, by its refractive index. The refractive index ratio of a particle relative its surrounding medium determines the amount of light that passes through (no scattering) or gets reflected by the sample. A refractive index ratio of 1 indicates no scattering while a complete reflection occurs as the ratio approaches infinity (Chowdury, VanGelder, Lawler, & Moran, 1999). For measurements to be made based on the Mie theory, only a single particle type should exist in the dispersion and the refractive indices of the particle and the surrounding medium, as well as the absorption of transmitted light by the particles need to be known (Dodds, 2013). In this study, the refractive index used for water and starch were 1.31 and 1.52 respectively and this was based on published literature (Wilson, Bechtel, Todd, & Seib, 2006). The obscuration level is maintained at 10 % and this represents the amount of light that is lost upon the introduction of the sample to the laser beam (Malvern Instrument, 2007). The differences in the assumptions of the two theories gives Mie theory a higher accuracy of measuring particles size over a large range of size.

3.4 Solid state NMR

Interactions between starch and non-starch polysaccharides are usually deduced from rheological data and thermal analysis. Nuclear Magnetic Resonance spectroscopy (NMR) offers several methods of detecting interactions between molecules and the effect these interactions have on the molecular motion of each molecule (Newman & Hemmingson, 1998; Zhang, Do, Hoobin, & Burgar, 2006). Nuclear Magnetic Resonance spectroscopy (NMR) is a technique that is most commonly used to structure unknown molecules. The principle of the techniques is based on the nuclear spin of an atom that can be transferred to a higher energy level when a magnetic field is applied. In NMR, fluctuations in the magnetic field of the excited nucleus occur as a result of molecular motion and this allows the molecule to return to its equilibrium state (Apperley, Harris, & Hodgkinson, 2012). This process is known as magnetic relaxation and it involves two mechanisms: Spin-spin relaxation (T_2) and spin-lattice relaxation (T_1). The two mechanisms describe the direction of the decay of the excited magnetic spin with the former corresponding to a decay perpendicular to the applied field, and the latter in the direction of the magnetic field. If T_2 and T_1 relaxation are measured in the right conditions, then any variation in their relaxation constants can be directly related to changes in the molecular motion of the molecules, which may or may not be caused by interactions.

In solid-state proton NMR, the spectra obtained are typically broad when compared to liquid-state NMR. This is a result of inequivalent nuclear site within a powdered sample, which causes patterns to overlap (chemical anisotropy). In liquid state NMR, chemical anisotropy is usually averaged out by molecular motion. Additionally, two magnetic dipoles may interact to cause dipole coupling, which further broadens the NMR spectrum. This makes it difficult to distinguish the individual components of a semi-solid such as a gel (starch and polysaccharides) from the water. In order to obtain a high-resolution spectrum using solid state NMR, Magic-Angle Spinning (MAS) is used, whereby the sample is spun at an angle of 54.74° to reduce the effect of chemical anisotropy and dipole coupling. High spectral resolution can, therefore, be obtained. For semi-solids, this method is defined as high resolution MAS (HR-MAS) and is the chosen method in this study.

The interaction between two polymers can be identified using an NMR technique known as proton ROESY, which allows the determination of spatial proximity (up to less than 5 Å) between two polymers. 1D-NMR spectra obtained from this technique contains diagonal peaks, which are then phased to obtain 2D-ROESY spectra containing cross peaks. These cross peaks indicate signals arising from protons that are close in space, typically indicative of interactions. An example of a 1D and 2D-NMR spectra containing diagonal and cross peaks respectively is shown in Figure 3-17. The interaction between two polymers can then be further investigated by looking at their spatial heterogeneity at different scales (1 micron to 1 Å) by measuring the molecular mobility of their protons or carbons. Homogeneity is typically observed for interacting polymers and, hence their molecular mobility cannot be distinguished at specific scales.

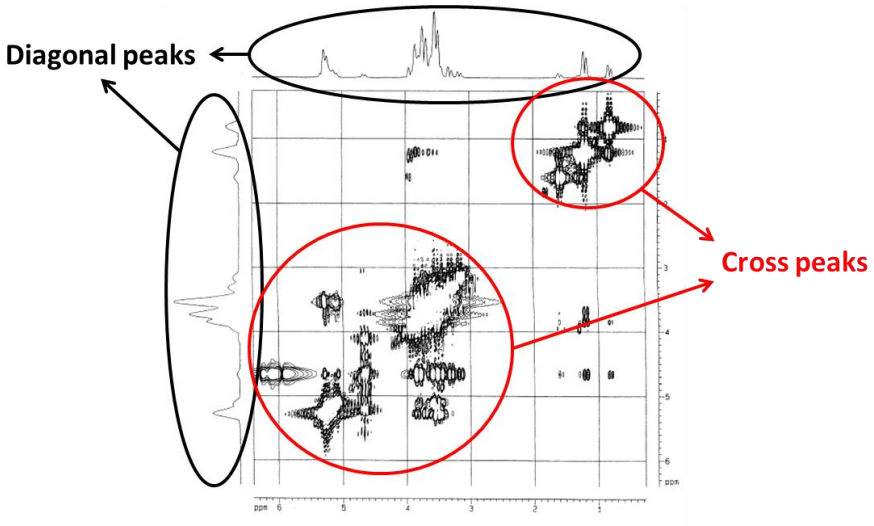

Figure 3-17 Proton ROESY spectra of a maltodextrin-SDS mixture (Wangsakan, McClements, Chinachoti, & Dickinson, 2004).

3.5 *In vitro* digestion

The digestibility of many foods is often predicted using *in vitro* rather than *in vivo* studies. The reason being that *in vitro* techniques are relatively quick and less costly. Nevertheless, it is inevitable that an *in vitro* method will fail to accurately demonstrate the body's response to an ingested food for its lack of complexity as compared to the human digestive system. However, the aim of an *in vitro* digestion method is to provide relative comparisons between foods and obtain results that are as close as possible to an *in vivo* system, provided the compromises that need to be made. Apart from a consensus that digestion is carried out at 37°C, there are great variations between *in vitro* digestion models, with enzyme source and concentrations being the factors that are widely varied. The number of digestive phases (oral, gastric and small intestine), pH, digestate residence time, shearing parameters and sampling method are among the other parameters that differ between studies.

Three types of *in vitro* digestion models exist: static, semi-dynamic and dynamic. The difference among these models is in the simulation of physiological peristaltic movements, rate at which the pH of the digestate is adjusted, the rate at which enzymes are added, and the rate of which substrates are removed. In the human body, changes in the pH of the digestate and the enzyme released occur over a period of time, and the same applies to the removal of digestate from the gut into the small intestine and the substrate into the blood. In order to simulate this *in vitro*, dynamic models (Gouseti *et al.*, 2014; Villemejane *et al.*, 2016) have been developed whereby pH adjustments, enzymes addition, gastric emptying and substrate removal are monitored and carried out gradually so as to achieve a condition as similar as possible to the human gut system. In a dynamic model, peristaltic movements of the gut are also often simulated using equipment that imitate the contraction of the gut. While dynamic models are ideal, the setup is often complicated, time consuming and not feasible for many. Compromises are normally made, and semi-dynamic models are used in place of dynamic models. In a semi-dynamic model, peristaltic movements and digestate transit time are usually neglected, but gradual pH adjustments and enzymes addition are typically maintained. However, most studies have chosen to use static models as they are less time consuming and the setup is typically simple. In a static model, while some form of shearing is typically applied, physiological peristaltic movements are not simulated. The adjustment of pH and enzyme addition occurs at the start of a digestive phase (oral, gastric or intestinal), rather than gradually. Hence the ideal pH for maximum enzyme activity is achieved immediately and enzymes are typically concentrated at the start of the phase.

The lack of consistency between methods makes it difficult for digestion results to be compared and validated (Woolnough, Monro, Brennan, & Bird, 2008) and, hence, the need for standardisation. This

became the drive for the development of a standard digestion method known as the Infogest model by Minekus *et al.* (2014). An overview of the static model is shown in Figure 3-18.

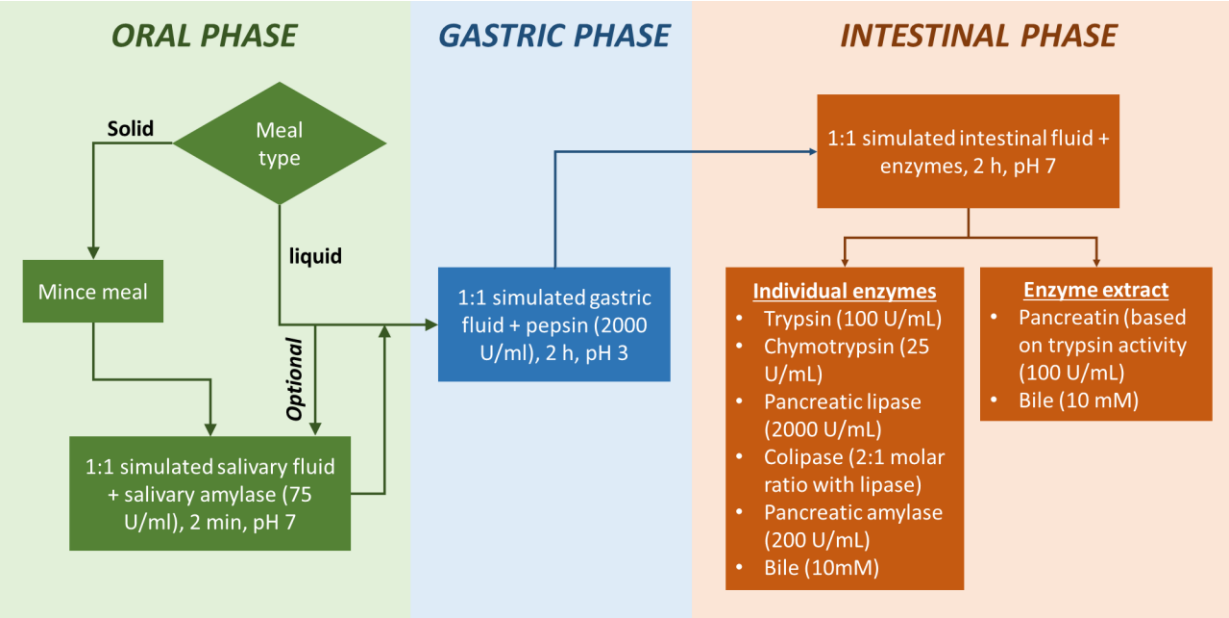

Figure 3-18 Overview of the Infogest *in vitro* static digestion model developed by Minekus *et al.* (2014).

The Infogest model have been used widely in the digestion of protein and lipid (Egger *et al.*, 2016; Mat, Le Feunteun, Michon, & Souchon, 2016) and less on the digestion of starch (Bustos, Vignola, Pérez, & León, 2017). One of the most distinct difference between the Infogest model and other *in vitro* starch digestion models (Englyst, Englyst, Hudson, Cole, & Cummings, 1999; Mishra, Monro, & Hedderley, 2008) is the absence of amyloglucosidase (AGU) in the former. This enzyme is found in the brush border of the human small intestine and is involved in starch digestion. The addition of AGU in the model has two advantages: (i) it increases the proportion of starch digestion that can be completed in the experimental timeframe and (ii) the conversion of all α-amylase products into glucose for ease of analysis using glucose quantification assays (Warren, Zhang, Waltzer, Gidley, & Dhital, 2015). Hence, while the Infogest model may provide standardisation of digestion methods, this study has chosen to use a static model that has been developed by Mishra *et al.* (2008) with the addition of an oral phase (Figure 3-19). In comparison to the Infogest method, the chosen static method is faster and has been used specifically for starch digestion, hence includes all the starch digestive enzymes in the mix.

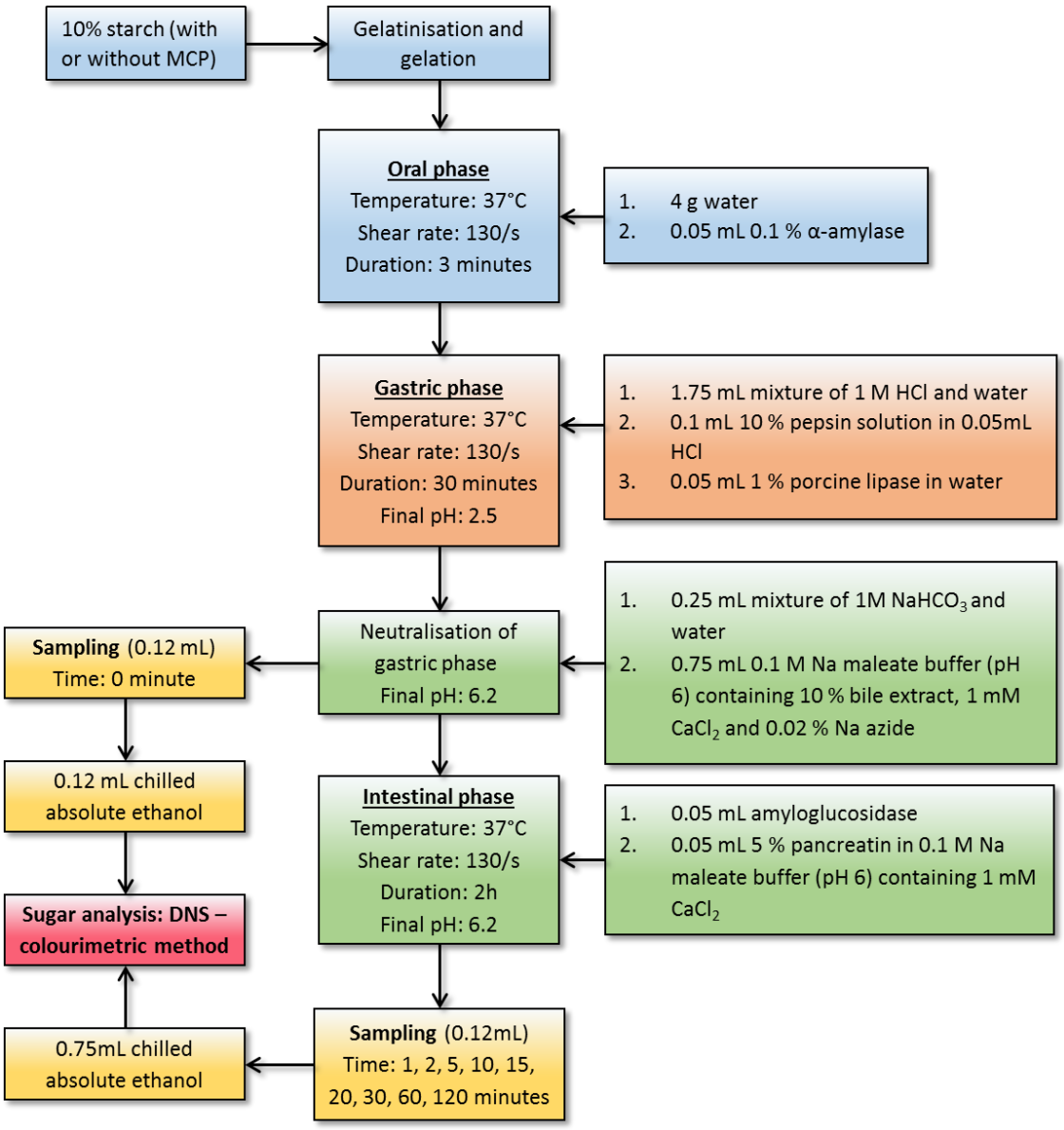

Figure 3-19 Modified static digestion model based on Mishra *et al.* (2008).

3.6 Dinitrosalicylic (DNS) assay

The product of starch digestion is composed of short chain oligosaccharides, disaccharides and monosaccharides. The inclusion of amyloglucosidase in the digestion model converts most of the product into glucose, which makes the analysis much easier. Quantification of glucose obtained from starch digestion can be done using chromatography techniques, but it is more common to use assays such as the D-glucose assay (GOPOD) or the 3,5-dinitrosalicylic acid assay (DNS). The method of glucose quantification that is used in this study is the DNS assay as it was the quickest and most cost-effective method. The DNS assay is a colourimetric method that is based on the redox reaction between DNS acid and glucose under alkaline condition at high temperature (Farinas, Damaso, &

Couri, 2013). The reaction results in the formation of a brownish red coloured compound that can be detected using the spectrophotometer (Figure 3-20).

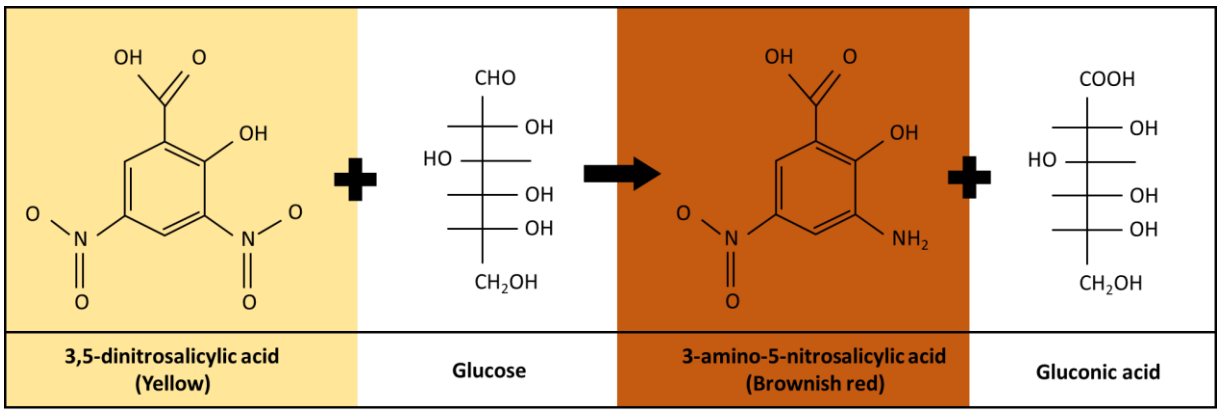

Figure 3-20 Reaction between DNS acid and glucose (adapted from Nag (2016)).

Despite being a quick and simple technique, the DNS assay tends to overestimate results due to the harsh conditions (alkaline and high temperature) of the method that leads to polysaccharide degradation (Gusakov, Kondratyeva, & Sinitsyn, 2011). In particular, the use of DNS assay for the activity of pectinase against pectin (esterified polygalacturonase) is not recommended as the polysaccharide degrades at the non-reducing side of the methoxylated galacturonide residue (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). Since this study involves the use of MCP, a polysaccharide that has a similar structure to pectin, precautionary measures were taken: (1.) the results from the assay were validated using HPLC and (2.) the use of appropriate blanks to eliminate any overestimation resulting from the degradation of the polysaccharide. The blanks used contained MCP without the glucose released from the digestion. These were obtained by carrying out the digestion in the absence of amylolytic enzymes.

3.7 Enzyme inhibitory assays

The inhibitory activities of MCP against α-amylase and α-glucosidase were investigated using α-amylase and α-glucosidase inhibitory assays. The principle of both methods is based on the quantification of the product released as the enzymes hydrolyse the substrate for a fixed period of time, with increasing concentrations of substrate and inhibitors. The kinetics of an enzyme are governed by the following equation:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P$$

Where S = substrate, E = enzyme, ES = enzyme-substrate complex, P = product, k = rate constants. The rate of product formation is directly proportional to the formation of EA, which depends on the concentration of the reactants. Therefore, assuming appropriate constants, an enzymatic reaction would proceed with time in three phases:

1. A fast initial phase of no product formation. EA is instantly formed and there is a decrease in free enzyme.
2. This is followed by the medium phase, whereby product formation is at its maximum and the concentration of EA remains constant.
3. Upon substrate exhaustion, the system enters the depletion phase and the concentration of EA decreases up to a point where product formation reaches 0.

The rate constants k_1, k_{-1} and k_2 are typically combined into a common constant known as the Michaelis constant K_m, which is defined using the following equation:

$$K_m = \frac{(k_{-1} + k_2)}{k_1}$$

The fundamental equation of enzyme kinetics (Michaelis-Menten equation) was developed by Leonor Michaelis and Maud Leonora Menten (Cornish-Bowden, 2004). This equation assumes steady state and the rate of product formation (v) can be calculated as follow:

$$\boldsymbol{v = \frac{V_{max}}{K_m + S}}$$

Where V_{max} = maximum rate of reaction of system, K_m = Michaelis constant, and S = substrate concentration. In a reaction, the highest possible rate of substrate to product conversion is defined as the maximum velocity (V_{max}) and this occurs when all of the enzyme molecules in the system are involved in the reaction.

Plotting of the Michaelis-Menten equation gives a curve of second order reaction. Rewriting this equation by taking the reciprocals of both sides allows results to be plotted on a straight line that creates a plot known as the Lineweaver-Burk plot (Figure 3-21):

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{S}$$

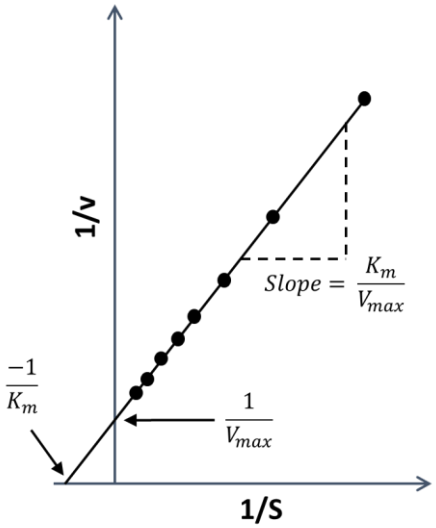

Figure 3-21 Lineweaver-Burk plot

K_m represents the concentration of the substrate at which ½ of the enzymes' active sites are occupied by the substrate. Thus when [S] = K_m, half of the enzyme is in the form of the enzyme-substrate complex and the rate of reaction can be described as $v = 0.5V_{max}$.

The kinetics of an enzyme are influenced by the presence of inhibitors. There are four classes of inhibitions and these are known as competitive, non-competitive, uncompetitive and mixed inhibitions. Competitive inhibitors typically have structures resembling the substrate and, therefore, compete with the substrate for the active site of the enzyme, thereby making the site unavailable for substrate binding. Non-competitive inhibitors, on the other hand, have equal affinities towards an enzyme and enzyme-substrate complex, and this creates either an enzyme-inhibitor or enzyme-substrate-inhibitor complex, which are inactive. In the case of unequal affinity towards the enzyme or enzyme-substrate complex, the nature of the inhibition is termed as mixed type. Uncompetitive inhibitors have no affinities towards the enzymes but will bind only to enzyme-substrate complex, making them inactive. Regardless of the type of inhibition, the presence of an inhibitor decreases the activity of the enzyme, altering the kinetic of the enzyme. The nature of this inhibition is determined based on the changes in the parameters, K_m and V_{max}. In the past, these parameters are obtained from the Lineweaver-Burk plot, which transforms data into straight lines so that linear regressions can be carried out. Data transformation (reciprocals) distorts the experimental error, which means that the values of K_m and V_{max} cannot be accurately determined using the Lineweaver-Burk plot. With advancement in computer technology, the values of K_m and V_{max} can be obtained accurately from experimental data using non-linear regression (Motulsky & Christopoulos, 2004). Therefore, the type of inhibitor present in the system can be determined by looking at the changes in these parameters, as outlined in Table 3-1.

Table 3-1 The effect of different type of inhibitors on the K_m and V_{max} of an enzyme

Type of inhibition	K_m	V_{max}
Competitive	Increase	No change
Non-competitive	No change	Decrease
Mixed	Increase	Decrease
Uncompetitive	Decrease	Decrease

Chapter 4 [1]Molecular interactions between wheat starch and *Mesona chinensis* polysaccharide

4.1 Introduction

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful technique that can be used to identify interactions between polymers. NMR relaxation techniques have been used to study the mobility of water in food products such as bread containing hydrocolloids in order to understand the interaction between starch, hydrocolloid and protein (gluten) (Linlaud, Ferrer, Puppo, & Ferrero, 2011). The use of NMR to study polymer interactions has also been reported for surfactants and polymers (Sharma *et al.*, 2011), LBG and cellulose (Newman & Hemmingson, 1998), amylose and fatty acids (Godet, Tran, Delage, & Buleon, 1992), and protein and starch (Zhang, Do, *et al.*, 2006). Using the solid-state NMR, polymer interactions can be detected at molecular level by: (i) observing the proximity of the molecules in space with Nuclear Overhauser Effects (NOE) or the Rotating Frame Nuclear Overhauser Effects (ROE) and (ii) detecting the changes in the molecular motion of polysaccharides at different spatial resolutions with T_1 and T_2 relaxation.

The addition of *Mesona chinensis* polysaccharide (MCP) to starches results in an increase in the viscosity and strength of the starch paste/gel depending on the concentration of the polysaccharides (Feng *et al.*, 2010a; Feng *et al.*, 2014). Based on rheological and DSC results, interaction between the two has been proposed to cause the synergistic increase in starch gel strength/viscosity (Lai & Chao, 2000b). However, evidence of molecular interaction between the two has never been reported. Hence, the objective of this chapter is to use solid state NMR to identify the interaction between wheat starch and Mesona chinensis polysaccharide (MCP) and to determine how MCP influences the molecular mobility of wheat starch. In combination with other characterisation techniques that are used in this thesis, the solid-state NMR is a powerful tool that can be used to elucidate the interaction between wheat starch and MCP.

This study involves a series of experiments which differed in terms of their spatial resolution. The proton ROESY allows the identification of spatial proximity of less than 5 Å between starch and MCP.

[1] Parts of this chapter are published in Yuris, A., Matia-Merino, L., Hardacre, A. K., Hindmarsh, J., & Goh, K. K. T. (2018). Molecular interactions in composite wheat starch-Mesona chinensis polysaccharide gels: Rheological, textural, microstructural and retrogradation properties. *Food Hydrocolloids*, 79, 1 - 12.

The relaxation measurements detect heterogenic spatial variation in molecular motion depending on the frequency of motion probed and the level of signal diffusion. The spatial heterogeneous detection scales for the relaxation mechanisms are as follows:

- Proton spin-lattice relaxation (T_1) in tens of micron scale
- Spin-spin relaxation (T_2) in 1 to 10 micron scale.
- Proton spin–lattice relaxation in 10 to 50 nano-scale.
- Carbon spin-lattice relaxation in the 2 to 3 bond length from the central nuclei (1-2 Å).

All of the above relaxation methods were employed to determine if there was any spatial heterogeneity between wheat starch and MCP after gelatinisation and if there was any change in the molecular motion of the starch due to interaction with MCP. Therefore, it is the objective of this chapter to determine whether wheat starch and MCP interacted at a molecular level.

4.2 Materials and methods

4.2.1 Preparation of starch and MCP extract suspensions

Mesona chinensis powder (Xi'an, China) was dissolved on a dry weight basis in MilliQ (Millipore, Massachusetts, United States) water and left to hydrate for at least 8 hours at 20°C under constant stirring. Wheat starch was purchased from Penford (New South Wales, Australia) and its moisture content on a dry weight basis was determined by oven drying at 108°C. All starch suspensions were prepared on a dry weight basis. MCP solutions were added to calculated quantities of wheat starch to obtain suspensions containing 0 – 8 % w/w MCP and 10 % w/w starch.

4.2.2 Preparation of starch and MCP gels

Starch-MCP suspensions were dispersed in the RVA (Rapid Visco Analyser, Perten Instruments, Massachusetts, USA) at a constant vane speed of 960 rpm for 1 minute at 25°C. The speed was then reduced to 160 rpm and starch was gelatinised by heating the suspension up to 25, 45, 50, 60, 70, 90 and 95°C at a rate of 2°C/min. The pastes were then removed and cooled to room temperature and used for proton relaxation T_1(H) analysis. For the remaining relaxation analysis ($T_{1\rho}$ and T_1(C)), the gels were frozen using liquid nitrogen and then grounded to produce fine solid samples. For proton ROESY analysis, starch-MCP suspensions were prepared using heavy water (deuterium oxide) in place of water. This was done to reduce the signal from the proton of water (that obstructs starch and MCP signals) and also to reduce broadening of starch and MCP proton peaks, which occurs as a result of proton exchange with water. The suspensions were then gelatinised by heating up to 95°C and the gels were directly used for analysis.

4.2.3 Solid state NMR

The association of water with the polysaccharides in the gels was analysed using MAS NMR. MAS NMR spectra were acquired on a Bruker BioSpec spectrometer (Elektronik GmbH, Rheinstetten, Germany) operating at a ^{1}H frequency of 200.32 MHz. The samples were measured in a Bruker 7-mm double resonance H/X SB-MAS (small bore-magic angle spinning) probe with temperature control. Typically, 150 – 200 mg of sample (dry weight) was packed into a sealed 7 mm rotor. The 90° pulse of ^{1}H was 5.54 µs. During all experiments, a 15 kHz dipolar proton decoupling was employed and the decoupling sequence was "spinal64". The rotor spinning speed was maintained at 3000 Hz ± 10 Hz. Chemical shifts were referenced externally to glycine. All spectra processing and relaxation constant curve fitting were undertaken with MNova version 9.02 (Mestrelabs, Spain).

4.2.3.1 Proton ROESY

The association of water with the polysaccharides in the gels was analysed using MAS NMR. ^{1}H-^{1}H HR-MAS ROESY NMR spectra were acquired using the standard Bruker pulse sequence "roesyphr". Sample temperature was regulated at 55°C to improve peak narrowing. The measuring conditions for the 2D spectra were: spectral width 6000 Hz; data size 4096/256; relaxation delay 2 s, 4 dummy scans and 8 scans with a mixing time of 200 ms. Phase sensitive spectra were acquired using TPPI scheme. The water signal was suppressed with presaturation.

4.2.3.2 Proton Relaxation T_1(H) and T_2(H)

All proton spectra were acquired with a spectral width of 12 kHz, 4096 data points, 8 scans and a recycle time of 10 sec. For each sample of the wet gels, the proton transverse relaxation constants (T_2) and longitudinal relaxation constants (T_1) were measured. The T_2 was measured with the Carr–Purcell–Meiboom–Gill (CPMG) sequence with a 90 – 180 echo spacing of 250 µs, this was used to match the MAS spinning period (Callaghan, 1993). The number of echoes was varied to produce 70 echo times between 0.5 to 960 ms. The proton longitudinal relaxation constants (T_1) were measured with an inversion recovery method. The inversion time was varied with 14 steps between 30 ms and 15 sec. Proton spectra were zero filled to 8192 data points, baselined corrected and windowed with an exponential factor of 5 Hz. The correlation times were estimated from the T_1/T_2 ratio with the generalised fits derived by Carper and Keller (1997).

4.2.3.3 Rotating Frame Spin–lattice Relaxation $T_{1\rho}$

Slow motions on the microsecond time-scale were probed with ^{1}H $T_{1\rho}$ rotating- frame spin–lattice relaxation times. The $T_{1\rho}$ was measured using the variable spin-lock method (Kolodziejski & Klinowski, 2002). The cross polarisation (CP) contact time was 1000 ms and spin-lock times were varied for 5 µs to 9.5 ms with 12 steps. A recycle delay of 2 s was inserted between successive data acquisitions. Free induction decays (FIDs) were acquired with a sweep width of 40 kHz; 1216 data points were collected over an acquisition time of 15 ms. Carbon spectra were zero filled to 2048 data points, baselined corrected and windowed with an exponential factor of 45 Hz.

4.2.3.4 Carbon Relaxation T_1(C)

All carbon spectra were acquired with 894 data points, spectral width of 18 kHz, 4096 data points, 2048 scans, a ^{1}H-^{13}C CP contact pulse of 1000 ms and a recycle time of 1 sec. During all acquisitions, a 55 kHz dipolar proton decoupling was employed. The proton longitudinal relaxation inversion recovery with CP detection constants (T_1(^{1}H-^{13}C)) was measured with the standard Bruker pulse sequence "cpht1". The inversion time was varied from 50 ms to 15 s with 12 steps. The carbon

longitudinal relaxation constants ($T_1(^{13}C)$) were measured with the standard Bruker pulse sequence "cpxt1" which uses the CP inversion recovery (Torchia) method. The inversion time was varied from 50 ms to 15 s with 8 steps. Carbon spectra were zero filled to 2048 data points, baselined corrected and windowed with an exponential factor of 30 Hz unless stated otherwise.

4.3 Results and discussion

4.3.1 Proton ROESY

Figure 4-1 contains the HR-MAS proton NMR spectra of the gelatinised samples of 10 % w/w wheat starch, 10 % w/w MCP and 10 % w/w wheat starch with 5 % w/w MCP in D_2O spun at 3 kHz. In these spectra, the water resonance at 4.3 ppm has been suppressed. Partial assignment of the starch and MCP peaks are taken from previously published text and known characteristic proton chemical shifts (Feng, Gu, & Jin, 2007; Nilsson, Bergquist, Nilsson, & Gorton, 1996; Soderberg, 2016). The spectra of the MCP indicates that it is mainly made up of a polysaccharide mixture (hydroxyl peaks 3.5 – 5.5 ppm) (Feng *et al*., 2007) and contains fractions of oils (alkyl peaks 0.9 – 1.3 ppm) and proteins (amide peaks 7 ppm) (Figure 4-1b).

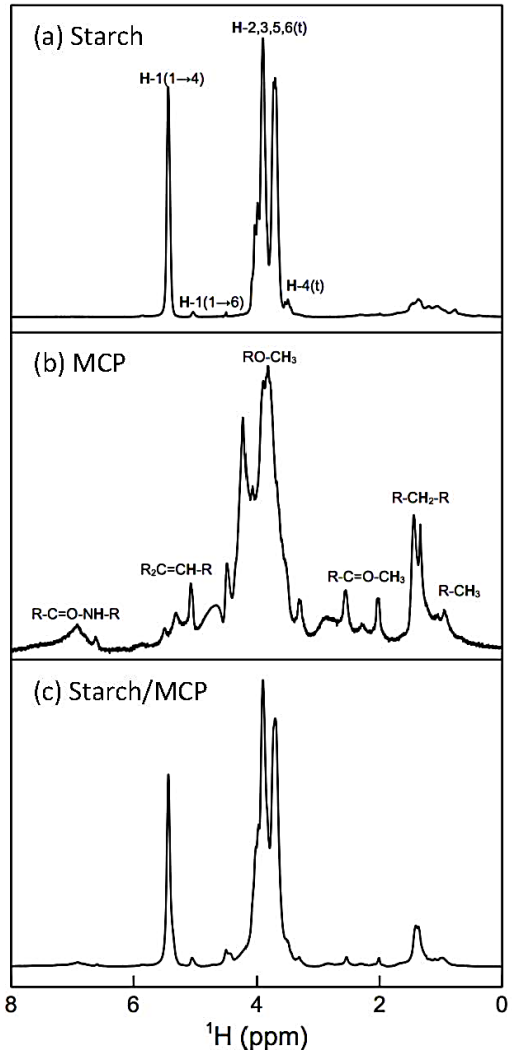

Figure 4-1 ^{1}H HR-MAS spectra of gels of wheat starch (a), MCP (b) and wheat starch/MCP (c) in D_2O. (t) indicates terminal proton on glucan monomer.

The spatial proximity of molecules can be detected with NMR using the Nuclear Overhauser Effects (NOEs). Sizeable NOEs are only observed between two nuclei when they are close in space by 5 Å. ^{1}H-^{1}H Two-dimensional (2D) rotating frame Overhauser effect spectroscopy ROESY experiment yields through space correlations via spin-spin relaxation (Otting, 2007). ^{1}H-^{1}H ROESY is useful for determining which signals arise from protons that are close in space even if they are not bonded. Figure 4-2 contains the HR-MAS 2D-ROESY spectra of hydroxyl region (3 – 6 ppm) of the starch, MCP and starch/MCP D_2O gels at 55°C. For each 2D-ROESY spectra, they contain a diagonal and cross peaks (off the 45° diagonal). The diagonal consists of the 1D proton spectrum of each sample (as in Figure 4-1). The cross peaks are signals arising from protons that have ROE correlations. This means they are close in space (spatial proximity of 5 Å). The spectra have been phased such as the diagonal is a positive intensity (black) and all the ROE signals are negative (red) (Otting, 2007).

When comparing the three 2D-ROESY spectra, unique ROE peaks appeared in the starch/MCP gel, which are not present in the individual spectra of the starch and MCP (Figure 4-2a and b). These peaks are indicated in Figure 4-2c by the dashed ovals. This shows that after gelatinisation, the starch and MCP were in close proximity to each other. No ROE peaks were observed between the oils (alkyl peaks), the proteins (amide peaks) and the starch peaks (not shown), demonstrating that no interaction was detected between the oil or protein components present in the *Mesona chinensis* extract and starch. All of the detectable starch/MCP interactions were between the polysaccharide components of the *Mesona chinensis* extract and glucan protons of the starch. NOEs were not detected in the starch-MCP mixture before gelatinisation (data not shown), indicating that gelatinisation was required for the interaction to occur. There appeared to be no site-specific interaction between the starch and MCP—all MCP protons appeared to be in close proximity to the separate proton groups on the glucan monomers of the starch. These results clearly show that the starch glucan polymers do interact with MCP at molecular level, as this interaction occurs at a proximity of less than 5 Å, which has never been demonstrated before.

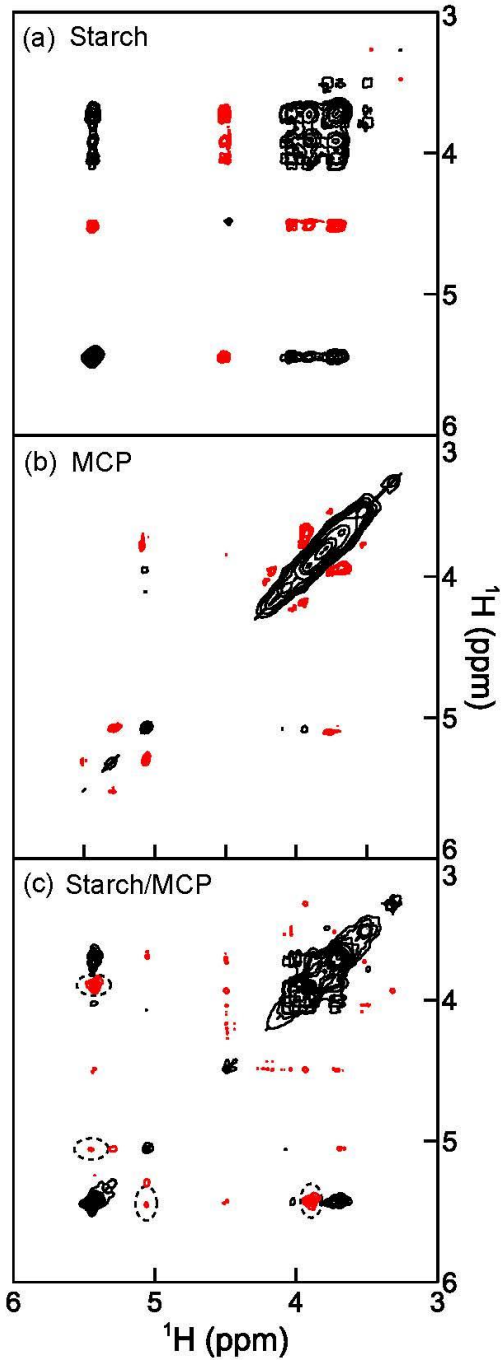

Figure 4-2 ^{1}H-^{1}H HRMAS 2D ROESY spectra of wheat starch (a), MCP (b) and wheat starch/MCP (c) gels in D_2O at 55°C with red peaks indicating ROEs between proton nuclei. The dashed ovals indicate unique ROE peaks for the starch/MCP gel mixture. Black and red spectra indicate positive and negative phase respectively.

4.3.2 The effect of MCP on water mobility within wheat starch gel

Correlation time is defined as the average time it takes for a molecule to rotate by one radian to achieve a different orientation (Cavanagh, Fairbrother, Palmer III, & Skelton, 1996). It is directly related to the T_1/T_2 ratio and provides a better indication of molecular mobility in a system where the

T_1 relaxation is changing. For a molecule to progress between two molecular orientations, overcoming an energy barrier is required and, therefore, the correlation time is typically defined by the following equation:

$$\tau_c = \tau_0 exp\left(\frac{E_a}{RT}\right)$$

where τ_0 = correlation time constant, E_a = activation energy, R = gas constant and T = temperature. The equation suggests that molecular reorientation is slowed down when the system is cooled, meaning that a larger correlation time corresponds to a slower motion (Bakhmutov, 2015). The effect of changing MCP to wheat starch ratio on the correlation time of the water and carbohydrate fractions in the gels is shown in Figure 4-3.

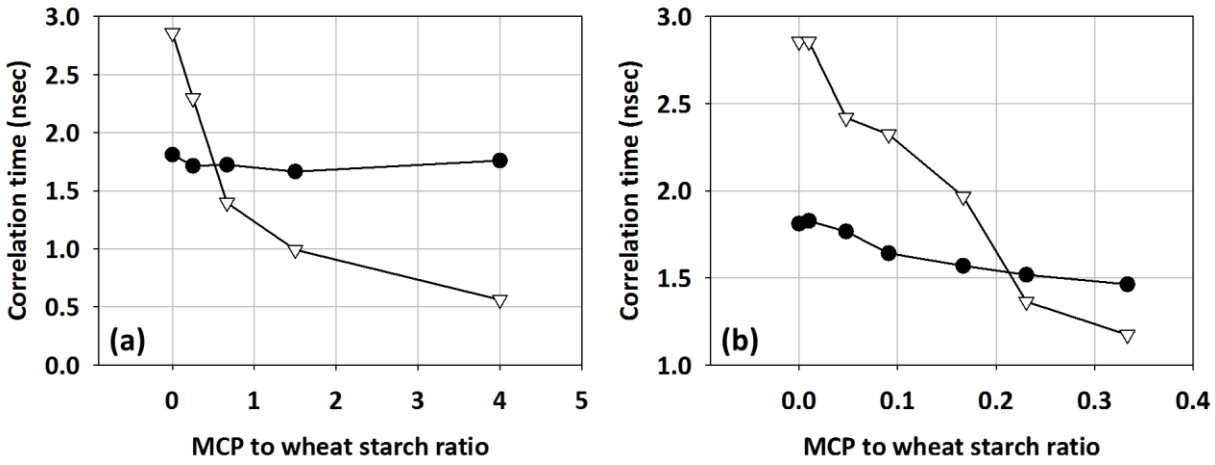

Figure 4-3 The average proton correlation time of the water (●) and carbohydrate (▽) population within the starch gels system as affected by the changing MCP to wheat starch ratios through substitution (a) and addition (b) of MCP.

The correlation time of starch and MCP were unable to be observed separately as their peaks were broadened into a single peak. Thus, the result obtained was an average of the overall mobility of each population. The mobility of water molecules within the system (average of starch and MCP) was unaffected when starch was partially substituted with MCP to alter their ratio while maintaining the total solid content of 10 % w/w (Figure 4-3a black symbol). However, a slight decrease in the correlation time of the water population can be observed when MCP was added rather than substituted (Figure 4-3b black symbol). This can be explained by the Stokes-Einstein-Debye theory, which is defined by the following equation:

$$\tau_c = \frac{4\pi a^3 \eta}{3kT}$$

where a = molecular radius, η = viscosity. The correlation time of a given system is influenced by the size of a molecule as shown above and since MCP is much smaller than starch, it would possess higher mobility. Increasing the MCP concentration meant that more protons would be in exchange between water and the polysaccharide, thus making the system more mobile on average, due to MCP being a smaller molecule than starch. Bearing in mind that the result measures an average of the population, a higher proportion of water exchanging protons with MCP would result in a higher fraction of mobile water and, hence, a decrease in the correlation time. An increase in water mobility has also previously been reported for doughs containing xanthan (Linlaud *et al.*, 2011) and was attributed to the polysaccharide's ability to exchange more protons with water.

The presence of MCP, regardless of whether it was by addition or substitution, resulted in an increase in the mobility of the carbohydrate population. This means that the carbohydrate population was more flexible and moved more readily in the presence of MCP. Such may represent a situation whereby the interaction between MCP and starch prevented associations between starch polymers such as amylose, hindering them from interlocking and hence increasing their molecular mobility. It is also noteworthy to mention that the increase in carbohydrate mobility (or decrease in correlation time) follows an exponential decay when starch was increasingly substituted by MCP. This means that the effect of substituting starch with MCP reached a plateau and further substitution did not result in any more increase in the mobility of the carbohydrate population. This reflects a situation whereby high MCP concentration and low starch concentration was not favourable for gel formation and hence, making the carbohydrate portion more mobile. The lack of gel structure was visually observed when there was a higher ratio of MCP to starch in the system (Figure 4-4). The strength of these gels were found to decrease from a maximum of ~2000 Pa in the presence of 10 % w/w starch without MCP to a minimum of ~20 Pa in the presence of 2 % w/w starch and 8 % w/w MCP.

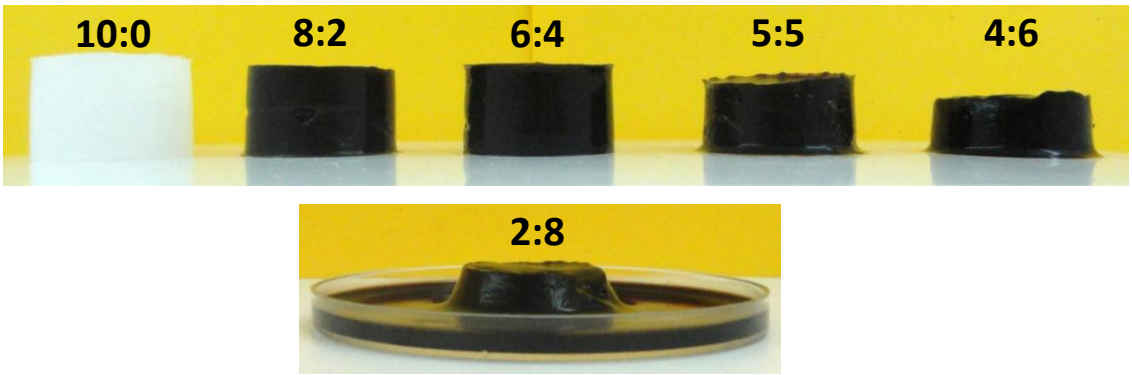

Figure 4-4 Gels formed using different ratios of starch to MCP.

These results suggest that the interaction between wheat starch and MCP did not affect the water phase of the gel. Therefore, the changes in the properties of the resulting gels, which will be discussed

in a later chapter, is a result of starch and MCP interaction rather than the viscosity changes associated with the continuous phase.

4.3.3 The effect of MCP on the gelatinisation of wheat starch

4.3.3.1 Proton relaxation $T_1(H)$ and Rotating Frame Spin–lattice Relaxation $T_{1\rho}$

Figure 4-5 shows the changes in the correlation time of 10 % wheat starch suspension that have been heated up to a range of temperatures in the absence and presence of MCP. In this case, since the sample was freeze dried, the correlation time detects the molecular mobility of the proton population on the starch and MCP. No differences in the relaxation behaviour of MCP and starch could be perceived and hence the result shows the macroaverage of the proton mobility of the entire system. This suggests that within the approximate diffusion distance of NMR signal (50 µm) wheat starch and MCP cannot be distinguished as two separate components with independent molecular mobility, indicative of interaction between the two.

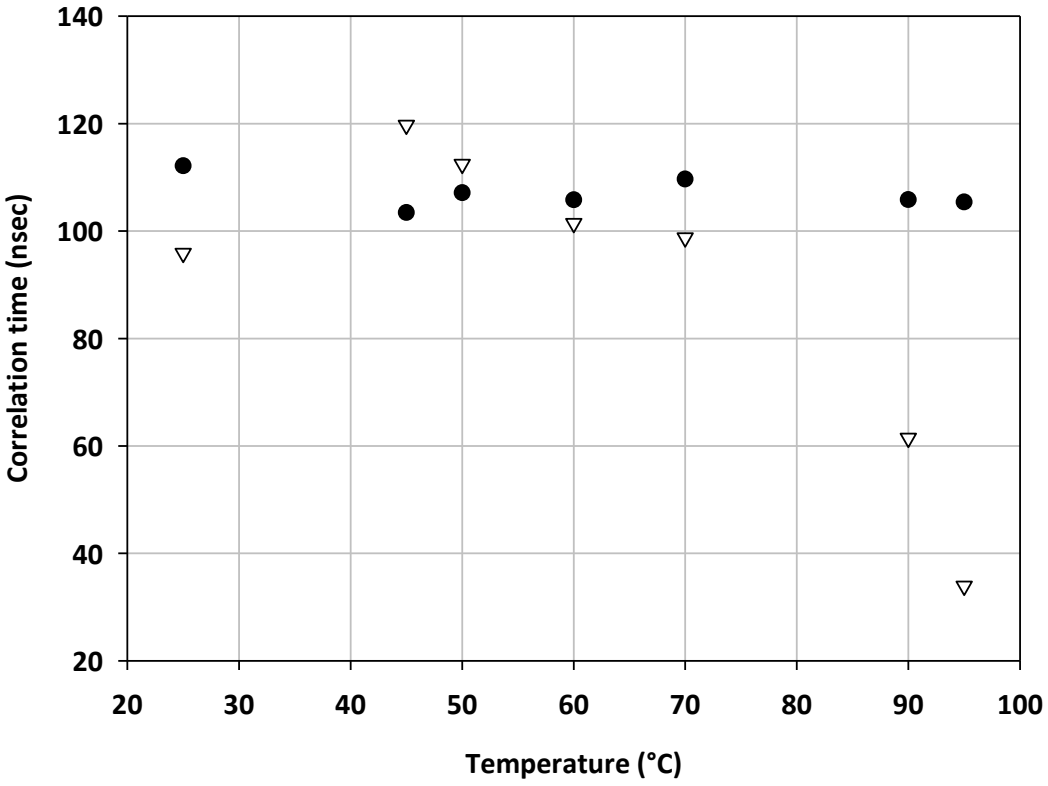

Figure 4-5 The average correlation time of wheat starch protons (carbon detected) in the absence (●) and presence (▽) of MCP that have been heated up to different temperatures.

In the absence of MCP, the correlation time of the protons was unchanged as starch was gelatinised by heating. This means that the gelatinisation of starch did not affect the mobility of the protons in the carbohydrate chains. The addition of MCP, on the other hand, resulted in a reduction in the

correlation time, suggesting that the long range mobility of the protons increased. The increase in the proton mobility began at about 50°C, a temperature at which wheat starch began to gelatinise and this was accompanied by amylose leaching out of the granules (as it will be demonstrated in Chapter 5). This suggests that the interaction between wheat starch and MCP has occurred during gelatinisation. Similar increase in starch chain mobility have been observed for starch gels containing glycerol, whereby it was demonstrated that glycerol acted as a plasticiser by interacting with amylose and amylopectin, hence preventing the associations between starch chains (Smits, Kruiskamp, van Soest, & Vliegenthart, 2003).

The motion of wheat starch and MCP in the 10 to 50 kHz frequency range were monitored by observing proton rotating frame spin–lattice relaxation ($T_{1\rho}$). Due to spin diffusion, the relaxation is averaged over approximately 50 nm, meaning that it only detects heterogeneous separation of components larger than 50 nm. Figure 4-6 shows the normalised spectral intensity of the wheat starch gels in the absence and presence of MCP. In this case, the magnetic field applied to the sample was spin-locked so that a radio frequency magnetic field perpendicular to the static magnetic field can be generated (Apperley *et al.*, 2012). Different normalised intensities within a system indicates that different components exist which have different relaxation pathways. However, it can be seen from Figure 4-6 that for starch gels both in the presence and absence of MCP, the normalised intensities were overlapping. This means the system appears homogenous at a distance of less than 50 nm. The motion of the starch and MCP cannot be separated when the relaxation is averaged over a length scale of approximately 50 nm. Hence, the result suggests that starch and MCP were closely bound, further indicating interaction between the two.

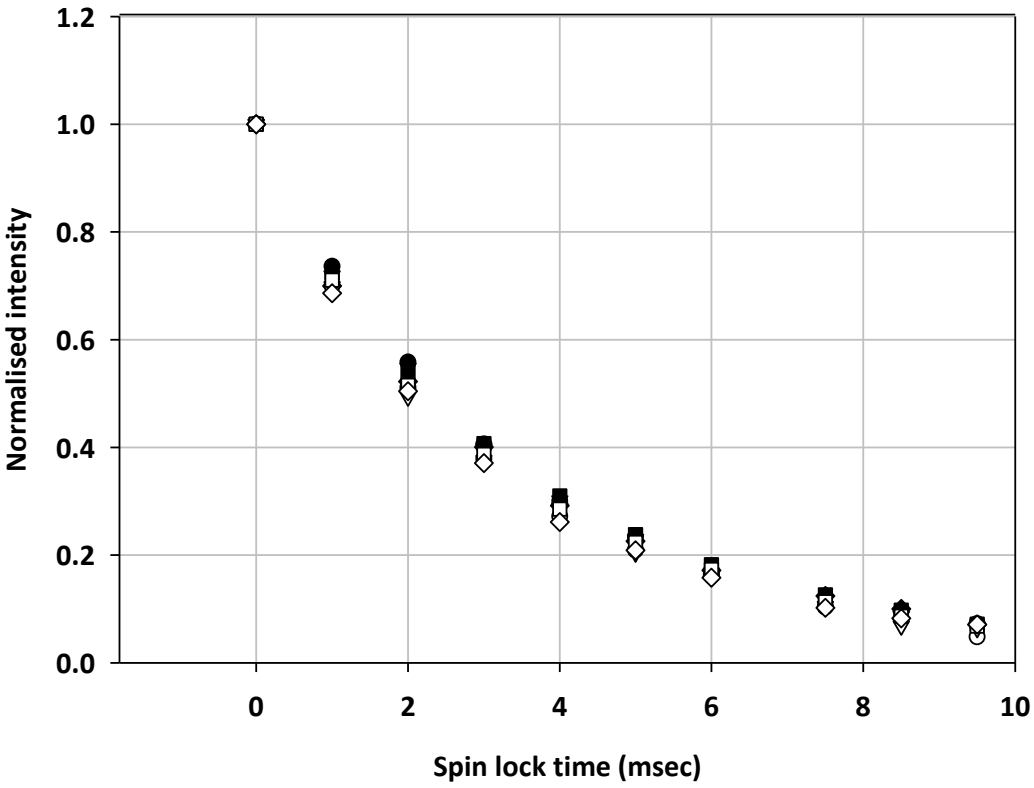

Figure 4-6 The proton-carbon detected rotating frame spin-lattice relaxation $T_{1\rho}$(H) of wheat starch suspension in the absence (black) and presence (white) of MCP heated to 25°C (●), 50°C (▼), 70°C (■) and 95°C (◆).

4.3.3.2 Carbon Relaxation T_1(C)

The carbon relaxation behaviour of 10 % w/w wheat starch gel is shown in Figure 4-7, whereby the correlation time is normalised so that the temperature behaviour of each carbon site can be compared on the same plot. For the method (Carper & Keller, 1997) used to calculate the correlation times, only values between 1 to 1200 ns could be determined. Heating the starch suspension up to a temperature between 50°C and 60°C resulted in a decrease in the mobility (increase in T_c) of all the carbon chains (carbon 1 – 6) and this was followed by an increase in chain mobility (decrease in T_c) at higher temperatures. At low temperatures, amylopectin helices dissociate off the crystallites but retain their double helical configuration, resulting in partial loss of crystallinity. Upon cooling, these helices relocate to the remaining crystals that were not dissociated (Waigh *et al.*, 2000). This may result in double helices associating with neighbouring crystals, hence resulting in a tightened structure. Therefore, the decrease in chain mobility at the early stages of heating can be associated with the relocation of amylopectin double helices following partial loss of crystallinity. When starch is heated up to a higher temperature, there is a complete loss of molecular order, whereby the dissociation of helix is followed by a transition into coils (Waigh *et al.*, 2000). This process is irreversible and, upon

cooling, the flexible coils do not reform helices, therefore resulting in increased chain mobility within the system.

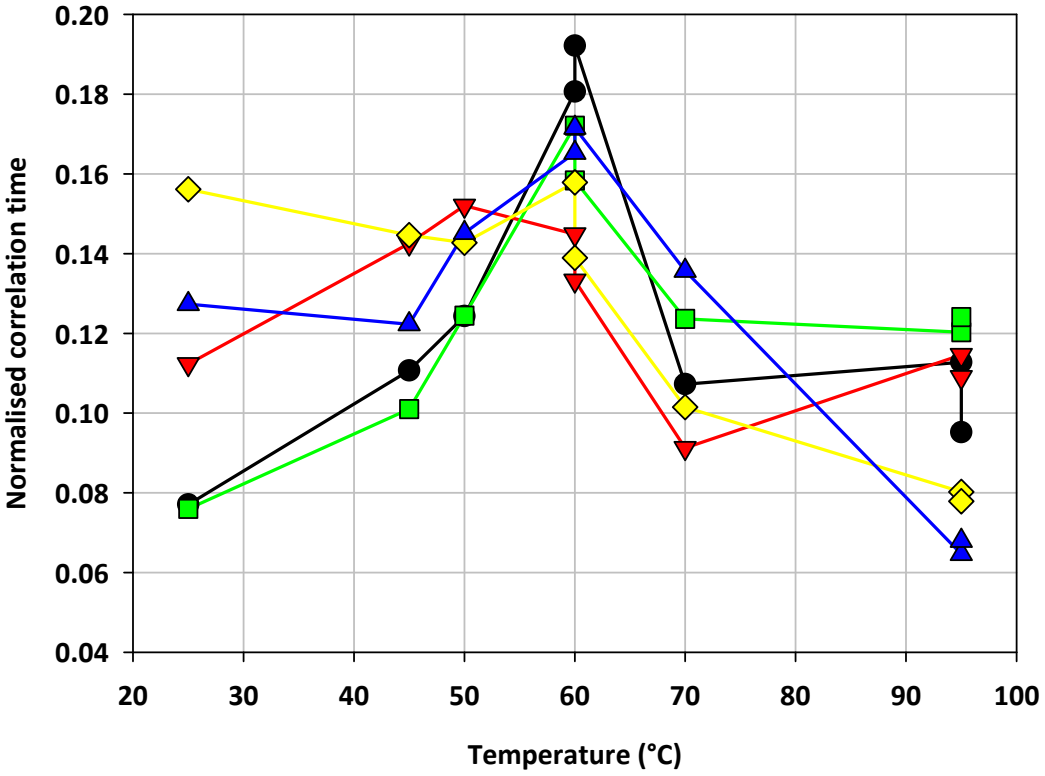

Figure 4-7 Normalised correlation time of the starch carbons C1 (●), C2, 3, 5 (■), C 4(▼), C6a (◆) and C6b (▲) in freeze dried 10 % w/w wheat starch that have been partially gelatinised to various temperatures and cooled.

The relaxation behaviour of most of the carbon molecules remained similar when 2 % w/w MCP was added (Figure 4-8)—a decrease in mobility up to a temperature of 50°C to 60°C followed by an increased mobility when gelatinised further. Two populations of different mobilities were detected for the carbon 6 (C6a and C6b) and are likely to correspond to: (1.) the C6 found in both amylose and linear fraction of amylopectin and (2.) the C6 involved in chain branching of amylopectin. For one of these fractions (C6b), the presence of MCP increased its mobility when the sample was gelatinised. This suggests that the interaction between MCP and starch is most prominent at the carbon 6b, hence resulting in them becoming more flexible upon heating. As previously mentioned, most of the carbon chains had very low mobility, hence resulting in very large correlation times. The exception was for C6b whereby their movement was relatively fast and, hence their correlation time could be quantified; this is shown in the insert of Figure 4-8. In the presence of MCP, it could be seen that the mobility of this carbon fraction increased upon gelatinisation and the difference in their mobility is particularly prominent when the sample has been heated to 60°C—correlation time of about 400 nsec and 100

nsec in the absence and presence of MCP respectively. In the presence of MCP, heating the mixture up to a low temperature (50°C – 60°C) increased C6b's mobility, which was an opposite effect to when MCP was absent (Figure 4-7). This may indicate that the presence of MCP is preventing dissociated double helices from reassociating with the neighbouring crystals upon cooling, hence making them more mobile. Further gelatinisation to a temperature above 60°C resulted in the helix transitioning into coils, increasing the flexibility of the carbon molecules. The flexibility of C6b plateaued in the presence of MCP after heating to 60°C, which could indicate the unwinding of amylopectin double helices at a lower temperature. However, since it cannot be concluded whether MCP was affecting the linear or branched fraction of C6, the only deduction that can be derived from the result is that the presence of MCP interacted with or prevented interaction at carbon 6, resulting in an increase in its mobility.

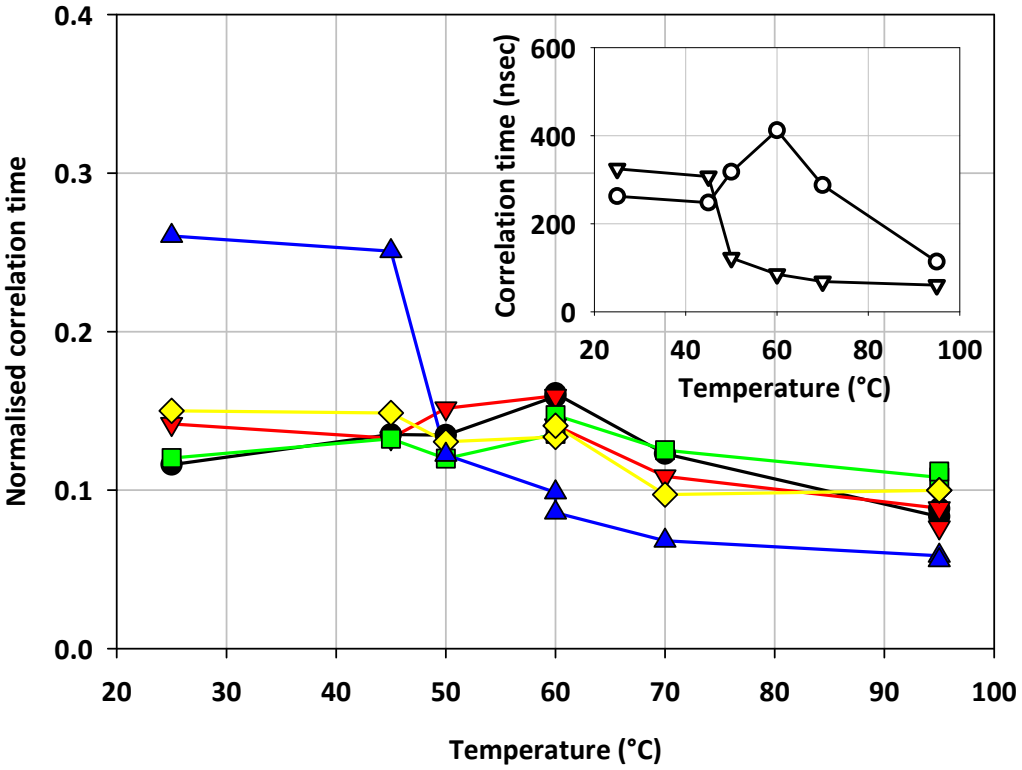

Figure 4-8 Normalised correlation time of the starch carbons C1 (●), C2, 3, 5 (■), C 4(▼), C6a (◆) and C6b (▲) in freeze dried 10 % w/w wheat starch in the presence of 2 % w/w MCP that have been partially gelatinised to various temperatures and cooled. Insert: Correlation time of C6b for freeze dried 10 % w/w wheat starch in the absence (○) and presence (▽) of 2 % w/w MCP.

4.4 Conclusion

The interaction between wheat starch and MCP was established using proton ROESY, which showed that MCP was in close proximity (<0.5 nm) with wheat starch following gelatinisation. The relaxation properties of the water and carbohydrate population found in the composite gel (wheat starch and MCP) could not be distinguished when their proton relaxation behaviour was measured, indicating that the two components were not separable at the micron scale. Analysis of the relaxation properties of the carbohydrate and water populations revealed that the changes associated with the gel properties were a result of their interaction rather than changes in the viscosity of the continuous phase. Differences in the molecular motion of the samples with and without MCP could only be identified at a distance of approximately less than 5 nm. The carbon 6 of the starch was observed to have increased flexibility in the presence of MCP. This indicates that MCP may interact with wheat starch specifically at the carbon 6 or inhibit interaction of the starch polymer chains at C6.

Chapter 5 [2]The effect of MCP on the gelatinisation properties of wheat starch in a dilute system

5.1 Introduction

Polysaccharides such as xanthan, guar, carrageenan, and cellulose are commonly added into food products for improved functionality. Polysaccharides have various roles in food products, such as providing mouthfeel and texture, increasing viscosity, forming gels and delaying starch retrogradation. These functionalities are determined by the interactions between polysaccharides and many food ingredients and generally include various types of interactions including electrostatic bonding and physical entanglement. The interaction between polysaccharides and starches, a major component of many food products, has been widely studied over the last decade. However, the results of these studies have not clearly elucidated the complex interactions involved, partially because of the wide range of starch and polysaccharide types that have been used (BeMiller, 2011).

Typically, interactions between starches and polysaccharides are characterised by changes in the pasting properties of the starches including pasting temperature and paste viscosities along with their amylose leaching behaviour. In wheat starch for instance, the peak viscosity increases in the presence of guar, tara, locust bean and konjac gums (Funami *et al.*, 2005a) but decreases in the presence of gum arabic and soluble polysaccharides from soybean (SSPS) (Funami *et al.*, 2008). Interactions between polysaccharides (guar, tara, locust bean, and konjac) and wheat starch molecules are thought to play a role in increasing the peak viscosity of wheat starch suspension (Shi & BeMiller, 2002) but the mechanisms behind these interactions have never been clearly explained. On the other hand, in the case of gum arabic and SSPS, it was proposed that the polysaccharides adsorb onto starch granules to create a coat that prevents polymer leaching, which led to a decrease in peak viscosity (Funami *et al.*, 2008). The ability of polysaccharides to coat starch granules has also been observed for xanthan gum (Gonera & Cornillon, 2002; Mandala & Bayas, 2004), for which the coat was thought to increase the rigidity of starch granules (Mandala & Bayas, 2004), hence increasing the viscosity of the starch paste due to less granule disintegration (Achayuthakan & Suphantharika, 2008). The specificity of starch and polysaccharide interactions is further demonstrated by the ability of gum arabic and

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xanthan to decrease and increase the amount of amylose leached respectively (Funami *et al.*, 2008; Mandala & Bayas, 2004). In order to explain the variations in the pasting properties of wheat starch-polysaccharide systems, several mechanisms have been previously proposed: (i) thermodynamic incompatibility which leads to an increase in the effective starch concentration in the continuous phase (Alloncle & Doublier, 1991; Funami *et al.*, 2005a), (ii) interactions between starch and polysaccharides resulting in polysaccharide adhesion on granule surface (Abdulmola *et al.*, 1996) and (iii) interactions between leached starch polymers (especially amylose) and polysaccharides resulting in an increase in viscosity (Shi & BeMiller, 2002).

The polysaccharide fraction from the herb *Mesona chinensis* (denoted as MCP) is among the less common polysaccharides that seems to show an interesting interaction with starches. *Mesona chinensis* is a herb of the mint family that is used in Asia to make a gel dessert known as "grass jelly". The extract of this herb has been used as traditional medicine to treat diseases such as diabetes, hypertension, heatstroke, and muscle joint pain (Feng *et al.*, 2012). The health benefits associated with *Mesona chinensis* and its unique taste make the gel dessert popular in many Asian countries. Studies on MCP have suggested its use as a fat-substitute in sausages (Feng *et al.*, 2013), salad dressings (Lai & Lin, 2004), and in extruded rice formulations (Zhuang *et al.*, 2010). The extract of *Mesona chinensis* is black in colour, has low viscosity and does not form a gel on its own when heated and cooled. In order to make "grass jelly", the extract of this herb needs to be heated with starch to a high temperature and then cooled. The gel formed from this mixture is stronger in comparison to gels formed by starch alone. Such strong gels have only been reported to form when the extract is cooked with non-waxy starches (Lii & Chen, 1980). The ability of the extract to form strong gels with starches has been attributed to the presence of an anionic polysaccharide found in the extract. This polysaccharide has been reported to contain galactose, glucose, mannose, xylose, arabinose, rhamnose and galacturonic acid (Feng *et al.*, 2008). The structure of MCP is made up of α-(1$\rightarrow$4)-galacturonan backbone with insertions of α-1,2-Rha*p* residues on which all of the other sugars are attached to (Feng *et al.*, 2008). The interaction between wheat starch and MCP has been reported as synergistic resulting in an increased viscosity and formation of a strong gel (Feng *et al.*, 2010a). However, the nature of this interaction has not been established.

The high viscosity imparted by the addition of MCP to starch systems can be used to modify texture and stability. Increasing the viscosity of a system is also considered to reduce the hydrolysis of starch due to limitations of enzymes accessibility (Brennan *et al.*, 2008). This means that MCP may be used to formulate starch based food products with lower digestibility. In order to use MCP as an ingredient in food formulations, there is a need to understand how MCP interacts with starch when they are processed i.e. heated and cooled. While the synergism between MCP and starches has been reported,

the interaction between the two has not been studied in detail. With the exception of pasting curves generated from the Rapid Visco Analyser, the majority of studies that have been done on starches and MCP focus on the final gel properties (rheology and microstructure of the gel) rather than the gel properties during its formation. Since the interaction between starches and MCP is thought to take place during heating and cooling, this study will follow the events (amylose leaching, granular swelling and development of *G'* during cooling) that occur during gelatinisation and gelation, which are not available in the current literature. This will provide a novel understanding on the interaction between wheat starch and MCP as the mixture is heated into a paste and cooled to form a gel. Therefore, this chapter aimed to better understand the mechanism of interaction between MCP and wheat starch by looking at the rheological, gelatinisation, granular swelling and amylose leaching properties of wheat starch in the presence of increasing concentrations of MCP.

5.2 Materials and methods

5.2.1 *Mesona chinensis* polysaccharide

Dried crude *Mesona chinensis* powder was purchased from Xi'an Hua Rui Bio-Engineering Co. Ltd. (Xi'an, China). The composition of the extract was analysed by an accredited chemical laboratory (Massey University Nutritional laboratory, Palmerston North, New Zealand). Protein, fat, dry matter, ash and starch contents were measured using the DUMAS combustion method (AOAC 991.36), convection oven method (AOAC 930.15, 925.10), ashing at 600°C (AOAC 942.05), and amyloglucosidase-α-amylase method (AOAC 996.11) respectively. The total sugars were measured using the phenol-sulphuric acid method (Hall, Hoover, Jennings, & Webster, 1999). Total carbohydrate content of the extract was calculated based on 100 % - (Moisture + Ash + Protein + Fat) %. Non-starch polysaccharide content was calculated by difference [total carbohydrate % – (Starch + Free sugar) %].

5.2.2 Preparation of starch-MCP suspension

Food grade *Mesona chinensis* powder purchased from Xi'an HuaRui Bio-Engineering Co., Ltd. (Xi'an, China) was dissolved on a dry weight basis in MilliQ (Millipore, Massachusetts, United States) water and left to hydrate for at least 8 hours under constant stirring at 20°C. A range of *Mesona chinensis* powder concentrations (0.1 – 7 % w/w) was selected to determine the concentration at which MCP and wheat starch interact to increase the viscosity of the suspension. The *Mesona chinensis* solution was centrifuged at 4000 g for 30 minutes to remove all insoluble materials. The insoluble materials were then oven dried and the total soluble solids in the supernatant were determined to be between 0.9 -60.3 mg/g solution. The total carbohydrate of the supernatant was measured using the phenol sulphuric acid method. The free sugar present in the solution was obtained by precipitating NSP overnight from the water extract using ethanol. The ethanolic extract was then centrifuged and free sugar in the supernatant was measured using the phenol sulphuric acid method. The total soluble NSP (MCP concentrations) was then calculated by difference (total carbohydrate – free sugar). For ease of reading, the concentration of raw *Mesona chinensis* extract will be used in place of concentration of *Mesona chinensis* polysaccharide and this is reported in the thesis as % w/w MCP. The actual amount of MCP found in 0.1, 0.5, 1, 2, 3, 5 and 7 % w/w raw *Mesona chinensis* solution was 0.2, 1.1, 2.3, 4.5, 6.8, 11.3 and 15.8 mg MCP/g suspension respectively.

Wheat starch was purchased from Penford (Sydney, NSW, Australia) and its moisture content was determined by oven drying method. All starch suspensions were prepared by weight on a dry weight basis. MCP solutions were mixed with wheat starch to obtain suspensions containing 2 – 10 % w/w starch suspensions.

5.2.3 Rheological measurements

Rheological measurements were carried out using a controlled-stress rheometer (MCR302, Anton Paar Physica, Graz, Austria) fitted with a starch cell (C-ETD160/ST) and spindle (ST24-2D/2V/2V-30). Based on preliminary trials, stirring conditions were selected to prevent starch granule sedimentation. Twenty millilitres of the wheat starch/MCP suspensions were added into the rheometer cup and the sample was thoroughly mixed by rotation at a shear rate of $800s^{-1}$ for 3 minutes at 20°C. The shear rate was then reduced to $100s^{-1}$ for 2 minutes while maintaining the temperature at 20°C to equilibrate the system. The temperature was then increased to 95°C at 2°C/minute and held at 95°C for 5 minutes. At this point, the rheometer was switched to small strain oscillation mode using a strain of 1 % (within the linear viscoelastic range) at a frequency of 1Hz and cooling was commenced at 4°C/min to 20°C and held there for 30 minutes as the gel formed. Small strain oscillation was used to prevent destruction of the gel as it sets.

5.2.4 Starch swelling: visualisation using microscopy

A light microscope (HM-Lux, Leitz Wetzlar, Wetzlar, Germany) fitted with a digital camera (ScopeTek MDCC200) and a hot-stage was used to take images of the starch granules as they swelled during heating in excess water and the presence or absence of MCP. Two self-adhesive ring enforcements were laid, one on the other, on a microscope slide to create a well that allowed the granules to swell without restriction. Suspensions of wheat starch (1 % w/w) with between 0 to 5 % w/w MCP suspensions were prepared and a drop (40 µL) was placed in the centre of the well. Evaporation was minimised by carefully placing a cover slip over the sample, ensuring that air bubbles were excluded. The cover slip was sealed using nail polish and the assembly placed in the chamber of the hot stage. Imaging was carried out at 12.5x magnification. In order to gelatinise the starch, the temperature was increased from 30 to 95°C at about 5°C/min and the swelling process recorded using ScopePhoto (Version 3.1.268, ScopeTek). Images of starch granules at specific temperatures (monitored manually using a probe in the hot stage) were then extracted from the data files.

5.2.5 Particle size of starch granules

Microcentrifuge tubes containing 2 mL of 2 % w/w wheat starch suspension with and without MCP were placed in a water bath. The temperature of the bath was increased from 20 to 95°C at about 1°C/min and held at 95°C for 60 minutes with frequent inversion of the tubes to ensure that starch granules remained evenly dispersed in the suspension. Individual microcentrifuge tube containing the sample was removed at the following temperature intervals for a total of 12 samples: 25, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95°C and after 60 minutes at 95°C. Upon removal from the water bath, the tube was immediately submerged in cold water to prevent further swelling of the granules. The volume

weighted mean size of the starch granules collected at different temperatures was measured using a laser diffraction particle size analyser (Malvern MasterSizer Hydro 2000MU, Malvern Instruments Ltd., Malvern, UK). Samples from the cooled microcentrifuge tubes were dispersed in the MasterSizer whereby they were directly measured or continuously stirred at 3000 rpm for 16 minutes and 20 seconds and their D (4,3) values were recorded with time. The refractive indices used were 1.31 and 1.52 (Wilson *et al.*, 2006) for water and starch, respectively and the obscuration level was maintained at 10 %.

5.2.6 Amylose leaching quantification

The apparent amount of amylose leached from the starch granule suspensions was determined using a modified iodine binding method (Kaufman, Wilson, Bean, Herald, & Shi, 2015). Microcentrifuge tubes containing 0.25 mL of 2 % w/w wheat starch and MCP were placed in a water bath at room temperature and heated as for the particle size measurements (section 5.2.5). In order to cool the samples, 0.75 mL of chilled MilliQ water was quickly added to the tubes as they were removed from the water bath in order to halt amylose leaching. The tubes were then centrifuged at 10000 g for 15 minutes. Using a disposable 96-well plate, 100 µL of the supernatant and 100 µL of iodine (3.04 g/L iodine in 0.9 mL/mL DMSO) were mixed in a well for 2 minutes and 20 µL of each mixture was dispensed into an empty well using an autopipettor. An aliquot of 180 µL of distilled water was added to this and the diluted solutions were mixed for another 2 minutes. Absorbances at 510 nm and 620 nm were measured using a spectrophotometer (SPECTROstar Nano Microplate Reader, BMG LABTECH, Offenburg, Germany). MCP solutions of appropriate concentration were used as blanks. All measurements were carried out in triplicate. The amylose content of the samples was calculated using the following equation:

$$\text{Amylose} = ((\text{Abs}_{620\text{nm}} - \text{Abs}_{510\text{nm}}) - \text{y-intercept})/\text{slope}$$

Where $\text{Abs}_{620\text{nm}}$ = absorbance of sample at 620 nm, $\text{Abs}_{510\text{nm}}$ = absorbance of sample at 510 nm, y-intercept = y-intercept of regression, and slope = slope of regression. Standard curves were plotted using a mixture of amylose (A0512, Sigma-Aldrich, St. Louis, MO, USA) and amylopectin (10120, Sigma-Aldrich, St. Louis, MO, USA).

5.2.7 Differential Scanning Calorimetry

Approximately 30 mg of the starch-MCP suspension (2 % w/w starch and 0 – 5 % w/w MCP) was weighed into an aluminium pan. The pan was sealed and equilibrated to 20°C in the DSC chamber (Q2000 DSC, TA Instruments, New Castle, DE, USA). The temperature was then increased to 95°C at 10°C/min and held at 95°C for 5 minutes before being cooled to 20°C at 10°C/min. An empty

aluminium pan was used as the reference. The onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) and heat enthalpy were recorded and analysed using TA software (TA Universal Analysis 2000, TA Instruments, New Castle, DE, USA).

5.2.8 Statistical analysis

All measurements were done in three replicates. Means, standard deviations and curve fits were calculated using Excel spreadsheets (Microsoft Excel, Microsoft Corp., Redmond, WA, USA) and graphs were plotted using SigmaPlot (Systat Software Inc., San Jose, CA, USA). To find differences among the gelatinisation temperatures of starch-MCP suspensions, one-way analysis of variance (ANOVA) was performed with Tukey's Honest Significant Difference test as the post-hoc test using Minitab (Minitab 16, Minitab Inc., Sydney, NSW, Australia).

5.3 Results and discussion

5.3.1 *Mesona chinensis* powder composition

The composition of the raw *Mesona chinensis* powder determined in this study is shown in Table 5-1 alongside previously reported data. The result obtained from this study varied slightly from that previously reported by Feng *et al.* (2014) and Feng et al. (2010b). This could be due to the difference in the raw materials used (*Mesona chinensis* vs. *Mesona blumes*).

Table 5-1 Composition of raw *Mesona chinensis* powder.

Chemical composition		This study (g/g)	(Feng *et al.*, 2010b) (g/g)	(Feng *et al.*, 2014) (g/g)
Ash		0.282	0.309	0.309
Protein		0.090	0.097	0.097
Fat		0.001	-	0.008
Carbohydrate	Total	0.582	0.422	-
	Starch	0.011	-	-
	Sugars	0.092	-	-
	Non-starch polysaccharide	0.479	-	-
	Fibre	-	0.030	0.030
Moisture content		0.047	0.142	0.051

5.3.2 Starch pasting behaviour

The pasting and viscoelastic behaviour of wheat starch (2 % w/w) in the presence of various concentrations of MCP is shown in Figure 5-1. It is important to note that the flow behaviour of 10 % w/w solution exhibited Newtonian profile with a relatively low viscosity of ~1.7 mPa.s at 20°C. Pasting of pure MCP solutions resulted in neither an increase in viscosity during heating nor development of viscoelasticity during cooling (Figure 7-4). As observed in Figure 5-1, the low viscosity of MCP had little contribution to the suspension viscosity at the beginning of the pasting cycle when the temperature was 20°C. The presence of MCP, however, becomes important as the suspension was heated. At 95°C, the viscosity of the starch suspension was 9.0 mPa.s in the absence of the MCP but almost 4 times greater at 35.7 mPa.s when 5 % w/w MCP was present.

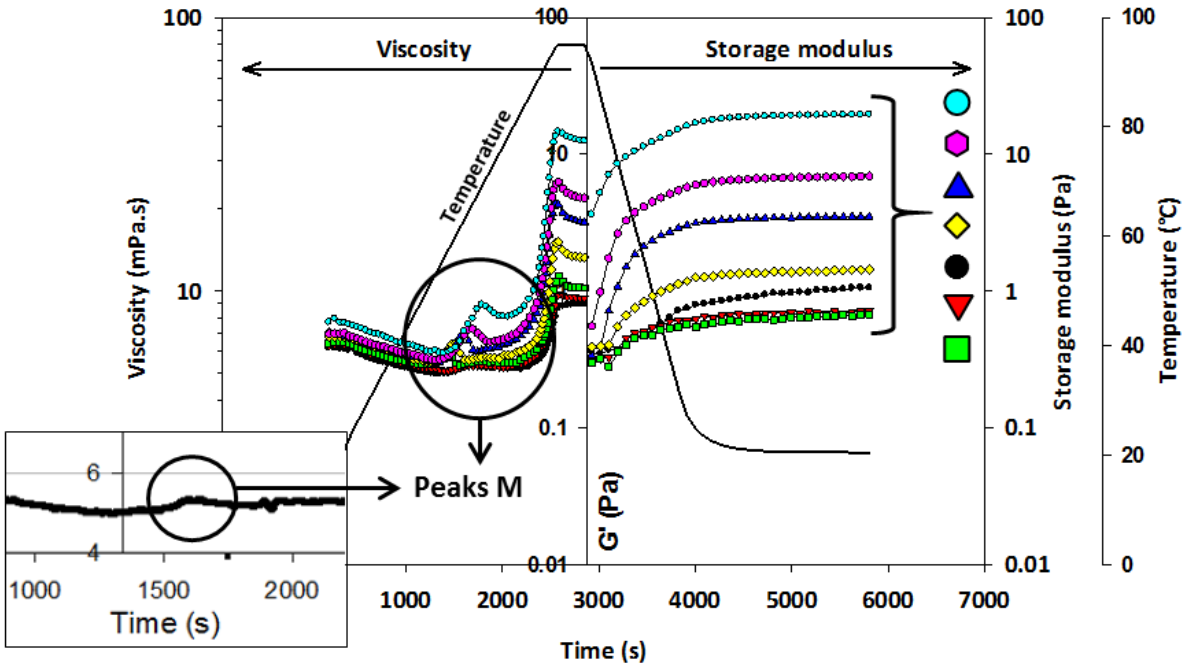

Figure 5-1 Pasting curve of wheat starch suspension (2 % w/w) containing 0 (●), 0.1 (▼), 0.5 (■), 1 (◆), 2 (▲), 3 (⬢) and 5 (●) % w/w MCP. The left side of the graph represents change in viscosity during heating, and the right side of the graph represents storage modulus recorded during cooling under small strain oscillation. Insert image: Peak "M" of wheat starch alone.

The viscosity of the wheat starch suspensions increased in two phases during heating whereby a small concentration dependent transient peak (peak "M") was initially detected at about 60°C followed by a rapid increase at about 86°C (Figure 5-1). The distinction between these two phases became more pronounced when MCP was added and as its concentration was increased. The onset of peak "M" decreased from about 60°C to 51°C when 0.5 % w/w MCP was present and subsequently increased with increasing MCP concentration, up to 57°C when 5 % w/w MCP was present. The presence of peak "M" has been observed during the pasting of various starches in the presence of *Mesona blumes* extract (Feng *et al.*, 2014), however, no author has discussed the significance of this peak. Regardless, the earlier onset of the increase in viscosity during heating (observed here as peak "M") has been previously reported for starches in the presence of a range of polysaccharides including extracts from yellow mustard, sodium alginate, carboxymethylcellulose, gellan, xanthan, guar gum and *Mesona blumes* (Feng *et al.*, 2014; Liu *et al.*, 2003; Shi & BeMiller, 2002).

The peak viscosity (PV), measured as the maximum viscosity detected during the heating phase (Figure 5-1), increased from 8.62 mPa.s (starch alone) to 37.2 mPa.s in the presence of 5 % w/w MCP. An increase in the PV for starch in the presence of polysaccharides is well known (BeMiller, 2011) and two possible mechanisms have been proposed: (i) an increase in the effective concentration of polysaccharides in the continuous phase due to depletion of water in the continuous phase as the

starch gelatinises (Chaisawang & Suphantharika, 2006), and (ii) physical or chemical interaction between the starch and polysaccharide (Liu *et al.*, 2003) perhaps resulting in an increased effective molecule size and, hence, viscosity.

The gels produced during this study were all very weak due to the low concentrations of starch present, however, the dominance of *G'* over *G''* (Figure 5-1) and the frequency independence of these moduli at frequencies between 0.1 and 1 Hz indicated that the final system was considered in rheological terms as a weak gel (Ross-Murphy, 1995). During cooling, the *G'* of wheat starch gel increased by 3.5 fold from 0.3 Pa at 95°C to 1.1 Pa after 30 minutes at 20°C. Under the same cooling conditions, the presence of 3 % w/w MCP enhanced the increase in *G'* by 14.4 fold (0.5 Pa to 7.2 Pa). Although the increase in *G'* was reduced to 6.9 fold when the concentration of the MCP was increased to 5 % w/w, the final *G'* was the greatest—over 20 Pa compared to 1.1 Pa for the starch gel alone. At low concentrations of MCP (below 0.5 % w/w), despite an increase in PV, the final *G'* values were lower than for starch alone.

5.3.3 Amylose leaching

The amount of amylose leached from wheat starch granules during gelatinisation was determined using dual wavelength iodine binding method. The use of two wavelengths to measure the amount of amylose present corrects for any interference from amylopectin-iodine complex that often leads to an overestimation of amylose content (Kaufman *et al.*, 2015). For wheat starch alone, two phases of amylose leaching were observed (Figure 5-2). However, when MCP was present only one phase was evident. In the absence of MCP, the first phase of amylose leaching for wheat starch occurred at a temperature corresponding to the first onset of viscosity increase that was characterised by peak "M" in Figure 5-1. This further emphasised the importance of peak "M" whereby the interaction between MCP and starch may involve the amylose that leaches out of the starch granules. The absence of the first phase of amylose leaching in the presence of MCP indicates that the polysaccharide delayed or prevented amylose from leaching out of the granules or perhaps sequestered the amylose as it was released in the early stages of starch gelatinisation. As the concentration of MCP was increased to 5 % w/w MCP, the total amount of amylose leached from the wheat starch granules was reduced by about 40 % when the mixture was held at 95^{o}C for 60 minutes (Figure 5-2). The total amount of amylose leached from wheat starch alone after 60 minutes of heating was about 32 %, which was slightly greater than the 28 % previously reported (Kaufman *et al.*, 2015; Swinkels, 1985; Tester *et al.*, 2004). In general, the quantity of amylose leached seemed to reach a plateau between 10 minutes (in the absence of MCP) and 30 minutes (in the presence of MCP) after heating at 95°C.

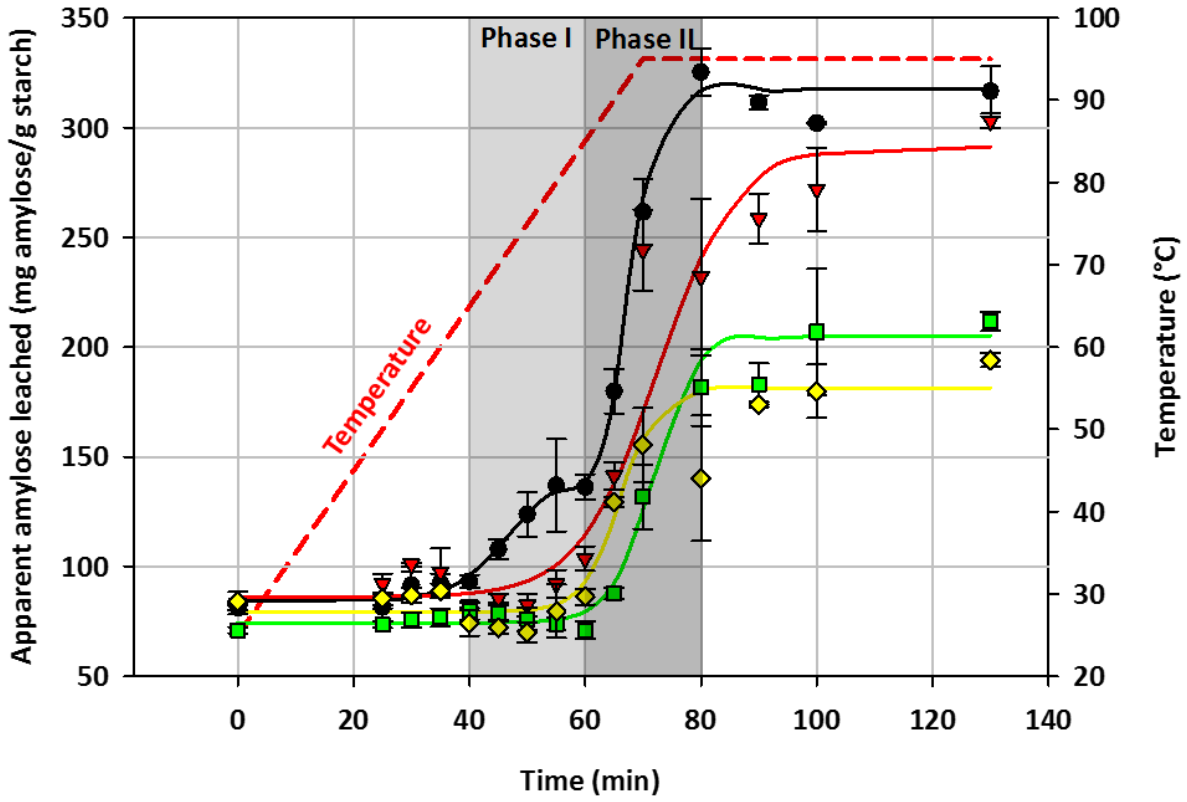

Figure 5-2 Apparent amylose leached (mg amylose/g starch) detected in the supernatant of wheat starch suspension (2 % w/w) containing 0 (●), 2 (▼), 5 (■) and 7 (◆) % w/w MCP with increasing temperature. Values are the mean of three replicates with error bars representing standard deviations.

Increases in PV and decreases in the amount of amylose leached from starch in the presence of polysaccharides have previously been reported (Funami *et al.*, 2005a; Funami *et al.*, 2008; Liu *et al.*, 2003). These were considered to be the result of an increase in the effective polysaccharide concentration in the continuous phase; hence increasing the viscosity and reducing the diffusibility of amylose (Funami *et al*, 2005). This was unlikely to be the case for MCP due to the non-viscous nature of the extract. Alternative explanations concluded that coating of the granules by SSPS or gum Arabic (Funami *et al.*, 2008) or binding of amylose within the granule by yellow mustard mucilage (Liu *et al.*, 2003) reduced amylose leaching from wheat and rice starch.

In the presence of MCP, the earlier onset of viscosity during pasting (peak "M"), the greater PV values (Figure 5-1) and the absence of a first phase of amylose leaching in Figure 5-2 suggest that the polysaccharide may interact with amylose as it is released during heating at or near the granule surface so forming a highly hydrated MCP-amylose complex that increases the overall size of the starch granule. This would cause the viscosity of the suspension to increase and would appear as a shift in the onset of viscosity increase to lower temperatures—since granules would be larger at low

temperatures due to the presence of MCP-amylose coat. With increasing MCP concentrations, transient peak "M" and the onset of viscosity increase shifted to a higher temperature. This is probably due to restricted water movement into the granules in the presence of MCP-amylose complex saturating on the surface of the granules. Hence more energy—higher temperature—would be required to dislodge the complex into the continuous phase and allow water to penetrate into the granules for swelling.

5.3.4 Swelling of wheat starch granules

The swelling of starch granules was investigated using the hot-stage microscope. Clear micrographs of starch granules were obtained up to a temperature of 80°C, after which ghost granules began to crowd and the clarity of the image was reduced drastically. The size of starch granules in the presence or absence of MCP was similar at room temperature, prior to heating up to 90°C on the hot stage of the microscope (see micrographs a.i., b.i. & c.i. in Figure 5-3). However, wheat starch granules aggregated into clusters when MCP was present at all concentrations used in this study. Aggregation happened prior to gelatinisation and granules remained aggregated throughout heating, indicative of some form of phase separation between MCP and wheat starch prior to and during gelatinisation. The nature of the phase separation between wheat starch and MCP (associative or segregative) is unclear. Segregative phase separation as a result of thermodynamic incompatibility between polymers is frequently reported for gelatinised starch and NSP (Alloncle & Doublier, 1991). However, the dark areas around the aggregated granules prior to any heating (Figure 5-3b and c) raise the possibility of the MCP being associated to the granules in some sort of bridging aggregation. While most associative phase separations are the result of electrostatic interactions between two biopolymers, surface adsorption between anionic polysaccharides and hydrophobic protein aggregates have also been suggested (Doublier, Garnier, Renard, & Sanchez, 2000). Therefore, prior to gelatinisation, starch aggregation as a result of (i) the interaction between surface protein of wheat starch and MCP or (ii) some sort of starch-MCP interaction mediated by hydrogen bonds, cannot be excluded. Nevertheless, this starch granule aggregation induced by the presence of mesona seems to be a common phenomenon also observed in other types of starches (see appendix A).

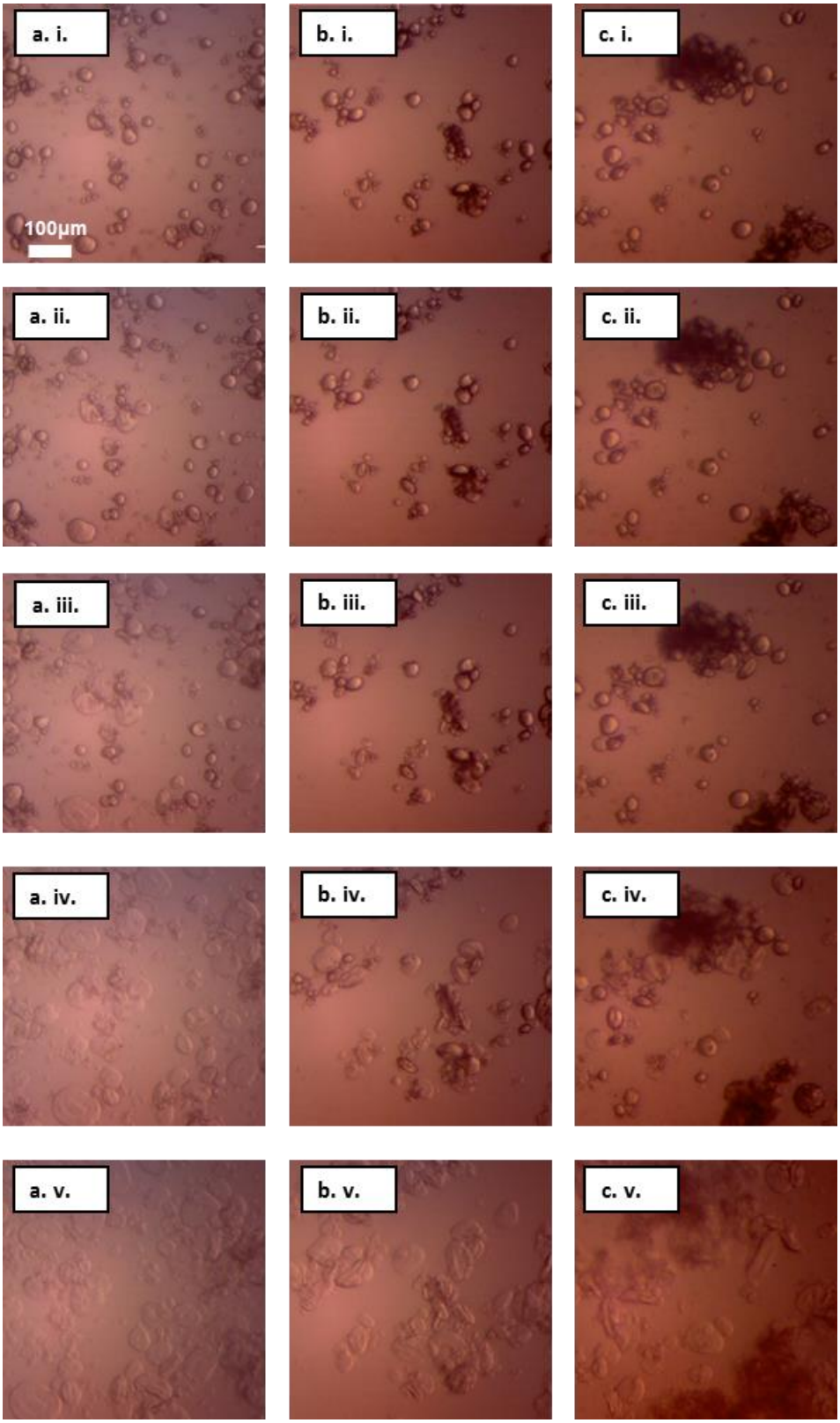

Figure 5-3 Hot stage microscope imaging of wheat starch granules in the presence of 0 (a), 2 (b) and 5 (c) % w/w MCP at temperatures 30°C (i), 70°C (ii), 72°C (iii), 75°C (iv) and 80°C (v). Scale bar on the first image denotes 100µm.

In the absence of MCP, Figure 5-3 showed that swelling occurred at 70°C, a temperature higher than the onset for viscosity increase (Figure 5-1, 60°C) and the onset of amylose leaching (Figure 5-2, 65°C). The higher temperature recorded for the hot-stage microscopy was probably due to greater heat loss i.e. a temperature gradient existed between the sample on the glass slide and the heating element where the temperature probe measures. Nevertheless, the micrographs showed a prominent delay in the overall starch granular swelling when MCP was present in the suspension. At 70°C, no swelling was noticeable with MCP added (see micrographs a.ii., b.ii. & c.ii. in Figure 5-3). The difference in the degree of swelling was clear between 72°C (see micrographs a.iii. versus b.iii. & c.iii.) and 75°C (see micrographs a.iv. versus b.iv. & c.iv.). Most granules were swollen for the sample containing only wheat starch, whereas many remained intact in the presence of the MCP. A delay in starch granular swelling in the presence of polysaccharides such as konjac glucomannan, guar, tara and locust bean gum has previously been reported and was attributed to the polysaccharides' ability to either thicken the continuous phase (Funami *et al.*, 2005a; Krüger, Ferrero, & Zaritzky, 2003) or bind onto water (Tester & Sommerville, 2003) to limit water availability or mobility for granular swelling. In our case, since MCP solution had a relatively low viscosity, restriction of water available for the starch is unlikely. A probable explanation for the retardation of starch granular swelling is the formation of a polysaccharide coating by MCP around the starch granules.

Particle volume fraction determines viscosity in dilute systems such as those used in this work. During starch gelatinisation, the viscosity is largely influenced by granule volume as they swell. In the presence of MCP, granule volume is visually reduced despite an increase in viscosity. This can be explained by an increase in the effective granule volume fraction as a result of (i) coating of the granules by MCP and/or (ii) loose aggregation of the starch granules.

Results from the Mastersizer confirmed that starch granules aggregated in the presence of MCP (Figure 5-3). The filled circle markers (Figure 5-4) represent the mean size of the starch granules prior to and during gelatinisation without prolonged shearing. The mean D (4,3) values of the ungelatinised starch granules in the absence of MCP increased from 21.5 μm to 35.5 μm as they gelatinised and were unaffected by shear as indicated by the open circle markers (mean D (4,3) after shearing for 16 minutes and 20 seconds). These data are considered to represent the mean granule size for wheat starch when not aggregated. The increase in particle size during gelatinisation occurred in two phases beginning at about 55°C with an intermediate plateau at about 75°C followed by a further increase beginning at about 80°C. These two phases of granular swelling have previously been reported (Tester & Morrison, 1990) and may represent the two phase amylose leaching observed in Figure 5-2.

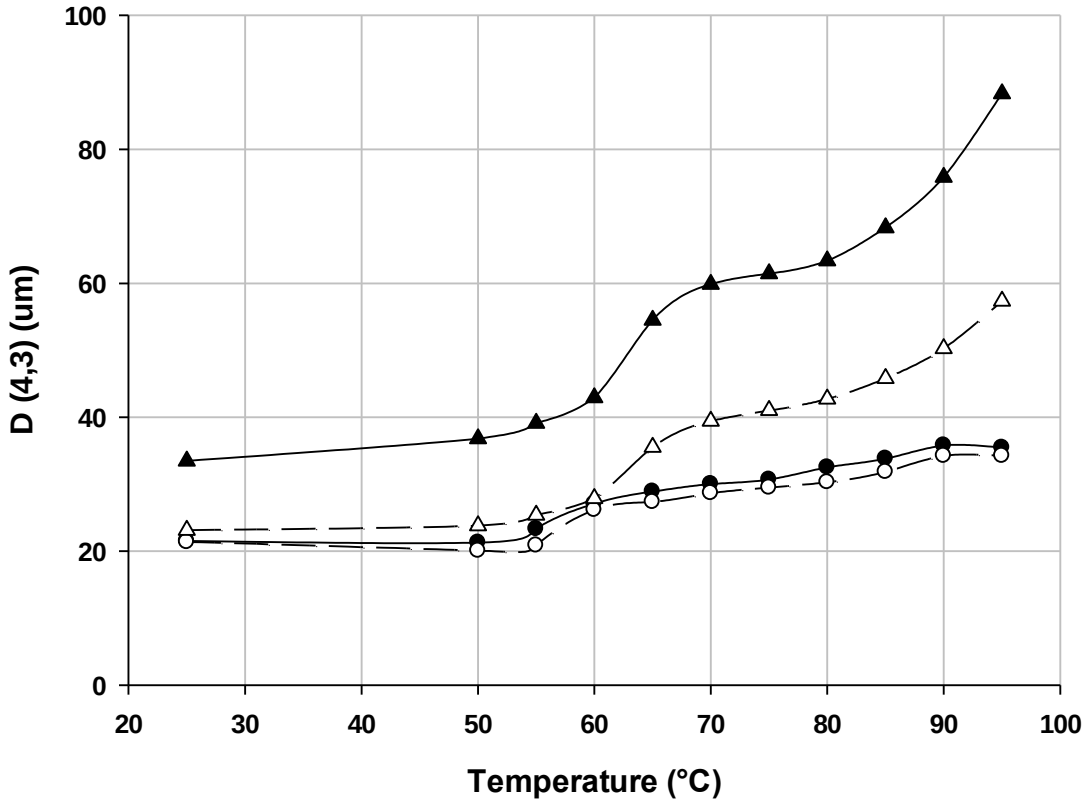

Figure 5-4 Volume weighted mean diameter D (4,3) of wheat starch (2 % w/w) with (○) and without prolonged shear (●) and wheat starch (2 % w/w) + 5 % w/w MCP with (△) and without prolonged shear (▲) during gelatinisation.

The mean particle size increased markedly when MCP was added in the absence of shear (filled triangle symbols), which indicates that either aggregation of particles (Figure 5-4) and/or the presence of a MCP-amylose coat had increased the particle volume. These aggregates could not be broken up using sodium dodecyl sulphate acting as surfactant, but shearing resulted in a decrease in the D (4,3) value. Shearing the suspension containing MCP almost halved mean D (4,3) values (open triangle symbol) suggesting disaggregation of the basic particles and/or removal of the MCP-amylose coat. Further evidence for the disaggregation of particles with shear time is shown in Figure 5-5, whereby the decreases in the size of the aggregates was most prominent in the first 200 seconds of shear, especially for the starch-MCP sample gelatinised at 95°C (Figure 5-5 open squares). This suggests that prior to gelatinisation, the interaction between MCP and wheat starch was weak enough to be at least partially broken up by mechanical force. However, when starch was gelatinised in the presence of MCP, shear could not reduce the D (4,3) values to those of the original starch granule mean size suggesting that a tightly bound component may be present and this could contribute to the solid volume fraction of the suspension. It is also worth noting that starch granules retained their structural integrity when heated up to 95°C. As observed in Figure 5-5 (a.i. and a.ii.), these granules appeared as

ghost granules as they have swelled and released a portion of their polymers. Staining of iodine within the granule indicates retention of starch polymers, amylose and amylopectin, within the ghost granule.

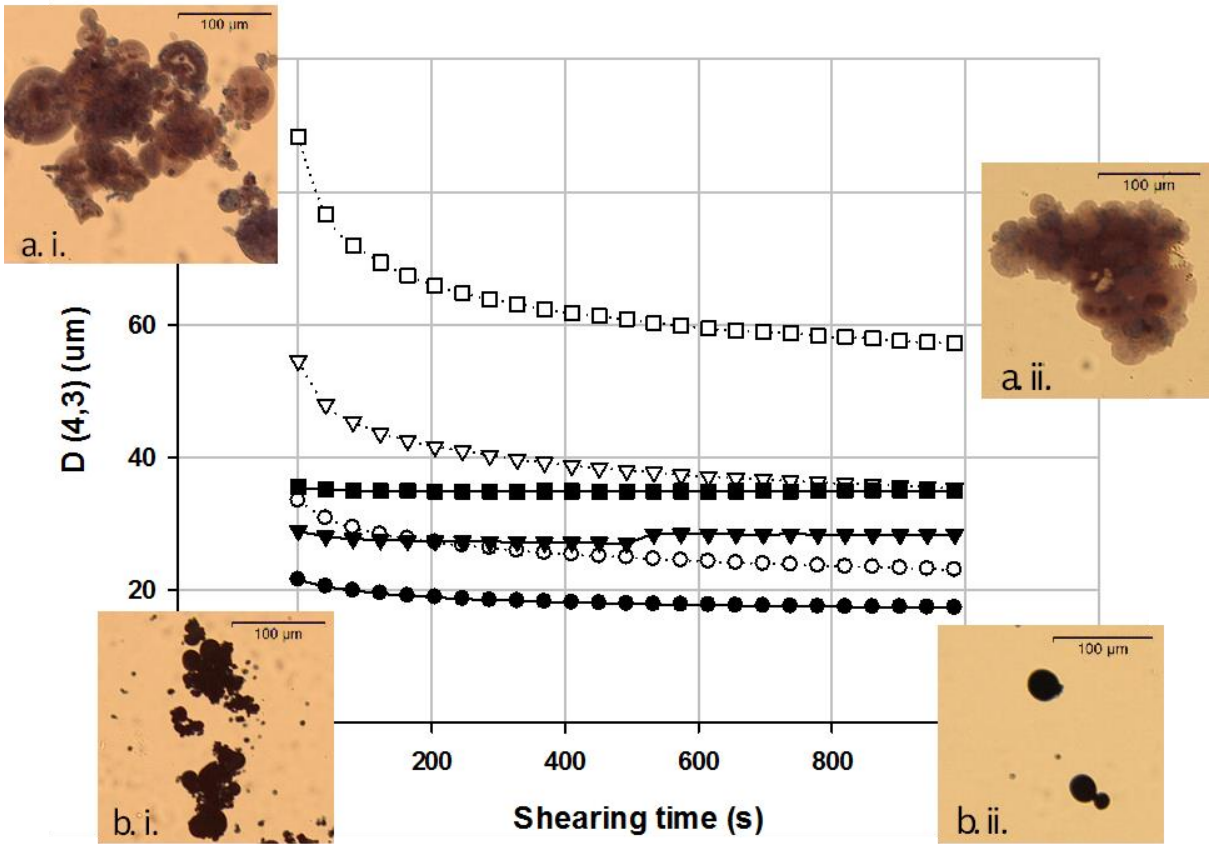

Figure 5-5 Change in the volume weighted mean diameter D (4,3) of wheat starch (2 % w/w) in the absence (filled symbols) and presence of 5 % w/w MCP (open symbols) with prolonged shear at 25°C (●), 65°C (▼) and 95°C (■). Insert images: 2 % w/w wheat starch + 5 % w/w MCP at 95°C (a) and 25°C (b) before (i) and after (ii) shearing at 3000 rpm for 16 minutes and 20 seconds. Granules were stained with iodine for visualisation under the light microscope.

5.3.5 Differential Scanning Calorimetry

Gelatinisation of wheat starch alone began (T_o) at 54.8°C and is within the range (51 – 57°C) previously reported (Ghiasi, Hoseney, & Varriano-Marston, 1982; Jenkins & Donald, 1998; Ratnayake & Jackson, 2007). As increasing concentrations of MCP were added to the starch, all of the temperatures used during calorimetry to characterise gelatinisation (onset, T_O; peak, T_P; and conclusion, T_C) increased slightly but consistently (Table 5-2). However, the endotherm ΔH (J/g) was similar for all samples indicating that the energy ΔH required to disrupt the semi-crystalline amylopectin structure is not significantly affected by the addition of MCP. The slight increase in the starch gelatinisation activation temperatures in the presence of MCP is not uncommon for a starch-polysaccharide system (Khanna

& Tester, 2006; Yoshimura, Takaya, & Nishinari, 1996; Zhou, Wang, Zhang, Du, & Zhou, 2008). This has been attributed to the ability of polysaccharides to imbibe water, reducing its availability for starch gelatinisation (Yoshimura *et al.*, 1996). While this may be the case for MCP as well, it does not rule out the possibility of the formation of MCP-amylose barrier around the starch granules that limits water mobility into the granules. A decrease in T_o for rice starch in the presence of 0.5 % w/w *Mesona blumes* gum from 66.6 to 61.2°C has been reported (Feng *et al.*, 2012). This seems to be due to differences in the interaction between wheat and rice starch with MCP. In support, Feng *et al.* (2010a) have reported that suspensions containing 6 % w/w wheat starch and 0.1 – 0.5 % w/w *Mesona blumes* gum had greater *G'* than similar suspensions with rice starch.

Table 5-2 The gelatinisation temperatures and enthalpy of wheat starch in the presence of increasing MCP concentration as obtained from Differential Scanning Calorimetry. Values are the mean ± standard deviation of n = 3; different letters within a column represents significant differences (P ≤ 0.05).

MCP (% w/w)	Onset temp T_O (°C)	Peak temp T_P (°C)	Conclusion temp T_C (°C)	Enthalpy ΔH (J/g)
0	54.8 ± 0.4 ^{a}	61.1 ± 0.9 ^{a}	67.2 ± 0.1 ^{a}	13.2 ± 1.4 ^{a}
0.2	55.4 ± 0.3 ^{ab}	61.3 ± 0.4 ^{a}	68.3 ± 0.2 ^{b}	13.4 ± 0.8 ^{a}
1.1	56.5 ± 0.4 ^{bc}	62.9 ± 0.2 ^{b}	69.5 ± 0.3 ^{c}	13.1 ± 0.6 ^{a}
2.3	57.2 ± 0.7 ^{cd}	63.3 ± 0.1 ^{b}	70.3 ± 0.1 ^{d}	13.5 ± 0.6 ^{a}
4.5	57.8 ± 0.6 ^{de}	64.0 ± 0.4 ^{bc}	70.8 ± 0.1 ^{de}	13.8 ± 0.3 ^{a}
6.8	58.1 ± 0.2 ^{de}	64.9 ± 0.3 ^{cd}	71.4 ± 0.3 ^{ef}	13.3 ± 0.2 ^{a}
11.3	58.7 ± 0.4 ^{e}	65.8 ± 0.3 ^{d}	72.1 ± 0.5 ^{f}	13.7 ± 1.4 ^{a}

5.3.6 Mechanisms of wheat starch and *Mesona chinensis* polysaccharide interaction

While the presence of MCP increased the final *G'* of wheat starch gels (Figure 5-1), the total amount of amylose leached was reduced (Figure 5-2). Amylose is the key polymer contributing to gel formation when gelatinised non-waxy starches are cooled (Lii, Tsai, & Tseng, 1996). In order for amylose to form a cross-linked network, a critical coil overlap concentration of 1.5 % has been reported (Miles (1985) as cited in Biliaderis (1991)). At low concentrations of MCP, the reduction in the amount of amylose leached could hinder the formation of a network resulting in a decrease in the *G'* of the starch gel. However, at high MCP concentration, the increase in *G'* was probably a result of the association

between the MCP and the leached amylose to create an amylose-MCP network with the starch granules entrapped within. It is well established that the viscosity of a dilute suspension is dependent on the volume fraction of solids that it contains. Hence, the increase in viscosity of a suspension containing starch could imply an increase in the volume fraction of starch granules present. In the presence of MCP, starch granule swelling appears to be reduced (Figure 5-3). However, the overall increase in viscosity could indicate an association between the MCP molecules and ghost granules leading to the formation of some network between the two. Furthermore, at the temperature at which amylose leached from the starch granules, the presence of MCP causes increases in viscosity and at sufficiently high concentration of MCP, formed a weak gel.

At all MCP concentrations used in this work, starch granules tended to aggregate when the polysaccharide was present, although the aggregates could be partially broken up by shear. Effects such as depletion due to excess xanthan gum have previously been proposed (Abdulmola *et al.*, 1996; Achayuthakan & Suphantharika, 2008). However, in the case of MCP, aggregates were seen at concentrations as low as 0.005 % w/w MCP suspension (see Appendix A) and no critical MCP concentration for starch aggregation was detected, making a depletion effect unlikely. An alternative mechanism as mentioned earlier could be bridging of starch granules by mesona via hydrogen bonds. While previous studies have focused on the interaction between MCP and amylose (Lai & Chao, 2000b), the results of the present study suggest the involvement of the starch granules themselves in wheat starch-MCP interactions.

The interaction between MCP and wheat starch granules became stronger as gelatinisation proceeded. Prior to gelatinisation at about 54°C, the MCP mediated starch aggregates could be broken down with shear in the Mastersizer. However, at temperatures greater than 60°C, aggregate size D (4,3) of wheat starch granules in the presence of MCP was always greater than that of wheat starch alone. Furthermore, at about 60°C, peak “M” was observed in Figure 5-1, which corresponded to the onset of amylose leaching in Figure 5-2. The release of amylose appeared to facilitate interaction of the granules with MCP so creating stronger aggregates. This signified the importance of peak “M” marking the onset of interaction between MCP and starch granules prior to the detection of the rapid viscosity increase. MCP may interact with amylose either by diffusing into the starch granules during hydration—though this cannot explain granule aggregation—or as the amylose leaches from the granules during heating. The data presented in this work supports the second hypothesis as the onset of peak “M”, the absence of the first phase of amylose leaching and the increase in D (4,3) of starch granules in the presence of MCP can be explained by initial weak binding of MCP at the surface of the starch granules followed by the development of a stronger more extensive network as amylose leaches out from the granules. Additionally, as the concentration of MCP was increased, the amount

of free amylose detected in the suspension decreased (Figure 5-2); this indicates that MCP associated with amylose on the granule surface and prevented it from leaching out of the granules—being these MCP-coated granules sedimented during centrifugation. Therefore, it is proposed that MCP interacted with amylose chains as they emerged from the granules, resulting in the formation of a network of aggregated granules interconnected by MCP and amylose.

The concentration of MCP is important in determining the characteristics of starch gels containing the polysaccharide. The addition of an increasing MCP concentration resulted in an increase in PV, which can be explained by the presence of heterogeneous clusters of ghost granules acting as larger particles. However, the final *G'* of the gel (or gel strength) seemed to be affected by the concentration of MCP present in the system. At high concentrations of MCP (above 1 % w/w), an extended network may be formed throughout the suspension as MCP interacts with amylose from different granules, creating an interlinked network of amylose, MCP and ghost granule clusters. This would result in the formation of a stronger gel despite a reduction in the amount of leached amylose detected. At low MCP concentrations (below 0.5 % w/w), the final *G'* of the MCP-starch gels was lower than for starch gels alone, suggesting the presence of a less extensive network. This is possibly due to: (i) a decrease in the amount of amylose leached as MCP binds onto the amylose on the granule surface—hence insufficient amylose is available to form a stronger 3D network and (ii) insufficient MCP available to interlink ghost granule clusters. So ultimately, MCP would be acting as a disrupting agent by preventing amylose connections, therefore decreasing the gel strength. All of this means that the effect of MCP on the final gel strength (*G'* values) is dependent on two inversely related factors: the total MCP available for interaction and a reduction in the total amount of amylose leached. The proposed mechanism between wheat starch and MCP is schematically represented in Figure 5-6.

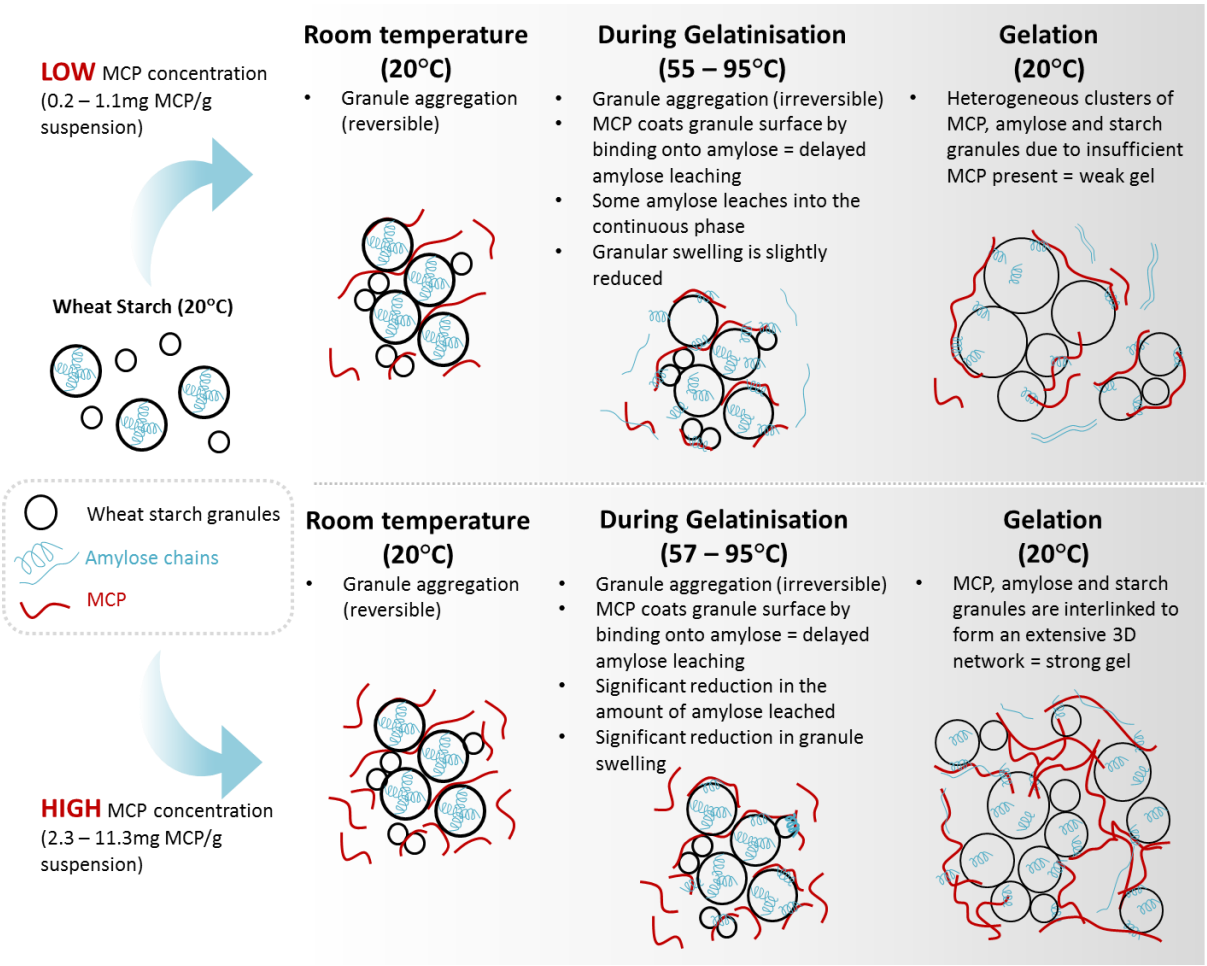

Figure 5-6 Schematics for the mechanism of interaction between wheat starch and high and low concentrations of *Mesona chinensis* polysaccharide.

5.4 Conclusion

This study has shown that at room temperature, interactions between starch and MCP were consistent with a weak aggregation of starch granules. When gelatinised, and particularly at the point when amylose was shown to leach from the granules, the viscosity of the suspension increased, providing evidence of a stable amylose-MCP complex that may have increased the effective volume of solids in suspension. The reduced swelling of starch granules argues for possible binding between starch and MCP on the granule surface, resulting in restricted granule swelling during gelatinisation. Binding of MCP at the starch granule surface prior to gelatinisation results in small increases in viscosity and much larger increases as amylose leaching occurs. On cooling of the hot pastes, the presence of MCP above 1 % w/w results in the formation of much stronger gels (>> *G'*) than for wheat starch (2 % w/w) alone due to the formation of an extensive 3D network. However, at low concentrations of MCP, the final gel strength was weaker compared to wheat starch alone since insufficient MCP was available to form an extensive network with amylose and granule clusters. These results are important in determining how MCP functions to increase viscosity and gel strength, which may allow the formulation of food products containing less starch. Since MCP has the ability to increase gel strength while restricting granule swelling, it may have the potential to decrease the digestibility of fully gelatinised starch and hence assist in reducing glycaemia when starchy foods are consumed.

Chapter 6 [3]Rheological, textural, microstructural and retrogradation properties of wheat starch-MCP gels

6.1 Introduction

Non-starch polysaccharides are often added to starchy foods to increase freeze-thaw stability, provide a higher viscosity, reduce starch digestibility or to decrease starch retrogradation (Brennan *et al.*, 1996; Funami *et al.*, 2005b; Lee *et al.*, 2002; Yoshimura *et al.*, 1996). These properties are determined by the type and concentration of polysaccharide and starch used, as well as by the interactions between both polymers.

Mesona chinensis polysaccharide (MCP) is an anionic plant polysaccharide found in the extract of the herb *Mesona chinensis,* and is widely used in Asian countries to prepare a gel dessert known as "grass jelly". The crude extract of *Mesona chinensis* synergistically increases the strength of wheat starch gels (Lai *et al.*, 2003) although the mechanism for this is not well understood. The extract itself consists of two fractions of MCP, one neutral and one acidic. Both of these polysaccharides are composed of galactose, glucose, mannose, xylose, arabinose and rhamnose, with galacturonic acid present only in the acidic MCP. The structure of acidic MCP consists of α-(1→4)-galacturonan backbone with insertions of α-1,2-Rhap residues on which galactose, glucose, mannose, xylose, arabinose, rhamnose and galacturonic acid are attached to (Feng *et al.*, 2008)—a structure that is similar to that of the rhamnogalacturonan I found in pectin, which has a backbone made of repeating units of rhamnose and galacturonic acid, with neutral sugars side chains substituted at the rhamnose residues (Voragen, Coenen, Verhoef, & Schols, 2009). The presence of MCP can increase the viscosity of starch pastes and the strength of wheat starch gels as the starch is gelatinised. Thermodynamic incompatibility resulting in phase separation between the MCP and starch glucan polymers (Lai *et al.*, 2003) as well as starch-MCP interactions via hydrogen bonds (Feng *et al.*, 2012) have been suggested as possible mechanisms by which MCP increases the viscosity of wheat starch pastes. Unlike many other polysaccharides, MCP is unique because it neither is viscous nor forms a gel upon heating and cooling at a concentration range of 0.1 to 10 % w/w. Therefore, increases in viscosity of the starch paste in the presence of MCP

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cannot be explained by just an increase in MCP concentration in the continuous phase for a model invoking phase separation.

The strength of a starch gel is influenced by several factors including the solid volume fraction and rigidity of the gelatinised granules, the rheological properties of the continuous phase and the interaction between the granules and the continuous phase (Savary, Handschin, Conde-Petit, Cayot, & Doublier, 2008). Since MCP affects the gelatinisation of starch granules and the formation of the amylose matrix in the continuous phase, its presence alters the rheological and textural properties of starch gels depending on the concentration of both MCP and starch (Lai *et al.*, 2003; Yuris, Goh, Hardacre, & Matia-Merino, 2017). We have previously shown that a reduction in the amount of amylose leached and restriction in starch granular swelling occur when native wheat starch is gelatinised in the presence of MCP (Yuris *et al.*, 2017). The effect of MCP and starch concentrations on the properties of the composite gel has only been studied at low starch concentrations (< 4 % w/w) (Lai *et al.*, 2003; Yuris *et al.*, 2017), where the viscosity of the paste is largely determined by the volume fraction of the starch granules. However, the effect of MCP in the presence of higher starch concentration gels has not been previously reported.

The presence of polysaccharides influences starch retrogradation and syneresis properties (Ferrero, Martino, & Zaritzky, 1994; Katsuta, Miura, & Nishimura, 1992; Lee *et al.*, 2002). Syneresis occurs in freeze-thawed starch gels as a result of an increase in amylose associations and ice crystal formation (Charoenrein *et al.*, 2011; Ferrero *et al.*, 1994). A decrease in starch syneresis has been reported in the presence of guar, sodium alginate, xanthan, locust bean gum (LBG) and konjac glucomannan (Charoenrein *et al.*, 2011; Funami *et al.*, 2005a; Lee *et al.*, 2002). This was thought to be due to: (i) retardation of amylose retrogradation—due to polysaccharide-amylose associations rather than amylose-amylose interactions (Ferrero *et al.*, 1994) and/or (ii) retardation of ice crystal formation—due to polysaccharides immobilising water molecules (Lee *et al.*, 2002). On the other hand, retrogradation studies using DSC (often associated with the long-term amylopectin retrogradation) have shown contradicting results. Some non-starch polysaccharides (NSPs) such as guar gum, tara gum, locust bean gum and konjac glucomannan (Funami *et al.*, 2005b) have been shown to accelerate retrogradation while others like xanthan, tea polysaccharides and carboxymethyl cellulose delays retrogradation (Lee *et al.*, 2002; Zhou *et al.*, 2008). To the best of the authors' knowledge, there are no studies on the retrogradation and syneresis of wheat starch gels in the presence of MCP.

From all of the above, it is clear that there are no studies that have been carried out on : (i) the effect of MCP on the final viscoelastic polymer network at gel-forming starch concentrations and (ii) the effect of MCP concentration on the retrogradation properties of wheat starch. This study utilised a combination of techniques including rheology, microscopy and differential scanning calorimetry with

the aim to characterise the rheological, textural, microstructural and retrogradation properties of these gels.

6.2 Materials and methods

6.2.1 Preparation of starch-MCP suspension

Wheat starch-MCP suspension was prepared according to section 5.2.2. The *Mesona chinensis* powder was analysed by an accredited chemical laboratory (Massey University Nutritional laboratory, Palmerston North, New Zealand) and by the Ferrier Research Institute, Wellington, New Zealand. It was found to contain 47.9 % non-starch polysaccharide [GalA (59.1 %), Gal (11.6 %), Glc (8.7 %), Rha (6.2 %), Ara (4.5 %), Man (3.6 %), Xyl (3.0 %), GluA (1.9 %) and Fuc (1.5 %)], 28.2 % ashes (K, Mg, P, Ca, Na, Fe and traces of Cu, I, Mn, Zn and Se), 9.0 % protein, 9.2 % free sugars (mainly mono and disaccharides), 1.1 % starch, 4.7 % moisture and 0.1 % fat.

6.2.2 Rheological measurements

6.2.2.1 Gelatinisation and gelation parameters

Studies on the gelatinisation and gelation properties of starch-MCP mixtures were carried out as per section 5.2.3 using a controlled-stress rheometer (MCR302, Anton Paar Physica, Graz, Austria) fitted with a starch cell (C-ETD160/ST) and spindle (ST24-2D/2V/2V-30). Measurements were carried out in triplicate.

6.2.2.2 Addition of MCP solution to wheat starch suspension during gelatinisation

Wheat starch suspensions (6.25 % w/w) and MCP solutions of 5 and 15 % w/w were prepared separately. Gelatinisation of 16 mL of the wheat starch suspension was carried out in the rheometer. As the starch suspension was heated to 95°C, 4 mL of either water or MCP solution were added at various temperatures to bring the final concentration of the mixture to 5 % w/w wheat starch containing either water, 1 % w/w MCP or 3 % w/w MCP. MCP or water was added at the start of the cycle in the rheometer when the temperature was 20°C, prior to the onset of gelatinisation (50°C), at the onset of gelatinisation (55°C), during gelatinisation (60, 75 and 90°C) and at the peak temperature (95°C). All additions were carried out under constant rotational shear to ensure even mixing of the suspension. During the rotational phase (shear rate of 100/s), apparent viscosity was recorded and during small strain oscillation (1 % strain and 1 Hz frequency), *G′* was recorded. Measurements were carried out in triplicate.

6.2.3 Textural measurements

6.2.3.1 Gel preparation

Starch suspensions (5 % w/w, 8 % w/w and 10 % w/w) containing between 0 – 5 % w/w MCP (25 mL) were placed in an RVA cup (Rapid Visco Analyser, Perten Instruments, Massachusetts, USA) and dispersed at a vane speed of 960 rpm for 1 minute at 25°C. The speed was then decreased to 160 rpm and the suspension equilibrated at 25°C for 2 minutes. The starch was gelatinised by heating the suspension to 95°C at a rate of 2°C/min and was then held at 95°C for 5 minutes. The hot paste was immediately removed from the RVA and poured into a cylindrical mould with a diameter of 3.2 cm and height of 2 cm. Samples were then stored overnight (16 h) at 20°C prior to texture analysis.

6.2.3.2 Compression test

The gels were removed from their moulds and equilibrated at room temperature for 30 minutes. They were then were then subjected to a single compression cycle test using the TA.XT plus texture analyser (Stable Micro Systems, England). The test was carried out using a 61 mm plastic cylindrical probe and a load cell of 50 kg. The pre-test and post-test speeds were set at 1 mm/s and 5 mm/s respectively. The target deformation was set to 50 % of the gel's initial height with a trigger force of 0.05 N. Measurements were carried out in triplicate. The hardness of the gels was defined by the peak force obtained after the compression.

6.2.4 Granule size measurements

Suspensions containing 1 % w/w wheat starch and 0 – 5 % w/w MCP (40μL) were placed in wells made out of two self-adhesive rings that were stuck onto microscope slides. Cover slips were placed over the samples and the edges were sealed with nail polish to minimise evaporation during heating. The slides were heated up to 95°C at a rate of 5°C/min using the hot stage microscope (HM-Lux, Leitz Wetzlar, Wetzlar, Germany) fitted with a digital camera (ScopeTek MDCC200). Starch granular swelling was recorded using ScopePhoto (Version 3.1.268, ScopeTek) and images of the granules at specific temperatures were obtained from the recording. Twenty five starch granules were randomly selected and their diameters were measured manually throughout the heating process.

6.2.5 Amylose leaching

The amount of amylose leached out of 2 % w/w wheat starch during gelatinisation was measured as per section 5.2.6.

6.2.6 Scanning electron microscopy

Wheat starch (5 % w/w and 10 % w/w) gels containing 0 – 5 % w/w MCP were prepared in the starch cell (section 3.3.1). The gels were removed from the rheometer once they had been cooled to 20°C and then were suspended in a modified Karnovsky's fixative (3 % gluteraldehyde and 2 % formaldehyde in 0.1 M phosphate buffer, pH 7.2) for at least 12 hours at room temperature. The fixative was then removed and the samples were washed 3 times (10 – 15 minutes each) with phosphate buffer (0.1 M, pH 7.2) followed by dehydration in graded ethanol series (25 %, 50 %, 75 %, 95 %, 100 %) for 10 – 15 minutes each and a final 100 % ethanol wash for 1 hour. All of the samples were centrifuged for 2 minutes at 1075 g between each ethanol dehydration step to condense the sample and prevent sample loss as the waste ethanol was removed. At each ethanol dehydration step, the samples were resuspended to allow maximum contact with the solvent. Samples were further dried to their critical point (CP) using liquid CO_2 as the CP fluid, and 100 % ethanol as the intermediary fluid (Polaron E3000 series II critical point dryer, Quorum technologies, England). Samples were fractured to expose the inside of the gel and mounted on to aluminium stubs using double sided tape. They were then sputter coated to a thickness of approximately 100 nm with gold (Baltec SCD 050 sputter coater) and viewed using the FEI Quanta 200 Environmental Scanning Electron Microscope (Oregon, United States) at an accelerating voltage of 20 kV.

6.2.7 Retrogradation studies

6.2.7.1 Syneresis measurement

The syneresis of starch gels (10 % w/w) in the presence and absence of MCP was measured according to the method of Charoenrein *et al.* (2008) with modifications. Hot starch paste was prepared as in section 3.4.1, poured into a 15 mm diameter cylinder and left to set for 2 h. The cooled gel was then cut into cylindrical blocks of approximately 1 cm long, weighing approximately 2 g. The gel was then placed into a cylindrical tube that is attached onto the cap of a centrifuge tube (Figure 3-12). The cylinder was then covered with a perforated plastic film that is lined with a filter paper. The setup was then frozen at -18°C for 22 h prior to thawing to room temperature over 2 h and then centrifugation at 100 g for 15 minutes. Water that is released from the gel was absorbed by the filter paper that prevents reabsorption into the gel and collected at the bottom of the centrifuge tube. The water loss (syneresis) was determined by measuring the weight of the gels before and after the freeze-thaw cycle and the percentage of syneresis was calculated as follows:

$$\% \text{ syneresis} = (W_g - W_{ft})/ W_g \times 100$$

Where W_g = weight of gel before the freeze-thaw cycle and W_{ft} = weight of gel after centrifugation following freeze-thaw cycles. The freeze-thaw cycle was repeated 5 times for each gel sample and syneresis determined for each cycle. Measurements were done in triplicate.

6.2.7.2 Differential scanning calorimtery (DSC)

Starch suspensions (25 % w/w) containing 0, 1 and 5 % w/w MCP were prepared as per section 5.2.2. Using an autopipetter, approximately 30 µL of the suspension was weighed into a Tzero hermetic aluminium pan (TA Instruments, Delaware, United States). The pan lid was fitted and hermetically sealed before temperature cycling in the DSC. The sample and reference (empty) pans were placed in the DSC Q2000 (TA Instruments, Delaware, United States) and equilibrated at 20°C for one minute before increasing the temperature to 110°C at 10°C/min and then lowering it back to 20°C at the same rate. The pans were then stored at 4°C for 5, 7 and 14 days before rescanning them with the same temperature profile. During the temperature cycle thermal energy flow was measured and the magnitude of these endotherms was used to estimate the degree of retrogradation, which was calculated as:

$$Retrogradation\ ratio = \frac{\triangle H_2}{\triangle H_1}$$

Where ΔH_1 = gelatinisation enthalpy in the first scan and ΔH_2 = regelatinisation enthalpy in the second scan up to 14 days after the first scan. Duplicate were measured for each cycle.

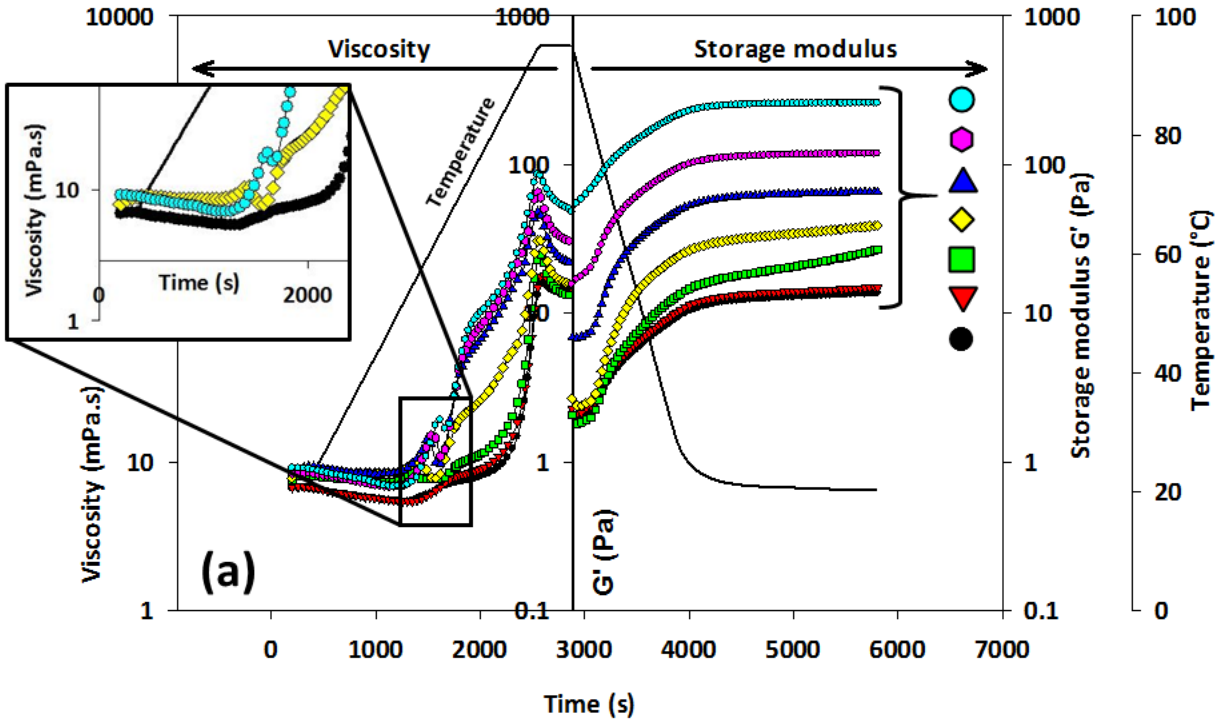
6.2.8 Statistical analysis

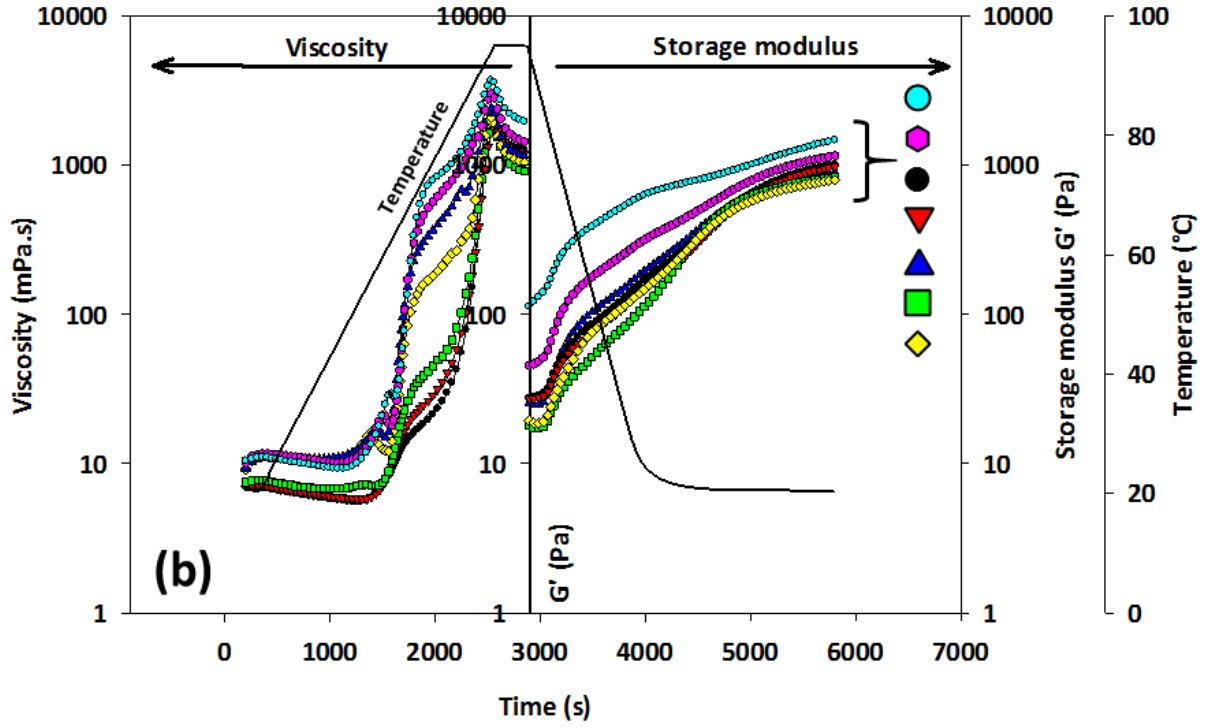
An analysis of variance (ANOVA) with Tukey's range test as the post-hoc test was performed using Minitab 16.

6.3 Results and discussion

6.3.1 Pasting, gelation and granular properties of wheat starch and MCP

The pasting properties of wheat starch gels in the presence of increasing concentrations of MCP are shown in Figure 6-1. Three starch concentrations were used in this study to form set gels and these ranged from low (5 % w/w) (Figure 6-1a), to medium (8 % w/w) (Figure 6-1b) to high (10 % w/w) (Figure 6-1c). For all of the starch concentrations, increases in the peak viscosities (PV) of the starch pastes were observed with increasing MCP levels. The highest apparent peak viscosity (over 7000 mPa.s) was obtained at maximum quantities, when 10 % w/w wheat starch was pasted in the presence of 5 % w/w MCP. The increase in starch PV in the presence of a non-starch polysaccharide (NSP) has been reported for many other NSPs such as guar gum, tara gum, locust bean gum and konjac glucomannan (Funami *et al.*, 2005b). All of the reported NSPs are either viscous or form a gel upon heating, hence a common explanation for this effect is an increase in the polysaccharide concentration in the continuous phase as starch gelatinises (Alloncle & Doublier, 1991; Funami *et al.*, 2005b). However, MCP on its own neither forms a gel upon heating nor is viscous. Therefore, it is unlikely that the extent of increase in the PV of wheat starch in the presence of MCP was due to the viscosity contributed by the MCP. An alternative explanation was proposed in the previous chapter, whereby it was suggested that MCP interacted with amylose as it leached out of starch granules to form an amylose-MCP coat around the granules. This was thought to increase the volume fraction (influenced by granule size and rigidity) of starch granules, which resulted in an increase in the PV of the starch pastes. In the present study, the effect of MCP on the PV of increasing concentrations of wheat starch was also compared. The largest magnitude of increase in PV was observed at the lowest starch concentration of 5 % w/w, whereby the PV of the starch pastes increased by 5.4 times, which was then followed by 2.1 times and 1.7 times for 8 % w/w and 10 % w/w wheat starch respectively. This appeared to indicate that with increasing starch concentrations, MCP's contribution towards increasing the apparent peak viscosity decreases. This can be explained by the fact that at higher starch concentrations (≥8 % w/w), the apparent viscosity of a starch paste becomes less affected by the starch volume fraction but increasingly dependent on the interactions between leached polymers and starch granules (Okechukwu & Rao, 1995).


Viscosity
Storage modulus
Temperature
Viscosity (mPa.s)
Time (s)
G' (Pa)
Storage modulus G' (Pa)
Temperature (°C)
(a)


Viscosity
Storage modulus
Temperature
Viscosity (mPa.s)
Time (s)
G' (Pa)
Storage modulus G' (Pa)
Temperature (°C)
(b)

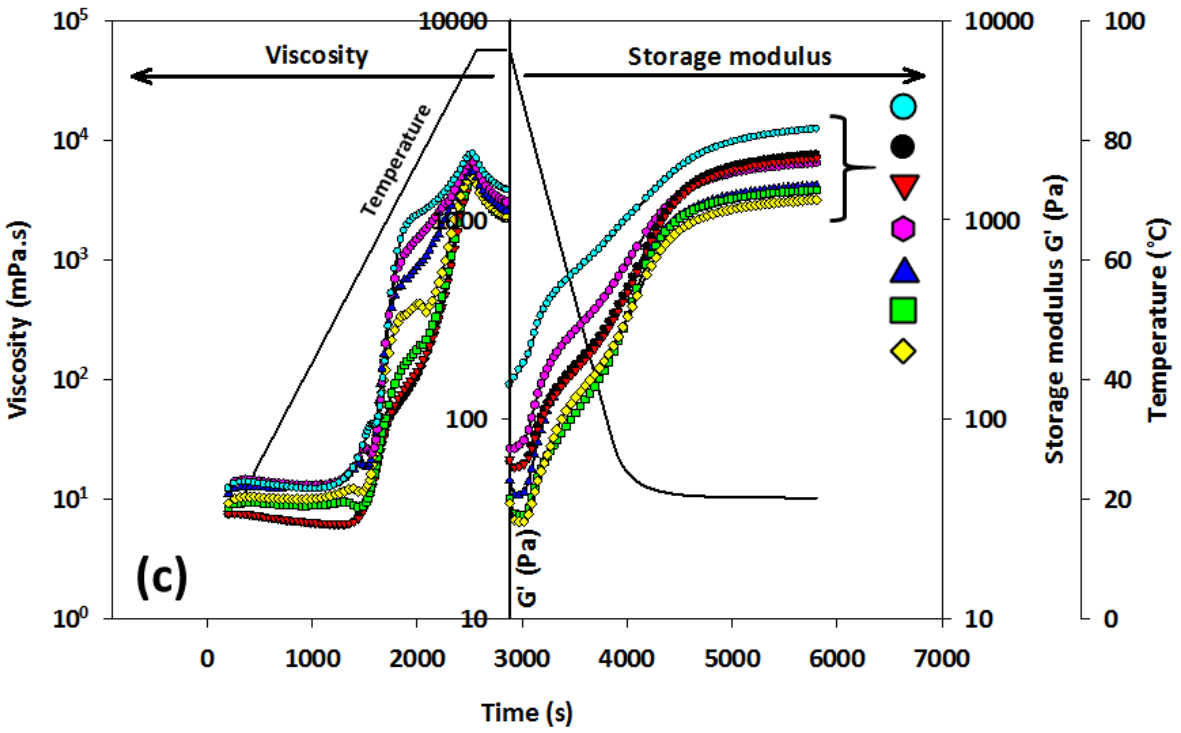


Figure 6-1 Pasting and rheological properties of 5 % (a), 8 % (b) and 10 % (c) w/w wheat starch with 0 % (●), 0.1 % (▼), 0.5 % (■), 1 % (◆), 2 % (▲), 3 % (⬢) and 5 % (○) w/w MCP. Symbols on the right indicate the concentration of MCP from the highest to the lowest *G'*. Insert: peaks M of 5 % w/w wheat starch in the absence (●) and presence of 1 % (◆) and 5 % (○) w/w MCP. Values are averages of triplicate.

Measurements of starch granule diameter indicated a restriction in granular swelling (Figure 6-2), whereby in the presence of 2 % w/w and 5 % w/w MCP, the increase in granule diameter was reduced by 14 % and 29 % respectively. Reduction in starch granular swelling in the presence of NSPs have been previously reported (Tester & Sommerville, 2003). However, we believe that it is not possible to model the change in granule volume fraction based on the change in starch granule diameter in the presence of MCP since the actual size of the MCP-coated granules could not be accurately determined because MCP is not visible under the microscope. Hence, while the size of the starch granules themselves decreased in the presence of MCP, it does not necessarily correspond to a decrease in the volume fraction of the starch granules. Therefore, it is possible that the observed change in the magnitude of PV increase represents an increase in the volume fraction of starch granules as a result of MCP coating formed around the starch granules. Furthermore, in the presence of MCP, the increased PV of wheat starch may indicate (i) increased granule rigidity possibly as a result of restricted granular swelling and (ii) increased interaction between starch granules, mediated by amylose-MCP interaction.

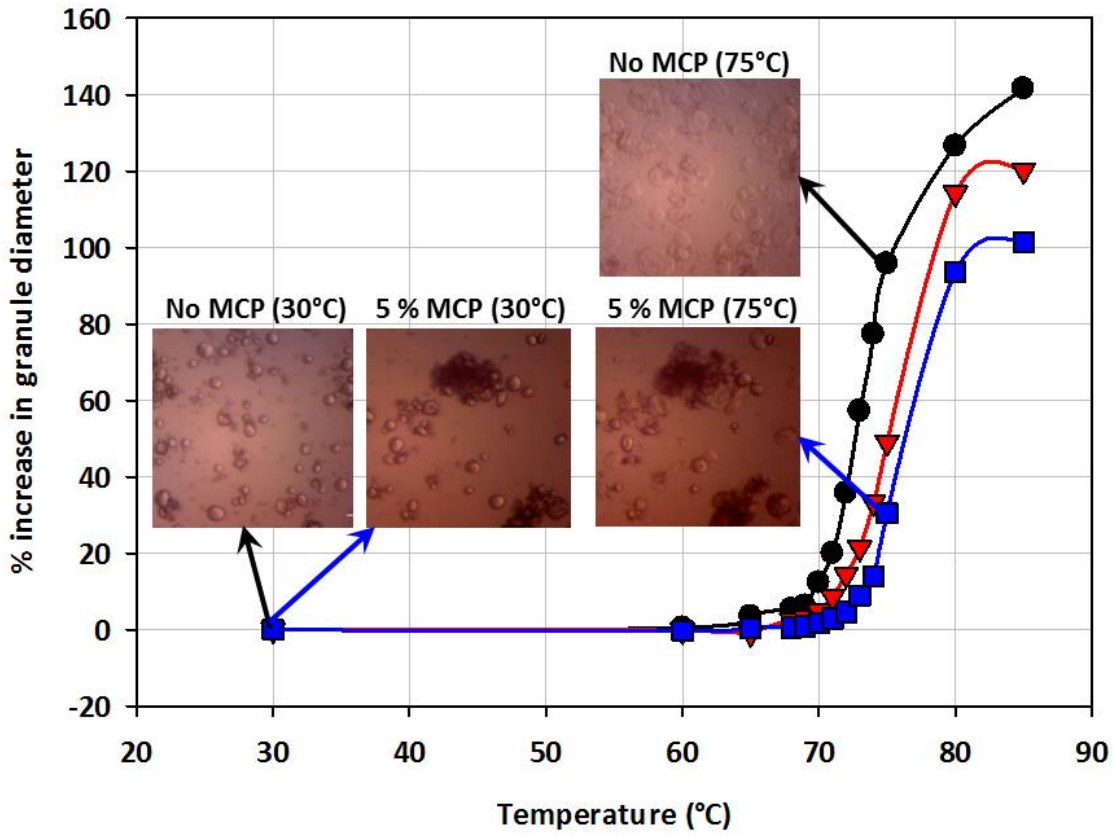


Figure 6-2 The increase in wheat starch granule diameter during heating in the absence (●) and presence of 2 % (▼) and 5 % (■) w/w MCP.

The gelation of wheat starch in the absence and presence of MCP was studied by switching the system into oscillatory mode to monitor the development of *G'* as the paste cooled to form a gel. For the range of starch and MCP concentrations used in this study, the storage modulus (*G'*) was always greater than the loss modulus (*G''*) indicating that all of the systems behaved as gels. As the starch-MCP mixtures set upon cooling, there was an increase in *G'* and all of the gels displayed frequency independent storage and loss moduli (See appendix B)—indicating the formation of a strong gel (Clark & Ross-Murphy, 1987). The development of *G'* as the paste cooled from 95°C to 20°C represents the formation of cross-linking amylose networks and is typical of starch gelation upon cooling (Hansen, Hoseney, & Faubion, 1991; Keetels, van Vliet, & Walstra, 1996). At low starch concentration (5 % w/w), there was a consistent increase in the final strength (*G'*) of the gel with increasing MCP concentrations and this reached a maximum of over 250 Pa when 5 % w/w MCP was present from ~14Pa in its absence. However, increasing the starch concentration to ≥ 8 % w/w resulted in a decrease in the final *G'* of the gel at low MCP concentrations (between 0.1 % w/w to 3 % w/w), before following a similar increasing trend with further addition of MCP up to 5 % w/w— reaching over 1000 Pa and 2000 Pa for 8 % w/w and 10 % w/w starch respectively. These results show that the impact of MCP on starch gel

strength depends on the starch concentration present and it could be negative (weaker gels) at starch concentrations greater than 8 % w/w in the presence of low quantities of MCP.

The interaction between leached starch polymers and polysaccharides has been previously suggested as a mechanism to increase the PV and *G'* of starches (Shi & BeMiller, 2002). We have further hypothesised, in the previous chapter, that the interaction between MCP and starch can take place specifically when amylose leaches out of the starch granules. In order to investigate this in depth, MCP solution was added to starch suspensions at various temperatures during gelatinisation to obtain a final concentration of 5 % w/w wheat starch and 1 % w/w or 3 % w/w MCP. The final *G'* of the cooled gels was measured and shown in Figure 6-3. The addition of water at different temperatures during gelatinisation (between 20°C and 95°C) did not affect the final *G'* of the starch gel. However, in the presence of MCP, the final *G'* of the gel showed dependency on the time at which MCP was added. The addition of MCP at temperatures ≥ 55°C resulted in the weakening of the starch-MCP gels as indicated by a decrease in the final *G'* of the gel, with the weakest gel formed when MCP was added at 95°C—the strength of these gels was reduced by 33.6 % and 65.1 % at the addition of 1 % w/w and 3 % w/w MCP respectively. This result indicates that to increase the final *G'* of wheat starch gel, MCP must be present in the suspension at temperatures below 55°C for any interaction to occur. This temperature corresponds to the temperature at which amylose leached out into the supernatant (Figure 6-3 filled symbols). This observation has never been reported and it proves that the interaction occurred as the polymer exited the starch granules. Interactions between polysaccharides and leached starch polymers have been previously suggested due to an earlier onset of viscosity increase (Christianson, Hodge, Osborne, & Detroy, 1981; Shi & BeMiller, 2002), increase in the final viscosity (Bahnassey & Breene, 1994) and network formation (Funami *et al.*, 2005b; Lai & Liao, 2002b; Liu *et al.*, 2003). In the presence of MCP, wheat starch presented an earlier onset of viscosity increase as a characteristic transient peak "M" occurring in the pasting curve (Figure 6-1 at about 55°C). The transient peak "M" showed a rise and fall in apparent viscosity that can be explained by an increase in the starch volume fraction due to the formation of MCP-amylose coats around the granules, followed by the detachment of this complex from the granule surface to the continuous phase, hence decreasing the starch volume fraction (Yuris *et al.*, 2017). However, at high concentration of MCP, the presence of excess polysaccharide maintained the presence of amylose-MCP coats around the granules, hence sustaining the viscosity of the suspension. This could explain the disappearance of peak "M" (characterised by only an increase in the apparent viscosity without a subsequent fall) as the concentration of MCP was increased (Figure 6-1 insert).

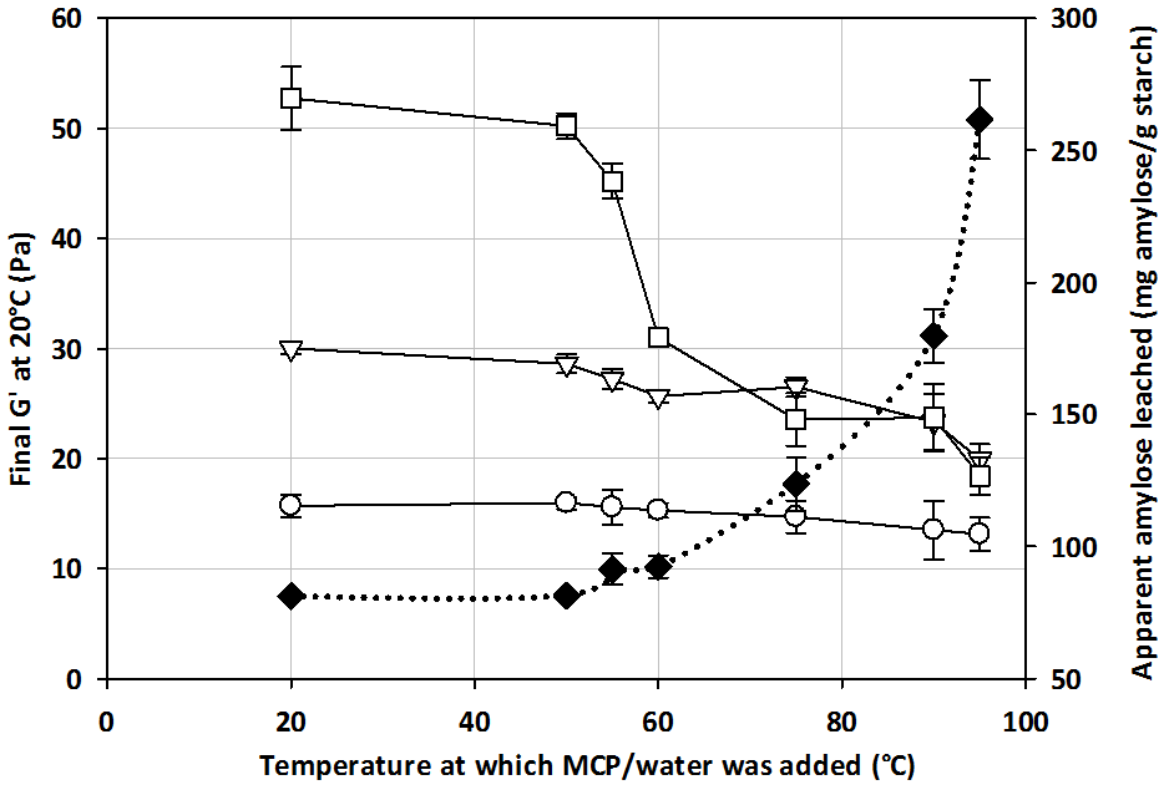


Figure 6-3 The final *G'* (empty symbols) of 5 % w/w wheat starch gels in the presence of water (○), 1 % (▽) or 3 % (□) w/w MCP added at different temperatures during starch gelatinisation and the apparent amylose leached (filled symbols) out of 2 % w/w wheat starch.

The effect that MCP had on the final gel strength of wheat starch can be explained further in relation to the amount of amylose that leached out of the granules. With increasing starch concentration, there seems to be a decrease in the amount of amylose leached out of individual starch granules (Palav & Seetharaman, 2006). The strength of a starch gel is dependent on the amount of amylose present in the supernatant, which associates to create a 3D network (Biliaderis & Juliano, 1993). The presence of MCP is known to restrict amylose leaching (Yuris *et al.*, 2017) and hence, at high starch concentration, the effective amount of amylose leached would be further reduced. This may have contributed to the formation of a weak heterogeneous gel network as indicated by a decrease in the final *G'* of the gel at low MCP concentration. However, at high MCP concentration, despite a decrease in the amount of amylose present, the presence of excess MCP may bridge starch granules by interacting with multiple amylose chains from different granules, hence enhancing the strength of the gel by creating a homogenous network.

6.3.2 Rheological, textural and microstructural properties of wheat starch-MCP gels

The textural properties of wheat starch gels in the absence and presence of MCP were investigated in relation to their rheological (Figure 6-5a) and microstructural properties (Figure 6-4). At ≥ 8 % w/w starch concentration the changes in gel hardness with the addition of MCP aligned with the changes in the gel strength (final *G'*) whereby an initial decrease in the hardness of the gels was observed at low MCP concentrations before subsequently increasing. All of the gels made using 8 % w/w and 10 % w/w starch, regardless of the MCP concentration, were firm with a hardness value of > 10 N and did not collapse under the compression test. The structure of these gels can be seen using the SEM (Figure 6-4a), whereby at high starch concentration, the addition of low MCP concentration (1 % w/w) led to the formation of larger pores within the gel. Pores within the gel structure are representative of the location of water within the network prior to the gel being dried out for SEM analysis. The presence of larger pores indicates that water within the network was present in larger bulks, probably due to the absence of a homogeneous network. This could explain the decrease in gel hardness of 10 % w/w wheat starch gels when 1 % w/w MCP was present. The effect that gel porosity has on textural hardness has been previously reported by Sae-kang and Suphantharika (2006), where they found that a reduction in the network membrane porosity resulted in strengthening of starch gels. When the concentration of MCP was further increased, a denser network was formed and the pores of the gels were reduced (Figure 6-4), consequently the gels' hardness increased (Figure 6-5a).

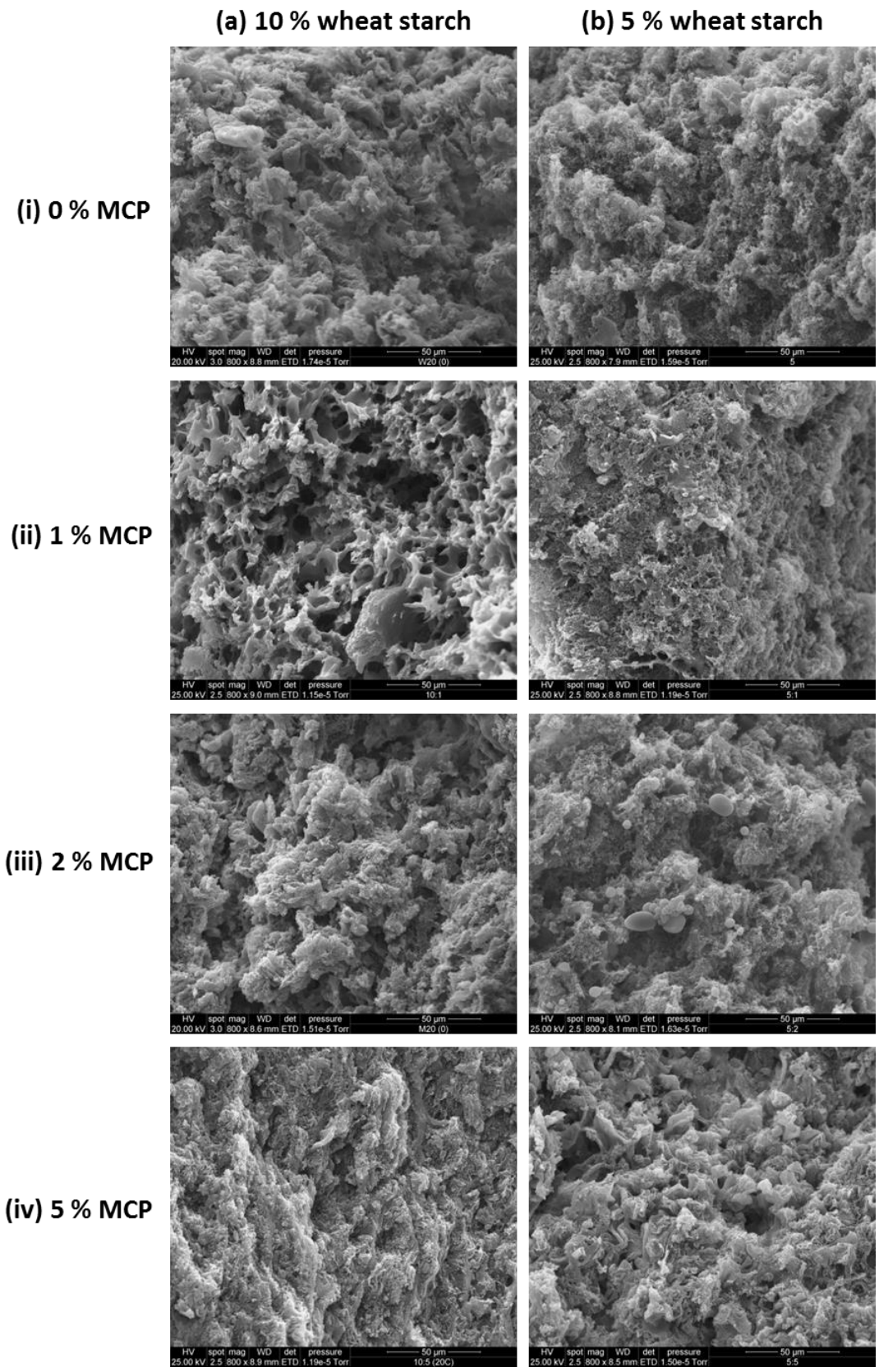


Figure 6-4 SEM of 5 % (a) and 10 % (b) w/w wheat starch gels in the presence of 0 % (i), 1 % (ii), 2 % (iii) and 5 % (iv) w/w MCP.

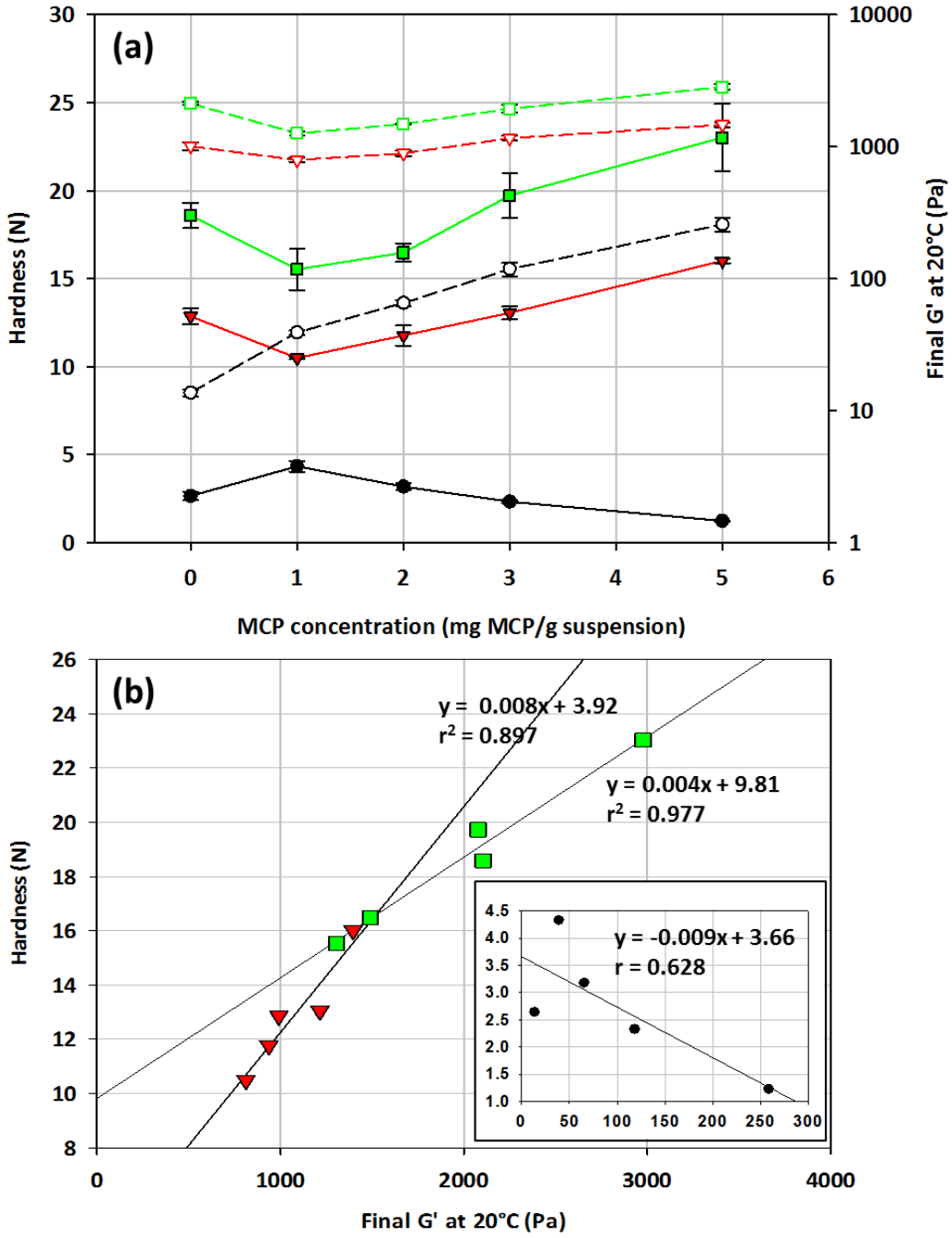


Figure 6-5 The effect of increasing MCP concentration on the hardness (solid lines) and final *G'* (broken lines) of 5 % (●), 8 % (▼) and 10 % (■) w/w wheat starch gels (a) and the relationship between the final *G'* and hardness of these gels (b).

However, when the starch concentration was decreased to 5 % w/w, the final *G'* and hardness of the gels deviated when the concentration of MCP was above 1 % w/w. While the final *G'* of the gels constantly increased with increasing concentration of MCP up to 259 Pa in the presence of 5 % w/w MCP, the opposite was observed for the hardness of the gel—an increase in the presence of 1 % w/w MCP followed by a subsequent decrease to a minimum of 1.2 N in the presence of 5 % w/w MCP. The

addition of 1 % w/w MCP to low concentration of starch (5 % w/w) was found to create finer networks with smaller pores (Figure 6-4b.ii.), resulting in a homogenous and denser structure, hence increasing the gels' hardness (Figure 6-5a). In contrast, further increase in MCP concentration (2 and 5 % w/w) resulted in not only the formation of larger pores within the gel due to a less dense network but also incomplete gelatinisation of granules. While the presence of 1 % w/w MCP may facilitate network formation with amylose, further increases in MCP concentration could have led to insufficient amylose leaching out of the granules to create a homogenous network (Yuris *et al.*, 2017). This could lead to micro-phase separation due to a lack of a dense network to hold the starch gel together. Hence, the hardness of wheat starch gel in the presence of 2 % and 5 % w/w MCP decreased as a result of a decrease in network density and homogeneity within the gel matrix. Images of low (5 % w/w) and high (10 % w/w) concentrations of wheat starch gels in the absence and presence of MCP are shown in Figure 6-6. In order to emphasise the effect of high concentrations of MCP on the gel structure, gels containing 10 % w/w MCP were included in the image. While the addition of 1 % w/w MCP to 5 % w/w wheat starch increased the hardness of the starch gel (Figure 6-5a), further increases in MCP concentration resulted in the gel gradually losing its structural integrity and collapsing as seen in Figure 6-6a. Loss of structural integrity, however, was not observed at the medium and high starch concentration (Figure 6-5) in the presence of MCP, possibly due to the close packing of starch granules at these concentrations. This result could also indicate that while MCP facilitated the formation of a denser amylose-MCP network (higher *G'*), this was not as strong as an amylose-amylose network resulting in a collapse of the structure under pressure. This was more prominent for lower starch concentration, whereby the textural strength of the gel depends largely on amylose associations to hold the structure together.

The relationship between the gel strength (final *G'*) and the hardness of the starch-MCP gels is shown in Figure 6-5b. Positive correlation with Pearson coefficients of 0.90 and 0.98 were obtained for 8 % w/w and 10 % w/w wheat starch respectively in the absence and presence of MCP, showing that at high starch concentration ($\geq$ 8 % w/w), small deformation measurements (final *G'*) reflect textural properties (hardness) as measured by large deformation. Such agreement between small and large deformation measurements have also been shown in milk protein gels (skim milk powder and milk protein concentrate) containing increasing concentrations of κ-carrageenan (Hemar, Hall, Munro, & Singh, 2002) and gels of dietary fibre blends containing carboxymethylcellulose, hydroxypropylmethylcellulose, locust bean gum, high ester pectin, fructo-oligosaccharide and gluco-oligosaccharide (Angioloni & Collar, 2009). Nonetheless, the correlation between the two parameters (*G'* and hardness) did not stand at low starch concentration (5 % w/w)—this resulted in a negative correlation with Pearson coefficient of 0.63. Similar discrepancy in the rheological and textural

properties have been previously reported in WPI/κ-carrageenan gels whereby a high final *G'* was observed despite a low fracture stress (Çakır & Foegeding, 2011). The negative correlation between the hardness and *G'* of 5 % w/w wheat starch in the presence of MCP can be explained in terms of their interaction. The *G'* of a gel is increased when the strength and/or number of crosslinks in the system is increased (Draget, Moe, Skjak-Bræk, & Smidsrod, 2006). With the addition of MCP, the number of crosslinks between the polysaccharide and amylose is likely to increase due to their interaction, hence raising the *G'* of the gel. However, due to starch granule aggregation in the presence of MCP (Yuris *et al.*, 2017), the resulting network became heterogeneous—composing of amylose-MCP network with granule aggregates interspersed—making the gel structurally weak (decrease in hardness). However, the same was not observed at higher starch concentration (≥ 8 % w/w) due to the close packing of starch granules that prevented heterogeneity within the gel.

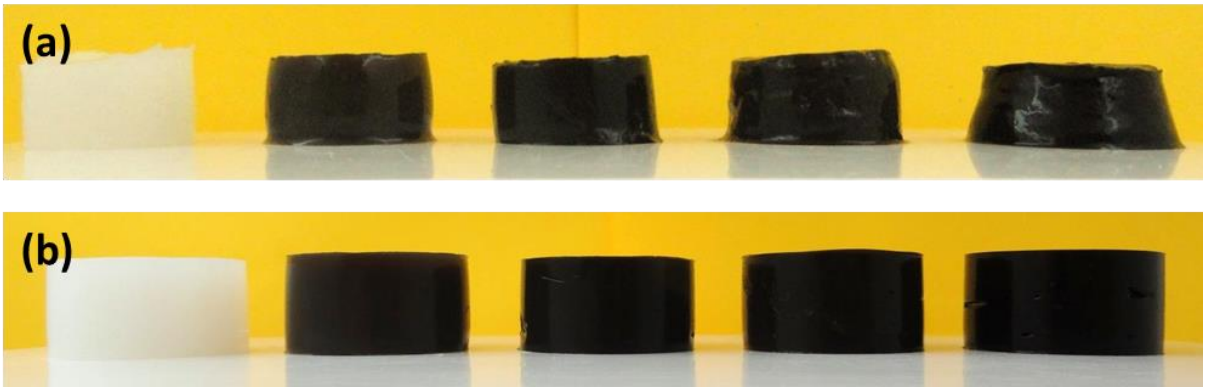


Figure 6-6 5 % (a) and 10 % (b) w/w wheat starch gels in the presence of increasing concentrations of MCP. From left to right: 0, 1, 3, 5 and 10 % w/w MCP.

6.3.3 Retrogradation

6.3.3.1 Syneresis

Regardless of its concentration, the presence of MCP reduced syneresis of the 10 % w/w wheat starch gels following the first freeze-thaw cycle (Figure 6-7). In this case, it is likely that MCP retarded amylose retrogradation by interacting with amylose, as evidenced by the results from MAS NMR (Chapter 4) and rheology. However, retardation of ice crystal formation as a result of water molecules being immobilised by polysaccharides cannot be excluded (Lee *et al.*, 2002). For the subsequent second and third freeze-thaw cycles, this effect was no longer observed and there seemed to be no difference in the amount of syneresis in the absence or presence of MCP. Subsequent freeze-thaw cycles increased the amount of syneresis of the gels when 1 % w/w MCP was present—up to 20.6 % in the presence of 1 % w/w MCP at the 5th cycle. Nonetheless, this increase was not observed at high concentration of MCP (5 % w/w). Therefore, it would seem that syneresis was only affected at low concentration of

MCP. While the presence of 1 % w/w MCP retarded the retrogradation of amylose and hindered water release at the first freeze-thaw cycle, subsequent freezing and thawing may result in the formation of larger ice crystals, which could disrupt the porous amylose-MCP network (as observed in the micrographs and the lower *G'* for the gels containing 1 % w/w MCP), thereby releasing water. This has been previously observed in tapioca starch-xanthan gels at pH 3 whereby large ice crystal formation resulted in the disruption of the weak network, thereby increasing syneresis after 5 freeze-thaw cycles (Sae-kang & Suphantharika, 2006). At higher MCP concentration, the presence of excess MCP resulted in a less porous network and this may trap water within the structure, preventing it from being released out of the gel, hence reducing syneresis. While other polysaccharides such as curdlan, gellan and carrageenan (Lee *et al.*, 2002) have also been reported to increase starch gel syneresis, variation in effects depending on polysaccharide concentration have never been observed. Nevertheless, previous studies have shown that concentration influences a polysaccharide's effectiveness in reducing syneresis (0.3 % w/w xanthan was more effective than 0.3 % w/w guar but 0.6 % w/w guar was more effective than 0.6 % w/w xanthan in reducing syneresis) (Lee *et al.*, 2002). It is also interesting to note that while not quantified, for 5 % w/w wheat starch gels, high concentration of MCP (5 % w/w) resulted in more syneresis based on visual observation (see appendix B). This, again, can be explained by the weak and heterogeneous network that allowed the water to be released.

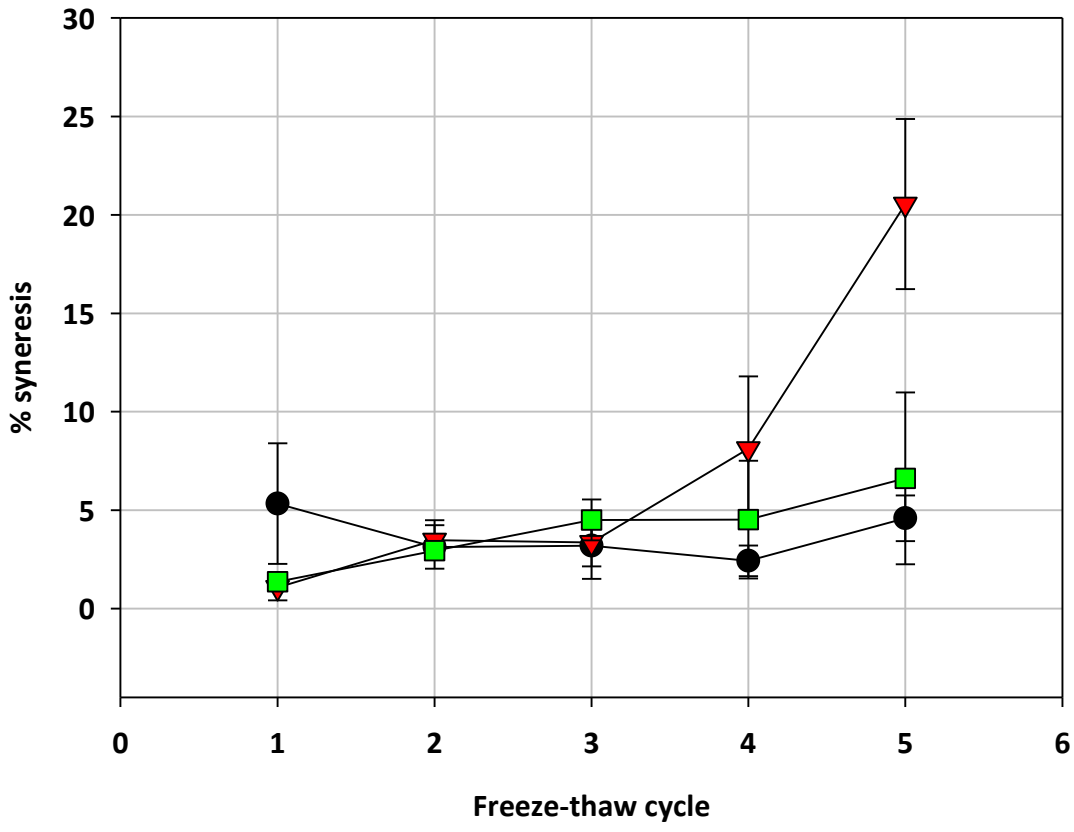


Figure 6-7 Syneresis of 10 % w/w wheat starch in the absence (●) and presence of 1 % (▲) and 5 % (■) w/w MCP after 5 freeze-thaw cycles.

6.3.3.2 Differential scanning calorimeter

DSC was used to determine the reordering behaviour of wheat starch in the absence and presence of MCP during storage at 4°C. The endothermic enthalpy and retrogradation ratio of wheat starch in the absence and presence of MCP is shown in Table 6-1. The endothermic enthalpy reported (beginning at about 40°C) corresponds to the amount of energy required to break amylopectin chains that have reordered during cooling—a higher enthalpy corresponds to a higher degree of amylopectin reordering (more retrograded) during storage. The presence of 1 % w/w MCP was found to accelerate retrogradation as indicated by a higher endothermic enthalpy and retrogradation ratio than wheat starch alone. Similar observations have been reported for wheat starch retrogradation in the presence of other polysaccharides such as guar gum, tara gum, locust bean gum and konjac glucomannan (Funami *et al.*, 2005b). This was thought to be due to a decrease in starch chain mobility as a result of associations between amylopectin and polysaccharide during storage; hence a larger amount of energy is required to disrupt the structure.

Table 6-1 The endothermic enthalpy and retrogradation ratio of 25 % w/w wheat starch in the absence and presence of 1 % and 5 % w/w MCP on reheating at day 5, 7 and 14. Values are the mean ± standard deviation of duplicate.

Samples	Day 5, ΔH_2 ($\Delta H_2/\Delta H_1$)	Day 7, ΔH_2 ($\Delta H_2/\Delta H_1$)	Day 14, ΔH_2 ($\Delta H_2/\Delta H_1$)
25 % w/w wheat starch	1.34 ± 0.22 (0.11 ± 0.01)	2.92 ± 0.20 (0.23 ± 0.02)	5.43 ± 0.40 (0.44 ± 0.03)
25 % w/w wheat starch + 1 % w/w MCP	2.93 ± 0.30 (0.23 ± 0.03)	4.10 ± 0.30 (0.33 ± 0.02)	6.27 ± 0.04 (0.49 ± 0.02)
25 % w/w wheat starch + 5 % w/w MCP	1.99 ± 0.36 (0.15 ± 0.03)	3.21 ± 0.09 (0.25 ± 0.02)	5.25 ± 0.07 (0.41 ± 0.01)

The addition of high concentrations of MCP (5 % w/w) did not affect both the endothermic enthalpy and retrogradation ratio. Most studies that looked into starch retrogradation in the presence of polysaccharides reported either an increase or decrease in the retrogradation ratio of starches in the presence of a polysaccharide (Funami *et al.*, 2005b; Lee *et al.*, 2002; Zhou *et al.*, 2008). It is very rare that the presence of a polysaccharide does not have an effect on the retrogradation property of the starch—the exception being freeze-thawed sweet potato starch in the presence of 0.3 % w/w guar

gum (retrogradation ratio of 0.82 in the absence and presence of 0.3 % w/w guar) (Lee *et al.*, 2002). Nevertheless, our DSC data are in concordance with the syneresis result—increased retrogradation in the presence of 1 % w/w MCP but no difference between the absence or presence of 5 % w/w MCP. This result further confirms the importance of MCP concentration on the retrogradation properties of wheat starch gels. The effect that MCP concentration has on the retrogradation properties of wheat starch could be explained by the existence of two mechanisms: (i) polysaccharides associating with starch polymers, resulting in an increase in the amount of energy required for melting of structure (increased retrogradation) and (ii) polysaccharides binding onto water, which limits their availability for starch polymer (amylose and amylopectin) crystallisation (decreased retrogradation) (Katsuta *et al.*, 1992; Lee *et al.*, 2002).

6.4 Conclusion

Rheological data strongly suggests that the interaction was primarily between MCP and amylose. The effect of this interaction on the rheological and textural properties of the composite gels was dependent on the amount of amylose that was leached from the granules and the amount of MCP available. Heterogeneity within the gels' microstructure was found when there was insufficient amylose and/or MCP available for interaction and this weakened the gel (decrease in gel hardness). Starch gel retrogradation was also found to increase when the microstructure of the gel was heterogeneous as evidenced by increased syneresis and retrogradation ratio, measured by DSC. Clearly, MCP could be used as a co-texturizer of starch as MCP concentration can be used to manipulate starch properties. An impact on starch digestibility is also envisaged and it will be the subject of future publications.

Chapter 7 Factors influencing the rheological properties of wheat starch-MCP gels

7.1 Introduction

The interaction between wheat starch and MCP is reflected in the rheological changes during gelatinisation and gelation behaviour as has been presented in previous chapters. A distinct characteristic, particularly the formation of a peak M at the onset of gelatinisation is an indicator of the interaction between starch and MCP. Based on the current literature, it is known that the interaction between MCP and starch varies depending on the starch type (waxy, normal and non-waxy). Due to the differences in the starch and MCP concentrations used in different studies, these results cannot be compared. The interaction between starch and MCP is difficult to quantify but by measuring changes in the rheological properties of starches—that vary in their chemical properties—as they are gelatinised in the presence of MCP, factors that influence the formation of starch MCP gels may be understood.

MCP is an anionic polysaccharide and, like many other anionic polysaccharides, its conformation is likely to be affected by changes in pH and the presence of salt. Typically, anionic polysaccharides adopt a more expanded helical-coiled conformation at high pH, whereas at low pH it adopts a compact conformation, resulting in aggregates as a result of charge screening (Horinaka, Kani, Hori, & Maeda, 2004). Therefore, it is expected that changes in conformation with pH would affect the interaction of MCP with wheat starch and, hence, alter the rheological properties of the gel. A previous study (Lai & Chao, 2000a) has considered the effect of salts such as NaCl, KCl, $MgCl_2$ and $CaCl_2$ on the pasting properties of wheat starch-MCP paste at dilute concentrations (2 % w/w), using the RVA. The result of the study showed that increasing concentrations of monovalent salts (0 – 75 nM) resulted in an increase in the peak and final viscosity of the composite paste, whereas the effect divalent salt has on the paste viscosity was salt concentration dependent. However, the effect of salt on wheat starch-MCP mixtures at concentrations above 5 % w/w that will form solid gels following gelatinisation and subsequent cooling has, to our knowledge, not been reported. To add to this, no studies have been found that measure the rheological properties of wheat starch-MCP gels at various pH levels. This chapter considers the effect of starch type, polysaccharide to starch ratio, and the effect of pH and salt concentration on the rheological properties of wheat starch-MCP gels. Due to time constraint, this chapter does not explore in depth the changes associated with the gel texture, amylose leaching, granule swelling and the molecular interaction between the polymers. However, it is hypothesised

that the results from this work will assist with further understanding of the interaction between starch and MCP, and will be an important basis for future studies.

7.2 Materials and Methods

7.2.1 Preparation of starch-MCP gels

7.2.1.1 Starch type

Various types of starch (maize, wheat, potato and waxy maize) and MCP suspensions were prepared as per section 5.2.2.

7.2.1.2 Starch to MCP ratio

The concentration of wheat starch and MCP was varied to obtain different starch to MCP ratios with a total solid content of 10 % w/w.

7.2.1.3 pH adjustment

The pH of 10 % w/w wheat starch suspensions in the absence or presence of 2 % w/w MCP was adjusted using sodium hydroxide and hydrochloric acid to attain a pH between 4 and 9. The suspension was then topped up with MilliQ water to make up the final concentration.

7.2.1.4 Salt addition

In order to investigate the effect of salt on the properties of 5 % w/w wheat starch suspensions in the absence or presence of 5 % w/w MCP, 0.5, 1.0 or 1.5 % w/w NaCl, KCl, $MgCl_2$ or $CaCl_2$ were dissolved in the MCP solution and left to hydrate overnight prior to mixing with wheat starch suspension.

7.2.2 Rheological measurements

The rheological properties of starch-MCP mixtures during gelatinisation and gelation were measured as per section 5.2.3. All measurements were carried out in duplicate.

7.3 Results and discussion

7.3.1 Effect of starch type

The interaction between starches and MCP can be characterised by the rheological properties of the composite gels. Interaction resulting in an increase in the viscosity of the cooled paste (measured using RVA) has been previously reported for rice, wheat, pea and sweet potato starches (Feng *et al.*, 2014). On the other hand, the viscosities of some starch pastes such as corn, mung bean, potato and tapioca (6 % w/w) are MCP concentration dependent whereby at low MCP concentration their viscosities were reduced, but increased with further addition of MCP. As mentioned in Chapter 5, the interaction between the starch and MCP was hypothesised to involve specifically the amylose fraction of starch as the starch is gelatinised, which is key in the formation of a 3D network during starch gelation. Hence, measurement of starch paste viscosity as an indicator of starch and MCP interaction may be inaccurate as amylose and/or amylose-MCP networks are being broken up by shearing during rotational viscosity measurements. The involvement of amylose in the polymer interactions with MCP can be established by measuring the strength of the composite gel by oscillatory non-destructive methods. The gel mechanical spectra (*G'* and G") obtained by oscillatory measurement can infer the formation of bonds between MCP and starch during gelation.

The rheological properties of 5 % w/w maize and wheat starch gels containing MCP are shown in Figure 7-1a and b respectively. For these starches, the addition of MCP at concentrations of 1, 3 and 5 % w/w resulted in an increase in both the peak viscosity of the paste during the heating cycle and in the final strength of the gel upon cooling. Peak M (onset of viscosity increase) was formed at the onset of gelatinisation for both starches in the presence of MCP and this is characteristic of many non-waxy starch pastes containing MCP. As previously discussed in Chapter 5, this is likely to signify the interaction between MCP and amylose, resulting in an increase in the solid volume fraction of the granules in suspension. The magnitude of increase in gel strength was larger for wheat starch, whereby the addition of 5 % w/w MCP resulted in ~1800 % increase in the *G'* compared to ~400 % increase in *G'* for maize starch. While both wheat and maize starch contain similar proportions of amylose (~21 %), the rate at which amylose is leached from the granules during gelatinisation is significantly faster for wheat compared to maize starch (Eliasson, 1986). Furthermore, gelatinisation of wheat starch results in the leaching of mostly amylose molecules, whereas for maize starch, about a third of the polymer leached is made up of amylopectins (Tester & Morrison, 1990). The fact that more amylose is leached at a faster rate for wheat starch is likely to facilitate its interaction with MCP to form a more extensive network that increases the *G'* of the gel.

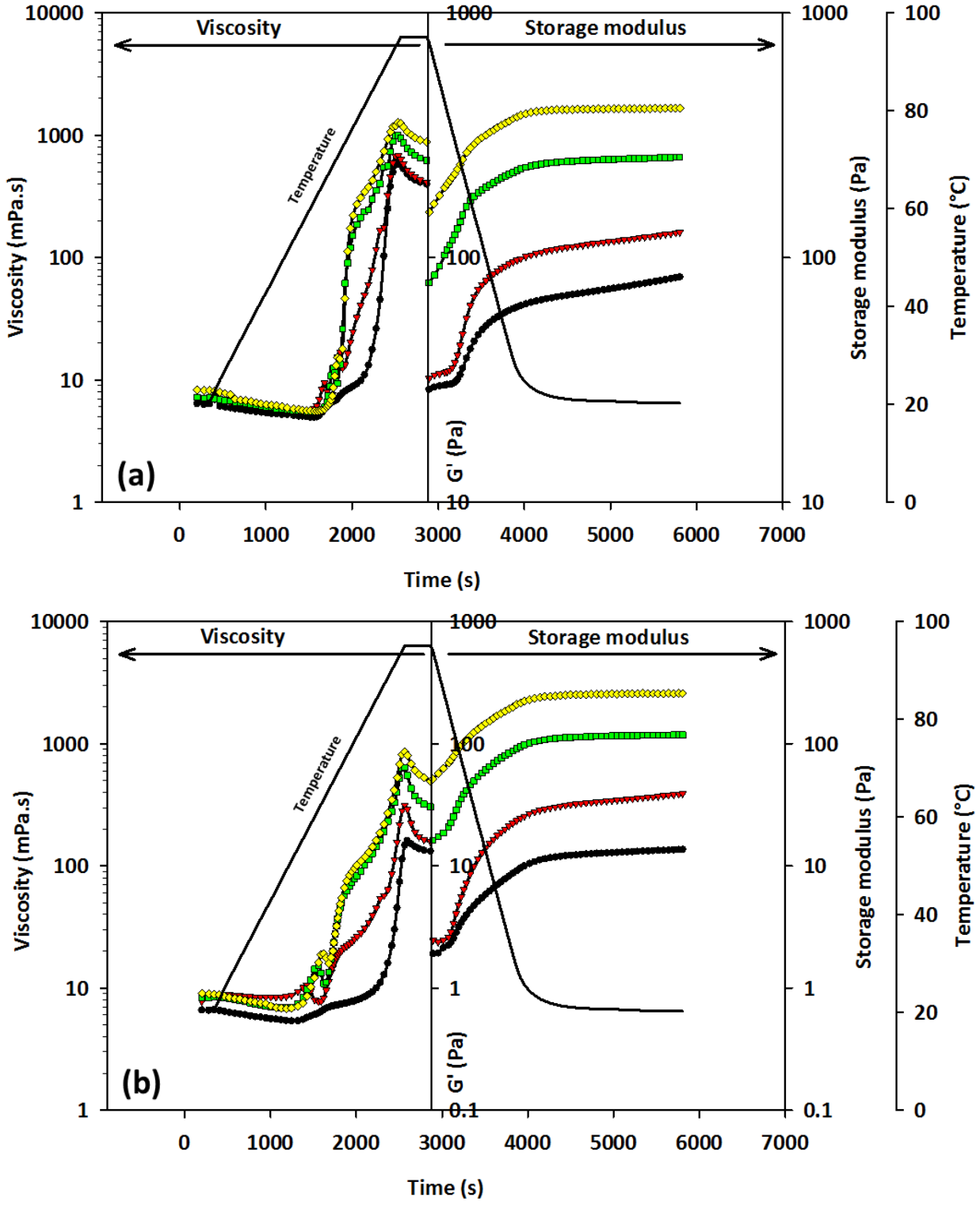


Figure 7-1 Rheological properties of 5 % w/w maize (a) and wheat starch (b) mixtures containing 0 % (●), 1 % (▼), 3 % (■) and 5 % (◇) w/w MCP.

In contrast to wheat and maize starch, the addition of MCP to potato and waxy maize starches resulted in the peak viscosity of the paste to decrease in the case of potato starch and unchanged in the case of waxy maize starch (Figure 7-2). For both starches, peak M was absent at the onset of gelatinisation.

However, for waxy maize starch (Figure 7-2b), the onset of viscosity increase appears to still occur at a lower temperature in the presence of MCP. Waxy maize and non-waxy potato starches are similar in that they produce viscous pastes upon gelatinisation and do not form a firm gel upon cooling as do gels from wheat or maize starch. In the case of potato starch, the formation of a strong gel does not occur as the granules soften and their deformability increase due to the large proportion of water that was absorbed. This creates a viscoelastic gel that is less firm due to the absence of rigid granules to provide structural strength (Eliasson, 1986). For waxy maize, the lack of gel formation is simply due to the lack of amylose present to create a network that is essential for starch gelation (Biliaderis, 2009).

While maize and potato starched contain similar proportions of amylose and amylopectin, the addition of MCP resulted in different effects on the strength of the resulting gels. For potato starch, increasing the concentrations of MCP reduced the peak viscosity (Figure 7-2a) and this was consistent with the results reported by Feng *et al.* (2014), whereby a decrease in the peak viscosity of 6 % w/w potato starch paste was found when MCP (0.1 – 0.5 % w/w) was added to the suspension. The decrease in the peak viscosity was suggested to be due to the presence of negatively charged phosphate groups in potato starch that reduced the granular swelling and gelatinisation of the starch. Based on our results, the presence of MCP has facilitated the formation of a gel as indicated by the increase in the final *G'* of the gel from 60 Pa up to a maximum of 600 Pa in the presence of 5 % w/w MCP. The mechanism that was proposed in Chapter 6 can be used to explain the changes in the properties of potato starch in the presence of MCP: (1) reduced starch granular swelling, (2) reduced amylose leaching and (3) amylose-MCP interacts to form a network. The reduction in starch granular swelling for potato starch resulted in a large decrease in the peak viscosity, since the size of the granules largely influence this. The rapid increase in the viscosity of potato starch in the presence or absence of MCP is likely to mask any small changes in the initial viscosity, hence peak M was not observed. This was also accompanied by a reduction in the proportion of amylose leached, which increases the rigidity of the starch granules. The increase in granule rigidity was observed as the lack of breakdown (decrease in viscosity at high temperature) of potato starch in the presence of high MCP concentration at 95°C. Upon gelation, the presence of MCP facilitated the formation of a more extended network comprising of both amylose and MCP chains that results in an increase in the *G'* of the starch gel.

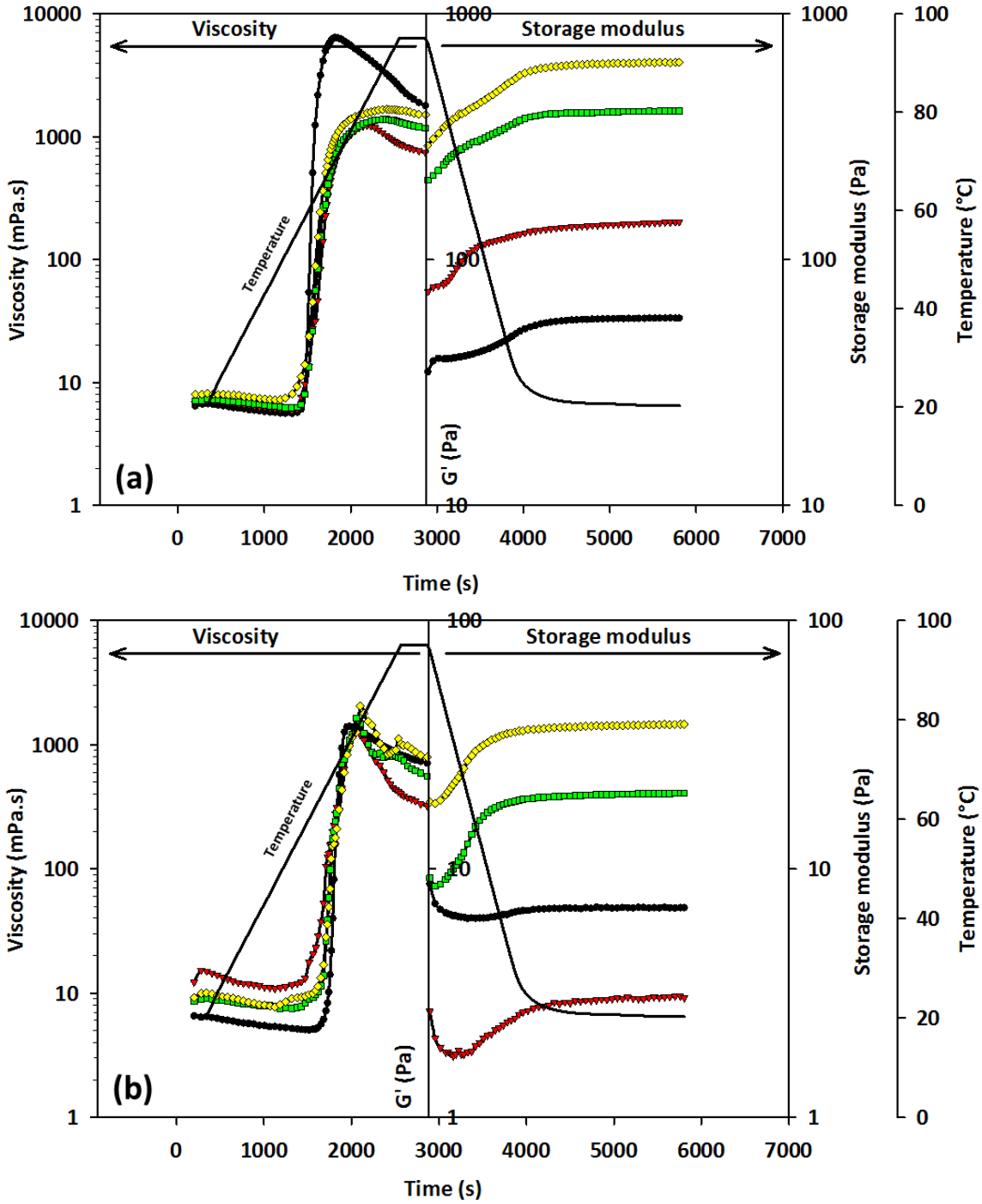


Figure 7-2 Rheological properties of 5 % w/w potato (a) and waxy maize (b) starch gels containing 0 % (●), 1 % (▼), 3 % (■) and 5 % (◆) w/w MCP.

A similar effect was observed for waxy maize starch. At lower MCP concentration, 1 % w/w in this case, the gel strength was reduced. Visually, the addition of 1 % w/w MCP to waxy maize starch produced a paste with lower viscosity and gel strength (Figure 7-2b) and this was apparent in the image presented in Figure 7-3. However, it is interesting that further addition of MCP up to 5 % w/w increased the final *G'* of waxy maize, creating a gel network that was strong enough to maintain an unsupported structure (Figure 7-3). A previous study by Lii and Chen (1980) suggested that the

formation of starch-MCP gels was only possible using non-waxy starches. Our study has shown that the formation of a starch-MCP gel is possible using a waxy starch, provided that the concentration of MCP is sufficiently high since waxy starches are not devoid of amylose, but contains markedly lower concentrations of amylose. In Chapter 6 it was pointed out that the concentrations of both amylose and MCP are critical in creating a strong gel. At low concentrations of MCP, a strong gel for waxy maize cannot be created due to the sparse distribution of both amylose and MCP, hence MCP-starch aggregates exist rather than an interconnected network. At higher MCP concentrations, there is sufficient amount of MCP to form bridges among the starch granules despite the low amylose concentration, thereby creating a gel network. Studies on MCP and waxy starches are almost non-existent due to the idea that the two do not interact to form a gel. This result opens up avenues for research on MCP and waxy starches, which can build further understanding of the interaction between MCP and starches, apart from the ability to control gel texture for different food applications.

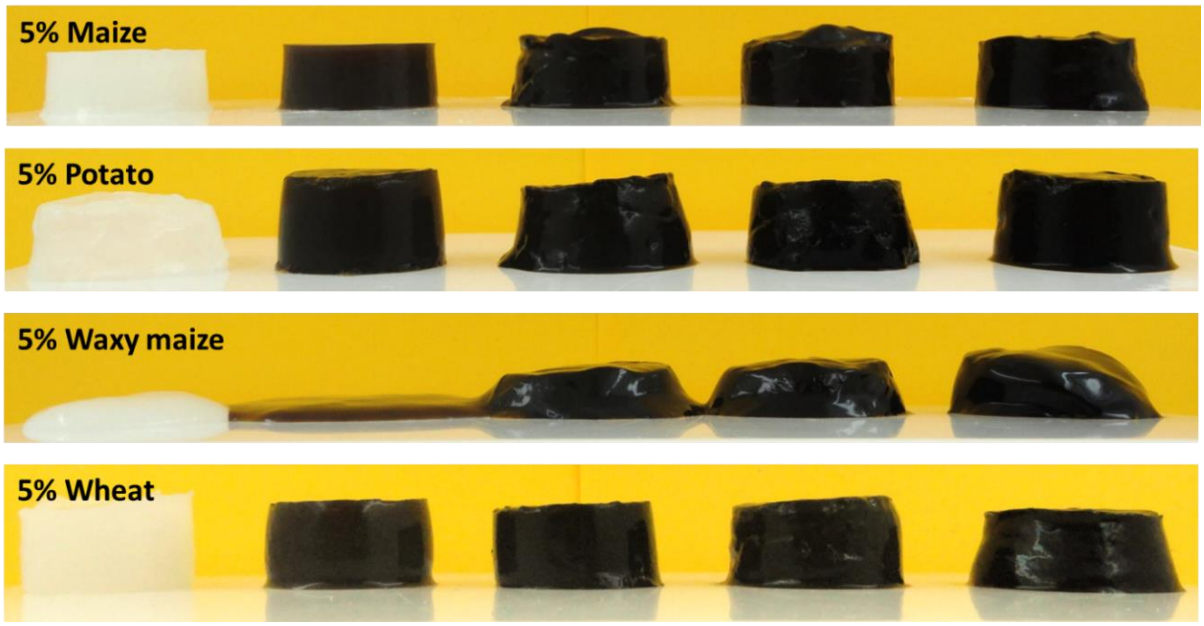


Figure 7-3 Starch gels (5 % w/w) in the presence of 0 %, 1 %, 3 %, 5 % and 10 % w/w MCP (from left to right).

7.3.2 Effect of starch to MCP ratio

The effect of wheat starch to MCP ratio is shown in Figure 7-4. Partial substitution of starch with MCP resulted in a decrease in the peak viscosity of the composite paste and the strength of the resulting gel. This was expected due to a decrease in the starch concentration, meaning that starch granules were less closely packed and the total amount of amylose available for interaction was reduced. The formation of a strong starch-MCP gel as discussed in Chapter 6 can only be achieved when there is sufficient amylose and MCP available. For example, gelatinisation of 2 % w/w wheat starch resulted

in the formation of a smooth viscous paste with a *G'* of about 1 Pa (despite being a gel as determined by oscillatory rheological measurements, whereby *G'* > *G''*), whereas the addition of 8 % w/w MCP to the same concentration of wheat starch resulted in the formation of non-interconnected gel aggregates (gel aggregates distributed in a viscous paste). Gel strengthening can only occur when the starch concentration is sufficiently high, whereby the close packing of starch granule facilitates the interaction between the two polymers (amylose and MCP). Decreasing the ratio of starch also resulted in a visually identifiable phase separation (Figure 4-4), due to the lower volume fraction and the lack of amylose available for interaction. This result indicates that MCP by itself, cannot be used to substitute wheat starch to obtain a gel of similar strength at comparable total solids concentration.

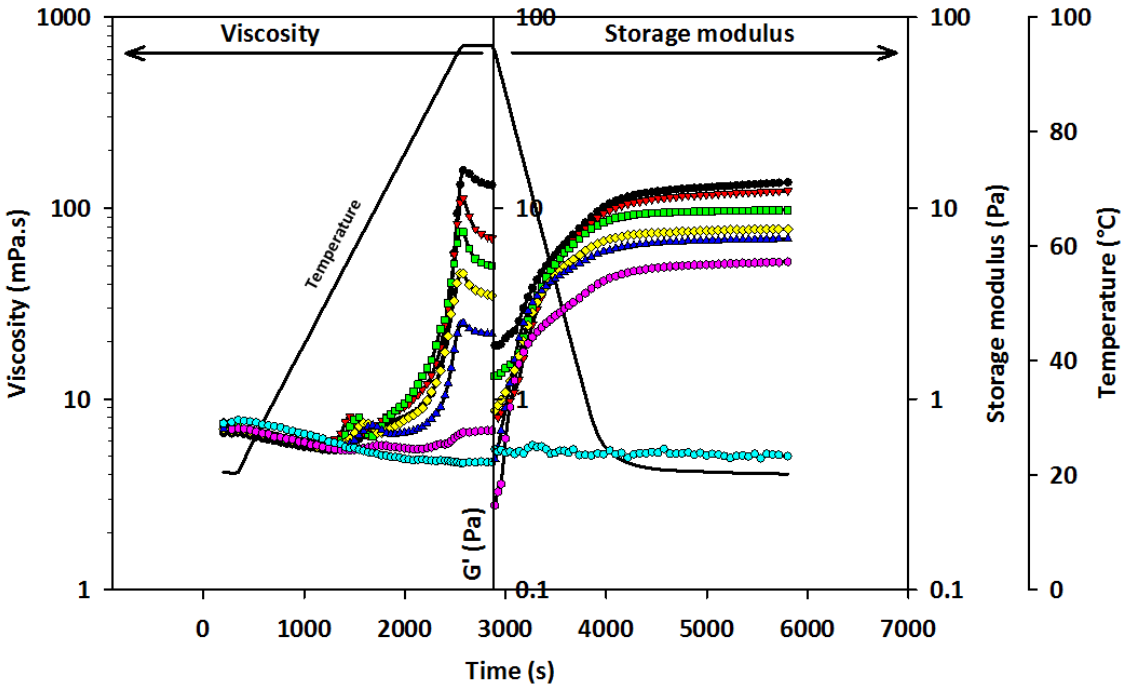


Figure 7-4 Rheological properties of wheat starch-MCP gels in the ratio of 10:0 (●), 8:2(▼), 6:4(■), 5:5(◆), 4:6 (▲), 2:8 (⬢) and 0:10 (●).

7.3.3 Effect of pH

The effect of pH adjustment on the rheological properties of 10 % w/w wheat starch is shown in Figure 7-5. The native pH of wheat starch was 5.8 and its final gel strength was ~2000 Pa. Decreasing the pH of the suspension below 5.8 resulted in a slight decrease in the gel strength (~1500 Pa at pH 4), probably as a result of starch hydrolysis as previously reported for tapioca starch at pH 3 (Sae-kang & Suphantharika, 2006). On the other hand, increasing the pH of the suspension by adding sodium hydroxide did not largely affect the final strength of the gel.

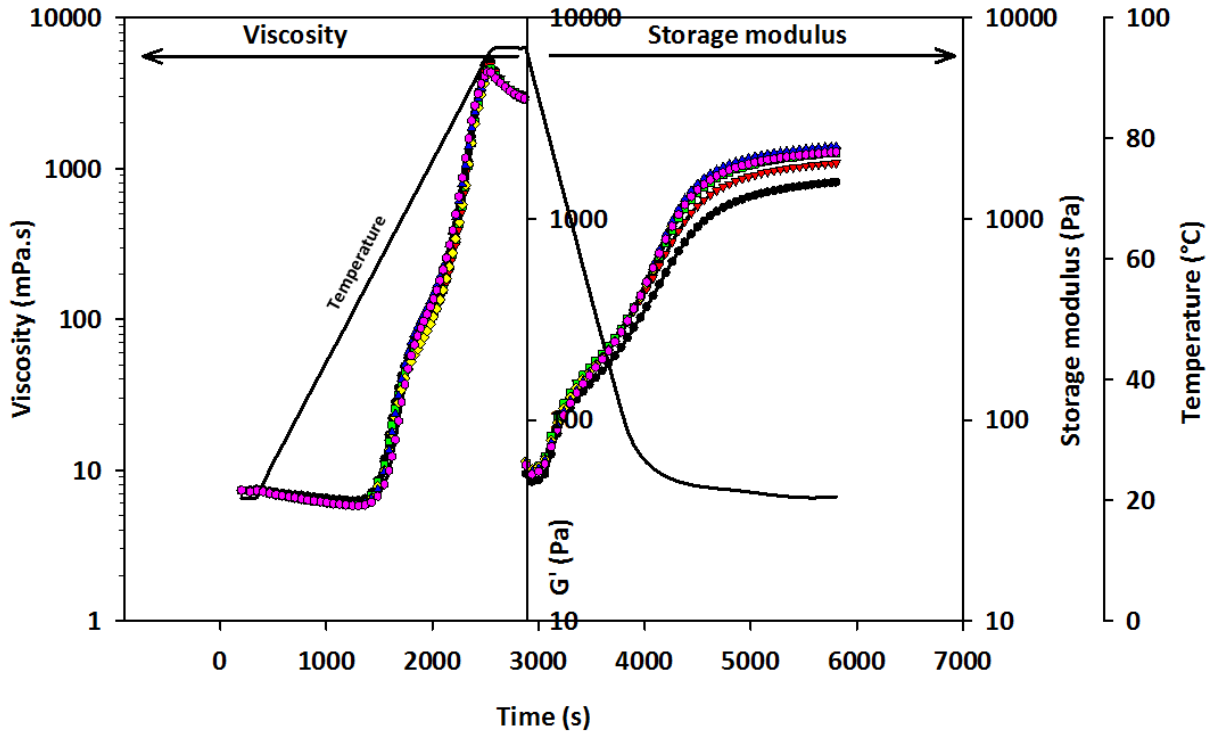


Figure 7-5 The rheological properties of 10 % w/w wheat starch at pH 4 (●), 5 (▼), 5.8 – native (■), 7 (◆), 8 (▲) and 9 (⬢).

A similar trend was observed in the presence of 2 % w/w MCP (Figure 7-6), whereby the strength of the composite gel was lower at pH 4 (~800 Pa) than at its native state at pH 5.5 (~1500 Pa). In comparison to wheat starch only (18 %), there was a larger (41 %) decrease in the final strength of the composite gel when the pH of the suspension was decreased to 4 in the presence of MCP. This indicates that the decrease in the pH did not only affect starch granules, but also likely affected the interaction between MCP and starch. This is further supported by the disappearance of peak M at the initial stages of heating in the presence of MCP. At low pH, it is possible that MCP undergoes intermolecular aggregation as the electrostatic repulsion within the polysaccharide is screened, as previously reported for other anionic polysaccharides (Horinaka *et al.*, 2004).

Between the pH of 5 and 9, the addition of MCP resulted in an increase in the gel strength, reaching a maximum of ~2600 Pa at pH 9. The interaction between wheat starch and MCP was maintained as indicated by the presence of peak M at pH above 5. Increasing the pH of the suspension seemed to promote interaction between the two polymers, resulting in the strengthening of the gel. The changes in the interaction between wheat starch and MCP at different pH could be attributed to the changes in the conformation of MCP as the pH was altered. MCP is an anionic polysaccharide that contains a large proportion of negatively charged groups. Decreasing the pH probably resulted in a decrease in the electrostatic repulsions between MCP polymers and hence causing them to aggregate

(McClements, 2015). On the other hand, the increase in pH by the addition of NaOH would have increased electrostatic repulsion within the polysaccharide, leading to a more expanded conformation, as proposed by Feng *et al.* (2007), whereby they reported an increase in the apparent viscosity of *Mesona blumes* gum solution at pH 10. The expansion of random-coiled chains as a result of an increase in pH have also previously been observed for other anionic polysaccharides (Horinaka *et al.*, 2004). Chain expansion would allow more sites for interaction between MCP and amylose to form a more extended network (possibly via hydrogen bonds), hence increasing the strength of the gel.

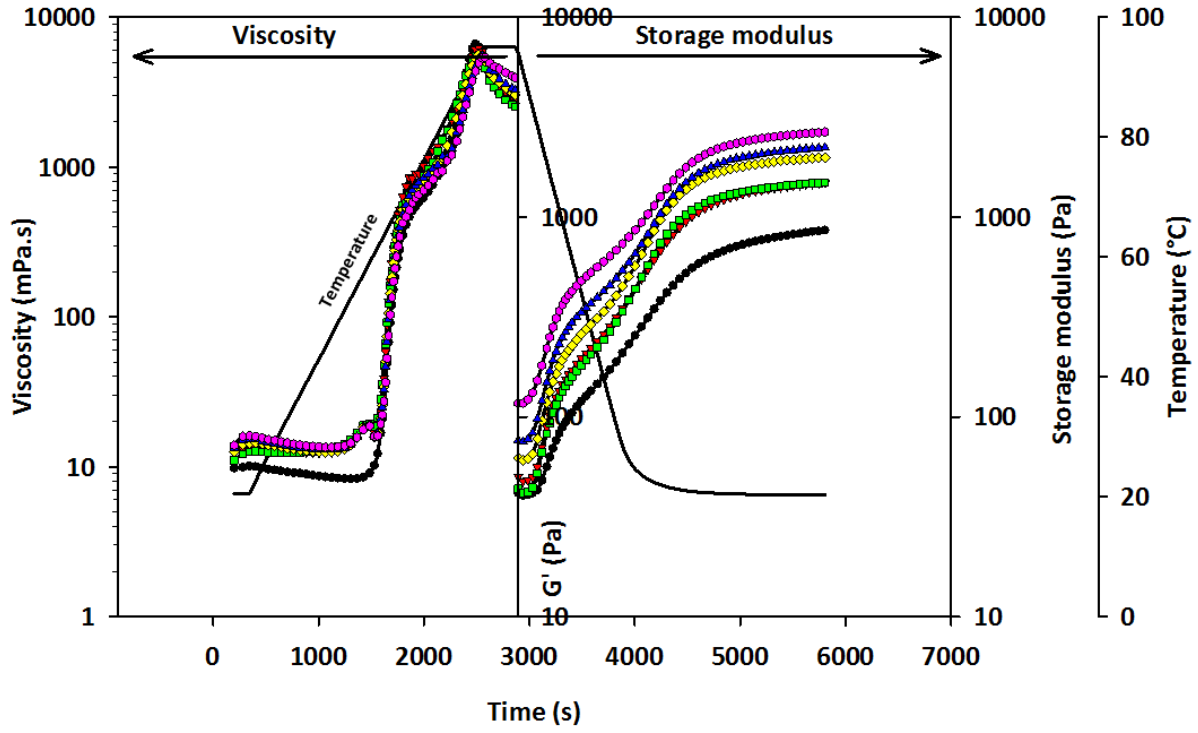


Figure 7-6 The rheological properties of 10 % w/w wheat starch + 2 % w/w MCP gels at pH 4 (●), 5 (▼), 5.8 - native (■), 7 (◇), 8 (▲) and 9 (⬢).

7.3.4 Effect of salt

The effect of adding increasing concentrations of monovalent and divalent salts to 5 % w/w wheat starch in the presence and absence of MCP was investigated. Due to time constraint, the effect of salt at equivalent ionic strength was not investigated. Regardless, within the same concentration, monovalent salts such as NaCl and KCl have lower ionic strength than divalent salts such as $MgCl_2$ and $CaCl_2$. In the range that was tested (0.5 – 1.5 % w/w), salt concentration did not have an effect on the final strength of wheat starch gel alone. Figure 7-7 shows the pasting curve of 5 % w/w wheat starch in the presence of the highest concentration of salts used (1.5 % w/w). The addition of KCl did not alter the final strength of wheat starch gel on its own, whereas NaCl resulted in a slight increase in the

final gel strength. The effect of sodium chloride on the gelatinisation properties of starch have been widely studied, whereby it is known that its presence reduces the extent of starch granule swelling (Rumpold & Knorr, 2005), hence enhancing granule integrity and rigidity (Olkku & Rha, 1978), which results in an increase in the peak viscosity of the paste. On the other hand, the addition of divalent salts ($CaCl_2$ and $MgCl_2$), resulted in a decrease in wheat starch gel strength. Similar findings have been reported for cassava starch, whereby the addition of calcium chloride decreased the viscosity of the resulting paste due to a reduction in starch granular swelling (Jyothi, Sasikiran, Sajeev, Revamma, & Moorthy, 2005). While there is no definite explanation for this phenomenon, it has been proposed that divalent salts bind onto water and, hence, reduce the proportion available for starch gelatinisation. A decrease in starch gelatinisation meant that granule swelling and polymer leaching are reduced and, therefore, the final gel weakens.

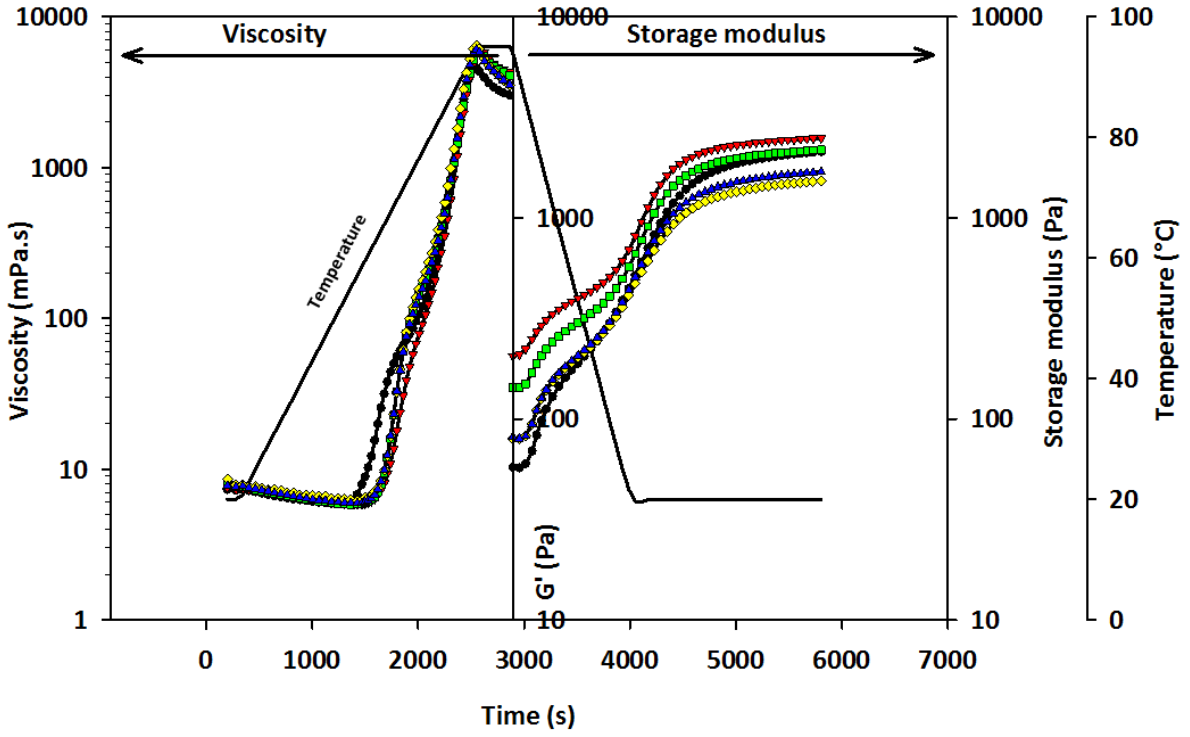


Figure 7-7 The rheological properties of 10 % w/w wheat starch in the absence (●) and presence of 1.5 % w/w NaCl (▼), KCl (■), $CaCl_2$ (◆) and $MgCl_2$ (▲).

The addition of monovalent salts (NaCl and KCl) to wheat starch-MCP gels resulted in a slight increase in their gel strength (Figure 7-8). This result was consistent with that reported by Lai and Chao (2000a) and Feng *et al.* (2016) and was thought to be due to the ability of these monovalent cations to facilitate the formation of junction zones between starch and MCP. The increase in gel strength was concentration dependent for NaCl, whereas for KCl, changes in the concentration of the salt did not seem to have an effect on the magnitude of *G'* increase. At higher concentrations of monovalent salts

(1.5 % w/w), peak M seemed to diminish. This is likely due to the salt reducing starch granular swelling during gelatinisation (Jyothi *et al.*, 2005). An increase in the final starch paste viscosity upon the addition of NaCl have also been reported in other systems such as that of rice starch and xanthan, and this was thought to be due to the monovalent cation promoting network formation and, at the same time, reducing the intermolecular repulsion (Samutsri & Suphantharika, 2012).

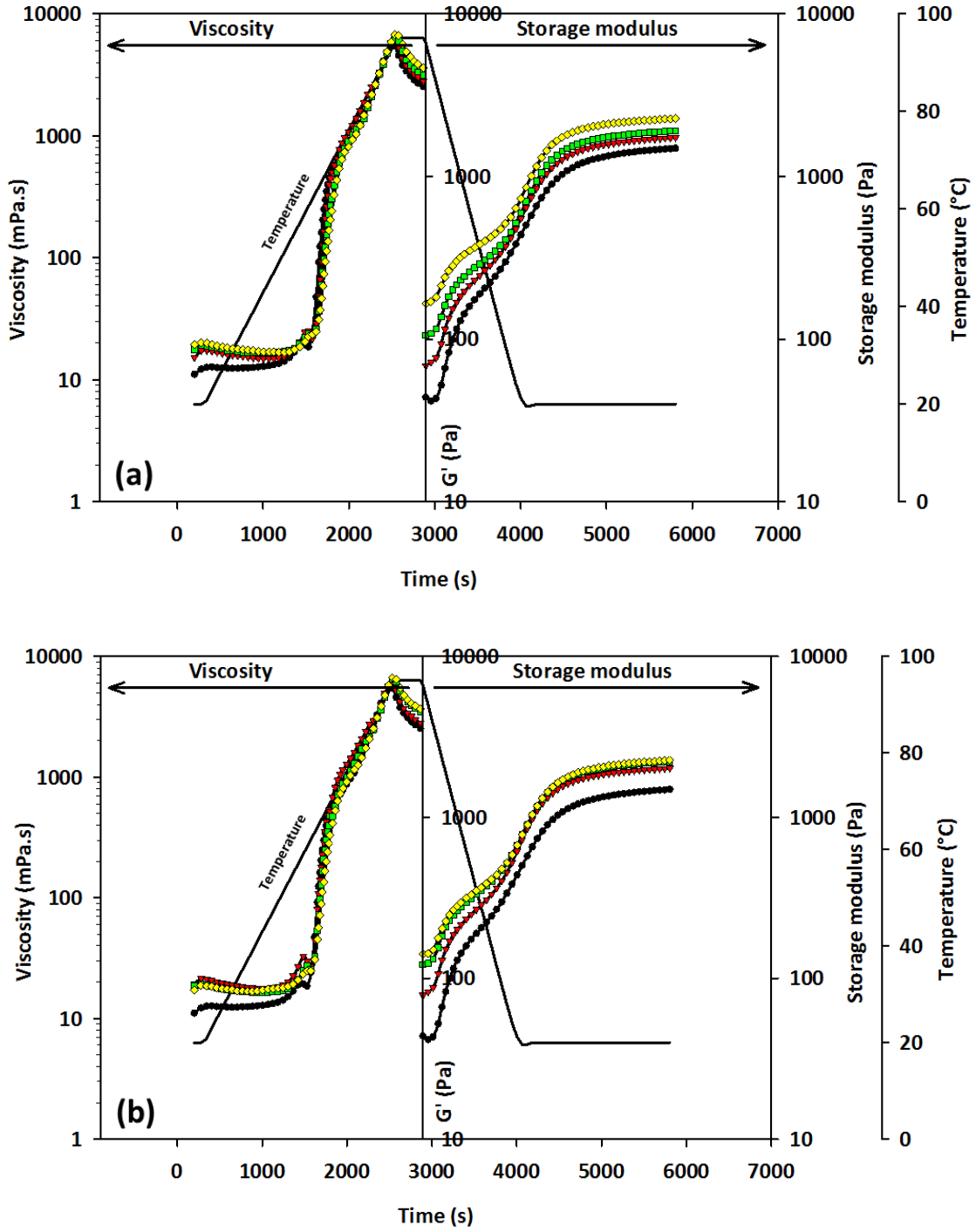


Figure 7-8 The rheological properties of 10 % w/w wheat starch + 2 % w/w MCP in the presence of 0 (●), 0.5 (▼), 1 (■) and 1.5 % w/w (◆) NaCl (a) and KCl (b).

In contrast to monovalent salts, the addition of divalent salts resulted in a slight decrease in the strength of starch-MCP gels (Figure 7-9). Again, this result was consistent with the effect of calcium chloride on the pasting properties of rice starch gels containing *Mesona blumes* gum (Feng *et al.*, 2016). The weakening of the gel was more prominent for $CaCl_2$ (~25 % decrease) than for $MgCl_2$ (~13 % decrease). It is also interesting to note that the addition of $CaCl_2$ resulted in the disappearance of peak M and this peak M was also not observed in the presence of 1.5 % w/w $MgCl_2$. Past studies on the effect of salt on the interaction between starch and NSPs have shown opposing results where the authors reported increases in the viscosities of the starch-MCP (Lai & Chao, 2000a) and starch-xanthan (Samutsri & Suphantharika, 2012) pastes when $CaCl_2$ was added. However, the magnitude of the increase was found to be dependent on $CaCl_2$ concentration. It is likely that the difference in the results was due to the difference in the concentration of starch used in the case of starch-MCP system (10 % w/w in this study and 2 % w/w in the other study) and a different type of polysaccharide used in the case of starch-xanthan system. The role of divalent and monovalent cations on starch gelatinisation properties have only been studied in depth at high ionic concentrations, whereby divalent cations (calcium) were found to induce starch gelatinisation at the periphery, whereas gelatinisation began at the hilum in the presence of monovalent cation (potassium) (Jane, 1993). The effect was thought to be dependent on the charge density of the cations. The mechanisms by which this occur involve the ability of the cation to interact with water and with the hydroxyl groups of starch. However, in dilute solutions, salts are dissociated completely into their ionic components and this is likely to have a greater effect on the charged NSP present in the system— MCP in this case. It is known that the extract of MCP used in this study contained a large amount of calcium that is likely to be bound onto the anionic polysaccharide (see Chapter 9). The role of the calcium in the extract is not fully understood but may be involved in the intermolecular associations between MCP polymers. The addition of more divalent cations (calcium or magnesium) may result in greater charge screening, causing MCP chains to adopt a more compact configuration, preventing it from forming an extended network with starch. Therefore, it is likely that a maximum calcium concentration exists where the interaction between MCP and wheat starch will be at its maximum. The role of calcium in the interaction between wheat starch and MCP will be further explored in Chapter 9.

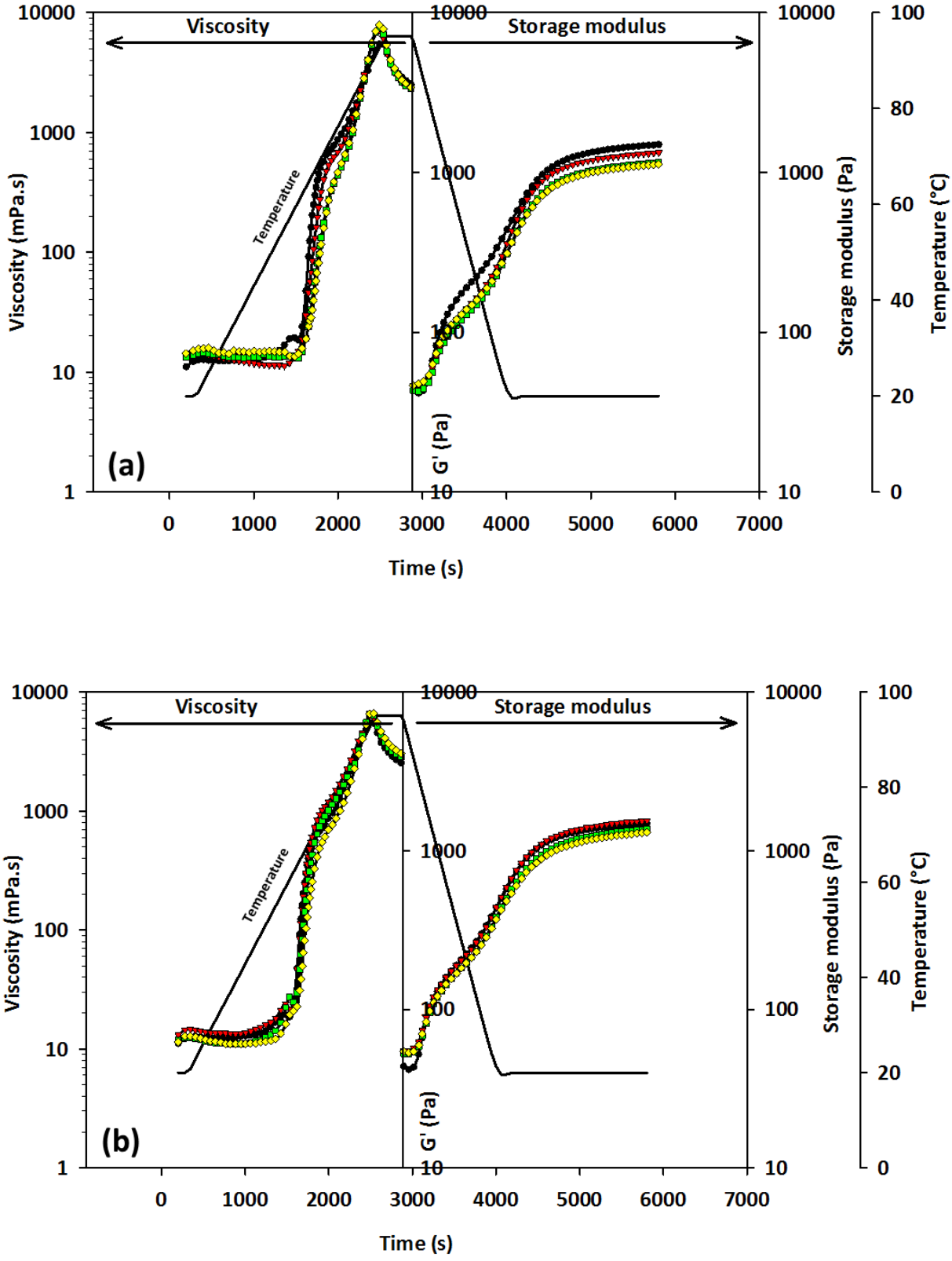


Figure 7-9 The rheological properties of 10 % w/w wheat starch + 2 % w/w MCP in the presence of 0 (●), 0.5 (▼), 1 (■) and 1.5 % w/w (◇) $CaCl_2$ (a) and $MgCl_2$ (b).

7.4 Conclusion

The rheological properties of starch-MCP gels were affected by the starch type, polysaccharide ratio, changes in pH and addition of salt. MCP was thought to interact with amylose and decrease granule swelling, which led to different effects on the hot peak viscosity and gel strength of different starches. For wheat and maize starches, both their peak viscosity and gel strength were increased with increasing additions of MCP. However, for potato starch, there was a reduction in the peak viscosity despite an increase in the gel strength. This was likely due to the reduction in starch granule swelling—an important factor that determines the viscosity of potato starch paste. Interestingly, at high MCP concentration, a gel was formed for waxy maize, which has very low proportions of amylose. The ratio of starch to MCP determines the formation of wheat starch-MCP gels, whereby decreasing the proportion of starch in the mixture resulted in phase separation and weakening of the gel due to the sparse packing of starch granules.

The interaction between wheat starch and MCP was also found to be influenced by changes in pH and salt concentrations. While the addition of both monovalent and divalent salts slightly alters the strength of starch-MCP gels, the strength of these gels seemed to be more sensitive to the changes in the pH of the system. It is proposed that both the addition of salts and changes in the pH of the system cause MCP to adopt a different conformation (compact or open coil) allowing either more or less interactions between starch polymers and MCP. While the results are clear, more studies need to be done to understand the mechanisms by which the factors explored in this study affect the interaction and, consequently, the gel strength of starch-MCP systems

Chapter 8 [4]*In vitro* digestibility of wheat starch-MCP gels – Role of gel structure

8.1 Introduction

Starch is the most important source of carbohydrate in the human diet and is obtained from a large variety of plant sources. Wheat starch is found in many staple food products including bread, breakfast cereals, and pasta. In the human gastrointestinal tract, wheat starch is broken down by the salivary and pancreatic amylase into glucose, which is absorbed in the small intestine into the blood stream termed as blood glucose. Under the regulation of insulin, blood glucose is metabolised or stored. However, an over consumption of easily digestible starchy foods will result in elevation of blood glucose, which can lead to Type II diabetes (Lehmann & Robin, 2007; Miao, Jiang, Cui, Zhang, & Jin, 2015).

Digestion of starch can be divided into three types depending on their degree of digestibility: rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) (Englyst *et al.*, 1992). The amount of RDS corresponds to the amount of glucose released after 20 minutes, SDS between 20 and 120 minutes, and RS the remaining starch (total starch minus the amount of glucose released after 120 minutes) (Singh *et al.*, 2010). High proportions of RDS in the diet result in rapid increase in blood glucose and insulin levels. Resistant starch, on the other hand, is scarcely digested in the small intestine but is fermented in the colon. In the case of SDS, which has attracted considerable research interest in recent years, it is digested less rapidly with a small and gradual increase in blood sugars closely matching a normal glucose absorption, hence eliminating or reducing postprandial hyperglycaemia (Lehmann & Robin, 2007).

The rate of starch digestion can be controlled by the addition of certain non-starch polysaccharides (NSPs) to the diet. The mechanisms by which this occurs vary depending on the type of NSP with the most common explanation being an increase in the viscosity of the gut contents (Brennan *et al.*, 2008). An increase in the viscosity of digesta slows down mixing in the lumen of the small intestine (Lentle & Janssen, 2008), reduces the mass transfer of enzyme and their substrate (Dartois *et al.*, 2010) and prolongs the time it takes for glucose to be absorbed (Vaugelade *et al.*, 2000). The presence of highly

[4] This chapter has been submitted as Yuris, A., Matia-Merino, L., Hardacre, A. K., & Goh, K. K. T. (In submission). The effect of gel structure on the *in vitro* digestibility of wheat starch-Mesona chinensis polysaccharide gels. *Food & Function*.

viscous NSPs is also known to reduce starch granular swelling by reducing water availability (Krüger *et al.*, 2003). Consequently, gelatinisation of these granules decreases, which leads to a lower degree of starch hydrolysis. It has been shown that completely ungelatinised starch granules were digested about 40 % less than fully gelatinised granules after 60 minutes *in vitro* in excess of porcine pancreatic α-amylase (Holm, Lundquist, Bjorck, Eliasson, & Asp, 1988). This is often associated with the presence of a more crystalline structure of the starch granules, making them less susceptible to enzymatic attack (Chung, Lim, & Lim, 2006). Furthermore, certain NSP such as xanthan (Gonera & Cornillon, 2002) has been shown to encapsulate starch granules by surface association and this has been proposed as a possible mechanism to inhibit or reduce starch hydrolysis due to the presence of a physical barrier for enzyme accessibility (Brennan *et al.*, 2004; Dartois *et al.*, 2010). Alternatively, an NSP may non-competitively bind onto amylase to prevent substrate binding, which hinders digestion (Slaughter *et al.*, 2002), therefore reducing postprandial glycaemia.

Mesona chinensis polysaccharide (MCP), obtained from the extract of the *Mesona chinensis* herb, is an anionic polysaccharide that, unlike many other hydrocolloids, has a very low viscosity in solution. It is unique in that it synergistically increases the viscosity and gel strength of certain non-waxy starches including wheat, maize and rice during the pasting process (Feng *et al.*, 2014). However, synergistic increase in starch gel strength can only be attained at specific concentrations of starches and MCP; for instance, for a 10 % w/w wheat starch suspension, at least 5 % w/w MCP is required to increase the strength of the starch gel (Yuris, Matia-Merino, Hardacre, Hindmarsh, & Goh, 2018). Concentration dependency of starch gel properties in the presence of MCP has also been observed for corn and mung bean starches, whereby at a concentration below 0.35 % w/w MCP, a decrease in the cooled paste viscosity (measured using the rapid visco analyser) was observed (Feng *et al.*, 2014). In the former, where a synergistic increase in gel strength is observed at specific starch and MCP concentrations, the interaction between amylose and MCP creates a homogenous network as the gel sets (Lai & Chao, 2000b; Yuris *et al.*, 2017). In the latter, heterogeneity within the gel network occurs when insufficient amylose or MCP is available for interaction. Therefore, for high starch-low MCP or low starch-high MCP gels, the decrease in the elastic moduli of the gels is probably due to the formation of a heterogeneous gel network (Yuris *et al.*, 2018). Although MCP has been traditionally used in Asia to produce a firm black coloured starch-based gel dessert known as “grass jelly”, the impact of its structure on starch digestibility is not known. The consumption of a solution of *Mesona chinensis* extract following a meal containing a high proportion of carbohydrates has been shown to reduce postprandial hyperglycaemia as a result of α-glucosidase inhibition by the polyphenols found in the extract (Chusak *et al.*, 2014). However, no study quantified the digestibility of the gels formed from starch that has been gelatinised together with MCP. The aim of this study was to compare the

digestibility of MCP-wheat starch gels of various macrostructures (intact gels, fragmented gels and macerated gels) to elucidate whether different gel structures could influence the digestibility of wheat starch gels in the presence of MCP. Xanthan, guar, locust bean gum (LBG) and agar were also included in this study to compare the effectiveness of MCP relative to other polysaccharides in reducing the digestibility of wheat starch gels.

8.2 Materials and methods

8.2.1 Starch-polysaccharide suspension preparation

Starch-polysaccharide suspensions were prepared as per section 5.2.2. The polysaccharides used in this study were MCP, guar (Danisco, Copenhagen, Denmark), xanthan (Sigma-Aldrich, St. Louis, MO, USA), locust bean gum (Danisco, Copenhagen, Denmark) and agar (Hawkins Watts, Auckland, New Zealand). For *Mesona chinensis* powder, the resulting solution was centrifuged at 4000g for 30 minutes to remove insoluble materials. Wheat starch (Penford, Sydney, NSW, Australia) was suspended in water and added to the hydrated polysaccharide solution to make suspensions containing 10 % w/w starch and 2 – 5 % w/w MCP, 0.5 % w/w guar, 1 % w/w xanthan, 1 % w/w LBG or 0.3 % w/w agar. The NSP concentrations were selected to match the hardness of starch gels containing 2 % w/w (16.5 ± 0.5 N) and 5 % w/w (23.0 ± 1.9 N) MCP gels. The use of gels with similar hardness allow for the assumption that for all gels, the extent of which enzymes could penetrate into the gels are similar.

8.2.2 Rheology

The rheological properties of the starch and polysaccharide suspensions were measured using a combination of rotational and oscillatory techniques as per section 5.2.3. Measurements were carried out in triplicate.

8.2.3 Texture analysis

Starch-polysaccharide gels were prepared as per section 6.2.3.1 and a single compression test was carried out as per section 6.2.3.2. Measurements were carried out in triplicate.

8.2.4 *In vitro* digestion

The digestibility of wheat starch-MCP gels were measured in two ways: (i) in a rheometer and (ii) a shaking water bath. Both methods measured the digestibility of gels under constant shear (rheometer) and the digestibility of intact, fragmented and macerated gels under conditions of minimal shear (shaking water bath). All digestions were carried out in triplicate.

8.2.4.1 Digestion in the rheometer

Fifteen millilitres (15 mL) of 10 % w/w wheat starch suspension containing MCP (2 % w/w and 5 % w/w) was added into the starch cell. The sample was gelatinised as per section 5.2.3 and the gels were allowed to set at 20°C overnight. The weight of the system was measured before and after gelatinisation to determine water loss due to evaporation. Appropriate quantities of water were then added to compensate for that loss during the pasting process.

In vitro digestion of the gels formed in the starch cell was carried out according to Mishra, Monro, and Hedderley (2008) with modifications. Three phases of digestion: oral, gastric and small intestinal were simulated in the rheometer under constant shearing (130 s^{-1}) and a temperature of 37°C during which the gels were broken by the imposed shear. Firstly, 4 g of water were added to the set gel sample that was left overnight in the rheometer to act as a medium for enzyme dispersion. The temperature of the gel was increased to 37°C and allowed to equilibrate for 2 minutes at a constant shear rate of 130 s^{-1}. These conditions were maintained throughout the digestion process. The oral phase commenced with the addition of 0.05mL of 0.1 % w/w of a-amylase (A6255, Sigma-Aldrich, St. Louis, MO, USA) dispersed in milliQ water and digestion (in the oral phase) was allowed to proceed for a minute. The gastric phase was then initiated by the addition of 1.75 mL mixture of 1 M HCl and MilliQ water to reduce the pH to 2.5 ± 0.5. The ratio of water to HCl added was predetermined for each gel in order to obtain the correct pH while maintaining the same final volume. This was followed by the addition of 0.1 mL of 10 % w/w pepsin (P7000, Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.05 M HCl and 0.05 mL of 1 % w/w lipase (L3126, Sigma-Aldrich, St. Louis, MO, USA) dissolved in MilliQ water. These conditions were maintained for 30 minutes to complete the gastric phase. To begin the small intestinal phase, 0.25 mL of a mixture of water and 1 M $NaHCO_3$ were added to increase the pH to 6.2 ± 0.5. This was followed by the addition of 0.75 mL of 10 % w/w bile extract (B8631, Sigma-Aldrich, St. Louis, MO, USA) suspended in 0.1 M sodium maleate buffer containing 1 mM $CaCl_2$ and 0.02 % w/w sodium azide. An aliquot (0.12 mL) was then removed and immediately dispensed into a preweighed micro-centrifuge tube containing 0.12 mL of chilled absolute ethanol (time = 0 minutes). Following this, 0.05 mL of amyloglucosidase (E-AMGDF, Megazyme, Wicklow, Ireland) and 0.05 mL of 5 % w/w pancreatin (P7545, Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M sodium maleate buffer were added into the starch cell. Samples were removed into microcentrifuge tubes containing chilled ethanol to halt digestion at 1, 2, 5, 10, 15, 20, 30, 60 and 120 minutes after the addition of amyloglucosidase and pancreatin. Blanks were prepared by carrying out the digestion in the absence of amyloglucosidase and pancreatin to eliminate interference from the colour of MCP.

8.2.4.2 Digestion in shaking water bath

Sample of the starch-polysaccharide gels prepared using the RVA (Rapid Visco Analyser, Perten Instruments, Massachusetts, USA) as described in section 6.2.3.1 and were each poured into a cylindrical mould (diameter = 3.2 cm and height = 0.8 cm) to allow the gel to set. The set gels were then weighed (~7 g) and the water content determined with the oven drying method and the total starch content was calculated by subtracting the weight of the water from the weight of the gel. Three types of gels were prepared: intact cylindrical gel, fragmented gel and macerated gel. Intact cylindrical gels were prepared as described above. The intact gel was then passed twice through a woven wire

mesh with a mesh diagonal aperture of 0.5 mm to create fragmented gels. Macerated gels were prepared by adding water to the fragmented gels and passing the mixture through the mesh five times to produce a dispersed gel pulp. The amount of water (10 mL) used to macerate the gels equate to the amount of water that was added for enzyme dispersion in intact and fragmented gels in order to prevent sample dilution. Digestion of the gels was carried out in a cylindrical screw top container with a 4.1 cm diameter and 5.8 cm height. The containers were placed in a 37°C water bath (BS-11, Lab companion, Seoul, Korea) under constant linear shaking of 130 rpm. Digestion was initiated by adding 10 mL of MilliQ water (for enzyme dispersion) to all samples except for the macerated gels where 10mL of water was already added during its preparation. Images of these gels are shown in Figure 8-1. *In vitro* digestion was then carried out by scaling up (2.5 times) the volumes described in section 8.2.4.1.

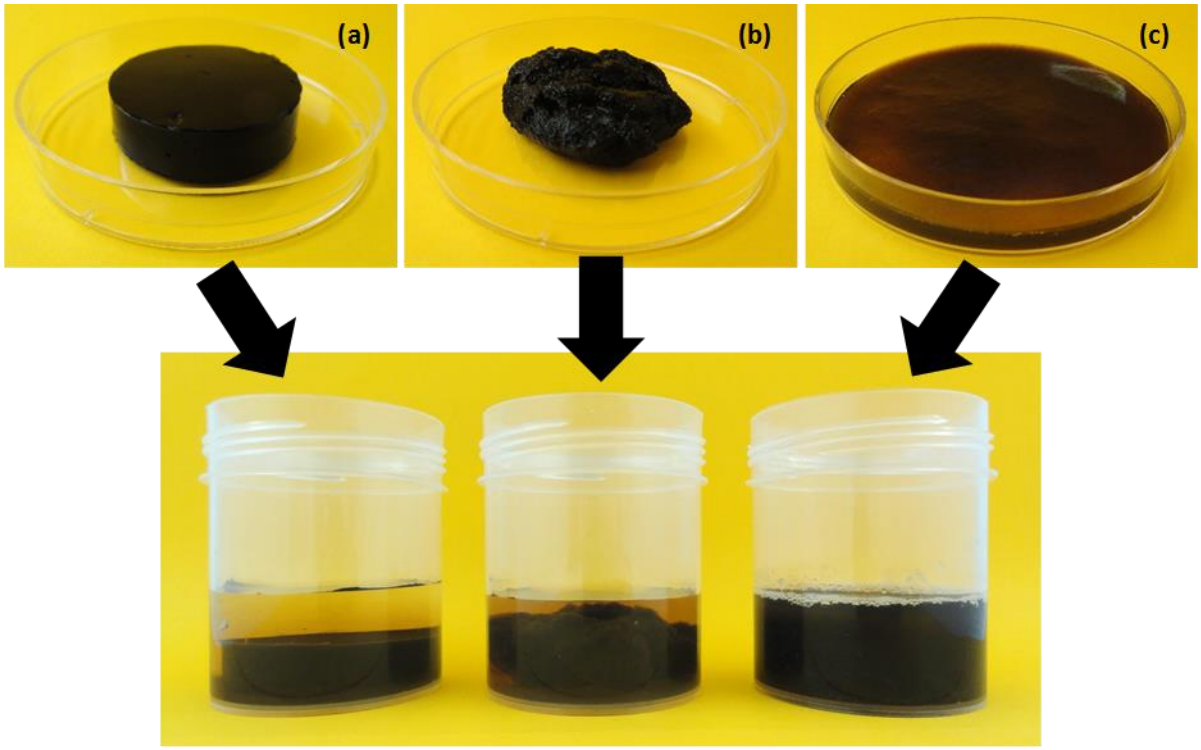


Figure 8-1 Images of prepared intact (a), fragmented (b) and macerated (c) wheat starch-MCP gels (top) and after addition of a fixed amount of water prior to the gastric phase (bottom).

8.2.5 Sugar analysis

The amounts of glucose resulting from the digestion of starch in the samples described above were determined using a modified DNS method (Mishra *et al.*, 2008). The tubes containing the digestate in ethanol were centrifuged at 10000 g for 15 minutes to remove solids. An aliquot (0.05 mL) of the supernatant was transferred into a 10 mL Kimax tube and 0.25 mL of 1 % v/v amyloglucosidase (E-AMGDF, Megazyme, Wicklow, Ireland) and 1 % v/v invertase concentrate (Fisher Scientific, Hampton,

NH, USA) both in 0.1 M sodium acetate buffer pH 5.2 was added to the tube to convert any remaining carbohydrates to glucose. The mixture was incubated in a 37°C water bath for 10 minutes before the addition of 0.75 mL of DNS mixture containing, in a ratio of 1:1:5 of the following: 0.5 mg/mL glucose, 4 M NaOH and DNS reagent (10 g 3,5-dinitrosalicylic acid, 16 g NaOH and 300 g Na K tartrate dissolved in 1 L water). The tubes were then capped and placed in a water bath at 95°C for 15 minutes for colour development. The tubes were then removed from the water bath and cooled to room temperature over 15 minutes. Water (4 mL) was then added to the mixture and the tubes were inverted several times to ensure even mixing. Absorbances at 530 nm were measured using a spectrophotometer (Helios Epsilon spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA).

8.2.6 Statistical analysis

An analysis of variance (ANOVA) with Tukey's range test as the post-hoc test was performed using Minitab 16.

8.3 Results and discussion

8.3.1 Physical properties of wheat starch-MCP gels

The rheological and textural properties of 10 % w/w wheat starch gels with and without MCP are shown in Table 8-1. Increasing the concentrations of MCP increased peak viscosity (PV) of the hot paste. This is characteristic of many viscous NSPs when added to starch (Funami *et al.*, 2005a), whereby phase separation results in the localisation of the NSP in the continuous phase, hence increasing the overall viscosity (Alloncle & Doublier, 1991). Due to the low viscosity of MCP, it is likely that the rise in PV was a result of the interaction between the starch and polysaccharide, forming a network structure. Despite the formation of stronger gels when 5 % w/w MCP was present (higher final *G'* of 2843 ± 144 Pa and hardness of 23 N), low MCP concentration (2 % w/w) resulted in a weaker gel as indicated by a lower final *G'* (1489 ± 8 Pa versus 2133 Pa) and hardness (16.5 ± 0.5 N versus 18.6 ± 0.7 N). We have previously shown that the formation of strong starch-MCP gels was attributed to the interaction between MCP and amylose as it leached from the granules during pasting, hence forming a homogenous and dense network (Yuris *et al.*, 2018). However, when the concentration of MCP was lowered, the limited availability of MCP and amylose for interaction resulted in heterogeneity within the network, so a low MCP concentration disrupted/weakened the structure of the gel. Detailed explanations on the interaction and physical properties of wheat starch and MCP gels can be found in Chapter 5 and Chapter 6. Due to the effect that MCP concentration has on the hardness of wheat starch gels (weakening and strengthening), this study investigated the digestibility of both, the weaker gel (containing 2 % w/w MCP) and stronger gel (containing 5 % w/w MCP) to ascertain the role of their interaction and the importance of gel strength in reducing starch-MCP digestibility.

Table 8-1 The peak viscosities, final *G'* and hardness of 10 % w/w wheat starch gel in the absence and presence of MCP. Values are the mean ± standard deviation of n = 3; different letters within a column represents significant differences (P ≤ 0.05).

Samples (w/w)	PV of hot paste (mPa.s)	Final *G'* of set gels (Pa)	Hardness of set gels (N)
10 % wheat starch	4601 ± 75 [a]	2133 ± 60 [a]	18.6 ± 0.7 [a]
10 % wheat starch + 2 % MCP	5580 ± 290 [b]	1489 ± 8 [b]	16.5 ± 0.5 [a]
10 % wheat starch + 5 % MCP	7667 ± 573 [c]	2843 ± 144 [c]	23.0 ± 1.9 [b]

8.3.2 Wheat starch digestibility in the presence of various polysaccharides

The digestibility of fragmented wheat starch gels in the absence and presence of various concentrations of NSPs that had similar strength to wheat starch gel containing 2 % w/w and 5 % w/w MCP is shown in Figure 8-2 (non-gelling NSP) and Figure 8-3 (gelling NSP) respectively. Fragmented gels were chosen for this experiment since they better represent the state of gels that enter the gut (after chewing). Three viscous NSPs, 1 % w/w xanthan (17.8 ± 1.8 N), 0.5 % w/w guar (17.3 ± 1.4 N) and 1 % w/w LBG (16.9 ± 1.9 N), were used to decrease the gels' hardness to a level that is comparable to starch gels containing 2 % w/w MCP (16.5 ± 0.5 N). Despite the hardness of these gels being lower than that of wheat starch alone, starch digestibility was reduced in the order of MCP (~10 % digested at 20 min) < xanthan < guar < LBG (~18 % digested at 20 min) in comparison to wheat starch gels alone (~25 % digested at 20 min). While no data is available on the digestibility of LBG, similar results have been previously reported for xanthan and guar, whereby xanthan was found to be more effective in reducing starch digestibility than guar (Sasaki & Kohyama, 2012). This was attributed to the ability of xanthan to encapsulate starch granules, which reduces enzyme accessibility (Brennan *et al.*, 2004). Since the gels were fragmented, the viscous NSPs added to the gels were leached into the digestion mixture. The increase in viscosity of the digestion mixture due to leached NSPs may be a contributing factor to the reduction in the digestibility of these gels compared to the starch gel alone (though this is not substantial for MCP). It is expected that with increased viscosity, the enzyme substrate interaction would be decreased due to reduced mobility and therefore, the rate of starch digestion will be reduced (Dartois *et al.*, 2010).

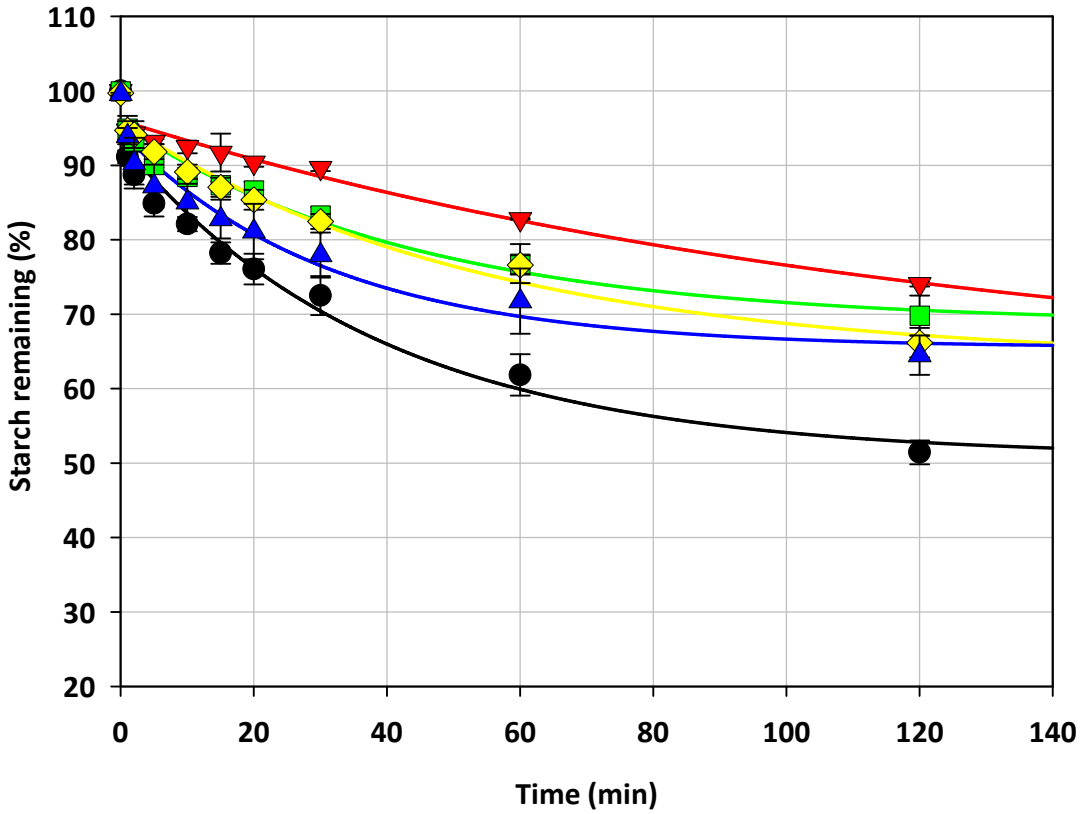


Figure 8-2 The digestibility of fragmented 10 % w/w wheat starch gels in the absence (●) and presence of 2 % w/w MCP (▼), 1 % w/w xanthan (■), 0.5 % w/w guar (◆) and 1 % w/w LBG (▲).

A large reduction in starch digestibility (80 % starch remained after 120 minutes of digestion) was also observed when 5 % w/w MCP was present in the starch gel (Figure 8-3). However, the addition of agar, a gelling polysaccharide, did not reduce starch digestibility, despite an increase in the gel hardness to a level similar to the gel containing 5 % w/w MCP. This was likely due to its gelling nature, which results in agar gels fragments being present in the digestate rather than it being leached into the digestate, therefore not contributing to a rise in viscosity. This result was consistent with the report by Sasaki and Kohyama (2011) for milled rice starch-agar gels. However, they reported reduced digestibility for intact rice starch-agar gels, suggesting that gel strengthening by the addition of agar could contribute to decreased enzyme accessibility. This highlights the fact that agar would not be the ideal NSP to be used for manipulating starch digestion in starchy food since the chewing process would fragment the gels, resulting in no reduction in starch digestibility. The fact that the digestion rates of fragmented starch gels were unaffected by the addition of agar suggests that interaction between the two is unlikely. The ability of MCP to reduce starch digestibility despite the disruption of the gel structure indicates that gel hardness was not the sole factor in determining the digestibility of starch-MCP gels. The fact that MCP has a low viscosity further shows that viscosity increase as a result of polymer leaching did not influence the digestibility of starch-MCP gels.

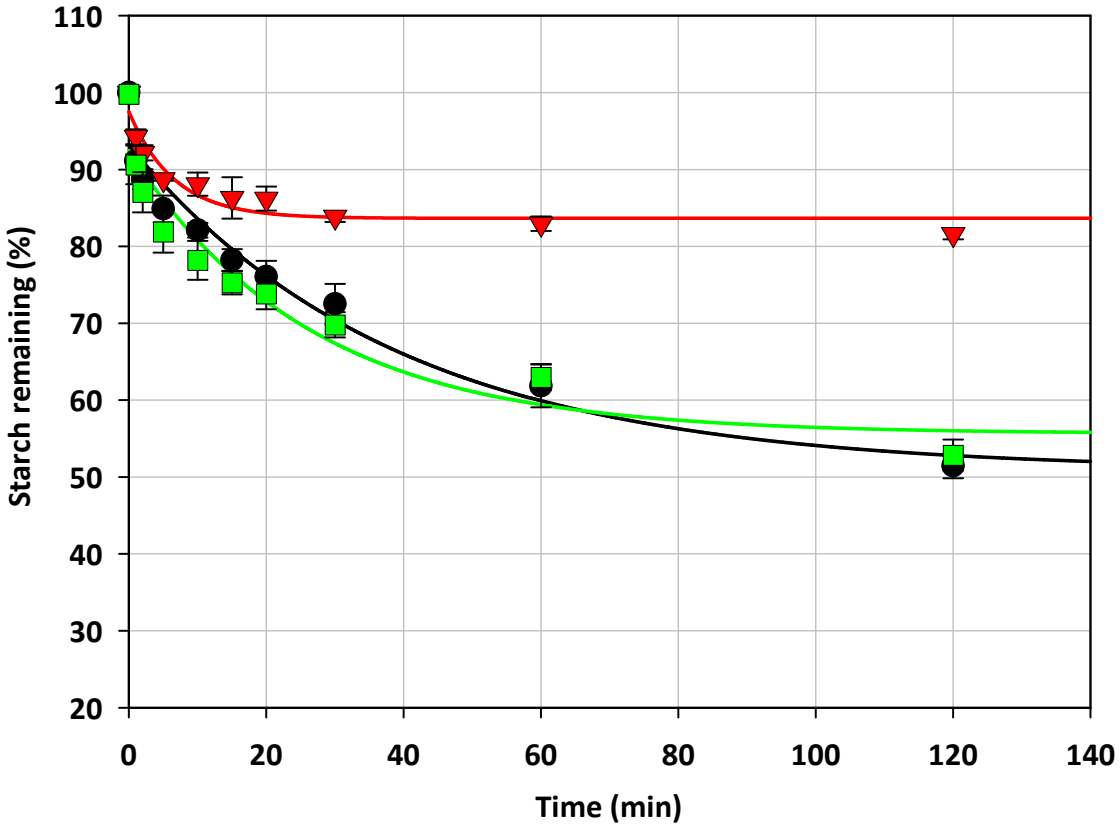


Figure 8-3 The digestibility of fragmented 10 % w/w wheat starch gels in the absence (●) and presence of 5 % w/w MCP (▼) and 0.3 % w/w agar (■).

8.3.3 Digestion of wheat starch-MCP gels under constant shear

The digestibility of wheat starch-MCP gels was also studied using a rheometer. The setup employs a constant shear and temperature during digestion. The shear rate used was 130 s^{-1} in order to ensure that the enzymes were evenly dispersed throughout the system and even mixing was achieved. The digestibility of 10 % w/w wheat starch in the presence and absence of MCP under constant shear rate is shown in Figure 8-4. The addition of MCP did not result in any change to the amount of RDS in wheat starch gel. However, the amount of SDS was increased in the presence of 5 % w/w MCP as indicated by a decrease in the digestibility of wheat starch between 20 and 120 minutes (41 ± 0.2 % versus 58 ± 2.6 % starch remaining at 120 minutes in the absence and presence of 5 % w/w MCP). On the other hand, the addition of 2 % w/w MCP did not alter the digestibility of the starch gel. Reduced digestibility of wheat starch gel in the presence of 5 % w/w MCP can be explained by in terms of the hardness of the gel itself. Due to the constant shearing in the rheometer, gels are broken up and their particle size was dependent on the gel hardness—stronger gels were less susceptible to being broken up by shear. Therefore, for a stronger gel (containing 5 % w/w MCP), shearing in the rheometer would result in the formation of larger gel pieces as compared to the fine gel pieces produced from shearing a weaker gel (containing 2 % w/w MCP). Starch digestibility is reduced for larger gel particles due to their smaller

surface area as a result of an overall slower rate of enzyme diffusion into the gel (Mahasukhonthachat, Sopade, & Gidley, 2010). Interestingly, despite the weakening of starch gel when 2 % w/w MCP was present, the digestibility of the gel was not increased. It can be deduced from this result that at a constant shear rate, the digestibility of wheat starch gels containing MCP was influenced by not only the hardness of the gel but also other factors. Despite the reduction in starch digestibility when gel strengthening occurs at high MCP concentration (5 % w/w), there is uncertainty towards the exact mechanism by which MCP reduces starch digestion due to the uneven sizes of broken gels as a result of shear in the rheometer. Hence, the effect of gel hardness on starch-MCP digestibility was further investigated by using gels of different macrostructure.

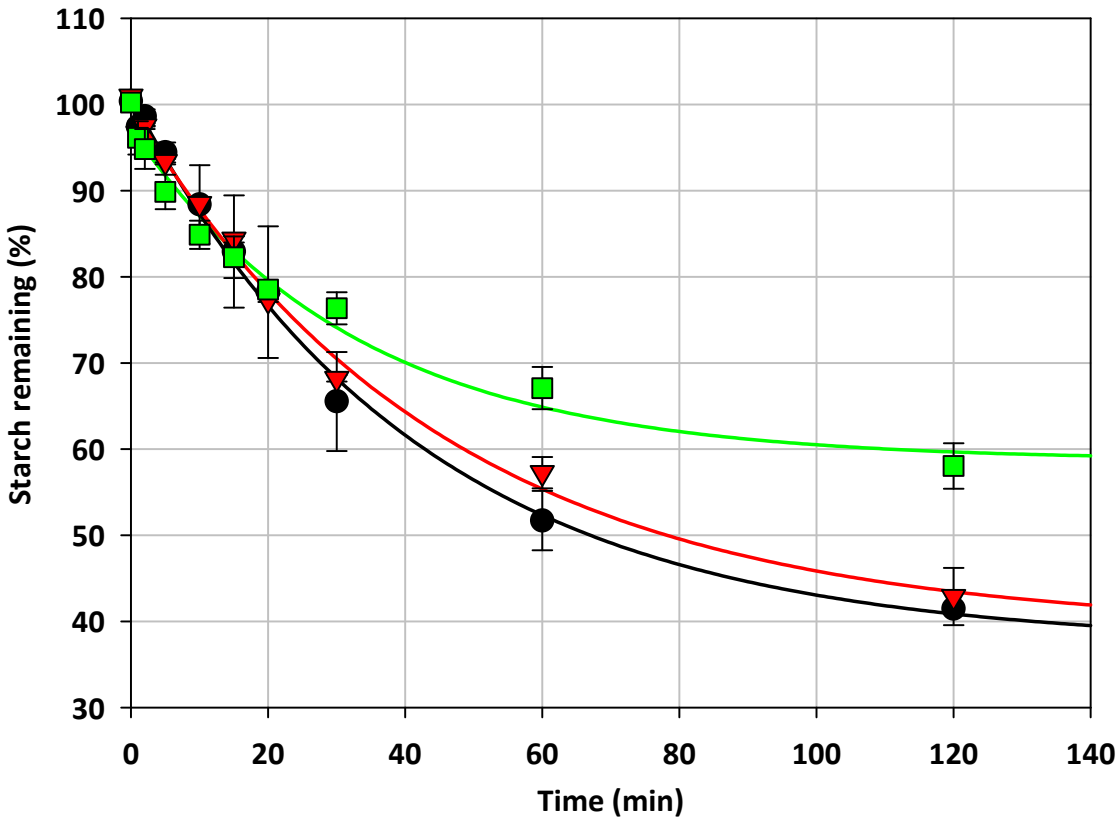


Figure 8-4 The digestibility of 10 % w/w wheat starch gels in the absence (●) and presence of 2 % w/w (▼) and 5 % w/w (■) MCP under constant shear rate.

8.3.4 The effect of wheat starch-MCP gel structure on starch digestibility

The effect of the physical properties of wheat starch-MCP gels on their digestibility was further explored using three distinctive gel types: an intact cylindrical gel (Figure 8-5a), a fragmented gel (Figure 8-5b) and a macerated gel (Figure 8-5c). The use of these gels allows for the comparison of the physical structure of the gel on its digestibility under linear shearing that does not disrupt the gel structure. For intact gels, the rate of hydrolysis was much lower than that for the fragmented and macerated gels. This was expected due to a lower surface area for enzymes to hydrolyse the starch in

the gel. Higher rates of digestion were observed, particularly in the first 20 minutes when macerated gels were used (31 ± 1.0 % starch remaining versus 76 ± 2.1 % and 92 ± 0.3 % starch remaining for fragmented and intact gels, respectively). The same observation was reported by Sasaki and Kohyama (2011) for intact and milled rice starch whereby more starch hydrolysis was observed for the milled sample.

The digestibility of intact wheat starch gel in the presence or absence of MCP is shown in Figure 8-5a, whereby a decrease in starch digestibility was observed for wheat starch gel containing 5 % w/w MCP. For an intact gel, the digestibility of the gel is dependent primarily on the ability of the enzymes to penetrate into the gel to access the substrate. Hence, the structure and network of the gels influences the rate and extent of digestion. Therefore, in this instance, the digestibility of wheat starch gel containing 5 % w/w MCP may reflect the higher strength of the gel due to the presence of a stronger network. Despite a decrease in the gel hardness when 2 % w/w MCP is present, the digestibility of the gel was slightly lower or comparable to 10 % w/w wheat starch on its own. These findings conclusively indicated that while the gel strength could affect an enzyme's accessibility to its substrate, it was not the sole factor that affected the digestibility of wheat starch-MCP gels, as initially suggested from the results in the previous section.

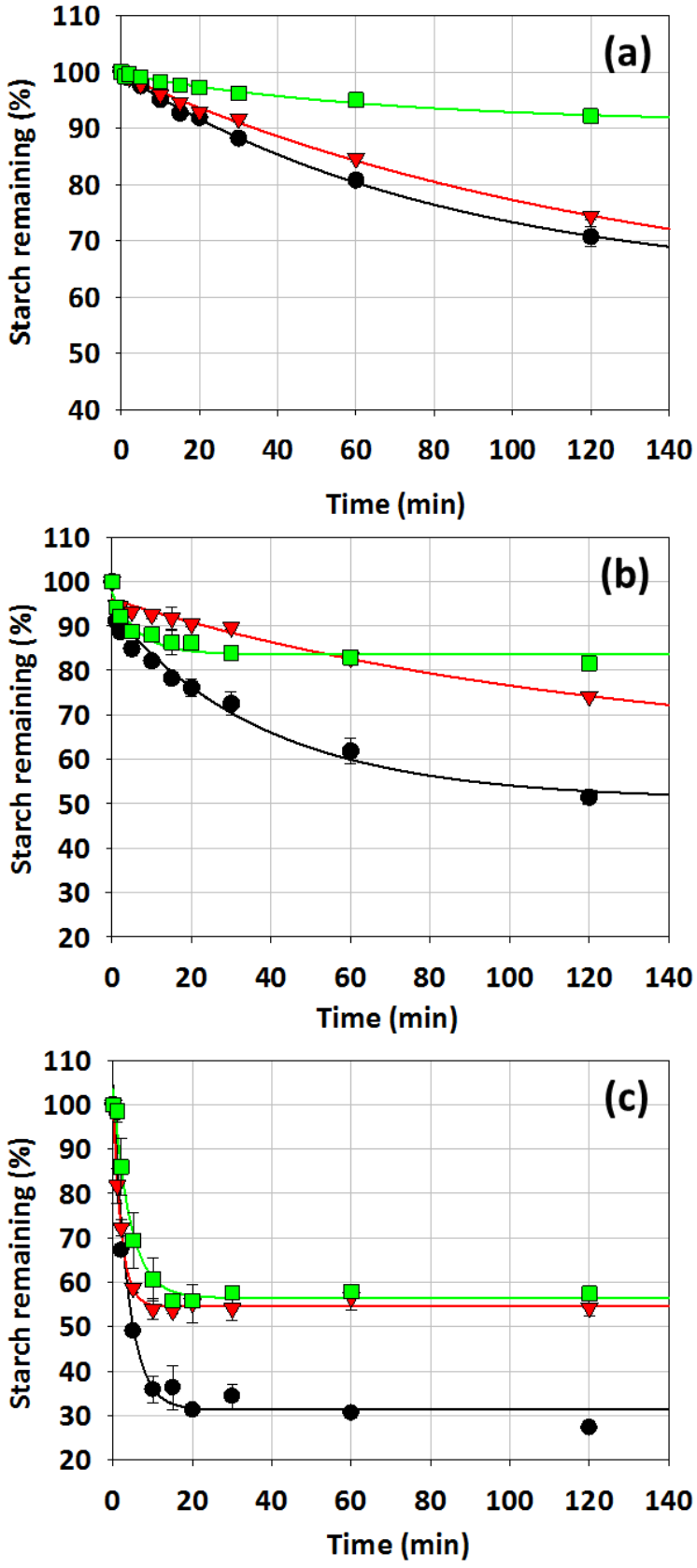


Figure 8-5 The digestibility of 10 % w/w wheat starch intact cylindrical gels (a), fragmented gels (b) and macerated gels (c) in the absence (●) and presence of 2 % w/w (▼) and 5 % w/w (■) MCP.

The digestibility of fragmented gels is shown in Figure 8-5b. The total amount of starch remaining after 120 minutes of digesting these gels were lower than that of the intact gels and this was due to an increase in the surface of the gels, which allowed enzymes to access their substrate more easily. For a fragmented gel, the addition of 5 % w/w MCP reduced starch digestibility by 60 % after 120 minutes as compared to a 30 % reduction for intact gels (Figure 8-5a and b), indicating that the ability of MCP to reduce starch digestibility was more prominent in a fragmented gel. The digestibility of fragmented gels is affected not only by the size of the gel fragments but more so by the release of NSPs from the matrix into the digestate, resulting in an increase in viscosity as in the case of xanthan and konjac glucomannan (Sasaki & Kohyama, 2011). In such instance, starch digestibility can be hindered as a result of restricted enzyme mobility due to the high viscosity of the digestate in the presence of the NSP (Brennan *et al.*, 2008). This is unlikely for MCP due to its low viscosity and, hence, its release from the gel matrix into the digestate would not greatly impact the digestate viscosity.

In the absence of MCP, the total amount of starch digested (after 120 minutes) was 30 % higher when the intact gels were fragmented. In contrast, this remained at a similar level when MCP was present despite gel fragmentation. This suggests that in the presence of MCP, the degree of starch hydrolysis by enzyme remained limited despite the disruption of the gel structure. Such result may reflect a situation in which MCP encapsulate the starch granules, thereby limiting enzyme access, as hypothesised by Brennan and Tudorica (2008) for other NSPs. The ability of MCP to encapsulate starch granules is not known but its interaction with amylose has been hypothesised to occur as the polymer leaches out, creating an amylose-MCP coat around the granule (Yuris *et al.*, 2017). The formation of barriers around starch granules, together with NSP's hydration capacity (Tester & Sommerville, 2003), could limit water mobility, which would consequently result in reduced granular swelling. This effect is often accompanied by reduced starch hydrolysis as a result of not only limited access to the granule, but also the unavailability of substrate due to the tight packing of starch polymer within the partially gelatinised granules (Chung *et al.*, 2006).

Interestingly, the initial rate of digestion (first 30 minutes) of fragmented gels was lowest when a low concentration of MCP (2 % w/w) was present, despite being a weaker gel. We hypothesised that the presence of MCP affected the stickiness of the fragmented gels so that those containing low concentration of MCP (2 % w/w) tended to stick more and form large gel clumps that had lower surface area, resulting in an initial reduction in digestibility. The hypothesis was proposed based on the fact that the stickiness of a starch gel is primarily dependent on the cohesive effect of amylopectin. The formation of an amylose network is known to interfere with amylopectin-amylopectin interactions, therefore creating a less sticky gel (Iturriaga, de Mishima, & Añon, 2006). At higher concentrations of MCP, there was an increase in the extent of network formation as indicated by the higher *G'* of the

gel. This may have interfered with amylopectin-amylopectin interaction, therefore reducing the stickiness of the gel. The opposite was observed for starch gels containing 2 % w/w MCP, whereby a decrease in its *G'* indicates a less extensive network formation. In this instance, interference to the amylopectin-amylopectin interaction would be reduced, and hence gels would become stickier. With time, these aggregates were loosened up and the gel containing 2 % w/w MCP was digested more readily than that containing 5 % w/w MCP, suggesting that multiple factors were involved which affected the overall digestibility of wheat starch-MCP gels. This phenomenon will need to be further investigated in order to validate the suggested hypothesis.

In order to eliminate the effect of stickiness on the gel digestibility, the fragmented gels were macerated in water to disperse them completely (consistency of a paste). In this case, the rate of starch digestion was then not limited by the gel hardness as the macrostructure was disrupted. The rate and total amount of starch digested was increased greatly as a result of an increased enzyme accessibility due to a larger surface area. For the macerated gel (Figure 8-5c), it was observed that the presence of MCP resulted in a reduction in the rate of starch digestion and, more importantly, the amount of starch digested. Starch digestion ceased after 10 minutes, with 27 ± 0.6 % starch remaining in the absence of MCP and this was increased to 57 ± 1.0 % when 5 % w/w MCP was present, suggesting that there was a reduction in the amount of starch available for digestion.

8.3.5 Mechanisms by which MCP reduce starch digestibility

Among the NSPs used in this study (xanthan, guar, LBG and agar), MCP was found to be the most effective in reducing starch digestibility. One of the mechanisms by which this could occur may be related to the strength of the gels, whereby stronger gels limited enzyme diffusion into the gel to access its substrate. This was observed at high MCP concentration regardless of its macrostructure. At a low MCP concentration (2 % w/w), starch digestibility was more dependent on the macrostructure of the gel whereby, reduction in starch digestibility was more prominent when the gels were fragmented. In a fragmented starch gel system containing polysaccharides, the reduction in starch digestibility can generally be attributed to the release of NSPs into the digestate, resulting in an increase in its viscosity, hence limiting enzyme mobility (Brennan *et al.*, 2008). However, this was unlikely to be pertinent for MCP due to its low viscosity but its leaching into the digestate may have had other effects that influenced starch digestion.

Our previous studies have shown that granule swelling was reduced in the presence of MCP, with intact granules being present at 5 % w/w starch in the presence of high MCP concentrations following gelatinisation. Nevertheless, at high starch concentration, such as that used in this study (10 % w/w), the close packing of gelatinised starch granules would result in many granules being broken up by

shear during gel preparation. However, we cannot discount the fact that starch-MCP composite gel is likely to contain a higher proportion of granules that are not fully gelatinised, meaning that they are more rigid and resistant to shear. In the presence of these less gelatinised granules, starch digestion would consequently be reduced due to the tight packing of the crystalline structure that reduces enzyme accessibility.

Another factor that influenced starch digestibility, which was hypothesised in this study was the tendency of the fragmented gels to stick together, especially when 2 % w/w MCP was present. The rate of digestion for these gels was reduced due to the fragmented gels clumping together. This result may have positive implications on the digestibility of a starchy food product that is produced using low MCP concentration, since fragmented gels resulting from mastication may reassociate to form larger clumps that are digested with less ease. This showed that gel adhesiveness was, in part, contributing to the digestion rate of the fragmented starch gel and should be further investigated.

A much clearer deduction on the effect of MCP on starch digestibility is based on the dispersion of fragmented wheat starch-MCP gels in water (macerated gels), which resulted in decreasing starch digestibility with increasing MCP concentration. Digestion of macerated gels seemed to cease after 10 minutes both in the absence and presence of MCP. Roughly 27 % of starch remained after 120 minutes of digestion and this increased by 30 % when 5 % w/w MCP was present. The results thus far suggested that the association between amylose and MCP forming complexes, which have previously been proposed, could account for the 30 % of undigested starch in the presence of MCP implying that these MCP-starch complexes are somewhat resistant to the enzymes. Resistance of amylose complexes to enzymatic hydrolysis is not unreported and has been shown for amylose-lipid complexes (Ai, Hasjim, & Jane, 2013; Cui & Oates, 1999). While other studies have suggested that the decrease in the rate of starch digestion in the presence of a non-starch polysaccharide was due to the ability of certain NSPs such as guar gum to inhibit enzyme activity (Slaughter *et al.*, 2002), the ability of MCP to do so is still unknown. There is also the added possibility for other compounds present in the extract of *Mesona chinensis* such as polyphenols that may possess inhibitory activities against digestive enzymes. Hence, these possibilities cannot be excluded and should be studied in future work.

8.4 Conclusion

The digestibility of 10 % w/w wheat starch gels containing xanthan, guar, LBG, agar and MCP were compared. Despite their similar gel hardness, it was found that MCP was the most effective polysaccharide in reducing wheat starch digestibility, making it a potential ingredient to formulate starchy products with low glycaemic load. The mechanism by which MCP reduces starch digestibility was found to be complex, possibly consisting of several different factors. The hardness of the gel, in part, contributed to reducing enzyme accessibility to starch trapped within the gel. Consequently, reduced digestibility was observed for the intact strong gels (high MCP concentration) but not for the weak gels (low MCP concentration). When the gel structure was fragmented, starch digestibility was reduced in the presence of both low and high MCP concentration. The stickiness of the fragmented gel pieces contributed to reducing starch digestibility, whereby at low MCP concentration, fragmented gels pieces aggregated to form larger clumps, leading to more reduction in digestibility for the first 30 minutes. Finally, macerated gels dispersed in water showed that ~30 % of the starch in gels containing MCP was not available for digestion. This is likely to be largely attributed to the formation of MCP-amylose that survived enzymatic hydrolysis.

Chapter 9 [5]*In vitro* digestibility of wheat starch-MCP gels – Enzyme inhibition and role of calcium

9.1 Introduction

Mesona chinensis polysaccharide (MCP) is obtained by hot water-extraction from the leaves of the herb *Mesona chinensis* (MC). The crude extract contains an ionic polysaccharide that presents a high concentration of minerals and is coloured almost black by pigments. The extract does not form a gel and shows low viscosity at concentrations up to 10 % w/w. Gelatinisation of non-waxy starches (containing amylose) in the presence of this extract, results in a synergistic increase in the strength of the composite gel. Hence, it is used in many Asian countries to produce a black gel dessert known as "grass jelly". The synergistic increase in gel strength is attributed to the presence of MCP in the extract, which interacts with amylose during gelatinisation, forming a homogenous dense network (Yuris *et al.*, 2018).

The extract itself is composed of two polysaccharide fractions known as the neutral and acidic MCP. The acidic MCP contains a large amount of galacturonic acid residues within its backbone structure with insertions of rhamnose residues (Feng *et al.*, 2008), largely resembling the structure of the rhamnogalacturonan I that is found in pectin (Voragen *et al.*, 2009). Analysis of the crude *Mesona chinensis* extract reveals the presence of a relatively high proportion (0.9 %) of calcium (data in this chapter). The importance of calcium for polysaccharide gelling has previously been established for low methoxy pectin and alginate, resulting from the presence of a high proportion of galacturonate and guluronate residues respectively in their structure—these form junction zones that accommodate calcium ions (Fang *et al.*, 2008). The gelling of pectin in the presence of calcium mainly involves the homogalacturonan chains, which form crosslinking via calcium ions in blocks of more than 10 galacturonic acid residues (Voragen *et al.*, 2009). While the rhamnogalacturonan I of pectin is not typically involved in the formation of pectin gels, the presence of galacturonan residues within its backbone could form crosslinking at specific points via calcium ions.

The effect of adding calcium into aqueous starch-MCP mixtures has previously been studied at low starch (2 % w/w) and MCP (1 % w/w) concentrations (Lai & Chao, 2000a), whereby an increase in the

[5] This chapter has been submitted as Yuris, A., Hardacre, A. K., Goh, K. K. T. & Matia-Merino, L., (In submission). *In vitro* digestibility of wheat starch-MCP gels – Enzyme inhibition and role of calcium. *LWT - Food Science and Technology*.

final viscosity of the paste was observed in the presence of calcium (Lai & Chao, 2000a). However, the extent of the increase was dependent on calcium concentration, with the highest viscosity obtained in the presence of 5.1 mM calcium chloride, and further additions resulted in a decrease in viscosity. At higher starch (8 % w/w) and MCP (4 % w/w) concentrations, the addition of calcium increased the melting temperature of the gel, and this was regarded as a consequence of more junction zones forming in the presence of calcium ions (Lai & Chao, 2000b). The role of the calcium that is naturally present in the MC extract has never been studied and, hence, its influence on the interaction between wheat starch and MCP is unknown.

The digestibility of wheat starch gels is known to be reduced in the presence of MCP and some of the mechanisms by which this occurs have been investigated (Yuris, Matia-Merino, Hardacre, & Goh, In submission). A combination of the following factors have been proposed to be involved in reducing the digestibility of the gel: (1.) reduction in granular swelling, (2.) increased gel strength, (3.) the formation of an amylose-MCP complex that is resistant to enzymatic digestion and/or (4.) the inhibition of starch hydrolysing enzymes by MCP. The latter has previously been investigated using rat intestinal α-glucosidase and porcine pancreatic α-amylase (Chusak *et al.*, 2014), whereby MC extract was found to inhibit α-glucosidase activity but not that of α-amylase. However, the nature of inhibition has never been studied. While enzymatic inhibition of plant extracts are commonly attributed to the polyphenolic compounds (Rubilar *et al.*, 2011; Shobana, Sreerama, & Malleshi, 2009), the inhibitory activities of non-starch polysaccharides from plants such as *Camellia sinensis*, corn silks and cluster beans (guar) have also been reported (Chen *et al.*, 2013; Slaughter *et al.*, 2002; Wang *et al.*, 2010).

Understanding the role of the calcium present in the extract will add to the current understanding of the interaction between MCP and starch and, subsequently, the properties of the resulting gels. Therefore, it was the aim of this study to understand how the calcium that was present naturally in the extract of *Mesona chinensis* influenced the interaction between MCP and wheat starch during gelatinisation. This study will also investigate the digestibility of starch-MCP gels with reduced calcium levels and quantify the polysaccharide's ability to inhibit amylolytic enzymes before and after calcium reduction.

9.2 Materials and methods

9.2.1 Preparation of starch-MCP suspension

Wheat starch-MCP suspension was prepared according to section 5.2.2.

9.2.2 *Mesona chinensis* extract purification

Mesona chinensis extract was prepared as described in section 9.2.1 and freeze dried to obtain soluble MCP (after removal of insoluble and aggregated material). To 25 g of soluble MCP, 25 mL of ethanol were added, followed by 500 mL of pancreatic α-amylase/amyloglucosidase mixture containing 0.334 g porcine pancreatic α-amylase (E-PANAA, Megazyme, Wicklow, Ireland) in 500 mL of 50 mM, pH 6 sodium maleate buffer (contains 2 mM $CaCl_2$ and 0.02 % sodium azide) and 1.05 mL amyloglucosidase (E-AMGDF, Megazyme, Wicklow, Ireland). The mixture was placed in a 37°C shaking incubator (150 rpm) for 16 hours. To terminate the reaction, 50 mL of 0.75 M tris base solution was immediately added to increase the pH of the mixture to 7.9 – 8. The mixture was then heated to approximately 60°C before the addition of 2.4 mL of protease (E-BSPRT, Megazyme, Wicklow, Ireland) and incubated at 60°C for 30 minutes. Acetic acid (2M) was then added to adjust the pH to 7. The mixture was centrifuged and the supernatant was retained. The material comprising the residual pellet was washed three times with water and the resulting supernatant liquid was combined with the original supernatant. Four volumes of denatured ethanol were added to the pooled supernatant and the liquid covered and left to stand overnight at 4°C. Precipitated polysaccharide was then obtained as pellets by centrifugation at 4000 g for 20 min at 8°C. The pellets were re-dissolved in water and dialysed against milliQ water to remove salts present. The dialysate was refreshed every hour for 5 hours and, subsequently, once every 12 hours for 24 hours. The dialysed sample was then freeze dried and designated “purified MCP” (MCP_p) for further analysis.

9.2.3 Proximate analysis

Before and after purification, the composition of MCP was quantitatively determined by an accredited chemical laboratory (Massey University Nutritional laboratory, Palmerston North, New Zealand) using the following methodology: Protein was determined using the DUMAS combustion method (AOAC 991.36), fat using the convection oven method (AOAC 930.15, 925.10), dry matter and ash by kilning at 600°C (AOAC 942.05) and non-starch polysaccharides by the enzymatic-gravimetric method (AOAC 991.43). The total carbohydrate was calculated by difference [total carbohydrate % – (Starch + Free sugars) %]. Minerals (calcium, magnesium, potassium, sodium, iron, copper, manganese and zinc) present in the sample were measured using inductively coupled plasma optical emission spectrometry (ICP-OES). The sugar composition was determined by the Ferrier Research Institute (Wellington, New

Zealand) and was found to comprise of Galacturonic Acid (59.1 %), Galactose (11.6 %), Glucose (8.7 %), Rhamnose (6.2 %), Arabinose (4.5 %), Mannose (3.6 %), Xylose (3.0 %), Glucuronic Acid (1.9 %) and Fucose (1.5 %).

9.2.4 Calcium content

Calcium present in the raw or purified MCP was reduced using a weakly cationic resin (Amberlite IRC-50 (H), BDH Laboratory, USA) that had been charged using 1 M sodium chloride solution. Excess resin (20 g charged resin to 1 g calcium present in MCP solutions) was added to various concentrations of MCP solutions (raw and purified sample) and the mixtures were left overnight at 4°C before filtering to recover the resin. This process reduced the calcium content of the raw MCP by 29 % and the purified MCP by 74 %. Calcium-reduced MCP solutions were designated as MCP_{red}. The calcium content of 5 % w/w aqueous raw MCP_{red} solution (7.4 mmol/L Ca) was then increased by the addition of calcium chloride to 9.4 and 14.8 mmol/L, the former (9.4mmol/L) being the approximate calcium ion concentration in the original 5 % w/w aqueous raw MCP solution prior to partial calcium removal. The solutions were then used to prepare starch-MCP suspensions as described in section 9.2.1, for the rheological measurements (section 9.2.7) and for the starch digestion experiments (section 0).

The calcium content of MCP solution before and after partial calcium removal was measured using the O-Crescolphthalein method (JaICA, 2015) with modifications. Briefly, 20 µL of MCP solution was mixed with 0.65 mL 1 M ethanolamine buffer for 25 seconds. To the mixture, 0.25 mL of a reagent containing 0.3 mmol/L O-Cresolphthalein complexone, 13.8 mmol/L, 8-hydroxyquinoline and 122 mmol/L hydrochloric acid was added and left to stand for 2 minutes before reading the absorbance at 550 nm (Helios Epsilon spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA).

9.2.5 Inhibitory assays

9.2.5.1 α-amylase activity

The inhibition of α-amylase activity by purified MCP before and after partial calcium removal was measured using the 3,5-dinitrosalicylic acid (DNS) colourimetric assay for reducing sugars (Gowri, Tiwari, Ali, & Rao, 2007) with modifications by Pranprawit, Heyes, Molan, and Kruger (2015). Porcine pancreatic α-amylase (200 µL of 4 U/mL) (A6255, Sigma-Aldrich, St. Louis, MO, USA) was added to 200µL of MCP_p solutions and the mixture incubated at 25°C for 5 minutes. The assay was initiated by the addition of 400 µL of 0.1 – 0.5 % w/v soluble starch (AL0715, Scharlau, Barcelona, Spain) solution. Starch hydrolysis was allowed to proceed for exactly 3 minutes at 25°C before terminating it by adding 400 µL of DNS reagent (1 g DNS, 30 g sodium potassium tartrate and 20 mL 2 N sodium hydroxide in 100 mL milliQ water). The mixture was placed in a boiling water bath for 10 minutes for colour

development and then cooled to room temperature over 15 minutes. Finally, the mixture was diluted by adding 4.2 mL of milliQ water and absorbances read at 550 nm (Helios Epsilon spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Mixtures containing no enzymes were used as blanks and controls were prepared using 200 µL of water in place of the MCP solution. At least two replicates were carried out for each treatment.

9.2.5.2 α-glucosidase activity

The α-glucosidase inhibitory activity of purified MCP before and after partial calcium removal was measured using a method reported by Schäfer and Högger (2007) and modified by Pranprawit *et al.* (2015). Yeast α-glucosidase (50 µL, 0.6 U/mL) (G5003, Sigma-Aldrich, St. Louis, MO, USA) in 100 mM of phosphate buffer at pH 6.9 was added to 50 µL of MCP_p solutions and the mixture incubated in a water bath at 37°C for 10 minutes. Enzymatic reaction was initiated by adding 125 µL of 0.1 – 1 mM chromogenic substrate, *p*-nitrophenyl α-D-glucopyranoside (*p*NPG) in 100 mM pH 6.9 phosphate buffer and the mixture incubated for a further 30 minutes at the same temperature. In order to terminate the reaction, 590 µL of 1 M Na_2CO_3 was added and the absorbance of the mixture read at 405 nm (Helios Epsilon spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Mixtures containing no enzymes were used as blanks and controls were prepared using 50 µL of water in place of the MCP solution. For each set of measurements at least two replicates were carried out.

9.2.6 *In vitro* digestion

Fragmented wheat starch-MCP gels were prepared and digested in the shaking water bath as per section 8.2.4.2.

9.2.7 Rheology

Aqueous raw MCP (5 % w/w) contains 9.4 mmol/L calcium, which was reduced (raw MCP_{red}) to 7.4 mmol/L using a cationic resin. Calcium chloride solution was added into the raw MCP_{red} to make solutions containing 9.4 mmol/L and 14.8 mmol/L of calcium. Starch (10 % w/w) was then dispersed in the solution at 20°C for 15 minutes and the pasting properties of the starch and MCP suspensions were measured as per section 5.2.3.

9.2.8 Zeta potential

Purified MCP (0.02 % w/w) prior to and after calcium reduction were prepared as per section 9.2.1 and 9.2.4 and filtered through 0.22 µm filter using a disposable syringe. The zeta potential of MCP_p prior to and after calcium reduction was measured at 20°C using the Zetasizer (Malvern ZetaSizer Nano ZS, Malvern Instruments Ltd., Malvern, UK).

9.3 Results and discussion

9.3.1 Composition of *Mesona chinensis* extract

The composition of MC extract before and after purification is shown in Table 9-1. Despite the protease treatment, it was found that the amount of protein in MCP remained unchanged following purification. This could mean that the proteins found in MCP were intrinsically bound to the polysaccharide and, therefore, were inaccessible for enzymatic hydrolysis. The MC extract was found to contain a high proportion of non-starch polysaccharides (NSPs), which increased upon purification. Purification also led to a reduction in monovalent cations in the MCP_p—potassium was reduced by 98 % and sodium by 70 %. However, the concentrations of divalent metal ions increased and, in particular, calcium ions concentration increased by 200 %, due to the large reductions in solid content during the purification process. Magnesium ions increased by a smaller amount (70 %) but was also found at a relatively high concentration following purification. While the concentrations of other divalent metal ions (iron, manganese and zinc) also increased by more than 200 %, they were not present in large quantities. The increase in the concentration of divalent metal ions following purification is counterintuitive and suggests that these divalent ions may be bound since they were not removed following dialysis. It is likely that the calcium in the extract was bound onto the MCP as it contains high proportions of uronic acids (59 % galacturonic acid and 2 % glucuronic acid), which have been shown to be directly related to calcium binding in plants (James, Branch, & Southgate, 1978).

Table 9-1 The composition of raw and purified MC extract powders on a dry weight basis.

		MC extract	Purified MC extract
Ash (% w/w)		29.5	12.7
Protein (% w/w)		9.4	9.6
Fat (% w/w)		0.1	0.7
Carbohydrate (% w/w)	Non-starch polysaccharides (% w/w)	50.1	67.8
	Free sugar (% w/w)	10.8	9.2
Minerals (g/kg)	Ca	9.31	29.41
	Mg	11.64	19.85
	K	161.76	2.16
	Na	7.97	2.30
	Fe	0.75	2.82
	Mn	0.40	1.46
	Zn	0.02	0.09

Partial calcium removal from the extract (both raw and purified) was carried out using weak cationic resin that has been charged with sodium chloride. The calcium content of the extract measured using the O-Cresolphthalein method (Table 9-2) was found to be lower than measurements obtained using the ICP-OES method (Table 9-1), however the differences were small and the speed and convenience of the latter method was preferred for subsequent work. Based on the O-Cresolphthalein method, the cationic resin removed about 70 % of the calcium from MCP_p but less than 30 % from the raw MCP. This suggests that while calcium was bound to the polysaccharide, the purification process may have resulted in weakening of the bonds and, hence, the ions could be more easily exchanged by the ionic resin. Calcium displacement by sodium has previously been reported for polyglucuronate, suggesting a weak electrostatic interaction between the polymer and calcium, in contrast to the strong electrostatic interaction between calcium and polygalacturonan (Braccini, Grasso, & Perez, 1999). The same mechanism for calcium binding has also been proposed for high methoxy pectin (Fang *et al.*, 2008). It is probable that the calcium is bound to the glucuronate residues in MCP and, hence, could be displaced by sodium. While it is not clear whether this was definitely the case, it is likely that calcium was not tightly bound onto the polysaccharide after purification.

Table 9-2 Calcium content of raw and purified MCP before and after partial calcium removal measured using the O-Crescolphthalein method (values are averages of two replicates).

Sample	Before calcium removal (g/kg)	After calcium removal (g/kg)
Raw MCP (MCP)	6.20 ± 0.05	4.5 ± 0.01
Purified MCP (MCP_p)	24.34 ± 0.13	6.4 ± 0.11

9.3.2 Rheology of starch-MCP gels

The pasting properties of gels made using 10 % w/w wheat starch with or without 5 % w/w raw MCP and containing various concentrations of calcium are shown in Figure 9-1. The presence of MCP resulted in an increase in both the peak viscosity (>70 % increase) and strength of the resulting gel (>30 %). This increase is associated with the interaction between MCP and amylose to form a homogenous network of amylose-MCP chains with gelatinised ghost granules comprised largely of amylopectin distributed throughout (Yuris *et al.*, 2018).

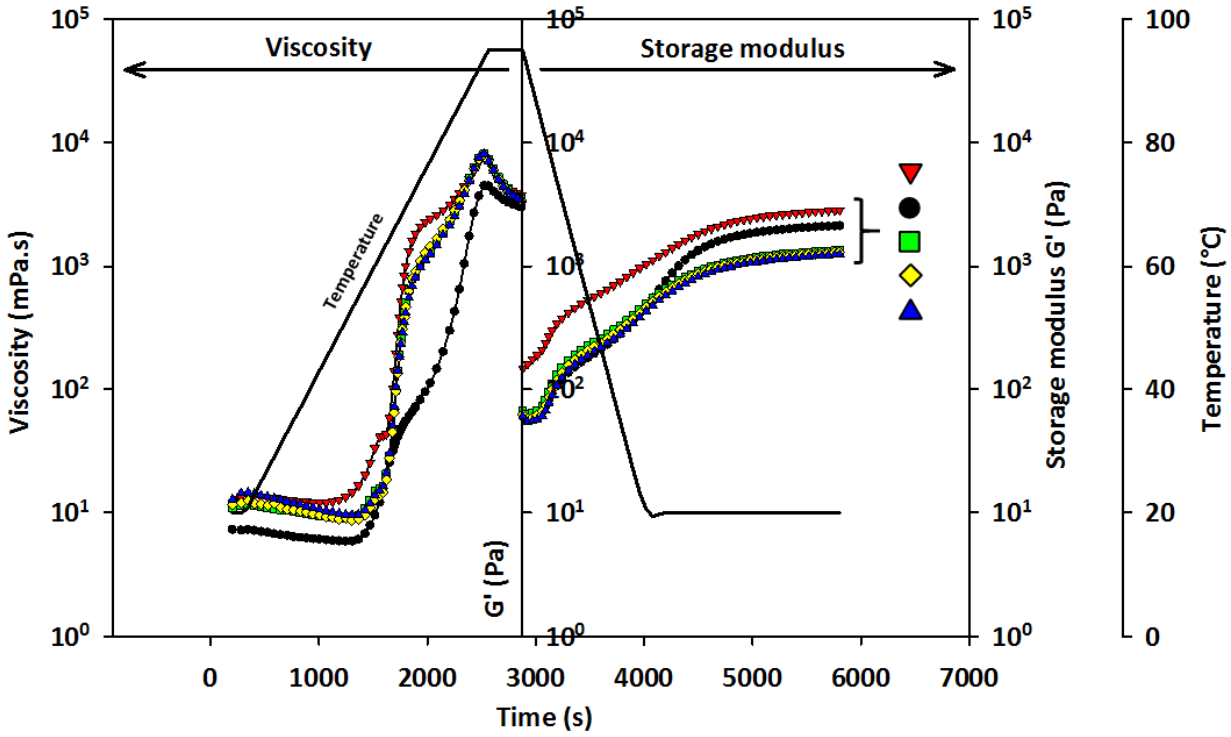


Figure 9-1 Pasting and rheological properties of 10 % w/w wheat starch in the absence (●) or the presence of raw 5 % w/w MCP (▼) or 5 % w/w MCP_{red} containing 7.4 mmol/L Ca (■), 9.4 mmol/L Ca (◇) or 14.8 mmol/L Ca (▲). Values are averages of triplicate. Note: 9.4 mmol/L Ca corresponds to the amount of calcium naturally present in raw 5 % w/w MCP.

The onset of this interaction is typically characterised by an earlier onset of viscosity increase below 55°C, which appears as a very reproducible peak in the viscosity curve, termed as peak M (Figure 9-2a arrow). The formation of peak M is a result of an increase in the starch granule volume fraction as MCP interacts with amylose to form a coat around the granules, which subsequently dislodges from the granule surface as more amylose is leached. The presence of MCP helps to stabilise the matrix between the swollen ghost granules (Yuris *et al.*, 2017).

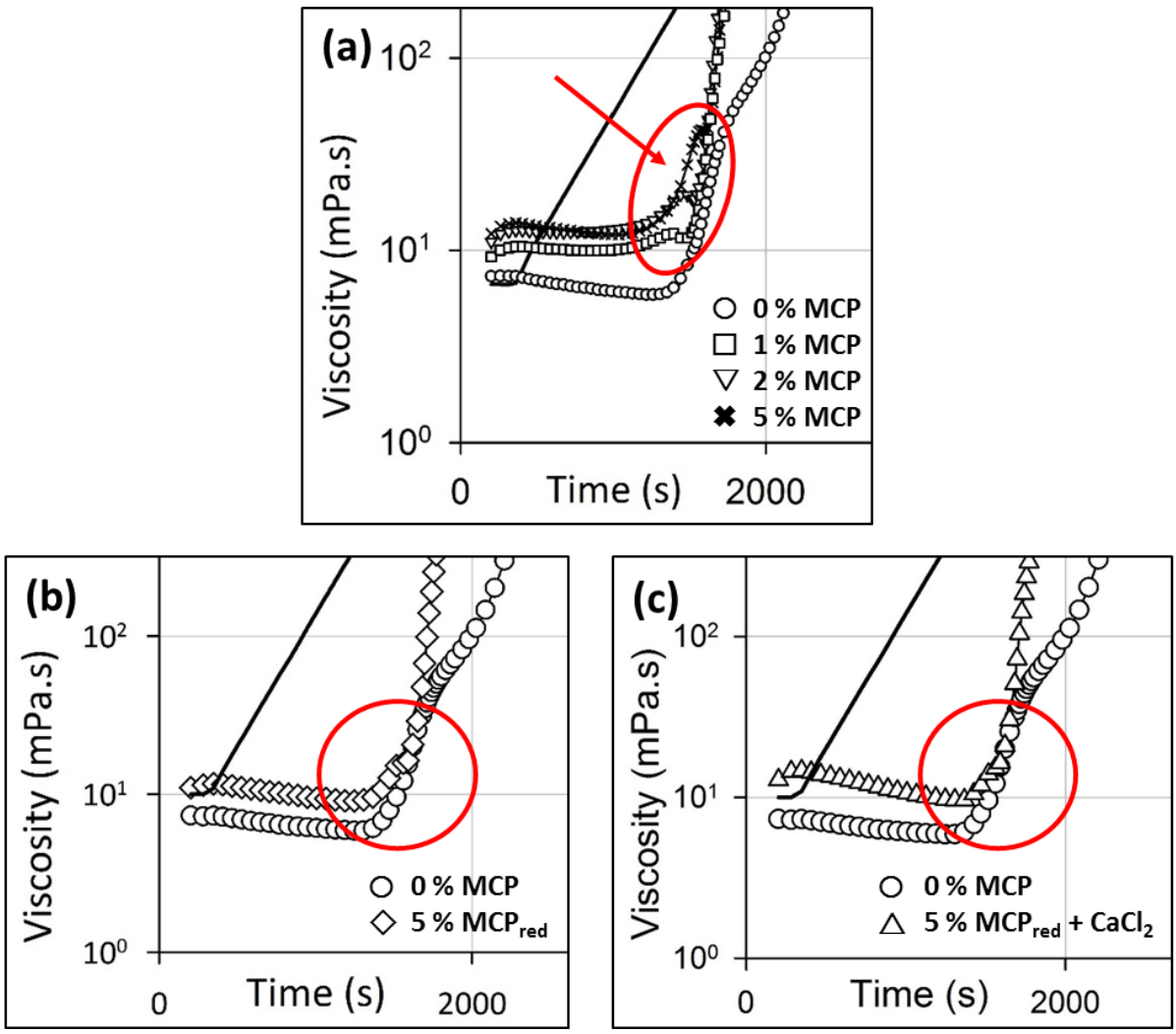


Figure 9-2 Peaks M appearing during gelatinisation of 10 % w/w wheat starch suspensions containing various concentrations of raw MCP (a). Disappearance of the peak "M" of 10 % w/w wheat starch + 5 % w/w raw MCP when calcium was reduced (b) and then added back to the solution (c).

Partial removal of calcium from MCP using cationic resin resulted in the disappearance of peak M when 10 % w/w wheat starch was gelatinised with MCP_{red} solution (Figure 9-2b). While not shown on the graph, adding calcium to wheat starch gels to the level present in MCP did not alter the pasting properties of the gels made with starch only. This suggests that calcium primarily affects the properties of MCP and not starch. Calcium ions naturally present in the extract would allow chains of polysaccharides to be held together by crosslinking at specific points in the solution. Partial removal of the calcium ions would reduce polymer cross linking due to an increase of electrostatic repulsion, which would prevent MCP chains from associating in solution and when interacting with the starch granules. This is supported by the zeta potential measurements of MCP_p prior to and after calcium reduction. The reduction in the calcium level of the purified extract increased the zeta potential of

MCP_p from -21.2 mV to -28.7 mV, indicating an increase in the negative charge density of the polysaccharide following calcium reduction.

Therefore, as amylose is leached during gelatinisation, the interaction between amylose and chains of MCP would be less likely to form large clusters of interconnected polysaccharide at the granule surface to increase the granule volume fraction and hence viscosity. This proposed mechanism is supported by the reduction in peak M when calcium was reduced using the resin. Despite the disappearance of peak M, the viscosity of starch suspensions containing MCP increased in a similar fashion during heating regardless of the reduction in calcium level, reaching a peak viscosity of about 8000 mPa.s. This could be explained by the relatively high starch concentration used, which meant that the viscosity of the suspension is dominated by starch gelatinisation. The reduction of MCP-MCP interaction upon the removal of calcium was evident following gelation, whereby the final gel strength decreased by more than 50 % from 2800 Pa to about 1300 Pa.

The storage modulus of a gel is influenced by the number and strength of bonds present in the system (Feng & Ye, 2013). In this case, a decrease in the *G'* of the gel would indicate fewer bonds interconnecting MCP and starch and hence a weaker gel. Interestingly, the addition of calcium ions to the MCP_{red} did not result in the recovery of peak M (Figure 9-2c) or its gel strength (Figure 9-1)—the resulting *G'* was lower than that of the gel containing MCP regardless of the proportion of calcium added. One of the reasons that may explain this was that the removal of calcium resulted in an irreversible structural or conformational change, which prevented the polysaccharide from binding calcium ions into their original active sites.

9.3.3 *In vitro* digestion

The *in vitro* digestibility of wheat starch gels containing MCP has been reported in our previous study (Yuris *et al.*, In submission), whereby the presence of MCP was found to reduce starch digestion in fragmented gels. Figure 9-3 shows the *in vitro* digestibility of fragmented 10 % w/w wheat starch gels (representing masticated gels) with and without 5 % w/w of added MCP following calcium reduction and addition. As a control, calcium chloride was added to wheat starch suspensions to increase the calcium ions to a level (9.4 mmol/L) similar of that found in MCP (Figure 9-3 dotted line). The presence of calcium chloride in the starch gels resulted in 10 % more starch being digested after 120 minutes. This is likely to be due to an activation of α-amylase in the presence of calcium and chloride ions. Past studies have shown that the removal of metal ions by dialysis results in a decrease in α-amylase activity, which can be restored upon the addition of calcium ions (Buonocore, Poerio, Silano, & Tomasi, 1976). This was attributed to the ability of the cation to stabilise α-amylase. Chloride ions, on the other

hand, have been shown to be essential for the activation of α-amylase (Caldwell & Kung, 1953). Hence, both calcium and chloride ions are required for the activation and stability of the enzyme.

The addition of 5 % w/w MCP resulted in a decrease in the rate of digestion of wheat starch gels (80 % starch remaining after 120 minutes as compared to 50 % in the absence of MCP). However, upon partial removal of calcium from MCP, the rate of starch digestion was slightly increased (65 % starch remaining after 120 minutes). There are two possible explanations for this; the first of which is the increase in starch gel strength in the presence of MCP, which would result in a reduction in the accessibility of enzymes to their substrate. In contrast, MCP_{red} had decreased strength (Figure 9-1) and, consequently would increase the accessibility of enzymes to their substrate. Hence, the digestibility of MCP_{red} gels was increased (Figure 9-3 green square) compared to the calcium-rich gels made using raw MCP (Figure 9-3 red triangle). Secondly, the removal of calcium is likely to affect the interaction between amylose and MCP as the polymer was leached out during gelatinisation. In Chapter 8, it was hypothesised that the presence of enzyme resistant amylose-MCP complexes reduced the digestibility of these gels. Here, it is shown that this interaction is influenced by the presence of calcium and that its removal irreversibly decreased the extent of amylose and MCP interaction. We propose that when calcium is reduced, the decrease in the interaction between MCP chains results in smaller fractions of the polysaccharide chains being bound onto amylose and, hence, leaving some areas of the amylose chains susceptible to enzymatic attack, so increasing digestibility. The digestibility of these gels remained high when calcium ions were added back to MCP_{red}. This was consistent with the rheological data, whereby the addition of calcium back into the MCP_{red} did not restore the interaction between wheat starch and MCP and, hence, the digestion was not reduced.

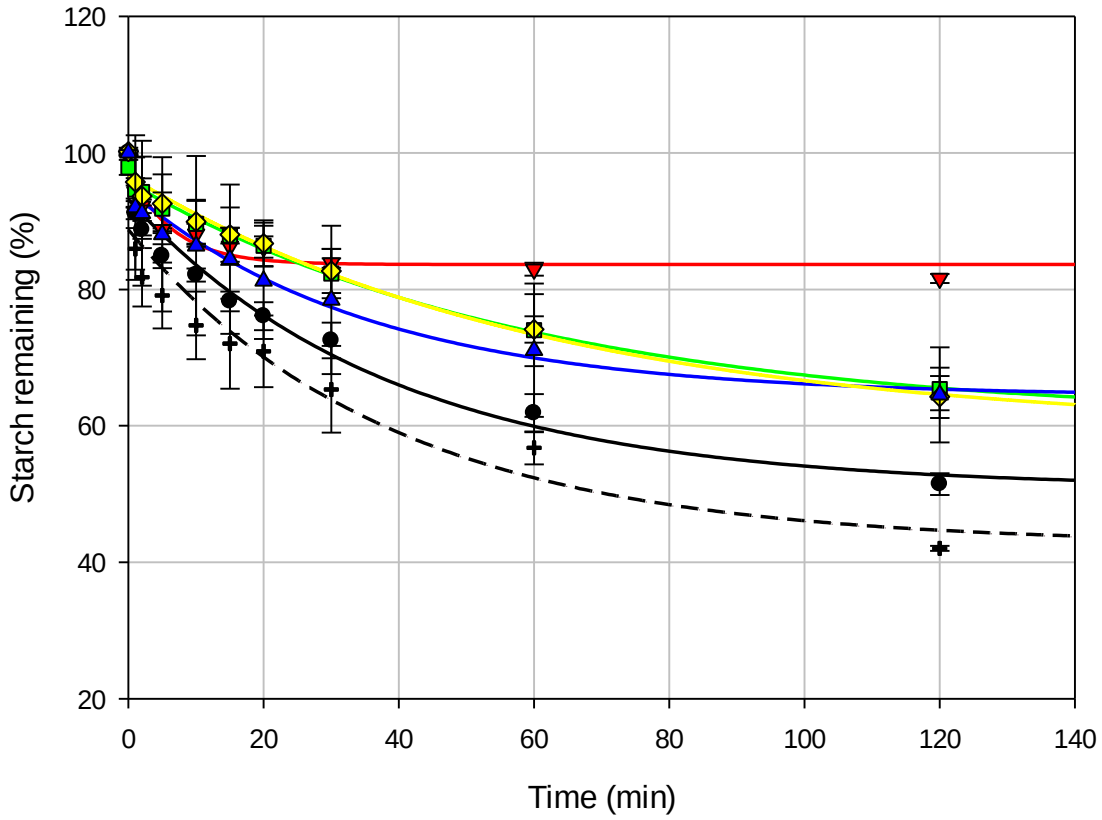


Figure 9-3 *In vitro* digestibility of fragmented 10 % w/w wheat starch in the absence (●) and presence of 5 % w/w raw MCP (▼) or MCP_{red} containing 7.4 mmol/L Ca (■), 9.4 mmol/L Ca (◆) or 14.8 mmol/L Ca (▲). Control: 10 % wheat starch + 9.4 mmol/L Ca (dotted line ✚). Values are averages of triplicate.

9.3.4 Inhibitory activity of MCP

The inhibition of α-amylase and α-glucosidase hydrolysis activity in the presence of MCP was investigated using purified MCP (MCP_p), since it contains a higher proportion of the non-starch polysaccharide. α-Amylase hydrolyses the α-1,4-glycosidic linkages in starch polymers to produce glucose, maltose and dextrins, while α-glucosidase breaks down oligosaccharides and disaccharides to produce monosaccharides that can be absorbed into the bloodstream (Rubilar *et al.*, 2011). Therefore, inhibition of either enzyme could lead to a reduced postprandial blood glucose levels.

Figure 9-4 shows the activity of α-amylase in the absence or presence of MCP_p. There was no inhibition of α-amylase as shown in Figure 9-4a and the slight increase in the K_m and V_{max} values (Table 9-3) indicate a possible slight increase in the activity of the enzyme. The absence of α-amylase inhibition by MCP was consistent with a previous report (Chusak et al., 2014). Calcium ions are essential for the activity of amylase as they provide structural integrity for the enzyme (Buisson, Duee, Haser, & Payan,

1987), hence its presence in MCP_p may have led to a slight increase in the enzyme's activity (Figure 9-4a). However, when MCP_p was significantly reduced in calcium, there was a decrease in the enzymatic activity of α-amylase as indicated by a decrease in the amount of maltose released with increasing MCP concentrations (Figure 9-4b). There are two possible explanations for this; the first is that the removal of calcium resulted in MCP_p inhibiting α-amylase. The nature of this inhibition was uncompetitive as indicated by a decrease in the values of both V_{max} and K_m (Table 9-3) with increasing MCP concentration. Uncompetitive inhibition occurs when inhibitors only bind to its specific site when a substrate is bound on the enzyme and the resulting enzyme-inhibitor-substrate complex is inactive (Copeland, 2000). While this type of inhibition is relatively uncommon, it has been previously reported for quercetin against finger millet malt amylase (Chethan, Sreerama, & Malleshi, 2008). Binding of galactomannans onto α-amylase has been demonstrated Slaughter *et al.* (2002) and regions within an enzyme that may have affinity towards NSPs have been proposed (Slaughter, Ellis, & Butterworth, 2001). The removal of calcium (purified MCP_{red}) increased intramolecular electrostatic repulsion within MCP as demonstrated by an increase in its zeta potential, causing the polysaccharide to adopt a more open conformation. This could result in the exposure of sites that can readily bind onto α-amylase, hence inhibiting the enzyme. The second possible explanation is that the reduction in the amount of calcium present in the purified extract caused a reduction in the stability of α-amylase, as previously reported Buonocore *et al.* (1976), hence causing its activity to be reduced. The latter explanation seemed to be less likely since α-amylase remained active in the absence of MCP (Figure 9-4 black circle), demonstrating the fact that the enzyme was stable enough to hydrolyse starch in the absence of additional calcium.

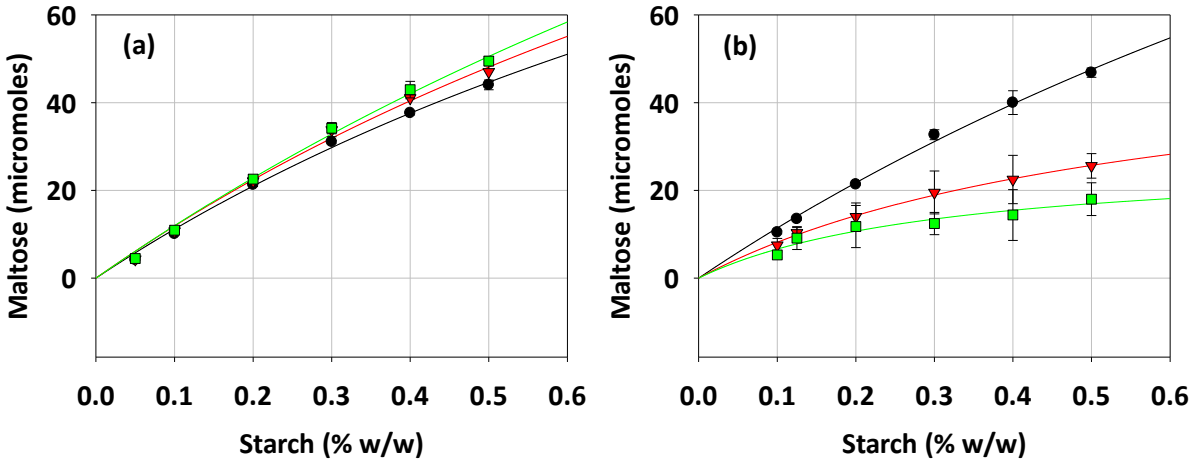


Figure 9-4 Plot of the activity of α-amylase in the absence (●) or presence of 0.005 % (▼) and 0.01 % (■) w/w MCP_p before (a) and after (b) calcium reduction. Note that in the absence of purified MCP, no calcium is present.

Table 9-3 The effect of reducing calcium from MCP_p on the V_{max} and K_m of α-amylase.

MCP_p concentration (% w/w)	Before calcium removal		After calcium removal	
	V_{max}	K_m	V_{max}	K_m
0	181.12	1.53	227.41	1.89
0.005	203.58	1.62	55.56	0.58
0.01	270.40	2.18	25.21	0.25

Unlike α-amylase, MCP_p inhibited α-glucosidase independently of calcium reduction (Figure 9-5), although when calcium was partially reduced (Figure 9-5b) the extent of this inhibition was reduced. Reduction in the calcium level of MCP_p resulted in an increase in V_{max} and a decrease in K_m (Table 9-4)—this corresponds to an increase in the maximum velocity of the reaction, yet a decrease in the substrate affinity. The nature of the inhibition was uncompetitive as shown by a decrease in both K_m and V_{max} with increasing MCP concentration. The calcium ions present in MCP are likely to be bound onto the polysaccharide, keeping a compact conformation by reducing intramolecular electrostatic repulsion. As mentioned in section 9.3.3, partial removal of these ions is likely to cause a part of its structure to adopt a more open coil conformation (due to greater intramolecular repulsive forces) and, therefore, increasing the intermolecular repulsion between MCP_p and α-glucosidase, hence preventing the two from binding. Consequently, the lack of inhibitors binding onto the enzyme would result in an increase in the activity of α-glucosidase as shown in Figure 9-5b.

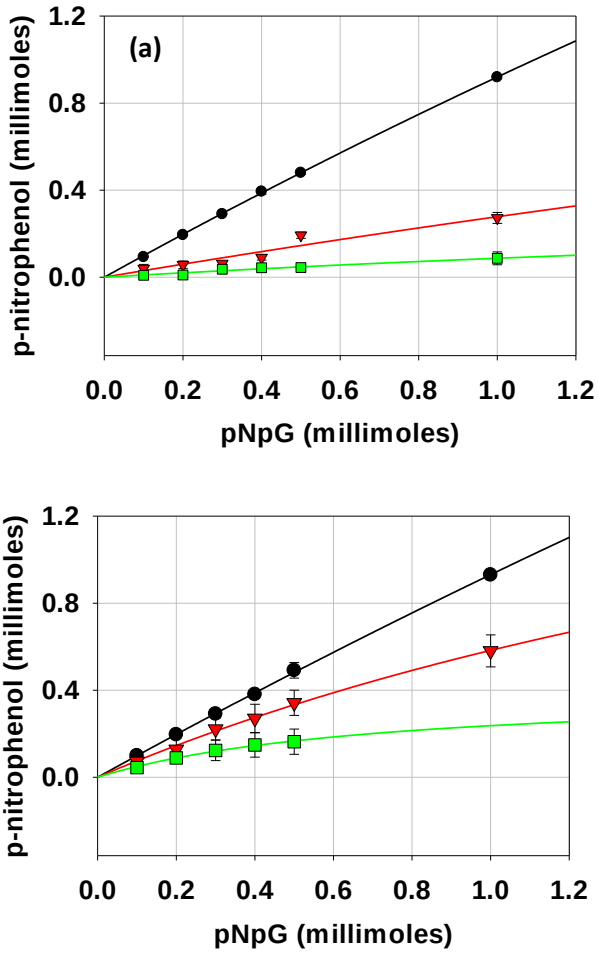


Figure 9-5 Plot of the activity of α-glucosidase in the absence (●) or presence of 0.002 % (▼) and 0.003 % (■) w/w MCP_p before (a) and after (b) calcium reduction. Note that in the absence of purified MCP, no calcium is present.

Table 9-4 The effect of reducing calcium from MCP_p on the V_{max} and K_m of α-glucosidase.

MCP_p concentration (% w/w)	Before calcium removal		After calcium removal	
	V_{max}	K_m	V_{max}	K_m
0	11.15	11.12	13.73	13.74
0.002	3.13	10.27	2.34	3.01
0.003	0.51	4.83	0.41	0.72

9.4 Conclusion

The divalent ions, in particular calcium, present in *Mesona chinensis* extract were found to be bound to MCP, which results in an increase in their concentration following purification. This study found that calcium ions naturally present in the extract play an important role on the interaction between wheat starch and MCP. When calcium was reduced using ion exchange, the strength of the composite gel decreased and this could not be recovered by adding calcium ions back into the system. Starch gels made with MCP that have reduced calcium levels were significantly more digestible than gels made with raw MCP. It is concluded that starch gels containing MCP will have lower glycaemic potential compared to similar gels made with calcium-reduced MCP and much lower than those made without MCP. This effect may be due to the high strength of the starch-MCP gels preventing enzyme access or the formation of amylose-MCP complexes resistant to enzymatic digestion.

In terms of the MCP extract itself, inhibition of α-amylase was observed for purified MCP that has been significantly reduced in calcium. This was attributed to the exposure of sites (as a result of intramolecular electrostatic repulsion) that bind onto the enzyme-substrate complex, resulting in a decrease in the enzymatic activity. In contrast, inhibition of α-glucosidase was observed for purified MCP and partial removal of calcium resulted in a decrease in its inhibitory ability. This was thought to be due to the intermolecular electrostatic repulsion between MCP and α-glucosidase that occurred as a result of partial calcium removal. Consequently, “calcium-reduced purified MCP” may be consumed as a functional herb to reduce postprandial glycaemia by inhibiting α-amylase and α-glucosidase, the enzymes that are required for starch hydrolysis.

Chapter 10 Overall conclusion and recommendations

10.1 Overall conclusion

This thesis uses a combination of wheat starch and *Mesona chinensis* (MC) extract to study the interaction between starch and *Mesona chinensis* polysaccharide (MCP). The herbal extract was used in place of the pure polysaccharide since it better represents a typical food system that contains MCP—whereby MC extract (in its impure form) in combination with starch is used to create a composite gel. The interaction between wheat starch and MCP was established and its effects on wheat starch digestibility, gelatinisation, gelation, retrogradation and gel textural and microstructural properties were studied. The following research questions were answered:

10.1.1 How does MCP interact with wheat starch?

The interaction between wheat starch and *Mesona chinensis* polysaccharide was established at molecular level using solid state NMR, whereby starch glucan chains and MCP were in close proximity of less than 5 Å. MCP was found to affect the molecular mobility of the carbon 6 in starch glucan polymer, either by interaction or prevention of polymer association (Figure 10-1).

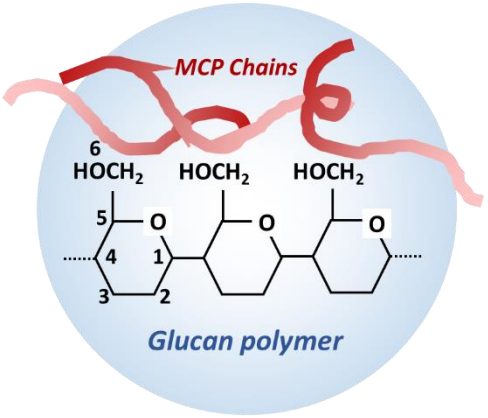


Figure 10-1 Interaction between MCP and wheat starch glucan polymer at carbon 6.

Heating of starch-MCP suspension resulted in an earlier onset of viscosity increase that was characterised by a peak "M". This was also accompanied by a delayed granular swelling and a reduction in amylose leaching. Based on rheological and amylose leaching data, interaction was thought to occur when amylose leaches out of wheat starch granules forming an MCP-amylose barrier, increasing the apparent size of the granules.

10.1.2 How does MCP alter the gelatinisation, gelation, retrogradation and textural properties of wheat starch gels?

The interaction between wheat starch and MCP resulted in an increase in the composite paste viscosity (Figure 10-2) and, depending on the concentration of starch and MCP, the strength of the final gel (Figure 10-3).

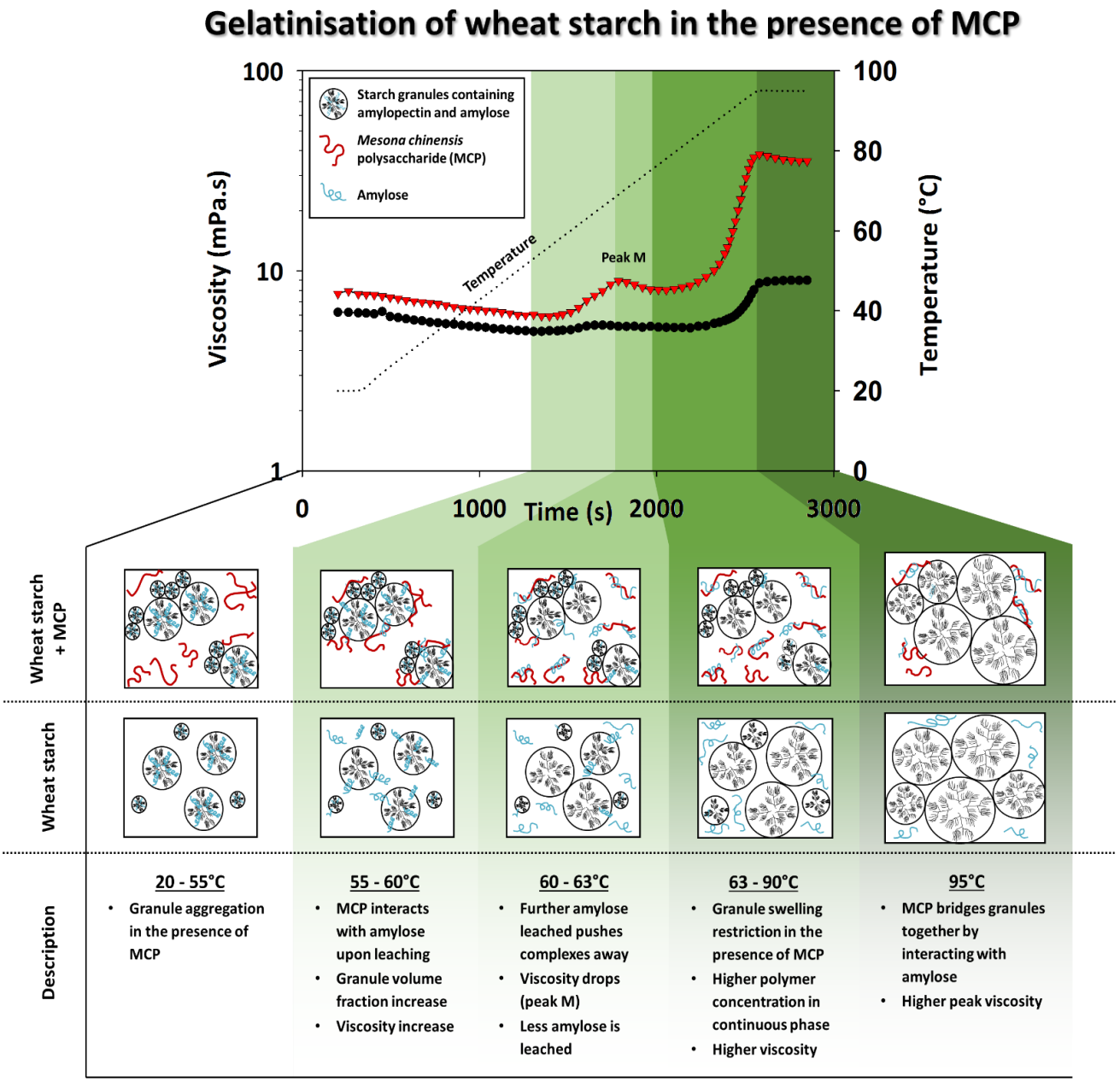


Figure 10-2 The effect of adding MCP (▼) on the gelatinisation of wheat starch (●).

Homogeneity of amylose-MCP network was found to play an important role in determining the textural properties of the composite gel, whereby a heterogeneous network leads to a weaker gel. This was influenced by the amount of polymers (amylose and MCP) available for interaction, as high starch-low MCP and low starch-high MCP mixtures led to the formation of a heterogenous network, as evidenced by their microstructure. The final gel properties were dependent on the concentration of MCP present, whereby an extensive MCP-amylose network was required to stabilise the starch granule aggregates despite a reduction in the amount of amylose leached. Syneresis and retrogradation (based on DSC results) were accelerated for 10 % w/w wheat starch in the presence of low MCP concentration but not at high MCP concentration. This was thought to be due to the heterogeneity of the network, which allows water to be expelled from the matrix during freeze-thaw cycles. The retrogradation properties of the gels were likely to be the result of a combination of (i) MCP associating with starch polymers, hence increasing the amount of energy required to melt the structure and (ii) MCP binding onto water (as supported by the increase in the mobility of water in the presence of MCP when measured using solid-state NMR), hence limiting starch polymer crystallisation.

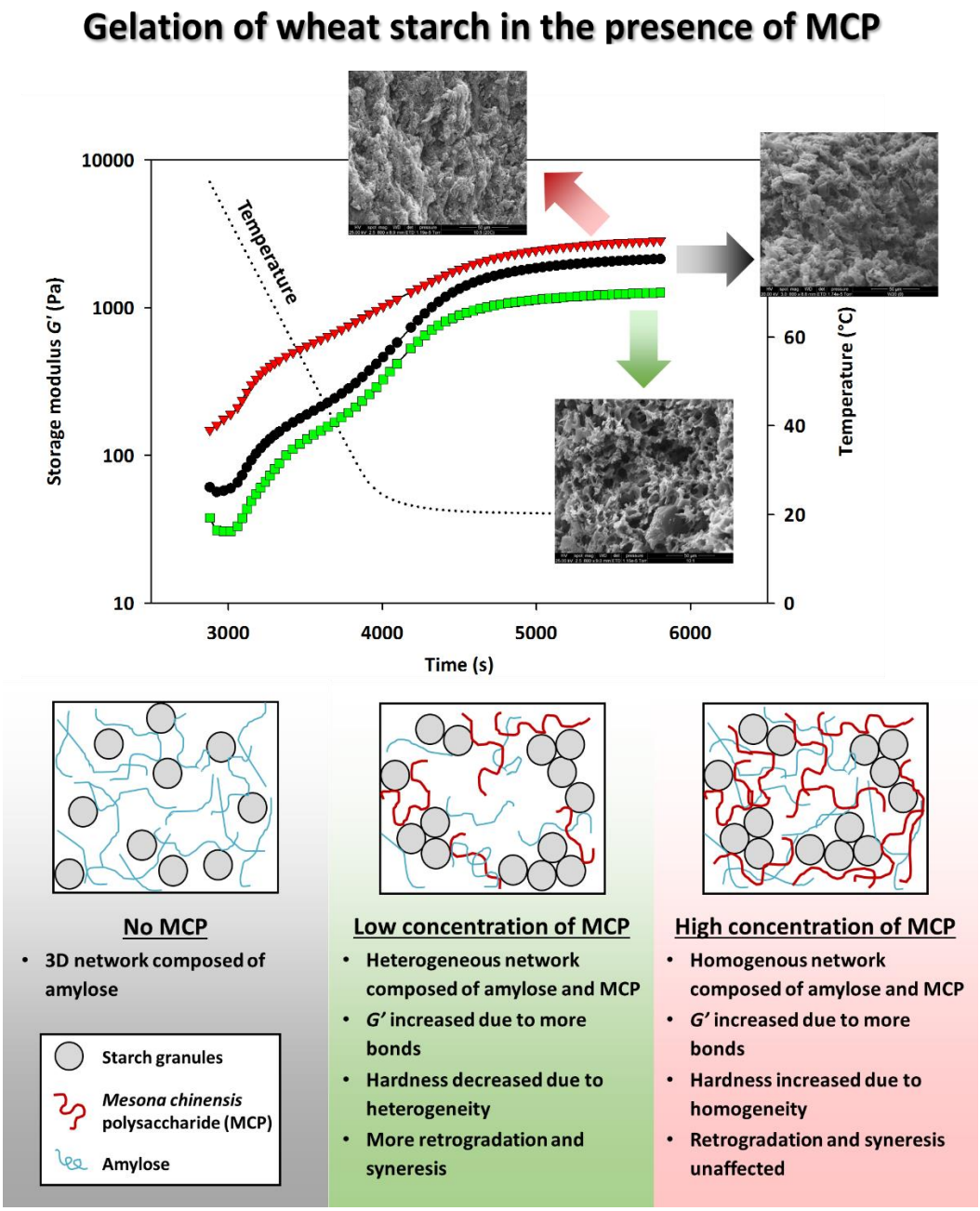


Figure 10-3 The effect of adding low (■) and high (▼) concentrations of MCP on wheat starch (●) gelation and retrogradation properties.

10.1.3 What are the factors that influence the interaction between starch and MCP?

The factors influencing the rheological properties of starch-MCP gels were studied (Figure 10-4). While several observations were made, more in depth studies need to be done in order to understand and explain the underlying mechanisms that alters the rheological properties of these gels. The following factors were considered:

1. **Starch type.** For maize and wheat starch, the peak viscosity and strength of the gel were increased with increasing concentrations of MCP. The same was observed for the strength of potato starch gel; however, its peak viscosity was reduced in the presence of MCP. For waxy maize starch, gels were only formed at high MCP concentration. A combination of factors such as the amount of amylose present, the swelling power and the packing of starch granules were thought to influence the properties of the final starch-MCP gels.
2. **Starch to MCP ratio.** A higher starch to MCP ratio was found to be essential for the formation of a firm gel. Decreasing the starch content led to a decrease in the amount of granules to enforce the 3D amylose matrix and insufficient amylose to interact with MCP.
3. **pH.** The strength of starch-MCP gel was reduced at pH 4 compared to gels with native MCP (pH 5.5); the strength subsequently increased with increasing pH.
4. **Salts.** The addition of monovalent salts (NaCl and KCl) was found to increase the strength of starch-MCP gels while the opposite effect was observed for divalent salts like $CaCl_2$.

The changes in the strength of starch-MCP gels when the pH of the system was adjusted or when salt was added were thought to be related to a decrease or increase in repulsive forces, which affect the conformation of the polysaccharide. This, in turn, was thought to influence the interaction between MCP and starch. However, more studies are required to validate this hypothesis.

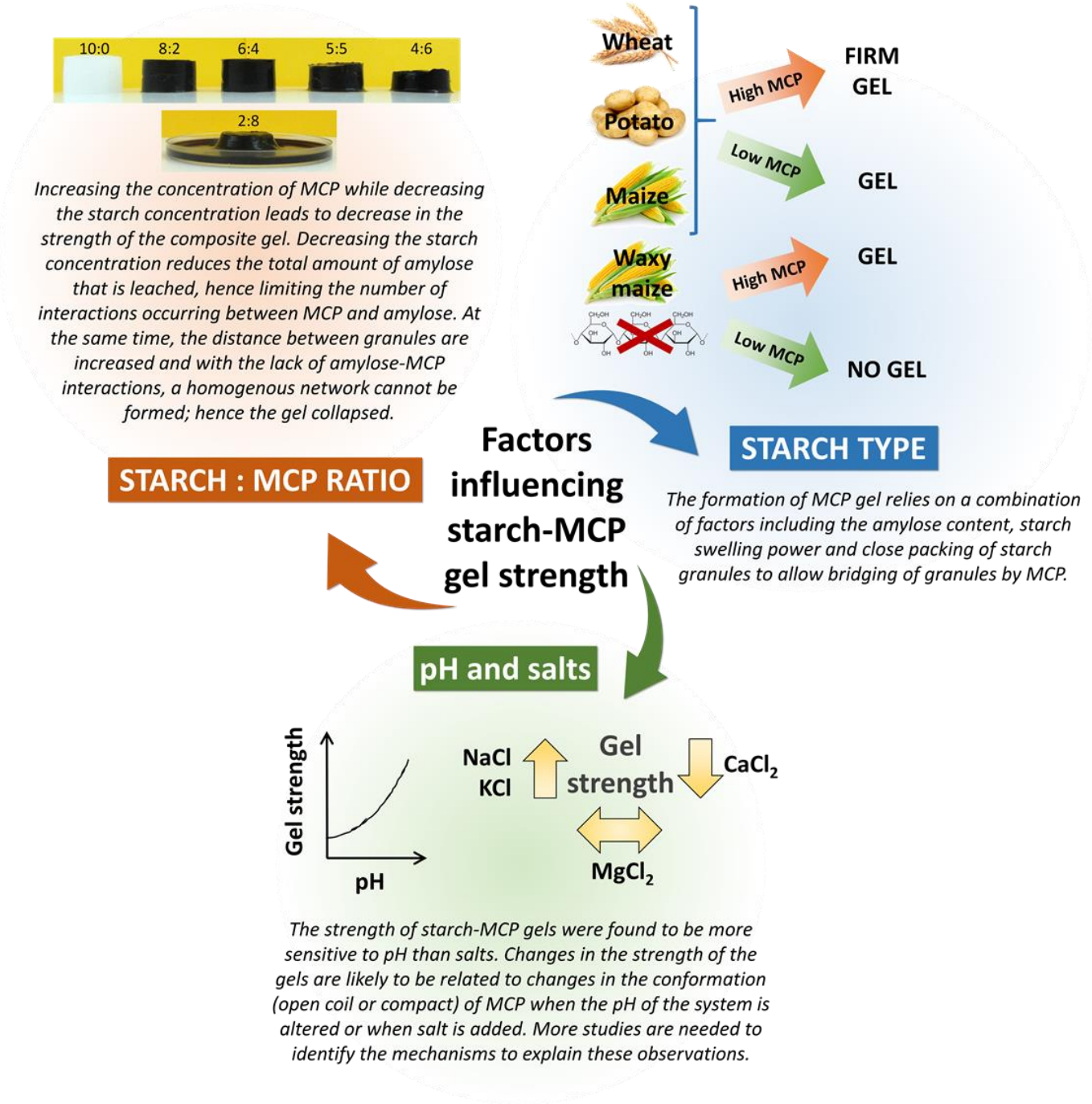


Figure 10-4 Factors influencing the rheological properties of wheat starch-MCP gels.

10.1.4 Can MCP reduce wheat starch digestibility and what are the mechanisms by which MCP reduce starch digestibility?

In vitro studies showed that the addition of MCP reduced the digestibility of wheat starch gels (Figure 10-5). This is likely to be the result of a combination of factors including: (i) an increase in the starch gel strength, (ii) a reduction in starch granular swelling, (iii) the formation of indigestible amylose-MCP complex and (iv) the ability of MCP to inhibit the activity of α-glucosidase.

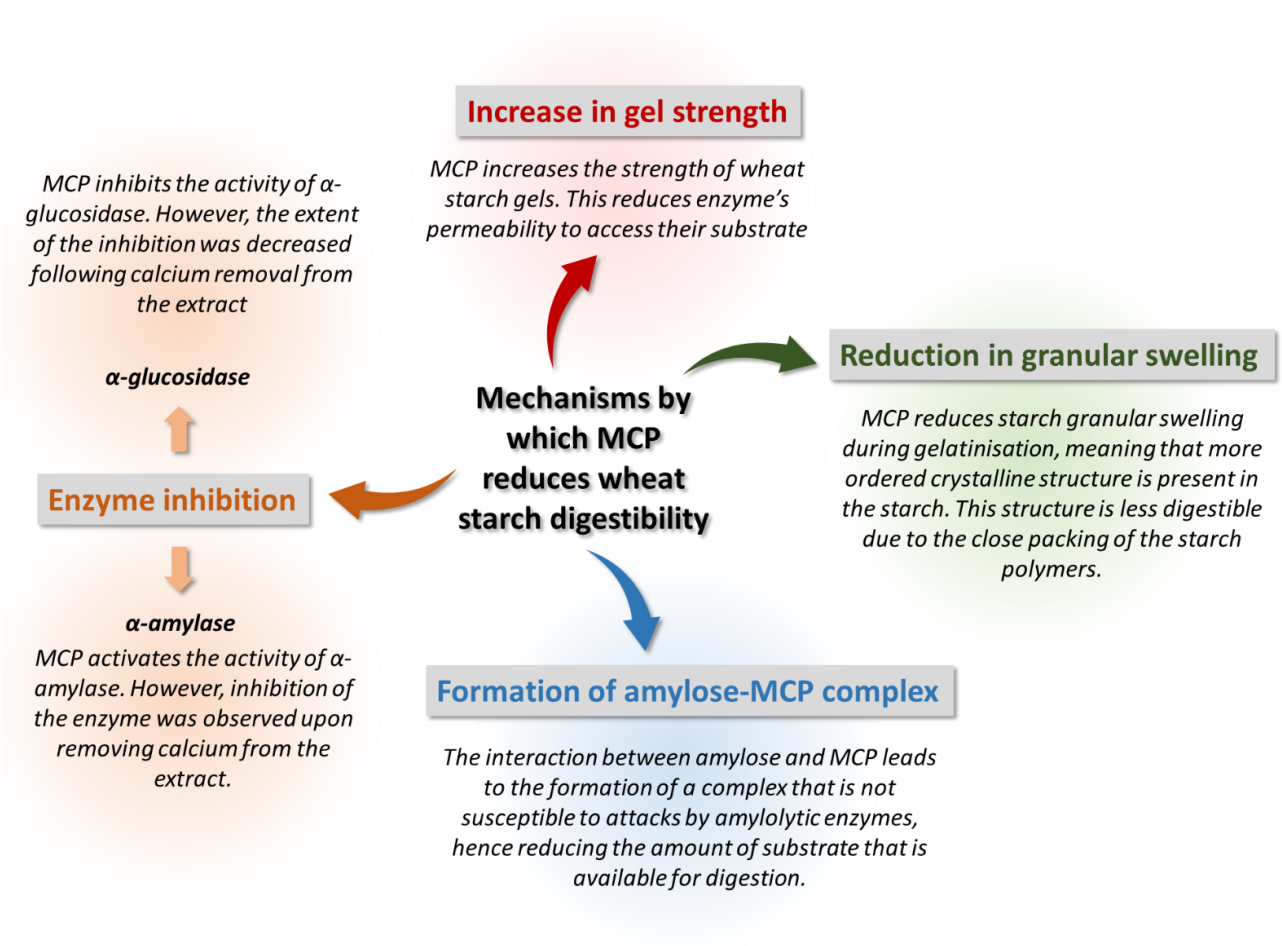


Figure 10-5 The mechanisms by which MCP reduces wheat starch digestibility.

10.1.5 What is the role of calcium in the interaction and digestibility of wheat starch and MCP gels?

Following partial calcium reduction, MCP was found to possess inhibitory activities against α-amylase. This was accompanied by an increase in the zeta potential of MCP, suggesting that its conformation may be less compact due to the presence of negatively charged groups on the polysaccharide and this was thought to expose sites that could bind onto α-amylase, hence inhibiting the enzyme. However, starch gels containing the calcium-reduced MCP were found to be more digestible compared to those containing raw MCP extract. This was thought to be due to the decrease in gel strength as a result of a decrease in their interaction that is mediated by calcium ions. Therefore, MCP may be used as an ingredient to formulate wheat starch based product with lowered digestibility.

10.1.6 Prospects of MCP in food application

The result of this thesis indicates that MCP can be used to increase the viscosity and strength of starch gels. Through modifying the concentrations of MCP and starch, the microstructure of the composite gel can be altered, leading to a change in their textural and retrogradation properties. This meant that

MCP may be used as a co-texturizer in starch based food products to alter their textural properties. This thesis also envisages the formulation of a low GI starchy food product based on the ability of MCP to reduce starch digestibility in a composite system. Furthermore, MCP itself was found to possess inhibitory activities against α-amylase and α-glucosidase, which opens up avenues for the polysaccharide to be used independently as a functional herb to help manage diabetes.

10.2 Recommendations

1. **Purification of MCP**

The MCP that is used in this study has not been purified and contains many other compounds including proteins, lipids, phenolics and minerals. In order to identify the specific nature of the interaction between starch glucan polymers and MCP, a purer polysaccharide is needed. Techniques such as Fourier-transform infrared spectroscopy or Raman spectroscopy can then be used to identify the bonds that are involved in the interaction between starch polymers and MCP. Hence, its purification will allow more studies to better understand the factors that influence starch-MCP interactions.

2. **Structural characterisation of MCP**

While the composition of MCP is reported in this study, the understanding of its structure is based on the polysaccharide of a similar plant, *Mesona blumes* that is previously report by Feng *et al*. (2008). Structural characterisation of the polysaccharide should be carried out and its molecular weight and the types of linkages needs to be determined. This is essential to understand the nature of their interaction with starch.

3. **Decolourisation of MCP**

The MC extract containing MCP is dark brown in colour, limiting its use as a food ingredient. In order to make MCP acceptable for commercial purposes, there is a need to decolourise the polysaccharide. Several techniques such as the use of activated charcoal and refluxing in sodium bicarbonate have been attempted in this study but with no success.

4. **Interactions between MCP and waxy starches**

One of the most interesting discoveries of this study was the formation of gel when waxy maize is gelatinised in the presence of high concentrations of MCP. Past studies have reported that MCP only interacts with non-waxy starches. Further studies should be carried out to investigate the granule swelling, polymer leaching and textural properties of waxy starches containing MCP. The interaction between the two should also be investigated using the solid-state NMR.

5. **Further studies on the effect of pH and salt on starch-MCP interaction and MCP on its own**

In order to further understand the effect of pH and salt on starch-MCP interaction, NMR techniques could be applied to identify whether the disappearance of peak “M” at low pH and addition of calcium was a result of MCP and starch polymers not interacting. The effect of pH and salt on the conformation of MCP itself also needs to be investigated by measuring their zeta potential to validate the hypothesis that have been proposed in this study.

6. Dynamic *in vitro* digestion

While the static *in vitro* digestion results obtained in this study are promising, a more dynamic setup is required to better simulate the human gut. This includes the gradual adjustments of pH, addition of enzymes and removal of substrate. The use of physiological shear rate (10 s^{-1}) should also be considered.

7. *In vivo* and human studies

In vivo and, subsequently, human intervention studies are needed to verify the results obtained from the *in vitro* study reported in this thesis. At present, the only reported study involving human subjects that looked at MCP focuses on the ability of the extract itself to reduce postprandial rise in blood glucose after the consumption of a high carbohydrate meal (Chusak *et al*., 2014). There is a no human intervention trials carried out on the digestibility of starchy products that have been prepared together with MCP.

8. Formulation of starchy food product with MCP

While MCP is commercially used to produce a gel dessert with starch, its viability as an ingredient in other products such as bread, noodle and pasta should be considered due to its ability to lower starch digestion. Previous studies have investigated the possibility of including MCP in the formulation of rice extrudates (Zhuang *et al*., 2010), low fat sausages (Feng *et al*., 2013) and low fat salad dressing (Lai & Lin, 2004). Based on the *in vitro* results of this thesis, MCP has a potential to be used in the formulation of low GI starchy food products such as pasta and bread.

References

Abdel-Aal, E. S. M., Hucl, P., Chibbar, R. N., Han, H. L., & Demeke, T. (2002). Physicochemical and structural characteristics of flours and starches from waxy and nonwaxy wheats. *Cereal Chemistry, 79*, 458 - 464.

Abdulmola, N. A., Hember, M. W. N., Richardson, R. K., & Morris, E. R. (1996). Effect of xanthan on the small-deformation rheology of crosslinked and uncrosslinked waxy maize starch. *Carbohydrate Polymers, 31*, 65 - 78.

Achayuthakan, P., & Suphantharika, M. (2008). Pasting and rheological properties of waxy corn starch as affected by guar gum and xanthan gum. *Carbohydrate Polymers, 71*, 9-17.

Ahmad, F. B., & Williams, P. A. (1999). Effect of sugars on the thermal and rheological properties of sago starch. *Biopolymers, 50*, 401-412.

Ai, Y., Hasjim, J., & Jane, J. L. (2013). Effects of lipids on enzymatic hydrolysis and physical properties of starch. *Carbohydrate Polymers, 92*, 120 - 127.

Alloncle, M., & Doublier, J. L. (1991). Viscoelastic properties of maize starch/hydrocolloid pastes and gels. *Food Hydrocolloids, 5*, 455 - 467.

Angioloni, A., & Collar, C. (2009). Small and large deformation viscoelastic behaviour of selected fibre blends with gelling properties. *Food Hydrocolloids, 23*, 742-748.

Apperley, D. C., Harris, R. K., & Hodgkinson, P. (2012). *Solid-state NMR: Basic principles and practice*. USA: Momentum Press.

Atkin, N. J., Abeysekera, R. M., & Robards, A. W. (1998). The events leading to the formation of ghost remnants from the starch granule surface and the contribution of the granule surface to the gelatinization endotherm. *Carbohydrate Polymers, 36*, 193-204.

Bahnassey, Y. A., & Breene, W. M. (1994). rapid-visco analyzer (RVA) pastign profiles of wheat, corn, waxy corn, tapioca and amaranth starches (*A. hypochondriacus* and *A. cruentus*) in the presence of konjac flour, gellan, guar, xanthan and locust bean gums. *Starch - Stärke, 45*, 134 - 141.

Bakhmutov, V. I. (2015). *NMR spectroscopy in liquids and solids*. USA: CRC Press.

Bean, M. M., & Yamazaki, W. T. (1978). Wheat starch gelatinization in sugar solutions. I. sucrose-microscopy and viscosity effects. *Cereal Chemistry, 55*, 936 - 944.

BeMiller, J. N. (2011). Pasting, paste, and gel properties of starch–hydrocolloid combinations. *Carbohydrate Polymers, 86*, 386-423.

Benmoussa, M., Hamaker, B. R., Huang, C. P., Sherman, D. M., Weil, C. F., & BeMiller, J. N. (2010). Elucidation of maize endosperm starch granule channel proteins and evidence for plastoskeletal structures in maize endosperm amyloplasts. *Journal of Cereal Science, 52*, 22-29.

Berdanier, C. D. (Ed.). (2000). *Advance nutrients: Macronutrients* (2nd ed.). Florida: CRC Press LLC.

Biliaderis, C. G. (1991). The structure and interactions of starch with food constituents. *Canadian Journal of Physiology and Pharmacology, 69*, 60 - 78.

Biliaderis, C. G. (2009). Structural transitions and related physical properties of starch. In J. N. BeMiller, & R. L. Whistler (Eds.), *Starch: Chemistry and Technology* (3rd ed., pp. 293-372). New York: Academic Press.

Biliaderis, C. G., & Juliano, B. O. (1993). Thermal and mechanical properties of concentrated rice starch gels of varying composition. *Food Chemistry, 48*, 243 - 250.

Bordoloi, A., Singh, J., & Kaur, L. (2012). In vitro digestibility of starch in cooked potatoes as affected by guar gum: Microstructural and rheological characteristics. *Food Chemistry, 133*, 1206-1213.

Bourne, M. (2002). *Food texture and viscosity: Concept and measurements* (2nd ed.). Florida: Academic Press.

Braccini, I., Grasso, R. P., & Perez, S. (1999). Conformational and configurational features of acidic polysaccharides and their interactions with calcium ions: a molecular modeling investigation. *Carbohydrate Research, 317*, 119 - 130.

Brennan, C. S. (2005). Dietary fibre, glycaemic response, and diabetes. *Molecular Nutrition & Food Research, 49*, 560-570.

Brennan, C. S., Blake, D. E., Ellis, P. R., & Schofield, J. D. (1996). Effects of Guar Galactomannan on Wheat Bread Microstructure and on the In vitro and In vivo Digestibility of Starch in Bread. *Journal of Cereal Science, 24*, 151-160.

Brennan, C. S., Kuri, V., & Tudorica, C. M. (2004). Inulin-enriched pasta: effects on textural properties and starch degradation. *Food Chemistry, 86*, 189-193.

Brennan, C. S., Suter, M., Luethi, T., Matia-Merino, L., & Qvortrup, J. (2008). The Relationship Between Wheat Flour and Starch Pasting Properties and Starch Hydrolysis: Effect of Non-starch Polysaccharides in a Starch Gel System. *Starch - Stärke, 60*, 23-33.

Brennan, C. S., & Tudorica, C. M. (2008). Evaluation of potential mechanisms by which dietary fibre additions reduce the predicted glycaemic index of fresh pastas. *International Journal of Food Science & Technology, 43*, 2151-2162.

Brown, M. E. (Ed.). (2001). *Introduction to thermal analysis: Techniques and applications* (2nd ed.). Netherlands: Kluwer Academics Publishers.

Buisson, G., Duee, E., Haser, R., & Payan, F. (1987). Three dimensional structure of pocine pancreatic α-amylase at 2.9 Å resolution. Role of calcium in structure and activity. *The EMBO Journal, 6*, 3909 - 3916.

Buleon, A., & Colonna, P. (2007). Physiochemical behaviour of starch in food applications. In P. Belton (Ed.), *The chemical physics of food* (pp. 20-67). Oxford: Blackwell Publishings.

Buonocore, V., Poerio, E., Silano, V., & Tomasi, M. (1976). Physical and catalytic properties of α-amylase from Tenebrio molitor L. larvae. *Biochemical Journal, 153*, 621 - 625.

Bustos, M. C., Vignola, M. B., Pérez, G. T., & León, A. E. (2017). In vitro digestion kinetics and bioaccessibility of starch in cereal food products. *Journal of Cereal Science, 77*, 243 - 250.

Çakır, E., & Foegeding, E. A. (2011). Combining protein micro-phase separation and protein–polysaccharide segregative phase separation to produce gel structures. *Food Hydrocolloids, 25*, 1538-1546.

Caldwell, M. L., & Kung, J. T. (1953). A study of the influence of a number of factors upon the stability and upon the activity of pancreatic amylase. *Journal of the American Chemical Society, 75*, 3132 - 3135.

Callaghan, P. T. (1993). *Principles of Nuclear Magnetic Resonance Microscopy*. USA: Oxford University Press.

Carper, W. R., & Keller, C. E. (1997). Direct determination of NMR correlation times from spin− lattice and spin− spin relaxation times. *The Journal of Physical Chemistry A, 101*, 3246 - 3250.

Cavanagh, J., Fairbrother, W. J., Palmer III, A. G., & Skelton, N. J. (1996). *Protein NMR Spectroscopy*. USA: Academic Press.

Chaisawang, M., & Suphantharika, M. (2006). Pasting and rheological properties of native and anionic tapioca starches as modified by guar gum and xanthan gum. *Food Hydrocolloids, 20*, 641 - 649.

Champ, M. (2004). Resistant starch. In A.-C. Eliasson (Ed.), *Starch in Food: Structure, function and applications* (pp. 560 - 574). England: Woodhead Publishing.

Chanamai, R., & McClements, D. J. (2001). Depletion flocculation of beverage emulsions by gum arabic and modified starch. *Journal of Food Science, 66*, 457 - 463.

Charoenrein, S., Tatirat, O., & Muadklay, J. (2008). Use of centrifugation–filtration for determination of syneresis in freeze–thaw starch gels. *Carbohydrate Polymers, 73*, 143 - 147.

Charoenrein, S., Tatirat, O., Rengsutthi, K., & Thongngam, M. (2011). Effect of konjac glucomannan on syneresis, textural properties and the microstructure of frozen rice starch gels. *Carbohydrate Polymers, 83*, 291 - 296.

Cheetham, N. W. H., & Tao, L. (1998). Variation in crystalline type with amylose content in maize starch granules- an X-ray powder diffraction study. *Carbohydrate Polymers, 36*, 277 - 284.

Chen, S., Chen, H., Tian, J., Wang, Y., Xing, L., & Wang, J. (2013). Chemical modification, antioxidant and α-amylase inhibitory activities of corn silk polysaccharides. *Carbohydrate Polymers, 98*, 428-437.

Chethan, S., Sreerama, Y. N., & Malleshi, N. G. (2008). Mode of inhibition of finger millet malt amylases by the millet phenolics. *Food Chemistry, 111*, 187 - 191.

Chowdury, Z. K., VanGelder, A., Lawler, D., & Moran, M. (1999). *Particle count method development for concentration standards and sample stabilization*. USA: AWWA Research Foundation and American Water Works Association.

Christianson, D. D., Hodge, J. E., Osborne, D., & Detroy, R. W. (1981). Gelatinization of wheat starch as modified by xanthan gum, guar gum, and cellulose gum. *Cereal Chemistry, 58*, 513 - 517.

Chung, H.-J., Lim, H. S., & Lim, S.-T. (2006). Effect of partial gelatinization and retrogradation on the enzymatic digestion of waxy rice starch. *Journal of Cereal Science, 43*, 353 - 359.

Chusak, C., Thilavech, T., & Adisakwattana, S. (2014). Consumption of Mesona chinensis attenuates postprandial glucose and improves antioxidant status induced by a high carbohydrate meal in overweight subjects. *American Journal of Chinese Medicine, 42*, 315-336.

Copeland, R. A. (Ed.). (2000). *Enzymes: A practical introduction to structure, mechanism, and data analysis* (2nd ed.). USA: Wiley-VCH.

Cornish-Bowden, A. (2004). *Fundamentals of enzyme kinetics* (3rd ed.). UK: Portland Press Ltd.

Cui, R., & Oates, C. G. (1999). The effect of amylose–lipid complex formation on enzyme susceptibility of sago starch. *Food Chemistry, 65*, 417 - 425.

Dartois, A., Singh, J., Kaur, L., & Singh, H. (2010). Influence of guar gum on the in vitro starch digestibility - Rheological and microstructural characteristics. *Food Biophysics, 5*, 149-160.

Debet, M. R., & Gidley, M. J. (2007). Why do gelatinized starch granules not dissolve completely? Roles for amylose, protein, and lipid in granule "ghost" integrity. *Journal of Agriculture and Food Chemistry, 55*, 4752-4760.

Dodds, J. (2013). Techniques to analyse particle size of food powders. In B. Bhandari, N. Bansal, M. Zhang, & P. Schuck (Eds.), *Handbook of food powders: Processes and properties* (pp. 309 - 338). UK: Woodhead Publishing Limited.

Donovan, J. W. (1979). Phase transitions of the starch-water system. *Biopolymers, 18*, 263-275.

Doublier, J. L., Garnier, C., Renard, D., & Sanchez, C. (2000). Protein-polysaccharide interactions. *Current Opinion in Colloid & Interface Science, 5*, 202-214.

Draget, K. I., Moe, S. T., Skjak-Braœk, G., & Smidsrod, O. (2006). Alginates. In A. M. Stephen, G. O. Phillips, & P. A. Williams (Eds.), *Food polysaccharides and their applications* (2nd ed., pp. 289-334). Florida: Taylor & Francis Group.

Ebewele, R. O. (2000). Polymer science and technology.

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., Barberá, R., Brodkorb, A., Cattenoz, T., Clemente, A., Comi, I., Dupont, D., Garcia-Llatas, G., Lagarda, M. J., Le Feunteun, S., JanssenDuijghuijsen, L., Karakaya, S., Lesmes, U., Mackie, A. R., Martins, C., Meynier, A., Miralles, B., Murray, B. S., Pihlanto, A., Picariello, G., Santos, C. N., Simsek, S., Recio, I., Rigby, N., Rioux, L.-E., Stoffers, H., Tavares, A., Tavares, L., Turgeon, S., Ulleberg, E. K., Vegarud, G. E., Vergères, G., & Portmann, R. (2016). The harmonized INFOGEST in vitro digestion method: From knowledge to action. *Food Research International, 88*, 217 - 225.

Eliasson, A. C. (1986). Viscoelastic behaviour during the gelatinization of starch I. Comaprison of wheat, maize, potato and waxy-barley starches. *Journal of Texture Studies, 17*, 253 - 265.

Eliasson, A. C. (2017). Starch: Physicochemical and functional aspects. In A. C. Eliasson (Ed.), *Carbohydrates in food* (3rd ed.). USA: CRC Press.

Ellis, H. S., & Ring, S. G. (1985). A Study of Some Factors Influencing Amyiose Gelation. *Carbohydrate Polymers, 5*, 201-213.

Englyst, H. N., Kingman, S., & Cummings, J. H. (1992). Classifications and measurements of nutritionally important starch fractions. *European Journal of Clinical Nutrition, 46*, S33-S50.

Englyst, H. N., Veenstra, J., & Hudson, G. J. (1996). Measurement of rapidly available glucose (RAG) in plant foods: a potential *in vitro* predictor ofthe glycaemic response. *British Journal of Nutrition, 75*, 327 - 337.

Englyst, K. N., Englyst, H. N., Hudson, G. J., Cole, T. J., & Cummings, J. H. (1999). Rapidly available glucose in foods: an in vitro measurement that reflects the glycemic response. *American Journal of Clinical Nutrition, 69*, 448 - 454.

Espinosa-Dzib, A., Ramírez-Gilly, M., & Tecante, A. (2012). Viscoelastic behavior and microstructure of aqueous mixtures of cross-linked waxy maize starch, whey protein isolate and κ-carrageenan. *Food Hydrocolloids, 28*, 248-257.

Fang, Y., Al-Assaf, S., Phillips, G. O., Nishinari, K., Funami, T., & Williams, P. A. (2008). Binding behavior of calcium to polyuronates: Comparison of pectin with alginate. *Carbohydrate Polymers, 72*, 334 - 341.

Farinas, C. S., Damaso, M. C. T., & Couri, S. (2013). *Methods to determine enzymatic activity* A. B. Vermelho, & S. Couri (Eds.),

Feng, T., Gao, L., Zhuang, H., Ye, R., Xu, Z., Liu, Y., & Bing, F. (2016). Effects of NaCl and CaCl2 on physical properties of Mesona blumes gum/rice starch mixed gel. *Advance Journal of Food Science and Technology, 12*, 138 - 144.

Feng, T., Gu, Z., Jin, Z., & Zhuang, H. (2010a). Rheological properties of cereal starch gels and *Mesona Blumes* gum mixtures. *Starch - Stärke, 62*, 480-488.

Feng, T., Gu, Z., Jin, Z., & Zhuang, H. (2010b). Rheological Properties of Rice Starch-*Mesona Blumes* Gum Mixtures. *Journal of Texture Studies, 41*, 685-702.

Feng, T., Gu, Z. B., & Jin, Z. Y. (2007). Chemical composition and some rheological properties of *Mesona blumes* gum. *Food Science and Technology International, 13*, 55-61.

Feng, T., Gu, Z. B., Jin, Z. Y., & Zhuang, H. N. (2008). Isolation and characterization of an acidic polysaccharide from *Mesona Blumes* gum. *Carbohydrate Polymers, 71*, 159-169.

Feng, T., Su, Q., Zhuang, H. N., Ye, R., Gu, Z. B., & Jin, Z. Y. (2014). Ghost structures, pasting, rheological and textural properties between mesona blumes gum and various starches. *Journal of Food Quality, 37*, 73-82.

Feng, T., & Ye, R. (2013). Rheological behavior of biopolymer systems. In S. Thomas, D. Durand, C. Chassenieux, & P. Jyotishkumar (Eds.), *Handbook of biopolymer-based material* (pp. 673 - 698). Germany: Wiley VCH.

Feng, T., Ye, R., Zhuang, H., Fang, Z., & Chen, H. (2012). Thermal behavior and gelling interactions of *Mesona Blumes* gum and rice starch mixture. *Carbohydrate Polymers, 90*, 667-674.

Feng, T., Ye, R., Zhuang, H., Rong, Z., Fang, Z., Wang, Y., Gu, Z., & Jin, Z. (2013). Physicochemical properties and sensory evaluation of *Mesona Blumes* gum/rice starch mixed gels as fat-substitutes in Chinese Cantonese-style sausage. *Food Research International, 50*, 85-93.

Ferrero, C., Martino, M. N., & Zaritzky, N. E. (1994). Corn starch-xanthan gum interaction and its effect on the stability during storage of frozen gelatinized suspensions. *Starch - Stärke, 46*, 300 - 308.

Funami, T., Kataoka, Y., Omoto, T., Goto, Y., Asai, I., & Nishinari, K. (2005a). Effects of non-ionic polysaccharides on the gelatinization and retrogradation behavior of wheat starch. *Food Hydrocolloids, 19*, 1-13.

Funami, T., Kataoka, Y., Omoto, T., Goto, Y., Asai, I., & Nishinari, K. (2005b). Food hydrocolloids control the gelatinization and retrogradation behavior of starch. 2b. Functions of guar gums with different molecular weights on the retrogradation behavior of corn starch. *Food Hydrocolloids, 19*, 25-36.

Funami, T., Nakauma, M., Noda, S., Ishihara, S., Asai, I., Inouchi, N., & Nishinari, K. (2008). Effects of some anionic polysaccharides on the gelatinization and retrogradation behaviors of wheat starch: Soybean-soluble polysaccharide and gum arabic. *Food Hydrocolloids, 22*, 1528-1540.

Gabbott, P. (2008). A practical introduction to Differential Scanning Calorimetry. In P. Gabbott (Ed.), *Principles and applications of thermal analysis* (pp. 1 - 51). Oxford, UK: Blackwell Publishing

Ghiasi, K., Hoseney, R. C., & Varriano-Marston, E. (1982). Gelatinization of wheat starch. III. Comparison by differential scanning calorimetry and light microscopy. *Cereal Chemistry, 59*, 258 - 262.

Gidley, M. J. (1989). Molecular mechanisms underlying amylose aggregation and gelation. *Macromolecules, 22*, 351-358.

Godet, M. C., Tran, V., Delage, M., & Buleon, A. (1992). Molecular modelling of the specific interactions involved in the amylose complexation by fatty acids. *International Journal of Biological Micromolecules, 15*, 11 - 16.

Gonera, A., & Cornillon, P. (2002). Gelatinization of starch gum sugar systems studied by using DSC, NMR, and CSLM. *Starch - Stärke, 54*, 508-516.

Gouseti, O., Jaime-Fonseca, M. R., Fryer, P. J., Mills, C., Wickham, M. S. J., & Bakalis, S. (2014). Hydrocolloids in human digestion: Dynamic in-vitro assessment of the effect of food formulation on mass transfer. *Food Hydrocolloids, 42*, 378 - 385.

Gowri, P. M., Tiwari, A. K., Ali, A. Z., & Rao, J. M. (2007). Inhibition of alpha-glucosidase and amylase by bartogenic acid isolated from Barringtonia racemosa Roxb. seeds. *Phytother Res, 21*, 796 - 799.

Gusakov, A. V., Kondratyeva, E. G., & Sinitsyn, A. P. (2011). Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities. *International Journal of Analytical Chemistry, 2011*, 283658.

Han, X.-Z., & Hamaker, B. R. (2002a). Association of starch granule proteins with starch ghosts and remnants revealed by confocal laser scanning microscopy. *Cereal Chemistry, 79*, 892-896.

Han, X. Z., & Hamaker, B. R. (2002b). Location of Starch Granule-associated Proteins Revealed by Confocal Laser Scanning Microscopy. *Journal of Cereal Science, 35*, 109 - 116.

Hannan, J. M., Ali, L., Rokeya, B., Khaleque, J., Akhter, M., Flatt, P. R., & Abdel-Wahab, Y. H. (2007). Soluble dietary fibre fraction of *Trigonella foenum-graecum* (fenugreek) seed improves glucose homeostasis in animal models of type 1 and type 2 diabetes by delaying carbohydrate digestion and absorption, and enhancing insulin action. *British Journal of Nutrition, 97*, 514-521.

Hansen, L. M., Hoseney, R. C., & Faubion, J. M. (1991). Oscillatory rheometry of starch-water systems: effect of starch concentration and temperature. *Cereal Chemistry, 68*, 347 - 351.

Hemar, Y., Hall, C. E., Munro, P. A., & Singh, H. (2002). Small and large deformation rheology and microstructure of κ-carrageenan gels containing commercial milk protein products. *International Dairy Journal, 12*, 371-381.

Hizukuri, S., Shirasaka, K., & Juliano, B. O. (1983). The influence of amylose and amylopectin characteristics on gelatinization and retrogradation properties of different starches. *Starch - Stärke, 35*, 348-350.

Hizukuri, S., Takeda, Y., & Yasuda, M. (1981). Multi-branched nature of amylose and the action of debranching enzymes. *Carbohydrate Research, 94*, 205-213.

Holm, J., Lundquist, I., Bjorck, I., Eliasson, A. C., & Asp, N. G. (1988). Degree of starch gelatinization, digestion rate of starch in vitro, and metabolic response in rats. *American Journal of Clinical Nutrition, 47*, 1010 - 1016.

Horinaka, J., Kani, K., Hori, Y., & Maeda, S. (2004). Effect of pH on the conformation of gellan chains in aqueous systems. *Biophysical Chemistry, 111*, 223 - 227.

Hu, P., Zhao, H., Duan, Z., Linlin, Z., & Wu, D. (2004). Starch digestibility and the estimated glycemic score of different types of rice differing in amylose contents. *Journal of Cereal Science, 40*, 231-237.

Huber, K. C., & BeMiller, J. N. (2000). Channels of maize and sorghum starch granules. *Carbohydrate Polymers, 41*, 269-276.

Ishiguro, K., Noda, T., Kitahara, K., & Yamakawa, O. (2000). Retrogradation of sweet potato starch. *Starch - Stärke, 52*, S13 - 17.

Iturriaga, L. B., de Mishima, B. L., & Añon, M. C. (2006). Effect of amylose on starch pastes viscoelasticity and cooked grains stickiness in rice from seven argentine genotypes. *Food Research International, 39*, 660 - 666.

JaICA. (2015). Calcium assay kit: O-Cresolphthalein (OCPC) chromogenic method. In Japan Institute for the Control of Aging (Ed.), *MC Reagent Biochemical Assay Series*. Japan: Nikken SEIL Co., Ltd.

James, W. P. T., Branch, W. J., & Southgate, D. A. T. (1978). Calcium binding by dietary fibre. *The Lancet, 311*, 638 - 639.

Jane, J. L. (2009). Structural features of starch granules II. In J. N. BeMiller, & R. L. Whistler (Eds.), *Starch: Chemistry and Technology* (3rd ed., pp. 193-236). New York: Academic Press.

Jane, J. L. (1993). Mechanism of starch gelatinization in neutral salt solutions. *Starch - Stärke, 45*, 161-166.

Jane, J. L., Kasemsuwan, T., Leas, S., Zobel, H., & Robyt, J. F. (1994). Anthology of starch granule morphology by Scanning Electron Microscopy. *Starch - Stärke, 46*, S121 - 129.

Jenkins, D. J. A., Kendall, C. W. C., Augustin, L. S. A., Franceschi, S., Hamidi, M., Marchie, A., Jenkins, A. L., & Axelsen, M. (2002). Glycemic index: Overview of implications in health and disease. *The American Journal of Clinical Nutrition, 76 (suppl)*, 266S-273S.

Jenkins, P. J., & Donald, A. M. (1998). Gelatinisation of starch: a combined SAXS/WAXS/DSC and SANS study. *Carbohydrate research, 308*, 133 - 147.

Jyothi, A. N., Sasikiran, K., Sajeev, M. S., Revamma, R., & Moorthy, S. N. (2005). Gelatinisation Properties of Cassava Starch in the Presence of Salts, Acids and Oxidising Agents. *Starch - Stärke, 57*, 547 - 555.

Jyoti, B. V. S., & Baek, S. W. (2014). Formulation and comparative study of rheological properties of loaded and unloaded ethanol-based gel propellants. *Journal of Energetic Materials, 33*, 125 - 139.

Katsuta, K., Miura, M., & Nishimura, A. (1992). Kinetic treatment for rheological properties and effects of saccharides on retrogradation of rice starch gels. *Food Hydrocolloids, 6*, 187 - 198.

Kaufman, R. C., Wilson, J. D., Bean, S. R., Herald, T. J., & Shi, Y. C. (2015). Development of a 96-well plate iodine binding assay for amylose content determination. *Carbohydrate Polymers, 115*, 444-447.

Kaur, L., Singh, J., McCarthy, O. J., & Singh, H. (2007). Physico-chemical, rheological and structural properties of fractionated potato starches. *Journal of Food Engineering, 82*, 383-394.

Keetels, C. J. A. M., van Vliet, T., & Walstra, P. (1996). Gelation and retrogradation of concentrated starch systems: 1 Gelation. *Food Hydrocolloids, 10*, 343 - 353.

Khanna, S., & Tester, R. (2006). Influence of purified konjac glucomannan on the gelatinisation and retrogradation properties of maize and potato starches. *Food Hydrocolloids, 20*, 567-576.

Khursheed, A. (2011). *Scanning Electron Microscope optics and spectrometers*. Singapore: World Scientific.

Kolodziejski, W., & Klinowski, J. (2002). Kinetics of cross-polarization in solid-state NMR: A guide for chemists. *Chemical Reviews, 102*, 613 - 628.

Krüger, A., Ferrero, C., & Zaritzky, N. E. (2003). Modelling corn starch swelling in batch systems: effect of sucrose and hydrocolloids. *Journal of Food Engineering, 58*, 125 - 133.

Kumar, R. (2012). *Interactions between extract from grass jelly herb (Mesona chinensis) & starch*. Honours report. Massey University. Singapore.

Lai, L. S., & Chao, S. J. (2000a). Effects of salts on the thermal reversibility of starch and hsian-tsao (*mesona procumbens* hemsl) leaf gum mixed system. *Journal of Food Science, 65*, 954-959.

Lai, L. S., & Chao, S. J. (2000b). A DSC study on the gel-sol transition of a starch and hsian-tsao leaf gum mixed system. *Journal of Agricultural and Food Chemistry, 48*, 3267-3274.

Lai, L. S., & Chiang, H. F. (2002). Rheology of decolorized hsian-tsao leaf gum in the dilute domain. *Food Hydrocolloids, 16*, 427-440.

Lai, L. S., & Liao, C.-L. (2002a). Steady and dynamic shear rheological properties of starch and decolorized *Hsian-tsao* leaf gum composite systems. *Cereal Chemistry, 79*, 58-63.

Lai, L. S., & Liao, C.-L. (2002b). Dynamic rheology of structural development in starch/decolourised hsian-tsao leaf gum composite systems. *Journal of the Science of Food and Agriculture, 82*, 1200-1207.

Lai, L. S., & Lin, P.-H. (2004). Application of decolourised hsian-tsao leaf gum to low-fat salad dressing model emulsions: a rheological study. *Journal of the Science of Food and Agriculture, 84*, 1307-1314.

Lai, L. S., Liu, Y.-L., & Lin, P.-H. (2003). Rheological/textural properties of starch and crude hsian-tsao leaf gum mixed systems. *Journal of the Science of Food and Agriculture, 83*, 1051-1058.

Lai, L. S., Tung, J., & Lin, P. S. (2000). Solution properties of hsian-tsao (*Mesona procumbens* Hemsl) leaf gum. *Food Hydrocolloids, 14*, 287-294.

Lee, M. H., Baek, M. H., Cha, D. S., Park, H. J., & Lim, S. T. (2002). Freeze-thaw stabilization of sweet potato starch gel by polysaccharide gums. *Food Hydrocolloids, 16*, 345 - 352.

Lehmann, U., & Robin, F. (2007). Slowly digestible starch – its structure and health implications: a review. *Trends in Food Science & Technology, 18*, 346-355.

Lentle, R. G., & Janssen, P. W. (2008). Physical characteristics of digesta and their influence on flow and mixing in the mammalian intestine: a review. *Journal of Comparative Physiology B, 178*, 673 - 690.

Lentle, R. G., & Janssen, P. W. M. (2011). *The physical processes of digestion*. London: Springer.

Li, X., Gao, W., Wang, J., Jiang, Q., & Huang, L. (2010). Comparison of the morphological, crystalline, and thermal properties of different crystalline types of starches after acid hydrolysis. *Starch - Stärke, 62*, 686-696.

Lii, C. Y., & Chen, L. H. (1980). *The factors in the gel forming properties of hsian-tsao (Mesona procumbens Hemsl). I. Extraction conditions and different starches.* Paper presented at the National Science Council ROC.

Lii, C. Y., Tsai, M. L., & Tseng, K. H. (1996). Effect of amylose content on the rheological property of rice starch. *Cereal Chemistry, 73*, 415 - 420.

Lin, L., Shen, M., Liu, S., Tang, W., Wang, Z., Xie, M., & Xie, J. (2017). An acidic heteropolysaccharide from *Mesona chinensis*: Rheological properties, gelling behavior and texture characteristics. *International Journal of Biological Micromolecules, 107*, 1591 - 1598.

Linlaud, N., Ferrer, E., Puppo, M. C., & Ferrero, C. (2011). Hydrocolloid interaction with water, protein, and starch in wheat dough. *Journal of Agricultural and Food Chemistry, 59*, 713-719.

Liu, H., Eskin, N. A. M., & Cui, S. W. (2003). Interaction of wheat and rice starches with yellow mustard mucilage. *Food Hydrocolloids, 17*, 863 - 869.

Liu, J.-M., & Zhao, S.-L. (1990). Scanning Electron Microscope study on gelathization of starch granules in excess water. *Starch - Stärke, 42*, S96 - 98.

Madar, S., & Shomer, I. (1990). Polysaccharide composition of a gel fraction derived from fenugreek and its effect on starch digestion and bile acid absorption in rats. *Journal of Agriculture and Food Chemistry, 38*, 1535-1539.

Mahasukhonthachat, K., Sopade, P. A., & Gidley, M. J. (2010). Kinetics of starch digestion in sorghum as affected by particle size. *Journal of Food Engineering, 96*, 18 - 28.

Malvern Instrument. (2007). Mastersizer 2000 user manual. Retrieved 12/03/2018, from https://www.labmakelaar.com/fjc_documents/mastersizer-2000-2000e-manual-eng1.pdf

Mandala, I. G. (2012). Viscoelastic properties of starch and non-starch thickeners in simple mixtures or model food. In J. D. Vicente (Ed.), Viscoelasticity - From theory to biological applications. Retrieved from https://www.intechopen.com/books/viscoelasticity-from-theory-to-biological-applications/viscoelastic-properties-of-starch-and-non-starch-thickeners-in-simple-mixtures-or-model-food. doi: 10.5772/50221

Mandala, I. G., & Bayas, E. (2004). Xanthan effect on swelling, solubility and viscosity of wheat starch dispersions. *Food Hydrocolloids, 18*, 191-201.

Manners, D. J. (1989). Recent developments in our understanding of amylopectin structure. *Carbohydrate Polymers, 11*, 87-112.

Mat, D. J. L., Le Feunteun, S., Michon, C., & Souchon, I. (2016). In vitro digestion of foods using pH-stat and the INFOGEST protocol: Impact of matrix structure on digestion kinetics of macronutrients, proteins and lipids. *Food Research International, 88*, 226 - 233.

McClements, D. J. (2000). Comments on viscosity enhancement and depletion flocculation by polysaccharides. *Food Hydrocolloids, 14*, 173 - 177.

McClements, D. J. (2015). *Nanoparticle- and microparticle-based systems: Encapsulation, protection and release of active compounds*. USA: CRC Press.

Mezger, T. G. (Ed.). (2011). *The rheology handbook* (3rd ed.). Germany: Vincentz Network.

Miao, M., Jiang, B., Cui, S. W., Zhang, T., & Jin, Z. (2015). Slowly digestible starch - a review. *Critical Reviews in Food Science and Nutrition, 55*, 1642 - 1657.

Miles, M. J., Morris, G. A., Orford, P. D., & Ring, S. G. (1985). The roles of amylose and amylopectin in the gelation and retrogradation of starch. *Carbohydrate Research, 135*, 271-281.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le

Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Menard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S., Weitschies, W., & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food and Function, 5*, 1113 - 1124.

Miri, T. (2011). Viscosity and oscillatory rheology. In I. T. Norton, F. Spyropoulos, & P. Cox (Eds.), *Practical food rheology: An interpretive approach* (pp. 7-28). UK: Blackwell Publishings.

Mishra, S., Monro, J., & Hedderley, D. (2008). Effect of Processing on Slowly Digestible Starch and Resistant Starch in Potato. *Starch - Stärke, 60*, 500-507.

Monro, J. A. (2002). Glycaemic glucose equivalent: combining carbohydrate content, quantity and glycaemic index of foods for precision in glycaemia management. *Asia Pacific Journal of Clinical Nutrition, 11*, 217 - 225.

Morris, E. R. (1990). Shear-thinning of 'random coil' polysaccharides: Characterisation by two parameters from a simple linear plot. *Carbohydrate Polymers, 13*, 85-96.

Morris, E. R., Cutler, A. N., Ross-Murphy, S. B., & Rees, D. A. (1981). Concentration and shear rate dependence of viscosity in random coil polysaccharide solutions. *Carbohydrate Polymers, 1*, 5-21.

Morris, E. R., Rees, D. A., & Welsh, E. J. (1980). Conformation and dynamic interactions in hyaluronate solutions. *Journal of Molecular Biology, 138*, 383-400.

Morris, V. J. (2007). Gels. In P. Belton (Ed.), *The chemical physics of food* (pp. 151-198). UK: Blackwell Publishing.

Motulsky, H., & Christopoulos, A. (2004). *Fitting modes to biological data using linear and nonlinear regression: A practical guide to curve fitting*. USA: Oxfrod University Press.

Mua, J. P., & Jackson, D. S. (1997). Relationships between functional attributes and molecular structures of amylose and amylopectin fractions from corn starch. *Journal of Agriculture and Food Chemistry, 45*, 3848 - 3854.

Nag, A. (2016). *Analytical and instrumental techniques in agriculture, environnmental and food engineering*. India: PHI Learning Private Limited.

Newman, R. H., & Hemmingson, J. A. (1998). Interactions between locust bean gum and cellulose characterized by 13C NMR spectroscopy. *Carbohydrate Polymers, 36*, 167 - 172.

Noda, T., Takigawa, S., Matsuuraendo, C., Suzuki, T., Hashimoto, N., Kottearachchi, N., Yamauchi, H., & Zaidul, I. (2008). Factors affecting the digestibility of raw and gelatinized potato starches. *Food Chemistry, 110*, 465-470.

Okechukwu, P. E., & Rao, M. A. (1995). Influence of granule size on viscosity of cornstarch suspension. *Journal of Texture Studies, 26*, 501 - 516.

Olkku, J., & Rha, C. (1978). Gelatinisation of starch and wheat flour starch—A review. *Food Chemistry, 3*, 293 - 317.

Otting, G. (2007). *Encyclopedia of Magnetic Resonance*. UK: John Wiley & Sons Ltd.

Palav, T., & Seetharaman, K. (2006). Mechanism of starch gelatinization and polymer leaching during microwave heating. *Carbohydrate Polymers, 65*, 364 - 370.

Perez, S., Baldwin, P. M., & Gallant, D. J. (2009). Structural features of starch granule I. In J. N. BeMiller, & R. L. Whistler (Eds.), *Starch: Chemistry and Technology* (3rd ed., pp. 149 - 192). New York: Academic Press.

Pérez, S., & Bertoft, E. (2010). The molecular structures of starch components and their contribution to the architecture of starch granules: A comprehensive review. *Starch - Stärke, 62*, 389-420.

Piculell, L. (2006). Gelling carrageenans. In A. M. Stephen, G. O. Phillips, & P. A. Williams (Eds.), *Food polysaccharides and their applications* (2nd ed., pp. 239-288). Florida: Taylor & Francis Group.

Pranprawit, A., Heyes, J. A., Molan, A. L., & Kruger, M. C. (2015). Antioxidant activity and inhibitory potential of blueberry extracts against key enzymes relevant for hyperglycemia. *Journal of Food Biochemistry, 39*, 109 - 118.

Ratnayake, W. S., & Jackson, D. S. (2007). A new insight into the gelatinization process of native starches. *Carbohydrate Polymers, 67*, 511-529.

Richardson, P. H., Jeffcoat, R., & Shi, Y.-C. (2011). High-amylose starches: From biosynthesis to their use as food ingredients. *MRS Bulletin, 25*, 20 - 24.

Ring, S. G., Colonna, P., I'Anson, K. J., Kalichevsky, M. T., Miles, M. J., Morris, V. J., & Orford, P. D. (1987). The gelation and crystallisation of amylopectin. *Carbohydrate Research, 162*, 277-293.

Ross-Murphy, S. B. (1995). Structure–property relationships in food biopolymer gels and solutions. *Journal of Rheology, 39*, 1451.

Rubilar, M., Jara, C., Poo, Y., Acevedo, F., Gutierrez, C., Sineiro, J., & Shene, C. (2011). Extracts of Maqui (Aristotelia chilensis) and Murta (Ugni molinae Turcz.): sources of antioxidant compounds and α-Glucosidase/α-Amylase inhibitors. *Journal of Agriculture and Food Chemistry, 59*, 1630 - 1637.

Rumpold, B. A., & Knorr, D. (2005). Effect of salts and sugars on pressure-induced gelatinisation of wheat, tapioca, and potato starches. *Starch - Stärke, 57*, 370 - 377.

Sablani, S. S. (2009). Gelatinization of starch. In M. S. Rahman (Ed.), *Food properties handbook* (2nd ed., pp. 287 - 314). USA: CRC Press.

Sae-kang, V., & Suphantharika, M. (2006). Influence of pH and xanthan gum addition on freeze-thaw stability of tapioca starch pastes. *Carbohydrate Polymers, 65*, 371 - 380.

Salmeron, J., Manson, J. E., Stampfer, M. J., Colditz, G. A., Wing, A. L., & Willett, W. C. (1997). Dietary Fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women. *Diet and Diabetes, 277*, 472 - 477.

Samutsri, W., & Suphantharika, M. (2012). Effect of salts on pasting, thermal, and rheological properties of rice starch in the presence of non-ionic and ionic hydrocolloids. *Carbohydrate Polymers, 87*, 1559 - 1568.

Sarikaya, E., Higasa, T., Adachi, M., & Mikami, B. (2000). Comparison of degradation abilities of a- and b-amylases on raw starch granules. *Process Biochemistry, 35*, 711-715.

Šárka, E., & Dvořáček, V. (2017). New processing and applications of waxy starch (a review). *Journal of Food Engineering, 206*, 77 - 87.

Sasaki, T., & Kohyama, K. (2011). Effect of non-starch polysaccharides on the in vitro digestibility and rheological properties of rice starch gel. *Food Chemistry, 127*, 541-546.

Sasaki, T., & Kohyama, K. (2012). Influence of non-starch polysaccharides on the in vitro digestibility and viscosity of starch suspensions. *Food Chemistry, 133*, 1420-1426.

Saulnier, L., & Thibault, J. F. (1999). Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *Journal of the Science of Food and Agriculture, 79*, 396-402.

Savary, G., Handschin, S., Conde-Petit, B., Cayot, N., & Doublier, J.-L. (2008). Structure of polysaccharide-starch composite gels by rheology and confocal laser scanning microscopy: Effect of the composition and of the preparation procedure. *Food Hydrocolloids, 22*, 520-530.

Schäfer, A., & Högger, P. (2007). Oligomeric procyanidins of French maritime pine bark extract (Pycnogenol®) effectively inhibit α-glucosidase. *Diabetes Research and Clinical Practice, 77*, 41 - 46.

Sharma, K. P., Choudhury, C. K., Srivastava, S., Davis, H., Rajamohanan, P. R., Roy, S., & Kumaraswamy, G. (2011). Assembly of polyethyleneimine in the hexagonal mesophase of nonionic surfactant: effect of pH and temperature. *The Journal of Physical Chemistry B, 115*, 9059 - 9069.

Shi, X., & BeMiller, J. N. (2002). Effects of food gums on viscosities of starch suspensions during pasting. *Carbohydrate Polymers, 50*, 7-18.

Shobana, S., Sreerama, Y. N., & Malleshi, N. G. (2009). Composition and enzyme inhibitory properties of finger millet (Eleusine coracana L.) seed coat phenolics: Mode of inhibition of α-glucosidase and pancreatic amylase. *Food Chemistry, 115*, 1268 - 1273.

Singh, J., Dartois, A., & Kaur, L. (2010). Starch digestibility in food matrix: a review. *Trends in Food Science & Technology, 21*, 168-180.

Singh, N., Singh, J., Kaur, L., Singh Sodhi, N., & Singh Gill, B. (2003). Morphological, thermal and rheological properties of starches from different botanical sources. *Food Chemistry, 81*, 219-231.

Slaughter, S. L., Ellis, P. R., & Butterworth, P. J. (2001). An investigation of the action of porcine pancreatic α-amylase on native and gelatinised starches. *Biochimica et Biophysica Acta, 1525*, 29-36.

Slaughter, S. L., Ellis, P. R., Jackson, E. C., & Butterworth, P. J. (2002). The effect of guar galactomannan and water availability during hydrothermal processing on the hydrolysis of starch catalysed by pancreatic α–amylase. *Biochimica et Biophysica Acta, 1571*, 55-63.

Smits, A. L. M., Kruiskamp, P. H., van Soest, J. J. G., & Vliegenthart, J. F. G. (2003). Interaction between dry starch and plasticisers glycerol or ethylene glycol, measured by differential scanning calorimetry and solid state NMR spectroscopy. *Carbohydrate Polymers, 53*, 409 - 416.

Steeneken, P. A. M. (1989). Rheological properties of aqueous suspensions of swollen starch granules. *Carbohydrate Polymers, 11*, 23 - 42.

Stephen, A. M., & Churms, S. C. (2006). Introduction. In A. M. Stephen, G. O. Phillips, & P. A. Williams (Eds.), *Food polysaccharides and their applications* (2nd ed., pp. 1-24). Florida: Taylor & Francis Group.

Sun, T., Lærke, H. N., Jørgensen, H., & Knudsen, K. E. B. (2006). The effect of extrusion cooking of different starch sources on the in vitro and in vivo digestibility in growing pigs. *Animal Feed Science and Technology, 131*, 67-86.

Swinkels, J. J. M. (1985). Composition and properties of commercial native starches. *Starch - Stärke, 37*, 1 - 5.

Szczesniak, A. S., Brandt, M. A., & Friedman, H. H. (1963). Development of standard rating scales for mechanical parameters of texture and correlation between the objective and sensory methods of texture evaluation. *Journal of Food Science, 28*, 397 - 403.

TA Instruments. (2015). Understanding Rheology of Structured Fluids. Retrieved 06/03/2018, 2018, from http://www.tainstruments.com/pdf/literature/AAN016_V1_U_StructFluids.pdf

Takeo, K., Tokumura, A., & Kuge, T. (1973). Complexes of starch and its related materials with organic compounds. *Starch - Stärke, 25*, 357-362.

Tester, R. F., Karkalas, J., & Qi, X. (2004). Starch—composition, fine structure and architecture. *Journal of Cereal Science, 39*, 151-165.

Tester, R. F., & Morrison, W. R. (1990). Swelling and gelatinization of cereal starches. I. Effects of amylopectin, amylose, and lipids. *American Association of Cereal Chemists, 67*, 551-557.

Tester, R. F., & Sommerville, M. D. (2003). The effects of non-starch polysaccharides on the extent of gelatinisation, swelling and α-amylase hydrolysis of maize and wheat starches. *Food Hydrocolloids, 17*, 41-54.

Uthumporn, U., Zaidul, I. S. M., & Karim, A. A. (2010). Hydrolysis of granular starch at sub-gelatinization temperature using a mixture of amylolytic enzymes. *Food and Bioproducts Processing, 88*, 47-54.

Vaclavik, V. A., & Christian, E. W. (2008). *Essentials of Food Science* (3rd ed.). USA: Springer.

van de Velde, F., Weinbreck, F., Edelman, M. W., van der Linden, E., & Tromp, R. H. (2003). Visualisation of biopolymer mixtures using confocal scanning laser microscopy (CSLM) and covalent labelling techniques. *Colloids and Surfaces B: Biointerfaces, 31*, 159-168.

Van Hung, P., Maeda, T., & Morita, N. (2006). Waxy and high-amylose wheat starches and flours—characteristics, functionality and application. *Trends in Food Science & Technology, 17*, 448 - 456.

Vaugelade, P., Hoebler, C., Bernard, F., Guillon, F., Lahaye, M., Duee, P., & Darcy-Vrillon, B. (2000). Non-starch polysaccharides extracted from seaweed can modulate intestinal absoprtion of glucose and insulin response in the pig. *Reproduction Nutrition Development, 40*, 33 - 47.

Villemejane, C., Denis, S., Marsset-Baglieri, A., Alric, M., Aymard, P., & Michon, C. (2016). In vitro digestion of short-dough biscuits enriched in proteins and/or fibres using a multi-compartmental and dynamic system (2): Protein and starch hydrolyses. *Food Chemistry, 190*, 164 - 172.

Voragen, A. G. J., Coenen, G.-J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry, 20*, 263 - 275.

Voragen, A. G. J., Pilnik, W., Thibault, J. F., Axelos, M. A. V., & Renard, C. M. G. C. (1995). Pectins. In A. M. Stephens (Ed.), *Food polysaccharides and their applications* (pp. 287 - 340). USA: Marcel Dekker.

Waigh, T. A., Gidley, M. J., Komanshek, B. U., & Donald, A. M. (2000). The phase transformations in starch during gelatinisation: A liquid crystalline approach. *Carbohydrate Research, 328*, 165-176.

Wang, S., Blazek, J., Gilbert, E., & Copeland, L. (2012). New insights on the mechanism of acid degradation of pea starch. *Carbohydrate Polymers, 87*, 1941-1949.

Wang, S., & Copeland, L. (2013a). Effect of acid hydrolysis on starch structure and functionality: A review. *Critical Reviews in Food Science and Nutrition*, 130925130049008.

Wang, S., & Copeland, L. (2013b). Molecular disassembly of starch granules during gelatinization and its effect on starch digestibility: a review. *Food & Function, 4*, 1564-1580.

Wang, S., Li, C., Copeland, L., Niu, Q., & Wang, S. (2015). Starch retrogradation: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety, 14*, 568 - 585.

Wang, Y., Yang, Z., & Wei, X. (2010). Sugar compositions, α-glucosidase inhibitory and amylase inhibitory activities of polysaccharides from leaves and flowers of *Camellia sinensis* obtained by different extraction methods. *International Journal of Biological Macromolecules, 47*, 534-539.

Wangsakan, A., McClements, D. J., Chinachoti, P., & Dickinson, L. C. (2004). Two-dimensional rotating-frame Overhauser spectroscopy (ROESY) and (13)C NMR study of the interactions between maltodextrin and an anionic surfactant. *Carbohydrate Research, 339*, 1105 - 1111.

Warren, F. J., Zhang, B., Waltzer, G., Gidley, M. J., & Dhital, S. (2015). The interplay of alpha-amylase and amyloglucosidase activities on the digestion of starch in in vitro enzymic systems. *Carbohydrate Polymers, 117*, 192 - 200.

Wilson, J. D., Bechtel, D. B., Todd, T. C., & Seib, P. A. (2006). Measurement of wheat starch granule size distribution using image analysis and laser diffraction technology. *Cereal Chemistry, 83*, 259-268.

Woolnough, J. W., Monro, J. A., Brennan, C. S., & Bird, A. R. (2008). Simulating human carbohydrate digestionin vitro: a review of methods and the need for standardisation. *International Journal of Food Science & Technology, 43*, 2245-2256.

Yeh, A. I., & Li, J. Y. (1996). A continuous measurement of swelling of rice starch during heating. *Journal of Cereal Science, 23*, 277 - 283.

Yoshimura, M., Takaya, T., & Nishinari, K. (1996). Effects of konjac-glucomannan on the gelatinization and retrogradation of corn starch as determined by rheology and differential scanning calorimetry. *Journal of Agriculture and Food Chemistry, 44*, 2970 - 2976.

Yuris, A., Goh, K. K. T., Hardacre, A. K., & Matia-Merino, L. (2017). Understanding the interaction between wheat starch and Mesona chinensis polysaccharide. *LWT - Food Science and Technology, 84*, 212 - 221.

Yuris, A., Matia-Merino, L., Hardacre, A. K., & Goh, K. K. T. (In submission). The effect of gel structure on the in vitro digestibility of wheat starch-Mesona chinensis polysaccharide gels. *Food & Function*.

Yuris, A., Matia-Merino, L., Hardacre, A. K., Hindmarsh, J., & Goh, K. K. T. (2018). Molecular interactions in composite wheat starch-Mesona chinensis polysaccharide gels: Rheological, textural, microstructural and retrogradation properties. *Food Hydrocolloids, 79*, 1 - 12.

Zeeman, S. C., Kossmann, J., & Smith, A. M. (2010). Starch: Its metabolism, evolution, and biotechnological modification in plants. *Annual Review of Plant Biology, 61*, 209 - 234.

Zhang, G., Venkatachalam, M., & Hamaker, B. R. (2006). Structural basis for the slow digestion property of native cereal starches. *Biomacromolecules, 7*, 3259-3266.

Zhang, X., Do, M. D., Hoobin, P., & Burgar, I. (2006). The phase composition and molecular motions of plasticized wheat gluten-based biodegradable polymer materials studied by solid-state NMR spectroscopy. *Polymer, 47*, 5888 - 5896.

Zhou, Y., Wang, D., Zhang, L., Du, X., & Zhou, X. (2008). Effect of polysaccharides on gelatinization and retrogradation of wheat starch. *Food Hydrocolloids, 22*, 505 - 512.

Zhuang, H., Feng, T., Xie, Z., Toure, A., Xu, X., Jin, Z., & Su, Q. (2010). Effect of *Mesona Blumes* gum on physicochemical and sensory characteristics of rice extrudates. *International Journal of Food Science & Technology, 45*, 2415-2424.

Appendices

Appendix A: Supplementary material for Chapter 5

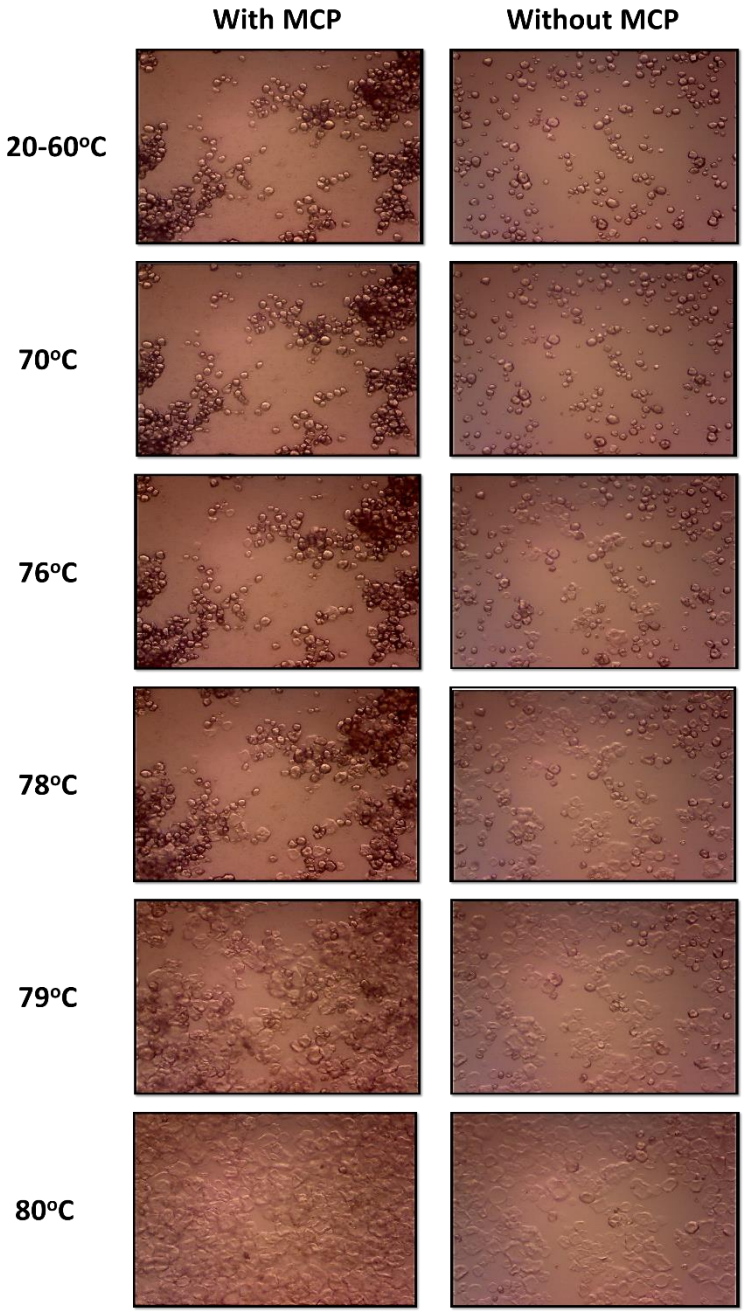


Figure A-1 Micrographs of 1.5 % w/w maize starch with and without 3.5 % w/w MCP at different temperatures (Micrographs credited to Ramesh Krish Kumar).

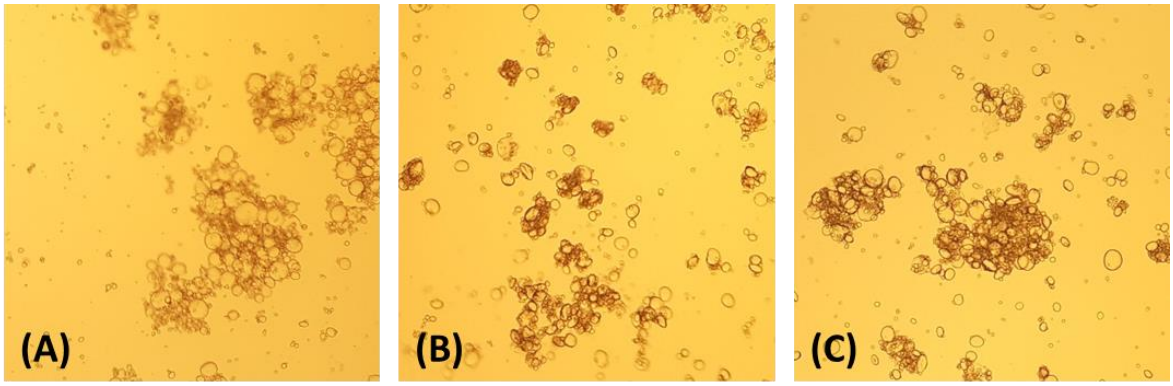


Figure A-2 Micrographs of 1 % w/w wheat starch containing 0.05 % w/w (A), 0.01 % w/w (B) and 0.005 % w/w (C) MCP.

Appendix B: Supplementary material for Chapter 6

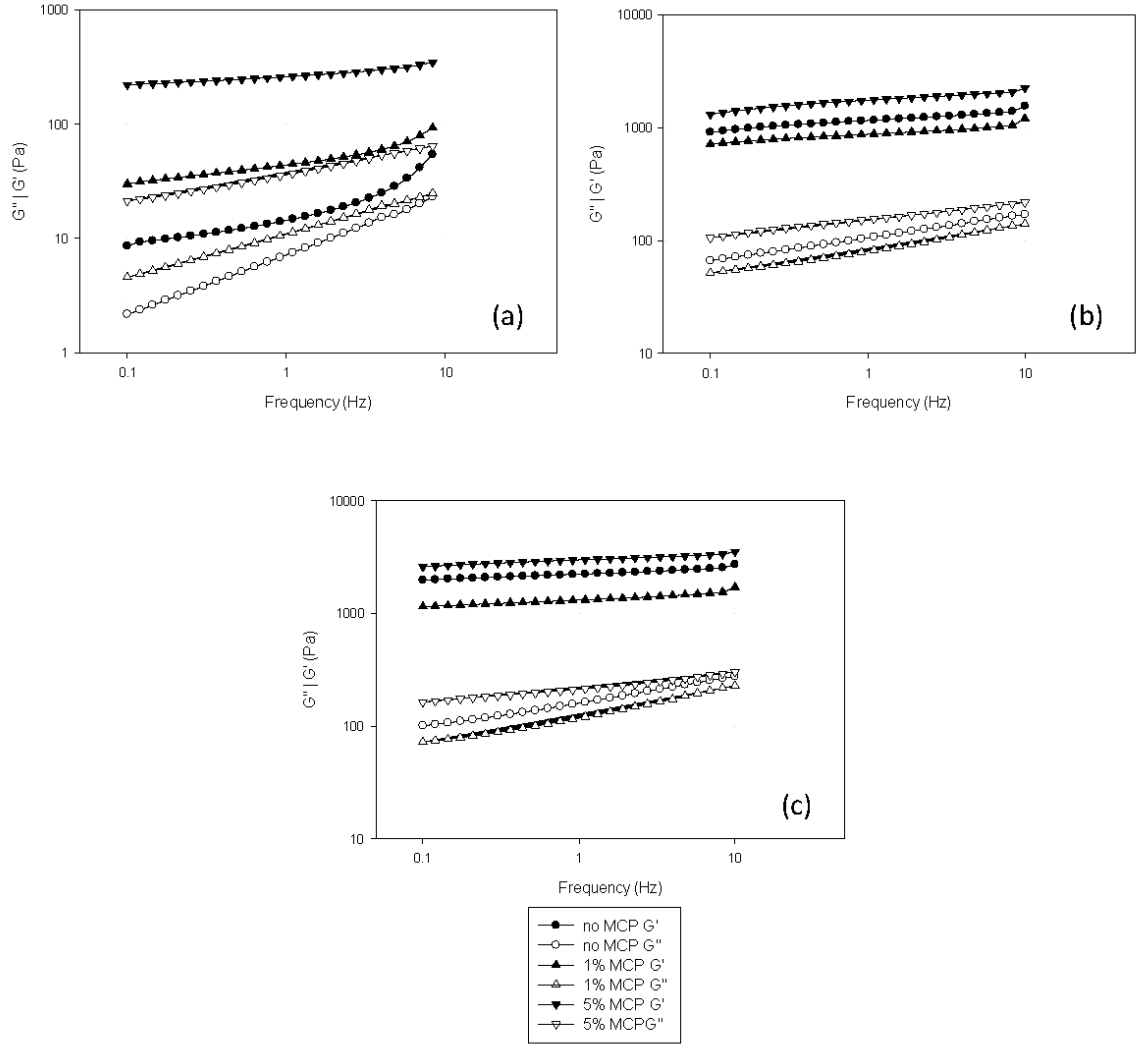


Figure B-1 Frequency sweep of 5 % (a), 8 % (b) and 10 % (c) w/w wheat starch in the presence of 0 – 5 % w/w MCP.

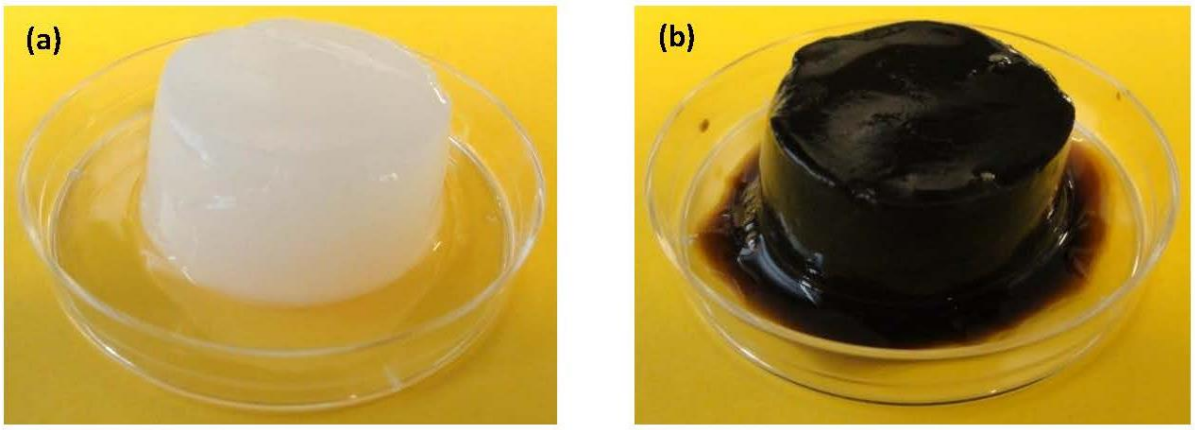


Figure B-2 5 % wheat starch gels in the absence (a) and presence (b) of 5 % MCP after storage at 20°C for 24h.

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We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Anynda Yuris

Name/Title of Principal Supervisor: Lara Matia-Merino/Senior lecturer

Name of Published Research Output and full reference:

Yuris, A., Matia-Merino, L., Hardacre, A. K., Hindmarsh, J., & Goh, K. K. T. (2018). Molecular interactions in composite wheat starch-Mesona chinensis polysaccharide gels: Rheological, textural, microstructural and retrogradation properties. Food Hydrocolloids, 79, 1 - 12.

In which Chapter is the Published Work: Chapter 4 and 6

Please indicate either:

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  and / or

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  The candidate has carried out the majority of the experimental work (with the exception of NMR). Input on the draft manuscript was provided by the supervisors.

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Name/Title of Principal Supervisor: Lara Matia-Merino/Senior lecturer

Name of Published Research Output and full reference:

Yuris, A., Goh, K. K. T., Hardacre, A. K., & Matia-Merino, L. (2017). Understanding the interaction between wheat starch and Mesona chinensis polysaccharide. LWT - Food Science and Technology, 84, 212 - 221.

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  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The experimental work and writing were carried out by the candidate with input from the supervisors on the draft manuscript

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