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Development of Food Ingredients for Modulation of Glycemia

Thesis presented by

Anthony Paul Kett, B.Sc.

for the degree of

Doctor of Philosophy

in

Food and Nutritional Sciences
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Declaration

The research presented in this thesis was performed at Teagasc Food Research Centre and the Pig Development Unit, Moorepark, Fermoy, Co. Cork and at University College Cork, unless otherwise stated. The work has not been submitted to any other University or higher education institute or for any other academic award within this University.

__________________________  __________
Anthony P. Kett            Date
Abstract

Starches are a source of digestible carbohydrate and are frequently used in formulated food products in the presence of other carbohydrates, proteins and fat. This thesis explored the effect of addition of neutral (Konjac glucomannan) or charged (milk proteins) polymers on the physical characteristics and digestion kinetics of waxy maize starch. The aim was to identify mechanisms to modulate the pasting properties and subsequent susceptibility to amylolytic digestion. Addition of $\alpha_s$- or $\beta$-caseinate protein fractions to waxy maize starch restricted granular swelling during gelatinisation, increasing granule integrity. It was shown that, while $\beta$-caseinate can adsorb to starch granules during pasting, $\alpha_s$-caseinate can be absorbed into maize starch granules. The resultant effect was a reduction in granule size after heating, more intact granules and a subsequent decrease in starch digestion in vitro as determined by analysis of reducing sugars. The ability of $\alpha_s$-caseinate to reduce the level of amylolytic digestion was confirmed through in vivo pig (Teagasc, Moorepark) and human (University of Surrey, UK) trials. The scope of the thesis extended to the development of a new automated cell for attachment to a rheometer to measure digestion kinetics of starch-protein mixtures.

In conclusion, the thesis offers new approaches to modulation of the physical characteristics of unmodified starch during gelatinisation and suggests that the type of protein and/or polysaccharide used in starch-based food systems may influence the ability of the food to modulate glycemia. This is an important consideration in the design of foods with positive health benefits.
Chapter 1

Literature Review
1. Literature Review

1.1 Starch structure

Pure starch consists predominantly of α-glucan in the form of amylose and amylopectin (Figure 1). Amylose is a roughly linear molecule containing ~99% α- (1-4) and ~1% α- (1-6) glycosidic bonds while amylopectin is a much larger branched molecule with ~95% α- (1-4) and ~5% α- (1-6) glycosidic bonds. Each amylose chain is of the order of 1000 glucose units in length whereas amylopectin ranges from ~12 to 120 anhydroglucose units. Starches can be classified according to their amylose to amylopectin ratio and this ratio characterises the starch in relation to digestibility, rheological properties, thermodynamic properties and structure formation (gelatinisation and retrogradation). Starches are defined as waxy when the ratio of amylose to amylopectin is low (<10%), normal when amylose represents ~10-35% and high amylose when the ratio of amylose to amylopectin exceeds 36% (Tester, Karkalas and Qi, 2004). Native starch is a semi-crystalline polysaccharide synthesised as roughly spherical granules in many plant tissues including pollen, leaves, stems, roots, tubers, fruits and seeds. Commercial starch is extracted from a variety of sources with maize being the predominant source. Other sources include wheat, rice, potato, tapioca, sago, barley, oats and yam to mention but a few.

No other single food ingredient compares with starch in relation to versatility of application in the food industry. Second only to cellulose in natural abundance, this polymeric carbohydrate was designed by nature as a plant energy reserve (Taggart, 2004). Starches have been adapted to create competitive advantage in new products, enhance product viability, reduce production costs, increase product throughput, extend product shelf life and ensure product consistency.
1. Literature Review

1.1.1 Starch gelatinisation

Researchers have tried to define accurately the term starch gelatinisation for many years. Gelatinisation and retrogradation are two extremely important physical phenomena that starch undergoes when heated and cooled, respectively, in excess water. Gelatinisation is a thermal process that disrupts the molecular order of starch, resulting in granule rupture. Some researchers claim that gelatinisation starts when granular birefringence is lost, others believe it is
initiated when an increase in viscosity occurs. A definition of starch gelatinisation emanating from the Starch Science and Technology Conference in 1988 proposed that:

“Starch gelatinisation is the collapse (disruption) of molecular orders within the starch granule manifested in irreversible changes in properties such as granular swelling, native crystalline melting, loss of birefringence and starch solubilisation.” The point of initial gelatinisation and the range over which it occurs is governed by starch concentration, method of observation, granular type and heterogeneities within the starch granule population under observation (Atwell et al., 1988).

Granular starch is insoluble in cold water and even when added to water at ambient temperature there is no difference in granule morphology until heat is applied. The combination of heat and water, however, causes uncooked starch granules to undergo unique and irreversible changes altering granular structure by (i) disrupting the semi-crystalline structure as evidenced by a loss of birefringence (maltese cross pattern observed under light microscope) and (ii) an increase in granule size. It is important to note, not all starch granules swell at the same rate or to the same extent, and as a result do not dissolve completely resulting in granule “ghost” integrity (Debet and Gidley, 2007). Heating of starch in water causes disruption of the hydrogen bonds between polymer chains, thereby weakening the starch granules. It is postulated that the initial swelling takes place in the amorphous regions of the granule where hydrogen bonds are less numerous and the polymers are more susceptible to dissolution. When peak viscosity is reached on heating, the process is considered to be ‘cooked out’ creating a paste. The process of pasting follows gelatinisation, whereby,
continued heating of starch granules in the presence of excess water results in continued granule swelling. Additional leaching of dissolved starch polymer molecules and disruption of the fragile swollen starch granules results in a viscoelastic mass, known as a paste, consisting of a continuous phase that is a molecular dispersion of once dissolved starch polymer molecules that now form a network, and a discontinuous phase of swollen granules, granule ghosts and granule fragments (Ratnayake and Jackson, 2009).

Starch pastes in general are thixotropic and can follow a shear thinning pattern (Nguyen, Jensen and Kristensen, 1998; Kett et al., 2009). On cooling, starch gels can still be metastable (Slade and Levine, 1987; Biliaderis and Zawadzki, 1990) and, as a result, undergo further chain aggregation and partial crystallisation upon storage (Morris, 1990).

Amylose/amylopectin polymers and remaining insoluble granular fragments reassociate on cooling. This reassociation is called retrogradation and is responsible for structure formation of the starch and increases in final viscosity, depending on the source. The retrogradation rate and extent are mainly affected by the molecular and crystalline structure of the starch, by storage conditions, i.e., temperature, time and water content (Park, Baik and Lim, 2009). Amylopectin contributes to the retrogradation occurring in long term rheological and structural changes, while amylose may be responsible for the short term, rapid changes in a food mixture (Sathaporn and Jane, 2007). Upon cooling, less energy is available to keep the solubilised starch molecules apart. Retrogradation results in the formation of crystalline aggregates. Although amylopectin molecules also retrograde upon cooling, linear amylose molecules have a greater
tendency to reassociate and form hydrogen bonds than larger amylopectin molecules.

Gelatinisation involves different loss-of-order transitions and because granules in a population from a single source are heterogeneous with regards to imperfections in crystallites (Ji et al., 2004), gelatinisation occurs over a temperature range. This gelatinisation (phase-transition) temperature range not only varies from granule to granule within a starch matrix, but also with varying botanical sources (Fredriksson et al., 1998; Ji et al., 2004).

In most cases, the viscous paste produced after the gelatinisation process makes starch a very functional food ingredient. The degree of gelatinisation also has a major impact on the digestibility and rheological properties of a starch; the greater the extent of gelatinisation of starch the easier it is for digestive enzymes (e.g., amylase) to degrade enzymatically the starch to its reducing sugars (Holm et al., 1988; Tester et al., 2004).

1.1.2 Starch retrogradation

On heating, starch undergoes irreversible swelling and even complete disruption of the starch granules depending on the severity of heat applied and the duration. The behaviour of gelatinised starches on cooling, i.e., the realignment of amylose and amylopectin polymers, and storage, generally termed retrogradation is of great importance to food scientists as many have found that it affects quality and shelf-life stability of many starch containing foods (Biliaderis, 1991).

During the gelatinisation of starch, the crystalline structure of amylopectin disintegrates and the polysaccharide chains take up a random configuration thus
causing the swelling and rupturing of the starch granules (Singh et al., 2007). Upon cooling of the heated starch, re-crystallisation of the starch chains starts occurring. The aggregation and realignment of amylose is almost instantaneous and has been reported to be complete within the first few hours after pasting while aggregation and crystallisation of amylopectin occur at later stages (Singh, McCarthy and Singh, 2006; Singh et al., 2008). Amylopectin recrystallises at a much slower rate than that at which amylose forms its single or double helices, allowing for the formation of aggregates (Baik et al., 1997). Consequently, the duration of the first stage of retrogradation depends on the amylose content of the starch. The linear chains of amylose facilitate the cross-linkages through hydrogen bonds whereas the branched chains of amylopectin delay recrystallisation. The retrogradation properties of starches are also influenced by the structural arrangement of starch chains within the amorphous and crystalline regions of the un-gelatinised granule. The structural arrangement influences the extent of granule breakdown during gelatinisation and also influences the interactions that occur between starch chains during storage (Kaur et al., 2007).

In starches with high amylopectin content, i.e., waxy starches, amylopectin molecules can associate to form weak gels (Cameron, Durrani and Donald, 1994; Durrani and Donald, 1995). The chain lengths affect the rate of retrogradation of amylopectin molecules, with longer chain lengths resulting in faster retrogradation rates (Kalichevsky, Orford and Ring, 1990). There is a general consensus from studies in the past that starch retrogradation contributes significantly to staling and firming in bread and other starch based products (D'appolonia and Morad, 1981; Kulp and Ponte, 1981; Iten, Escher and Petit, 2003).
The retrogradation properties of starch can also be beneficial in modifying the structural properties of certain starch based foods. In the production of breakfast cereals and parboiled rice, retrogradation results in hardening and reduced stickiness (Colonna, Leloup and Buléon, 1992). Many methods for the study of starch retrogradation have been developed, i.e., rheological (measurement of pasting properties, oscillatory, creep and stress recovery tests), thermal analysis (differential scanning calorimetry) and spectroscopic (nuclear magnetic resonance, infra-red).

An early indicator of starch retrogradation is an increased formation of resistant starch, i.e., an increased resistance of the starch to hydrolysis by acid or amylolytic enzymes i.e., α- and β-amylase and amyloglucosidase (Matsukura, Matsunaga and Kainuma, 1983; Berry, 1986; Rosin, Lajolo and Menezes, 2002). Higher values for indices of retrogradation such as higher melting temperatures, higher melting enthalpies and higher crystalline activity, of stored (48 h at ambient temperature) gelatinised waxy maize starch increased resistance to enzymatic hydrolysis of α-amylase and amyloglucosidase (Eerlingen, Jacobs and Delcour, 1994).

1.1.3 Modified and resistant starches

Modified starches have become a vital ingredient in the food industry to maximise functionality in final food products. Starch is one of the most abundant natural food resources available to the food industry. Modified starches provide functional attributes such as thickening power, they can create a smooth texture (extremely important for mouthfeel), imbibe or inhibit moisture, can be used in the development of a soft or crisp coating and stabilise an emulsion, an attribute
of paramount importance to some food industries. Modified starches can provide all these attributes that most native starches cannot. Gelatinising a 5% (w/w) native waxy maize starch solution results in a weak bodied, cohesive paste (Kett et al., 2009) not suitable for imparting structure but can provide stability to food matrices. Starch modification can improve tolerance to rigorous processing conditions such as heat and shear, provide desirable texture and more importantly prolong stability. Modified starches are important functional ingredients in a number of food systems providing applications in the area of adhesion, antistaling, binding, emulsion stabilisation, encapsulation, gelling, moisture retention, stabilising and thickening. There are many different methods applied for starch modification which include physical modification (pregelatinisation and hybridisation) and chemical modification (conversion, crosslinking and stabilisation).

1.1.3.1 Hybridisation

Hybridisation is a process whereby corn is selectively bred to yield differing ratios of amylose to amylopectin. High amylose starches have unique properties for gelling and film forming and can be used to provide structure (confectionery industry) and also reduce oil pick-up in fried foods. Waxy maize starches, composed almost entirely of amylopectin (Tester et al., 2004) provide stability due to the branched nature of the polymer. Waxy maize is useful in a wide range of applications in the food industry due to its stabilising attribute but can also retard the on-set of syneresis, something high amylose starches are prone to over a long shelf life (Hermansson and Svegmark, 1996).
1. Literature Review

1.1.3.2 Physical modification

Starch can be physically modified using a number of techniques to provide desirable properties. Pregelatinisation is an extremely popular method in the food industry to impart quick viscosity development in instant systems (Taggart, 2004). Pregelatinisation and subsequent drying leads to cold water swelling starches. Heat is not needed to solubilise the starch granules. Cold water swelling starches are used in more process tolerant food products where microwaves are needed.

1.1.3.3 Conversion

Conversion is a process that is used to reduce the viscosity of native starches. The process allows for higher concentrations of starch to be used, increases water solubility, controls gel strength and modify the stability of the starch. Generally, native starch cannot be used >6% solids as it imparts a high viscosity. However, in the confectionery industry in a product such as a soft gum candy, a low-viscosity starch at high solids is needed to obtain the desired gel strength. This is achievable using a converted starch. Methods of conversion include acid hydrolysis, oxidation, dextrinisation and enzyme conversion (Taggart, 2004).

1.1.3.4 Crosslinking

Crosslinking yields starch granules with increased resistance to overcooking and other variations in processing conditions. Crosslinking is a process whereby small amounts of compounds that can react with more than one hydroxyl group are added to the starch polymers to form covalent bonds which can increase resistance to heat, shear, temperature and acid.
1.1.3.5 Stabilisation

Stabilisation involves blocking groups reacting with starch polymers to inhibit retrogradation (Section 1.1.2), the alignment of polymers that causes a change in the structure of a food product. Inhibiting retrogradation imparts textural and freeze thaw stability prolonging the shelf life of the food product. Stabilisation is important in frozen foods due to the accelerated retrogradation of starch polymers at cold temperatures which can eventually lead to syneresis (Karim, Norziah and Seow, 2000).

1.1.4 Applications of modified starch products

Modified starches can be used in dry mixes, confectionaries, low-calorie foods, emulsion stabilisation and encapsulation.

1.1.4.1 Dry mixes

Dry mix foods include both cook-up and instant food products. An example of a modified starch in a dry mix cook-up product would be cheese sauce or white sauce whereby the starch is able to tolerate the heat applied in the cooking step, finally adding the desired texture after a period of cooking.

1.1.4.2 Emulsion stabilisation

Reactions of certain lipophilic substances with starch polymers impart a slightly hydrophobic character to the starch resulting in a product that can be used for emulsion stabilisation. The objective of the starch is to provide viscosity and stability. In liquid emulsions, e.g., beverage emulsions for the drinks industry, a concentrated flavour emulsion is used to prepare soft drinks. In the preparation,
an emulsion of a particular flavour is added to a solution of lipophilic starch that also contains citric acid, sodium benzoate and colour. The emulsion is then homogenised to obtain a desired particle size. A known amount of this emulsion is then used to flavour a finished beverage that contains mostly sugar and water. In a liquid system like this, emulsion stability is extremely important as the flavour can separate over time (Taggart, 2004).

1.1.4.3 Confectionaries

Modified starches are used for a number of applications in the confectionery industry. Two popular areas of starch based sweets are hard gums and soft jellies. In the case of soft jelly gums, the retrogradation properties of the starch are utilised to give a desired texture that includes chewy bites to soft clean bites. Gum drops and jelly centres are popular soft jellies, other include lozenges, chewy cough drops and wine gums.

1.1.4.4 Low-calorie foods

Modified starches can be used to replace super sweeteners such as sucrose reducing the calorie intake but still imparting the desired sweetness level sugar provides. Modified starches can be used as fat replacers (Tharanthan, 2005). Fat can be replaced with resistant starches that are not digested in the gastrointestinal tract. A special maltodextrin, (Instant N-Oil II, National Starch, U.K) when dissolved in water results in a gel like consistency with fat-like textural characteristics. One gram of this special maltodextrin gel at 25% solids provides one calorie compared to nine calories for the equivalent weight of fat (Atwell et al., 1988).
1. Literature Review

1.1.5 Starch digestibility

The physical state of starch has a major impact on its digestibility. Factors such as granule morphology, amylose to amylopectin ratio, molecular structure and degree of branching all have an effect on digestibility and subsequent glycemic responses on blood sugar levels. Predicting and controlling the glucose absorption following starch digestion is of great interest in the context of worldwide health. The majority of starches contain portions that digest rapidly, digest slowly and that are resistant to digestion. Resistant starch is not hydrolysed by the enzymes in the small intestine and passes into the large intestine (Englyst et al. 1999). The three classifications of starch are determined by a widely used in vitro digestion method which simulates the conditions of the stomach and intestine to measure glucose release at different stages of digestion over time (Englyst, Kingman and Cummings, 1992). Based on this method, starch fractions are defined as either: rapidly digested starch (RDS), i.e., concentration of glucose liberated after 20 min, slowly digested starch (SDS), i.e., amount of glucose liberated between 20 and 120 min and resistant starch (RS), i.e., the difference between total starch and the amount of glucose liberated after 120 min of in vitro digestion.

Physico-chemical and structural characteristics of starch vary among different botanical origins (Singh et al., 2007). Modified starches (i.e., pre-gelatinised, cross-linked, acid/enzymatic hydrolysis) are commonly used in the food industry for their improved functional properties and previous studies have reported that chemically modified starches exhibit greater resistance towards intestinal enzymes responsible for starch degradation, i.e., α-amylase (Han and BeMiller, 2007). Digestible starch is converted to maltose and maltotriose in the duodenal
cavity by secreted pancreatic α-amylase and converted to glucose by brush border enzymes in the intestinal lumen. Salivary amylase plays a minor role in the first stage of the conversion during mastication of the food but salivary amylase is rapidly degraded in the acidic environment of the stomach. Most of the starch hydrolysis is performed by pancreatic amylase released in the small intestine via the pancreatic duct. α-amylase catalyses the hydrolysis of α-(1-4) glycosidic bonds in amylase and amylpectin (Lehmann and Robin, 2007). Amylose and amylpectin polymers are hydrolysed by virtue of binding of their five glucose residues adjacent to the terminal reducing glucose unit to specific catalytic subsites of α-amylase, followed by cleavage between the second and third α-1-4 linked glucosyl residues (Gray, 1992). The final hydrolysis products from starch digestion are mainly the disaccharide, maltose and the trisaccharide, maltotriose. However, some microbial sources of α-amylase may produce other forms, i.e., maltohexose and maltoheptose as well as maltotriose (Yook and Robyt, 2002). It is well established that α-amylase has no affinity for α-1-6 glucosidic linkages in amylpectin and only has specificity for α-1-4 glucosidic linkages (Tester et al., 2004). The resulting oligosaccharides (maltose, maltotriose and α-dextrins) are further hydrolysed efficiently into glucose by the action of brush border enzymes in the intestine. Absorptive epithelial cells which line the intestinal villi produce a number of these brush border enzymes that allow for digestion of oligosaccharides and disaccharides (Tester et al., 2004). Brush border enzymes include sucrase, an enzyme that converts sucrose to glucose and fructose, and lactase, an enzyme that converts lactose to glucose and galactose but neither of these enzymes are relevant to starch hydrolysis. However, maltase (which converts maltose to glucose from α-amylase activity)
and isomaltase (which hydrolyses the $\alpha$-1-6 bonds of isomaltose and $\alpha$-dextrins) continue the digestive process of starch with glucose as an end product.

Structural differences among the various types of starch, such as degree of branching and molecular weight may influence the rheological characteristics of their solutions which may affect the rate of starch hydrolysis. It has been reported that the hydrolysis kinetics of glucoamylase on two types of starches differed due to differences in the apparent viscosity of the starch dispersions. In one of the starches, the hydrolysis kinetics corresponded to Michaelis-Menten behaviour while the hydrolysis rate for the other starch increased with the concentration up to a maximum and then decreased once a critical concentration was surpassed (Sanromán, Murado and Lema, 1996). A higher degree of branching allows an increase in the number of available sites for enzymatic attack (Sanromán et al., 1996). Branching may increase the steric hindrance and, consequently the mass transfer resistance. These together may explain the behaviour of starches at higher concentrations when the positive effect of branching is generally overcome by the diffusional restrictions towards the enzymatic action due to higher viscosity. The molecular weight distribution of the starches may also affect the enzymatic hydrolysis; a higher molecular weight can result in an increased hindrance towards active centres of the enzyme.

The surface characteristics of starch granules have been observed to affect their enzymatic digestion. A study of the enzymatic digestibility of native uncooked starches from different sources reported that cereal starches had higher digestibility compared to tuber or legume starches (Dreher, Dreher and Berry, 1984). This may be attributed to the presence of numerous pin-holes on the surface layer and pores that penetrate towards the interior of the starch granules
from cereal sources such as waxy maize starch (the starch studied in this thesis). The pores on the surface of the starch granules may facilitate easier access to digestive enzymes (e.g., α-amylase). This type of hydrolytic attack may be termed endo-corrosion (Dhital et al. 2010)

Figure 1.2: Surface pores, channels and cavities illustrating possible diffusion of digestive enzymes, (A) maize starch showing pores, channels and cavities, (B) maize starch hydrolysed by amylase with enlarged pores and channels, (C) potato starch lacking surface pores, channels and cavities and (D) potato starch showing evidence of exo- and endo-corrosion by amylase (Dhital, Shretha and Gidley, 2010)
Native or uncooked potato starch granules, due to their smooth surface and the absence of any pores, have been observed to have high resistance towards enzymatic hydrolysis and are digested less rapidly compared to native maize starch (Dhital et al., 2010). The presence of proteins on the granule surface may also limit the rate of enzymatic hydrolysis. Granule surface proteins and lipids can reduce surface accessibility of enzymes by blocking adsorption sites influencing enzyme access and binding (Oates, 1997).

Amylose content of starch granules varies with botanical source which may attribute to variances in enzyme activity involved in the biosynthesis of the linear and branched chains. Amylose content varies from 14 to 31% for normal starches, up to 70% for high amylose starches and waxy starches are almost devoid of amylose (Hoover and Zhou, 2003). Legumes contain 30-40% amylose and 60-70% amylopectin in their starch granules, while most other food starches contain 25-30% amylose and 70-75% amylopectin (Hoover and Zhou, 2003). It has been reported that legumes (high amylose content) are less digestible than corn starch (high amylopectin content) in rats (Thorne, Thompson and Jenkins, 1983). In addition, legumes contain twice as much protein as cereals and anti-nutrients which may affect starch digestibility. Moreover, the glucose chains of amylose are strengthened by hydrogen bonds making them less susceptible to amylolytic attack compared to the branched chains of amylopectin (Thorne et al., 1983). The physical nature of starch in carbohydrate containing foods is important to consider for digestibility and subsequent glycemic response.
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1.1.5.1 Food processing and starch digestibility

Processing leads to an alteration in food structure but in particular the granular integrity of starch granules. This alteration subsequently affects the nutritional properties of starch containing foods including digestibility of starch. The principal process facilitating starch availability for water penetration and subsequent $\alpha$-amylase action involves physical processing by means of heating the starch (>90°C) for a defined period of time. This physical ‘cooking’ increases the hydrolysis rate of the starch as a consequence of gelatinisation allowing easier access for digestive amylases. Extrusion cooking significantly increases the in vitro digestibility of starches (Alonso, Aguirre and Marzo, 2000; Altan, McCarthy and Maskan, 2009). The increase in digestibility of the starch may be explained by the physical disruption of the starch granules structural integrity by increased shearing and kneading in the extruder barrel subsequently increasing the starches susceptibility towards amylolytic breakdown. It has been reported that extrusion decreased the particle size of a range of starches (maize, wheat, barley, oats and peas) compared to the raw samples and thus affecting digestibility (Anguita et al. 2006). Processing of cereals such as dehulling, soaking and germination may result in an enhancement of digestibility due to the loss of phytic acid, tannins and polyphenols which can inhibit $\alpha$-amylase activity decreasing starch digestibility. It has been suggested that the removal of tannins and phytic acid creates a large space within the starch matrix, increasing susceptibility to enzymatic attack (Rehman and Shah, 2005).

The increase in the sheeting cycles of pasta dough has been reported to enhance the in vitro starch digestibility due to pulling away of protein from the starch granules (Kim et al., 2008). The reduction in cohesiveness between the starch
and proteins in the dough may increase accessibility of $\alpha$-amylase resulting in greater digestibility.

### 1.1.5.2 Viscosity and starch digestibility

Several factors affect the rate of starch digestibility and the viscosity of a food matrix is no exception. The viscosity and physical texture of food may affect starch digestion and absorption of hydrolysis products. Polysaccharide based gums are classified as water-soluble non starch polysaccharides (NSPs) and their effects on human metabolism are reported to be beneficial because they decrease postprandial glycemia following ingestion of starchy food (Kaur and Singh, 2009). A study into the effect of guar gum addition at different concentrations to meals of growing pigs resulted in a significant reduction in the rate of glucose absorption (Ellis et al. 1995). Postprandial, the guar gum increased the viscosity of the jejuna digesta within the gastrointestinal tract, due to the enlargement of fully hydrated galactomannan chains. This subsequent increase in viscosity reduced the rate of digestion and absorption of carbohydrates therefore lowering the post-prandial rise in blood glucose levels. The presence of galactomannans in starch mixtures restricts swelling of the granules during gelatinisation which results in a reduction in starch granule remnant size in the starch paste. Moreover, some of the starch granules may not gelatinise properly due to limited water availability, as a result of water competition between the starch and galactomannan, increasing resistance to enzymatic hydrolysis (Kaur et al., 2008). Gums contribute by decreasing water activity during hydrothermal processing of starch subsequently influencing starch swelling and the activity of $\alpha$-amylase. Chaisawang and Suphantharika (2005) demonstrated that xanthan gum inhibited
swelling of tapioca starch and prevented amylose leach out and also reported on strong electrostatic interactions between cationic starch (tapioca) and anionic gum (xanthan) resulting in instantaneous aggregation of starch granules. The addition of sodium alginate to rice dough decreased starch hydrolysis during in vitro digestion (Koh et al. 2009). Alginate forms a continuous network by suspending the starch granules in a coherent gel that acts as a barrier during amylolytic breakdown from enzymes. Studies postulate that the high level of viscosity retards many of the physiological processes associated with the digestion of food and absorption of nutrients subsequently aiding in the management and control of obesity and glucose absorption.

1.1.5.3 Lipids and starch digestibility

The effect of free fatty acids (lauric, myristic, palmitic, stearic and oleic acids lysolecithin and cholesterol) on the hydrolysis of starch, amylose and amylopectin enzymatically digested by $\alpha$-amylase and amyloglucosidase were elucidated and findings show that 60% of amylose was converted to glucose in 1 h, reaching as far as 90% after 6 h (Crowe, Seligman and Copeland, 2000). The addition of lauric, palmitic, myristic and oleic acids reduced the enzymatic hydrolysis of amylose by 35%. Lauric acid had no effect on the enzymatic breakdown of amylopectin, whereas the enzymatic cleavage of whole starch was inhibited (12%) by lauric acid (Crowe et al., 2000). These experiments suggest that only the hydrolysis of the amylose fraction of starch is affected by lauric acid. Complexes between fatty acids such as lauric acid and amylose can form rapidly under physiological conditions which contribute to the formation of resistant starch (Seligman et al., 1998). The formation of such complexes with
lipids may result in significant changes in the physical properties of the starch including decreased solubility, increased gelatinisation temperature, delayed retrogradation and further resistance to digestive enzymes.

1.1.5.4 Modified starch and digestibility

Another type of chemical modification to starch involves the introduction of functional groups into the starch molecule. This process increases the functional properties of the native starch and can profoundly impact on the gelatinisation and retrogradation behaviour of the starch (Singh et al., 2007). Modifications such as cross-linking and substitution can reduce the extent of enzymatic hydrolysis of modified waxy corn starch (Han et al., 2007). It has been reported that chemical modifications involving substitution and oxidation on maize starch contributes to increasing the resistant starch content whereas cross-linking does not affect digestibility of the starch to the same extent (Chung, Shin and Lim, 2008). These findings are not in agreement with another study on the in vitro digestibility of modified (cross-linked and hydroxypropylated) waxy and non-waxy rice starches which showed no significant changes in resistant starch content compared to the control (unmodified starch samples) (Hwang et al., 2009). The author did note that resistant starch assays may not be appropriate to measure the resistant starch content of chemically modified starches. Chemically modified starches are increasingly being used as fat replacers (Tharanthan, 2005) as they are only partially or digested or completely undigested in the food system therefore contributing zero calories to the food during gastrointestinal digestion. It has also been reported that the chemical modification of potato starch by acetylation improves the satiating, glycemic and insulinemic properties
of a test meal (Raben et al. 1997). Furthermore, phosphorylation of high amylose
(~70%) corn starch results in a resistant starch fraction that survives cooking, is
slowly digestible and may provide nutritional benefits for humans (Sang and
Seib, 2006). Modified starches used in food products presently contain only
small amounts of substituent groups and have been deemed safe as food
ingredients. The legislative approval for use of modified starches in processed
food products is still under debate but several tailor made starch derivatives with
multiple modifications are being prepared (Tharanthan, 2005).

1.2 Milk Proteins

Bovine milk has a protein content of ~3.5 wt %. This protein can be
divided into two categories: those precipitating upon adjustment of the milk to
pH 4.6, the caseins; and those that remain soluble at pH 4.6, the whey proteins.
Most (~80 %) of the protein is casein occurring in large (50 - 300 nm diameter),
approximately spherical, particles known as casein micelles. The main protein
constituents of the micelles are $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$- and $\kappa$- casein, with molecular
weights of, respectively, 23.6, 25.2, 24.0 and 19.0 kDa (Fox and McSweeney,
1998). All caseins have a large proportion (35 - 45 %) of non-polar
(hydrophobic) amino acids, but are solubilised by non-protein appendages. The
$\alpha_s$- and $\beta$-caseins have a high content of phosphate, attached by ester linkages to
serine residues of the protein chains. In the $\alpha$-caseins, the phosphoseryl groups
are clustered around the middle of the protein chain, in a hydrophilic region
which is flanked by hydrophobic sequences at the C-terminal and N-terminal
ends. The corresponding hydrophilic region in $\beta$-casein is at the N-terminal end
of the molecule, with a hydrophobic region at the C-terminal end. In $\kappa$-casein
this distribution is reversed, with a predominantly hydrophobic \(N\)-terminal region and a hydrophilic, charged, \(C\)-terminal region, which contains only one phosphate ester group, but is extensively glycosylated.

1.2.1 Caseins

Caseins have been described as rheomorphic proteins (Holt and Sawyer, 1993). A rheomorphic protein is one with an open conformation and therefore has a considerable degree of side chain, and possibly also backbone, conformational flexibility (De Kruif and Holt, 2003). Casein micelles (Figure 1.3) have a protein content of \(\sim94\%\); the remaining 6\% consists mainly of calcium and phosphate, with some magnesium and citrate. These small species are known collectively as "colloidal calcium phosphate" and stabilise the micelle structure by balancing the charge on the phosphate groups of casein. The \(\alpha_s\)- and \(\beta\)-caseins are clustered in the centre of the micelle, the \(\kappa\)-casein molecules lay predominantly at the surface, with the glycosylated \(C\)-terminal sequences protruding to form an expanded "hairy layer" which acts as a barrier to aggregation (Walstra, 1990; Holt and Horne, 1996). In cheese-making, the protruding \(C\)-terminal region, known as the \(\kappa\)-casein "macropetide", is removed by enzymatic cleavage by chymosin from rennet, with consequent association of casein micelles into a gel. In yoghurt, the charge on the macropetide is suppressed by reduction in pH, which causes collapse of the "hairy layer" and eliminates the steric, entropic and electrostatic barrier to agglomeration.

A typical bovine micelle contains \(10^4\) individual casein molecules in colloidal association with calcium phosphate and small quantities of magnesium, sodium, potassium and citrate (Holt, 1992). The proteins are held together by a mixture of
hydrophobic and electrostatic interactions that are intrinsic to the individual caseins and their interaction with calcium phosphate. Moreover, it has been reported that the presence of calcium phosphate is critical for maintaining micelle structure. In addition, hydrophobic associations between the individual components appear to be important for maintaining micellar integrity (Payens, 1965; Schmidt, 1982; Farrell and Thompson, 1988). Dalgleish et al. (1989) investigated the size of micellar fractions isolated by ultracentrifugation using protein and mineral analysis and light scattering measurements. The authors reported that the surface of the micelle contains equal amounts of $\alpha$- and $\kappa$-casein (as well as small amounts of $\beta$-casein), while the interior is comprised of equimolar amounts of $\alpha$- and $\beta$-casein (Dalgleish, Horne and Law, 1989).

At temperatures above ~8°C the caseins are insoluble at their isoelectric point of pH ~4.6. Sodium caseinate, the water-soluble form of casein most commonly used in food, is usually prepared by neutralisation of acid-precipitated casein with NaOH. Partial separation of the constituent caseinates can be achieved by a range of procedures, as described by Mulvihill and Ennis (2003). Storage of milk at low temperatures (4°C) can cause micelles to partially dissociate by releasing a proportion of their $\beta$-casein into the serum, which may suggest that $\beta$-casein is associated within the micelle by hydrophobic interactions (Dalgleish, 1997). The casein micelle (Figure 1.4) is also altered on heating (Rollema and Brinkhuis, 1989), when denaturation of the milk serum proteins looses their binding capacity to $\kappa$-casein of the micelles (Dalgleish, 1990).
The major caseins (αs1- and β-caseins) contain no cysteine and as a consequence lack the capacity to form inter- or intramolecular disulfide bonds. However, the minor caseins (αs2- and κ-casein) each contain two cysteine residues which are known to give rise to intermolecular bonds (Dalgleish, 1997). The two αs-caseins and β-casein are calcium (Ca)-sensitive caseins because they are precipitated relatively easily by millimolar concentrations of Ca$^{2+}$. In contrast, κ-casein is insensitive to Ca$^{2+}$, and if added in sufficient amount will protect the other caseins against precipitation by this ion (De Kruif et al., 2003).

All the caseins are hydrophobic proteins, containing many amino acids with non-polar side chains. More important is the distribution of these amino acids especially in β- and κ-casein. In β-casein most of the charge of the molecule, and the polar groups are contained in the first 40 amino acids from the N-terminal with the remainder of the molecule being strongly hydrophobic. Consequently,
this makes β-casein an amphipathic protein, i.e., having hydrophilic and hydrophobic ends (Dalgleish, 1997). Caseins are relatively flexible and form extended chains in solution. They demonstrate an ability to hold considerable amounts of water and can exhibit high viscosity compared to similar concentrations of whey proteins (Dalgleish, 1997) and also impart increased viscosity in the presence of other polysaccharides compared to whey proteins.

**Figure 1.4:** Field emission scanning electron micrograph of an individual casein micelle, scale bar 100 µm (Dalgleish, Spagnuolo and Goff, 2004).
1.2.1.1 $\alpha_{s1}$-Casein

$\alpha_{s1}$-casein is a polypeptide chain which usually contains 199 amino acid residues depending on the genetic variant (Swaisgood, 1982; Swaisgood, 2003). Bovine $\alpha_{s1}$-casein is highly phosphorylated existing as one major and one minor form containing 8 and 9 bound phosphate groups per mole of protein (Eigel et al., 1984). All the $\alpha_{s1}$-caseins contain multiple phosphorylation sites including one major site at residues 100-110. Phosphorylation occurs at serine or threonine residues comprising part of the motif Ser/Thr-X-Y (where X is any amino acid and Y is an acidic residue). When glutamate or aspartate occupies the third position of this sequence, the site is termed a primary phosphorylation site (Mercier, 1981).

The genetic variants include A, B, C, D and E. Holsteins have been reported to have $>95\%$ of B variant and Jersey cattle contain approximately 60% of the B variant along with 40% of the C variant (Ng-Kwai Hang and Grosclaude, 1992; Fitzgerald, 1997). $\alpha_{s1}$-Casein has no cysteine residues and as a result no disulphide linkage in its polypeptide structure (Swaisgood, 1982; Swaisgood, 2003). $\alpha_{s1}$-Casein contains 17 proline residues per mole and as a result it contains very little $\alpha$-helix but contains some $\beta$-sheet and $\beta$-turns in its limited secondary structure (Kumosinski and Farrell, 1994). It also contains 14 lysine residues per mole of protein. $\alpha_{s1}$-casein has three distinct hydrophobic regions within its structure, residues 1-40, 90-113 and 132-199 and a highly charged polar zone between residues 41 and 89 and most of the phosphate groups are located within this polar zone (Swaisgood, 1982; Walstra, Jeness and Badings, 1984).
Of the calcium-sensitive caseins, the $\alpha_s$-caseins are characterised by their greater solubility in the presence of calcium. The distinction between $\alpha_{s1}$- and $\alpha_{s2}$-caseins is based on their amino acid sequences (Holt and Sawyer, 1988; Swaisgood, 2003). $\alpha_{s1}$-Casein can bind up to 9 moles of $\text{Ca}^{2+}$ per mole of protein depending on genetic variant. $\alpha_{s1}$-Casein has a molecular weight of 24,000 Da in monomeric form but can form polymeric units, which is dependent on pH, ionic strength and protein concentration (Swaisgood, 2003).

1.2.1.2 $\alpha_{s2}$-Casein

$\alpha_{s2}$-Casein consists of approximately 207 amino acid residues and like $\alpha_{s1}$-casein is highly phosphorylated. Bovine milk contains four differentially phosphorylated isoforms containing between 10 and 13 moles of phosphorous per mole of protein (Brignon et al., 1977; Eigel et al., 1984). There are 4 genetic variants of $\alpha_{s2}$-Casein and unlike $\alpha_{s1}$-Casein, $\alpha_{s2}$-Casein contains two sulphhydryl groups and can therefore form disulphide linkages (Swaisgood, 1982; 2003). $\alpha_{s2}$-Casein contains 10 moles of proline per mole of protein (Fox et al., 1998; Swaisgood, 2003). $\alpha_{s2}$-casein contains 24 lysine residues within its primary structure and is the most hydrophilic of the caseins. It has a dipolar structure with a large concentration of negative charges near the $N$-terminal and positive charges near the $C$-terminal region of the protein (Swaisgood, 1982; Walstra et al., 1984). The hydrophobic regions of the protein are located between residues 90-120 and 160-207 (Swaisgood, 1982; Walstra et al., 1984; Modler, 1999). $\alpha_{s2}$-Casein can bind up to 13 moles of $\text{Ca}^{2+}$ per mole.
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1.2.1.3 β-Casein

β-Casein consists of 209 amino acid residues and can contain up to 5 moles of phosphorous per mole of protein. β-Casein contains no sulphydryl groups and therefore cannot form any disulphide linkages. It contains approximately 35 moles of proline, 11 moles of lysine and up to 5 moles of arginine per mole of protein (Swaisgood, 1982; 2003). As much as 77% of the β-casein molecule is in unordered structures, 13% in β-sheets and 10% in α-helices (Fox, 1989; Kumosinski et al., 1994). β-Casein is the most hydrophobic of all the caseins, its N-terminal section (residues 1-21) is highly charged and it is in this part of the molecule that most of the phosphoseryl residues are also contained (Swaisgood, 1982; Walstra et al., 1984). The remainder of the molecule has no net charge at pH 6.6. β-casein usually exists in the form of monomeric subunits at or below 4°C but above this temperature can aggregate into large polymeric units. This association can occur in the presence or absence of Ca\(^{2+}\) but in the presence of Ca\(^{2+}\) at temperatures above 8.5°C, β-casein precipitates out of solution (Fox et al., 1998).

β-Casein has been reported to have high heat stability (Swaisgood, 1993) and is one of the commonly used substrate proteins for analysis of proteolytic activity in vitro (Hu et al. 1998; Faccio et al. 2000). β-Casein contains a hydrophilic N-terminal domain and a hydrophobic C-terminal domain, and is able to form micelle-like oligomeric structures (Swaisgood, 1993; Ginger and Grigor, 1999). Moreover, it has been reported that β-casein is able to bind to 1-anilinonaphthalene-8-sulfonate (ANS), a commonly used probe for detecting hydrophobic surfaces on folded or more often unfolded proteins (Slattery, Sood
and Chang, 1989; Javor et al. 1991; Bu et al. 2003). Furthermore, it has been reported that β-casein is able to prevent the thermal- and chemical-induced aggregation of various substrate proteins (insulin, lysozyme, alcohol dehydrogenase and catalase), forming stable β-casein-substrate complexes (Zhang et al. 2005). In the same study, it was reported that β-casein was able to solubilise substrate proteins already aggregated. In addition, β-casein seemed to exhibit a chaperone-like activity (molecular chaperones are a family of proteins that prevent protein aggregation under favourable conditions by blocking the hydrophobic surfaces exposed from the denaturing substrate proteins) that was significantly higher to that of α-casein (Zhang et al., 2005).

1.2.1.4 κ-Casein

κ-Casein consists of 169 amino acids residues and contains at least one serine phosphate at position 149 (Banks, Dalgleish and Rook, 1985). κ-Casein contains two cysteine residues at positions 11 and 88, and at least 20 proline residues per mole of protein; it also contains nine lysine residues per mole (Swaisgood, 2003). κ-Casein is thought to be the most highly structured of the caseins and molecular modelling studies indicate that it contains 23% of its helices in α-helices, 31% in β-sheets and 24% in β-turns (Fox and McSweeney 1998). κ-Casein isolated from bovine milk consists of a mixture of polymers held together by intermolecular disulphide bonds, these polymers range in molecular weight from 60,000 Da to more than 150,000 Da.

κ-Casein differs from the calcium sensitive caseins in a number of respects. It is the only casein that is soluble in the presence of calcium ions and has a much smaller phosphate component than any of the other caseins. κ-Caseins ability to
remain soluble in the presence of Ca\(^{2+}\) at all concentrations up to those at which general salting out occurs is an extremely important property of the protein molecule as it helps to stabilise the other caseins from precipitating in the presence of Ca\(^{2+}\) (Fox et al., 1998). The phosphorylation sites are confined to the C-terminal region of the molecule and are present as single sites rather than the clusters found in the Ca-sensitive caseins. The signal peptide of \(\kappa\)-casein is 21 residues in length (compared to 15 in Ca-sensitive caseins). \(\kappa\)-casein is the only eutherian casein that has been conclusively shown to contain carbohydrate moieties. These features have led to the conclusion that \(\kappa\)-casein is not related to the Ca-sensitive caseins (Jolles, Loucheux-Lefebvre and Henschen, 1978; Bonsing and Mackinlay, 1987; Mercier and Vilotte, 1993).

\(\kappa\)-casein is susceptible to cleavage by the aspartate protease, chymosin. It has been reported that cleavage occurs at a specific phenylalanine-methionine bond in the C-terminal portion of bovine \(\kappa\)-casein (Delfour et al., 1965; Jolles, Alais and Jolles, 1968). The products of this cleavage are the highly charged and glycosylated C-terminal fragment (termed the macropeptide or glycomacropeptide) and the hydrophobic N-terminal peptide, \(para\)-\(\kappa\)-casein. The \(\kappa\)-caseins are the only caseins from eutherian milk at least that are glycosylated. Glycosylation is increased during the colostral period and in response to infection such as mastitis, but decreases with successive periods of lactation (Dziuba and Minkiewicz, 1996). Human \(\kappa\)-casein is more highly glycosylated than the bovine and carbohydrate residues may account for up to 55% of the weight of the molecule (Dev et al. 1993). The carbohydrate portion of bovine \(\kappa\)-casein consists of galactose, \(N\)-acetyl galactosamine and \(N\)-acetyl neuraminic
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acid (Jolles and Fiat, 1979). Human κ-casein is more complex and contains fucose, galactose, \(N\)-acetyl galactosamine, \(N\)-acetyl glucosamine and \(N\)-acetyl neuraminic acid (Fiat et al. 1980; Yamauchi et al. 1981; Dev et al., 1993).

1.2.2 Whey Proteins

In addition to casein, milk contains other proteins which remain soluble at pH values low enough to cause aggregation of casein, these are known as whey proteins. Whey is a by-product of the cheese-making and caseinate production industries. For many years it was viewed as waste and was simply disposed of by dumping it untreated into rivers or the sea, or by feeding it to farm animals. In recent years the nutritional benefits of whey were realised. As well as their nutritional benefits, whey proteins have desirable functional properties as thickening or gelling agents. Whey proteins are used as ingredients in a wide variety of food products, from the baking and confectionary to the dairy industry. The principal whey proteins are \(\beta\)-lactoglobulin (\(\beta\)-lg) and \(\alpha\)-lactalbumin (\(\alpha\)-lac), which constitute, respectively, \(~50\%\) and \(~20\%\) of the total whey proteins in bovine milk (Fox and McSweeney 1998). Both have compact globular structures, and their content of hydrophobic amino acid residues is substantially lower than in caseins. At pH values above \(~7.5\) and below \(~3.5\) \(\beta\)-lg exists as a monomer with a molecular weight of 18,400 Da, but it forms dimers in the pH range 5.5 - 7.5 and octamers in the pH range 3.5-5.2. Each monomer has two intramolecular disulfide (\(\text{–S–S–}\)) bonds and a free–SH group. On heating, \(\beta\)-lg undergoes partial unfolding (denaturation). Under favourable conditions of concentration and ionic environment the denatured molecules associate to form a gel (Fox et al., 1998).
Association is promoted by rearrangement of disulfide linkages from intramolecular to intermolecular, triggered by the free thiol group. In \( \alpha \)-lac, there are four intramolecular disulfide bonds, but no free thiol group, and it therefore does not gel on heating. \( \alpha \)-lac has somewhat lower molecular weight than \( \beta \)-lg (14,200 Da), and it is much less soluble at room temperature (Fox, 1989).

### 1.2.2.1 \( \beta \)-Lactoglobulin

\( \beta \)-Lactoglobulin is the major whey protein in bovine milk making up \( \sim \)50% of the total whey protein; it was first isolated in 1934 (Palmer, 1934). It has a molecular weight of 18,362 Da, and contains 162 amino acid residues (Braunitzer et al., 1972). There are eight genetic variants of \( \beta \)-lg in bovine milk, A, B, C, D, E, F, G and D. The most commonly occurring are A and B. These variants only differ by two amino acids: variant A has an aspartic acid residue in position 64 and a valine residue in position 118, variant B has glycine and alanine in these positions, respectively. The amino acid composition means that \( \beta \)-lg (and also \( \alpha \)-lac) is of nutritional benefit.

\( \beta \)-lg is not found in human milk and as with other bovine milk proteins, some infants and adults are allergic to bovine \( \beta \)-lg (Järvinen et al., 2001). There are seven epitopes for IgE and six for IgG on \( \beta \)-lg. The structure of \( \beta \)-lactoglobulin contains nine \( \beta \)-strands making up two \( \beta \)-sheets which face each other (Brownlow et al., 1997). There is an \( \alpha \)-helix located at the C-terminal. There are five cysteine residues, located at positions, 66, 106, 119, 121 and 160. These cysteines give rise to two disulphide bonds, between Cys\textsubscript{66} and Cys\textsubscript{160}, and between Cys\textsubscript{106} and Cys\textsubscript{119}. On heating >70°C, extremes of pH or high pressure,
β-lactoglobulin can partially unfold, exposing the sulphhydryl group and making it available for reaction with other sulphhydryl groups.

The exact biological function of β-lg is unclear. It is of nutritional value because of its amino acid composition, but it has other properties which have led to the theories that it may have another role. β-lg is structurally similar to retinol binding protein (RBP) (Papiz et al., 1986). Bovine β-lg binds retinol and fatty acids in a hydrophobic pocket on its surface (Perez and Calvo, 1995; Kontopidis, Holt and Sawyer, 2002). The ability to bind fatty acids is unlikely to be the biological function as porcine and equine β-lg does not bind fatty acids (Puyol et al., 1991; Ugolini et al., 2001). The binding of retinol increases the stability of β-lg (Creamer, 1995; Shimoyamada et al., 1996). β-lg also binds vitamin D₂ (Wang, Allen and Swaisgood, 1997).

Given that native β-lg is highly resistant to acid and to pepsin digestion (Miranda and Pelissier, 1983; McAlpine and Sawyer, 1990; Guo et al. 1995), it has been hypothesised that it is involved in the transport of retinol (Puyol et al., 1991). A study has shown that β-lg increases the uptake of retinol in the gut of neonate calves (Papiz et al., 1986).

1.2.2.2 α-Lactalbumin

α-Lactalbumin is the second most abundant whey protein in bovine milk. It is present in bovine milk at concentrations between 1.0 and 1.5 g/L. It was first purified in 1939 (Sørensen and Sørensen, 1939). α-Lac controls the synthesis of lactose by regulating the lactose synthase enzyme system in the mammary gland. α-Lac has a molecular weight of 14,174 Da and contains 123 amino acids. It
contains 8 cysteine residues forming four disulphide bridges between: Cys$_6$ and Cys$_{120}$, Cys$_{28}$ and Cys$_{111}$, Cys$_{61}$ and Cys$_{77}$, and Cys$_{73}$ and Cys$_{91}$. There are no cysteines with free sulphydryl groups in $\alpha$-lac. Another interesting feature of $\alpha$-lac is its high content of tryptophan residues; there are three in total, located at positions 26, 104 and 118. Tryptophan is a precursor of serotonin, which plays an important role in the regulation of anger, aggression, mood and sleep (Young and Leyton, 2002). A diet rich in $\alpha$-lac increases tryptophan levels in blood plasma, leading to improved memory in subjects who were stressed or recovered from depression (Markus, Olivier and de Haan, 2002; Booij et al., 2006). It has also been shown that it aids sleep and improves morning alertness (Markus et al., 2005).

The presence of calcium increases the stability of $\alpha$-lac, allowing it to refold after the protein has been heated. If the pH of the protein solution is reduced below 5.0 the aspartic residues become protonated and can no longer bind Ca$^{2+}$. This apo $\alpha$-lac can be denatured at low temperatures and does not refold after heat treatment. $\alpha$-Lac denatures at a lower temperature than $\beta$-lg. However, due to it having no free sulphydryl group, it does not polymerise, but forms molten globules (Considine et al., 2007). Heating above 100°C in the absence of calcium gives rise to disulphide-linked aggregates. When heated in the presence of whey proteins containing a free sulphydryl group, such as $\beta$-lg or bovine serum albumin, $\alpha$-lac forms homo- and heteropolymers (Considine et al., 2007).
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1.2.2.3 Bovine Serum Albumin

Bovine serum albumin (BSA) is the most abundant protein in blood plasma, where it helps to maintain blood pH. It is also present at lower concentrations (0.1-0.4 g/L) in bovine milk, probably due to leakage from the blood. BSA shows about 80% homology with human serum albumin (HSA). The first large scale purification of a serum albumin was carried out during the second world war (Cohn et al., 1946), when it became necessary to have stable blood substitutes for transfusions. Initially BSA was purified from cow blood and transfused to patients. However, this caused an immune response in the patients, so human serum albumin (HSA) purified from donated human blood was used instead. The cold ethanol procedure established by Cohn et al. (1946) is still used for the commercial production of serum albumins. BSA binds a large number of ligands, including Ca$^{2+}$, Cu$^{2+}$, oleate, stearate, linoleate, and palmitate (Peters, 1985).

Due to their large size it took some time before a sequence for BSA or HSA was determined (Behrens, Spiekerman and Brown, 1975; Brown, 1975). BSA contains 582 amino acids and has a molecular weight of 66,430 Da. As is characteristic for albumins, BSA is low in tryptophan and methionine content, but it contains a high number of cysteines and charged amino acids such as aspartic acid, glutamic acid, lysine and arginine. BSA contains 35 cysteines, giving rise to 17 disulphide bonds and one free sulphydryl group. BSA contains two tryptophan residues while HSA contains only one. The cysteine in position 34 has the free sulphydryl.

BSA denaturation is reversible across a wide range of pH, 2-12, provided that the period of time at extreme pH is short. As well as bringing about the exposure of
the free sulphydryl group, altering the pH of BSA leads to other conformational changes. The different isomers are called expanded, fast, normal, basic and aged (Foster, 1977).

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**Figure 1.5:** The different isomeric forms of bovine serum albumin (Foster 1977)

The change from the normal isomer to the fast isomer leads to increased viscosity and reduced solubility. Further reduction of pH causes the formation of the expanded isomer and an increase in viscosity. Using DSC, conformational changes in BSA were observed to occur at 58.1°C (Poole, West and Fry, 1987). Denaturation of BSA can be divided into two stages. The first stage which is reversible occurs at temperatures up to 65°C. Above 65°C the second, irreversible, stage occurs.

**1.3 Starch-protein interactions**

The textural and rheological properties of food and the presence of other food components like proteins, lipids and non-starch polysaccharides and the molecular changes on their interaction that occur during processing have a major impact on the subsequent digestibility of starch. In food systems where starch
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and milk proteins are present, the importance in understanding their interaction and synergistic effects are becoming increasingly popular. The addition of starches to dairy systems is increasing due to their high availability and relatively low cost. Starch interacts with the two milk proteins, casein and whey, but to differing extents in relation to the physicochemical properties of the starch, i.e., swelling, gelatinisation, retrogradation and ionic bonding.

Lelievre and Husbands (1989) reported an increase in viscosity of gelatinised maize (corn) starch on the addition of sodium caseinate. The authors suggested that the addition of the caseinate altered the swelling volume of the starch, subsequently affecting rheological behaviour (Lelievre and Husbands, 1989).

Kelly et al. (1995) compared the rheological effects of sodium caseinate on the pasting properties of potato and maize starch and observed that the addition of a very low (0.01%) caseinate level to potato starch (1%) was sufficient for a significant decrease in viscosity (Kelly et al., 1995, Mitchell, 1995). The same authors experimented on the role of associated ions in sodium caseinate and there subsequent effect on viscosity. The addition of low levels of sodium chloride (0-0.1%) resulted in a decrease in the viscosity of potato starch but the same viscosity decrease was not observed for maize starch (Kelly et al., 1995). These authors concluded that potato starch had a unique response to low levels of electrolytes. The high phosphate content of potato starch (Noda et al., 2006) gives it a high polyelectrolyte character and behaves in a similar manner to a super swelling polyelectrolyte gel when gelatinised (Kelly et al., 1995). A study by Bertolini et al. (2005) examined the influence of sodium caseinate addition to a range of starches from different botanical sources, i.e., cassava, potato, waxy corn, wheat and rice starches. These authors reported an increase in the storage
modulus and viscosity in all starches with potato starch being the exception (Bertolini et al. 2005), which is in agreement with Kelly et al. (1995). Bertolini et al. (2005) also observed homogenous matrices using light microscopy, in gels made from potato and cassava starch, but more importantly, from waxy corn starch with and without the addition of sodium caseinate (Bertolini et al., 2005). Similar homogenous matrices for waxy maize starch were observed in this thesis (Chapter 2; Kett et al. 2009).

It has been suggested that casein micelles contribute significantly to the storage modulus of skim milk-starch systems because they are excluded from the swollen starch granule (Matser and Steeneken, 1997) (as was the case with β-caseinate, Chapter 2). This leads to an increase in the concentration of protein between the swollen starch granules and an increase in the concentration of starch between the milk proteins. Matser and Steeneken (1997) examined the interaction of skim milk with highly cross-linked waxy maize starch and concluded that the salts, lactose and whey proteins added at concentrations found in skim milk had no effect on the swelling capacity of differing starch dispersions (0-8 g/100g). The final storage modulus of waxy maize starch was higher in a system with skim milk compared to water at similar concentrations of starch. Furthermore, concentrating the skim milk increased the final storage modulus indicating that milk components influenced the storage modulus of the starch (Matser and Steeneken 1997).

Competition for available water on heating between starch granules and sodium caseinate has been reported in a number of studies (Noisuwan et al. 2007) (see also Chapters 2 and 7 of this thesis). Noisuwan et al. (2007) investigated the interaction between waxy or normal rice starch suspensions containing sodium
caseinate, whey protein isolate (WPI) and skim milk protein (SMP) during the early stages of pasting. The authors reported that the addition of sodium caseinate and SMP affected the swelling characteristics of both starches by increasing onset temperature (by 2°C and 4°C respectively). The addition of WPI did not affect swelling onset temperature or viscosity (Noisuwan et al., 2007).

Figure 1.6: Proposed pasting profile of starch in the presence of milk protein ingredients showing granule swelling on heating, and rupture when heated to 95°C under shear. On cooling, a homogenous phase separated mixture is achieved whereby the aggregated protein is embedded in the continuous starch phase. Dotted line represents temperature profile (Noisuwan et al. 2009).
The authors concluded that the increase in swelling onset temperature was due to competition for available water between the starch granules and sodium caseinate. Furthermore, Noisuwan et al. (2007) postulated that adsorption of milk proteins to the starch granule, through hydrophobic interactions, may restrict the diffusion of water into the starch granules, which may subsequently reduce swelling in the granule. Recent research by the same author confirms this hypothesis (Noisuwan et al., 2011). More studies investigated the effect of several milk protein products, i.e., milk protein concentrate (MPC), SMP, WPI and sodium caseinate on the pasting behaviour of normal and waxy rice starch (Noisuwan et al. 2008). The author’s results suggested that the composition of the added product, i.e., the protein content and associated ions affects the pasting behaviour of starch. The association between ions and starch has already been elucidated in previous studies by Bertolini et al. (2005). Noisuwan et al. (2008) reported that the type of protein present influenced the pasting behaviour, for example, the addition of sodium caseinate and SMP increased the temperature of peak viscosity in normal rice starch but no such increase in temperature was observed on the addition of MPC or WPI. The addition of sodium caseinate, SMP and WPI increased the temperature of peak viscosity of waxy rice starch (Noisuwan et al., 2008). Protein concentration plays a major role in rheological characteristics of starch, especially in relation to peak viscosity. The addition of sodium caseinate (10%) increased the peak viscosity of normal rice starch (10%) (Noisuwan et al., 2008).

The addition of the same concentrations of casein hydrolysate, did not however, influence the swelling or gelatinisation temperature of corn starch dispersions (Goel, Singhal and Kulkarni, 1999). Goel et al. (1999) postulated that casein and
its hydrolysates interacted with amylose and outer branches of amylopectin through non-covalent hydrogen bonding. The pasting behaviour of both normal and waxy rice starch were affected by the addition of some milk protein ingredients, with an increased effect observed for normal rice starch. Noisuwan et al. (2008) attributed this increased effect to the amylose:amylopectin ratio of normal to waxy rice starch stating that normal rice starch with its higher amylose content (14%) forms a stronger gel.

The interaction of starch and casein in cheese systems has also been extensively reported in recent years. Results suggest that the rheological properties and microstructure of model cheese systems are influenced by the interaction between starch addition and milk proteins. Mounsey and O’Riordan (2008a) reported that the replacement of casein with increasing concentrations of pre-gelatinised maize starch resulted in increased processing time to form a well emulsified imitation cheese structure. Furthermore, the authors suggested that competition for available water between the starch and casein resulted in a less homogenous protein matrix, with the protein matrix taking on a ‘honeycomb’ structure which may have been an indication of protein dehydration (Mounsey and O’Riordan, 2008a). High amylose starches increase the hardness of imitation cheeses more so than low amylose starches due to the retrogradation process during storage (Mounsey and O’ Riordan, 2001; Guinee, Caric and Kalab, 2004). Moreover, the hardness of imitation cheese increased linearly with the concentration of added starch (Montesinos-Herrero et al., 2006; Mounsey and O’Riordan 2008a). The storage modulus (G’) of imitation cheese was shown to increase as a result of added starch. The authors postulated that this was due to the binding of water by the starch (Montesinos-Herrero et al., 2006). The
interaction between casein and starch affected the microstructure of imitation cheese as a result of the mutual exclusion of each component (Montesinos-Herrero et al., 2006; Mounsey and O' Riordan, 2008a,b). An increase in starch concentration resulted in a less homogenous protein structure, as a result of the exclusion effect, and an increase in the concentration of each component, subsequently resulting in phase separation (Mounsey and O' Riordan, 2008a).

A study investigating the effect of maize starches with varying amylose-amylopectin ratios (i.e., normal, waxy and high amylose maize) concluded that the effect of starch on the properties of processed cheese was strongly influenced by the phase behaviour of the starch-protein network (Ye, Hewitt and Taylor, 2009). The addition of low levels of starch or non-gelatinised maize starch to the model cheese resulted in confinement of the starch to a dispersed phase, behaving as inert filler, increasing gel strength within the cheese. Furthermore, increasing the starch concentration in the system resulted in the formation of a stranded network, subsequently leading to phase separation between the starch and protein phase, which affected the meltability of the processed cheese (Ye et al., 2009).

In cultured/acidified milk systems the release of caseinomacropeptide (renneting) and/or acidification is usually a prerequisite for gelation. Polysaccharides present in the system have a significant effect on the formation of the gel structure (Corredig, Sharafbafi and Kristo, 2011). The addition of normal rice starch (pasted at low temperature as not to induce granule disruption) or modified waxy maize starch to milk, results in the starch granules acting as inert fillers that absorb water increasing the concentration of the casein micelles. This increase strengthens the continuous casein network formed during fermentation or
acidification (Zuo et al. 2008; Azim et al. 2010). Addition of a low concentration (1%) of modified waxy maize starch did not affect the gelation pH during milk acidification but increasing the starch concentration (2.5%) resulted in a higher pH and shorter time of gelation, most probably due to the volume fraction effect of starch granules distributed in the continuous phase (Azim et al., 2010).

Acidified skim milk gels prepared with native starch can, as an end product, differ from gels prepared with cross linked starch. Heating and homogenisation of milk results in leaching of amylose and amylopectin from the native starch into the continuous phase causing phase separation in the milk (Corredig et al., 2011).

Whey proteins can form gels under certain conditions of temperature, salt and concentration and the formation of these gels can be beneficial especially when heated in the presence of starch dispersions. Whey protein and starch can form unique gels when mixed upon heating, and a synergistic effect can occur because the two components are structurally compatible. Proteins contain many hydrophilic groups such as -OH, -NH₂, -COOH and –SH in the alkyl side chains which are compatible with starch (Sopade et al., 2006). Goel et al. (1999) showed that casein addition to corn starch dispersions influenced swelling and gelatinisation temperature and also increased viscosity of the starch-casein system. The presence of hydrophilic groups in the alkyl side chains of proteins which are capable of forming crosslinks with starch is one of the theories for this interaction. The structural differences in individual protein and protein hydrolysates could be attributed to differences in their ability to crosslink with starch. Proteins subjected to various modes and degrees of hydrolysis have very
differences in surface characteristics, making crosslinking possible. In a study involving corn starch and whey protein, the authors postulated that, on heating, the increased viscosity observed in the starch-protein system was not due to swelling of the starch but the entanglement of the whey proteins (Ling, 1984). It has been well reported in literature that increasing the temperature of an aqueous dispersion of starch, above the gelatinisation temperature, results in the continuous disruption of hydrogen bonds between polymers with subsequent migration of water into the interior. A study investigating the structural properties of heat-induced WPI-cassava starch gels to 85°C yielded a composite gel which comprised of a continuous WPI phase filled with swollen hydrated cassava starch granules (Aguilera and Baffico, 1997). The authors calculated the effective concentration of the gelling WPI solution by subtracting the water removed from the swollen starch granules. Aguilera and Baffico (1997) showed that as the concentration of cassava starch increased, the water uptake decreased rapidly, subsequently slowing much more thereafter. They hypothesised that different granule sizes within starch dispersions will imbibe and swell at different rates, leaving granules not swollen exposed to amylose and amylopectin, therefore reducing the degree of swelling within the starch granules. Finally, prior to WPI gelation, water imbibed from the system would result in a more concentrated WPI solution that gelled later adsorbing the starch granules as a continuous phase.

Recent studies investigating the interactions of WPI and cross-linked waxy maize starch involved a systematic rheological study (Fitzsimons, Mulvihill and Morris, 2008) and flow behaviour of starch-WPI dispersions (Vu Dang, Loisel, Desrumaux and Doublier, 2009). Vu Dang et al. (2009) reported that the flow
behaviour of WPI (0.5, 1 and 1.5%)-cross linked waxy maize starch (3 to 4%) systems was significantly different from that of starch alone. The viscoelastic behaviour of the starch-protein system changed from solid-like (3-4% starch) to a liquid-like (3-4% starch/1.5% protein) dispersion on increasing concentration of WPI. Confocal laser scanning microscopy (CLSM) observations showed swollen starch granules dispersed in a protein continuous phase. Vu Dang et al. (2009) also postulated that protein aggregates were of different sizes and some appeared to adsorb onto swollen starch granules.

Fitzsimons et al. (2008) performed a systematic rheological study on co-gels of WPI and phosphate cross-linked waxy maize starch dispersions and reported that a continuous WPI network containing a dispersed starch phase was obtained at high (10%) WPI concentrations, while at low (1%) WPI concentrations a continuous starch network was formed, fragmented by the gelation of WPI.

The selectivity in interaction between proteins and starch can be seen in results of thermal and rheological studies. Interactions depend on the molecular structure of the starch and protein, the amylose/amylopectin ratio of the starch, the composition of the starch and protein and environmental factors such as pH, temperature and ionic strength. In many biopolymer mixtures, the destabilisation forces are greater than the stabilisation forces resulting in phase separation. Starch and proteins are thermodynamically different polymers and their presence in a dispersed system may lead to phase separation, inversion or mutual interaction with significant consequences on texture (Morris, 1990), but, in the context of the studies performed in this thesis, affect in vitro and in vivo hydrolysis. An aqueous starch-protein dispersion can be either (i) thermodynamically compatible (associative, i.e., biopolymers attract each other)
or (ii) thermodynamically incompatible (segregative, i.e., biopolymers repel each other). A two phase system is obtained in which the two polymers are mostly in two separate phases. If they are compatible, a homogenous and stable system is achieved coexisting in a single phase (co-soluble) or a two phase system is achieved whereby the molecules interact and both are in concentrated phases (Tolstoguzov, 1991). In the single phase, increasing the concentration of biopolymers in the system may result in instability, depending on thermodynamic compatibility and the type of interactions. In general, biopolymers tend to segregate (Tolstoguzov, 1991; Grinberg and Tolstoguzov, 1997). Exceeding a certain concentration of either or both polymers leads to phase separation into distinct protein and polysaccharide phases. A mixture of proteins and polysaccharides can be unstable when associative interactions are occurring and may subsequently lead to adsorption of the polysaccharides onto the protein surfaces. If the concentration of polysaccharide is not sufficiently high to completely cover the protein molecules, polysaccharide molecules may adsorb onto more than one protein surface, thereby bridging two or more protein particles, resulting in a process called complex coacervation (Tolstoguzov, 1991).

A study performing small amplitude oscillatory rheology on cassava-WPI starch gels, for the development of viscoelastic properties as a function of time and starch fraction, reported higher mechanical (E, elastic modulus) and rheological (G’, storage modulus) properties for mixed cassava-WPI gels than that of protein or starch gels alone (Aguilera and Rojas, 1996). The increase observed in G’ has been reported to be due to the formation of a network by aggregation of unfolded
protein molecules through non-specific hydrophobic interactions (McSwiney, Singh and Campanella, 1994).

Olsson, Stading and Hermansson (2000) studied the effect of non-gelling genetically engineered potato amylpectin (high and low viscosity) at various concentrations (0-2%) on the gelling properties of particulate β-lg (6%) by comparing modulus in shear and modulus in compression (parallel plate) deformation rheology and light microscopy. Both the viscoelastic and failure properties of β-lg gels changed upon addition of amylpectin even at low concentrations (0.25%). Higher viscosity potato amylpectin had a shear-thinning behaviour and a small yield stress, while the lower viscosity potato amylpectin had a lower viscosity with Newtonian behaviour. The gels containing the lower viscosity potato amylpectin increased in shear modulus with increasing potato amylpectin concentration.

The effect of a range of proteins, albeit non-dairy proteins (i.e., gliadin, glutelin, glutenin and zein), on the rheological properties of three starches from different botanical sources with differing amylose-amylopectin ratios was investigated at a range of temperatures (64-100°C). The gelatinisation of amioaca starch (98% amylopectin, the same starch used throughout all studies in this thesis) resulted in a synergistic increase in viscosity with the addition of all proteins except zein. The addition of zein yielded no difference in viscosity, and the authors attributed this to zein being the most hydrophobic of the four proteins studied, resulting in the lowest compatibility or interaction potential with starch (Chedid and Kokini, 1992). Furthermore, increasing the gelatinisation temperature to 100°C (at 64% moisture), resulted in an increase in viscosity of all amioaca-protein samples with amioaca-gliadin and amioaca-glutenin showing the highest complex viscosity
values. Consistent with the viscosity result at 72°C (64% moisture), the more hydrophobic corn proteins zein and glutelin resulted in the lowest degree of synergistic behaviour, having lower viscosities than gliadin- and glutenin-starch systems. Moreover, the addition of all proteins increased the temperature at which peak viscosity was reached on heating for amioaca. The authors postulated that the presence of protein retards the gelatinisation process, possibly as a result of competition for available water (Chedid and Kokini 1992). The use of dairy proteins to modulate the physical characteristics of starch have been highlighted (Considine et al., 2010), but as the previous study has shown (Chedid and Kokini 1992), proteins from other sources can have similar synergistic effects with starch as dairy proteins.

Cooked pasta, a food reported to have a low glycemic index is generally described as a compact matrix with starch granules entrapped in a protein network (Pagani, Resmini and Dalbon, 1989; Cunin et al., 1995). A unique feature of pasta is that it contains slowly digestible starch (Jenkins et al., 1981; Monge et al. 1990). Modifying either the manufacturing or cooking process parameters could affect the pasta structure and therefore potentially alter the digestibility of the starch and protein fractions (Simonato et al., 2004). In durum wheat pasta gelatinisation of the starch and protein coagulation are the main changes to pasta structure during the cooking process. Protein coagulation and interaction lead to a continuous and strengthened network in the interspaces between granules trapping the starch. Gelatinisation and subsequent swelling of the granules occludes these interspaces (Cuq, Abecassis and Guilbert, 2003) consequently protecting the starch granules. Structural changes in starch and protein during heating (gelatinisation and retrogradation) are approximately in
the same temperature (approximately 70°C) and moisture range, although proteins appear to react at a slightly lower moisture level (Cuq et al., 2003). These changes are both competitive (both components compete for available water) and antagonistic (swelling of starch granules is opposed to the formation of the protein network). The faster the starch swells, the slower the rate of protein interaction, hence, the weaker the protein network (Pagani et al. 1986, Resmini, 1986). The protective role of protein networks and even fibres on starch has been reported by several authors. The encapsulation of starch granules by fibres (Tudorica, Kuri and Brennan, 2002) and protein (Colonna et al., 1990; Fardet et al., 1998) limits the accessibility of α-amylase to the starch reducing hydrolysis. Inclusion of insoluble fibres, i.e., pea fibres, may disrupt the protein network resulting in a highly porous structure. The starch granules consequently become more accessible increasing susceptibility to enzymatic degradation (Tudorica et al., 2002). In contrast, adding soluble fibres (e.g., guar gum) may induce the entrapment of starch granules within a viscous protein-fibre-starch network, acting as a protective coat subsequently reducing glucose release (Tudorica et al., 2002). The protective role of proteins in cooked pasta has been studied and results indicate that by removing the protein by proteolytic enzymes results in increased starch degradation (Colonna et al., 1990). Colonna et al. (1990) reported that starch encapsulation by proteins could limit water absorption by starch granules, subsequently reducing granular swelling limiting enzyme diffusion, decreasing amylolytic breakdown. However, it has been reported that starch granules are not fully encapsulated by the protein network, whose pore diameter porosity is high enough (0.5-40 µm) to allow α-amylase to diffuse freely into the starch granule (Fardet et al., 1998). These authors suggested that
the strength and flexibility of the protein network decreases $\alpha$-amylase efficiency, this and the presence of some high molecular weight starch polymers naturally resistant to enzymatic degradation are hypothesised as being responsible for the low glycemic index (GI) content of pasta.

Many studies have reported on the interactions of starch and protein in relation to viscosity increases, phase separation, oscillatory and deformation rheological properties. Starch interaction (or non-interaction as the case may be) can be significantly affected by the presence of protein. The type and concentration of the protein present is of major importance, as is, the amylose-amylopectin ratio of the starch, the temperature of the biopolymer system, coupled with residence time and moisture content. Of the many studies involving low amylose/high amylopectin starches (i.e., waxy starches), amylopectin starches increase in viscosity above gelatinisation temperature. This process can be modified by the presence of proteins during gelatinisation, with subsequent restriction in swelling of the starch granules, which may be a prerequisite for reduced enzymatic hydrolysis and furthermore starch digestibility.

1.3.1 Starch and protein digestibility

Many studies have reported that the presence of protein in a starch based food matrix may influence physical characteristics and subsequent digestion rates of starch (Jenkins et al. 1987c; Hamaker and Bugusu, 2003; Brennan, Kuri and Tudorica, 2004; Ezeogu et al., 2008; Kim et al., 2008; Kett et al., 2009). Protein fractions such as albumin, globulins and glutenins aid in the bonding of protein bodies into a matrix surrounding starch granules that may act as a barrier towards digestive enzymes reducing starch digestibility (Hamaker et al., 2003; Brennan et
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Evidence suggests that the removal of the protein barrier, by enzymatic hydrolysis surrounding the starch, by the addition of a proteolytic enzyme (pronase), significantly increased digestibility of the starch in vitro allowing amylase and amyloglucosidase to hydrolyse starch more readily (Rooney and Pflugfelder, 1986).

Thermal processing (such as cooking) may sometimes reduce the digestibility of starch as conformational changes in proteins may occur that could facilitate the formation of disulphide linked polymers (Oria, Hamaker and Shull, 1995). Effects of the protein matrix on in vitro starch digestibility of processed starch products such as pasta have previously been reported (Brennan et al., 2004; Kim et al., 2008). Jenkins et al. (1987c) studied the effect of starch-protein interaction in wheat and the subsequent affect on starch digestibility and reported that the starch-protein interaction in white flour may account for a reduced rate of digestion and decreased glycemic response (Jenkins et al., 1987c). The same authors postulated that the reduced rate of digestion, and subsequent glycemic response, was due to the presence of a protein network surrounding the starch core of wheat flour which may have inhibited the rate of hydrolysis in the lumen of the small intestine.

1.4 Starch-hydrocolloid interactions

Recent studies reporting on the influence of hydrocolloid addition to starch observed a common trend in that starch-hydrocolloid mixtures increased paste viscosity or gel strength of the starch compared to starch alone (Lafargue, Lourdin and Doublier, 2007; Achayuthakan and Suphantharika, 2008; Funami et al., 2008; Pongsawatmanit and Srijunthongsiri, 2008; Sikora and Krystyjan,
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2008; Techawipharat, Suphantharika and BeMiller, 2008; Viturawong, Achayuthakan and Suphantharika, 2008; Huang, 2009; Kaur et al., 2009; Wang et al., 2009). Moreover, it has been reported that the effect of a hydrocolloid on a particular starch was a function of the botanical source of the starch and the concentration of the hydrocolloid (Kaur et al., 2008; Sikora and Krystyjan 2008).

Several reports have shown a reduction in granule swelling of maize and wheat starches (Tester and Sommerville, 2003), corn starch (Kruger et al., 2003) and non-waxy rice starch (Song et al., 2006) in the presence of hydrocolloids. Shi and BeMiller (2002) reported on a specific inhibition in potato starch granule swelling; the authors attributed the inhibition by anionic hydrocolloids (i.e. xanthan and alginate gum) to repelling forces between the high concentration of phosphate groups in potato starch and the negative charges on the hydrocolloid molecules.

A study reporting on the interaction between waxy maize starch and xanthan postulated that xanthan assisted in maintaining the integrity of waxy maize starch granules during gelatinisation (Abdulmola et al., 1996). Gonera and Cornillon, (2002) observed an association between xanthan and corn starch in the form of adsorption onto the surface of starch granules using confocal laser scanning microscopy (CLSM). Similar findings were observed by Chaisawang and Suphantharika (2006) with the aid of a scanning electron microscope (SEM). The authors showed that xanthan molecules wrapped around tapioca starch granules coating them in comparison to guar gum which did not show the same effect.

Interaction between cationic tapioca starch and anionic xanthan molecules was observed by Chaisawang et al. (2005) using SEM; the authors concluded that compared to non-ionic guar gum, the strong electrostatic interactions between the
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starch and xanthan resulted in instantaneous aggregation of granules. Moreover, the non-ionic guar formed a sheet structure and only loosely wrapped the starch granules. The authors related the decrease in granule swelling, peak viscosity and solubility index and increase in pasting temperature to the strong interaction between the cationic starch and anionic xanthan.

Inhibition of granule swelling has been shown to reinforce a rice starch-hydrocolloid gel matrix upon gelatinisation (Liu and Lelievre, 1992). Furthermore, inhibition of swelling may be related to reduced peak viscosity as was the case with xanthan addition to non-waxy rice starch (Song et al., 2006). Granule swelling is likely related to dissolution and leaching of starch polymers, i.e., amylose and amylopectin. Studies have reported on the inhibition of starch polymer (primarily amylose) leaching with addition of certain hydrocolloids. Funami et al. (2005) reported that the addition of certain hydrocolloids (konjac, locust bean gum, tara gum and guar gum) to wheat starch resulted in restricted leaching at a hydrocolloid concentration of 0.5% with konjac having the greatest effect. The authors related the increase in viscosity in the continuous phase to decreased leaching of amylose (Funami et al. 2005). A more recent study in 2008 by the same author reported inhibition of wheat starch polymer leaching again with the use of other hydrocolloids. Funami et al. (2008) and Nagano et al. (2008) reported inhibition of polymer leaching from maize starch during gelatinisation on increasing concentration of guar gum. Interestingly, it has been reported that xanthan increased the leaching of amylose from wheat starch at temperatures <80°C, but reduced leaching at higher temperatures (Mandala and Bayas, 2004). Mohammed et al. (1998) showed the effect of agarose (agaran) on waxy maize starch and reported that at a low (2%) starch concentration, swollen
granules were present within the continuous hydrocolloid network. At concentrations of 3-5%, a bicontinuous network formed, and at higher concentrations (6%), waxy maize starch became the dominant component (Mohammed et al., 1998) which is in agreement with work by Kaur et al. (2008) and Sikora and Krystyjan (2008) who showed that the effect of a hydrocolloid on a starch was a function of both the starch used and the concentration of hydrocolloid present.

The fact that hydrocolloid addition to starch could strengthen granular structure may present a possible mechanism of decreasing starch digestibility and subsequently lower the GI value of a food mixture. Numerous studies have reported on restriction of granule swelling and retardation of starch polymer leaching subsequently affecting granule stiffening. One such study proposed that the addition of hydrocolloids to waxy maize starch retarded gelation kinetics. The authors observed a decrease in storage modulus (G’) development of amylopectin-hydrocolloid gels. The same effect was not observed for wheat starch rheology with addition of the same hydrocolloids (Biliaderis et al., 1997). Tester et al. (2003) reported that hydrocolloids (arabic, carrageenan, guar, pectin and xanthan) restrict swelling of starch granules around their gelatinisation temperature. Furthermore, the same authors concluded that the partially gelatinised starch granules, due to restriction of water availability and decreased swelling, inhibited amylolytic hydrolysis of the starch (Tester and Somerville 2003). Recently, correlations between starch digestibility and physicochemical properties of starch on addition of certain hydrocolloids were established. Gularte and Rosell (2011) reported that hydrocolloid addition to either corn or potato starch affected the in vitro hydrolysis of the starch as a direct result of
changes in the physicochemical nature (viscosity, water holding capacity and pasting temperature). The addition of guar gum to potato starch decreased enzymatic hydrolysis and GI of the starch (Gularte and Rosell, 2011). The addition of guar gum to waxy maize starch reduced starch hydrolysis by 25% during the first ten minutes and by 15% at the end of in vitro intestinal digestion compared to starch alone. The viscosity of digesta was also affected, the viscosity during digestion of starch alone decreased but the extent of decrease observed in the presence of guar was quite low (Dartois et al., 2010). Furthermore, the authors concluded that the addition of guar significantly increased viscosity within the system and the action of guar on the water availability and effect on granule swelling may ultimately have affected starch hydrolysis (Dartois et al., 2010).

It is suggested that a hydrocolloid may form hydrogen bonds with starch polymers within starch granules (Liu, Eskin and Cui, 2003), assuming that hydrocolloids may penetrate into starch granules. Such a phenomenon may sound highly unlikely due to the molecular weight of these non-starch polysaccharides. There is evidence (Savary et al., 2008) to suggest that hydrocolloids may penetrate into starch granules subsequently inhibiting granule swelling and amyllose/amylopectin dissolution. Hongsprabhas, Israkarn and Rattanawattanapratik (2007) is in agreement, reporting that the presence of certain hydrocolloids (alginate and carrageenan) maintained granular structure under thermal conditions (heating to 80°C and holding for 30 min) of amylose-rich swollen granules. Further evidence of starch granule strengthening by addition of hydrocolloids has been reported. Gonera et al. (2002) presented evidence that xanthan molecules become associated with the external granule
surface and hypothesised that xanthan molecules absorbed water from the
surrounding environment and the starch granules resulting in strengthening of the
granule. Other authors are in agreement with the strengthening property due to
available water competition between xanthan and starch granules (Chaisawang et
al., 2006; Achayuthakan et al., 2008).

There is little doubt that the addition of certain hydrocolloids to starch affects the
physicochemical properties especially under thermal conditions, i.e.,
gelatinisation and retrogradation. The fact that hydrocolloid addition in some
cases strengthens granular structure, reduces swelling and subsequently limits the
degradation action of amylolytic enzymes is of major importance as a possible
mechanism of lowering starch digestibility and GI.

1.5 Gastro-intestinal digestive enzymes

Many factors influence the rate of gastro-intestinal digestive enzymes and
subsequent enzymatic hydrolysis \textit{in vitro}, for example, temperature and pH,
concentration, stability, activators, inhibitors and incubation time all affect
enzyme activity (Boisen and Eggum, 1991). Furthermore, several factors have
significant results on \textit{in vitro} digestion methods which include but are not limited
to, sample characteristics, digestion times, enzyme activity and applied
mechanical stresses. Therefore, \textit{in vivo} conditions can never be completely
replicated under \textit{in vitro} conditions (Boisen et al., 1991). It has been reported
that using a single purified enzyme, compared to a complex mixture of enzymes,
is often advantageous because it facilitates the standardisation of \textit{in vitro}
digestion models, which can subsequently result in more consistent inter-
laboratory comparisons (Coles, Moughan and Darragh, 2005). However, in
simulating *in vivo* conditions, using purified enzymes is inconclusive as the digestion of one nutrient is often influenced by the digestion of another nutrient resulting in the need for a complex mixture of enzymes for complete digestion (Boisen *et al.*, 1991). The choice of enzymes and incubation conditions are of utmost importance in the digestion of substrates and the resulting hydrolysis products are usually determined by the objectives of the study. Single enzyme methods can be useful for predicting digestibility of single substrates, e.g., protease activity of pepsin or corolase on protein, amylolytic activity of amylase on starch or the lipolytic activity of lipase on lipids.

1.5.1 *Amylases*

Amylases are enzymes responsible mainly for the cleavage of glycosidic bonds and are widely distributed in nature. Amylases act on starch, glycogen and derived polysaccharides to hydrolyse the $\alpha$-1-4 glycosidic linkages of amylose and amylopectin converting them into oligosaccharides and monosaccharides (i.e., glucose). Amylases can be divided into three main groups, (i) $\alpha$-amylases, which split the bonds in the interior of the substrate (endoamylases), (ii) $\beta$-amylases, which hydrolyse units from the non-reducing end of the substrate (exoamylases) and (iii) glucoamylases, which liberate glucose units from the non-reducing terminal of the substrate molecules (Kulp, 1975). $\alpha$-Amylase is the representative member of family 13 glycosidases, several of which are used in starch processing. This family of enzymes is characterised by having at least three separate domains within the protein, one for catalysis, another to serve as a granular starch binding site and the third to provide for calcium binding and to link the other two domains (van der Maarel *et al.*, 2002). The molecular size of
the enzyme from various sources (over 70 sequences have been reported) typically ranges from 50-70 kDa. α-Amylases bind Ca$^{2+}$ at multiple sites and Ca$^{2+}$ is tightly bound and serves to broaden the pH stability of the enzyme between pH 6 and 10 (Parkin, 2008). Thermal stability of α-amylase is dependent on source and there are several sources of α-amylases. Most are microbial, although malt (barley or wheat) amylases are available (Pandey et al., 2000). The typical end products of α-amylase activity are branched α-limit dextrins and maltooligosaccharides of 2-12 glucose units, predominantly in the upper end of this range (Wong and Robertson, 2003; Parkin, 2008).

![Schematic representation of how a potato starch tuber is built-up.](image)

**Figure 1.7:** Schematic representation of how a potato starch tuber is built-up. A, tuber; B, electron microscopic image of starch granules; C, slice of a starch granule showing the growth rings consisting of semi-crystalline and amorphous regions; D, detail of the semi-crystalline region; E, organisation of the amylopectin molecule into the tree-like structure and F, two glucose molecules with an α1-4 glycosidic bond (van der Maarel et al., 2002).
Salivary $\alpha$-amylase (present in the mouth) and pancreatic $\alpha$-amylase are responsible for starch degradation in humans and most animals and both act on amylase and amylopectin in an essentially random (endo) manner with subsequent production of reducing sugars. $\alpha$-amylase is routinely used for *in vitro* studies involving hydrolysis of starch (Fredriksson *et al.*, 2000; Brennan *et al.*, 2004; Evans and Thompson, 2004; Kong and Singh, 2008). $\alpha$-amylases from microbial sources (*Aspergillus oryzae*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*) are used in the food processing industry for the production of sugars and syrups and also in brewing (Nigam and Singh, 1995).

1.5.2 Proteases

Catalytic action of enzymes is markedly affected by change in temperature and pH and proteolytic enzymes have a strict specificity to substrates. Proteases are mainly present in the stomach and intestine and are responsible for breaking down proteins and large peptides into smaller peptides and amino acids. Pepsin degrades proteins in the stomach on ingestion of food and trypsin/chymotrypsin degrades the proteins/peptides further in the small intestine. The daily pepsin secretion in adults is approx. 20-30 kU of enzyme activity at 37°C, equivalent to around 10 mg, while a typical adult dietary intake of protein comprises around 75 g/24 h giving a pepsin/protein ratio of 1:7500 (Mills *et al.* 2004, Shewry, 2004; Bublin *et al.*, 2008). Pepsin is a gastric acid protease and has activity in extremely acidic conditions, being active at pH 1. Pepsin is formed from a precursor, pepsinogen, which is found in the stomach mucosa of humans and animals (Yamamoto, 1975). Pepsinogen is autocatalytically converted to pepsin
at pH <5.0 by pepsin itself. Pepsin is relatively stable at pH 5.0 and is active between pH values 1.0 and 4.0, with maximum activity between 1.8 and 2.0 (Yamamoto, 1975).

Trypsin and chymotrypsin are both serine proteases. Trypsin is one of the major proteases of the pancreas which has been highly purified and is used in the food industry for the production of hydrolysates in its crude form, pancreatin. Trypsin has a rather narrow specificity for certain peptide bonds and is formed in the intestinal tract from an inactive precursor (trypsinogen) and is inhibited by naturally occurring proteinaceous inhibitors. Trypsinogen and other occurring precursors (chymotrypsin and procarboxypeptidase A) are excreted by the pancreas but activated in the intestinal tract (Yamamoto, 1975).

Chymotrypsin, another serine protease is stable at acid pH values, but, in contrast to trypsin, its stability extends to pH values near neutrality. Similar to trypsin, the enzyme shows optimum activity in the pH range 7 to 9, which conforms to the natural action and environment of the small intestine. Chymotrypsin has specific activity on peptides containing aromatic amino acids, such as tyrosine, phenylalanine and tryptophan. Chymotrypsin also has affinity for the peptides containing amino acids with bulky hydrophobic side chains, although the enzyme hydrolyses these peptides at a much slower rate (Yamamoto, 1975). Matoba and Hata (1972) attributed the development of the bitter taste produced from proteolytic hydrolysates to the presence of peptides with a high content of hydrophobic amino acids. Furthermore, the same authors reported that amino acids in a peptide chain independently contribute to bitterness regardless of amino acid sequences (Matoba and Hata, 1972).
The choice of proteolytic enzymes, digestion conditions and methodologies used for analysis of protein hydrolysates have considerable impact on the digestibility of proteins (Abdel-Aal, 2008). Abdel-Aal (2008) reported that a one-step digestion of protein using trypsin, chymotrypsin and peptidase resulted in higher protein digestibility (approx. 39-66%) compared to a two-step digestion using pepsin and pancreatin. Abdel-Aal (2008) suggested that the three enzyme (trypsin, chymotrypsin and peptidase) digestion method is more comparable to in vivo conditions, concluding that in vitro digestion methods involving a mix (saliva, gastric juice, duodenal juice or bile juice) have the advantage of being more reproducible than those digestion methods using single enzymes.

1.5.3 Lipases

Lipases constitute an important group of enzymes associated with fat metabolism. Lipases are present in the stomach (gastric lipase) and pancreas (pancreatic lipase) where they adsorb at the surface of emulsified lipids and oils (Hur et al., 2011) and liberate free fatty acids and partial glycerides which are essential for fatty acid transport, oxidation and resynthesis of glycerides and phospholipids (Shahani, 1975). The activity of pancreatic lipase depends on the presence of bile salts and an absolute requirement for calcium (Zangenberg et al., 2001). Pancreatic lipase binds in a stoichiometric ratio of 1:1 to the lipid substrate (Carey, Small and Bliss, 1983). Calcium reacts with liberated free fatty acids by means of ionic complexation, removing them from the surface of the lipid droplets and preventing them from inhibiting the lipase (Fatouros and Mullertz, 2008). Fatouros and Mullertz (2008) reported that when calcium is
added at the start of lipolysis it results in a very fast lipolysis rate followed by a plateau over longer times, which was attributed to product inhibition by free fatty acids and possibly precipitation of bile salts with calcium (Fatouros et al., 2008). Therefore, Fatouros et al. (2008) proposed that it is better to add calcium continuously throughout the in vitro digestion process, rather than adding it all at the beginning of hydrolysis.

Lipases are frequently used enzymes, usually commercial preparations are crude that may contain other enzymes, which in many cases can have different activities and make interpretation of results difficult (Segura et al., 2004). Segura et al. (2004) also found that the three main components of porcine pancreatic lipase extract exhibit very different catalytic properties.

1.5.4 Models systems used to simulate digestion

Various in vitro gastro-intestinal models currently exist trying to replicate as accurately as possible the in vivo response to food ingredients or foods of varying macro- and micro-nutrient composition. One such model is the TNO gastro-Intestinal Model (TIM), a dynamic multi-compartmental, computer-controlled model that closely simulates all in vivo conditions of the upper gastrointestinal tract (stomach and small intestine) of humans. The model has been used in many peer reviewed studies (Verwei et al. 2003; Avantaggiato, Havenaar and Visconti, 2004; Mitea et al., 2008) to assess digestion kinetics in a range of disciplines from pharmaceutical and food to toxicology.
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Figure 1.8: Front panel of the TIM system and schematic diagram showing the stomach compartment (A) and three small-intestinal compartments: duodenum (C), jejunum (E), and ileum (G) connected by vertical peristaltic valves, including the pyloric sphincter (B) and the ileo-cecal valve (H). Each compartment with secretion tubes, such as gastric acid and pepsin in the stomach (I), and pancreatin and bile in the duodenum (J), pH electrodes (P), pressure (S) or level sensors (Q) and temperature sensors (R). Connected to the jejunum (left) and ileum (right) are semi-permeable hollow-fiber membrane units (M) for continuous absorption of digested products and water absorption (N) (Verwei et al., 2003).

Peristaltic mixing and transit activity of TIM is accomplished hydraulically with the pumping of water in and out of jackets surrounding flexible compartments. Heating of the water in the jackets maintains physiological body temperature inside the compartments. The gastric emptying and intestinal transit time of the contents are controlled between each compartment with a series of three peristaltic valves which open and close in sequence to move a specific volume.
with each cycle. The frequency of transit is computer-controlled for the specific conditions to be simulated and secretions of the different body fluids (e.g., saliva, gastric juice, bile, and pancreatin) are made by pumps connected to each compartment and controlled by a computer. Absorption of released and digested compounds (water or fat soluble) is accomplished with separate dialysis/filtration devices for the jejunal and ileal compartments. During the TIM experiments, samples can be taken from the different compartments during the digestion process.

Other groups involved in in vitro digestion models are the Leatherhead Food Research group (UK) and the NIZO food research group (Netherlands) using their model, Simulation of physiological digestion (SIMPHYD platform). The NIZO model has been used in a study aimed to develop a basic computer model for first quantitative interpretation of results obtained from in vivo intestinal experiments with bacteria (de Jong et al. 2007). In all the models mentioned above, one parameter not measured throughout digestion is viscosity of the food bolus as it is being digested. The viscosity of a food before and during digestion has an impact on the rate and accessibility of enzymes to digestion (Hur et al., 2011). In Chapter 6, a novel design is proposed for digestion of food and beverage ingredients while simultaneously measuring viscosity throughout digestion. Viscosity measurement is possible as the digestion cell is incorporated into a concentric cylinder attached to a controlled stress rheometer allowing for accurate measurement in viscosity changes during digestion.
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1.6 Glycemic index

Glycemic index (GI) is a measure of the change in blood glucose levels following ingestion of carbohydrate-containing foods. Some carbohydrate foods result in a rapid increase followed by a more or less rapid fall in blood glucose, whereas other types of carbohydrates (i.e., resistant starches) produce a more sustained release along with a more gradual decline in blood glucose levels. GI is a ranking of carbohydrate exchanges according to their effect on postprandial glycemia, and a means of quantifying the relative blood glucose response to carbohydrates in individual foods. Comparison is on a weight per weight basis per gram of carbohydrate. The amount of carbohydrate is an important determinant of the glycemic effect of a given food or meal. At equal concentrations, the carbohydrates in some foods have much greater effects on postprandial glycemia than others. For this reason, the concept of GI was introduced in 1981 as a method for classifying food carbohydrates according to their effect on postprandial glycemia (Jenkins et al., 1981). The GI is methodologically defined as the blood glucose response of a 50 g (or 25 g) carbohydrate portion of food, expressed as a percentage of the same amount of carbohydrate from a reference food, either glucose or white bread (Wolever et al., 1991). The GI of a food is influenced by many different factors including, (i) the type of sugar (i.e., fructose vs. glucose) present, (ii) the type of starch (i.e., amylose:amylopectin ratio), (iii) the extent of gelatinisation of the starch, (iv) presence of soluble or insoluble fibre, (v) whether the food has been thermally processed or not and (vi) the presence and type of fat and/or protein in the food system. Studies have suggested that the body’s glycemic response to carbohydrates when co-ingested with proteins is lower than that from ingestion
of carbohydrate alone (Spiller et al., 1987). Although there are many methods for GI determination, it cannot be easily predicted or measured in vitro, and is more a measure of carbohydrate digestibility (physical hydrolysis) rather than the true in vivo response of a food on blood glucose and insulin levels.

Studies have reported significant evidence demonstrating the benefits of low GI diets (Bjorck, Liljeberg and Ostman, 2000; Jenkins et al., 2002; Accurso et al., 2008). These benefits include (i) improvements in glycemic control reducing insulin fluctuations, (ii) improvements in insulin resistance, (iii) management of hyperlipidemia and (iv) improvements in blood glucose control and glucose tolerance. Although a high carbohydrate diet has been reported to elevate many of the features of insulin resistance, including postprandial glycemia and insulinemia, this is not seen when the carbohydrate comes from low GI sources. High GI meals result in a rapid increase in blood glucose and insulin levels followed by reactive hypoglycemia, counter regulatory hormone secretion, and elevated free fatty acid concentrations, which may then lead to excessive food intake, consequently leading to dyslipidemia and beta cell dysfunction. Over time, this could lead to increased risk of obesity, type 2 diabetes and cardiovascular disease (Ludwig, 2002).

1.6.1 Glycemic Load

While GI provides a basis for ranking foods based on their blood glucose response, it does not take into account the effect of a typical amount of carbohydrate in a food portion on glycemia. The overall blood glucose response to a food or meal is determined by both the quantity and quality (i.e., nature or source) of the carbohydrate consumed; thus, the concept of glycemic load was
put forward in 1997 (Salmerón et al., 1997a). It is a measure of the overall effect of food on blood glucose and insulin levels. As defined, the glycemic load (GL) of a particular food is the product of the GI of the food and the amount of carbohydrate in that serving. The overall GL of a meal can therefore be calculated using the individual values of the meal (Salmerón et al., 1997a). The higher the GL, the greater the expected elevation in blood glucose and in the insulinogenic effect of the food. The long term consumption of a diet with a relatively high GL has been reported to be associated with an increased risk of type 2 diabetes and coronary heart disease (Liu et al., 2000). Reducing the dietary GL could be done in two ways, (i) lowering the GI of the carbohydrate or (ii) by reducing the total carbohydrate in the diet, but the metabolic responses of these methods are likely to be different. In 2002, a revised International GI and GL table was published with values for many different food types (Foster-Powell, Holt and Brand-Miller, 2002).

1.6.1.1 Glucose transport

Fluctuations in glycemia are finely regulated so that normal glucose concentration varies between 4-5.5 mmol/L. Following ingestion of a carbohydrate rich meal (30 min postprandial), glucose concentration in the blood rises to 8-10 mmol/L. Insulin acts quickly to combat this postprandial surge and return glucose concentration to within the normal range.

The chief function of the digestive tract is to break down food macromolecules (fats, carbohydrates and proteins) into fatty acids, monosaccharides and amino acids so that they can be absorbed across the gastric wall. The digestive system achieves this by mechanical and enzymatic processes where ingested food is
mixed, crushed and ground by smooth muscle contractions into chyme and then hydrolysed and cleaved by acid and enzymes. This process converts digestible carbohydrates in a meal into simple sugars (i.e., glucose and galactose) which are then absorbed by mature enterocytes lining the intestinal villi (Wright, Martin and Turk, 2003; Drozdowski and Thomas, 2006). Specifically, glucose is transported from the intestinal lumen across the brush border membrane of these enterocytes primarily by the sodium dependant membrane transporter, SGLT1 (Hediger et al. 1987; Wright, Martin and Turk, 2003; Drozdowski and Thomson, 2006). SGLT1 is a 73 kDa membrane protein with 14 transmembrane domains that binds two sodium ions to one glucose molecule (Turk et al., 1996; Panayotova-Heiermann et al., 1997; Turk and Wright, 1997). Two sodium ions bind to SGLT1 resulting in a conformational change that allows one glucose molecule to bind. Once inside the enterocytes, glucose and the sodium ions dissociate from the transporter (Wright et al., 2003; Drozdowski et al., 2006). Internalised glucose then exits from the enterocytes across the basolateral membrane into the blood, principally by the facilitative glucose transporter, GLUT2. GLUT2, with 12 transmembrane domains, demonstrates a low affinity but high capacity for glucose, fructose, galactose and mannose (Maenz and Cheeseman, 1987; Cheeseman, 1993; Wu, Fritz and Powers, 1998). Although SGLT1 is the major player in glucose transport into the enterocyte, this transporter saturates at 30-50 mmol/l glucose. However, experiments have shown that glucose absorption continues linearly above this saturation (Fordtran, Rector and Carter, 1968). This suggests an alternative albeit secondary glucose transporter exists in the brush border membrane (Malo, 1988). It has been proposed that SGLT1 promotes the
recruitment of GLUT2 to the brush border membrane to aid with glucose uptake from the lumen (Kellett and Helliwell, 2000). Other sugar transporters that have recently been identified in the intestine, for example GLUT7 and SGLT4, have also been nominated as supplementary glucose transporters (Wright et al., 2003). Glucose exit from the cell may also involve a secondary mechanism independent of GLUT2. This mechanism is thought to necessitate a glucose phosphorylation step (Stumpel et al., 2001). Changes in intrinsic activity of the glucose transporters and alterations in abundance in the membrane have been reported for disease states such as glucose-galactose maladsorption (Wright et al., 2003), hyperglycemia (Maenz and Cheeseman, 1986; Cheeseman and Tsang, 1996) and diabetes (Cheeseman et al., 1996). In addition, certain gut peptides (e.g., oxyntomodulin) are known to effect intestinal glucose uptake (Collie et al., 1997).

Once food enters the alimentary canal, the gut must inform the body of the impending arrival of glucose to the blood and ensure insulin is produced and secreted to maintain glucose homeostasis. The intestinal mucosa achieves this primarily by secreting gut peptides termed incretin hormones into the bloodstream (Baggio and Drucker, 2007). These incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependant insulinotropic polypeptide (GIP), respond to carbohydrate or fat ingestion (Brubaker, 2006). GLP-1 is secreted from intestinal endocrine L-cells located in the ileum and colon. It is one of five peptides produced (Glicentin, Oxyntomodulin, GLP-1, IP-2, GLP-2) from the proteolytic processing, in the intestine, of the proglucagon protein (Baggio and Drucker 2007) and the only one with insulinotropic effects. The entire 37 amino acid sequence of GLP-1 is coded for in exon 4 of the proglucagon gene. GLP-1
secretion is stimulated by nutrients, neural and endocrine factors in a biphasic pattern with early GLP-1 release occurring within 10-15 min of meal ingestion followed by a later release after 30-60 min (Herrmann et al., 1995; Baggio et al., 2007). Early phase production is probably due to neural stimulation of the L-cells whereas late phase production conceivably results from direct contact of L-cells with nutrients (Roberge and Brubaker, 1991). Biologically active GLP-1 circulates at a concentration of 5-10 pmol/L plasma rising 2-3 fold after a meal (Orskov et al., 1994). It has a half life of 2 min and is degraded by dipeptidyl peptidase-4 (DPP-4) (Mentlein, Gallwitz and Schmidt, 1993; Kieffer, McIntosh and Pederson, 1995). Once GLP-1 reaches the pancreas it binds to its receptor GLP-1R and stimulates insulin secretion via mechanisms that include inhibition of potassium channels, increase in intracellular calcium, increase in mitochondrial ATP synthesis, closure of potassium channels and directly effects insulin storage granules. It also acts at the transcriptional level to produce and store more insulin (Drucker et al., 1987; MacDonald et al. 2002; Li et al., 2005; Baggio et al., 2007). The other incretin hormone, GIP is a 42 amino acid peptide released by post-translational modification of the 153 amino acid proGIP prohormone. GIP is secreted from intestinal K cells primarily present in the duodenum and jejunum (Baggio et al., 2007). The absorption of nutrients, particularly glucose and fatty acids, from the intestinal lumen result in GIP secretion. Circulating GIP has a half life of 5-7 min and similar to GLP-1 is inactivated by DPP-4 (Deacon et al., 2000). Postprandial concentrations in plasma range from 0.06 nmol/L to 0.20 nmol/L (Orskov, Wettergren and Holst, 1996; Vilsboll et al., 2001). Bioactive GIP mediates its insulinotropic effects by binding to its specific receptor on pancreatic β-cells. The mechanisms involved
appear somewhat similar to those for insulin stimulation by GLP-1 (Baggio et al., 2007).

Although glucose and fats are known to stimulate the incretin peptides, it must also be mentioned that certain amino acids/peptides in foods appear to stimulate insulin secretion (van Loon et al., 2003; Nilsson et al., 2004; Frid et al., 2005). In vitro studies using incubated pancreatic β cell lines demonstrate that different amino acids appear to have different ability to stimulate insulin production (Charles and Henquin, 1983; Brennan et al., 2002). The gut lumen is the site for glucose absorption and also the site for incretin secretion into the blood. The rate at which food passes through the gut has a major impact on glucose appearance in the blood (Chaikomin et al., 2006). Research into functional food design (i.e., starch/protein interactions) that delay gastrointestinal food passage and slow glucose absorption into the bloodstream would result in less dramatic fluctuations in blood glucose and decrease the need for insulin.

1.6.2 Glycemic index and the metabolic syndrome

An increasing number of studies have all reported the health benefits of a low GI diet. Increased consumption of diets with a high GI can increase the risk of a number of chronic diseases such as obesity and insulin resistance leading to type 2 diabetes, cardiovascular disease and even some cancers (Jenkins et al., 2002; Ludwig, 2002; Aston, 2006).

1.6.2.1 Glycemic index and diabetes

The link between high GI and high GL diets and diabetes may relate to glucose peaks and increased insulin demand (Figure 1.9). High GI foods lead to rapid
rises in blood glucose and insulin levels. Hyperinsulinemia (excess levels of insulin circulating in the blood), in turn, may down-regulate insulin receptors, reducing insulin efficiency and therefore result in insulin resistance (Virkamaki, Ueki and Kahn, 1999).

**Figure 1.9:** The potential relationship between high GI foods and insulin resistance (adapted from Jenkins *et al.*, 2000).

This condition may be self-propagating, increasing blood glucose concentrations and insulin secretion. Insulin resistance has been reported to be a risk factor for type 2 diabetes (Nijpels, 1998). Although there have been no long-term intervention studies looking at the effects of a low GI diet in the prevention of diabetes, two large epidemiological studies in men (Salmerón *et al.*, 1997a) and women (Salmerón *et al.*, 1997b) have associated low GI diets with reduced risk of type 2 diabetes. In both studies, the risk of developing diabetes was 37% higher in the highest quintiles (highest vs. lowest quintiles) of GI after adjusting
for confounding factors, including cereal fibre intake. A high dietary GL was also found to increase the risk of diabetes and was related to GI and not the amount of dietary carbohydrate. In a study feeding rats a high GI diet over the course of 18 weeks, results showed a significantly higher area under the curve (AUC) for blood glucose and insulin, higher triglyceride levels and severe disruption to islet cell structure. These symptoms together can increase the chances of developing type 2 diabetes (Pawlak, Kushner and Ludwig, 2004).

Low GI foods tend to delay glucose absorption, thereby resulting in reduced peak insulin concentrations and overall insulin demand. Several studies have found improvements in glycemic control with low GI diets and have also shown to benefit those with existing diabetes. Studies involving a total of 356 subjects with type 1 (203) and type 2 (153) diabetes found that low GI diets can improve blood glucose control to a similar extent as that seen with medications that target post-prandial hyperglycemia (Brand-Miller et al., 2003). A second study which investigated the effect of low versus high GI diets on markers for carbohydrate and lipid metabolism found that low GI diets significantly reduced fructosamine and total cholesterol levels and tended to reduce low-density lipoprotein (LDL) cholesterol levels in people with type 2 diabetes (Opperman et al. 2004). Further studies involving ingestion of uncooked cornstarch (slow release carbohydrate) at bedtime (Axelsen et al., 1999a) produced a substantial suppression of nocturnal free fatty acid levels and postprandial improvements in breakfast glucose levels, and a slow release carbohydrate food taken in the evening can also prevent nocturnal hypoglycemia in insulin-dependent diabetes sufferers (Axelsen et al., 1999b). Two main mechanisms of action may be involved in the regulation of insulin sensitivity and glucose levels by low GI diets: (i) free fatty
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acid levels and (ii) oxidative stress. Rapidly absorbed carbohydrate stimulates a large rise in insulin secretion followed usually by a rapid decline in blood glucose, often below basal levels. This could result in a counter-regulatory response with the release of free fatty acids, creating an insulin-resistant environment and reduced glucose tolerance (Piatti et al., 1991). While the use of GI is not universally accepted, a number of health organisations throughout the world now recommend consuming low GI foods in the management of type 2 diabetes (Buchorn, 1997).

1.6.2.2 Glycemic index and obesity

Diets with a lower GI or GL may assist in weight management by influencing appetite and fuel partitioning. Short-term feeding studies have generally found low GI foods to increase satiety, reduce hunger, or lower subsequent voluntary food intake, whereas high GI foods are associated with increased appetite and higher energy intake (Ludwig et al., 1999; Roberts, 2000). In a review involving 16 studies, it was reported that low GI foods increased satiety, delayed return of hunger or decreased adlibitum food intake in all but one case compared to high GI foods (Ludwig, 2000). High GI foods elicit, calorie for calorie, higher insulin levels and c-peptide excretion than low GI foods (Jenkins et al., 1987; Miller, 1994). Hyperinsulinemia associated with high GI diets may promote weight gain by preferentially directing nutrients away from oxidation in muscle and toward storage in fat (Ludwig, 2000). A study by Sigal et al. (1997) reported that, in humans, high acute insulin secretion after intravenous glucose tolerance tests, predicts weight gain among glucose-tolerant offspring of parents with diabetes mellitus (Sigal et al., 1997).
Generally, low GI foods are associated with greater satiety compared to high GI foods (Haber et al., 1977; SH Holt and Miller, 1994; Liljeberg, Akerberg and Bjorck, 1999). Studies performed showing the effect of high, medium or low GI breakfast meals on subsequent adlibitum food intake in obese subjects resulted in reductions in energy intake of up to 53% and 81% in the medium and low GI groups respectively, compared to the high GI group, 5 h post ingestion of breakfast (Ludwig et al., 1999). These results could suggest that, in isoenergetic meals, slowly digested carbohydrate rich foods may allow a sense of satiety to last longer than rapidly digested foods. The characteristic effects of high GI foods, i.e., fast carbohydrate absorption, rapid blood glucose and insulin fluctuations and reduced satiety may subsequently lead to overnutrition in the long run (Haber et al., 1977). High insulin and low glucagon levels, triggered by high GI foods, induce glucose storage inhibiting lipolysis and consequently reduce glucose availability for metabolic oxidation, (hypoglycemic undershoot) is a characteristic effect of high GI foods and may induce hunger. This metabolic state could be seen as a fasting state and would trigger glucagon release and hunger signals. Low GI foods tend to maintain glucose and insulin homeostasis avoiding the hypoglycemic state. Cholecystokinin (CCK), a gut peptide that induces satiety, is thought to be directly affected by gastric volume. Meal ingestion and its GI values has been reported to be inversely proportional to CCK response and satiety (Holt et al., 1992), suggesting a possible role of gastric volume in appetite suppression. There are no long term clinical trials examining the effects of dietary GI on body weight regulation. However, numerous studies involving animals or short term studies in humans have attempted to address this issue. A study whereby rats were fed amylopectin (with a high GI) compared
with amylose (with a low GI) under energy controlled conditions for up to 5 weeks exhibited physiological changes that would favour fat deposition, including larger adipocyte diameter, increased glucose incorporation into lipids and greater fatty acid and Glut 4 gene expression in fat tissue (Lerer-Metzger et al., 1996; Kabir et al., 1998). Another study where animals were fed a high GI diet showed increased epididymal fat mass by week 7 (Pawlak et al., 2001) and, according to preliminary data, developed obesity by 32 weeks (Pawlak, Denyer and Brand-Miller, 2000). A study including obese teenage boys given a high GI instant oatmeal, medium GI oatmeal or low GI ‘vegetable omelette’ with identical energy and macronutrient content at breakfast and lunch resulted in increased energy intake after adlibitum energy consumption was monitored throughout an afternoon; energy intake was 53% higher after the high GI compared with the low GI meal (Ludwig et al., 1999). The decreased circulating concentrations of metabolic fuels in the middle postprandial period post ingestion of a high GI meal would be expected to result in increased hunger and food intake as the body attempts to restore energy homeostasis. Modest transient decreases in blood glucose concentration, either spontaneous or insulin induced, were associated with hunger and initiation of feeding in rats and humans (Campfield and Smith, 1990; Campfield et al., 1996). Studies have reported that hyperinsulinemia (Rodin et al., 1985) and hypo-glycemia (Friedman and Granneman, 1983) may preferentially stimulate consumption of high GI foods, which can lead to cycles of hypoglycemia and hyperphagia. Weight loss issues may exacerbate this phenomenon, as demonstrated by relatively severe postprandial hypoglycemia after overweight subjects on very low calorie diets consumed high GI carbohydrate (Marsoobian et al. 1995).
The optimal diet for the prevention and treatment of obesity remains to be determined, that is, if one does not already exist. The effects of GI on body weight regulation must be explored in long term clinical trials. A growing body of experimental work suggests that diets designed to lower insulin response to ingested carbohydrate may improve access to stored metabolic fuels, decrease hunger and promote weight loss. One such diet would have to contain daily ingestion of vegetables, fruits and legumes and moderate amounts of protein and heart healthy fats, and decreased intake of high GI carbohydrates such as potatoes and refined grain products. The interaction of protein and starch in reducing size of starch granules during gelatinisation subsequently lowering GI of the starch is one such way of achieving this balance.

1.6.2.3 Glycemic index and cardiovascular disease

Postprandial hyperglycemia has been recognised as an important risk factor for cardiovascular disease (CVD) among the general population as well as those with diabetes and impaired glucose tolerance (IGT) (Coutinho et al., 1999; Lefebvre and Scheen, 1999; Ceriello, 2000). Analysis of 38 studies found a positive linear relationship between postprandial blood glucose levels and cardiovascular disease risk, even in nondiabetic patients (Levitan et al., 2004). High GI diets have been associated with oxidative stress; a novel factor implicated in the pathogenesis of cardiovascular disease (Hu et al., 2006), and studies suggest that oxidative stress may be a factor in the link between hyperglycemia and increased cardiovascular disease (Ceriello, 2000). A high GI diet may also increase insulin levels and excessive insulinenia can exacerbate blood pressure, endothelial function and coagulation factors (Ludwig, 2002).
Low GI diets on the other hand have been reported to reduce triglycerides (TG) as well as total and low density lipoprotein (LDL) cholesterol and improve high density lipoprotein (HDL)/total cholesterol ratio (Jenkins et al., 1985, 1987a; 1987b; Wolever et al., 1992a; 1992b; Jarvi et al., 1999; Luscombe, Noakes and Clifton, 1999; Opperman et al., 2004; Pereira et al., 2004). Research into the association between dietary GI and CVD risk have found higher HDL, lower TG or lower coronary heart disease risk in those with lowest dietary GI and GL (Frost et al., 1999; Liu et al., 2000; Ford and Liu, 2001). Liu et al. (2000) reported that the relative risk of heart disease was 2-fold higher during 10 years of follow up in those in the highest quintile of GL compared with those in the lowest quintile after adjustment for a range of factors including age, BMI, dietary factors and other coronary disease risk factors (Liu et al., 2000). The adverse effects of hyperglycemia on endothelial function and other CVD related outcomes occur rapidly following consumption of glucose or mixed meals that induce high postprandial glycemia (Ceriello et al., 1999; Title et al., 2000). Studies have shown that administration of antioxidants can prevent or reverse these adverse effects (Marfella et al., 1995; Title et al., 2000). It may be reasonable to postulate that increased consumption of high GI diets may increase the risk of CVD due to hyperglycemia induced oxidative stress.

1.6.2.4 Glycemic index and coronary heart disease

The possible beneficial effects of a low GI diet in the prevention of coronary heart disease (CHD) may be explained by improvements in blood lipid profiles, insulin levels, thrombotic factors and endothelial function. Long term studies aimed at determining the metabolic effects of isocaloric macronutrient balanced
diets with high vs. low GI foods, have been shown to significantly reduce serum cholesterol and triglyceride levels in hyperlipidemic and diabetic patients (Wolever et al., 1992 a,b). Hyperlipidemia is a risk factor for CHD and it is one of the most common metabolic dysfunctions associated with diabetes (Lotufo et al., 2001). There have been concerns that high carbohydrate intakes at the expense of fat, especially monounsaturated fat (Coulston, Hollenbeck, Swislolocki and Reaven, 1989; Garg et al., 1994) could result in a rise in triglycerides and LDL and a suppression of HDL levels, which may result in a higher risk of heart disease (Hokanson and Austin, 1996; Vega and Grundy, 1996). However, not all carbohydrate rich diets may produce the same effects on HDL levels, as low GI diets may confer a more favourable lipid profile compared with high GI diets. Lowering the dietary GI by at least 12 points reduced triglycerides by approximately 9% in 10 out of 11 studies (Brand-Miller, 1994) and recent data showed that a high carbohydrate diet made of low GI foods significantly increased HDL levels compared to an isocaloric high carbohydrate/high GI diet (Luscombe et al., 1999). Furthermore, data from two studies (Frost et al., 1999; Ford and Liu 2001) showed that dietary GI was inversely related to HDL cholesterol levels, which in turn were inversely related to triglycerides, and that GI was a stronger predictor of serum HDL levels than dietary fat (Frost et al., 1999). There is evidence to suggest that insulin plays a role in stimulating acute-phase proteins (i.e., HDL) (O'Riordain et al., 1995) which have been directly related to intra-abdominal fat and inversely related to insulin stimulated glucose disposal (Sites et al., 2002). Regulating insulin levels may be important not only in diabetic patients but also in healthy subjects, as hyperinsulinemia has been directly associated with CHD in healthy populations.
1. Literature Review

Hyperinsulinemia has recently been found to moderately increase cardiovascular mortality in middle aged men (Lakka, Lakka, Tuomilehto, Sivenius and Salonen, 2000) and insulin resistance, a risk factor for CHD (Reaven, 1993), has been shown to respond to changes of the dietary GI (Frost, Keogh, Smith, Leeds and Dornhorst, 1998). Patients with a history of CHD were randomised to either a low or high GI diet (15% GI difference). After a 4 week treatment period an oral glucose tolerance test was performed following an overnight fast and a fat biopsy was obtained to assess in vitro glucose uptake in adipocytes. Less insulin was needed to handle a standard glucose challenge and increased insulin stimulated glucose uptake was observed in the low GI group, suggesting an improvement in insulin resistance (Frost, Keogh, Smith, Akinsanya and Leeds, 1996).

1.6.2.5 Glycemic index and cancer

It has been reported that Western lifestyle may promote certain types of cancers (Bruning et al., 1992) and that high intakes of energy and refined carbohydrates along with low intake of vegetables, fruit, dietary fibre, lack of physical activity, high levels of insulin-like growth factors (IGF) and hyperinsulinemia have been implicated as factors in the various types of these cancers (Giovannucci, 1999). Low GI diets have been associated with a reduced risk of endometrial cancer (Augustin et al., 2003a), breast cancer (Augustin et al., 2001), colon cancer (Slattery et al., 1997; Franceschi et al., 2001), ovarian cancer (Augustin et al., 2003b) and prostate cancer (Augustin et al., 2004), all of which may be linked with high insulin levels and IGF and reduced IGF binding proteins.
Carbohydrates are among dietary factors that influence both glucose and insulin levels and also appear related to colorectal cancer risk. The main carbohydrate classes studied are starch and sugar. Studies have reported a direct association between starch/polysaccharide intake and colorectal cancer albeit in some cases there was not a significant difference (Tuyns, Haelterman and Kaaks, 1987; Zaridze et al., 1993; Franceschi et al., 1998). A positive association of sugar intake with colorectal cancer risk has also been observed in a number of cohort and case-control studies and of these; six reported a significant increase in the risk of colorectal cancer with high sugar consumption (Macquart-Moulin et al., 1987; La Vecchia et al., 1993; Bostick et al., 1994; Centonze et al., 1994; Franceschi et al., 1997; Slattery et al., 1997). Evidence from non-dietary factors also points to possible promoting roles of insulin. Type 2 diabetes, a condition resulting from long term exposure to high insulin levels leading to insulin resistance, has been found to significantly increase colorectal and colon cancer risk by 43% and 49%; respectively, in a cohort of women (Hu et al., 1999). Other studies postulate that hyperinsulinemia and insulin resistance may also promote colorectal cancer (McKeown-Eyssen, 1994; Giovannucci, 1995). Investigations into the insulin-colon cancer hypothesis by assessing the association between postprandial blood glucose levels and subsequent colorectal cancer mortality found positive associations in men but not in women after a 12 year follow up (Levine et al., 1990). These results were not confirmed by Smith et al. (1992) who conducted a similar study involving a larger cohort and a longer follow up of 18-20 years (Smith et al., 1992). Schoen et al. (1999) found a two fold increased risk in colorectal cancer after 77 months in subjects with high
baseline fasting glucose levels and high postprandial glucose and insulin levels after 2 hours (Schoen et al., 1999).

1.6.3 Effect of fat on the metabolic syndrome

There is always a debate concerning the effect macronutrients have in relation to energy balance and obesity. Most of the concerns involve dietary fat. Dietary fat is the most concentrated source of energy while being less satiating than carbohydrate and protein and is easily stored as body fat. Dietary fat is the most energy dense macronutrient, providing approximately 38 kJ/g compared with 17 kJ/g for carbohydrate or protein. Fats offer great flavour to foods and can increase the palatability which in turn could lead to greater consumption (Willett, 1998). Diets high in saturated fat are also associated with reduced insulin sensitivity (Lovejoy and DiGirolamo, 1992; Parker et al., 1993; Marshall, Bessesen and Hamman, 1997) and an increased risk of cardiovascular disease (Hu, Manson and Willett, 2001; Wolfram, 2003) and type 2 diabetes (Marshall, Hamman and Baxter, 1991; Colditz et al., 1992; Marshall et al., 1994; Marshall and Bessesen, 2002; van Dam et al., 2002). Reducing dietary fat however, usually leads to a concomitant increase in carbohydrate intake, which can increase postprandial blood glucose levels. This is particularly so with a typical western diet, which is based on carbohydrate foods with a high GI, including potatoes, bread, and processed cereals. It has been reported that the type of carbohydrate in the diet is important, as well as a significant amount of research indicating that postprandial glycemia has particular relevance in the prevention and management of obesity and chronic disease (Jenkins et al., 2002; Ludwig, 2002). There is evidence that foods high in fat either have weak control over
appetite, or has an opposite effect and actually stimulates appetite which can lead to a positive energy balance (Lawton et al., 1993). Excess body fat is the largest nutrition related problem in many affluent countries including the United States. Excess fat can account for thirty to forty percent of heart related diseases, including the onset of adult diabetes (Colditz et al., 1995). Diet and lifestyle among the population of developed countries especially, are said to be the main factors for the high rates of excess body fat. A prime example of this was observed in Japanese men living in San Francisco where the onset of obesity was three times greater than those living in Japan (Kato et al., 1973).
1.7 Thesis Outline

Chapters 2 to 4 examine the effects of physical modification and rheological behaviour of waxy maize starch (WMS) alone and in the presence of other polymers with a view to modulation of digestion kinetics. Chapters 5 and 6 describe \textit{in vitro} approaches, including enzymatic digestion of starch and starch-protein mixtures, paralleled by the development of a novel rheological digestion cell for simultaneous measurement of viscosity and digestion in situ. Chapter 7 describes \textit{in vivo} clinical trials which are based on results obtained from modification of starch in Chapter 2 and findings from \textit{in vitro} digestions performed in Chapters 5 and 6. Results observed in Chapter 5 and 7 show that the digestion kinetics of WMS and subsequent reducing sugars can be reduced by the addition of specific proteins.
1.8 References


1. Literature Review


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minimum amount of water giving products inside the granule. 


Chapter 2

Influence of milk proteins on the pasting behaviour and microstructural characteristics of waxy maize starch

Abstract
Gelatinisation of 5 wt % waxy maize starch (WMS) under shear (16.8 s⁻¹), alone and in mixtures with 5 wt % α-lactalbumin (α-lac), β-lactoglobulin (β-lg), α-s-caseinate or β-caseinate, showed reinforcement of the starch granule structure by both caseinates, but not by the whey proteins (α-lac and β-lg). Reinforcement was evident from (i) later onset of increase in viscosity on heating; (ii) higher gelatinisation temperature by differential scanning calorimetry; (iii) micrographs showing reduced swelling during heating and in the final pastes obtained on cooling; and (iv) elimination of a characteristic "secondary swelling peak" observed for WMS immediately after completion of heating to 95°C and attributed to fracture of a restricting layer of lipid and protein at the surface of the granules. A likely mechanism of reinforcement is binding of caseinate to the lipid–protein layer. Images from confocal laser scanning microscopy with fluorescent labelling of protein showed attachment of aggregated β-caseinate to the surface of WMS granules in mixtures that had been heated (under shear) to 70°C. Corresponding images for mixtures with α-s-caseinate (which is less aggregated) showed penetration of protein to the interior of the granules, which would allow binding to occur on the inside of the surface layer as well as the outside. The inability of the more hydrophilic whey proteins to reinforce the WMS granules suggests that binding of caseinates to the lipid–protein layer occurs predominantly by hydrophobic association. The understanding that caseinates make gelatinised WMS granules smaller and tougher could be useful in product formulation.
2. Influence of dairy proteins on the pasting behaviour of WMS

2.1 Introduction

Starch and milk proteins are used together in a variety of food products ranging from processed cheese to infant formulae. Their functionality and applications are varied to include stabilisation, emulsification and structuring ability.

Bovine milk has a protein content of ~3.5 wt %. Most (~80 %) of this protein occurs in large (50 - 300 nm diameter), approximately spherical, particles known as casein micelles. The main protein constituents of the micelles are $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$- and $\kappa$-casein, with molecular weights of, respectively, 23.6, 25.2, 24.0 and 19.0 kDa (Fox and McSweeney, 1998). All caseins have a large proportion (35 - 45 %) of non-polar (hydrophobic) amino acids. The $\alpha$- and $\beta$-caseins also have a high content of phosphate, attached by ester linkages to serine residues of the protein chains. In the $\alpha$-caseins, the phosphoseryl groups are clustered around the middle of the protein chain, in a hydrophilic region which is flanked by hydrophobic sequences at the C-terminal and N-terminal ends. The corresponding hydrophilic region in $\beta$-casein is at the N-terminal end of the molecule, with a hydrophobic region at the C-terminal end. The $\alpha$- and $\beta$-caseins occur predominantly in the centre of the micelle; the $\kappa$-casein molecules lie at the surface, and have glycosylated C-terminal sequences protruding to form an expanded "hairy layer" which acts as a barrier to aggregation (Holt and Horne, 1996; Walstra, 1990).

At temperatures above ~8°C the caseins are insoluble at their isoelectric point of pH ~4.6. Sodium caseinate, the water-soluble form of casein most commonly used in food, is usually prepared by neutralisation of acid-precipitated casein.
2. Influence of dairy proteins on the pasting behaviour of WMS

with NaOH. Partial separation of the constituent caseinates can be achieved by a range of procedures, as described by Mulvihill and Ennis (2003). In addition to casein, milk contains other proteins which remain soluble at pH values low enough to cause aggregation of casein micelles; these are known as whey proteins. The principal whey proteins are β-lactoglobulin (β-lg) and α-lactalbumin (α-lac), which constitute, respectively, ~50 % and ~20 % of the total whey protein in bovine milk (Fox and McSweeney, 1998). Both have compact globular structures, and their content of hydrophobic amino acid residues is substantially lower than in caseins. On heating, β-lg undergoes partial unfolding (denaturation). Under favourable conditions of concentration and ionic environment the denatured molecules associate to form a gel (Le Bon, Nicolai, and Durand, 1999). Association is promoted by rearrangement of disulfide linkages from intramolecular to intermolecular, triggered by a free thiol group. In α-lac, which has somewhat lower molecular weight than β-lg (14.2 kDa), there is no free thiol group, and it therefore does not gel on heating. Fourier transform infrared (FTIR) spectra (Fang and Dalgleish, 1998), however, indicate that it does form some intermolecular β-sheet structure.

Starch occurs widely in nature as an energy reserve, notably in cereals, legumes, tubers and roots, and is used extensively in industry as a thickener and gelling agent (Murphy, 2000; Zobel, 1984). It consists predominantly of two polysaccharides, amylopectin and amylose. Both are homopolymers of α-D-glucose and occur together in compact assemblies known as starch granules, which have diameters about 100 times greater than those of casein micelles (5 - 40 µm, depending on botanical source). Amylose is an essentially linear polymer. Its (1 → 4)-axial-equatorial linkage geometry promotes formation of
2. Influence of dairy proteins on the pasting behaviour of WMS

coaxial double helices which, being uncharged, associate readily (Gidley, 1989; Miles, Morris, and Ring, 1985) into large aggregates that remain stable to very high temperature (~140°C). In amylopectin, the α-D-glucose residues are also predominantly (1 → 4)-linked, but with extensive branching through 1 → 6 linkages (typically 15 - 30 residues between branch points). Adjacent branches can form double helices, which can associate further into regions of crystalline order in the starch granule. The amylopectin double helices have the same geometry as those of amylose but, since their length is restricted to the distance between branch points, they are shorter and therefore less stable.

Most normal starches contain ~25 % amylose, which is predominantly disordered within the granule. However, some starches available commercially have a much higher amylose content (up to ~75 %), and waxy starches contain essentially no amylose. In addition to amylose and amylopectin, starch also contains lipids (including phospholipids) and proteins, both within the granules and attached to their surface. The amount of these materials present varies widely between different starches (Morrison, 1995; Vasathan and Hoover, 1991), but it can be as high as 0.8 wt % lipid and 0.4 wt % protein.

When starch is heated in excess water the amylopectin double helices dissociate, with accompanying loss of crystallinity, and the granules swell (by imbibing water). This process is known as "gelatinisation" of the starch. When gelatinisation is carried out under quiescent conditions (e.g. low-amplitude oscillation) the swollen granules (which are also known as granule "ghosts") adhere to one another and form a continuous gel network at volume fractions far below the onset of close packing (Hasan, Fitzsimons, O'Neill, and Morris, 2008). In normal practical applications, however, starch slurries are stirred (sheared)
during gelatinisation, and the network structure is destroyed irreversibly. For non-waxy starches, disordered amylose can leach out from the swollen granules into the surrounding liquid. Amylopectin can also be released by fragmentation of granule ghosts on continued shearing at elevated temperature (Doublier, Llamas, and Le Meur, 1987; Steeneken, 1989), resulting in a starch paste.

Pasting behaviour is, of course, central to practical applications of starch as a thickener, and is characterised routinely by changes in viscosity on heating, holding at elevated temperature, and cooling. Parameters obtained from this procedure include the "pasting temperature" (the temperature at the start of the rapid increase in viscosity that accompanies the onset of gelatinisation) and the "peak viscosity" (the maximum value attained during pasting).

Different starches differ widely in their pasting behaviour (Steeneken, 1989). It has been proposed (Debet and Gidley, 2007) that these differences arise predominantly from two factors: (i) the amylose content of the starch, and (ii) restriction of swelling by a layer of lipid and protein on the surface of the granule. The role of amylose can be explained as follows (Debet and Gidley, 2007). Dissociation of amylopectin double helices and accompanying ingress of water allows disordered amylose chains to migrate within the swollen granule and associate with one another to form a crosslinked network. Because of the thermal stability of amylose double helices and their aggregates, this newly-created network allows the swollen granules to remain intact at very high temperatures. In waxy starches (which are devoid of amylose) a similar, though less thermally-stable, network may be formed by the longest branches of the amylopectin molecules (Atkin, Abeysekera, and Robards, 1998). Network structure develops over time, as progressively more chains find partners for
double-helix formation, and is therefore promoted by slow swelling of the starch granules on heating, and limited by rapid swelling.

In an earlier study, Debet and Gidley (2006) explored the effect of surface lipids and proteins on the rate of swelling, and the stability of the resulting granule ghosts, by removing them with sodium dodecyl sulfate (SDS). This procedure had little or no effect on high-amylose starches, indicating that the high concentration of amylose allows a thermally stable continuous network to form in the early stages of gelatinisation, obscuring any additional restriction of swelling by lipids and proteins at the surface of the granule. Other starches could be classified as "rapid-swelling", with comparatively low pasting temperatures, and "slow-swelling", where increase in viscosity occurred at higher temperatures (i.e. longer times) during heating. The rapid-swelling starches gave higher peak viscosities than the slow-swelling, but the subsequent decrease in viscosity on continued shearing at high temperature was much steeper, demonstrating that the swollen granules were less resistant to fragmentation. These differences in pasting characteristics correlate with the content of lipid and protein, the slow-swelling starches having ~0.7 - 0.8 % lipid and ~0.4 % protein while the corresponding values for the rapid-swelling starches were about four times lower.

Treatment with SDS had the striking effect of converting slow-swelling starches (wheat and non-waxy maize) to rapid-swelling (i.e. with a massive decrease in pasting temperature, and massive increase in peak viscosity), but had virtually no effect on rapid swelling (tapioca) starch, which is consistent with its low content of lipid (~0.2 %) and protein (~0.1 %). Of particular relevance to the present investigation, however, waxy maize starch (WMS), which also falls in the
"rapid-swelling" category, has about the same lipid content as tapioca starch but a higher content of protein (~0.2 %), and treatment with SDS decreased its pasting temperature by ~4°C. Although much smaller than the reductions seen for normal maize and wheat starch, the response of WMS to removal of surface lipid and protein suggests that these non-carbohydrate constituents may, to a limited extent, restrict granule swelling.

Most research on mixtures of starch with milk proteins has concentrated on the effect of the starch on gelation and heat stability of the protein (e.g. Nayak et al., 2004; Oh et al., 2007; Tziboula and Muir, 1993; Vu Dang et al., 2009). In the present work we have focussed on changes in the swelling and rheology of WMS when milk proteins are present during pasting and subsequent cooling, and have investigated the origin of these changes by differential scanning calorimetry (DSC), light microscopy, and confocal laser scanning microscopy (CLSM). Preliminary accounts of some aspects of the research have been presented previously (Kett, Fitzsimons, Morris, and Fenelon, 2009; Fitzsimons, and Fenelon, 2008).

2.2 Materials and methods

2.2.1 Materials

Waxy maize starch (AMIOCA Powder TF) was kindly donated by National Starch (Manchester, UK). Protein fractions enriched (> 75 %, as per suppliers’ certificates of analysis) in α-casein or β-casein were donated by Kerry Food Ingredients (Listowel, Co. Kerry, Ireland). Urea PAGE was performed as per the method of Andrews (1983) (see Appendix 1) to confirm purity of αs- and β-caseinate enriched fractions. For simplicity, the enriched fractions are termed,
αs- and β-caseinate throughout thesis. The α-casein preparation was supplied in the acid form (i.e., as acid casein) and was converted to sodium caseinate by titration to pH 7.5 with 2 N NaOH (using an overhead stirrer). The β-casein was supplied in the sodium caseinate form and was used as received. The whey proteins α-lac and β-lg were from Davisco (Le Sueur, Minnesota). Compositional analyses for the four protein samples are given in Table 2.1. Protein content was determined by Kjeldahl analysis (International Dairy Federation, 1993) and sodium and calcium content by atomic absorption. Tabulated values of moisture and ash are taken from the suppliers’ certificates of analysis, which also specified < 1 % lactose and < 1 % fat.

2.2.2 Preparation of starch and starch–protein dispersions

WMS was studied at a fixed concentration of 5 wt %, alone and in mixtures with 5 wt % α-lac, β-lg, αs-caseinate or β-caseinate. For preparation of the mixtures, dispersions of starch and protein were prepared individually at 10 wt % in Milli-Q water, and were combined in equal amounts to give the required final concentrations of 5 wt % WMS and 5 wt % protein. Control samples of starch alone were prepared directly at 5 wt %. WMS was dispersed by magnetic stirring for at least 1 h at ambient temperature. Dispersions of protein (caseinate and whey) were prepared in the same way, left to hydrate for at least 24 h, and stored overnight in a refrigerator at 4°C before use.

2.2.3 Pasting behaviour

Samples were pasted on an AR-G2 controlled stress rheometer (TA Instruments, Crawley, UK) fitted with a starch pasting cell. The internal diameter of the cell was 36 mm, the diameter of the rotor used was 32 mm, and the gap between the
two elements at the base of the geometry was 0.55 mm. Measurements were made at a fixed shear rate of 16.8 s\(^{-1}\). After loading onto the rheometer, all samples were tempered for 1 min at 35°C, heated from 35°C to 95°C over a period of 4 min, held at 95°C for 6 min, cooled from 95°C to 35°C over a period of 4 min and held for 5 min at 35°C, giving a total elapsed time of 20 min. As shown in Figure 2.1, there was some slight thermal overshoot at the end of the heating step, and a progressive reduction in the rate of cooling as the temperature approached 35°C, but the variation of temperature with time was highly reproducible between samples and between replicate measurements of the same sample.

**Table 2.1:** Composition (g/100 g) of milk-protein samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein</th>
<th>Moisture*</th>
<th>Ash*</th>
<th>Sodium</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-lac</td>
<td>93.4</td>
<td>&lt; 6.5</td>
<td>&lt; 3.5</td>
<td>1.38</td>
<td>79.0 × 10(^{-3})</td>
</tr>
<tr>
<td>β-lg</td>
<td>92.1</td>
<td>&lt; 6.5</td>
<td>&lt; 3.5</td>
<td>2.66</td>
<td>15.0 × 10(^{-3})</td>
</tr>
<tr>
<td>α(_s)-caseinate</td>
<td>84.1</td>
<td>&lt; 11.0</td>
<td>&lt; 5.0</td>
<td>0.28</td>
<td>12.5 × 10(^{-3})</td>
</tr>
<tr>
<td>β-caseinate</td>
<td>85.6</td>
<td>&lt; 8.0</td>
<td>&lt; 5.0</td>
<td>0.27</td>
<td>23.9 × 10(^{-3})</td>
</tr>
</tbody>
</table>

*From suppliers' certificates of analysis

2.2.4 *Differential scanning calorimetry*

Differential scanning calorimetry (DSC) measurements of 5 wt % WMS, alone and with 5 wt % α\(_s\)-caseinate or β-caseinate, were made using a Setaram DSC III microcalorimeter, with Milli-Q water as thermal reference. Sample and reference pans were balanced to within 0.5 mg (typical loading ~850 mg) and heated from 50°C to 98°C at 1.0°C/min.
2. Influence of dairy proteins on the pasting behaviour of WMS

2.2.5 Light microscopy

Gelatinisation of 5 wt % WMS, alone and in mixtures with 5 wt % of each of the milk proteins, was monitored using polarised light on an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan) fitted with a heating stage (CO 102; Linkam Scientific Instruments, Tadworth, Surrey, UK). Samples of WMS and WMS–protein mixtures taken from the pasting cell immediately after completion of the final holding period at 35°C (Section 2.2.3) were viewed using differential interference contrast (DIC 60) on the same microscope. The size of granule ghosts visible in the pastes was obtained from image capture analysis by the line intercept technique, using Fovea Pro Version 3.0 image analysis plug-ins (Reindeer Graphics, Asheville, North Carolina, USA).

2.2.6 Confocal laser scanning microscopy (CLSM)

Imaging was performed using a Leica TCS SP confocal laser scanning microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany), with fluorescent labelling of either WMS or milk protein. Staining of WMS in the presence or absence of protein was achieved by incorporation of 0.15 wt % fluorescein isothiocyanate (FITC) prior to gelatinisation. Excitation of the FITC probe was made at 488 nm; emission was within the range 500 - 525 nm. The images were acquired with $2048 \times 2048$ pixel resolution in tif format using an argon laser. Fluorescent labelling of milk proteins was carried out using 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA), purchased under the proprietary name of "ATTO-TAG" from Invitrogen, Dun Laoghaire, Ireland. CBQCA reacts specifically with primary amine groups of protein molecules (Scheme 2.1) to produce highly fluorescent 7-aza-1-cyano-5,6-benzisoindoles,
2. Influence of dairy proteins on the pasting behaviour of WMS

which are maximally excited at 450 nm and have emission maxima at ~550 nm.

Samples (~10 µL) of WMS–protein mixtures and WMS control with no added protein were taken at 60°C or 70°C during the pasting procedure described in Section 2.2.3, mixed with 5 µL CBQCA solution (3 mg/mL in DMSO) on a glass slide, and incubated for 2 h at 4°C before images were acquired, with excitation of the fluorescent label by an argon laser. To obtain representative micrographs, at least three replicates of each sample were studied.

2.2.7 Statistical analysis

Statistical analysis, where mentioned, was carried out using the Minitab 15 statistical software package. One way analysis of variance (ANOVA) by Fisher's individual error rate was used to determine whether a significant difference occurred or not. The level of significance was taken as $p < 0.05$.

![Scheme 2.1: Reaction of CBQCA with primary amine groups of protein](image)
2. Influence of dairy proteins on the pasting behaviour of WMS

2.3 Results and discussion

2.3.1 Pasting of WMS with and without milk proteins

The pasting curves obtained for 5 wt % WMS, alone and in mixtures with 5 wt % α-lac, β-lg, αs-caseinate or β-caseinate, are shown in Figure 2.1. Mean values of initial viscosity, peak viscosity, and viscosities recorded at the end of the holding period at 95°C, on completion of cooling to 35°C, and at the end of the final holding period at 35°C (Section 2.2.3) are listed in Table 2.2, along with the number of replicate measurements for each sample.

After reaching a pasting temperature of ~68.5°C, the measured values of viscosity for 5 wt % WMS in water, with no added protein, increased steeply (Figure 2.1a) on continued heating, passed through a shallow maximum towards the end of the heating step, and decreased progressively on holding at 95°C. Both effects are typical of starch pasting curves (Steeneken, 1989) and arise from, respectively, swelling of the starch granules during gelatinisation and subsequent fragmentation of swollen granules under shear.

Swelling of starch granules can be characterised by the "swelling volume", defined (Steeneken, 1989) as "the equilibrium volume of 1 g of dry starch after swelling under specified conditions in an excess amount of water". At low concentrations, where water is in excess, the granules are free to expand on gelatinisation. As the concentration of starch is increased, however, a point is reached at which their combined volume becomes equal to the total volume of the system. This occurs at $c q = 1$, where $c$ is the concentration of starch in units of g/mL and $q$ is the swelling volume in mL/g. Above this point, further swelling is impossible, and paste rheology is dominated by the rigidity of the individual
swollen granules (Evans, and Lips, 1992; Steeneken, 1989; Taylor, and Bagley, 1975).

Previous studies (Abdulmola, Hember, Richardson, and Morris, 1996) gave a swelling volume of 9.65 mL/g for WMS at 80°C. A substantially higher value ($q = 18.8$ mL/g) was obtained (Steeneken, 1989) at 97°C, which is consistent with the continued increase in viscosity observed (Figure 2.1) on heating above 80°C. Even at this higher degree of swelling, however, the value of $cq$ for 5 wt % (0.05 g/mL) WMS is below 1 ($cq = 0.94$) and the swollen granules at this concentration can therefore be regarded as a dispersed phase surrounded by a continuous liquid phase. The presence of polymers (and/or other materials) in the liquid phase has no effect on swelling volume until availability of water becomes a limiting factor, which does not occur until the starch concentration exceeds ~10 wt % (Tester, and Sommerville, 2003). The gelatinised granules in the systems studied (Section 2.2.3) would therefore be free to reach maximum swelling. As shown in Table 2.2, the final viscosity of 5 wt % WMS alone on completion of pasting (Figure 2.1) was 0.64 Pa s. The maximum value observed for any of the mixtures with milk proteins was only ~36 % higher (0.87 Pa s for 5 wt % WMS with 5 wt % $\beta$-lg). It is clear, therefore, that paste viscosity of the mixtures studied is dominated by the starch component. The same conclusion was reached by Goel, Singhal, and Kulkarni (1999), who found that partial replacement of normal maize (corn) starch by casein or casein hydrolysates caused a reduction in peak viscosity. Differences in viscosity between the various WMS–protein mixtures (Figure 2.1 and Table 2.2), however, show that the overall paste viscosity is also affected by the viscosity of the continuous phase, which in turn will be affected by increase in concentration as the phase volume is decreased by
swelling of starch, and by changes in protein structure (denaturation; aggregation) during pasting. A striking and unusual feature of the pasting curves (Figure 2.1) observed for WMS alone, which was highly reproducible for 36 replicate preparations (Table 2.2), is a sharp peak in viscosity in the early stages of the holding period at 95°C, superimposed on the progressive decrease. The sharp increase in viscosity suggests additional swelling of gelatinised granules, and we will refer to this feature as a "secondary swelling peak".

Table 2.2: Viscosity (Pa s) of 5 wt % WMS, alone and in mixtures with 5 wt % of each of the milk proteins studied, at various stages of the pasting regime shown in Figure 2.1. The values tabulated are means from $n$ replicate preparations.

<table>
<thead>
<tr>
<th>Stage of pasting</th>
<th>no protein</th>
<th>α-lac</th>
<th>β-lg</th>
<th>αs-caseinate</th>
<th>β-caseinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial viscosity</td>
<td>0.014b</td>
<td>0.015b</td>
<td>0.015b</td>
<td>0.023a</td>
<td>0.024a</td>
</tr>
<tr>
<td>Peak viscosity</td>
<td>0.72d</td>
<td>0.76c</td>
<td>0.89b</td>
<td>0.87b</td>
<td>1.00a</td>
</tr>
<tr>
<td>End of holding at 95°C</td>
<td>0.49d</td>
<td>0.51c</td>
<td>0.60a</td>
<td>0.55b</td>
<td>0.43c</td>
</tr>
<tr>
<td>End of cooling at 35°C</td>
<td>0.70b</td>
<td>0.68b</td>
<td>0.90a</td>
<td>0.92a</td>
<td>0.65c</td>
</tr>
<tr>
<td>Final paste</td>
<td>0.64b,c</td>
<td>0.64b,c</td>
<td>0.87a</td>
<td>0.86a</td>
<td>0.62c,b</td>
</tr>
<tr>
<td>$n$</td>
<td>36</td>
<td>8</td>
<td>8</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Values within each row that do not share a common superscript are significantly different from one another ($p < 0.05$)
Figure 2.1: Pasting curves (pH 7.5) for 5 wt % waxy maize starch (a) alone (*) and in the presence of 5 wt % α-lac (O) or β-lg (●) and (b) alone (*) and in the presence of 5 wt % αs-caseinate (Δ) or β-caseinate (▲). Measurements of viscosity were made at a fixed shear rate of 16.8 s⁻¹. The solid lines without symbols show temperature (right-hand axes).
Secondary swelling peaks are not common. They are, however, evident in pasting curves published by Debet, and Gidley (2006) for WMS and by Trivedi et al. (2008) for WMS (described as waxy corn starch) and potato starch, but are not mentioned in the accompanying text. We propose, tentatively, that they may arise from damage to the surface layer of lipid and protein, allowing further expansion to occur (with consequent sharp increase in viscosity) and decreasing resistance to fragmentation (which would explain the sharp drop in viscosity immediately after the sharp increase). Formation of an amylose network in slow-swelling starches (Debet, and Gidley, 2007) would prevent further swelling on damage to the lipid–protein layer, which could explain why secondary swelling peaks have been observed only for rapid-swelling starches (waxy maize and potato). The secondary swelling peak was still clearly evident (Figure 2.1a) for mixtures of WMS with the whey proteins (α-lac and β-lg). Both of these caused a significant increase in peak viscosity (Table 2.2), but had no significant effect ($p < 0.05$) on pasting temperature or on the temperature-dependence of viscosity during initial gelatinisation of the starch granules. For mixtures of WMS with caseinates (Figure 2.1b), the maximum viscosity attained before the decrease caused by fragmentation was substantially higher, and the secondary swelling peak was eliminated. Both of these effects suggest that the caseinates enhance the stability of the starch granules. There was also a significant ($p < 0.05$) increase in pasting temperature, and the subsequent increase in viscosity on heating through the temperature range of the amylopectin order–disorder transition was displaced to longer times (Figure 2.1b). The effect of caseinates on gelatinisation of WMS is demonstrated more clearly in Figure 2.2a, where viscosity is shown on a logarithmic axis and is plotted against temperature rather
than time. Before gelatinisation, the mixtures of WMS with caseinates have significantly higher viscosities (Table 2.2) than WMS alone, which can be readily explained by the direct contribution of the proteins to overall resistance to flow. The subsequent increase in viscosity on heating is displaced to higher temperature than for WMS alone, by \( \sim 3^\circ C \) for mixtures with \( \alpha_s \)-caseinate and \( \sim 4^\circ C \) for those with \( \beta \)-caseinate, suggesting that both proteins interact with the starch granules to increase their resistance to swelling during gelatinisation. Elevation of the onset temperature for granule swelling and associated increase in viscosity has also been observed for mixtures of sodium caseinate with normal and waxy rice starch Noisuwan et al. (2007).

2.3.2. Differential scanning calorimetry (DSC)

Figure 2.2b shows DSC heating curves recorded for 5 wt % WMS alone and in mixtures with 5 wt % \( \alpha_s \)-caseinate or \( \beta \)-caseinate. Both caseinates displace the gelatinisation endotherm of WMS to higher temperature, by amounts closely similar to the increases in pasting temperature shown in Figure 2.2a. The same direct comparison cannot be made for mixtures of WMS with \( \alpha \)-lactalbumin or \( \beta \)-lactoglobulin, because any changes in gelatinisation temperature would be masked by the endothermic transitions arising from denaturation of the whey proteins. However, previous studies (Fitzsimons, Mulvihill, and Morris, 2008) of mixtures of starch with whey protein isolate (WPI), whose principal constituents are \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin, showed close agreement between the composite endotherms of the mixtures and curves obtained by addition of the individual DSC traces for starch alone and protein alone. It seems clear, therefore, that \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin, unlike \( \alpha_s \)-caseinate and \( \beta \)-caseinate, have no discernable effect on gelatinisation of starch.
2. Influence of dairy proteins on the pasting behaviour of WMS

**Figure 2.2:** (a) Variation of viscosity (16.8 s\(^{-1}\)) with temperature during heating (Figure 2.1) from 60 to 90°C, and (b) DSC heating curves (1°C/min) for 5 wt % waxy maize starch alone (*) and in the presence of 5 wt % \(\alpha_s\)-caseinate (Δ) or β-caseinate (▲); vertical dashed lines in (b) indicate peak-maximum temperatures (T\(_{\text{max}}\))
2. Influence of dairy proteins on the pasting behaviour of WMS

2.3.3. Self-association and viscosity of milk proteins

The changes in viscosity observed for 10 wt % α-lac, β-lg, αs-caseinate and β-caseinate when subjected to the same time–temperature regime as the WMS–protein mixtures (Figure 2.1) are shown in Figure 2.3. The curves plotted are the mean of values obtained for three replicate preparations of each sample. Although α-lac is known to undergo denaturation on heating (Fang, and Dalgleish, 1998), it lacks the free thiol group necessary to initiate rearrangement of disulfide bonds from intramolecular to intermolecular (Section 2.1) and does not therefore form long-range networks. This is reflected in its observed viscosity (Figure 2.3a), which remained low (~0.015 Pa) and almost constant throughout, with only a slight decrease during the heating step and a corresponding slight increase on cooling (as would be observed for simple liquids or solutions of small molecules). The viscosity of 10 wt % β-lg, by contrast, showed a pronounced increase (Figure 2.3a) which began during heating and continued into the holding period at 95°C. A further large increase was then observed on cooling and holding at 35°C. These changes can be readily explained by the well-documented ability of thermally denatured β-lg to associate into a gel network, which consolidates on cooling (see, for example, Le Bon et al., 1999). Although the shear applied in the pasting cell would prevent formation of a continuous network, the underlying aggregation process would be unaffected, leading to formation of microgel particles (Vu Dang et al., 2009), and consequent increase in viscosity.

The initial viscosity of 10 wt % αs-caseinate (Figure 2.3b) was higher than any of the values recorded for the other protein samples at any point in the time–temperature regime. There was an initial sharp decrease, which became less steep
towards the end of the 1 min tempering period at 35°C, followed by a further sharp decrease during the heating step, which was reversed on subsequent cooling. These observations suggest self-association of $\alpha_s$-caseinate into aggregated assemblies that are disrupted by both shear and heat, but can re-form when temperature is decreased.

The initial steep decrease was unaffected by vigorous shearing (10 min at full speed on an overhead stirrer) immediately before loading onto the rheometer: replicate traces ($n = 3$) obtained by this procedure were highly reproducible and indistinguishable from those obtained without pre-shearing. It would appear, therefore, that the aggregates of $\alpha_s$-caseinate re-form rapidly, within the time needed to load the samples onto the rheometer (~1 min), before being disrupted again by the shear imposed during the initial tempering period. As might be expected, formation of aggregates appears to be promoted by increase in concentration, since there is no evidence of high initial viscosity at 35°C for the mixtures of $\alpha_s$-caseinate with WMS (Figures 2.1b and 2.2), where the protein concentration in the continuous phase is 5 wt % rather than 10 wt % until raised by swelling of the starch granules at higher temperatures.
2. Influence of dairy proteins on the pasting behaviour of WMS

Figure 2.3: Variations in viscosity (16.8 s^{-1}; pH 7.5) with temperature (——) for (a) 10 wt % α-lac (O) and β-lg (●), and (b) 10 wt % αs-caseinate (△) and β-caseinate (▲). The time–temperature regime used was identical to that shown in Figure 2.1 for starch and starch/protein mixtures.
Much higher initial viscosity at 10 wt % (Figure 2.3b) than at 5 wt % in the unheated mixtures with WMS (Figures 2.1b and 2.2) was also seen for β-caseinate. The subsequent changes in viscosity (Figure 2.3b) were similar to those observed for αs-caseinate, though smaller: an initial reduction in viscosity during tempering, a steeper decrease as temperature was raised, and a corresponding increase on cooling. Superimposed on these changes, however, there was a large, abrupt increase in viscosity towards the end of the heating period, followed by a steep decrease. In this region, the curves obtained for β-caseinate were erratic, and far less reproducible than those of the other proteins, suggesting formation of very large aggregates which were immediately and simultaneously broken down by shear in the pasting cell.

The ability of the various caseins to associate into supramolecular assemblies when present together is evident in the micellar structure of casein in milk. Self-association of individual caseins, however, also occurs, and has been documented extensively (Dauphas et al. 2005; De Kruif, and Grinberg, 2002; De Wit, and Klarenbeek, 1984; Farrer, and Lips, 1999; Horne, 2002; Leclerc, and Calmettes, 1997, 1998; O’Connell, Grinberg, and De Kruif, 2003; Payens, and Schmidt, 1966; Pellegrino et al. 1999; Qi, Wickham, and Farrell, 2004; Romella, 1992; Schmidt, 1982; Shunrokuro, Niki, and Takase, 1979; Snoeren, van Markwijk, and van Montfoord, 1980; Thurn, Burchard, and Niki, 1987).

Association is promoted by two factors: (i) interactions between hydrophobic sequences, and (ii) binding of calcium ions to charged, hydrophilic regions. Hydrophobic association is driven by the thermodynamic advantage of minimising exposure of water to apolar groups which decrease its entropy by decreasing the number of potential partners for hydrogen bonding. Since entropy
becomes increasingly significant as temperature is raised ($\Delta G = \Delta H - T\Delta S$),
hydrophobic association is promoted by heating (Tanford, 1980). Binding of
calcium ions promotes association by reducing negative charge density, and thus
decreasing intermolecular electrostatic repulsion (which is the principal role of
colloidal calcium phosphate in stabilising native casein micelles). Suppression of
repulsion then allows self-association of hydrophilic sequences to occur by non-
covalent bonding (Van der Waals attraction, attraction between permanent
dipoles, hydrogen bonding and calcium bridging). In contrast to hydrophobic
junctions, associations between hydrophilic sequences are normally destabilised
by heat (Rees et al., 1982). The sharp increase in viscosity of $\beta$-caseinate towards
the end of the heating period (Figure 2.3b) indicates an aggregation process
initiated by hydrophobic association; the subsequent sharp decrease indicates that
thermally labile, calcium-mediated associations between hydrophilic regions are
also involved.

Both caseinate samples used in the present work contained small, but
appreciable, amounts of calcium (Table 2.1). At a protein concentration of 10 wt
%, the calcium content of the $\beta$-caseinate fraction (23.9 mg/100 g) gives a $\text{Ca}^{2+}$
concentration of 0.60 mM; the corresponding value for the $\alpha_s$-caseinate (calcium
content 12.5 mg/100 g) is 0.31 mM.

Surfactants such as SDS and soaps, which have a charged, hydrophilic head-
group and a hydrophobic tail, form micelles in which the hydrophobic sequences
are clustered together in the centre, with the hydrophilic groups at the surface. As
described in Section 2.1, $\beta$-casein molecules also have a hydrophobic end and a
hydrophilic end, and form micelles in the same way. At very low temperature
(−0°C) β-casein exists as individual molecules, but micelles are formed on heating, in response to progressive hydrophobic association. Reported values for the hydrodynamic diameter of individual micelles range from ~20 to ~31 nm (de Kruif, and Grinberg, 2002). In the presence of calcium ions, however, the micelles associate to give much larger particles with diameters extending into the μm length scale. In an investigation by dynamic light scattering, using a β-casein concentration of 1 g/L and Ca$^{2+}$ concentration of 10 mM (Dauphas et al., 2005), these large particles became detectable only above ~30°C, where micellisation was almost complete, and then increased in size up to ~1.2 μm at 50°C, the highest temperature at which measurements were made.

The α-caseins, which have two hydrophobic sequences flanking a charged, hydrophilic region (Section 2.1), do not form micelles, and their self-association is limited to development of small aggregates comprising only a few protein molecules (Dalgleish, 1997). These are formed (Schmidt, 1982) in a series of reversible processes, involving association of both hydrophobic and hydrophilic sequences. Sensitivity to pH and ionic strength (Schmidt, 1982), however, indicates that the structural integrity of the aggregates is dominated by association of the charged, hydrophilic regions, which is consistent with the decrease in viscosity on heating and corresponding increase on cooling observed (Figure 2.3) for 10 wt % α$_{s}$-caseinate in the present work. As anticipated from its progressive reduction in viscosity on heating, α$_{s}$-caseinate showed only a monotonic decrease in particle size as temperature was raised from 25 to 80°C.

The maximum in viscosity of β-caseinate (Figure 2.3b) occurred at about the same point in the time–temperature regime as the maximum in the corresponding
pasting curve (Figure 2.1b) for the mixture of β-caseinate with WMS, which could explain, at least partially, why the peak viscosity for this mixture was significantly higher (Figure 2.1 and Table 2.2) than for mixtures of WMS with the other milk proteins studied.

Substantial enhancements in viscosity on incorporation of sodium caseinate have been reported previously for WMS and normal maize (corn) starch (Lelievre, and Husbands, 1989), normal and waxy rice starch (Noisuwan et al., 2008), wheat and potato starch (Doublier et al., 1994) and normal maize starch in deionised water (Kelly et al., 1995), and for mixtures of normal maize (corn) starch with casein or casein hydrolysates (Goel, Singhal, and Kulkarni, 1999).

2.3.4. Light microscopy

The effect of milk proteins on gelatinisation of WMS was explored further using polarised light on a microscope fitted with a heating stage. As expected, 5 wt % WMS alone at 25°C (Figure 2.4a) showed intact granules with the Maltese cross pattern characteristic of ungelatinised starch. On heating to 75°C, where the gelatinisation endotherm in DSC is close to completion (Figure 2.2b), only a few intact granules remained (Figure 2.4b). Closely similar micrographs were obtained for mixtures of 5 wt % WMS with 5 wt % α-lac (Figure 2.4c) or β-lg (Figure 2.4d) at the same temperature (75°C), but far more intact granules were visible in the mixtures with 5 wt % αs-caseinate (Figure 2.4e) or β-caseinate (Figure 2.4f), and it was only when the temperature was increased further to 80°C that the micrographs obtained for these mixtures (Figures 2.4g and 2.4h) became comparable to those observed at 75°C for WMS alone (Figure 2.4b) and in mixtures with the whey proteins (Figures 2.4c and 2.4d).
Thus dissociation of amylopectin double helices, as characterised by the gelatinisation endotherm in DSC (Figure 2.2b), loss of crystallinity, shown by the maltese cross pattern of surviving intact granules, and increase in viscosity, arising from granule swelling (Figure 2.2a), are all displaced to higher temperature by the caseinates, but not by the whey proteins.

Both caseinate fractions also had a significant effect on the size of the swollen granules remaining on completion of pasting. Images were recorded by differential interference contrast microscopy for fully-pasted samples of 5 wt % WMS alone and in mixtures with 5 wt % α-lac, β-lg, αs-caseinate or β-caseinate.

Four slides were examined for each sample, and the diameters of all granule ghosts visible on each slide (typically 10 - 14) were measured using the line intercept technique of image analysis. Average values for each slide, and the overall average diameter for each sample, are listed in Table 2.3.

**Table 2.3:** Average diameter (μm) of all granule ghosts visible in each of four replicate slides for 5 wt % WMS, alone and in mixtures with 5 wt % of each of the milk proteins studied, after completion of the pasting regime shown in Figure 2.1. The global average from all four slides for each sample is shown in the last column.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Slide 1</th>
<th>Slide 2</th>
<th>Slide 3</th>
<th>Slide 4</th>
<th>All Slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18.41</td>
<td>18.01</td>
<td>18.82</td>
<td>18.27</td>
<td>18.38</td>
</tr>
<tr>
<td>α-lac</td>
<td>17.24</td>
<td>17.55</td>
<td>17.04</td>
<td>18.08</td>
<td>17.48</td>
</tr>
<tr>
<td>β-lg</td>
<td>15.08</td>
<td>17.17</td>
<td>17.26</td>
<td>16.47</td>
<td>16.50</td>
</tr>
<tr>
<td>αs-caseinate</td>
<td>10.64</td>
<td>9.63</td>
<td>9.31</td>
<td>10.09</td>
<td>9.92</td>
</tr>
<tr>
<td>β-caseinate</td>
<td>10.88</td>
<td>11.34</td>
<td>11.86</td>
<td>11.79</td>
<td>11.47</td>
</tr>
</tbody>
</table>
2. Influence of dairy proteins on the pasting behaviour of WMS

2.3.5. Confocal laser scanning microscopy (CSLM)

Figure 2.6 shows CSLM images, obtained using fluorescent labelling of starch (Section 2.2.6), for 5 wt % WMS alone (Figure 2.6a) and in the presence of 5 wt % α-lac (Figure 2.6b), β-lg (Figure 2.6c), αs-caseinate (Figure 2.6d) or β-caseinate (Figure 2.6e) after heating to 70°C (centre column of micrographs) or 95°C (right-hand column). For comparison with granule size in the heated samples, the micrographs in the left-hand column of the figure were recorded at 30°C, before the onset of gelatinisation.

Appreciable swelling of WMS in the absence of protein (Figure 2.6a) is already evident at 70°C, and is accompanied (Figure 2.2a) by the onset of increase in viscosity during pasting. Comparable swelling at this temperature can be seen for the mixtures of WMS with the whey proteins, α-lac (Figure 2.6b) or β-lg (Figure 2.6c). In the mixtures with αs-caseinate (Figure 2.6d) or β-caseinate (Figure 2.6e), however, there is far less swelling, and some of the granules appear to be still completely ungelatinised, having the same size as at 30°C. These observations agree well with the later onset of increase in viscosity (Figure 2.2a) when WMS is heated in the presence of either caseinate.
Figure 2.4: 5 wt % WMS viewed under polarised light: (a) alone at 25°C; (b) alone at 75°C; (c) with 5 wt % α-lac at 75°C; (d) with 5 wt % β-lg at 75°C; (e) with 5 wt % αs-caseinate at 75°C; (f) with 5 wt % β-caseinate at 75°C; (g) with 5 wt % αs-caseinate at 80°C; and (h) with 5 wt % β-caseinate at 80°C. Scale bar 250 µm
Figure 2.5: Images from differential interference contrast microscopy (DIC 60) recorded at 35°C, immediately after completion of the pasting regime shown in Figure 2.1, for 5 wt % WMS (a) alone, (b) with 5 wt % αs-caseinate and (c) with 5 wt % β-caseinate. Scale bar: 25 μm
At 95°C, the maximum temperature reached in the pasting studies (Figure 2.1), the images obtained for WMS alone (Figure 2.6a) and in the mixtures with α-lac (Figure 2.6b) or β-lg (Figure 2.6c) indicate extensive fragmentation of the gelatinised granules, with consequent release of amylopectin (which can be seen as an amorphous fluorescent background in the micrographs), and the remaining intact granule ghosts are swollen further than at 70°C. For the mixtures with αs-caseinate (Figure 2.6d) or β-caseinate (Figure 2.6e), the visible ghosts are smaller and less diffuse, which is consistent with the conclusion (Section 2.4) that both caseinates interact with the WMS granules to increase their structural integrity and resistance to swelling during gelatinisation. Interaction of the caseinate fractions with WMS was explored further by fluorescent labelling of protein (Section 2.2.6) in mixtures that had been heated, under pasting conditions, to 60°C or 70°C. Representative micrographs at different magnifications are shown in Figure 2.7.

Figure 2.7b shows images obtained for a mixture of 5 wt % WMS with 5 wt % αs-caseinate after heating to 60°C, which is well below the pasting temperature of WMS (Figure 2.2a). The protein, stained red in the micrographs, can be seen predominantly in the continuous phase surrounding the ungelatinised granules, which are indistinguishable from those in images obtained for WMS alone (Figure 2.7a). However, on close inspection of Figure 2.7b, particularly at the highest magnification used (right-hand frame), a thin band of red staining can be seen around the periphery of the granules. The same effect was observed recently by Noisuwan et al. (2011) in a detailed investigation of adsorption of milk proteins to ungelatinised granules of normal or waxy rice starch. Surface staining could, in principle, arise from attachment (Scheme 2.1) of the fluorescent probe
to the indigenous surface protein of the starch. However, the absence of any significant staining of the control sample of WMS with no added protein (Figure 2.7a) strongly indicates that the fluorescent layer at the periphery of the ungelatinised granules (Figure 2.7b) comes from adsorbed caseinate.

The micrographs obtained for WMS–caseinate mixtures that had been heated to 70°C (Figures 2.7c and 2.7d) were entirely different. As already shown by CLSM images obtained with labelling of starch (Figures 2.6d and 2.6e), the WMS granules had increased in size by this temperature, demonstrating that gelatinisation had begun. In the mixtures of WMS with αs-caseinate (Figure 2.7c), the protein was no longer confined to the continuous phase or granule surface, but had penetrated the swollen granules. In some cases, penetration extends to the centre of the granules. In others, the labelled protein appears as a bright, fluorescent "halo" below the outer surface. No such penetration was observed for the mixtures of WMS with β-caseinate. The protein can be seen (Figure 2.7d) as large aggregates attached to the outside of the swollen granules, or between the granules, as well as being distributed throughout the continuous phase.
Figure 2.6: Confocal laser scanning micrographs of 5 wt % waxy maize starch, (a) alone and in mixtures with 5 wt % (b) α-lac, (c) β-lg, (d) αs-caseinate or (e) β-caseinate at (i) 30°C (left-hand column), (ii) 70°C (middle column) and (iii) 95°C (right-hand column). Scale bar: 25 µm
2. Influence of dairy proteins on the pasting behaviour of WMS

Figure 2.7: Images obtained by confocal laser scanning microscopy for 5 wt % waxy maize starch (pH 7.5) after gelatinisation (a) at 60°C in the absence of protein; (b) at 60°C with 5 wt % $\alpha_s$-caseinate; (c) at 70°C with 5 wt % $\alpha_s$-caseinate; and (d) at 70°C with 5 wt % $\beta$-caseinate. Fluorescent groups attached to the proteins appear as red areas in the micrographs. Arrows in (d) indicate $\beta$-caseinate attached to the surface of swollen starch granules or in dense aggregates between the granules. Scale bars: 50 $\mu$m.
Self-association of caseins (Dalgleish, 1997) involves two mechanisms: (i) interactions between hydrophobic sequences, which are promoted by heating (Tanford, 1980), and (ii) binding of calcium ions to charged, hydrophilic regions. Both caseinate samples used in the present work contained small, but appreciable, amounts of calcium (Table 2.1). At a protein concentration of 5 wt %, the calcium content of the β-caseinate fraction (23.9 mg/100 g) gives a Ca$^{2+}$ concentration of 0.30 mM; the corresponding value for the α$_{s}$-caseinate (calcium content 12.5 mg/100 g) is 0.15 mM.

As described in Section 2.1, β-casein molecules have a hydrophobic end and a hydrophilic end, and they form micelles in the same way as surfactants such as SDS and soaps. Reported values for the hydrodynamic diameter of individual micelles range from ~20 to ~31 nm (de Kruif, and Grinberg, 2002). In the presence of calcium ions, however, the micelles associate to give much larger particles with diameters extending into the µm length scale, which is consistent with the size of the aggregates visible in Figure 2.7d. The α-caseins, which have two hydrophobic sequences flanking a charged, hydrophilic region (Section 2.1), do not form micelles, and their self-association is limited to development of small aggregates comprising only a few protein molecules (Dalgleish, 1997). These are formed (Schmidt, 1982) in a series of reversible processes, involving association of both hydrophobic and hydrophilic sequences.

There have been numerous studies of mixtures of starch with hydrocolloids (BeMiller, 2011), including gelatin and the polysaccharide thickeners and gelling agents used extensively by the food industry, and it is well established that polymers of high molecular weight cannot penetrate gelatinised starch granules. The same, however, is not true of smaller molecules. Indeed, partially-swollen
maize starch has been used successfully as a stationary phase for size-exclusion chromatography, giving useful separation of materials with molecular weights up to ~150 kDa (Lathe, and Ruthven, 1956). This upper limit is well above the molecular weight of both α-caseins and β-caseins. Exclusion of β-caseinate from partially-gelatinised WMS (Figure 2.7d) can be readily explained by the extensive aggregation discussed above and evident in Figure 2.7d, whereas for αs-caseinate, where self-association is limited and reversible, no such large aggregates are visible in the micrographs (Figure 2.7c), and the protein can penetrate the swollen granules.

2.4 Conclusions

One conclusion from this investigation, which would have been expected from previous studies (e.g. Goel et al., 1999; Nayak et al., 2004; Vu Dang et al, 2009), is that the overall viscosity of the mixtures of WMS with milk proteins is determined by both the dispersed starch phase and the surrounding continuous phase, whose viscosity is altered during pasting by increase in protein concentration as the starch granules swell and occupy more of the total volume and by temperature-dependent changes in the extent of self-association/aggregation of the protein molecules. However, at the concentrations of WMS and milk proteins we have used (5 wt % of each), the overall viscosity (Table 2.2) comes predominantly from the starch.

The second, main, conclusion is that caseinates (αs-caseinate and β-caseinate), unlike whey proteins (α-lac and β-lg), reinforce the structure of WMS granules. This reinforcement is evident in (i) later onset of increase in viscosity on heating
2. Influence of dairy proteins on the pasting behaviour of WMS

(Figure 2.2a), which implies greater resistance to swelling during gelatinisation; (ii) increase in the gelatinisation temperature obtained by DSC (Figure 2.2b); (iii) direct observation of reduced swelling both during heating (Figure 2.4) and in the final pastes obtained on cooling (Table 2.3 and Figure 2.5); and (iv) elimination of the "secondary swelling peak" (Figure 2.1) attributed to fracture of the surface layer of lipid and protein.

A likely mechanism of reinforcement is binding of caseinate to the lipid–protein layer. For β-caseinate, which forms large aggregates incapable of penetrating the swollen granules (Figure 2.7d), binding would be confined to the exterior of the surface layer. For αs-caseinate, which does penetrate the granules (Figure 2.7c), binding could also occur on the inner surface of the layer, which is consistent with the smaller diameter (Table 2.3) of granule ghosts in WMS pastes incorporating αs-caseinate. The clear evidence that the changes in behaviour of WMS when mixed with caseinates are absent, or at least far less evident, in mixtures with the more hydrophilic whey proteins (α-lac and β-lg) suggests that binding to the lipid–protein layer occurs predominantly by hydrophobic association.

In summary, caseinates make gelatinised WMS granules smaller and tougher, which could be a useful insight in formulation of products where starch and milk (or milk-protein ingredients) are used together.

2.5 Acknowledgements

We thank Teagasc, Oak Park, Carlow, Ireland for the award of a Walsh Fellowship to one of us (A.P. Kett). We also thank Professor M.J. Gidley
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2.6 References


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Chapter 3

The effect of whey proteins on the pasting properties of microfluidised waxy maize starch


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Abstract

The aim of this study was to investigate the effect of the whey protein fractions, α-lactalbumin (α-lac) or β-lactoglobulin (β-lg) on gelatinisation of microfluidised waxy maize starch suspensions. Starch (2% w/w) suspensions were subjected to 0, 2, 5 or 10 passes through an M-110 EH Microfluidiser at a pressure of 170 MPa and ambient temperature. Pasting behaviour was examined using a controlled stress rheometer equipped with a starch pasting cell. Increasing the number of passes through the microfluidiser from 0 (control) to 10 resulted in a significant ($p < 0.05$) decrease in viscosity in all samples during pasting. The addition of 4% α-lac or β-lg to either the control or microfluidised starch samples increased viscosity during gelatinisation, with the addition of β-lg having the greatest effect. Scanning electron microscopy showed that high fluidic pressure during microfluidisation caused physical disruption of the starch granules resulting in altered swelling characteristics. The extent of disruption increased with increasing number of passes.
3. Effect of whey protein addition on microfluidised WMS

3.1 Introduction

Starch is widely used as an ingredient in the food industry for applications such as thickening, gelling and stabilisation. The gelatinisation properties of starch are strongly dependent on structure and properties of its granules. Starches have been adapted and modified to reduce production costs, create new functionalities, extend product shelf life and ensure product consistency. Mechanisms for starch modification involve cross-linking (to strengthen the starch granule and improve tolerance to heat and shear), stabilisation (prevent shrinkage of the starch granule providing stability at low temperatures) and biochemical modification (produce varied viscosity and sweetness providing a range of different textural and sensory attributes) (Taggart, 2004). The rheological properties of starch are important during processing, especially when heated in a solvent such as water. Such properties depend on concentration, type of starch (native or modified), processing conditions (rate of heating and cooling on pasting) and additives (proteins, sugars, gums, salts) (Abu-Jdayil, Mohameed and Eassa, 2004; Chang, Lim and Yoo, 2004).

An alternative approach to modify the structure and hence functionality of starch is to subject it to high fluidic pressures through a process known as microfluidisation. Microfluidisation involves the transfer of mechanical energy to fluid particles under high pressure (Figure 3.1). A liquid stream is split into two micro channels in an interaction chamber and accelerated using venturi geometry; these two jets of liquid are collided at high pressure and speed, causing high shear. The high shear forces can result in physical disruption of starch granules (Guraya and James, 2002; Augustin, Sanguansri and Htoon, 2008; Kasemwong et al. 2011). Microfluidisation
3. Effect of whey protein addition on microfluidised WMS

has been used in a number of studies which include, separation of rice starch from protein (starch-protein agglomerates are physically disrupted in the presence of water and separated on the basis of density) for the preparation of low-protein starch (Guraya et al., 2002), the incorporation of resistant starches (reduction in resistant starch content, from 58% to 30% due to decreased molecular weight after microfluidisation) into processed food products (Augustin et al., 2008) and the functional properties of hydrocolloids, e.g., xanthan gum (Lagoueyte and Paquin, 1998). Altering the structural integrity of starch granules prior to gelatinisation has been shown to cause physicochemical changes and subsequent reduction of enzymatic digestion (Tester, Karkalas and Qi, 2004).

The functionality and digestibility of starch may also be altered by the addition of proteins. Studies have shown that the addition of proteins (caseinate) to starch can reduce the rate of enzyme-induced hydrolysis, as measured by the release of reducing sugars in vitro (Brennan, Kuri and Tudorica, 2004; Kett et al. 2009), and postprandial sugars in vivo (Kett et al., 2012). Whey proteins and starch can form unique mixed gels upon heating and a synergistic effect can occur as the two components are structurally compatible (Sopade et al., 2006). Proteins contain many hydrophilic groups such as -OH, -NH₂, -COOH and –SH in the alkyl side chains which can interact and form links with starch (Sopade et al., 2006).

Whey proteins are known for their broad-ranging physicochemical and biological functionalities such as solubility, foaming, gelling and health benefits (McIntosh et al., 1998) and can form gels that range in properties from viscous fluids and pastes to gels (Kinsella and Whitehead, 1989). Whey proteins form viscoelastic gels upon
heating with thermal gelation involving an initial denaturation-unfolding step followed by aggregation into a protein network by hydrophobic and sulfhydryl-disulfide interactions (Mulvihill and Kinsella, 1987; Aguilera and Rojas, 1996; Zasypkin, Braudo and Tolstoguzov, 1997). Aggregation of unfolded protein molecules occurs principally by hydrophobic and sulfhydryl-disulfide interactions (McSwiney, Singh and Campanella, 1994; Mleko and Foegeding, 1999). The proteins α-lactalbumin (α-lac) and β-lactoglobulin (β-lg) represent approximately 70% of the total protein in sweet whey. β-lg has a molecular mass of 18.3 kDa and contains 162 amino acids with one free thiol group and two disulfide bonds (Cayot and Lorient, 1997). β-lg is the main contributor to gelling behavior in whey and as such controls its rheological behaviour (McSwiney et al., 1994). Association of denatured protein molecules is promoted by rearrangement of disulfide linkages from intramolecular to intermolecular, triggered by the free thiol group (Le Bon, Nicolai and Durand, 1999).

This work focuses on the effects of microfluidisation of starch on subsequent gelatinisation during heating and shearing. The role of whey protein type in modifying the gelatinisation characteristics of starch-protein mixtures is also investigated.
3. Effect of whey protein addition on microfluidised WMS

Figure 3.1: Schematic diagram of microfluidiser showing pressure chamber, interaction chamber and heat exchanger, adapted from Kasemwong et al. (2011)

3.2 Materials and Methods

3.2.1 Materials

Waxy maize starch (trade name AMIOCA Powder TF) was kindly donated by National Starch, Manchester, U.K. The proteins α-lactalbumin (α-lac) and β-lactoglobulin (β-lg) under the trade name Biopure were sourced from Davisco Foods
International Inc. (Davisco, Le Sueur, Minnesota, USA). The protein content of the α-lac and β-lg ingredients was 93.4% and 92.1%, respectively, as determined by Kjeldahl analysis (International Dairy Federation, 1993). Fat and ash content for both α-lac and β-lg was 1.0% and 3.5% respectively, as per supplier’s certificate of analysis.

3.2.2 Experimental Design

The main aim of this work was to investigate the effect of 4% (w/w) α-lac or β-lg addition on the gelatinisation properties of 2% (w/w) microfluidised waxy maize starch suspensions. These concentrations of starch and protein are approximate concentrations in beverages and semi-solid foods. The effect of increasing the number of passes through the microfluidiser was studied to understand the impact on viscosity during processing and subsequent gelatinisation. Starch samples were passed through the microfluidiser 0, 2, 5 or 10 times. All samples, including control (0 passes), were pasted on a controlled stress rheometer equipped with a starch pasting cell.

3.2.3 Microfluidisation

Prior to microfluidisation, 400 mL of a 4% (w/w) waxy maize starch suspension was prepared in distilled water with constant stirring in a beaker for 30 min using a magnetic stirrer to prevent the starch from falling out of suspension. A sub-sample of approximately 50 mL was removed as a control (diluted to 2% w/w with distilled water) and gelatinised. The remaining 350 mL was circulated through an M-110 EH
3. Effect of whey protein addition on microfluidised WMS

Microfluidiser (Microfluidics, Newton, MA). Continuous stirring was performed in the sample vessel of the microfluidiser. Each sample was subjected to 170 MPa pressure at a flow rate of 450 mL/min. The system consisted of a ceramic F 20 Y-shaped interaction chamber with a maximum operating pressure of 200 MPa. During microfluidisation, the temperature was controlled with the aid of a tubular heat-exchanger using chilled water to cool the microfluidised product and the reaction chambers covered with ice to prevent gelatinisation of the starch.

3.2.4 Rheological evaluation of pasting behavior (viscosity)

The starch-protein mixtures were prepared at double concentrations, i.e., 4% (w/w) starch and 8% (w/w) protein and mixed to give a final concentration of 2% (w/w) starch and 4% (w/w) protein. Protein prepared at double concentration was solubilised with the aid of an overhead stirrer and allowed to hydrate at 4°C for up to 16 h. Samples were pasted on a controlled stress rheometer (AR-G2, TA Instruments, Crawley, U.K.) equipped with a starch pasting cell. The internal diameter of the cell was 36 mm and the diameter of the rotor was 32 mm. The gap between the two elements at the base of the geometry was 0.55 mm. Samples were tempered at 35°C for 2 min then heated from 35 to 95°C (ramp rate of 14.5°C/min), held for 6 min and subsequently cooled from 95 to 35°C (ramp rate of 29°C/min). All samples were analysed in triplicate.
3.2.5 Oscillatory measurements of viscoelastic moduli

Oscillatory measurements were performed using a controlled stress rheometer (AR-2000, TA Instruments, Crawley, U.K.) using a 60 mm aluminium parallel plate with a geometry gap of 0.8 mm. Samples were equilibrated at 40°C for 1 min, heated from 40°C to 90°C (ramp rate of 1°C/min) then instantly cooled to 20°C (ramp rate of 1°C/min) using a strain of 0.5% and a single angular frequency of 1 rad/s. An aluminium solvent trap was used with tetradecane to avoid loss of moisture during heating due to evaporation.

3.2.6 Scanning Electron Microscopy

Starch and starch-protein samples were freeze dried in a Freezone 6 Vacuum Freeze Dryer (Labconco, Kansas City, Missouri) to give a sufficient amount of powdered sample to be viewed using a Scanning Electron Microscope (SEM). Powder samples were analysed on a Field Emission-SEM (Zeiss Supra 40VP Gemini, Cambridge, UK) at 1.0 kV. Samples were mounted onto aluminium SEM stubs with double sided carbon tape and then sputter coated with chromium (Emitech K550X, UK) using a secondary electron detector. Representative micrographs were obtained at various magnifications.

3.2.7 Light Microscopy

Starch samples were visualised under an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan) to investigate the effects of microfluidisation on the
3. Effect of whey protein addition on microfluidised WMS

starch granules. Up to six images from different areas on the glass slide were captured to get a representative view of the starch granules from each sample.

3.2.8 Statistical analysis

Data sets were subjected to one-way analysis of variance (ANOVA) using MINITAB® 15 (Minitab Ltd, Coventry, UK) statistical analysis package. Fisher’s individual error rate was used to determine statistical differences. The level of significance was accepted at  \( p < 0.05 \).

3.3 Results and Discussion

3.3.1 Microstructural analysis

The effect of microfluidisation on the starch granules can be seen in Figure 3.2. Some of the starch granules had fragmented after 2 passes (Figure 3.2b) but the structural damage was increasingly evident after 5 passes (Figure 3.2c). Examination of Figure 3.2c shows significant evidence of mechanical rupturing with a decreased density of intact starch granules throughout the slide. These light microscopy images revealed the Maltese cross pattern was still present in intact starch granules after 5 passes providing evidence that gelatinisation of the starch granules had not occurred during microfluidisation. Heat generated within the microfluidiser due to high fluidic friction can be sufficient for gelatinisation to occur. During microfluidisation the temperature was controlled with the aid of a heat-exchanger using chilled water and the reaction chambers covered with ice to prevent gelatinisation of the starch.
3. Effect of whey protein addition on microfluidised WMS

Scanning electron micrographs (SEM) (Figures 3.3 and 3.4) show the structural fragmentation of the starch granules during microfluidisation. Figures 3.3a and b show intact starch granules and Figures 3.3 c and d show the evidence of scission of starch granules from cavitation forces caused by microfluidisation. Studies have suggested that during heating and shearing, mechanical stress along the polymer chain causes the breakdown of covalent bonds, with amylose being less susceptible to mechanical degradation than amylopectin (Van den Einde et al. 2003). It has been reported that amylopectin polymers cannot realign effectively after high shear, (Davidson et al. 1984a) and Della Valle et al. (1996) suggested that the high sensitivity of amylopectin, compared to amylose, would be due to its higher molecular weight rather than its branched structure. Thus the high amylopectin content of waxy maize starch (Whistler and BeMiller, 1997) may make it more susceptible to mechanical degradation compared to other starches with higher amylose content.

Figure 3.2 (a): Light micrographs of 2% w/w starch before microfluidisation, (b) after 2 passes through the microfluidiser and (c) after 5 passes using a Y-shaped interaction chamber at a pressure of 170 MPa with a flow rate of 450 ml/min at ambient temperature.
3. Effect of whey protein addition on microfluidised WMS

Figure 3.3: Scanning electron micrographs of WMS (2% w/w) before microfluidisation (Figures 3.3 a and b) and after microfluidisation (Figures 3.3 c and d), 2 and 5 passes respectively, at a pressure of 170 MPa with a flow rate of 450 ml/min at ambient temperature

Figure 3.4 (a): Scanning electron micrographs of 2% w/w starch before microfluidisation (on the left) and (b) after 2 passes through the microfluidiser (on the right, with subsequent addition of 4% β-lg) at a pressure of 170 MPa with a flow rate of 450 ml/min at ambient temperature
3. Effect of whey protein addition on microfluidised WMS

3.3.2 Rheological evaluation of pasting behaviour

Native starch granules are insoluble in water and remain in such a state until sufficient heat is applied. On application of heat (≥55°C) and in the presence of excess water, starch granules undergo unique and irreversible changes altering granule structure, breaking hydrogen bonds and absorbing water, resulting in granule swelling and increased viscosity (Ratnayake and Jackson, 2009). The swelling phenomenon continues until the starch granules rupture leaching amylose and amylopectin into the continuous phase, leading to a consequent reduction in viscosity (Ratnayake et al., 2009). Pasting behaviour of 2% w/w starch, 2% w/w starch in the presence of 4% w/w α-lac or 4% w/w β-lg is shown in Figures 3.5 a, b and c respectively. All three samples show similar trends on pasting, i.e., decreasing viscosity with increasing number of passes through the microfluidiser. There were significant differences \( (p < 0.05) \) in final viscosity between all passes (0, 2, 5 or 10) for the starch only samples (Figure 3.5a); the final viscosities were 0.075, 0.064, 0.057 and 0.041 Pa.s, for 0, 2, 5 and 10 passes, respectively. The addition of α-lac or β-lg to starch that had been microfluidised with 0, 2, 5 or 10 passes resulted in a significant \( (p < 0.05) \) increase in viscosity. The final viscosity was 0.101, 0.078, 0.070 and 0.054 Pa.s, for 0, 2, 5 and 10 passes, respectively for starch samples containing α-lac and 0.120, 0.085, 0.073 and 0.060 Pa.s, for those containing β-lg.

Figure 3.6 shows the effect of increasing the number of passes through the microfluidiser on the mean final viscosity for each of the samples. The starch (0 passes) and β-lg sample had the highest final viscosity of 0.120 Pa.s followed by the starch (0 passes) and α-lac sample with a viscosity of 0.101 Pa.s, followed by starch
alone with a viscosity of 0.075 Pa.s at 0 passes. This trend was also evident for 2, 5 and 10 passes. The effect of microfluidisation on the gelatinisation properties of starch can be clearly seen by the reduction in final viscosity on increasing number of passes. The mechanical shear and cavitation forces caused scission of some starch granules (Figures 3.2 and 3.3) leading to a reduction in the number of granules swelling to maximum volume resulting in a reduction in peak viscosity. WMS is characterised by its high amylopectin content and granule size is between 2-30 µm (Whistler et al., 1997). It has been reported that amylopectin may only gel at high concentrations and after long periods (Ring et al., 1987; Kalichevsky, Orford and Ring, 1990); however, amylopectin forms a weak gel directly following dissolution of WMS granules by heat as observed by rheological characterisation (Cameron et al., 1994). In our study, the presence of either α-lac or β-lg in the starch dispersion increased the viscosity upon pasting. However, the viscosity was significantly (p < 0.05) higher in starch samples pasted with β-lg (Figure 3.5c) due to its ability to aggregate on heating (Mulvihill et al., 1987) and form gels through intra- and intermolecular sulphhydryl-disulfide interactions (Le Bon et al., 1999). A similar result was observed at higher starch and protein concentrations (5% w/w starch and 5% protein) in a study by Kett et al. (2013). It has been reported that α-lac does not form aggregates on heating due to its lack of a free thiol group (Havea and Singh, 2003). Subjecting solutions (4% w/w) of α-lac or β-lg to the starch gelatinisation temperature profile (Section 3.2.4) demonstrated that β-lg resulted in a higher final viscosity compared to α-lac, with a distinct peak in viscosity after ~ 5 min of heating for the β-lg solution, indicative of protein aggregation (Figure 3.7).
3. Effect of whey protein addition on microfluidised WMS

Figure 3.5: Viscosity profile of (a) 2\% w/w starch, (b) 2\% w/w starch in the presence of 4\% w/w α-lac or (c) 2\% w/w starch in the presence of 4\% w/w β-lg which had been microfluidised (with addition of protein after microfluidisation) at a pressure of 170 MPa and a flow rate of 450 ml/min at ambient temperature for 0, 2, 5, or 10 passes during heating (35°C-95°C), holding for 6 min at 95°C and cooling (95°C-35°C) at a shear rate of 16.78 1/s. Dashed lines represent temperature profile.
Figure 3.6: Average final viscosity as a function of number of passes (0, 2, 5, 10) through the microfluidiser at a pressure of 170 MPa and a flow rate of 450 ml/min at ambient temperature for 2% starch, (-♦-), 2% starch in the presence of 4% w/w α-lac (-□-) and 2% starch in the presence of 4% w/w β-lg (-▲-)
3. Effect of whey protein addition on microfluidised WMS

3.3.3 Oscillatory analysis

The storage ($G'$) and loss ($G''$) moduli in viscoelastic solids measure the stored energy, representing the elastic portion, and the energy dissipated as heat, representing the viscous portion, respectively. Oscillatory rheological measurements (Figure 3.8 and Table 3.1) show significant ($p < 0.05$) differences between non-

Figure 3.7: Viscosity profile of 4% w/w α-lac alone (−Δ-) or 4% w/w β-lg alone (−♦-) on heating (35°-95°C), holding for 6 min at 95°C and cooling (95°-35°C) at a shear rate of 16.78 l/s. Dashed line represents temperature profile.

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3. Effect of whey protein addition on microfluidised WMS

microfluidised starch samples with or without the presence of protein at peak and final G'. Starch alone had a $G'_\text{max}$ value of 7.54 Pa followed by the starch and $\alpha$-lac with a $G'_\text{max}$ value of 3.10 Pa and finally the starch and $\beta$-lg with a $G'_\text{max}$ value of 2.12 Pa. The absence of protein in the control (2% w/w starch) allowed the starch granules to swell freely and absorb available water upon heating resulting in a significant increase in G'. The addition of protein to the starch possibly restricted the uptake of water (due to competition for available water between the starch and protein) by the starch granules on heating and subsequently reduced the swelling of the starch (Kett et al., 2009; 2013), hence the lower G' values. Starch gelatinised in the presence of $\beta$-lg had an even lower G' value compared to starch gelatinised in the presence of $\alpha$-lac. $\beta$-lg, the dominant whey protein, aggregates on heating (Mulvihill and Donovan, 1987; Mulvihill and Kinsella, 1987) and this aggregation may have trapped available water, restricting swelling of the starch granules resulting in a lower $G'_\text{max}$ value compared to $\alpha$-lac. Final G' values were significantly ($p < 0.05$) different for the three samples and follow the same order as $G'_\text{max}$.

There was a significant ($p < 0.05$) difference in onset gelatinisation temperature between the starch alone (control) and starch-protein samples. Starch alone had a significantly lower onset gelatinisation temperature compared to starch and $\alpha$-lac or starch and $\beta$-lg which required the most energy to induce gelatinisation. Similar increases in onset gelatinisation temperature were observed by Kett et al. (2009) in the presence of $\alpha_s$- or $\beta$-caseinate at higher concentrations. The onset of gelatinisation reveals information about hydration and swelling of starch granules and the presence of either whey fraction increased the temperature of initial rise in
viscosity (Table 3.1). Studies have shown that the gelatinisation temperature of starch is altered by the presence of protein on the surface of granules and also by addition of protein to starch (Sumnu et al., 1999; Sopade et al., 2006; Noisuwan et al. 2008). Furthermore, it has been reported that milk proteins affect the gelatinisation of starch by forming complexes with starch molecules on the granule surface, preventing the release of amylose/amylopectin thereby increasing gelatinisation temperature of the starch (Olkku and Rha, 1978; Kett et al., 2009, 2013).

Table 3.1: Oscillatory measurements of 2% w/w starch and 2% w/w starch in the presence of either 4% w/w α-lac or 4% w/w β-lg.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Onset gelatinisation temperature (°C)</th>
<th>Storage modulus (G') Peak (Pa)</th>
<th>Storage modulus (G') Final (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Starch</td>
<td>65.6c</td>
<td>7.54a</td>
<td>2.18a</td>
</tr>
<tr>
<td>2% Starch + 4% α-lac</td>
<td>67.9b,a</td>
<td>3.10b</td>
<td>0.69b</td>
</tr>
<tr>
<td>2% Starch + 4% β-lg</td>
<td>68.3a,b</td>
<td>2.12c</td>
<td>0.51c</td>
</tr>
</tbody>
</table>

N= 5

(a,b,c) Columns not sharing a common superscript are significantly different (p < 0.05, One Way ANOVA: Fishers Test).
3. Effect of whey protein addition on microfluidised WMS

Figure 3.8: Storage modulus (G') of 2% w/w starch (-♦-), 2% w/w starch in the presence of 4% w/w α-lac (-☐-) and 2% w/w starch in the presence of 4% w/w β-lg (-▲-) on heating from 40°C to 90°C at 1°C/min using a strain of 0.5% and a single angular frequency of 1 rad/s.
Inset: Storage modulus (G') of 5% w/w waxy maize starch on heating from 40°C to 90°C at 1°C/min using a strain of 0.5% and a single angular frequency of 1 rad/s after microfluidisation at a pressure of 170 MPa with a flow rate of 450 ml/min at ambient temperature after 5 and 10 passes
3. Effect of whey protein addition on microfluidised WMS

The inset graph shows the effect of microfluidisation on swelling during heating of a 5% w/w starch sample after 5 and 10 passes, (reliable oscillatory rheological data could not be obtained for 2% w/w starch after the same number of passes). We postulate that the physical forces and mechanical rupture of the starch granules at such a dilute concentration resulted in insufficient swelling during heating/gelatinisation to measure storage modulus reliably. Average peak storage modulus of 5% w/w starch was 37.1 Pa (data not shown in inset graph). Peak storage modulus was significantly \( p < 0.001 \) reduced after 5 passes (2.17 Pa) through the microfluidiser and after 10 passes, viscosity could not be measured accurately possibly due to the complete disintegration of the starch granule. On heating after 10 passes the starch granules were extensively ruptured, largely unable to absorb water resulting in a low \( G' \). Final storage modulus did increase slightly at 90°C to a value of 0.182 Pa.

3.4 Conclusions

Microfluidisation at 2, 5, or 10 passes caused fragmentation/disintegration of waxy maize starch granules with the extent of disruption reflected in a significant decrease in viscosity on increasing microfluidisation passes. Addition of either 4% w/w \( \alpha \)-lac or 4% w/w \( \beta \)-lg increased final viscosity on pasting in all microfluidised starch samples (0, 2, 5 and 10 passes) with the addition of \( \beta \)-lg having the greatest effect.
3. Effect of whey protein addition on microfluidised WMS

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Chapter 4

The effect of konjac glucomannan addition on the pasting properties of waxy maize starch under different pH conditions

4. The effect of KGM addition on pasting properties of WMS

Abstract

Acid hydrolysis of starch disrupts its granular structure by cleaving glycosidic bonds altering the functional and rheological properties of starch. Waxy maize starch (5% w/w) with or without the addition of 0.1% w/w konjac glucomannan (KGM), adjusted to varying pH in the range 2 to 9, was gelatinised in a starch-pasting cell attached to a controlled stress rheometer to elucidate the effect of pH on the rheological properties of starch and starch-KGM mixtures. The presence of KGM increased viscosity of the starch pastes. Confocal laser scanning microscopy (CLSM) verified differences in granule size and morphology at varying temperatures and pH. Granule size at the end of pasting was smaller when the pH of the starch was adjusted to 3 compared to pH 7 or 9 at 65°C. The addition of KGM also resulted in a reduction in granule size measured at the end of pasting, with phase separation occurring at all pH’s, i.e., pH 3 to 9. It was found that decreasing pH significantly ($p < 0.05$) lowered peak viscosity of the starch during pasting with the emergence of a secondary peak in viscosity on heating in acidic conditions and clearly shown. It was clearly shown that, while reducing granule size, KGM increased viscosity throughout pasting at higher pH values, while reducing the pH to values less than 3 resulted in a significant decrease in paste viscosity.
4. The effect of KGM addition on pasting properties of WMS

4.1 Introduction

Starch is one of the most important carbohydrates in foods. It is an ingredient used in a range of food types from soups and sauces as a thickening agent to more complex systems such as infant formulae. Starch consists of two types of polymers, amylose, a linear polymer of α-D-glucose units linked by α-1,4 glycosidic bonds and amylopectin, a branched polymer of α-D-glucose units linked by α-1,4 and α-1,6 glycosidic bonds. The molecular structure of starch granules includes an arrangement of amorphous (amylose and branching points) and crystalline (amylopectin) lamella encapsulated in a layered semi-crystalline growth ring (Miao et al., 2011). The viscosity of starch pastes is dependent on the number of glucose chains leaching from the starch granule and the changes in the size of starch granules (Hirashima, Takahashi, and Nishinari, 2005). When the pH of aqueous starch suspensions is decreased, acid hydrolysis breaks long polysaccharide chains into smaller chains or into simple carbohydrates resulting in degradation of amylopectin (Hoover, 2000).

It has been reported that the addition of hydrocolloids increases the viscosity of starch (Christianson et al., 1981; Achayuthakan and Suphantharika, 2008; Funami et al., 2008; Wang et al., 2009). Konjac glucomannan (KGM) is a neutral polysaccharide found in roots and tubers of the Amorphophallus konjac plant. KGM has D-mannose (M) and D-glucose (G) in M/G molar ratio of 1.5-1.6 by β-1,4 glycosidic linkages with approximately one acetyl group every 17-19 sugar units at the C6 position (Liu and Xiao, 2004). It is widely used in China and Japan as a health food (Xiao, Gao, and Zhang, 2000). Native and modified (physically and
chemically) KGM has found application in a range of fields including pharmaceutical, chemical engineering and food formulation (Xiao, Gao, and Zhang, 2000). KGM is used as a food additive because of its ability to contribute to viscosity when dissolved in water at relatively low concentrations (Nishinari, Williams, and Phillips, 1992). It has been reported that KGM can be stable under acidic conditions albeit with minor changes occurring structurally. Infrared spectroscopy has shown that nanocrystals after acid hydrolysis of KGM were still composed of mannose and glucose as in native KGM but the amorphous state had changed to a highly crystalline state (Huang, Gao, and Shen, 2010).

Acidic modification of starch is usually performed using hydrochloric (HCl) or sulfuric (H$_2$SO$_4$) acid causing scission of the glycosidic linkages altering the granular structure of native starch (Hoover, 2000). Depending on the type of acid used, different end products are produced; hydrolysis with HCl (as in this study) produces lintnerised starches while hydrolysis with H$_2$SO$_4$ produces Nageli amylodextrins (Gérard et al., 2002; Hoover, 2000; Miao et al., 2011). In industry, acid modified starches are prepared by treating a starch suspension (30-40% w/w) with dilute HCl or H$_2$SO$_4$ at lower temperatures than those required for gelatinisation (normally 25-55°C) for various time periods (Hoover, 2000). When the starch suspension reaches the desired viscosity the paste is neutralised, and the granules are recovered by washing, centrifuging and subsequent drying. Industrial uses for acid-hydrolysed starches range from preparation of starch gum candies and manufacture of gypsum board for dry wall construction to paper and paperboard manufacture (Solarek, 1987). Pre-gelatinised starch (maize) is used in anti-reflux
4. The effect of KGM addition on pasting properties of WMS

Infant formulas to prevent regurgitation, a common problem in newborns. Partial pre-gelatinisation of the starch and subsequent drying results in a cold water swelling starch.

The objectives of this Chapter were (i) to determine the effect of KGM on the pasting properties of waxy maize starch under different pH conditions and (ii) to determine the impact of pH on rheological and microstructural properties of waxy maize starch during pasting.

4.2 Materials and methods

4.2.1 Materials

Waxy maize starch (WMS, trade name AMIOCA Powder TF) was kindly donated by National Starch (Manchester UK). Konjac glucomannan (KGM) was sourced from Deoxy Ltd. Cork, Ireland. All solutions were prepared in distilled deionised water.

4.2.2 Experimental Design

Two sets of experiments were performed to analyse the effect of pH (in the range pH 2 to 9) on the pasting properties of waxy maize starch. The first series was performed in the absence of KGM while the second series was performed in the presence of 0.1% KGM.
4. The effect of KGM addition on pasting properties of WMS

4.2.3 Sample Preparation

The concentration of waxy maize starch (5% w/w) and KGM (0.1% w/w; if used) in the mixtures was held constant throughout testing. Gelatinisation was performed on an aqueous starch suspension (5% w/w) in the absence or presence of KGM (0.1% w/w) between pH 2 and 8. pH was adjusted by adding different quantities of 0.1 N HCl or NaOH to the starch suspension under continuous agitation using an overhead stirrer followed by equilibration for 30 min at 20ºC. Starch and starch-KGM samples were readjusted, if necessary, to the target pH, and aliquots (28 g) were weighed into the starch pasting cell to undergo the pasting regime described in Section 4.2.4.

4.2.4 Rheological Behaviour during Pasting

Samples were gelatinised on a controlled stress rheometer (AR-G2, TA Instruments, Crawley, UK) fitted with a starch pasting cell. The internal diameter of the cell was 36 mm and the diameter of the rotor was 32 mm with a geometry gap of 0.55 mm. Samples were tempered at 35ºC then heated from 35ºC to 95ºC at a ramp rate of 14.5ºC/min, held for 6 min and cooled from 95ºC to 35ºC at a ramp rate of 29ºC/min and a shear rate of 16.78 1/s.

4.2.5 Differential Scanning Calorimetry (DSC)

Thermal gelatinisation of starch suspensions (5% w/w) in the absence or presence of 0.1% KGM, was performed using a DSC Q2000 (TA Instruments, Crawley, UK) at pH 2, 3, 7 and 9. Starch suspensions (20 µl) were weighed into the pans, hermetically sealed to avoid water loss through evaporation and allowed to
4. The effect of KGM addition on pasting properties of WMS

equilibrates for 30 min before analysis. Samples were scanned at 5°C/min from 25°C
to 95°C. An empty pan was used as a reference. Starch dry weight was determined
by weighing the perforated, oven-dried pans after analysis. The DSC was calibrated
by use of indium standards. Experiments were performed in triplicate. The
parameters, onset gelatinisation temperature (T₀), peak gelatinisation temperature
(Tₚ) and final gelatinisation temperature (Tₙ) were calculated from the analysis.

4.2.6 Confocal Laser Scanning Microscopy (CLSM)

An aqueous solution (0.5 g/100 g) of Nile blue (Sigma-Aldrich, Ireland) was
prepared to label the starch. An aliquot (20 µL) of the fluorescent staining solution
was applied to the surface of the starch and starch-KGM mixtures after transferring
to the microscope slides. Nile blue is a highly fluorescent dye which has been found
to fluoresce (and thus label) when excited in the 670-740 nm region (Green, 1990).
The dye can be used in the pH range 2-14. Transmitted light differential interference
contrast (DIC) was used in combination with CLSM. Imaging was performed using
a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems,
Heidelberg GmbH, Mannheim, Germany) using a 63X oil immersion objective.
Images (24 bit), 512 x 512 pixels in size, were acquired. A minimum of 4 z-stacks
were taken per sample with representative cross sections of micrographs shown.

4.2.7 Statistical Analysis

Statistical analysis where mentioned was performed using the Minitab 15 statistical
software package. One way analysis of variance (ANOVA) by Fisher's individual
error rate was used to determine significant differences. The level of significance was taken as $p < 0.05$.

### 4.3 Results and Discussion

#### 4.3.1 Effect of KGM addition and pH on pasting behaviour

The pasting behaviour of 5% WMS over the pH range 2 to 8 is shown in Figure 4.1., with parameters given in Table 4.1. Viscosity of WMS samples adjusted to various pH values in the range 4 to 8 increased at a similar rate as the temperature was increased from 35º to 95ºC. A second peak can be observed at the start of the holding interval at 95ºC (see Figures 4.1 and 4.2) for all samples regardless of pH or the presence of KGM. The initial decrease in viscosity observed after peak viscosity is a result of the amount of granules rupturing exceeding those swelling; therefore, the appearance of the secondary peak suggests some delayed granule swelling. This secondary peak during pasting of WMS was also observed in Chapter 2; Figure 2.1, where it is hypothesised that the effect may be due to damage of the surface layer of lipid and protein on WMS allowing further expansion to occur and hence the second peak in viscosity as shown in Figures 4.1 and 4.2 in the current Chapter. The size of this second peak was even more pronounced when KGM was present in the starch samples during pasting (compare Figure 4.2 with Figure 4.1). This is possibly due to increased competition for available water between the starch granules and KGM on heating. The addition of KGM resulted in an increase in viscosity throughout the pasting profile of WMS (compare Table 4.2 with Table 4.1). It is well documented that the addition of hydrocolloids to starch increases viscosity (Mali et al., 2003). Similar increases in viscosity during pasting of yam starch-xanthan gum and yam...
starch-guar gum mixtures were observed by Mali et al. (2003). Moreover, on pasting at pH 3 there was a rapid decrease in viscosity once the secondary peak was achieved on holding at 95°C with or without KGM (Figures 4.1 and 4.2). The decrease in viscosity continued during the holding step at 95°C and cooling with a viscosity plateau on holding at 35°C. An even greater decrease in viscosity was observed at pH 2, albeit the initial peak viscosity was lower compared to those samples at higher pH’s (Figure 4.1, Table 4.1). In fact, there was a significant ($p < 0.05$) difference in viscosity observed at pH 2 throughout pasting compared to all other pH values (Table 4.1); on pasting at pH less than 4, peak, end of heating, end of cooling and final viscosity all decreased significantly ($p < 0.05$) compared to equivalent viscosities greater than pH 4 (Table 4.1). Similar results were observed by Hirashima et al. (2005) in cornstarch pastes below pH 3.6. It is well established that acidic modification of starch influences its pasting behaviour. This has been shown by the significant difference observed in WMS between pH 3 and 2 (Table 4.1). The starch samples and starch-KGM samples at pH 2 had almost identical end of heating, end of cooling and final viscosity which may imply that the addition of KGM at pH 2 had no effect on the realignment of the amylopectin polymers during cooling (as reported in Tables 4.1 and 4.2).

Interestingly, peak and final viscosity of the starch-KGM samples decreased significantly ($p < 0.05$) between pH 7 and pH 8 (Table 4.2). Similar observations were reported for the peak viscosities of wheat and corn starch on increasing pH (Campbell et al., 1980; Dengate, 1988). Studies have shown that small fractions of amylopectin molecules are degraded at pH 9 (Sae-kang and Suphantharika, 2006).
Moreover, in terms of syneresis and textural properties, tapioca starch pastes at pH 9 are comparable with those at pH 7 (Sae-kang and Suphantharika, 2006). This observation agrees with results observed in this study as there was no significant difference in final viscosity in WMS (at 5% w/w) between pH 7 and pH 8 (Table 4.1).

Table 4.1: Viscosity of 5% waxy maize starch dispersions at pH in the range 2.0 to 8.0 at various stages of the pasting regime

<table>
<thead>
<tr>
<th>pH</th>
<th>On-set pasting temperature</th>
<th>Initial viscosity</th>
<th>Peak viscosity on heating</th>
<th>End of heating viscosity</th>
<th>End of cooling viscosity</th>
<th>Final viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(°C)</td>
<td>(Pa.s)</td>
<td>(Pa.s)</td>
<td>(Pa.s)</td>
<td>(Pa.s)</td>
<td>(Pa.s)</td>
</tr>
<tr>
<td>2.0</td>
<td>72.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.03&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.0</td>
<td>70.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.0</td>
<td>70.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.0</td>
<td>70.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.0</td>
<td>70.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.0</td>
<td>70.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8.0</td>
<td>70.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>(a-f) Values within a column with different superscripts are significantly (<i>p</i> < 0.05) different.</sup>
4. The effect of KGM addition on pasting properties of WMS

**Figure 4.1**: Gelatinisation profiles of 5% waxy maize starch in the pH range 2 to 8. (pH 2 -♦-, pH 3 -○-, pH 4 to 8 -■-). Dashed line represents temperature profile.

**Table 4.2**: Viscosity of 5% waxy maize starch dispersions with added 0.1% KGM at pH in the range 2.0 to 8.0 at various stages of the pasting regime

<table>
<thead>
<tr>
<th>pH</th>
<th>On-set pasting temperature (°C)</th>
<th>Initial viscosity (Pa.s)</th>
<th>Peak viscosity on heating (Pa.s)</th>
<th>End of heating viscosity (Pa.s)</th>
<th>End of cooling viscosity (Pa.s)</th>
<th>Final viscosity (Pa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>68.5(^a)</td>
<td>0.01(^a)</td>
<td>0.69(^c)</td>
<td>0.03(^c)</td>
<td>0.03(^f)</td>
<td>0.03(^d)</td>
</tr>
<tr>
<td>3.0</td>
<td>68.5(^a)</td>
<td>0.01(^a)</td>
<td>0.79(^a)</td>
<td>0.27(^b)</td>
<td>0.48(^e)</td>
<td>0.46(^c)</td>
</tr>
<tr>
<td>4.0</td>
<td>68.5(^a)</td>
<td>0.01(^a)</td>
<td>0.76(^a)</td>
<td>0.47(^a)</td>
<td>0.79(^e,b)</td>
<td>0.73(^a)</td>
</tr>
<tr>
<td>5.0</td>
<td>68.5(^a)</td>
<td>0.01(^a)</td>
<td>0.77(^a)</td>
<td>0.50(^a)</td>
<td>0.83(^a)</td>
<td>0.76(^a)</td>
</tr>
<tr>
<td>6.0</td>
<td>68.5(^a)</td>
<td>0.01(^a)</td>
<td>0.78(^a)</td>
<td>0.49(^a)</td>
<td>0.80(^b,c)</td>
<td>0.75(^a)</td>
</tr>
<tr>
<td>7.0</td>
<td>66.5(^a)</td>
<td>0.01(^a)</td>
<td>0.77(^a)</td>
<td>0.48(^a)</td>
<td>0.80(^b,c)</td>
<td>0.74(^a)</td>
</tr>
<tr>
<td>8.0</td>
<td>66.5(^a)</td>
<td>0.01(^a)</td>
<td>0.74(^b)</td>
<td>0.47(^a)</td>
<td>0.75(^d)</td>
<td>0.70(^b)</td>
</tr>
</tbody>
</table>

\(^{(a-g)}\) Values within a column with different superscripts are significantly \((p < 0.05)\) different.
4. The effect of KGM addition on pasting properties of WMS

**Figure 4.2:** Gelatinisation profiles of 5% waxy maize starch in the presence of 0.1% KGM gelatinised in the pH range 2-8 (pH 2 -♦-, pH 3 -○-, pH 4 to 8 -■-). Dashed line represents temperature profile.

Table 4.3 shows that on reducing pH from pH 3.9 and pH 3, the final viscosities of 5% starch decreased progressively. At pH less than 4, the long amylopectin chains are hydrolysed at the intercrystalline regions to form segments of linear and smaller branched chains (Hoover, 2000). The resulting hydrolysis products are assigned dextrose equivalent (DE) values. The value of DE is related to the degree of hydrolysis. A DE value of 100 corresponds to complete hydrolysis of starch into pure glucose (Hoover, 2000).

The effect of pH change (in the range pH 4 to 3) on gelatinisation on the final viscosity of the starch and starch-KGM mixture is shown in Figure 4.3. Most of the starch granules are broken and the amylopectin degraded below pH 3. Similar results were reported by Wang *et al.* (2001) and Sae-Kang *et al.* (2006). The difference in
viscosity in the pH range of 3 to 2 was much greater on pasting of both starch alone and starch-KGM in peak, end of heating, end of cooling and final viscosities compared to the samples in the pH range of 3 to 9. At pH 2, the amylopectin chains are broken into simple carbohydrates. Acid hydrolysis of starch decreases molar mass and consequently viscosity, thereby increasing solubility of the starch granule (Whistler and Daniel, 1990).

Table 4.3: Viscosity of 5% waxy maize starch dispersions at pH in the range 3.0 to 4.0 at various stages of the pasting regime

<table>
<thead>
<tr>
<th>pH</th>
<th>On-set pasting temperature (°C)</th>
<th>Initial viscosity (Pa.s)</th>
<th>Peak viscosity on heating (Pa.s)</th>
<th>End of heating viscosity (Pa.s)</th>
<th>End of cooling viscosity (Pa.s)</th>
<th>Final viscosity (Pa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.65b</td>
<td>0.18d</td>
<td>0.26i</td>
<td>0.25g</td>
</tr>
<tr>
<td>3.1</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.65b</td>
<td>0.21h</td>
<td>0.33h</td>
<td>0.32f</td>
</tr>
<tr>
<td>3.2</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.66b</td>
<td>0.25g</td>
<td>0.42g</td>
<td>0.39e</td>
</tr>
<tr>
<td>3.3</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.65b</td>
<td>0.29f,e</td>
<td>0.47f</td>
<td>0.44d</td>
</tr>
<tr>
<td>3.4</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.67</td>
<td>0.30c,f</td>
<td>0.50e</td>
<td>0.47c</td>
</tr>
<tr>
<td>3.5</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.67</td>
<td>0.37d</td>
<td>0.59d,c</td>
<td>0.54b</td>
</tr>
<tr>
<td>3.6</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.67</td>
<td>0.39c</td>
<td>0.60d,c,d</td>
<td>0.54b</td>
</tr>
<tr>
<td>3.7</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.67</td>
<td>0.43b</td>
<td>0.66b</td>
<td>0.59a</td>
</tr>
<tr>
<td>3.8</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.68</td>
<td>0.45a</td>
<td>0.68a</td>
<td>0.60a</td>
</tr>
<tr>
<td>3.9</td>
<td>70.5a</td>
<td>0.01a</td>
<td>0.69a</td>
<td>0.45a</td>
<td>0.68a</td>
<td>0.61a</td>
</tr>
</tbody>
</table>

Values within a column with different superscripts are significantly (p < 0.05) different.
4. The effect of KGM addition on pasting properties of WMS

![Figure 4.3: Viscosity of 5% waxy maize starch alone (-▲-) and in the presence of 0.1% KGM (-Δ-) gelatinised in the pH range of 3-4](image)

**Figure 4.3:** Viscosity of 5% waxy maize starch alone (-▲-) and in the presence of 0.1% KGM (-Δ-) gelatinised in the pH range of 3-4

### 4.3.2 Differential Scanning Calorimetry (DSC)

The thermal properties of WMS alone and in the presence of KGM in water were measured by DSC at pH 2, 3, 7 and 9. The thermal characterisation showed no significant \((p < 0.05)\) differences for transition temperatures (onset, peak and final) between pH 9 and pH 3 but between pH 3 and pH 2 there is a significant \((p < 0.05)\) difference in all transition temperatures in both starch alone and in the presence of KGM. The hydrolysis of the starch polymers at such acidic conditions (pH 2-3) affects the gelatinisation properties of starch (alone and in the presence of KGM). At pH 2, the transition temperature data suggest that even the presence of KGM has no effect on onset, peak or final gelatinisation temperature compared to starch alone as seen in Table 4.4 and 4.5. Similar results were observed by Mali *et al.* (2003) who studied the influence of pH on yam starch pastes. Studies on extreme sample
4. The effect of KGM addition on pasting properties of WMS

preparation (treatment of waxy rice starch with 2.2 M HCl for 45 days) have been
performed by Shi and Seib (1992) which were more extreme than the treatment
applied in this study and they reported a decrease in gelatinisation temperature and
less energy requirement for gelatinisation of waxy rice starch (Shi and Seib, 1992).

Table 4.4: Onset gelatinisation temperature ($T_o$), peak gelatinisation temperature
($T_p$), final gelatinisation temperature ($T_f$) and gelatinisation enthalpy ($\Delta H$) of 5%
waxy maize starch dispersions at pH in the range 2 to 9

<table>
<thead>
<tr>
<th>pH</th>
<th>$T_o$ (°C)</th>
<th>$T_p$ (°C)</th>
<th>$T_f$ (°C)</th>
<th>$\Delta H$ (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>67.52$^a$</td>
<td>71.88$^a$</td>
<td>75.13$^a$</td>
<td>12.88$^a$</td>
</tr>
<tr>
<td>3.0</td>
<td>66.55$^b$</td>
<td>70.97$^b$</td>
<td>74.06$^b$</td>
<td>13.00$^a$</td>
</tr>
<tr>
<td>7.0</td>
<td>66.80$^b$</td>
<td>70.80$^b$</td>
<td>74.13$^b$</td>
<td>11.90$^b$</td>
</tr>
<tr>
<td>9.0</td>
<td>66.35$^b$</td>
<td>70.90$^b$</td>
<td>74.49$^b$</td>
<td>12.31$^b$</td>
</tr>
</tbody>
</table>

$^{(a-d)}$ Values within a column with different superscripts are significantly ($p<0.05$)
different.

Table 4.5: Onset gelatinisation temperature ($T_o$), peak gelatinisation temperature
($T_p$), final gelatinisation temperature ($T_f$) and gelatinisation enthalpy ($\Delta H$) of 5%
waxy maize starch in the presence of 0.1% KGM at pH in the range 2-9

<table>
<thead>
<tr>
<th>pH</th>
<th>$T_o$ (°C)</th>
<th>$T_p$ (°C)</th>
<th>$T_f$ (°C)</th>
<th>$\Delta H$ (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>67.46$^a$</td>
<td>72.03$^a$</td>
<td>75.75$^a$</td>
<td>12.69$^b$</td>
</tr>
<tr>
<td>3.0</td>
<td>66.29$^b$</td>
<td>71.00$^b$</td>
<td>74.50$^b$</td>
<td>12.54$^b$</td>
</tr>
<tr>
<td>7.0</td>
<td>66.56$^b$</td>
<td>71.04$^b$</td>
<td>74.59$^b$</td>
<td>14.28$^a$</td>
</tr>
<tr>
<td>9.0</td>
<td>66.44$^b$</td>
<td>71.20$^b$</td>
<td>74.77$^b$</td>
<td>13.13$^b$</td>
</tr>
</tbody>
</table>

$^{(a-d)}$ Values within a column with different superscripts are significantly ($p<0.05$)
different.
4. The effect of KGM addition on pasting properties of WMS

4.3.3 Confocal Laser Scanning Microscopy (CLSM)

CLSM images were taken at different stages within the pasting temperature profile (Section 4.2.4) to observe the effect of KGM addition at pH 3, 7 and 9 on the microstructure of WMS. Starch granules were observed before pasting (Figure 4.4a), during pasting at 65°C (Figure 4.4b), after pasting (Figure 4.4c) and with added KGM during pasting at 65°C (Figure 4.4d) and after pasting (Figure 4.4e). Intact starch granules can be clearly seen in Figure 4.4a (only shown for pH 3 and 9). On heating to 65°C at pH 7 the granules appear swollen (Figure 4.4b) with the remains of ‘granule ghosts’ cells clearly evident after pasting (Figure 4.4c). Visual analysis of the CLSM images would suggest that the addition of KGM did not affect granule size during or at the end of pasting at pH 3, 7 or 9 (Figures 4.4d and 4.4e). However, KGM appears to have led to phase separation on completion of pasting as shown by the dark regions in Figure 4.4e at all pH’s; most likely pockets of KGM embedded within the continuous phase of ruptured WMS granules/amylopectin.

WMS adjusted to pH 3, with or without KGM present after pasting, (Figures 4.4c and 4.4e; see left hand column) appeared to have a greater degree of gelatinisation compared to the same samples at pH 7 (middle column) or pH 9 (right hand column). This result may be in agreement with the lower pasting viscosities for WMS and WMS in the presence of KGM at pH 3 after heating shown in Figures 4.1 and 4.2. The amorphous regions of starch granules are more susceptible to acid hydrolysis than the crystalline regions (Hoover, 2000), and with WMS primarily composed of amylopectin (Kulp, 1975), thus may have resulted in an increased resistance to acid hydrolysis. The granular structure of the starch observed in Figure
4. The effect of KGM addition on pasting properties of WMS

4.4b at pH 9 (right hand column) heated to 65°C would suggest that the swelling is more advanced compared to that at pH 7. Interestingly, on completion of pasting of the WMS (Figure 4.4c) there was some granular structure evident at pH 7 (middle column) and pH 9 (right hand column) but very little evidence of granular structure at pH 3 (left hand column). Even though the crystalline region of starch is more resistant to acid hydrolysis, the acidic conditions, coupled with the temperature regime (Section 4.2.4) applied, may have completely gelatinised the starch resulting in complete loss in granular structure.

The CLSM image in Figure 4.4b of 5% starch at pH 7 (middle column) heated to 65°C shows partially gelatinised and ruptured starch granules compared to Figure 4.4d in the presence of 0.1% KGM at pH 7 heated to 65°C. The starch granules heated to 65°C in the presence of KGM appear intact and smaller in size. At this neutral pH, the smaller granule size may be due to competition for available water between the starch and KGM. The addition of hydrocolloids such as KGM to starch has been reported to increase competition for available water and as a consequence reduce the size of starch granules increasing granular integrity (Funami et al., 2005; Khanna and Tester, 2006; Kruger, Ferrero, and Zaritzky, 2003; Song et al., 2006; Tester and Sommerville, 2003).
4. The effect of KGM addition on pasting properties of WMS

Figure 4.4: Confocal scanning laser micrographs of 5% waxy maize starch, (a) before pasting, (b) at 65°C during pasting, (c) after pasting (d) in the presence of 0.1% KGM at 65°C during pasting, (e) in the presence of 0.1% KGM after pasting, Left hand column = pH 3, Middle column = pH 7 and right hand column = pH 9. Scale bar: 50 µm.
4.4 Conclusions

Pasting of WMS at pH 2 significantly reduced peak viscosity during heating compared to that of the same pastes in the pH range 3-8. The lower viscosity may be attributed to acidic hydrolysis of the glycosidic bonds within the starch, assisted by heating during pasting, thus reducing the ability of polymer realignment during cooling. The addition of KGM increased peak viscosity of all WMS pastes, in the pH range 2-8. The presence of a secondary peak in the pasting profiles of WMS on heating at 5% concentration was identified by this study. This secondary peak in viscosity was more pronounced in starch pastes adjusted to pH 2, either with or without the presence of KGM, compared to 5% starch alone.

In conclusion, KGM at the concentration used in this study, i.e., 0.1%, disrupted the development of starch polymer networks during pasting and subsequent cooling through phase separation.

4.5 Acknowledgements

The authors would like to acknowledge the National Food Imaging Centre, Teagasc Food Research Centre, Moorepark, Co. Cork for access to the confocal laser scanning microscopy facilities.
4. The effect of KGM addition on pasting properties of WMS

4.6 References


4. The effect of KGM addition on pasting properties of WMS


4. The effect of KGM addition on pasting properties of WMS

(Dioscorea alata) starch pastes stability. *Food Science and Technology*, 36, 475-481.


4. The effect of KGM addition on pasting properties of WMS


Chapter 5

Simulated gastro-intestinal digestion of starch-protein mixtures and isolation of peptides involved in the modulation of GLP-1 and GIP

Abstract

This study demonstrated that caseinates have the ability to alter the digestion properties of waxy maize starch while the composition of peptide material in protein digests can result in different cellular response of incretin hormones in vitro.

Bovine αs- or β-caseinate were subjected to simulated gastro-intestinal digestion alone or in the presence of waxy maize starch and their resulting peptide material assessed for glucagon-like peptide-1 (GLP-1) and glucose-independent insulinotropic polypeptide (GIP) release in vitro using bio-assays. The protein digests were fractionated and the more hydrophobic fractions of αs-caseinate resulted in secretion of greatest levels of GLP-1 while the less hydrophobic fractions of αs- or β-caseinate induced greatest secretion in GIP. Using high performance liquid chromatography (HPLC), it was shown, that caseinate proteins/peptides can reduce the release of the reducing sugars maltose and glucose from starch.
5. SGID of starch-protein mixtures

5.1 Introduction

The rate of glucose absorption in the gastrointestinal tract governs the rate of appearance of glucose in the blood and consequently the hormonal balance such as insulin levels which effect satiation, fat metabolism, and ultimately weight gain. The rate and extent of blood glucose rise after eating is dependent on several factors including physiological status of the body and the interactions between/structures formed by food constituents.

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). In recent years research has been increasing in the area of bioactive peptides derived from dietary proteins. Bioactive peptides are normally inactive within the sequence of their parent protein but can be released in a number of ways. Enzymatic hydrolysis (using proteolytic digestive enzymes) of the parent protein is a very common method of production.

Milk proteins are an important source of bioactive peptides and an increasing number of bioactive peptides have been identified in milk protein hydrolysates and fermented dairy products (Kilara and Panyam, 2003; Matar et al., 2003; Korhonen and Pihlanto, 2003a; Fitzgerald, Murray and Walsh, 2004). Some of the most common bioactive peptides, i.e., angiotensin-converting enzyme (ACE) inhibitory peptides and calcium-binding phosphopeptides (CPPs) are produced from the action of trypsin, a gastrointestinal protease (Yamamoto, Ejiri and Mizuno, 2003; Fitzgerald et al., 2004; Gobbetti, Minervini and Rizzello, 2004; Vermeirssen, van Camp and Verstraete, 2004). Moreover, ACE-inhibitory peptides have recently been identified in the tryptic hydrolysates of bovine $\alpha_{s2}$-casein (Tauzin, Miclo and Gaillard, 2002) and in bovine, ovine and caprine $\kappa$-casein macropeptides (Manso and López-Fandino, 2004).
5. SGID of starch-protein mixtures

Peptides derived from casein have been shown to affect the cardiovascular, nervous, immune and digestive systems (Silva and Malcata, 2005; Shahidi and Zhong, 2008; Haque, Chand and Kapila, 2009). Bioactive peptides have been generated from intact protein using various enzymes including alcalase, chymotrypsin, pancreatin, trypsin and thermolysin (Kilara and Panyam, 2003; Korhonen and Pihlanto, 2003b; Fitzgerald et al., 2004).

The objective of this study was to perform simulated gastro-intestinal digestion on $\alpha_s$- and $\beta$-caseinate and their mixtures with waxy maize starch with a view to screening the resultant peptide fractions for their impact on in vitro expression of the gastro-intestinal hormones GLP-1 and GIP. In addition, the effect of protein-carbohydrate interactions on the kinetics of $\alpha$-amylase hydrolysis of starch will be determined by measuring the release of reducing sugars using HPLC analysis. It is thought that absorption/adsorption of protein on the surface of the starch granules (see Chapter 2) and interactions between the starch and caseinates may affect peptic and amylolytic hydrolysis generating different peptide profiles from the same parent protein.

5.2 Materials and Methods

The $\alpha_s$- and $\beta$-caseinate enriched protein fractions (trade name Ultranor) were kindly donated by Kerry Food Ingredients (Listowel Co. Kerry) and were used in the Na caseinate form. The protein content of the $\alpha_s$-caseinate and $\beta$-caseinate ingredients was 84.1% and 85.6%, respectively, as determined by Kjeldahl analysis (International Dairy Federation, 1993). Waxy maize starch (AMIOCA Powder TF) was kindly donated by National Starch (Manchester, UK).
5. SGID of starch-protein mixtures

5.2.1 Gelatinisation

Starch (5% w/w) and starch-protein mixtures were gelatinised using a controlled stress rheometer (AR-G2 rheometer, TA Instruments, Crawley, UK) fitted with a starch pasting cell. The internal diameter of the cell was 36 mm and the diameter of the rotor was 32 mm with a geometry gap of 0.55 mm. Samples were tempered at 35°C, then heated from 35°C to 95°C at a ramp rate of 14.5°C/min, held for 6 min at 95°C and subsequently cooled from 95°C to 35°C at a ramp rate of 29°C/min. A shear rate of 16.78 s⁻¹ was used throughout heating, holding and cooling.

5.2.2 Simulated Gastrointestinal Digestion (SGID)

Samples were subjected to a two stage SGID process incorporating the cellulose membrane for mono- and disaccharide and peptide transfer. Cellulose based dialysis membrane (Medicell International, London, UK) used during the digestion process had a MWCO of 12-14,000 Da. A cleaning step to remove the glycerol from the membrane was performed. The glycerol was removed by heating (60°C for 30 min) the membrane in a large volume (500 ml) of 2% sodium bicarbonate and 1 mM EDTA. The membrane was thoroughly rinsed with distilled water before use. pH adjustment was performed by means of a fully automated 842 Titrando titrator (Metrohm, Dublin, Ireland). The SGID process was based on methods as per Walsh et al. (2004) and Glycemic Index testing, as per Campden and Chorleywood Food Research Association group. Sodium phosphate buffer (20.6 ml), pH 6.9 was pre-incubated (37°C, 20 min) and adjusted using 2N HCl to 1.5; 4 ml of pepsin (145 U/ml Sigma code: P-7000) was added to the gently stirring buffer (110 rpm) followed by 4 g of substrate. Peptic digestion was performed at 37°C for 30 min and the reaction was stopped by adjusting the pH to 6.9 using 2N NaOH. The second stage involved a
further addition of 20.6 ml of buffer and 1 ml of α-amylase (250 U/ml Sigma code: A3176) and 1 ml of corolase PP. Aliquots of buffer (4 ml) containing the peptides (replaced with equal volume of fresh buffer) were removed from the buffer reservoir every 30 min for 180 min and incubated (80°C, 30 min) to terminate enzyme activity. Samples were cooled and then stored at -20°C for subsequent analysis. Three replicate digestions were performed for each treatment.

5.2.3 Analysis of enzymatically digested protein

Hydrolysate material was analysed by means of reversed phase HPLC using an Agilent 1200 series quaternary pump system (Agilent, Santa Clara, CA). Hydrolysates were filtered through a 0.2 μm syringe filter (polyethersulfone membrane) and 40 μl injected onto an Agilent (Poroshell, 5 μm) C18 reversed phase column (2.1 x 75 mm) equilibrated with solvent A (0.1 % trifluoroacetic acid in deionised water). Gradient elution with solvent B (0.1 % trifluoroacetic acid in 90 % acetonitrile/deionised water) was used with a flow rate of 0.8 ml/min. Peptides were eluted in a gradient of 5 to 35% solvent B over 55 min, increased to 60% over 5 min, increased again to 100% over the next 15 min and finally equilibrated back to 5% for a further 15 min. Peptides were continually assayed at 214 nm using an Agilent 1200 series UV dual-wavelength absorbance detector. Peptide fractions were generated on the basis of 15 min time intervals (Figure 5.1), i.e., fractions 1, 2, 3 and 4 were collected between 0-15 min, 15-30 min, 30-45 min and 45-60 min, respectively for use in bio-assays described in 5.2.5.
5. SGID of starch-protein mixtures

5.2.4 Analysis of enzymatically digested starch

Following the two stage SGID process, the reducing sugars of waxy maize starch which passed through the cellulose membrane were determined by HPLC analysis. Samples were filtered through a 0.2 µm syringe (nylon membrane) filter and injected onto a Carbopac PA20 anion exchange column (Dionex, Surrey, UK) fitted with an amino trap. Elution was achieved using 100 mM NaOH following regeneration with 200 mM (run time 40 min) and flow rate of 0.4 ml/min. Sugar analysis was conducted using an Agilent 1200 series binary pump (Agilent, Santa Clara, CA, USA) with pulsed electrochemical detection using a gold electrode target cell attached to a Coulchem III electrochemical detector (ESA, Buckinghamshire, UK).

5.2.5 GLP-1 analysis

Peptide fractions 1, 2, 3 and 4, generated in Section 5.2.3 were analysed using GLP-1 and GIP cell culture based bio-assays as described by Bruen et al. (2012).

5.3 Results and Discussion

Peptide profiles generated from the enzymatic digestion of starch and αs-caseinate or starch and β-caseinate are shown in Figure 5.1. It was demonstrated that the presence of starch and / or the process of gelatinisation altered the composition of peptide material released from either caseinate during SGID; three scenarios were tested in the study: 1) protein digested without starch present; 2) protein added to the starch after gelatinisation and digested and; 3) starch gelatinised in the presence of the protein and digested. The incretin hormone response to hydrolysates of protein alone or starch gelatinised in the presence of the protein differed significantly (Bruen et al., 2012; Appendix 3) and these differences were attributed to differences in the peptide
profiles shown in Figure 5.1. Differences were observed between those profiles for αs-caseinate and β-caseinate alone (Figures 5.1a and 5.1d), with more hydrophobic material present in the β-caseinate samples. A higher concentration of hydrophobic peptides, i.e., peptides with a retention time of 45-60 min, were present in samples where αs-caseinate was present during starch gelatinisation (Figure 5.1c) compared to αs-caseinate alone (Figure 5.1a); a trend not so clearly evident for β-caseinate. The inclusion of αs-caseinate during gelatinisation of starch (Figure 5.1c) resulted in a qualitatively different peptide profile compared to when the protein was added to the already gelatinised starch (Figure 5.1c); again the effect of gelatinisation was not as clear for those starch samples containing β-caseinate.

It is hypothesised that the gelatinisation process of starch may have afforded some protection to the protein (encapsulating effect observed in Chapter 2), thus restricting and/or altering the accessibility of pepsin to the protein during subsequent digestion. Certainly this may be evident where αs-caseinate was present during gelatinisation of starch, as αs-caseinate has the ability to penetrate the interior of WMS during heating/pasting (as reported in Chapter 2). In the case of β-caseinate, the aggregated state of the protein on heating (as reported in Chapter 2) may have offset any effect of starch granule swelling / rupture and disintegration on subsequent hydrolysis by pepsin.
Figure 5.1: Reversed-phase HPLC profiles of peptides after simulated gastrointestinal digestion of (a) 5% $\alpha_s$-caseinate, (b) 5% gelatinised starch + 5% $\alpha_s$-caseinate, (c) 5% starch gelatinised in the presence of 5% $\alpha_s$-caseinate, (d) 5% $\beta$-
5. SGID of starch-protein mixtures

caseinate, (e) 5% gelatinised starch + 5% β-caseinate and (f) 5% starch gelatinised in the presence of 5% β-caseinate.

In an extended study by Bruen et al. (2012) using the fractions 1 to 4 described in this study, it was shown that both αs-caseinate or β-caseinate present during gelatinisation of starch resulted in significantly lower levels of GLP-1 peptide in vitro, compared with gelatinised starch alone. Levels of GLP-1 peptide were also significantly lower in response to digested αs-caseinate alone compared with digested β-caseinate alone (Bruen et al., 2012). The significantly lower GLP-1 response to αs-caseinate is reflected in differences amongst peptide profiles for αs-caseinate (Figure 5.1a) compared to β-caseinate (Figure 5.1d). Peaks with retention times at approximately 22 and 25 min, were higher in profiles of β-caseinate (Figure 5.1d) digests compared with that for αs-caseinate digests (Figure 5.1a). In addition, a higher concentration of peptides was observed in the more hydrophobic region of the chromatogram from 45 min onwards for β-caseinate digests (Figure 5.1d) compared to the same region for αs-caseinate (Figure 5.1a).

Gelatinised starch and caseinate samples lowered levels of (i) GLP-1 secreted peptide, (ii) GIP mRNA transcripts and (iii) GIP secreted peptide, therefore a further step involved the fractionation of the caseinate hydrolysates after digestion to determine if a particular protein fraction/group of peptides was responsible for this effect. Four peptide fractions (fractionated based on 15 min intervals from 0-60 min, Figure 5.1), resolved on the basis of hydrophobicity by reversed phase HPLC, were generated for either αs-caseinate or β-caseinate.
Figure 5.2: STC-1 supernatant levels of GLP-1 peptide in response to 4 hour incubations with digested starch and caseinate mixtures (modified from Bruen et al., 2012).

The incretin responses of STC-1 cells to the caseinate fractions generated are detailed in Figure 6, Appendix 3. Levels of secreted GLP-1 were significantly lower in response to αs-caseinate fractions 1 (i.e., 0-15 min) and 2 (i.e., 15-30 min) compared to αs-caseinate fractions 3 (i.e., 30-45 min) and 4 (i.e., 45-60 min) (Figure 6(a) Appendix 3). αs-caseinate fraction 4 induced significantly higher levels of GLP-1 peptide compared to all of the other αs- and β-caseinate fractions (Bruen et al., 2012). Fractions 3 and 4 of the αs-caseinate peptide profile contained the more hydrophobic peptides with the most hydrophobic peptides in fraction 4 inducing the greatest response. Interestingly, β-caseinate fraction 4 induced the lowest response in GLP-1 secretion from the β-caseinate hydrolysate. In light of this observation it may be postulated that the response in GLP-1 secretion is to specific peptides/amino acids.
5. SGID of starch-protein mixtures

GIP gene expression results demonstrated that the β-caseinate fractions resulted in significantly lower levels of GIP mRNA transcripts than the α-caseinate fractions (Bruen et al., 2012). There were no significant differences in GIP gene expression between the α-caseinate fractions (Bruen et al., 2012). β-caseinate fraction 3 induced lower levels of GIP gene expression than β-caseinate fraction 4.

5.3.1 Analysis of enzymatically digested starch

Levels of glucose after 180 min of SGID of starch gelatinised alone or in the presence of αs-caseinate or β-caseinate are shown in Figure 5.3a. Starch gelatinised with αs-caseinate had the lowest level of glucose throughout the 180 min digestion. Similarly, levels of maltose were lower throughout digestion in the starch-αs-caseinate sample increasing slightly after 150 min. Moreover, all starch samples gelatinised in the presence of protein had a lower concentration of maltose throughout 180 min of digestion compared to the control (5% starch alone). Enzymatic hydrolysis of starch by α-amylase cleaves α-1-4 glycosidic linkages yielding maltose, maltotriose and α-limit dextrins (Wong and Robertson, 2003; Parkin, 2008) resulting in the higher concentrations of maltose seen in Figure 5.3b compared to that of glucose (Figure 5.3a) over the same time period.
Figure 5.3: Levels of glucose (A) and maltose (B) released from 5% waxy maize starch alone (♦) or in the presence of 5% α₃-caseinate (▲) or 5% β-caseinate (□) during 180 min of two stage simulated gastro-intestinal digestion.
5. SGID of starch-protein mixtures

5.4 Conclusions

Enzymatic hydrolysis of protein alone and starch-protein mixtures had a modulating effect on incretin hormone secretion and gene expression. The fractionation of caseinate hydrolysates by reversed phase HPLC yielded fractions with a more potent effect on GLP-1 and GIP secretion than crude caseinate hydrolysates. The more hydrophobic peptide fractions of enzymatically hydrolysed αs-caseinate resulted in secretion of greatest levels of GLP-1 while the less hydrophobic enzymatically hydrolysed αs- and β-caseinate fractions induced greatest secretion in GIP. Analysis of reducing sugars liberated on \textit{in vitro} digestion of gelatinised starch alone or in the presence of αs- or β-caseinate revealed that the presence of casein protein fractions impacted on the liberation of reducing sugars from starch; concentrations of both maltose and glucose were lowest in the starch-αs-caseinate sample after 180 min of digestion.

C.M.B. performed \textit{in vitro} cell line exposures and biological assays, statistically analysed the data and drafted the manuscript. A.P.K. was responsible for preparation of the gelatinised starch/casein mixtures, fractionation of the casein hydrolysates and for digestion of the samples. F.O.H. was involved in design of experiments and manuscript drafting. V.C. was involved in preparation of the starch/casein mixtures and fractionation of the casein hydrolysates. M.A.F. was responsible for production of starch/casein mixtures, was involved in design of experiments and secured funding for the project. K.A.C was involved in design of experiments and manuscript drafting. L.G. was involved in design of experiments, analysis of data and manuscript drafting.
5.5 References


5. SGID of starch-protein mixtures


Chapter 6

Development of an integrated digestion cell for simulated gastro-intestinal digestion (SGID) of foods

6. Development of integrated SGID cell

Abstract

During the course of this thesis it became apparent that no *in vitro* methodology exists which can simultaneously measure digestion and viscosity *in situ*. Thus, the aim of this chapter was to develop a novel digestion cell for evaluation of digestion kinetics and rheological changes during simulated gastro-intestinal digestion (SGID) of food matrices under different physiological conditions. The cell was designed to control temperature, pH, enzyme addition and mixing while simultaneously measuring viscosity. The main body of the cell was constructed from hastelloy, a nickel based alloy with a combination of acid resistance and high temperature strength. Cellulose membranes, with selected molecular weight cut off (12,000 Da), were fitted to the bottom and sides of the cell to allow transfer of soluble components and hydrolysis products to an outer chamber. The novel design was validated by determining the amount of peptides and reducing sugars released from enzymatically hydrolysed starch-protein dispersions during SGID. The invention differs from existing *in vitro* digestion methodologies in that the rate of transfer of molecular components across the membrane can be controlled. This, coupled with simultaneous *in situ* rheological measurement, gives this invention original design characteristics.
6. Development of integrated SGID cell

6.1 Introduction

Starch and protein are primary constituents of the western diet and their interaction, breakdown and physical characteristics are of major importance to the food industry. It is not surprising therefore that there is increasing interest in the area of in vitro starch digestion alone and the in presence of proteins, and also comparison to in vivo digestion (Englyst et al., 1996; Hasjim et al., 2010). Since the early 1990s, many methodologies have been published on the in vitro digestibility of carbohydrate containing foods and associated glycemic index, GI (Wolever et al., 1991), starch digestibility (Tester, Karkalas and Qi, 2004) and resistant starch and its different resistance categories (Englyst, Kingman and Cummings, 1992). A popular method currently used for assessing in vitro starch digestion was published by Brighenti et al. (1995). The method tested a range of food products and concluded that the effect of dietary fibre on glycemic response can successfully be predicted by integrating the compositional data with in vitro variables that simulate gastrointestinal events (Brighenti et al., 1995). More recent in vitro studies involving the digestion of starch include methods from a number of authors (Weurding et al., 2001; Evans and Thompson, 2004; Brennan, Cleary and Andersson, 2007; Mishra, Monro and Hedderley, 2008; Al-Rabadi, Gilbert and Gidley, 2009). A review by Hur et al. (2011) compiled information from a range of in vitro digestion systems used to test various foods. The review incorporates in vitro digestion methods from various researchers on a range of food products from plant and emulsion based foods to meat, marine, mineral and lipid related foods. One method reviewed in that article is a model stomach system used to investigate the kinetics of food disintegration under gastric conditions developed by Kong and Singh (2008). The authors reported a relationship between the kinetics of food disintegration and in vivo stomach emptying.
There are many methods available for determining the digestibility and GI of carbohydrate containing foods. In general, there are several common themes exhibited in these methods but the ways in which physiological conditions are implemented across methods differ considerably. The variation includes duration of gastric digestion, choice of amylolytic enzyme, incubation temperature, pH and stirring mode (Hur et al., 2011). Wolever et al. (2008) reported that GI values for foods can differ depending on the in vitro digestion methodology used. It is generally accepted that GI can be hard to predict and measure accurately in vitro and is more a measure of carbohydrate digestibility than an accurate in vivo response to the same food on blood sugar levels (Marsh and Brand-Miller, 2008). In the many in vitro methods available for determining GI, viscosity is a parameter that is often overlooked, most likely because of a lack of accurate means of measurement in situ. The link between viscosity and starch digestion/hydrolysis has been demonstrated; studies show that increases in viscosity within a food matrix can reduce starch hydrolysis (Dartois et al., 2010). Moreover, it is generally established that pasta, a low glycemic index (GI) food, releases reducing sugars by enzymatic hydrolysis of the starch slowly, due to the presence of a viscous protein network which, it is suggested, retards the action of α-amylase (Colonna et al., 1990). The addition of protein to starch has also been reported to increase the paste viscosity of starch suspensions and subsequently reduce the enzymatic release of sugars from the starch (Kett et al. 2009,2013). From these studies it is evident that viscosity plays a major role in the physicochemical properties of a food system but also in relation to enzymatic hydrolysis. Furthermore, rheological assessment of food matrices during simulated gastro-intestinal digestion may provide a greater understanding of the complex interactions between food components during digestion.
This chapter details the design and engineering of a rheological digestion cell for measurement of *in vitro* digestion kinetics while simultaneously measuring viscosity throughout the digestion process. The novel cell was used to study the effect of starch/protein interactions on structural properties, and the ensuing influence on the enzyme kinetic parameters (i.e., hydrolysis of carbohydrate). The new *in vitro* system was built as a tool for the evaluation of the rheology of food bio-polymer complexes under SGID conditions. Therefore the objective of this chapter was to design, build and validate a rheological digestion cell capable of simulating *in vitro* digestion with possible application as a tool for measurement of GI. The starch and protein ingredients used in previous chapters were used for validation.

### 6.2 Materials and Methods

#### 6.2.1 Design concept of integrated digestion cell

The digestion cell can be used for taking rheological measurements of a sample under dynamic conditions during which one or more compositional parameters of the sample are changed. The main body of the cell was constructed from hastelloy, a nickel-based alloy with a combination to oxidation resistance and low pH and high temperature strength. The cell consisted of a semi-permeable cellulose membrane incorporated into a sample vessel which can allow, for example, the transfer of maltose and glucose (reducing sugars of starch) to an adjacent buffer by means of osmosis, thus simulating transfer of glucose across the small intestine in the gut. The semi-permeable membrane may be chosen with a particular molecular weight cut-off (MWCO) to select those components in the sample fluid which are to be removed by osmosis; in this manner, the MWCO can be varied to select for the removal of solutes such as sugars, amino acids, peptides, and the like, thereby providing a means for
determining changes in the levels of these components under specific reaction conditions.

The digestion cell assembly (Figure 6.1) included (i) a buffer reservoir submerged within a temperature controlled water-bath providing a flow of buffer in a loop between the buffer reservoir and the buffer chamber, (ii) a peristaltic pump (Gilson Minipuls Evolution, Bedfordshire, UK; responsible for controlling flow and the level of buffer in the buffer chamber using 3.20 mm (internal diameter) x 0.86 mm wall tubing), (iii) concentric cylinder peltier attached to a rheometer (AR-G2, TA Instruments, Crawley, UK) which holds main body of cell and also enables accurate temperature control, (iv) hastelloy cylinder (custom manufactured, Somex Ltd., Macroom, Co. Cork, Ireland) containing various components (Figure 6.2-6.5) and (v) a Metrohm 842 Titrando titrator (Metrohm, Dublin, Ireland) adapted to dispense acid/base for pH control (needed for SGID process) and ingredient/buffer solutions. The titrator was connected to the reactor through feed tubes; a number of ports (Figure 6.2) were provided by means of a specially adapted cover on top of the digestion cell for this purpose. The total volume in the system was 500 ml. The internal volume of the digestion cell was 50 ml with an outer chamber volume of 90 ml. The remaining 360 ml was contained within the buffer reservoir (placed in the temperature controlled waterbath) which was continuously circulating between the outer chamber of the cell and the reservoir by means of the peristaltic pump.
Figure 6.1 Schematic representation of main assembly including rheometer, peristaltic pump and temperature controlled waterbath containing the sampling reservoir and computer for analysing viscosity data (titrator not included).
Figure 6.2 Side-view of cell contained within the hastelloy cylinder showing directional flow of buffer from the outside chamber and also directional flow inside the cell from angled rotor (actual rotor not shown in figure). Included also are ports for acid, base and enzyme addition.

6.2.2 Transfer of solutes/sugars/peptides

The digestion cell contained a selective dialysis membrane disc that was sealed into position by a specially designed gasket at its base. Additional membranes were fitted into the sidewalls of the cell using a clamping mechanism (see exploded view in Figure 6.5) to increase the surface area for transfer of solutes. These membranes (base
6. Development of integrated SGID cell

and side) were used to separate/isolate the molecular components by convection-assisted mass transfer. Selectivity of molecules can be achieved by changing the pore size of the membrane disc and thus the molecular size of the molecules that permeate the membrane. The dialysis membrane provides a desired MWCO (for this study a range of 12-14,000 Da was used) for separation of small molecules from digested material. The design creates conditions whereby mass diffusion can occur across the membrane and remove, for e.g., reducing sugars/peptides/amino acids from the digestion cell and transfer these to the outer chamber. This outer chamber contained a buffer which circulated from a temperature controlled reservoir located in an adjacent waterbath attached to a controlled stress rheometer (AR-G2, TA Instruments, Crawley, UK) (Figure 6.1). The buffer maintained a differential in sugar/peptide/amino acid concentration between the digestion cell and the outer chamber. The circulation system was designed to (i) control the level of the buffer in the outer chamber, (ii) regulate the diffusion of molecules through the membrane by increasing or decreasing removal of filtrate from the surface of the membrane and (iii) transfer permeate to the external reservoir for mixing, sampling and subsequent analysis. Molecules within the cell were kept in contact with the membrane by mass convection caused by the downward rotational movement of the angled rotor (made from Torlon 4301 with a rotor screw pitch of 80 mm, 54° over 12 mm with a diameter of 32.4 mm; see Figures 6.2 and 6.3, actual rotor not shown in schematics) attached to the rheometer used to take the viscosity measurements. The rotor was held at a constant shear rate of 15 l/s throughout all digestions.

The digestion cell differs from existing in vitro digestion methodologies in that the rate of transfer of molecular components across the membrane can be controlled by
6. Development of integrated SGID cell

altering the rate of agitation and the rate of circulation/type of buffer used in the outer chamber.

**Figure 6.3** Side view of digestion cell fully assembled showing outer chamber levels determined by draw-off tube
Figure 6.4 Cross sectional view of main components of integrated digestion cell

Figure 6.5 Schematic diagram of the major components making up the digestion cell
6. Development of integrated SGID cell

6.2.3 Ingredients

Waxy maize starch (AMIOCA Powder TF) was sourced from National Starch (Manchester, U.K). αs-Caseinate enriched protein ingredient was sourced from Kerry Ingredients (Listowel, Co. Kerry, Ireland). The protein content of the αs-caseinate ingredient was 84.1% as determined by Kjeldahl analysis (International Dairy Federation, 1993). Pepsin (Sigma code: P-7000) and α-amylase (Sigma code: A3176) were sourced from Sigma Aldrich (Arklow, Co. Wicklow, Ireland).

6.3 Analytical methods

6.3.1 Preparation of Dialysis Membrane

Cellulose based dialysis membrane (Medicell International, London, UK) used during the digestion process had a MWCO of 12-14,000 Da. A cleaning step to remove the glycerol from the membrane was performed prior to use. The glycerol was removed by heating (60°C for 30 min) the membrane in a large volume (500 ml) of 2% sodium bicarbonate and 1 mM EDTA. The membrane was thoroughly rinsed with distilled water before insertion into the cell base and sidewalls of the inner chamber.

6.3.2 Gelatinisation

Starch samples (5% w/w) were gelatinised on a controlled stress rheometer (AR-G2, TA Instruments, Crawley, U.K) fitted with a starch pasting cell. The internal diameter of the cell was 36 mm and the internal diameter of the rotor was 32 mm with a geometry gap of 0.55 mm. Samples were tempered at 35°C, then heated from 35°C to 95°C at a ramp rate of 14.5°C/min, held for 6 min at 95°C and subsequently cooled from 95°C to 35°C at a ramp rate of 29°C/min. A shear rate of 16.78 s⁻¹ was used
throughout heating, holding and cooling. The starch was subsequently used along with the proteins for validation of the digestion cell.

6.3.3 Simulated Gastrointestinal Digestion

Samples were subjected to a two-stage simulated gastro-intestinal digestion (SGID) process using the digestion cell fitted to the controlled stress rheometer (AR-G2, TA Instruments) incorporating the cellulose membrane for glucose transfer. Adjustment of pH was performed by means of a fully automated 842 Titrando titrator (Metrohm, Dublin, Ireland). Sodium phosphate buffer (20.6 ml), pH 6.9 was pre-incubated (37°C for 20 min) and adjusted using 2N HCl to pH 1.5; 4 ml of pepsin (145 U/ml) was added to the gently stirring buffer (110 rpm) followed by 4 g of substrate. Peptic digestion was performed at 37°C for 30 min and the reaction was stopped by adjusting the pH to 6.9 using 2N NaOH. The second stage involved a further addition of 20.6 ml of buffer and 1 ml of α-amylase (250 U/ml) and 1 ml of Corolase PP. Aliquots of buffer (4 ml) containing the reducing sugars of waxy maize starch (replaced with fresh buffer) were removed from the buffer reservoir every 30 min for 180 min and incubated (80°C for 30 min) to terminate enzyme activity. Samples were cooled and then stored at -20°C for subsequent analysis. Three replicate digestions were performed for proof of concept/validation of digestion cell (see Section 6.4).

6.3.4 Analysis of enzymatically digested protein

Hydrolysate material was analysed by means of reversed phase HPLC using an Agilent 1200 series quaternary pump system (Agilent, Santa Clara, CA). Hydrolysates were filtered through a 0.22 µm syringe (polyethersulfone membrane) filter and 40 µl injected onto an Agilent (Poroshell, 5 µm) C18 reversed phase column (2.1 x 75 mm)
equilibrated with solvent A (0.1% trifluoroacetic acid in deionised water). Gradient elution with solvent B (0.1% trifluoroacetic acid in 90% acetonitrile/deionised water) was used with a flow rate of 0.8 ml/min. Peptides were eluted in a gradient of 5 to 35% solvent B over 55 min, increased to 60% over the next 5 min, increased to 100% over the next 15 min and finally equilibrated back to 5% for a further 15 min. Peptides were continually assayed at 214 nm using an Agilent 1200 series UV dual-wavelength absorbance detector.

6.3.5 Analysis of enzymatically digested starch
Following the two stage SGID process, the reducing sugars of waxy maize starch which passed through the cellulose membrane were determined by HPLC analysis. Samples were filtered through a 0.22 µm syringe (nylon membrane) filter and injected onto a Carbopac PA20 anion exchange column (Dionex, Surrey, UK) fitted with an amino trap. Elution was achieved using 100 mM NaOH following regeneration with 200 mM NaOH (run time 40 min) and flow rate of 0.4 ml/min. Mono- and disaccharide analysis was conducted using an Agilent 1200 series binary pump (Agilent, Santa Clara, CA, USA) with pulsed electrochemical detection using a gold electrode target cell attached to a Coulchem III electrochemical detector (ESA, Buckinghamshire, UK).

6.4 Validation of integrated digestion cell
6.4.1 Reversed phase HPLC analysis of digested protein
Starch samples gelatinised in the presence of αs-caseinate were subjected to the two stage SGID process described in Section 6.3.3 and the peptides generated from the hydrolysis of αs-caseinate were analysed by reversed phase HPLC. The digestions
were performed in triplicate and three peptide profiles analysed to demonstrate that peptides generated from enzymatic hydrolysis could pass through the membrane and into the reservoir for subsequent analysis. All three profiles were highly reproducible; identifiable peaks and areas are marked on the profiles shown in Figure 6.6 a-c. The first identifiable peak marked (I) had a retention time of 6.5 min and peak height of approx. 441.13 mAU. This first peak can clearly be seen on Figure 6.6a as well as Figures 6.6b and c. The second identifiable peak had a retention time of 13.7 min and a height of 289 mAU. The third marking (III) is an area between a retention time of 22.75 min and 27.87 min. This area was marked because of its reproducibility between the three profiles. The three areas were almost identical, all having the same number of peaks albeit varying slightly in height. The fourth area (IV) marked is an area of retention time between 32 min and 46 min. Although these peaks were smaller in height, the region represented had the same number of peaks in all three profiles. Peaks V and VI are clearly identifiable on all three profiles with retention times of 49.51 and 52.1 min respectively and both were in the region of 360 mAU in peak height. Peptides were eluted from the column in order of increasing hydrophobicity. Peptides with greater hydrophobicity (generally non-polar amino acids) have longer retention time because of increased non-polar surface area; polar groups reduce retention time as they are more compatible with water. The profiles shown in Figure 6.6 demonstrate that the level and type of peptides produced was similar across the three replicate experiments.
Figure 6.6 (a-c): Peptide profiles generated from reversed-phase HPLC of $\alpha_s$-caseinate after enzymatic digestion in the cell.
6. Development of integrated SGID cell

6.4.2 Reducing sugar analysis

Waxy maize starch 5% (w/w) was first gelatinised using a starch pasting cell attached to the rheometer. The gelatinised starch was subjected to SGID in the cell (in triplicate) to investigate if the reducing sugars, glucose and maltose can permeate the cellulose membrane and transfer to the buffer reservoir (shown in Figure 6.2); sample aliquots were taken from the reservoir for analysis using a syringe. The results presented in Figure 6.7a clearly show the appearance of glucose 30 min post initiation of digestion, indicating permeation through the membrane and transfer to the reservoir. The graph showed an increase in glucose concentration as a result of enzymatic hydrolysis from the starch after 30 min (0.001 mg/ml) up to 180 min (0.008 mg/ml). A sample outside the cell (within the hastelloy chamber Figure 6.2) after 180 min was also analysed and found to have a glucose concentration of 0.010 mg/ml. The difference in concentration between the 180 min sample taken from the reservoir and the sample taken from outside the cell was 0.002 mg/ml; taking the concentration of glucose obtained from outside the cell and the sample taken from inside the reservoir after 180 min there was an 80% recovery of glucose from the digestion cell in the buffer reservoir.

Figure 6.7b shows the release of maltose from the starch after enzymatic hydrolysis over the 180 min period. An increase can also be observed for maltose concentrations, where, after 30 min of digestion, the concentration was 0.007 mg/ml and this increased to 0.042 mg/ml after 180 min. Again there was a sample taken from outside the cell on completion of the digestion and this sample had a maltose concentration of 0.050 mg/ml. The difference in maltose concentration between the outside of the cell and the buffer reservoir was just 0.008 mg/ml.
Figure 6.7 (a): Concentrations of glucose and (b) maltose released from 5% waxy maize starch at 0, 30, 60, 90, 120, 150 and 180 min after addition of α-amylase to the reactor cell contents.

6.5 Conclusions

The integrated rheological digestion cell developed in this chapter was successful in (i) performing in situ SGID while simultaneously measuring viscosity, (ii) allowing permeation of peptides and sugars across a 12-14,000 Da cellulose membrane to a temperature control reservoir for incremental sampling/analysis and (iii) incorporating an automated titrator system to effectively control acid/base addition during digestion.
The cell was successful in facilitating measurement of digestion kinetics of starch-protein dispersions. Such a system could be readily applied to other food bio-polymer systems in measuring digestibility and GI. The simultaneous measurement of viscosity throughout the SGID process was an issue due to the dilution effect on addition of buffer, hence accurate viscosity measurements could not be detected. Future work could involve the enzymatic digestion of certain foods where viscosity changes are detectable in both acidic and alkaline conditions. One such application may be the changes in viscosity in anti-reflux infant formula where the formula thickens in the acidic environment of the human stomach. A successful study showing the relationship between enzymatic breakdown and viscosity in model infant formulae was performed using the cell with samples of higher total solids and lower pH by Treacy (2012).

6.6 Acknowledgements

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6.7 References


6. Development of integrated SGID cell


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6. Development of integrated SGID cell


Chapter 7

The effect of αs- or β-caseinate addition to waxy maize starch on post prandial levels of glucose, insulin and incretin hormones in pigs as a model for humans

Abstract

Starch is an important source of glucose and energy in the human diet. The extent to which it is digested in the gastrointestinal tract plays a major role in variations in postprandial blood glucose levels. Interactions with other biopolymers, such as dairy proteins, during processing can influence both the duration and extent of this postprandial surge. The objective was to evaluate the effect of the addition of bovine αs- or β-caseinate to waxy maize starch on changes in postprandial blood glucose, insulin and incretin hormones (glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1(GLP-1)) in 30 kg pigs used as an animal model for humans. Gelatinised starch, starch gelatinised with αs-caseinate and starch gelatinised with β-caseinate were orally administered to trained pigs (n = 8) at a level of 60 g of available carbohydrate. Pre and postprandial glucose measurements were taken every 15 min for the first hour and every 30 min thereafter up to 180 min. Insulin; GIP and GLP-1 levels were measured in plasma samples up to 90 min postprandial.

Starch gelatinised with αs-caseinate had a significantly \((p < 0.05)\) lower peak viscosity on pasting and resulted in significantly lower glucose release at 15, 30 and 90 min postprandial compared to starch gelatinised with β-caseinate. During the first 45 min postprandial, the area under the glucose curve (AUC) for starch gelatinised with αs-caseinate was significantly \((p < 0.05)\) lower than that for starch gelatinised with β-caseinate. There was also a significant \((p < 0.05)\) difference at 30 min in GIP levels in response to the control compared to starch gelatinised with αs- or β-caseinate. Significant \((p < 0.05)\) increases in several free amino acid concentrations were observed on ingestion of either αs- or β-caseinate gelatinised with starch at 30 and 90 min postprandial compared to
7. The effect of $\alpha_s$- or $\beta$-caseinate addition to WMS

starch alone. In addition, plasma levels of 6 individual amino acids were increased on ingestion of starch gelatinised with $\alpha_s$-caseinate compared to ingestion of starch gelatinised with $\beta$-caseinate.

In conclusion, the presence of caseinate fractions ($\alpha_s$- or $\beta$-caseinate) in gelatinised waxy maize starch affects swelling characteristics, viscosity and subsequent *in vivo* digestion as determined by glucose levels in blood post-ingestion.
7. The effect of \( \alpha_s \)- or \( \beta \)-caseinate addition to WMS

7.1 Introduction

There is increasing interest in the role of carbohydrate ingestion in the metabolic syndrome and subsequent health effects relating to glycemia and type 2 diabetes (Accurso et al., 2008). Ingestion of foods which result in a decreased rate of carbohydrate absorption leads to a more controlled release of glucose into the bloodstream. These foods have a lower glycemic index (GI) value. The GI system ranks carbohydrate foods based on their post prandial glycemic concentrations (Jenkins et al., 1981). High GI foods are associated with a rapid rise in both glucose and insulin in blood after consumption (Augustin et al. 2002). Hyperinsulinemia can reduce insulin efficiency via down regulation of insulin receptors resulting in insulin resistance (Virkamaki, Ueki and Kahn, 1999) which, in addition to impaired pancreatic \( \beta \)-cell function, are risk factors for type II diabetes (Nijpels, 1998). Previous studies have reported on the benefits of low GI diets in relation to the metabolic syndrome, i.e., improved blood glucose control and glucose tolerance (Brand et al., 1991), improvements in insulin sensitivity (Jarvi et al., 1999) and a reduction of insulin resistance (Bjorck, Liljeberg and Ostman, 2000).

Upon ingestion, nutrients stimulate the secretion of various hormones and peptides by specialised enteroendocrine cells in the gastrointestinal tract. This informs the gut and pancreas of the impending arrival of glucose and ensures that insulin is produced and secreted by pancreatic \( \beta \)-cells to maintain glucose homeostasis. This signalling is primarily achieved by the incretin hormones, GLP-1 and GIP, secreted by the gut which act to stimulate insulin release and suppress glucagon (Baggio and Drucker, 2007). These hormones primarily respond to carbohydrate and fat ingestion but can also respond to proteins and
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amino acids (Brubaker, 2006). It is well established that proteins within a defined food matrix can affect the rate at which carbohydrates are metabolised *in vivo* (Nuttall *et al.* 1985; Nuttall and Gannon, 1991; Holt, Miller and Petocz, 1997; Lang *et al.*, 1999).

It is hypothesised that the addition of dairy protein fractions, $\alpha$- or $\beta$-caseinate, to waxy maize starch will have a post prandial glucose lowering effect. Preliminary results from rheological, microscopical and simulated gastrointestinal digestion studies showed a restriction in granule swelling and decreased maltose and glucose levels when starch is gelatinised with caseinate fractions. These results were not observed when starch was gelatinised with whey fractions ($\alpha$-lactalbumin and $\beta$-lactoglobulin) (Kett *et al.*, 2009).

The authors used the pig as an animal model for humans due to its omnivore status and its similarity to humans in digestive physiology, hormonal response, metabolism and organ size (Topping and Clifton, 2001; Spurlock and Gabler, 2008). The present study investigates the effect of adding enriched $\alpha$- or $\beta$-caseinate fractions to gelatinised waxy maize starch on post prandial blood levels of glucose, insulin and incretin hormone in 30 kg pigs.

### 7.2 Materials and Methods

The protein enriched $\alpha$- and $\beta$-caseinate fractions were sourced from Kerry Dairy Ingredients (Listowel Co. Kerry). The protein content of the $\alpha$-caseinate was 84.07 % and the $\beta$-caseinate 85.61 %, as determined by Kjeldahl (International Dairy Federation, 1993). Caseinate fractions (10 % w/w) on a protein basis were made up by dissolving the powders in Milli-Q water at room
7. The effect of αs- or β-caseinate addition to WMS

temperature, with the aid of an overhead stirrer and stored overnight at 4 °C to ensure complete hydration. Waxy maize starch (trade name AMIOCA Powder TF) was kindly provided by National Starch (Manchester, UK). All trial samples (starch alone or starch gelatinised with αs- or β-caseinate) were gelatinised by heating to 90 °C using in-direct steam heating (operating steam pressure of 1 bar) and held for 6 min to ensure complete gelatinisation; then cooled to 20 °C in a Stephen cooker (closed system) UMM/SK5 (Stephan U Sohne GMBH + Co, Hameln, Germany) using a mixing baffle operated at 100 % capacity and the main motor at 50 % capacity. Sample preparation was staggered each day so that each sample was fed to the pigs immediately after the pasting process to prevent further retrogradation of the starch.

7.2.1 Pasting behaviour

All samples were pasted as a model system using the same composition as the investigated samples on an AR-G2 Rheometer (TA Instruments, Crawley, U.K) fitted with a starch pasting cell (sample size 28 g). The internal diameter of the cell was 36 mm and the diameter of the rotor used was 32 mm with a gap of 550 μm. A thermal cycle was used to provide conditions for gelatinisation, pasting and subsequent molecular rearrangement on cooling. All samples were tempered at 35 °C, heated from 35 °C to 95 °C at a ramp rate of 14.5 °C/min, held for 6 min, subsequently cooled from 95 °C to 35 °C at a ramp rate of 29 °C/min and finally held for 10 min at 35 °C using a shear rate of 16.78 1/s.
7. The effect of $\alpha_s$- or $\beta$-caseinate addition to WMS

7.2.2 Experimental Design

A progeny of Large White x Landrace crossbred pigs ($n = 8$), (reared in the Pig Development Unit, Moorepark, Fermoy, Co. Cork) with a body weight of 30 kg ($\pm 2$), were trained 1 week before trial commencement to eat 10% w/w (600 g) gelatinised starch samples within a 10 min period. The control sample contained 60 g w/w (60 g available CHO) of waxy maize starch in 540 g of water. The starch and protein samples contained 60 g w/w of starch, 71.34 g of $\alpha$-caseinate and 468.66 g of water or 60 g w/w of starch, 70.08 g of $\beta$-caseinate and 469.92 g of water. (Note: all starch-protein samples were made up on a protein basis). There were no additional ingredients in the sample, i.e., fat, other carbohydrates or minerals. Additional constituents of a typical pig diet add caloric value and would have an effect on glucose and insulin levels. On completion of training, the pigs were catheterised in the lateral ear vein for blood sampling. Prior to commencement of sampling, a 24 h resting period allowed clearance of the anaesthetics from the body. The pigs were fasted from 16:00 h the previous day after their afternoon meal. Nine pigs were trained for the trial; however, due to problems with one catheter, one pig was removed from the trial post-catheterisation. The concentrations of the three semi-solid gelatinised foods ingested on a component level were (i) 10 % starch (control) (ii) 10 % starch + 10 % $\alpha_s$-caseinate and (iii) 10 % starch + 10 % $\beta$-caseinate. The protein and lipid concentrations naturally present in waxy maize starch were not accounted for as these concentrations are minimal (Debet and Gidley, 2006). The eight pigs were fed 600 g as a single meal of each gelatinised semi-solid food containing 60 g of available carbohydrate (CHO). Replicates for each sample were staggered across 3 days to exclude sampling day as a variable. All experiments complied with EU
7. The effect of α- or β-caseinate addition to WMS

Council Directive 91/630/EEC, which lays down minimum standards for the protection of pigs, and EU Council Directive 98/58/EC, which concerns the protection of animals kept for farming purposes. A licence to perform experiments on live animals under the Cruelty to Animals Act, 1876 Regulations 2002 and 2005 was obtained before commencing this work.

7.2.3 Catheterisation

A method for ear vein cannulation of pigs was successfully developed and it was determined from preliminary dose response feeding trials with starch as the main ingredient in the diet that using pigs with an average bodyweight of 30 kg would be most reproducible and appropriate for future studies involving study of the kinetics of starch metabolism in different foods formulations. The method was developed for pigs whereby point of entry was the lateral auricular vein, chosen because it is one of few visible superficial veins. Although the point of entry was the auricular vein, blood was drawn from the portal vein by means of a catheter running into the portal vein as regular blood sampling from the ear is not feasible. Blood samples obtained by venipuncture of the auricular vein are often too small for regular sampling and can result in swelling of the veins causing injury to the blood vessels and also distress to the animal. The procedure introduces a catheter into the vein via venipuncture as described below. This method allows for regular and substantial blood volumes to be collected for analysis. Ear vein catheterisation has its limits. The maximum time the catheter can stay in the ear is approximately 5-6 days after which it has to be carefully removed to avoid local infection at the point of insertion.
Insertion of catheters was performed on anaesthetised pigs according to bodyweight (30 kg ± 2) based on the procedure by Niiyama et al. (1985) with the following modifications: pigs were anaesthetised with detomidine (0.13 mg/kg), butorphanol (0.26 mg/kg) and ketamine (5 mg/kg). Prior to anaesthesia, the pigs were fasted for 24 h and the weight of the pigs recorded before administration of the drugs to ensure proper dosage. The lateral auricular vein was selected for catheterisation because of its smooth trajectory. The ear was occluded with a rubber band (at the base of the ear) to dilate the vein and removed after the venous access catheter was in place. Once the pig was under anaesthesia (after 15 min of drug administration) the ear was shaved and cleaned with 70 % ethanol and the vein was dilated by application of hot water to the ear. Catheterisation was performed using a 3FR 55 cm peripherally inserted central catheter (PICC) with trimmable placement wire (Arrow International Inc., Reading, PA, US) comprised of a venous access cannula (VAC) and 17 G peel away catheter over a 19 G needle. Only 40 cm was inserted and the tip of the catheter trimmed off. Venous access was obtained by inserting the VAC in the ear vein and removing the needle. The PICC was then inserted through the 17 G peel away catheter. When the insertion was complete, the guide wire was removed and the correct positioning assessed by sampling 2-3 mL of blood. After insertion, the peel away catheter was removed and the PICC flushed with isotonic saline solution. Pressure was applied at the insertion point to prevent haemorrhaging. The hub of the PICC was secured to the skin by suture with non-resorbable wire and an extension added to minimise the pigs’ manipulation during blood sampling. Several suture points were placed along the neck and back to hold the extension in place. Air was completely removed and the whole system flushed with
7. The effect of \( \alpha \)- or \( \beta \)-caseinate addition to WMS

isotonic saline and primed with heparin solution (0.01 units/L diluted to 2 %). The insertion point was disinfected thoroughly with 70 % ethanol and covered with Leukoplast to prevent infection. After the procedure the pigs were placed in a warm environment; in single pens and checked every 30 min until full recovery, usually within 2 h. Every pig was examined at least 3 times daily to ensure that the catheter was in position and to detect any associated health problems early.

7.2.4 Glucose analysis

Blood glucose was measured using a Glucomen Visio® glucometer (A. Menarini Diagnostics, Firenze, Italy) as per the manufacturer’s instructions. The glucometer was calibrated every morning before sampling using the Glucomen Visio control solution provided. The calibration region of the glucometer fell between 4.7 mmol/L and 6.4 mmol/L. The sampling times for glucose were 0, 15, 30, 45, 60, 90, 120, 150 and 180 min.
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Figure 7.1: Images of extension line and catheter for blood extraction including sampling vacutainers after catheterisation procedure
7. The effect of α- or β-caseinate addition to WMS

7.2.5 Blood sampling

Blood samples were collected at 3 time intervals; 0, 30 and 90 min, into pre-chilled vacutainers containing 18.0 mg K₂EDTA (BD Biosciences, Oxfordshire, England). For GLP-1 analysis at 0 and 90 min, dipeptidyl peptidase IV (DPPIV) inhibitor (Millipore Ltd., Watford, UK) was added within 30 s of collection, at a concentration of 0.01 µL/L of blood. Tubes were inverted several times to ensure a homogenous mix. Blood samples were kept on ice following collection, and then centrifuged at 2500 x g for 15 min at 4 °C within 1 h of collection. Plasma was obtained after centrifugation and stored at -80 °C until analysed.

7.2.6 Incretin hormones and Insulin Assays

Using commercial ELISA kits, plasma concentrations of active GLP-1 (GLP-1 (active) ELISA Kit, Millipore Ltd., Watford, UK), total GIP (rat/mouse GIP (total) ELISA Kit, Millipore Ltd., Watford, UK) and insulin (human insulin ELISA Kit, Millipore Ltd., Watford, UK) were measured. The sensitivity limits of the ELISA kits were 2.0 mU/L for intact insulin, 8.0 ng/L for total (active and inactive) GIP and 2.0 pmol/L for active GLP-1. Each plasma sample was assayed in triplicate. The concentrations of insulin, GIP and GLP-1 were derived by interpolation from a reference curve generated in each individual assay with reference standards of known concentrations of insulin, GIP and GLP-1 respectively.
7. The effect of αs- or β-caseinate addition to WMS

7.2.7 Amino acid levels in blood

Plasma samples were deproteinised by mixing equal volumes of 24 % (w/v) trichloroacetic acid (TCA) and sample, allowed to stand for 10 min and centrifuged at 14400 x g for 10 min. Supernatants were removed and diluted with 200 mmol/L sodium citrate buffer, pH 2.2 to give approximately 250 nmol of each amino acid residue. Samples were then diluted 1 in 2 with the internal standard, norleucine, to give a final concentration of 0.125 nmol/L. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, Herts, UK) fitted with a Jeol Na⁺ high performance cation exchange column.

7.2.8 Statistical analysis

Data sets were analysed using the mixed models procedure in SAS (Statistical Analysis Systems Institute Incorporated, Cary, NC) for an incomplete latin square design. The pig was the experimental unit. The model included the effects for treatment, time of sampling and the interactive effect between treatment and time of sampling. Animal weight was used as a covariant in the model. Treatment effect at particular times of sampling was determined using the slice statement in SAS. The results were presented as least square means ± SEM. Means separation was performed using the tukey-kramer test.
7. The effect of $\alpha_s$- or $\beta$-caseinate addition to WMS

7.3 Results

7.3.1 Pasting behaviour

The five stages of the gelatinisation/pasting process are outlined in Figure 7.2. Significant ($p < 0.05$) differences were observed between samples for each stage throughout pasting (Table 7.2). Significantly ($p < 0.05$) higher viscosity values were observed throughout pasting for all samples containing 10 % starch gelatinised with 10 % $\beta$-caseinate (Table 7.2) while 10 % starch gelatinised with 10 % $\alpha_s$-caseinate resulted in a significantly ($p < 0.05$) lower peak viscosity (1.7 Pa.s) compared to 10 % starch alone (3.2 Pa.s) or 10 % starch gelatinised with 10 % $\beta$-caseinate (4.6 Pa.s). Starch gelatinised with 10 % $\alpha_s$-caseinate had a significantly ($p < 0.05$) higher on-set pasting temperature (74.7 °C) compared to 10 % starch alone (68.4 °C) or 10 % starch gelatinised with 10 % $\beta$-caseinate (64.4 °C). The final paste viscosity increased in the following order: 10 % starch alone (1.7 Pa.s), 10 % starch gelatinised with 10 % $\alpha$-caseinate (4.8 Pa.s) and 10 % starch gelatinised with 10 % $\beta$-caseinate (7.6 Pa.s).
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Figure 7.2: Gelatinisation profile of 10% starch (♦), 10% starch + 10% $\alpha_s$-caseinate (▪), 10% starch + 10% $\beta$-caseinate (▴). (x) represents temperature profile. Point A on the curve represents the on-set stage of pasting, B represents peak viscosity on heating, C represents viscosity at end of heating, D represents viscosity at end of cooling and E represents the final viscosity of the paste.

Table 7.1: Viscosities of samples fed to pigs throughout stages (A-E) of pasting before feeding

<table>
<thead>
<tr>
<th></th>
<th>On-set pasting temp (°C)</th>
<th>Peak viscosity on heating (Pa.s)</th>
<th>End of heating viscosity (Pa.s)</th>
<th>End of cooling viscosity (Pa.s)</th>
<th>Final viscosity (Pa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Starch</td>
<td>n = 3</td>
<td>68.4$^b$</td>
<td>1.2$^b$</td>
<td>2.1$^c$</td>
<td>1.7$^c$</td>
</tr>
<tr>
<td>10% Starch + 10% $\alpha$-casein</td>
<td>n = 3</td>
<td>74.7$^a$</td>
<td>1.7$^c$</td>
<td>4.3$^b$</td>
<td>4.8$^b$</td>
</tr>
<tr>
<td>10% Starch + 10% $\beta$-casein</td>
<td>n = 3</td>
<td>64.4$^c$</td>
<td>0.5$^c$</td>
<td>6.7$^a$</td>
<td>7.6$^a$</td>
</tr>
<tr>
<td>df*</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

(a-c) Values within column with different lowercase letters are significantly different; ($P < 0.05$; Fishers test). * = degrees of freedom
7. The effect of $\alpha_s$- or $\beta$-caseinate addition to WMS

7.3.2 Glucose levels

The pre- and post prandial blood glucose concentrations in response to (i) 10% starch, (ii) 10% starch gelatinised with 10% $\alpha_s$-caseinate or (iii) 10% starch gelatinised with 10% $\beta$-caseinate over 3 h are illustrated in Figure 7.3. A rapid increase in glucose concentration was observed 15 min post prandial in response to gelatinised 10% starch, rising to a peak of 5.1 mmol/L. After the initial increase, an additional peak of 4.9 mmol/L was observed at 90 min with a subsequent decrease to 4.1 mmol/L at 180 min post prandial. Ingestion of 10% starch gelatinised with 10% $\beta$-caseinate also resulted in a rise in blood glucose concentration within 15 to 30 min, with a peak value of 5.4 mmol/L. A second peak in glucose concentration was observed at 90 min (5.2 mmol/L) with a decline to 3.6 mmol/L at 180 min post prandial. In contrast, ingestion of 10% starch gelatinised with 10% $\alpha_s$-caseinate resulted in a more gradual increase in blood glucose concentration reaching a peak of 4.6 mmol/L at 30 min. From 60 to 180 min, post prandial glucose concentrations decreased to 3.6 mmol/L. Starch gelatinised with $\alpha_s$-caseinate resulted in significantly ($p < 0.05$) lower glucose release at 15, 30 and 90 min post prandial compared to starch gelatinised with $\beta$-caseinate.

Area under the glucose curve (AUC) values for the initial rise (0-45 min) in blood glucose concentrations were 204, 194 and 226 mmol/L min on ingestion of 10% starch, 10% starch gelatinised with 10% $\alpha_s$-caseinate and 10% starch gelatinised with 10% $\beta$-caseinate, respectively. A significant difference ($p < 0.05$) was observed in AUC (0 – 45 min) between the 10% starch gelatinised with 10% $\beta$-caseinate and the 10% starch gelatinised with 10% $\alpha_s$-caseinate samples, with the latter being significantly lower.
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The second peak (60-120 min) resulted in AUC values of 141, 129 and 149 mmol/ (L min) for 10 % starch, 10 % starch gelatinised with 10 % αs-caseinate and 10 % starch gelatinised with 10 % β-caseinate and 180 min AUC values of 808, 752 and 846 mmol/ (L min) respectively.

Figure 7.2: Post prandial blood glucose concentrations following ingestion of 10 % starch (♦), 10 % starch + 10 % αs-caseinate (x), 10 % starch gelatinised with 10 % β-caseinate (Δ). (a-b) Values with different lowercase letters are significantly different, (p < 0.05).
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Table 7.3: Plasma GLP-1 concentrations (pmol/L) at 0 and 90 min following ingestion of 10 % starch, 10 % starch + 10 % $\alpha_s$-caseinate and 10 % starch + 10 % $\beta$-caseinate.

<table>
<thead>
<tr>
<th></th>
<th>10% Starch (pmol/L)</th>
<th>10% Starch +10% $\alpha_s$-caseinate (pmol/L)</th>
<th>10% Starch +10% $\beta$-caseinate (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>7.01</td>
<td>6.78</td>
<td>6.65</td>
</tr>
<tr>
<td>90 min</td>
<td>7.87</td>
<td>7.34</td>
<td>7.04</td>
</tr>
</tbody>
</table>

7.3.3 GLP-1 analysis

There were no significant differences in post prandial plasma GLP-1 concentrations following ingestion of (i) 10 % starch, (ii) 10 % starch gelatinised with 10 % $\alpha_s$-caseinate or (iii) 10 % starch gelatinised with 10 % $\beta$-caseinate (Table 7.2). Pre-prandial concentrations of plasma GLP-1 ranged from 6.65 pmol/L to 7.01 pmol/L. At 90 min these levels increased to between 7.04 and 7.87 pmol/L.

7.3.4 GIP analysis

Post prandial GIP concentrations in plasma over 90 min in response to (i) 10 % starch, (ii) 10 % starch gelatinised with 10 % $\alpha_s$-caseinate and (iii) 10 % starch gelatinised with 10 % $\beta$-caseinate are detailed in Figure 7.4a. Pre prandial plasma concentrations of GIP was 151 (S.E. 39) ng/L. The GIP concentration increased to 1509 (S.E. 117) ng/L at 30 min and decreased to 959 (S.E. 135) ng/L at 90 min in response to 10 % starch alone. Ingestion of 10 % starch gelatinised with 10 % $\alpha_s$-caseinate resulted in plasma GIP concentrations at 0, 30 and 90 min of
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155 (S.E 24), 2109 (S.E 180) and 1348 (S.E. 149) ng/L respectively. There was a significant ($p < 0.05$) difference at 30 min in GIP levels in response to the control compared to starch gelatinised with $\alpha$- or $\beta$-caseinate.

![Figure 7.4(a) Plasma GIP concentrations following ingestion of 10 % starch, 10 % starch + 10 % $\alpha$s-caseinate and 10 % starch + 10 % $\beta$-caseinate. Pre-prandial GIP concentrations [horizontal], 30 min post prandial [slanted] and 90 min post prandial [vertical].](image)

7.3.5 Insulin analysis

Post prandial plasma concentrations of insulin in response to (i) 10 % starch, (ii) 10 % starch gelatinised with 10 % $\alpha$s-caseinate and (iii) 10 % starch gelatinised with 10 % $\beta$-caseinate are detailed in Figure 7.4b. Ingestion of 10 % starch resulted in a rise from 21 (S.E. 3) mU/L to 111 (S.E 21) mU/L in 30 min. This was followed by a decline to 38 (S.E. 8) mU/L at 90 min. Ingestion of 10 % starch gelatinised with 10 % $\beta$-caseinate resulted in a rise from 18 (S.E. 2) mU/L to 151 (S.E. 25) mU/L within 30 min and decreased to 55 (S.E. 9) mU/L at 90
min. Ingestion of 10 % starch gelatinised with 10 % \( \alpha_s \)-caseinate resulted in a rise from 20 (S.E. 2) mU/L to 138 (S.E 23) mU/L within 30 min, decreasing to 74 (S.E. 13) mU/L at 90 min. Post prandial insulin levels at 30 min tended \((p < 0.1)\) to be higher for 10 % starch gelatinised with 10 % \( \beta \)-caseinate compared to 10 % starch and at 90 min tended \((p < 0.1)\) to be higher for 10 % starch gelatinised with 10 % \( \alpha_s \)-caseinate compared to 10 % starch. Plasma levels for both GIP and insulin peaked at the same time point (30 min).

Figure 7.4(b): Insulin concentrations (in plasma) following ingestion of 10 % starch, 10 % starch + 10 % \( \alpha_s \)-caseinate and 10 % starch + 10 % \( \beta \)-caseinate. Pre-prandial insulin concentrations [horizontal], 30 min post prandial [slanted] and 90 min post prandial [vertical].
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**Figure 7.4(c):** Total amino acid levels of 10 % starch, 10 % starch + 10 % $\alpha_s$-caseinate and 10 % starch + 10 % $\beta$-caseinate post prandial at 30 min (slanted line) and 90 min (vertical line). $^{(a-b)}$ Values with different lowercase letters are significantly different from each other ($p < 0.05$).

### 7.3.6 Amino acid composition

Total amino acid concentration in plasma (Figure 7.4c) decreased on ingestion of 10 % starch alone from a basal concentration of 1213 $\mu$mol/L to a 90 min concentration of 887 $\mu$mol /L. There was a significant ($p < 0.05$) increase in plasma amino acid concentration at 30 and 90 min post prandial in response to 10 % starch gelatinised with 10 % $\alpha_s$-caseinate and 10 % starch gelatinised with 10 % $\beta$-caseinate compared to basal levels. Not surprisingly, there was a significant ($p < 0.05$) increase in plasma concentrations of individual free amino acids, post prandial in response to 10 % starch gelatinised with 10 % $\alpha_s$-caseinate and 10 % starch gelatinised with 10 % $\beta$-caseinate at 30 and 90 min (Figures 7.5b and 7.5c) compared to basal levels. Exceptions to this were levels
of glycine, cysteine and asparagine. Moreover, there was a significant ($p < 0.05$) increase in 90 min plasma levels of leucine, phenylalanine, lysine, tyrosine, histidine and arginine post-ingestion of 10% starch gelatinised with 10% $\alpha_s$-caseinate compared to 10% starch gelatinised with 10% $\beta$-caseinate (Figure 7.5c).
Figure 7.5: Concentrations of free amino acids (AA) in (a) plasma pre-prandial (T0), (b) plasma post prandial (T30) and (c) plasma post prandial (T90) of 10 % starch (■), 10 % starch + 10 % αs-caseinate (■) and 10 % starch + 10 % β-caseinate (■). Values with different lowercase letters are significantly different from each other, \( p < 0.05 \).
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7.4 Discussion

Ingestion of starch gelatinised with $\alpha_s$-caseinate resulted in a lower post prandial glucose peak, lower glucose levels over 120 min and increased levels of several free amino acids than ingestion of starch gelatinised with $\beta$-caseinate. The difference in blood glucose levels may be explained by the lower peak viscosity of starch-$\alpha_s$-caseinate compared to that of the starch-$\beta$-caseinate equivalent. Lower peak viscosity may be indicative of reduced granule swelling. Previous work by Kett et al. (2009) showed that swelling characteristics of starch-protein mixtures were concentration dependent. The starch-protein concentration [compared to preliminary studies at 5 % (Kett et al., 2009)] in this study, i.e., 10 %, appeared to result in more extensive restriction in the starch-$\alpha_s$-caseinate mixture. During heating, starch granules absorb water and increase in size resulting in a concomitant increase in viscosity (Tester, Karkalas and Qi, 2004). We postulate that granule restriction specific to the starch and $\alpha_s$-caseinate mixture may be due to (i) competition between the starch and $\alpha_s$-caseinate for available water in the mixture or (ii) association of $\alpha_s$-caseinate with the surface proteins/lipids of the starch granules protecting granular structure (Noisuwan et al., 2011). Protection of starch granules, resulting in increased granular structure, may have resulted in reduced digestion by pancreatic $\alpha$-amylase activity and delayed glucose release into the blood. Several studies have demonstrated granule restriction of starch in the presence of various polymers including proteins (Christianson et al., 1981; Kelly et al., 1995). In addition, water competition has been reported in heated solutions of waxy rice starch and sodium caseinate (Noisuwan et al., 2007). Studies performed by the authors would suggest that reduced granular swelling has a role to play in inhibiting enzymatic
The effect of $\alpha_s$- or $\beta$-caseinate addition to WMS hydrolysis, delaying subsequent breakdown of the starch. Confocal and light microscopy indicated that waxy maize starch gelatinised in the presence of $\alpha_s$- or $\beta$-caseinate resulted in a significant reduction in granular swelling compared to starch gelatinised alone (Kett et al., 2009). Studies by Manders et al. (2005) showed that in healthy and diabetic patients, ingestion of a carbohydrate (CHO) (50:50 glucose-maltodextrin mixture) and casein hydrolysate-amino acid mixture lowers plasma glucose concentration compared to ingestion of CHO alone. Previous work by van Loon et al. (2000) with drinks containing CHO (50:50 glucose-maltodextrin mixture) and three different protein hydrolysates (i.e. whey, wheat and casein) reported that ingestion of the CHO-casein hydrolysate drink resulted in the lowest blood glucose levels 30 min post prandial.

Although the plasma levels of total amino acids did not differ between starch-caseinate samples, significant ($p < 0.05$) differences at 90 min in six (leucine, phenylalanine, lysine, tyrosine, histidine and arginine) individual amino acids postprandial in response to starch gelatinised with $\alpha_s$-caseinate compared to starch gelatinised with $\beta$-caseinate may be due to differences in (i) molecular state (Horne, 2002), (ii) hydrophobicity (Dalgleish, 1997; Patino, Sánchez and Nino, 1999), (iii) amino acid sequence and composition (Swaisgood, 2003) and (iv) serine phosphate groups (Fox, 2003) of the $\alpha$- and $\beta$-casein proteins. Although $\alpha_{s1}$-casein and $\beta$-casein are 31% homologous, $\alpha_{s1}$-casein consists of 199 amino acid residues (GenBank GI:30794348) whereas $\beta$-casein is composed of 209 amino acid residues (GenBank GI:162931) (Dalgleish, 1997). In addition, $\beta$-casein has less phosphate groups than $\alpha$-casein. In acidic conditions, such as in the stomach, partial dephosphorylation of casein occurs, reducing the size of protein precipitates, thus facilitating peptic digestion. Therefore, $\beta$-casein with its
lower degree of phosphorylation is more susceptible to peptic hydrolysis (Fox et al., 2004). Evidence of this susceptibility can be seen in Figure 7.4c whereby the percentage increase (from 0-180 min) in total free amino acids was statistically higher for the starch-β-caseinate mixture compared to the starch-αs-caseinate mixture. Assuming that the post prandial levels of the significant six amino acids were primarily as a result of the dietary source, ingestion of β-caseinate within the starch-β-caseinate mixture resulted in a reduction in the plasma levels of these amino acids after 90 min, which may imply that the starch-β-caseinate mixture was absorbed from the blood at a faster rate than starch-αs-caseinate equivalents. Of the six amino acids that were significantly different, five are essential amino acids. It has been reported that essential amino acids are absorbed more rapidly within a mixture of amino acids compared to the non-essential amino acids (Adibi, Gray and Menden, 1967).

The insulinotropic amino acids, arginine, leucine and phenylalanine (Floyd et al., 1966 a,b), were significantly (p < 0.05) higher in plasma 90 min post prandial in response to starch gelatinised with αs-caseinate compared to starch gelatinised with β-caseinate. van Loon et al. (2000) showed that these three amino acids promote insulin production when orally administered with carbohydrate. There was a significant (p < 0.05) difference at 30 min in GIP levels in response to the control compared to starch gelatinised with αs- or β-caseinate. Although there was no significant difference in plasma insulin levels between starch and αs-or β-caseinate, there was an observed tendency (p < 0.1) for an increase in plasma insulin levels 90 min postprandial in response to starch gelatinised with αs-caseinate albeit compared to starch alone.
Not surprisingly, the addition of caseinate to starch resulted in higher levels of free amino acids in the peripheral blood within 30 min compared to starch alone. Nilsson et al. (2004) reported that peak amino acid levels for milk proteins occurred 30-45 min post prandial, indicating that milk proteins are highly digestible and result in a rapid release of free amino acids into peripheral blood.

7.5 Conclusions

This study demonstrates that individual protein fractions can affect swelling characteristics, viscosity and subsequent digestion of waxy maize starch. The presence of 10% $\alpha_s$-caseinate during gelatinisation of the starch resulted in a significantly ($p < 0.05$) lower peak glucose concentration during subsequent digestion in an in vivo pig model compared to 10% $\beta$-caseinate. The difference in glucose release could be attributed to (i) restricted granule swelling during gelatinisation and/or (ii) differences in viscosity restricting $\alpha$-amylase activity during digestion. In addition, plasma levels of six individual amino acids were increased following ingestion of starch gelatinised with $\alpha_s$-caseinate compared to ingestion of starch gelatinised with $\beta$-caseinate. These findings suggest that the type of protein used in starch based food systems may influence the ability of the food to modulate glycemia. This is an important consideration in the design of foods with health benefits.

7.6 ACKNOWLEDGEMENT

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APK was responsible for pre-trial training of the pigs, performed animal trials, statistically analysed the data and drafted the manuscript. CMB participated in the animal trials and performed biological assays. FOH was involved in preliminary trials and performed biological assays. PL was involved in preliminary trials and design of experiments. LG was involved in design of experiments, analysis of incretin data and manuscript drafting. MAF was involved in preliminary trials, design of experiments and secured funding for the trials.
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7.7 References


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Please note that Appendices 1-5 (pp.302-345) are unavailable due to a restriction requested by the author.