Effect of plant proteins on *in vitro* digestibility of wheat starch

by

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ABSTRACT

The effect of plant proteins, specifically those from yellow field pea seeds on the *in vitro* digestibility of wheat starch was studied. Initially, the starch digestibility profile of pressure cooked pea seeds (dehulled and split), pea flour and isolated starches from pea and wheat was evaluated by determining the contents of RDS (rapidly digestible starch), SDS (slowly digestible starch) and RS (resistant starch). The RDS contents of cooked seed (80.3%) and flour (84.1%) samples were significantly lower than those of cooked starches (pea, 88.3%, and wheat, 88.6%), where the RDS content of cooked seeds was significantly lower than that of cooked flour. No significant difference was observed in the RDS contents of cooked starches from pea (88.3%) and wheat (88.6%). The SDS contents of pea seeds and flour generally were similar (12.2% and 13.2%, respectively), but significantly higher than that of isolated starches (pea, 6.4%, and wheat, 9.9%). The SDS content of isolated and cooked pea and wheat starches were significantly different. The RS content was significantly higher in split pea seeds (7.5%) than in pea starch isolate (5.3%), pea flour (2.7%) and wheat starch isolate (1.5%). Scanning electron microscopy (SEM) and differential scanning calorimetry (DSC) were used to characterize the morphological and thermal properties of the samples. The results indicated that endogenous protein and its interaction with starch during pressure cooking may be one of the factors that influence starch digestibility in pea seeds. In order to substantiate this preliminary finding, further research was performed using isolated plant proteins from different plant origins, and isolated wheat starch. The effect of protein isolates from wheat, corn, soybean, pea and rice grains in their native, heat-denatured and protease-hydrolysed forms on the *in vitro* amylase digestibility of wheat starch was investigated. Native proteins, except that from rice, did not cause significant reduction in the RDS content of cooked wheat starch when compared to controls. Heat-denatured proteins,

denatured either by boiling (except for corn and pea) or pressure cooking, resulted in significant reduction in RDS content. Protease- hydrolysed proteins, produced with or without denaturation by boiling or pressure cooking prior to their addition to wheat starch, caused significant reduction in RDS (except for pressure-cooked plus hydrolysed pea protein, and boiling-denatured and boiling-denatured plus hydrolysed corn protein). Differential scanning calorimetry studies and confocal laser scanning microscopy of selected starch-protein mixtures suggested that protein denaturation and protease hydrolysis promoted starch-protein interaction, and thus mitigated RDS content.

Further studies were conducted using digestion simulation models of the human gastro-intestinal system to understand the possible inhibitory effects of native and hydrolysed pea protein on the amylolysis of wheat starch in an extruded wheat snack matrix. A combination of an *in vitro* dynamic gastric model (DGM) and a static duodenal digestion model (SDM) was used in this study. The addition of native pea protein did not influence the release of soluble starch and glucose when compared to the control after complete gastric emptying. However, the addition of hydrolysed pea protein significantly reduced soluble starch at 0, 5, 20 and 40 min. Infrared (FTIR) scans of the extruded samples clearly indicated enhanced starch-protein interactions through hydrogen bonding, mainly in the blend with hydrolysed pea protein. The studies in general suggested the possibility of developing a protein-based strategy to formulate low-glycemic food products.

PREFACE

This thesis research was conducted under the supervision of Prof. Thava Vasanthan at the Department of Agricultural, Food and Nutritional Science (AFNS), University of Alberta. The research was financially supported by the discovery grant offered to Prof. Vasanthan by the Natural Sciences and Engineering Research Council (NSERC) of Canada and a graduate student scholarship offered to me by the Colombian Administrative Department of Science, Technology and Innovation (COLCIENCIAS).

All the work presented in chapters 3 and 4 was conducted at the University of Alberta. A version of chapter 3 will be submitted to Cereal Chemistry Journal. A version of chapter 4 has been published as López-Barón, N., Gu, Y., Vasanthan, T and Hoover, R. 2017. Plant proteins mitigate *in vitro* wheat starch digestibility. Food Hydrocolloids, Volume 69: 19-27. I was responsible for the experimental work, data collection, analysis and manuscript preparation. Yuchen Gu, assisted in the microscopic investigations, data analysis and manuscript preparation. Thava Vasanthan and Ratnajothi Hoover, assisted with scientific discussions and idea development, manuscript preparation, editing and publication.

The work presented in chapter 5 forms part of an international research collaboration between Prof. Andreas Blennow at the University of Copenhagen, and Prof. Thava Vasanthan at the University of Alberta. A version of chapter 5 has been published as López-Barón, N., Sagnelli, D., Blennow, A., Holse, M., Gao Jun, Saaby, L., Müllertz, A., Jespersen, B. and Vasanthan, T. 2017. Hydrolysed pea proteins mitigate *in vitro* wheat starch digestibility. Food Hydrocolloids, Volume, 79: 117-126. In this collaborative research, I was responsible for all major areas of experimental work (carried out both in Denmark and Canada), data analysis and

manuscript preparation. Domenico Sagnelli, Andreas Blennow (University of Copenhagen) and Lasse Saaby, assisted with scientific discussions for experimental planning, digestion model equipment setup and operation, sample analysis and manuscript revisions. Mette Holse (University of Copenhagen) provided training in extrusion processing and manuscript revision. Jun Gao, assisted in the statistical analysis of the data. Anette Müllertz and Birthe Jespersen provided and assisted in the operation of Dynamic Gastric Model – Static Duodenal Model and extruder, respectively. Thava Vasanthan generally facilitated and supervised the project throughout in concept formation, experimental planning, manuscript preparation, editing and publication.

DEDICATION

This doctoral dissertation is dedicated to my beloved mother Hilda Beatriz Barón Aranguren and the memory of my beloved father Pedro Léon López Castellanos who grew pulses (field pea and bean) and cereals (wheat, barley and oat) for many years of his life.

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First of all I would to thank God for all the blessings that I have received while doing my PhD and for letting me admire His perfect creation while learning and doing my research. I would like to thank Dr. Thava Vasanthan for giving me the opportunity to join his research group, for being my supervisor, but also my mentor and my friend. I am deeply thankful for his patient guidance, advice and support throughout the different stages of my PhD studies. I am very thankful for all his help and direction to successfully complete this thesis.

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In addition I would like to thank Jun Gao, Dr Yuchen Gu and Dr Kelvin Lien for their immediate help when I needed technical assistance, and my fellow labmates from labs: 2-54, 2-58 and 540 for giving me support and a friendly environment to work in all these years.

I am also grateful with my family especially my mother, Beatriz Barón Aranguren and my aunt Nelcy Barón Aranguren for their unconditional and endless love, support and encouragement. My appreciation is extended to my sister and my brother for always helping me. Lastly, I wish to thank my friends in Canada and Colombia for listening and being there for me while I was so far from home.

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LIST OF SYMBOLS AND ABBREVIATIONS

ANN	Annealing
AMG	Amyloglucosidase
AP	Amylopectin
APTS	8-aminopyrene-1,3,6-trisulfonic acid, trisodium salt
AUC	Area under the curve
Bfp	"Fingerprint" B-chains
BSmajor	Major group of B short chains
COOH	Carboxyl group
CL	Chain length
CLSM	Confocal laser scanning microscopy
DGM	Dynamic gastric model
DH	Degree of hydrolysis
DMSO	Dimethyl sulphoxide
DP	Degree of polymerization
DSC	Differential scanning calorimetry
FTIR	Fourier-transform infrared spectroscopy
GI	Glycemic index
GL	Glycemic load
GOPOD	Glucose oxidase peroxidase
GRAS	Generally regarded as safe

HCL	Hydrochloric Acid
HGS	Human gastric simulator
HMT	High moisture treatment
kDa	Kilodalton
NH_2	Amino group
NIR	Near infrared
PPi	Inorganic pyrophosphate
RDS	Rapidly digestible starch
RS	Resistant starch
SAXS	Small-angle X-ray scattering
SDS	Slowly digestible starch
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SME	Specific mechanical energy
TIM-1	Gastro intestinal model
Tc	Conclusion temperature
Tc – To	Transition temperature range
TNBS	2,4,6-trinitrobenzenesulfonic acid
To	Onset temperature
Tp	Peak temperature

UDP	Uridine diphosphate glucose
USP	The United States Pharmacopoeia
wt %	Weight percent
WSC	Wheat starch containing cellulose
ΔH	Enthalpy of gelatinization

CHAPTER 1. INTRODUCTION AND OBJECTIVES

1.1. INTRODUCTION

Starch is the main digestible carbohydrate found in cereal and pulse grains as well as tubers and roots; indeed, it is the primary nutritional source of energy for humanity. However, excessive consumption of starch-containing foods on a regular basis, especially those made with highly processed or refined ingredients such as white flour and purified starches, has been shown to have detrimental effects on human health worldwide. The digestion of a refined, starchy food is associated with the very efficient enzymatic hydrolysis of the starch and, subsequently, the rapid elevation of blood glucose levels following a meal.

The digestion of starch begins in the mouth, where it is moistened with saliva containing the starch enzyme, alpha-amylase. This initial hydrolysis of starch quickly stops once the bolus reaches the stomach, due to the very high acidity of the gastric juices. When the contents of the stomach pass into the small intestine, hydrolysis of starch continues through the action of pancreatic alpha-amylase converting starch to maltose, maltotriose, alpha-limit dextrins and glucose. Brush border enzymes in the intestinal mucosa, such as maltase-glucoamylase and sucrose-isomaltase, further hydrolyse the dextrins and sugars into glucose, which is absorbed and transported to the liver and then can be delivered to the body cells as a source of energy. Excessive consumption of refined starchy foods creates abnormal metabolic responses such as insulin resistance and the blockage of beta-oxidation of lipids, which are associated with the rise in diabetes and obesity, respectively (Frayn 2010).

Diabetes and obesity are two major global public health concerns because of their increasing prevalence among populations (Fryar et al. 2012; World Health Organization 2016). These health issues are paradoxically exacerbated by out-of-date dietary guidelines worldwide

recommending people to increase their consumption of grain products and decrease fat intake. These foods, such as breads, rice, pasta, etc., are considered rich in complex carbohydrates such as starch (digestible) and dietary fiber (indigestible). However, the consumption of grain products, such as biscuits, pastry, cakes and pancakes that are made from refined wheat flour (i.e. rich in rapidly digestible but complex carbohydrates) increased in the past decades, especially in western diets. Furthermore, the more recent trend toward gluten-free foods has potentiated these health problems since they are made with a variety of refined starches such as potato, tapioca, corn, cassava and rice. Gluten-free foods are consumed not only by people who certainly are suffering from celiac disease, an immunological reaction to gluten, but also by those who mistakenly believe that these foods are indeed healthier choices (Gaesser and Angadi 2012).

The rate of digestion of native starches has been related to different intrinsic factors such as morphology, the type and extent of starch crystallinity, the amylose/amylopectin ratio and molecular size, and the presence of channels within the starch granule. In this regard, research has shown that native tuber and pulse starches are more resistant to enzymatic hydrolysis compared to cereal starches. Potato starch, for example, possesses granules with a smooth surface that limits the easy entry of amylolytic enzymes, and contains a B-type crystal polymorph that is more resistant to amylase hydrolysis compared to the A-type crystal polymorph common in cereal starch. Pulse starches possess a smooth granule surface, C-type crystal polymorph (i.e. a combination of A-type and B-type crystals), and greater amylose content, which is associated with a slower amylolysis. In contrast, cereal starches have granules with a rougher surface, channels that facilitate the easy entrance of amylase enzymes and a greater content of amylopectin. Cereal starch has a molecular structure that arranges into the A-type crystallites that are typical of highly digestible starches (Singh et al. 2010). These intrinsic

factors in general can explain the higher resistance to amylase hydrolysis of purified tuber and pulse starches in their native state compared to cereal starches. However, a controlled preliminary study in our lab clearly showed that refined and purified starches of wheat, barley, field pea, fava bean and potato all become rapidly digestible once cooked and gelatinized. Here it was observed interesting discrepancies in the amylolysis of cooked flours that could not be explained by the intrinsic structural differences of the starches. For instance, after cooking/gelatinization, the starch in potato flour is rapidly hydrolysed, whereas the starch in pulse flour is slowly hydrolysed. Therefore, it remains an open question whether non-starch components such as protein in the flours influence the digestibility of gelatinized starches.

A number of *in vivo* studies have shown that endogenous proteins from wheat (Jenkins et al. 1987), and exogenous proteins and protein hydrolysates from yellow pea, wheat, rice and soybean, when added to starchy food matrices, generate faster insulin and glucagon hormonal responses that then reduce the blood glucose concentration (Smith et al. 2012; Claessens et al. 2009). These physiological findings are very useful from a clinical point of view; however, very little research so far, has elucidated the nature of the molecular interactions between starch and endogenous and exogenous plant proteins occurring during heat processing of foods. Understanding the way these proteins inhibit starch amylolysis may be critical to developing food systems that can better control starch digestibility. In addition, the effect of exogenous plant proteins in different states i.e. native, heat-denatured, enzymatically hydrolysed and denatured-hydrolysed, on reducing wheat starch enzymatic hydrolysis has not been investigated.

In vitro methods have been commonly used to study enzymatic starch hydrolysis. These methods resemble digestion of starch in the small intestine with addition of alpha-amylase and amyloglucosidase enzymes, and could be used as a tool to predict *in vivo* metabolic responses.

Bornet et al. (1989) correlated insulin and glycemic *in vivo* responses with *in vitro* alpha-amylase hydrolysis of native starches processed as raw ingredients, starch gels, and industrially processed foods. The results showed high similarities between the mean areas under glycemic and insulinemic curves, and the percentages of *in vitro* starch amylolysis at 30 min. Other biochemical methods using dialysis membranes, have allowed to measure sugars and oligosaccharides as products of carbohydrate digestion, to predict the *in vivo* glycemic index (GI) (Jenkins et al. 1982). Goñi et al. (1997) correlated the percentage of *in vitro* starch hydrolysis at 90 min (H_{90}) with *in vivo* glycemic responses, expressed as GI= 39.21+0.803(H_{90}). Also, the authors suggested the equation GI=39.71+0.549(HI), where HI is the hydrolysis index obtained as percentage, by dividing the percentage of area under the hydrolysis curve (AUC) between 0 to 180 min by the corresponding area of white bread as control. However, these empirical equations do not provide a complete kinetic analysis with regard to the rate and extent of starch digestion *in vivo*. In addition, these methods face challenges in terms of standardization of gastric residence times, digestive fluid secretions, etc., that will influence the prediction of *in vivo* blood glucose response and glycemic index (Dhital et al. 2017). To date, there are no studies reported on the effect of plant proteins on *in vitro* starch amylolysis using dynamic and more complex digestion models. Therefore, more *in vitro* research is needed as a primary tool to control experimental variables and thus better determine the efficacy of these proteins on lowering starch digestibility before effectively conducting *in vivo* studies. *In vivo* studies will further determine the real effectiveness considering a number of variables within individuals such as mastication time, rate of gastric processing, concentration of digestive enzymes and hormonal responses.

1.2. RESEARCH OBJECTIVES AND HYPOTHESIS

The primary focus of this thesis was to understand the effect of plant proteins on lowering *in vitro* digestibility of wheat starch, with particular focus on pea protein. The study investigated the potential interaction of native, heat-denatured, and protease-hydrolysed plant proteins with wheat starch. The specific objectives are presented below. The outcome of this thesis research may provide the foundation and the knowledge on how plant proteins could be used as a strategy to generate low blood glucose responses in healthy, diabetic and celiac individuals when consuming starch-containing foods.

Study 1:

Hypothesis: Endogenous protein within the split pea seeds has a significant effect on decreasing *in vitro* pea starch digestibility.

Objective: To investigate the effect of endogenous protein, on the *in vitro* starch digestibility profile [RDS (rapidly digestible starch), SDS (slowly digestible starch) and RS (resistant starch)] (Chapter 3).

Study 2:

Hypothesis: a) The rate of *in vitro* wheat starch digestibility can be decreased by plant proteins from different sources, such as pulse, cereal and oilseed, in their native, denatured and hydrolysed forms; b) "Protease-hydrolysed plant proteins" are more efficient than "native un-hydrolysed proteins" in mitigating the rate of *in vitro* wheat starch digestibility.

Objective: To investigate the effect of isolated plant proteins from wheat, corn, soybean, pea and rice in their native, heat-denatured and protease-hydrolysed states, on the *in vitro* porcine

pancreatic alpha-amylase hydrolysis (i.e. rapidly digestible starch content, RDS) of wheat starch (Chapter 4).

Study 3:

Hypothesis: Possible inhibitory effects on wheat starch amylolysis by native and hydrolysed pea protein, in an extruded wheat snack matrix, can be verified by using a combined *in vitro* dynamic gastric model (DGM) and a static duodenal digestion model (SDM) that simulate conditions in the human intestinal tract.

Objective: To determine the wheat starch amylolysis profile of an extruded snack matrix, prepared with blends of wheat flour and native or protease-hydrolysed pea proteins at 12% w/w (db), by measuring the time-dependent release of soluble starch and glucose during digestion using the DGM and SDM combined system (Chapter 5).

CHAPTER 2. LITERATURE REVIEW

2.1 STRUCTURE AND COMPOSITION OF CEREALS AND PULSES

Cereals are the oldest cultivated plants and are the main source of carbohydrates that provide energy to humans and animals. Botanically, cereals belong to the monocot family Poaceae or Gramineae and include plants such as wheat, corn, rice, barley, oat, sorghum, millet, rye and triticale (Koehler and Wieser 2013). Specifically, the wheat grain is a one-seeded fruit called a kernel or caryopsis (Figure 2.1). The caryopsis consists of a fruit coat (pericarp) that is strongly adhered to the seed coat (testa). The seed is composed of the germ, endosperm, aleurone layer, nucellar epidermis and a seed coat (testa) (Hoseney 1994). The pericarp, seed coat, nucellar epidermis and aleurone layer are together called bran. Cereal grains vary in shape, size, structure and chemical composition depending on the variety and soil and environmental conditions.

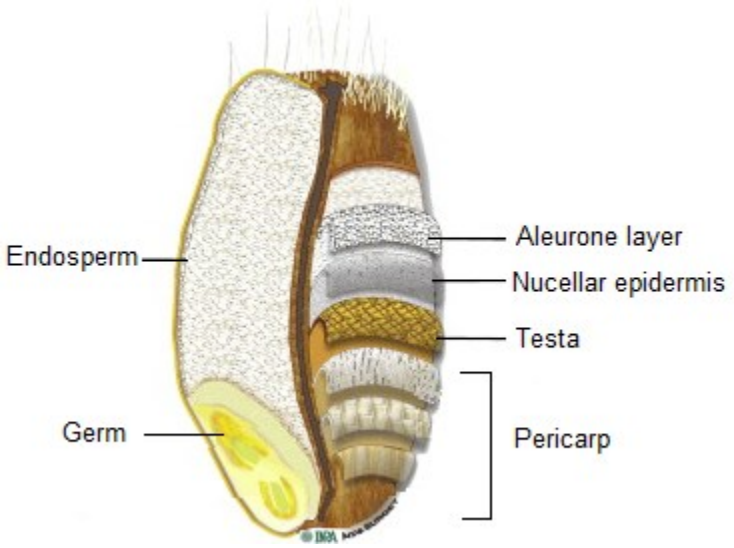


Figure 2. 1 Structure of wheat grains. Adapted from (Surget and Barron 2005), with permission of CGP Communication.

Cereal grains are rich in carbohydrates such as starch and dietary fiber. In particular, wheat contains ~ 60-80% of starch and it is mostly deposited in the endosperm. In other cereals, starch content varies from 66% for oat to 77% for barley. Wheat dietary fiber (~12.5%) is mainly located in the bran and is composed of a variety of carbohydrates such as arabinoxylans (1.5-2.0%), beta-glucans (~0.7%), cellulose (~1.0%) and glucofructans (~1.0%) (Koehler and Wieser 2013; Shewry et al. 2013). Protein is the second major component in the grains and varies between 6%-22% for wheat and ~7.5% for rice (Tacer-Caba et al. 2015). Based on the solubility, grain proteins are classified into four types: albumins soluble in water, globulins soluble in 0.5-1.0M salt solutions, prolamins soluble in 60-70% ethanol solutions and glutelins soluble in dilute acid or alkali (Osborne 1924). Cereal grains also possess minor components such as lipids, vitamins, minerals, sugars, enzymes, pigments, phytic acid and phenolics. Phytic acid and phenolic acids are considered as anti-nutritional factors. They have the capacity to chelate minerals that become unavailable for absorption, and cross-link proteins and fiber, decreasing digestibility and solubility, respectively (Hoseney 1994).

Pulse grains are dry dicotyledonous seeds that belong to the Leguminoseae (i.e. legume) or Fabaceae family (Hoover and Sosulski 1991), and include dry pea, chickpea, lentil, fava bean and dry bean, among others. Mature pulse seeds have three main components: cotyledons (80-95%), testa (5-15%) and embryo (1-2%) (Figure 2.2) (Chibbar et al. 2010). Pulses are good sources of carbohydrates and protein that also fluctuate by variety and soil and growing conditions. Carbohydrates are stored as the main energy reserve in the cotyledons. Starch is the most representative carbohydrate in pulse seeds and varies in concentration between 22-40% (Hoover and Ratnayake 2002).

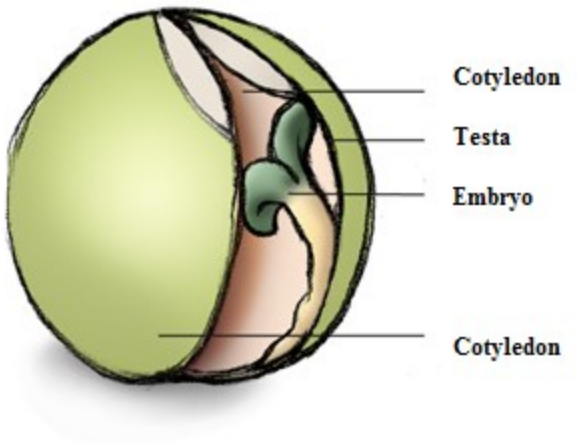


Figure 2.2 Structure of pulse grains.

Pulse proteins (18-40%) are usually albumin (~70%) and globulins (10-20%) and small amounts of prolamin and glutelin fractions. Albumins are composed of a heterogeneous group of enzymes, amylase inhibitors and lectins, whereas globulins are mainly composed of legumin and vicilin storage proteins (McCrory et al. 2010). Specifically, pea proteins mainly contain globulins (~70%) such as legumin, 11S, and vicilin/convicilin, 7S. In terms of essential amino acids, pulses are deficient in methionine but rich in lysine compared to cereals. In addition, pulses are a good source of fiber (from ~15.8% for lentil to 28.3% for chickpea), B-complex vitamins and minerals (Hoover et al, 2010; Majeed et al. 2017; Ramulu and Rao 1997).

2.2 STARCH

Starch is a carbohydrate and the major energy storage in plants. It is synthesized in many plant organs, including leaves, seeds, steams, tubers, roots and fruits. In essence, starch is a polymer of glucose formed by mainly linear amylose molecules that typically constitutes 15% to 20% of starch, and branched amylopectin molecules, as the main component of regular starch (Sajilata et al. 2006).

2.2.1 Biosynthesis

Starch is naturally synthesized by plants in the presence of water, carbon dioxide, soil nutrients and sunlight. It is found in chloroplasts, as a form of temporary storage in leaves, roots and stems, and in amyloplasts as long term energy storage in seeds and tubers (Keeling and Myers 2010). In storage organs, starch biosynthesis involves a series of reactions, starting from sucrose derived from photosynthesis (Figure 2.3).

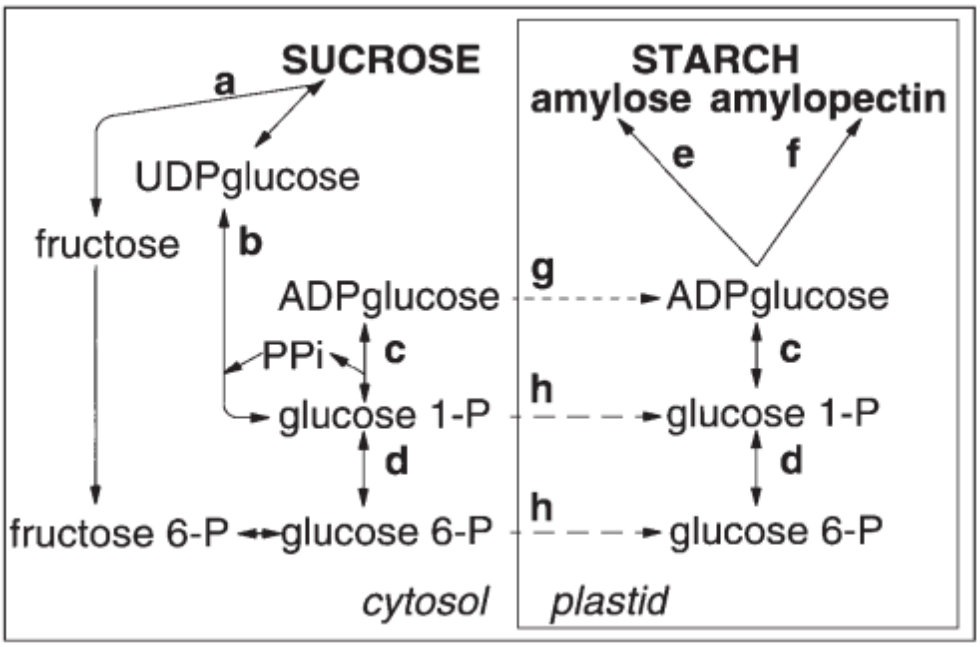


Figure 2.3. Biosynthesis of starch from sucrose in storage organs. Enzymes: a) sucrose synthase; b) UDPglucose pyrophosphorylase; c), ADPglucose pyrophosphorylase; d) phosphoglucomutase; e) starch synthase (GBSSI); f) starch synthase and starch-branching enzyme; g) ADP glucose transporter; h) hexose phosphate transporter. PPi: inorganic pyrophosphate. Adapted from Smith et al. (1997), with permission of Annual Reviews.

Sucrose is converted in the cell cytosol to fructose and uridine diphosphate glucose (UDP-glucose) by sucrose synthase. UDP-glucose, in the presence of inorganic pyrophosphate (PPi), is transformed by UDP-glucose pyrophosphorylase into glucose-1-phosphate (G-1-P), which is converted to glucose-6-phosphate (G-6-P) by phosphoglucomutase. G-6-P is then

transported into the amyloplast by hexose phosphate transporter and is converted to G-1-P by phosphoglucomutase. G-1-P is transformed to adenosine diphosphate glucose (ADP-glucose) by ADP glucose pyrophosphorylase (Smith et al. 1997). Evidence has shown that G-1-P in the cytosol of cereals may also have two additional pathways. The first is a direct translocation from the cytosol to the plastid, and the second is the conversion to ADP-glucose and further transportation into the plastid. ADP glucose formed by either pathway would provide glucose units as building blocks for the formation of amylose and amylopectin by granule-bound starch synthase, and soluble starch synthase along with starch branching enzyme, respectively (Tester et al. 2004).

2.2.2 Morphology

The morphology and size distribution of starch granules differ with botanical origin. Cereal starch granules are found to be round, polygonal and lenticular (Waterschoot et al. 2015), whereas most of the granules in pulse starches are oval (Hoover et al. 2010). In general, granules have a diameter that ranges from submicrons to more than 100μm, and show a "Maltese cross" that starts from the hilum when observed under cross-polarized light in an optical microscope. This Maltese cross suggests an organizational pattern in a radial fashion (French 1972) (Figure 2.4 a). In general, cereal starch granules contain surface pores (0.1μm) whereas most granules of tuber starches show a smooth surface.

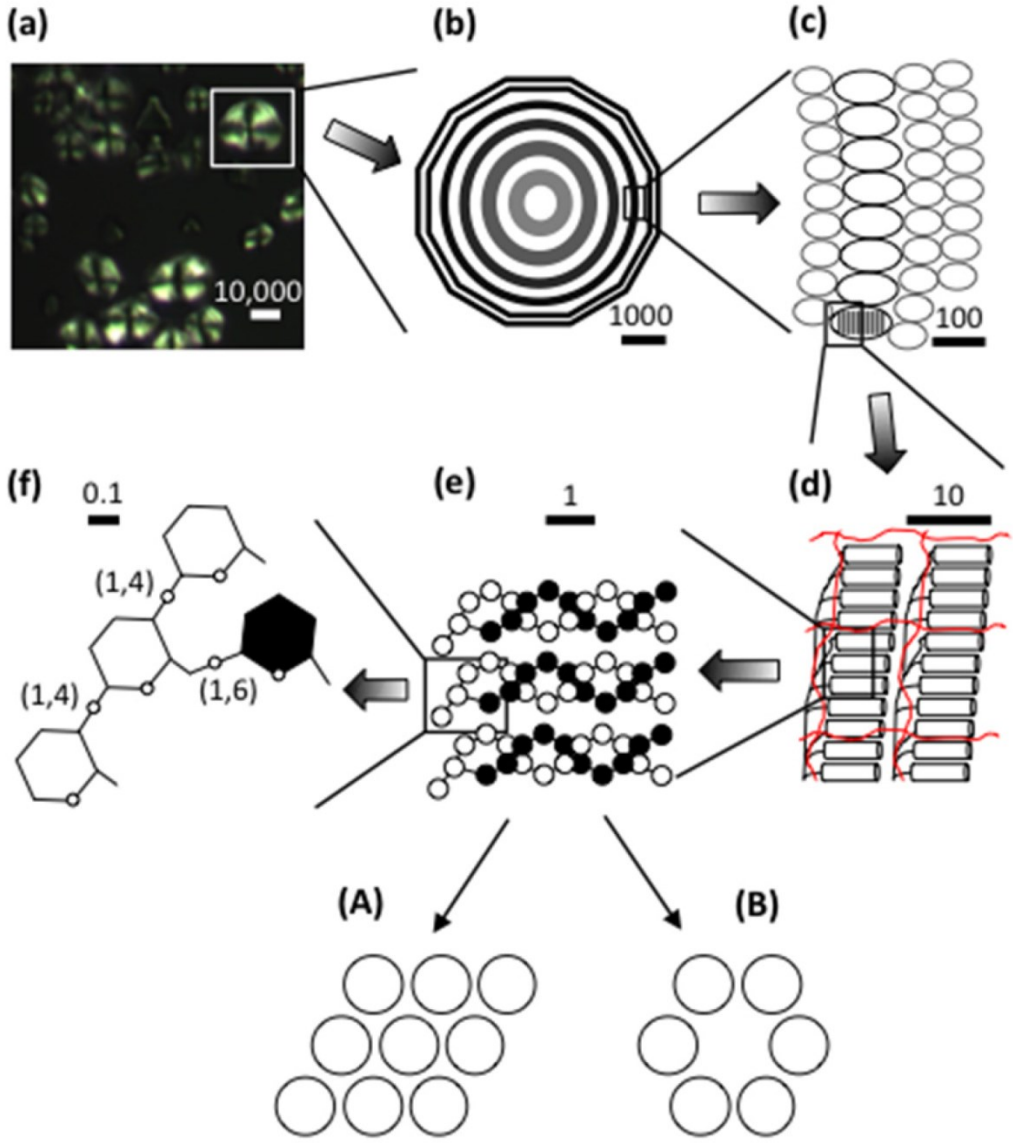


Figure 2. 4 Starch structure and architecture from granules to glucosyl units. (a) "Maltese cross" observed in maize starch granules under polarised light, (b) Growth rings extending from the hilum in a hypothetical polyhedral granule, (c) Blocklets located in amorphous (grey) and semicrystalline (black) rings, (d) Amorphous and crystalline lamellae formed by branched chains of amylopectin (black lines) and double helices (cylinders), respectively. Amylose chains (red lines) are distributed through the amorphous and crystalline lamellae, (e) Three double-helices formed by amylopectin short chains. Polymorphic crystals are formed by double helices: (A) A-type crystal and (B) B-type crystal (circles symbolize double helices observed from the top view), (f) α-(1,4)- and α-(1,6)-linkages of glucosyl units at the base of the double-helix. The dimensions in nm are only an approximation. Adapted from (Bertoft 2017), open-access article.

Confocal laser scanning microscopy (CLSM) has shown that depending on the botanical source, starches may contain voids inside the granules. Cereal starches show voids or pitting canals through the granules, whereas tuber starches exhibit a homogenous internal structure (Naguleswaran et al. 2011; Srichuwong and Jane 2007). Starch is composed of two types of glucan known as amylose and amylopectin. Amylose molecules are linear chains linked by α-(1-4) glycosidic bonds with few branches. Amylopectin molecules, the major component of most regular and waxy starches, are branched polymers of glucose linked by α-(1-4) and (1-6) linkages.

2.2.3 Granular structure and architecture

During the last century, significant progress has been made in the study of starch granular and molecular architecture primarily due to improvements in analytical techniques and instrumentation. Regardless of the variation in size and shape of starch granules from different plants, a remarkable similarity in their inner architecture despite the location (endosperm, root, stem, etc) has been observed. This granule architecture consists of semicrystalline and amorphous growth rings, blocklets, amorphous and crystalline lamellae, double helices, and crystals formed by amylose and amylopectin molecules (Figure 2.4).

Starch granules are composed of semicrystalline and amorphous "growth rings" that are observed with the microscope (Figure 2.4 b). Semicrystalline growth rings are hypothesized to be formed mainly by amylopectin chains (for which two models will be further explained), although amylose could also be involved (Koroteeva et al. 2007). Amorphous rings are assumed to be built by amylose molecules mainly (Atkin et al. 1999); however, amylopectin chains could play an important role since high-amylose starches still show growth rings (Bertoft 2015).

Growth rings are built by blocklets that are thought to be seen for the first time with an ordinary microscope, as small birefringent units (Hanson and Katz 1934). More recently, with advanced microscopic techniques such as scanning electron microscopy (SEM) and atomic force microscopy (AFM), blocklets were identified on the surface of the granules (Gallant et al. 1997; Ohtani et al. 2000). Blocklet sizes vary between 10-300 nm approximately, and have been found as small and large in amorphous and semicrystalline growth rings, respectively (Figure 2.4 c). Furthermore, it is hypothesized that blocklets in amorphous rings are "defective" and in semicrystalline rings "normal" (Tang et al. 2006). Due to a similarity in the dimensions of a blocklet with the amylopectin molecules, it is believed that blocklets in the semicrystalline ring are composed of amorphous and crystalline lamellae of amylopectin. However, amylose could also be part of the blocklet and/or could serve as connection between blocklets (Bertoft 2017) (Figure 2.4 d). The alternating crystalline and amorphous lamellae in amylopectin have been studied with X-ray diffraction and small-angle X-ray scattering (SAXS) and presents a repeat distance of 9-10 nm in all starch granules. Specifically, the crystalline lamellae are composed by double helices formed by short and external chains of amylopectin with approximately 11-15 glucose residues (Bertoft et al. 2008; Manners 1989) (Figure 2.4 e). However, although amylopectin is the principal component of these features, amylose chains also can participate (Tester et al. 2004). Double helices have a length of about 4-6 nm and crystallise in two different polymorphs; A-type for cereal starches and B-type for roots, tubers, high amylose cereal starches and retrogradated starches (Figure 2.4 A, B). However, pulse starches with the exception of wrinkled pea starch, exhibit a mixed pattern, so-called C-type (Bertoft 2017; Hoover et al. 2010).

In A-type starches, double helices are more densely packed in monoclinic unit cells (with dimensions a=2.124 nm, b=1.172 nm, c=1.069 nm) with the presence of eight water molecules (Imberty et al. 1988; Popov et al. 2009) (Figure 2.5 A). In contrast, B-type starches are formed by less closely packed double helices in the form of a hexagonal unit cell (a=b=1.85 nm and c=1.04 nm) with 36 water molecules (Imberty and Perez 1988) (Figure 2.5 B).

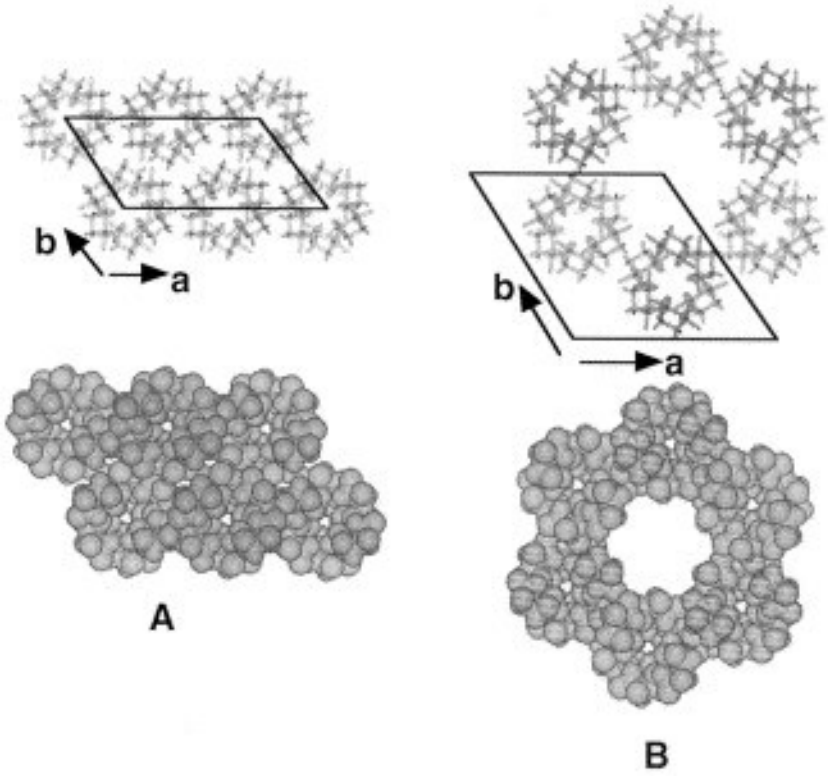


Figure 2.5 Crystalline structures of A- and B-type polymorphs. Adapted from (Bulon et al. 1998), with permission of Elsevier.

X-ray diffraction is generally used to identify the types of crystal structures within the granules, denoted A-, B- or C-type (Figure 2.6).

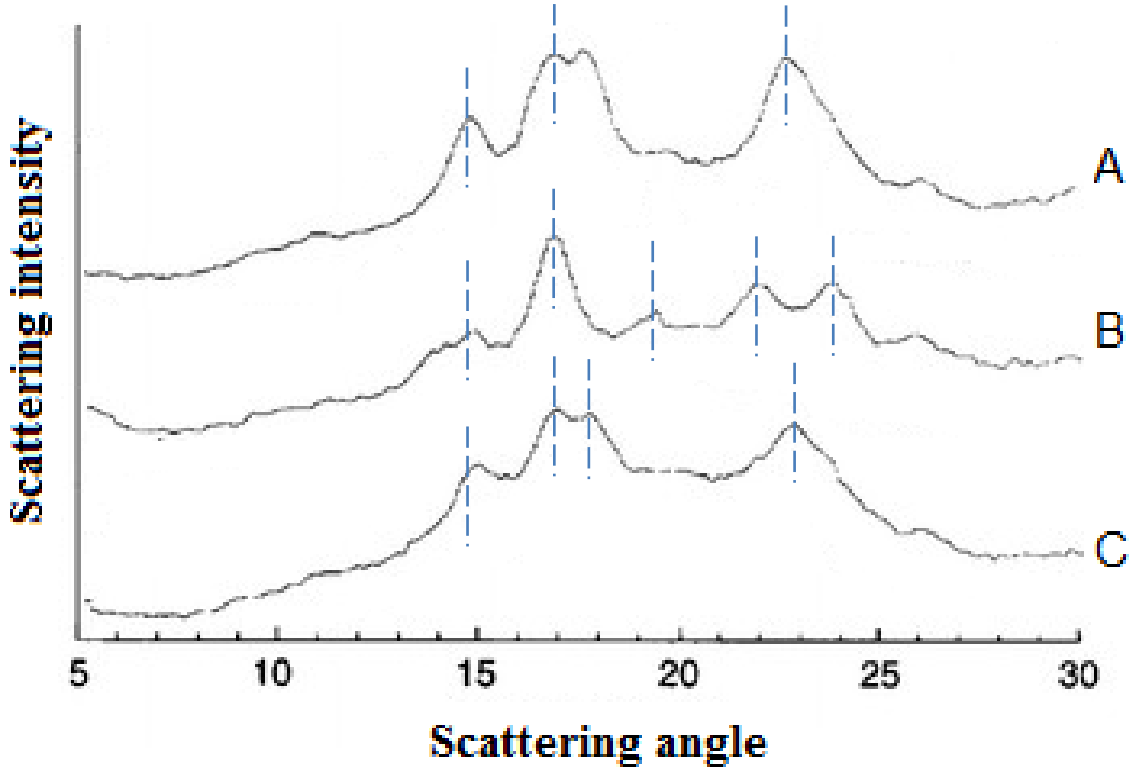


Figure 2.6 X-ray diffraction patterns of maize, potato and pea starch. The peak positions are characteristic of A-, B-, and C-type crystallinity, respectively. The peak locations for each crystalline type are marked by the dash lines. A-type polymorph: 15° (d=5.8Å), 17° (d=5.2Å), 18° and 23° (d=3.8Å) 2Θ. B-type polymorph: 5.6° (d=15.8-16Å), 15° (d=5.9Å), 17° (d=5.2Å), 22° (d=4.0Å) and 24° (d=3.7Å) 2Θ. C-type polymorph: 5.6°, 15°, 17°, 23° 2Θ. The peak at 20° (d=4.4Å) 2Θ is known as the V-complex. Adapted and modified from (Van Soest and Vliegenthart 1997), with permission of Elsevier.

The major starch components in the granule are two molecules: amylose and amylopectin (Figure 2.7)

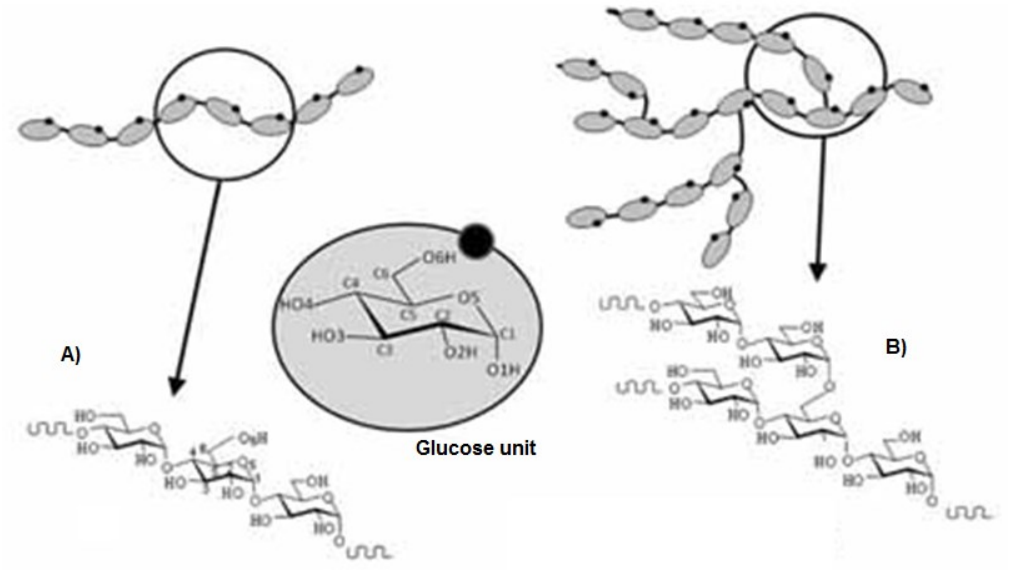


Figure 2.7 Basic molecular structure of (A) amylose and (B) amylopectin. Adapted and modified from Pérez and Bertoft (2010), with permission of John Wiley and Sons.

These two components can be isolated from the granules with different methods. First, granules must be completely dissolved in 90% dimethyl sulphoxide (DMSO) and further precipitated in ethanol (Klucinec and Thompson 1998). Then, precipitation of amylose can be accomplished by adding 1-butanol or a mixture of 1-butanol and isoamyl alcohol and further recovery of amylopectin after centrifugation of the supernatant (Klucinec and Thompson 1998; Schoch 1942).

Amylose is primarily a long linear polymer with several hundred glucose units linked by 99% α-(1-4) and 1% α-(1-6) bonds (Takeda et al.1987). It is the minor component of most starches and varies in content, structure and size depending on botanical source. In normal starches, the amylose content is about 20-35%, waxy starches have less than 15% amylose, and high amylose starches contain approximately more than 40% (Tester et al. 2004). Starch contains

both linear and branched amyloses (Peat et al. 1952). In a branched molecule, the number of chains is in the range of 5 to 21 (Pérez and Bertoft 2010). Each chain is built up with approximately 200-700 glucose residues. Branched amylose is determined by hydrolysis with β-amylase enzyme, where the linear chains are converted into maltose, whereas most of the branched chains form β-limit dextrins (Takeda et al. 1989; Takeda et al. 1992). Amylose has a degree of polymerization and molecular weight that ranges approximately between 324-4920 and $1x10^5$-$1x10^6$ g/mol, respectively (Tester et al. 2004). Cereal amyloses have been described to be small whereas potato amyloses are found to be the largest reported (Bertoft 2017). Amylose in pulse starches has not been completely characterized; however, studies have shown that the average chain length (CL) and degree of polymerization, vary between 240 to 568 and 650 to 2200 glucose units, respectively. The molecular weight is approximately $1.25x10^6$ – $6.30\ x10^6$ g/mol (Hoover et al. 2010).

Amylose can form single and double helices. Single helices are left-handed and interact with a variety of compounds (iodine, fatty acids or alcohols), creating inclusion complexes that crystallise into the so-called V-type polymorph. It is hypothesized that in the case of amylose-lipid complexes, the aliphatic part of the lipids is surrounded by the amylose helices (Bulon et al. 1998). Double helices precipitate in aqueous solutions of amylose and crystallise in the form of the B-type polymorph, as previously mentioned (Gidley 1989). Amylose in starch has been determined as apparent and absolute content. Apparent content is usually expressed by the affinity of defatted and native starch for iodine (Juliano 1971). However, in native starches, lipids also can form helical complexes with amylose, which do not have affinity for iodine, reducing the intensity of the blue color at 620nm after staining. In addition, the presence of long amylopectin chains influences the result, since they do have iodine affinity leading to an

overestimated result. Absolute content is determined by taking into consideration starch lipids and amylopectin concentration (BeMiller and Whistler 2009). There are other methods for amylose determination that include near infrared (NIR) spectroscopy, differential scanning calorimetry (DSC), size-exclusion chromatography (SEC), asymmetric flow field-flow fractionation, and recently a single-kernel based cut-grain dip method. However, the results of the analysis of amylose for one single sample can vary considerably between methods (Raja et al. 2017).

Amylopectin (AP) is the major component of starch and represents 65-85% of the matter in the starch granule (Fredriksson et al. 1998; Grard et al. 2001; Hoover 2001b). It is a highly branched and more complex molecule with shorter chains compared to amylose, and its molecular size, shape and structure vary with botanical origin. Amylopectin is composed of 95% α-(1-4) and 5% α-(1-6) linkages and it has a molecular weight of $1x10^7$–$1x10^9$ g/mol and a DP of approximately 9,600 to 15,900 (Tester et al. 2004). Since 1940, the amylopectin molecule has been defined as a branched polymer (Meyer and Bernfeld 1940). Peat et al. (1952) suggested a basic representation of the amylopectin chains: A, B and C (Figure 2.8).

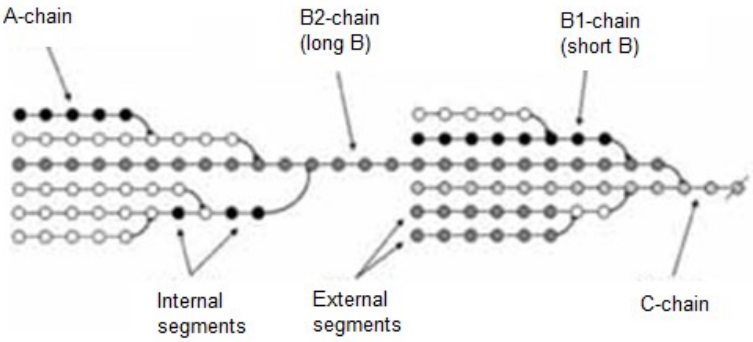


Figure 2.8 Simple labelling of amylopectin. Adapted and modified from Pérez and Bertoft (2004), with permission of John Wiley and Sons.

The A and B1 chains are the most external and are linked by α-(1-6) linkages to the rest of the macromolecule. B2 chains are long chains that connect to the C chain, which carries the sole reducing end group (Peat et al.1952).

Different molecular models for amylopectin were proposed in the 20th century (Larner et al. 1952; Lee et al. 1968; Meyer and Bernfeld 1940; Nikuni 1978; Peat et al. 1952), creating confusion to some extent in terms of nomenclature, concepts, structure, etc. However, the "cluster model", which has been redefined by a number of scientists (French 1972; Hizukuri, 1986; Manners and Matheson 1981; Nikuni 1978; Robin 1974), is widely accepted today and used as the traditional model. This model describes amylopectin as a molecule formed by chains of different lengths (Figure 2.9).

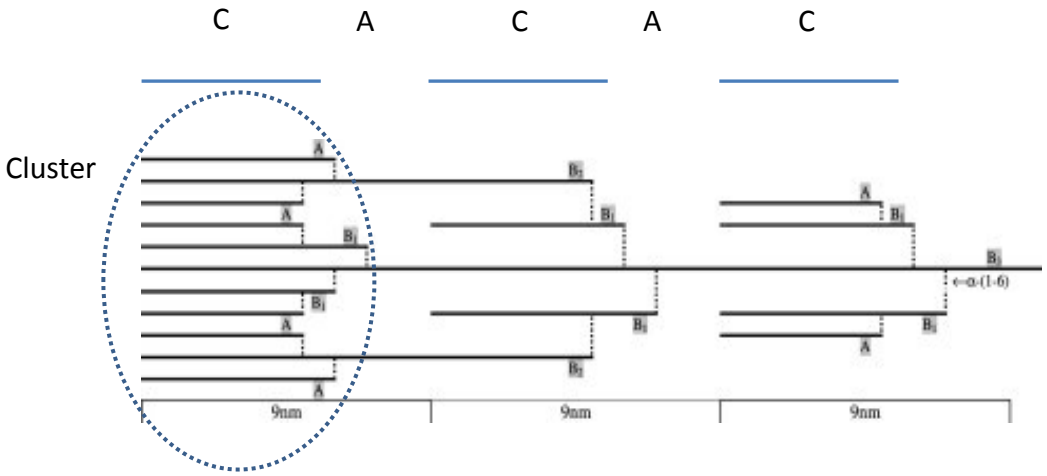


Figure 2.9 Representation of cluster model in the amylopectin molecule. Horizontal lines in the molecule symbolize α-(1-4) linkages and vertical lines symbolize α-(1-6) linkages. Long B chains are involved in the formation of (A) amorphous region and clusters in the (C) crystalline region. Adapted and modified from Hizukuri (1986), with permission of Elsevier.

A-chains and B1 chains are shorter chains (DP 11-14 and 20-24) and build the cluster. Longer chains are named B2-chains (DP 42-48), B3-chains (DP 69-75) and, eventually, B4-chains. This model suggests that there is a repetitive trend of one amorphous and one crystalline lamellae, each 9 nm long, and that larger B chains such as B2 and B3 interconnect two or three clusters, respectively. Furthermore, it is proposed that these B2 and B3 chains participate in the conformation of the cluster in the crystalline region and in the amorphous region (Hizukuri 1986).

The proposed cluster model of amylopectin that is 31 years old has been further refined in the past two decades. This was primarily facilitated by advancement in molecular purification and analytical techniques, especially enzyme technologies related to starch biosynthesis, and purification of debranching enzymes (pullulanase and isoamylase) from bacterial sources, etc. Furthermore, the development of techniques such as small angle X-ray scattering (SAXS) and gel-permeation chromatography, permitted the identification of the periodic length of the amorphus-crystalline lamellae and to classify the amylopectin chains as short and long chains after enzyme debranching (Bertoft 2017; Seetharaman and Bertoft 2013). Nevertheless, although this amylopectin model is generally accepted by the scientific community, this concept still remains uncertain.

Alternative to the cluster model, other models have been presented, which have in common a “backbone” definition (Bertoft 2013; Borovsky et al. 1979; Matheson and Caldwell 2008; Waigh et al. 2000). Consequently, the “building block backbone model” has been the most recently proposed concept based on experiments previously not available. When compared to the cluster model, where the B chains interconnect the clusters perpendicularly (Figure 2.10 a), the building block backbone model suggests that the long B chains are linked together forming a

backbone (Figure 2.10 b). This backbone builds mainly the amorphous lamellae rather than creating stacks of lamellae in the form of amorphous and crystalline rings. Furthermore, the clusters are located perpendicular to the backbone, not parallel as in the cluster model, and are supported by internal "building blocks" randomly distributed and connected to the backbone (Figure 2.10 b). Short chains of amylopectin associate, creating "double helices" that form a crystalline lamellae in both, the cluster model and the building block backbone model (Figure 2.10).

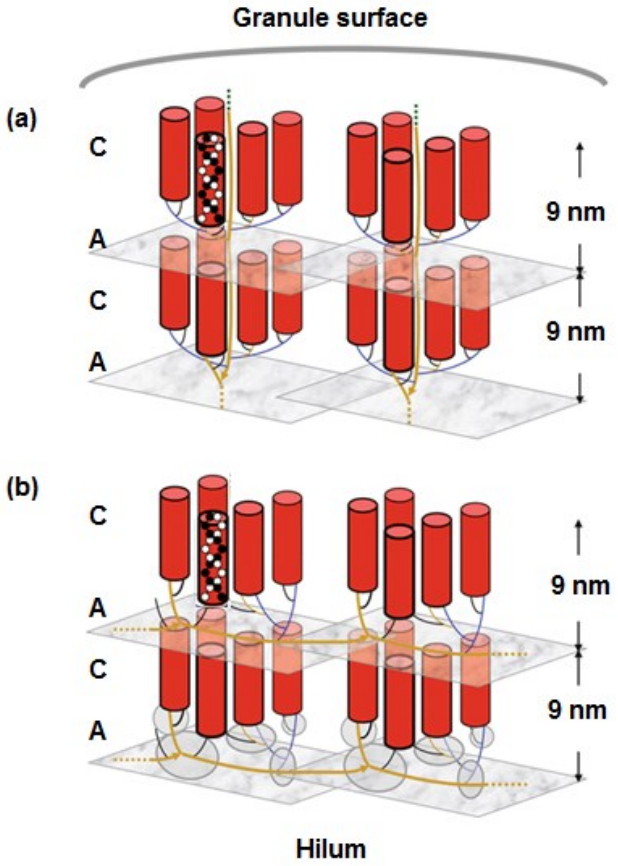


Figure 2.10 Cluster model (a) based on (Robin 1974) vs building block backbone model (b) based on (Bertoft et al. 2010). Thick yellow lines represent B long chains. Building blocks are circled in purple and red cylinders represent double helices. Crystalline lamellae (C) and amorphous lamellae (A). Adapted and modified from (Bertoft 2015), with permission of Springer Nature.

Detailed study of the molecular structure of amylopectin was made possible by using exo-acting amylolytic enzymes (phosphorylase-a and beta-amylase) and debranching enzymes (isoamylase and/or pullulanase). In the internal part, amylopectin presents short B chains that have been classified as "fingerprint" B-chains (Bfp) that differ considerably between plant species, and a major group of B short chains (BS_{major}). Based on this internal chain profile, amylopectin has been recently divided into four groups. Type 1 amylopectin has a small number of long chains (B2 and B3 chains) (<1%) and is mainly found in barley, rye and oat. Type 2 amylopectin that is characteristic in maize, rice and amaranth, has more B chains than type 1, specifically higher amounts of B2 chains but less BS_{major}. Type 3 amylopectin has more Bfp than types 1 and 2 and is abundant in cassava and mung bean starch. Lastly, type 4 amylopectin is characteristic of B-type starches, presenting the greatest amount of B3 chains (Bertoft et al. 2008).

2.2.4 Gelatinization and retrogradation

As previously described, native starch granules are composed of semi-crystalline and amorphous "growth rings" that are formed by amylose and amylopectin molecules. These polymers are placed in a form that ensures a tightly arranged architecture making the granule insoluble in cold water. When native starches are heated in excess water above their gelatinization temperature, intermolecular hydrogen bonds of amylose and amylopectin break down, altering the ordered structure of the granule significantly. The gelatinization temperatures at which these reactions occur, differ with starch origin. Water acts as a plasticizer and diffuses into the granules across the amorphous region by interacting with free hydroxyl groups through hydrogen bonding. The granules swell and the amorphous regions expand, generating disruptive forces that cause uncoiling of double helices and melting of amylopectin crystals. As the granule

continues to swell at high temperature, amylose molecules leach out of the granules resulting in an increased viscosity (Lelievre and Mitchell 1975; Lund and Lorenz 1984; Stevens and Elton 1971). Overall, the processes of granule swelling, melting of double helices and crystals, amylose leaching, loss of Maltese-cross birefringence and increase in starch solubility and viscosity are collectively described as gelatinization.

Many techniques have been used to study starch gelatinization, such as birefrigerance end point method (Watson 1964), viscosity method (Lund and Lorenz 1984), X-ray diffraction method (Owus-Ansah et al. 1982), enzymatic digestibility (Shetty et al. 1974), nuclear magnetic resonance (Lelievre and Mitchell 1975), X-ray and neutron scattering studies (Jenkins 1994) and differential scanning calorimetry (DSC) (Stevens and Elton 1971). However, DSC has emerged as the preferred method due to the accuracy in determining the endothermic transition temperatures of gelatinization [onset, T_o; peak, T_p; conclusion, T_c] and the enthalpy of gelatinization (ΔH).

Amylopectin is mainly responsible for granule swelling and viscosity. The amylose/amylopectin ratio (AM/AP) ratio of starch can influence its gelatinization transition temperatures (Schirmer et al. 2013). Higher gelatinization temperatures are related to the existence of greater amounts of long amylopectin chains that may stabilize the crystal structure increasing the degree of crystallinity (Gomand et al. 2010; Jane et al. 1999; Vandeputte et al. 2003). The water to starch ratio and heating conditions also have an effect on starch gelatinization. In excess water (>66 wt %), a single endotherm is observed normally between the range of 60-80°C for various native starches (Jacobs et al. 1995; Wang and Copeland 2013). When the amount of water becomes limited, starch gelatinization will not be completed at the typical temperature range, due to the reduced hydration and swelling of the amorphous regions.

Therefore, higher temperatures will be required for the melting of starch crystalline regions, i.e. a two stage melting pattern with two endotherms will be observed (Donovan 1979; Biliaderis et al.1980).

Different hypotheses have been proposed and investigated to understand the concept of enthalpy of gelatinization. Cooke and Gidley (1992) stated that ΔH represents the loss of double helical order rather than loss of crystallinity. Nevertheless, Jane et al. (1999) have postulated that ΔH represents the amount of energy needed to disrupt intermolecular bonds and gelatinize all the crystals. Waxy starches have been shown to have a higher ΔH, which is related to the dense and more ordered structure compared to regular starches. Waxy starches contain a higher percentage of amylopectin that requires more thermal energy to break down intermolecular hydrogen bonds (Schirmer et al. 2013). During gelatinization, amylose lipid complexes can form simultaneously, generating an exothermic reaction that lowers the ΔH (Eliasson 1986). In addition, these lipid complexes would melt at higher temperatures, i.e. a second endotherm between 95-125°C.

Upon cooling, amylose and amylopectin chains of gelatinized starches associate mainly through hydrogen bonds into partially ordered crystalline structures that differ from those in native granules, in a process known as retrogradation (Atwell et al. 1988). Starch concentrations higher than 6% will lead to the formation of a gel, where the gelatinized granules become surrounded into a continuous matrix of entangled amylose molecules. The initial texture of the gel is linked to the high rate of amylose retrogradation, whereas the final gel structure and crystallinity are influenced by the slower retrogradation (i.e. time dependent) of amylopectin that takes place over several days or weeks (Miles et al. 1985; Ring et al. 1987). Amylopectin gels have a soft texture as a result of fewer interactions of the branched molecules and have the capacity to hold more water. In contrast, gels of amylose are rigid due to the high interaction of

the essentially linear molecules (Zobel 1988). Cooled starches after long term storage, increase gel firmness and undergo phase separation, causing water loss (i.e. syneresis). This process is characterized by hydrogen bonding interactions of leached amylose and amylopectin chains, which leads to the formation of junction zones that reflect or scatter a significant amount of light (Perera and Hoover 1999). A B-type X-ray diffraction pattern is developed when amylopectin retrogrades transforming amorphous molecules to a fragile crystalline pattern (Srichuwong and Jane 2007).

2.2.5 Amylolysis

Starch amylolysis is a process by which starch is converted into smaller molecules/oligos/sugars by the action of amylase enzymes such as α-amylase, amyloglucosidase and debranching enzymes. These enzymes differ in their mechanism of action by cleaving specific bonds and generating products of different molecular weights and structure.

2.2.5.1 Types of amylases and their mechanism of action

The α-amylases (EC 3.2.1.1) are endo-acting enzymes that ramdomly cleave the α-1,4 glycosidic bonds in amylose and amylopectin molecules, producing mostly linear and branched oligosaccharides with varying length/degree of polymerization. The α-amylases have molecular weights ranging from 50 to 60 kDa and hydrolyse both amorphous and crystalline regions of native starch granules (Lauro et al. 1999; Leach and Schoch 1961). Alpha-amylase attacks the amorphous regions, and triggers uncoiling of crystalline chains, which facilitates hydrolysis of crystalline regions (Colonna et al. 1988). The temperature and pH for the optimal function of α-amylases vary with source. For example, α-amylases used in digestibility evaluation methods are active at 37°C and pH 6.0, whereas thermostable α-amylases from *Bacillus stearothermophilus* or *Bacillus licheniformis* industrially used in the liquefaction step of high glucose syrup

production, are active at temperatures above 100°C and pH above 5.9 (Van der Maarel 2002). Thermostable α-amylases help to reduce the viscosity of gelatinized starches as well as the time that amyloglosidase enzymes will take to further hydrolyse dextrins into glucose.

Alpha-amylases can be produced from various sources and have different specificities, generating diverse malto-oligosaccharides (maltose, maltotriose, α-limit dextrins) and glucose (James and Lee 1997). Human salivary α-amylase (ptyalin) is isolated from human saliva and has an average of three hydrolytic cleavages per encounter between amylose/amylopectin chains and the active site of the enzyme. Porcine pancreatic α-amylase is obtained from hog pancreas and has an average of seven hydrolytic cleavages per chain encounter. Microbial α-amylase is purified from *Aspergillus Oryzae,* among other species, and like human salivary α-amylase has an average of three hydrolytic cleavages per encounter (Robyt and French 1967). Thus, α-amylases, based on the origin, generate a variety of starch hydrolysis products, depending not only on the active-sites of the enzymes, but also on the substrates, i.e. amylose, amylopectin, linear or branched maltodextrins, etc. Alpha-Amylases require the presence of calcium ions for their activity and stability (Buisson et al. 1987).

Glucoamylase or amyloglucosidase (EC 3.2.1.3) is an exo-acting enzyme that cleaves α-(1-4) glycosidic bonds from the non-reducing ends of amylose and amylopectin molecules in a consecutive manner, producing only glucose as the hydrolysed product. It also hydrolyses α-(1-6) linkages of branched points of amylopectin but at a much slower rate (Pazur and Kleppe 1962). However, when added at higher concentrations, and also in the presence of higher amounts of substrate for long periods of time, it can resynthesize maltose and isomaltose from glucose. This enzyme is produced from various sources, especially from fungi, with molecular weights that range from 26 kDa to 112 kDa. Glucoamylase is active over pH range of 4.5-5.5,

stable at temperature range of 40-60°C, and does not require a metal ion to perform hydrolytic reactions (James and Lee 1997).

Debranching enzymes, such as isoamylase and pullanase, are endo-acting enzymes that cleave only α-(1-6) glycosidic bonds in amylopectin molecules, producing linear chain polysaccharides (Van der et al. 2002). Thus, they could enhance the rate of hydrolysis by glucoamylase on amylopectin molecules. In general, enzymes can attack the starch granule by endo-corrosion or exo-corrosion. Endo-corrosion is produced when the enzyme enters the granule through channels that radiate from the surface of starch granules, reaches the interior/center, and hydrolyses the surrounding area, as seen in wheat, barley, rye, triticale and some tropical tuber starches. Exo-corrosion occurs when enzymes corrode partially or completely the granule surface, as in the case of potato and legume starches (Gallant et al. 1992; Sujka et al. 2007).

2.2.5.2 Factors influencing amylolysis - granular structure, architecture, gelatinization and retrogradation

Native starch hydrolysis is influenced by a number of factors such as granule size, presence of channels/pores/pinholes, crystal polymorph types such as A, B or C, amylose/amylopectin ratio, and amylose lipid-complexes (Singh et al. 2010). Depending on the botanical origin, the size of the starch granules varies considerably. Large starch granules hydrolyse more slowly than small granules, possibly due to their smaller surface area to volume ratio (Tester et al. 2006). The rate and extent of α-amylase hydrolysis of different starches has shown the order wheat> maize> pea> potato, which partially reflects an increasing granule size (Ringet al. 1988). The presence of pores or pinholes on the surface of the granules facilitates easy entrance of enzymes toward the interior (Fannon et al. 1992). Native/un-gelatinized legume

and tuber starches are characterized by a lower hydrolysis rate that is linked to the smooth surface and absence of pores in the granules (Hoover and Sosulski 1985; Singh et al. 2010). In contrast, cereal starches such as normal corn, sorghum and millet, show circular pits, and wheat starch shows pores and channels mainly along the equatorial groove that allow a quick enzymatic hydrolysis (Dreher et al. 1984; Fannon 1992; Kim and Huber 2008). In addition, cereal starches show the presence of voids in the internal structure of the granules, whereas tuber starches possess a homogenous appearance, which impedes enzyme hydrolysis (Srichuwong and Jane 2007).

In regards to native starch crystallinity, amylopectin double helices that are packed as B-type (tubers) and C-type (legumes) patterns have been reported to be more resistant than A-type (cereals) toward amylolysis (Gallant et al. 1992; Jane et al. 1997). A-type starches have shorter double helices derived from short amylopectin branch chain lengths (DP 23-29), and crystallites that are hydrolysed faster. In contrast, B-type starches have longer amylopectin chains (DP 30-44) with longer and more stable helices (Jane et al. 1997; Singh et al. 2010). The amylose content of starch also has been directly related to starch resistance towards hydrolysis (Bertoft et al. 1993; Evans and Thompson 2004). Starches with longer linear chains are more susceptible to form crystalline structures compared to starches with shorter and branched amylopectin chains (Birt et al. 2013). Raw legume starches contain a high percentage of amylose (30-65%, as is basis) and have been shown to have lower *in vivo* and *in vitro* digestibility (Hoover and Zhou 2003). Amylose, with an average molecular weight of 10^4g/mol, has a smaller surface area per molecule than amylopectin with an average molecular weight of 10^5-10^6g/mol. Consequently, amylose molecules are less susceptible to be hydrolysed compared to amylopectin chains. Furthermore, amylose chains are more tightly linked to each other by hydrogen bonds, whereas

amylopectin molecules are loosely packed due to the branched chains of glucose (Hoover and Sosulski 1985; Singh et al. 2010). Amylose molecules can complex with lipids and enhance resistance to hydrolysis due to a smaller surface area available for the active site of the α-amylase enzyme (Tester et al. 2006). *In vivo* and *in vitro* digestibility studies have shown that amylose bound to lipids is digested to the same extent, but at a slower rate, compared to free amylose (Holm et al. 1983).

The degree of granule disruption during gelatinization (i.e. order to disorder transition by double-helical unravelling and crystal melting) has a major influence on starch digestibility, since most α-amylases possess a limited action on native starch granules (Lauro et al. 1993). After heat processing or cooking in excess water, swelled and hydrated granules become rapidly available for enzymatic hydrolysis regardless of the type or source, due to the loss of crystalline structure (Wang and Copeland 2013). However, the temperatures at which starches are gelatinized or the degree of starch gelatinization at a particular temperature-time combination differ, depending on the amylose content. For example, waxy, normal and high amylose starches gelatinize at temperature ranges of 63-72°C, 62-80°C and >110°C, respectively. *In vivo* studies have demonstrated that the degree of starch gelatinization is strongly correlated to higher glucose response (Holm et al. 1988; Parada and Aguilera 2009; Parada and Aguilera 2012) and insulin response (Holm et al. 1988). Indeed, a correlation coefficient of 0.96 between the degree of gelatinization and digestion rate was reported (Holm et al. 1988) . Likewise, Parada and Aguilera (2009) proposed correlations of 0.93 and 0.99 between the extent of gelatinization and blood glucose area under the curve and blood glucose concentration, respectively. This suggests that the degree of starch gelatinization could be proportional to the enzymatic digestion rate and the amount of glucose released into the bloodstream.

The extent of re-association among amylose and amylopectin molecules (amylose-amylose, amylose-amylopectin and amylopectin-amylopectin) through hydrogen bonding during retrogradation also has an impact on starch digestibility. In general, re-associated starch molecules have an ordered structure that becomes less accessible to salivary and pancreatic enzymes, lowering the amylolysis. Therefore, there is less availability of glucose for absorption and consequently a reduced glycemic response (Burton and Lightowler 2008; Morita et al. 2005).

In particular, starches with recrystallized amylopectin are loosely bound by associations of short branches (DP 15) (Ring et al. 1987) and as a result they are more susceptible to amylolysis. In contrast, retrogradated starches high in amylose form double-helical associations that are tightly packed (Hoover 2001), reducing the enzymatic hydrolysis with a moderated/slower release of glucose into the blood stream. Thus, amylose/amylopectin ratio is an essential factor in the kinetics of starch retrogradation (Sajilata et al. 2006)

2.2.6 Resistant starch (RS)

Resistant starch (RS) is the fraction of dietary starch that is resistant to digestion by human pancreatic amylase and passes into the colon to be metabolized/fermented by bacteria. The colonic fermentation of RS leads to the production of gas (methane, hydrogen and carbon dioxide), short chain fatty acids (butyrate, acetate, propionate and valerate) and small amounts of branched short chain fatty acids (isobutyrate and isovalerate), organic acids (succinate, lactate and formate) and alcohols (Birt et al. 2013; Sajilata et al. 2006; Tharanathan 2002). Initially, starch was assumed to be fully digestible in the human intestine. Later in 1982, Englyst et al (1982) reported for the first time that after treating gelatinized starch with α-amylase and pullulanase, a portion of starch resisted hydrolysis. Different mechanisms have been attributed to

the resistance of starch toward amylolysis, based on which resistant starch has been classified into five types: RS1, RS2, RS3, RS4 and RS5.

RS1 is starch physically entrapped by cell walls and other grain components, such as the protein matrix, and thus, remains relatively inaccessible to digestive enzymes, i.e. whole grains and seeds (Tovar et al. 1992). RS2 is amylopectin crystals in the native non-gelatinized/uncooked starch. The tight molecular packing of the crystalline structure restricts amylolysis, and based on the packing arrangement, native starch granules are classified into A- (regular and waxy cereals), B- (high amylose maize, potato, banana, etc) or C- (pulse) type polymorphs (Englyst and Cummings 1987; Wong and Louie 2016). RS3 is a portion of retrograded amylose, i.e. linear amylose molecules that associate to form heat stable crystal structures during retrogradation (Cummings et al. 1996; Tharanathan 2002). During retrogradation, amylose chains overlap at their chain ends/tips to form double helices (i.e. chain elongation). Folding of the elongated chains facilitates double-helical packing through hydrogen bonds, leading to crystal formation and restriction to the accessibility of glycosidic bonds by α-amylases. Depending on the nature and extent of retrogradation, amyloses form a complex that is very stable and can rehydrate only at very high temperatures (80-150°C) (Haralampu 2000). RS3 is 100% indigestible by amylase enzymes *in vitro* and *in vivo*, and is fermented in the hindgut, generating short chain fatty acids that are linked to multiple health benefits (Hoover and Zhou 2003). RS4 represents native starches that are chemically modified to improve their physicochemical properties. Chemically modified starches have been shown to enhance the proportion of RS (Donner et al. 2011). The main starch modifications are cationic, crosslinking, acetylation and hydroxypropylation. Cationic starches are obtained by treating native starches with cationic monomers such as 2,3-epoxypropyl trimethyl ammonium chloride or 3-chloro-2-

hydroxypropyl trimethyl ammonium chloride in dry or wet processes (Alcázar-Alay and Meireles 2015). Cross-linking of the hydroxyl groups of native starches has been done with reagents like phosphorus oxychloride, epichlorohydrin, sodium tripolyphosphate and sodium trimetaphosphate. Acetylation is an esterification process of native starch granules where part of the hydroxyl groups of glucose molecules is replaced by acetic anhydride or vinyl acetate in the presence of an alkaline catalyst such as sodium hydroxide (Ashogbon and Akintayo 2014; Zia-ud-Din et al. 2017). Hydroxypropylated starches are treated with propylene oxide in alkaline medium (Zheng et al. 1999). Among the chemically modified starches previously mentioned, all except cationic starches that are mainly used in the textile industry have shown an increased amount of RS4 and resistance towards enzymatic hydrolysis (Donner et al. 2011). RS5 represents complexes of lipids with amylose chains and long branched chains of amylopectin (Jane et al. 1997).

2.3 PROTEIN

Proteins are biological macromolecules formed via polymerization of amino acids in variable sequences (Ustunol 2015). Proteins are essential in human nutrition, mainly to provide building blocks for muscle and other tissue synthesis, transport biological molecules, and in immune functions (Ustunol 2015). In nature, there are 20 common amino acids that contribute to protein production, which are comprised of carbon, hydrogen, oxygen, nitrogen and sulfur atoms, in addition to other trace elements (Mojica et al. 2015). Proteins not only contribute to the nutritional value, but also impart functional properties to food products.

2.3.1 Amino acid composition and protein structural diversity

Amino acids are the fundamental structural unit and building block of proteins. The amino acids are formed by an α-carbon that provides covalent links to a carboxyl group (-

COOH), an amino group (-NH_2), a hydrogen atom and a side chain R group (Figure 2.11) (Kannan et al. 2012; Ustunol 2015).

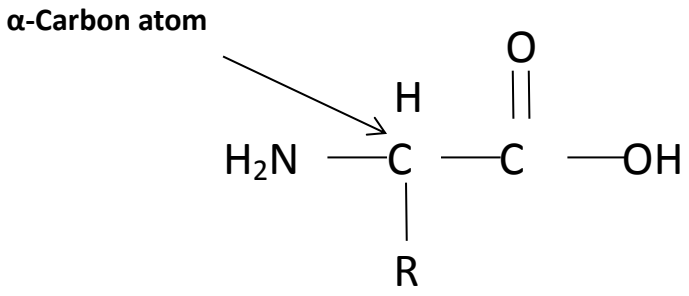


Figure 2.11 Amino acid structure.

The R group and its chemical nature influence the structural and functional properties of the amino acid, such as net charge, intra- and inter-molecular interactions, physicochemical properties (i.e. solubility), hydrogen bonding capacity and chemical reactivity (Kannan et al. 2012). Amino acids are classified into several categories based on the properties of their side chain R group at physiological pH (Nelson and Cox 2017). Arg, His and Lys behave as positively charged polar amino acids, whereas Asp and Glu are polar and negatively charged aminoacids at physiological pH. Polar uncharged amino acid group consists of Asn, Gln, Ser, Thr and Cys. Non-polar amino acids are further divided into two groups based on the presence of either aliphatic (Ala, Ile, Leu, Val, Gly, Pro, Met) or aromatic (Phe, Trp, Tyr) side chains (Nelson and Cox 2017; Srinivasan et al. 2007; Ustunol 2015). In addition, based on their nutritional and physiological roles, amino acids have been classified as essential and non-essential. Essential amino acids (i.e. Leu, Met, Phe, Lys, Val, Iso, Thr and Try) are not synthesized by the human body and therefore, must be obtained from the diet (Chopra and Panesar 2010).

Proteins show a hierarchical structure that exists on four ordered levels, namely primary, secondary, tertiary and quaternary structure. Primary structure refers to the linear sequence of amino acids that is covalently bonded by peptide linkages as the main binding force. The sequence of the amino acid residues and the chain length, influence the molecular conformation (secondary and tertiary structures), structural, physicochemical and biological properties of the protein (Nelson and Cox 2017; Srinivasan et al. 2007; Ustunol 2015). Peptides are classified based on the length of the amino acid chain as oligopeptides, with less than 50 amino acid residues, or polypeptides, when the chain in composed of 50 to 2000 amino acid residues (Berg et al. 2002).

Protein secondary structure is made by a periodic or aperiodic folding pattern with special arrangement at determined segments of the peptide chain. Random coil structures are the result of aperiodic protein folding, where helical and extended structures are generated due to periodic protein folding (Ustunol 2015). Helical structures such as α-helix, 3_{10}-helix and β-helix represent the major portion of protein secondary structure found in natural proteins. Among them, α-helix is more common than the 3_{10}-helix and β-helix in native proteins (Kannan et al. 2012; Nelson and Cox 2017). Hydrogen bonding is the main force that stabilizes α-helical structures via linking N-H backbone groups to the C=O group of the amino acid at the fourth preceding position. Although α-helices can exist in both right- and left-handed orientation, right-handed helices are more stable and abundant in nature (Srinivasan et al. 2007). In general, proteins with α-helical arrangements exhibit amphiphilic behavior because of the positional organization of amino acids within their secondary structure. Hydrophobic amino acid residues are located toward the interior of the protein, whereas hydrophilic residues are located toward the exterior (Srinivasan et al. 2007).

The β-sheet structure is an extended structure with either parallel or antiparallel alignment of two peptide chains stabilized by hydrogen bonds which perpendicularly link N-H backbone groups to C=O groups (Chopra and Panesar 2010). Parallel β-sheet has the amino acid chains located parallel to each other, whereas in antiparallel chains, the direction of one chain is the opposite of the other (Chopra and Panesar 2010). Proteins with higher β-sheet structures are generally more stable to heat treatments compared to α-helices (e.g. soy 11S globulin) (Srinivasan et al. 2007). Field pea protein isolate has been shown to have 30% β-sheet and 28% α-helices (Shevkani et al. 2015). Zein in maize approximately contains 45% α-helices and 15% β-sheet structures (Chopra and Panesar 2010). The main secondary structure in rice protein isolate is β-sheet (44.94%) (Wang et al. 2016).

The tertiary structure of a protein refers to the folding of the secondary structure into a three-dimensional form. Tertiary structures are mainly stabilized by hydrophobic interactions and hydrogen bonding by creating more compact spatial arrangements (Wang et al. 2016). In addition, disulphide bonding between cysteine residues, non-covalent interactions, salt linkages and dipole-dipole interactions also play an important role in the stabilization of protein tertiary structure (Chopra and Panesar 2010).

The quaternary structure represents a three-dimensional configuration of two or more polypeptide chains. Proteins with quaternary structures can exist as dimers, trimers, tetramers, etc. The structure of the oligomers is stabilized by non-covalent interactions such as hydrogen bonds and hydrophobic and electrostatic interactions. During the protein folding into quaternary structure, thermodynamically hydrophobic amino acid residues are placed toward the protein core (Srinivasan et al. 2007).

2.3.2 Protein denaturation

Alteration of the secondary, tertiary or quaternary protein structure without cleaving peptide bonds is considered as protein denaturation. The stability of the native protein structure is a result of different attractive and repulsive interactions generated from intermolecular and intramolecular interactions of polar groups with water (Srinivasan et al. 2007). The environment that surrounds the protein has an enormous impact on its stability and ordered structure. Several factors, including changes in pH, ionic strength, temperature and solvent composition, can influence the protein structural arrangement and denaturation (Kannan et al. 2012; Nelson and Cox 2017). Protein denaturation can be desirable or undesirable, depending on the application or the situation where is happens. Heat denaturation of trypsin inhibitors in legume products during food processing, increases the digestibility and biological availability of their proteins (Savelkoul et al. 1992). Partial denaturation of proteins can improve digestibility and other functionalities such as foaming, gelling and emulsifying properties depending on the food application (Mojica et al. 2015). On the other hand, protein denaturation generates undesirable outcomes when the biological activity and functional properties are compromised (Srinivasan et al. 2007).

Several internal and external factors can trigger protein denaturation. These factors can be either physical (temperature, hydrostatic pressure and shear), or chemical (pH, addition of organic solutes and salts) in nature (Mojica et al. 2015). Heat denaturation in a boiling aqueous solution can cause irreversible denaturation in most plant/animal proteins (Bull and Breese 1973). Heat denaturation increases the molecular vibration, destabilising the primary hydrogen bonds and hydrophobic interactions of the native structure (Arakawa et al. 2001; Hollar et al. 1995). As a result, the hydrophobic amino acids that were buried in the interior will become exposed and reorganize the secondary and tertiary structure of the protein. Heat-denatured

proteins may aggregate through electrostatic and hydrophobic interactions that may lead to precipitation, coagulation or gelation (Arakawa et al. 2001; Damodaran 1997). Similarly, hydrostatic pressure also affects the native conformation. Previous studies have shown that hydrostatic pressure within the range of 1-12 kbar has the potential to denature the protein at lower temperatures compared to traditional heat denaturation. However, pressure alone compared to heat generates a reversible denaturation, minimal impact on essential amino acids and does not produce toxic compounds (Srinivasan et al. 2007). Mechanical shear (shaking, kneading, whipping, etc.) is another factor that affects protein denaturation. Extrusion is an example of a unit operation that involves mechanical shear along with high temperature and high pressure, generating irreversible protein denaturation (Srinivasan et al. 2007). Most proteins are stable at neutral pH; however, proteins can unfold and swell at extreme pH values due to strong intramolecular electrostatic repulsions. In addition, organic solutes such as urea or guanidine hydrochloride and salts also can induce protein denaturation (Arakawa et al. 2001).

2.3.3 Proteolysis

Cleavage of larger protein molecules into polypeptides and/or amino acids via acid, alkali or enzymatic hydrolyzing agents is considered to be proteolysis (Arakawa et al. 2001; Ustunol 2015). Among the proteolysis methods, acid and alkaline hydrolysis provide simple and fast reaction conditions; however, they do exhibit technological and nutritional disadvantages in terms of process control and the maintenance of nutritional quality (Sarmadi and Ismail 2010). In contrast, enzymatic proteolysis has become a popular method due to mild reaction conditions (pH 6-8 and temperature 40-60^{o}C), avoiding the use of extreme treatments and organic solvents that potentiate side reactions and toxicity (Clemente 2000).

Enzymatic proteolysis can be influenced by several factors such as enzyme specificity, extent of protein denaturation, enzyme and substrate concentrations, pH, ionic strength, temperature, and absence or presence of enzyme inhibitory substances (Kilara 1985). Enzyme specificity determines the position and the direction of peptide cleavage and the number of peptide linkages that are hydrolysed within the protein. The percentage of peptide bonds cleaved is referred to as the extent of proteolysis, and it is quantified as degree of hydrolysis (DH) (Nielsen et al. 2001). Protein hydrolysis is commonly used for developing hypo-allergenic ingredients that have a higher DH, zero existence of peptides larger than 5 kDa and at least 90% of peptides smaller than 0.5 kDa (Mahmoud 1994). However, extensive hydrolysis generally can produce bitterness due to the exposure of hydrophobic amino acids in low molecular weight peptides. In contrast, hydrolysed proteins with lower DH are used to formulate protein supplements to reduce bitterness and other off-flavours (Panyam and Kilara 1996). Milk proteins such as casein or whey protein have been used as the conventional source of protein hydrolysates for nutritional applications. However, plant protein hydrolysates such as from soybean, pea and chickpea have demonstrated enormous potential for their functional and nutritional properties, in addition to their low cost and reduced environmental impact (Clemente 2000). Plant proteins, in particular pulse proteins are globular in nature and stabilized with disulphide bonds and hydrophobic interactions. During the enzymatic hydrolysis, proteins undergo three stages: reduction of molecular weight, augmentation of the number of ionisable groups and the exposure of hydrophobic amino acids (Panyam and Kilara 1996).

2.3.3.1 Type of proteases and their mechanisms of action

Proteases (peptidases, proteinases or proteolytic enzymes) are a group of enzymes that catalyze protein hydrolysis. Proteases are classified based on their mechanism of action into two

groups, exoproteases and endoproteases (Bender and Kezdy 1965; Fersht 2006). Exoproteases are further divided into two groups; amino and carboxypeptidases based on the starting point of peptide bond cleavage, either from the amino or carboxyl terminus of the protein, respectively. Carboxypeptidases are further sub-divided into serine peptidases, metallopeptidases and cysteine peptidases, based on the specificity of the enzyme to certain amino acids and the requirement of Zn^{2+} or Co^{2+} for their activity (Rao et al. 1998; Monteiro Desouza et al. 2015). Endoproteases randomly hydrolyze peptide bonds, generating relatively large peptides. Depending on their catalytic mechanism, endoproteases also are divided into serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Clark and Pazdernik 2016). Food-grade proteases can be obtained from plants, animal and microorganisms, and may require different pH and temperature conditions for their optimal function. However, microbial enzymes have dominated the industrial demand, due to their superior economic and technical advantages such as a lower production cost and susceptibility to genetic modification (Monteiro Desouza et al. 2015).

Microbial proteases are obtained using either bacteria or fungi. Bacterial proteases are mainly produced by bacteria that belong to the genus *Bacillus*. Bacterial proteases generally have working conditions at near neutral pH (pH 5 to 8) with low thermotolerance, or at alkaline pH (pH 8 to 10) with an optimal temperature of around 60°C (Rao et al. 1998). Neutral bacterial proteases are preferred in the food industry compared to animal proteases, due to the minimal bitterness generated during hydrolysis. Alkaline proteases are preferred in the detergent industry (Rao et al. 1998). Fungal proteases are obtained from filamentous fungi and their production has advantages compared to bacterial enzymes such as low manufacturing cost, high yield, faster production and convenience for modification (Vishwanatha et al. 2010). Fungal proteases have been extracted from a variety of strains belonging to the genera *Rhizopus, Mucor, Humicola,*

Thermoascus, Thermomyces, Aspergillus and *Penicilium*. The food industry utilizes a wide array of neutral enzymes produced by *Apergillus* species, such as *A. flavus, A. niger* and *A. oryzae*. In particular, protease from *A. oryzae* is preferred as it is generally regarded as safe (GRAS) and it has affinity for hydrophobic amino acids, preventing bitter taste of the hydrolysates (Sumantha et al. 2006). *A. Oryzae,* commercially known as Flavourzyme™, contains a mixture of endoproteases and exopeptidases for improved activity. Flavourzyme™ has been used as a debittering enzyme at a DH range of 10% to 20% and as a flavor enhancer at higher DH (>50%). Flavourzyme™ has an optimal pH range of 5.0-7.0 and an optimal temperature of around 50°C (Hamada 2000).

2.4 PROTEIN-STARCH INTERACTION

Starch digestibility and its effect on glycemic response may be influenced by starch structure, food processing conditions and individual components present in the food matrix i.e. lipids, fiber, antinutrients and protein. Specifically, proteins have been shown to affect the rate of starch hydrolysis in cereals (Ezeogu et al. 2008). Research has been focused mainly on understanding the endogenous protein-starch interactions in sorghum, corn and wheat and their effects on nutritional properties. The proposed mechanism in sorghum and corn matrices suggests that proteins strongly protect the starch granules by surrounding their surface, and impeding free access to amylolytic enzymes (Rooney and Pflugfelder 1986). Similarly, Jenkins et al. (1987) suggested a decrease *in vivo* glycemic response after consumption of wheat flour, due to the presence of gluten, surrounding the starch granules which could slow down the starch hydrolysis in the small intestine.

Legumes and pulses have higher protein contents that are in general, double than that of cereal grains, which is important as legumes show a slower digestion rate and low glycemic

response in healthy individuals and diabetics (Dilawari et al. 1981; Jenkins et al. 1980; Roy et al. 2010). However, there are limited studies reported in the literature on the mechanism of action of endogenous pulse proteins in lowering the digestibility of pulse starch and its consequent lower glycemic response. On the other hand, addition of exogenous plant proteins to food matrices has become an emerging technology, especially for food formulations targeted at celiac and diabetic patients. However, there is little evidence of the influence of protein structure and denaturation (i.e. native, denatured or hydrolysed) on the interaction with starch and its effect on lowering the glycemic response.

A study on the interactions of soy protein and wheat starch showed that heat and pressure treated proteins had a significantly higher binding affinity to starch granules compared to native soy protein (Ryan and Brewer 2007). Heat-denatured exogenous plant proteins may bind with starch via different mechanisms, depending on their conformational changes after denaturation. Protein denaturation will expose the hydrophobic amino acids, which were buried in the native state and which potentially may form hydrophobic interactions with non-polar components of the starch surface proteins (Ryan and Brewer 2007). In addition, the aggregated proteins formed during denaturation will likely generate polar interactions with starch through hydrogen bonding (Moore and Carter 1974). Also, protein aggregation facilitates the formation of disulphide bonds between exogenous proteins and starch surface proteins, thereby increasing the resistance to enzyme hydrolysis (Marshall and Chrastil 1992). Despite these findings, further research is required using advanced analytical and characterization techniques in order to understand the exact mechanism of interactions between exogenous heat-denatured pulse proteins and starch, and its influence on starch digestibility.

Studies on the interactions between hydrolysed proteins and starch have been focused mainly on understanding their effect on rheological properties within food matrices (Chen et al. 2012a, 2012b; Ribotta et al. 2011). Efforts have been made to understand the role of hydrolysed proteins on *in vivo* hormonal responses such as insulin and glucagon secretion and their effect on blood glucose concentration in healthy adults (Calbet and MacLean 2002; Claessens et al. 2008; Power et al. 2009) and diabetic patients (Van Loon et al. 2000). However, the majority of the research has focused mainly on wheat, soy, whey and casein proteins. Therefore, it is essential to conduct a systematic study on the mechanisms of interactions between hydrolysed pulse proteins and starch and its influence on digestibility and glycemic response.

2.5 *IN VITRO* DIGESTION MODELS

Human digestion is a complex process that involves two main events: mechanical transformation by reduction of food particle size, mainly in the mouth and stomach, and enzymatic degradation of food components to molecules absorbed mainly in the small and large intestines (Guerra et al. 2012). *In vitro* digestion models are commonly used as practical alternatives to human models, which are not always possible to conduct due to ethical, technical and financial reasons. *In vitro* digestion models are used in order to screen food ingredients in terms of structural changes, release of food components and digestibility (Hur et al. 2011). Although human models usually provide more accurate results, *in vitro* models are very useful tools to predict outcomes of the digestion *in vivo*. According to the complexity of the method, *in vitro* digestion models can be classified as simple, static or dynamic.

2.5.1 Simple lab models

In vitro simple models are rapid and easy methods to mimic the steps of carbohydrate digestion by using highly purified, commercially available enzymes to ensure specific hydrolysis

for starch determination. The most commonly simulated step is small intestinal digestion by using α-amylase and amyloglucosidase enzymes. Among the different methods proposed, the method 2002.02 has been accepted by AOAC INTERNATIONAL as the official method, for the measurement of solubilised starch (rapidly digestible starch and slowly digestible starch) and resistant starch (McCleary and Monaghan 2002). In this method, the sample is mixed with pancreatic α-amylase enzyme at pH 6.0 and 37°C in the presence of calcium as cofactor. Samples are subjected to continuous stirring for 20min, 120min or 16h for the determination of RDS, SDS and RS, respectively. The reaction is stopped by the addition of absolute ethanol followed by the addition of amyloglucosidase at pH 3.8-4.5 at a temperature of 50°C. With this method, starch is converted by α-amylase to maltose, maltotriose and α-limit dextrins that are further hydrolysed by amyloglucosidase into glucose molecules that react with GOPOD regent and are quantified spectrophotometrically. Although this method is simple and convenient in the obtention of consistent and reproducible results, it lacks of continuous stages, i.e. mouth, stomach and duodenum, and more realistic conditions in terms of the composition of digestive fluids, which will influence the digestion of starch.

2.5.2 Static models

Static models (also called biochemical models) are mainly used to study the digestibility of simple foods or isolated nutrients. Static models mimic the sequence of events that take place during human digestion (oral, gastric and intestinal) in single test tubes, by simulating physiological conditions such as temperature, agitation, pH, enzymes and chemical composition. However, static models do not simulate physical and dynamic processes that happen *in vivo* such as shear, hydration, mixing, changing pH and enzyme secretion conditions over time, or peristalsis (Wickham et al. 2009). In addition, static models do not allow determining uptake or

absorption of nutrients, nutrient competition at the location of absorption, or transporting kinetics, since it is not possible to simulate the complex gut mucosal barrier. However, they resemble physiological conditions in a more realistic manner compared to simple models (Alegría et al. 2015).

Different static *in vitro* methods have been used in order to simulate gastrointestinal human digestion. However, these methods differ in parameters such as type of enzymes added and enzyme activities, pH conditions, time of digestion, etc. (Kopf-Bolanz et al. 2012; Versantvoort et al. 2005; Woolnough et al. 2008), making extremely difficult to compare the results of different studies. Therefore, the network INFOGEST "Improving health properties of food by sharing our knowledge on the digestive process", funded by the European Cooperation in Science and Technology (COST) and financed by INRA, proposed different objectives to address these challenges (Dupont et al. 2011; INRA, 2011). Specifically the objectives were: 1) to harmonize in vitro protocols that simulate human digestion, 2) to develop a static model easy to set up and use, and 3) to have the possibility to compare results between laboratories. For that purpose, three studies on digestibility of skim milk powder were performed using different in vitro methods in different laboratories of the INFOGEST network (Egger et al. 2016). The results showed that the in vitro digestion method developed by (M. Minekus et al. 2014) was the most comparable and robust static model to study in vitro human digestion (Egger et al. 2016). This method was developed based on physiological in vivo enzyme and salt concentrations, and includes a standardized assay to determine the activities of added enzymes in order to minimize difference in the results between laboratories. This harmonized method consists of three digestion steps: oral, gastric and intestinal phases. Samples are mixed at a ratio of 1:1 with simulated salivary fluid (pH 7.0), calcium chloride and human salivary α-amylase (75 U/mL) at

37°C for 2 min. Then, simulated gastric fluid (pH 3) is added (ratio of 1:1) along with pepsin (2000 U/mL) and phospholipids if necessary, and mixed for 2 h. The oral and gastric hydrolytic processes are stopped by raising the pH to 7 by the addition of simulated intestinal fluid (ratio 1:1) and bile salts (Na-glycodeoxycolate and Na-taurocholate). Pancreatin or individual enzymes are then added to the hydrolysate/fluid and incubation continues for 2 h. The digestion reaction is stopped by either snap freezing at -80°C immediately or using enzyme inhibitors followed by freeze drying (Minekus et al. 2014).

Static models are useful screening tools to predict the *in vivo* digestibility of food components. However, it is urgently needed to ensure that static models have validated protocols in order to increase the reproducibility of the experiments before conducting more advanced trials such as dynamic *in vitro* models or *in vivo* studies (Alegría et al. 2015).

2.5.3 Dynamic models

Dynamic models simulate more complex *in vivo* conditions such as the constant change in biochemical environment and physical processes such as shear, hydration, mixing and gastric emptying. In addition, real food matrices can be used, unlike static models that are only able to process simple meals or purified nutrients (Wickham et al. 2009). Dynamic gastric models are further classified into monocompartmental, bicompartmental and multicompartmental, based on their method of digestion and parts involved (Guerra et al. 2012).

Dynamic monocompartmental models such as Human Gastric Simulator (HGS) and Dynamic Gastric Model (DGM) mimic the gradual acidification of gastric content, the slow flow rate of pepsin addition and the gastric emptying. HGS was developed by Kong and Singh (2010) from the University of California (Davis) in order to study gastric digestion of different foods. It consists of a latex vessel surrounded by a mechanical drive system composed of rollers and belts

that mimic the continuous peristaltic activity of the stomach, with similar amplitude and frequency to *in vivo* stomach forces (Figure 2.12). Secretion of simulated gastric juice and emptying of digesta are controlled using secretion tubes and peristaltic pumps. The system maintains a temperature of 37°C using light bulbs and isolating the system with plastic foam. A second replicate of HGS model is available at the Massey University in New Zealand.

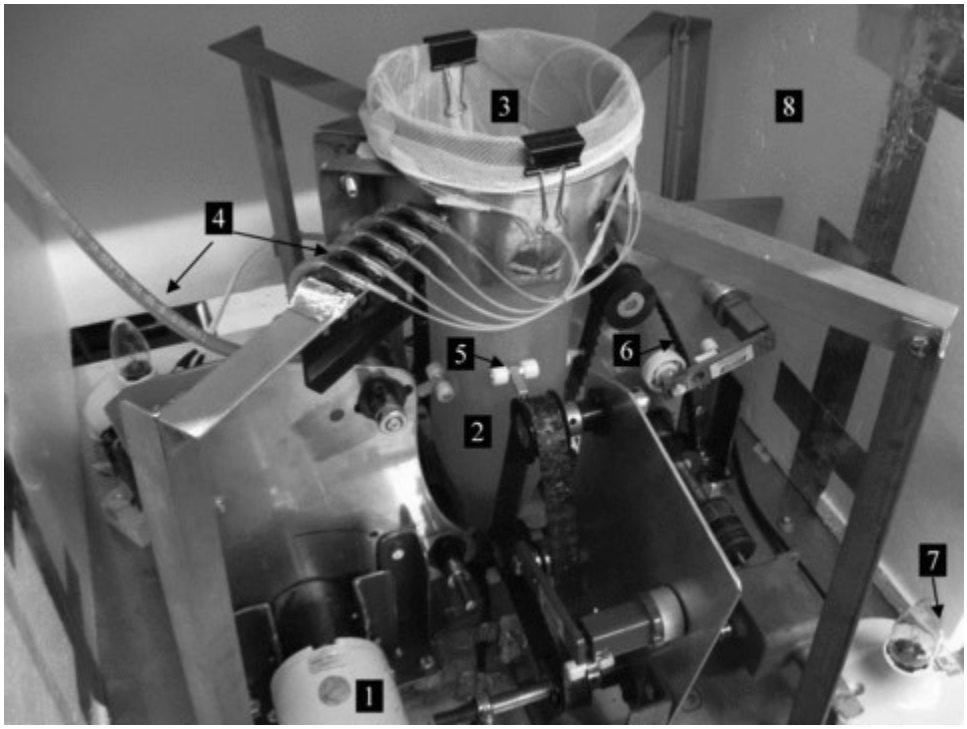


Figure 2.12 Human gastric simulator. (1) Motor; (2) latex lining; (3) mesh bag; (4) secretion tubing; (5) roller; (6) belt; (7) light bulb for temperature control; (8) plastic foam insulation. Adapted from Kong and Singh (2010), with permission of John Wiley and Sons.

Dynamic gastric model (DGM) was invented by the Institute of Food Research, U.K (Wickham et al. 2012) and it is available exclusively at Bioneer:FARMA (Copenhagen, Denmark) as an outsourced contract research facility. DGM was created with the purpose to simulate biochemical and mechanical characteristics of human gastric digestion and to study the

release, bioaccessibility and interactions of nutrients (Thuenemann et al. 2015) (Figure 2.13). It is composed mainly of a fundus/main body where acid and enzyme secretions are mixed with the bolus by gentle massaging and then, with the movement of a piston, sample passes to the antrum by the movement of a barrel. As the barrel moves downward and upward, antral content passes through a flexible annulus where physiological shear and grinding forces are reproduced. Acid and enzyme are controllably added from the top of the fundus through a perforated hoop connected to a pump. The pH is sensed in the fundus with an electrode that allows the control of acid addition. The rate of gastric enzyme addition is dependent on the bolus volume that remains in the fundus after ejection of sample from the antrum. The fundus and the antrum are surrounded by a water jacket that keeps the temperature at 37°C. Samples from the antrum are ejected through a valve assembly and collected for further analysis (Wickham et al. 2012).

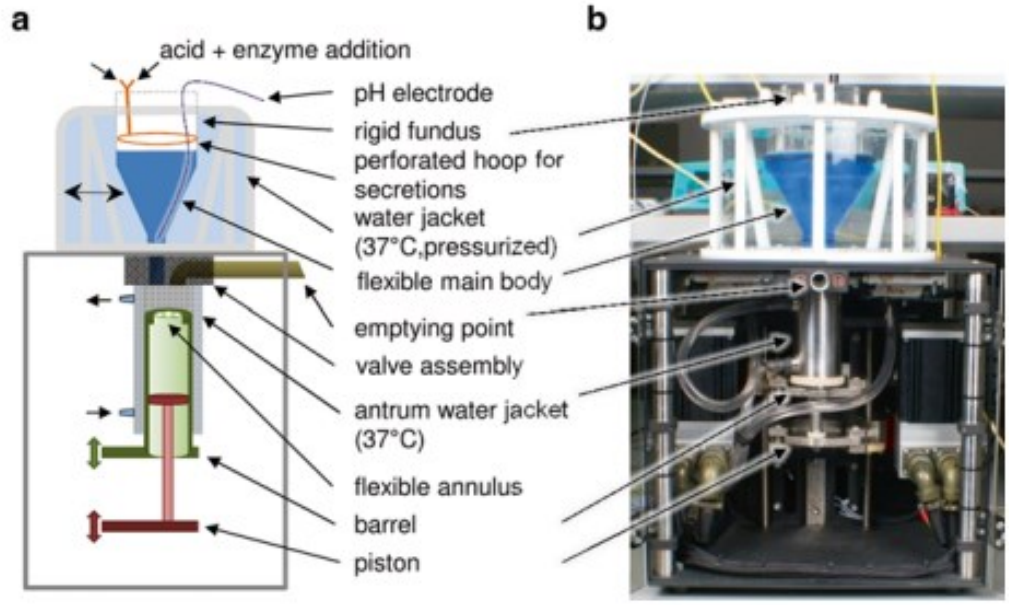


Figure 2.13 The dynamic gastric model (DGM). (a) Schematic representation of the main components of the DGM (side view), (b) Photographic image of the DGM (front view). Adapted from Thuenemann et al. (2015), open access article.

In a separate step, samples collected after each ejection from the antrum are immediately placed in a static USP (United States Pharmacopoeia) dissolution apparatus. USP dissolution apparatus is composed of a vessel (containing simulated intestinal fluid and pancreatin enzymes), placed inside a water bath that maintains the temperature at 37°C. The contents of the vessel are mixed by a paddle that can rotate at different speeds. Samples can be withdrawn at specific times for further analysis.

Dynamic bicompartmental models such the dynamic *in vitro* upper GI tract model system are designed to mimic the conditions of the stomach and small intestine in a continuous phase, based on *in vivo* data. This model simulates temperature, pH variations in the gastrointestinal compartments, addition of pepsin, gastric emptying, and addition of pancreatic juice or bile (Figure 2.14). The sample and acid are pumped into a first reactor (stomach) and heated and stirred with the presence of gastric enzymes. The emptying rate of the stomach vessel into the duodenum vessel; that contains intestinal enzymes, is controlled by a peristatic pump. Oxgall bile is gradually added by a peristaltic pump. This model has been mainly designed to study the antacid activity and the survival of probiotics during gastrointestinal digestion (Mainville et al. 2005).

Another simple bicompartmental dynamic gastrointestinal model is the DIDGI® digestion system, developed at the French National Institute for Agricultural Research (INRA) (Figure 2.15). The model was created to understand the mechanism by which infant formula was digested in the infant gastrointestinal tract, and to track the disintegration and the kinetics of hydrolysis of dairy, eggs, meat, bakery products, etc. DIDGI® model consists of two continuous compartments that resemble gastrointestinal transit times, pH and consecutive addition of digestive juices similar to *in vivo* conditions.

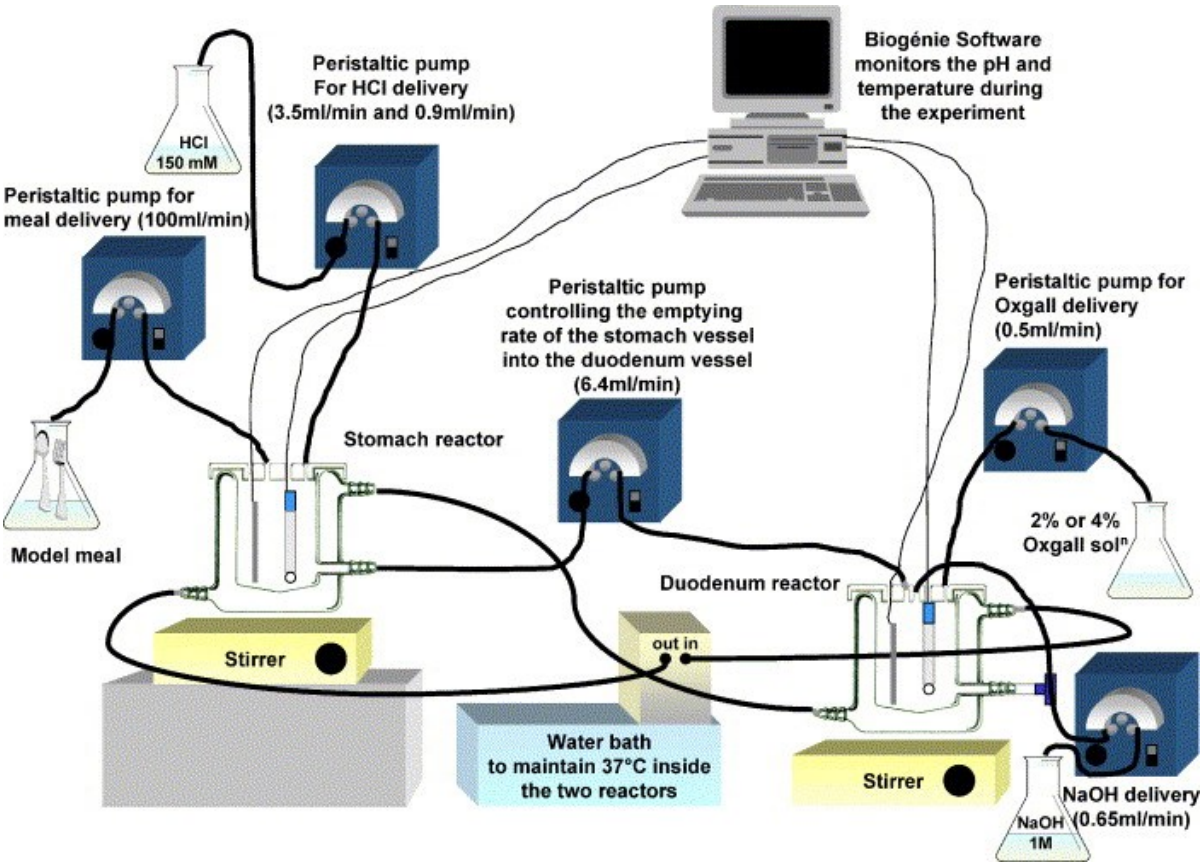


Figure 2.14 The dynamic *in vitro* upper GI tract model system. Adapted from Mainville et al. (2005), with permission of Elsevier.

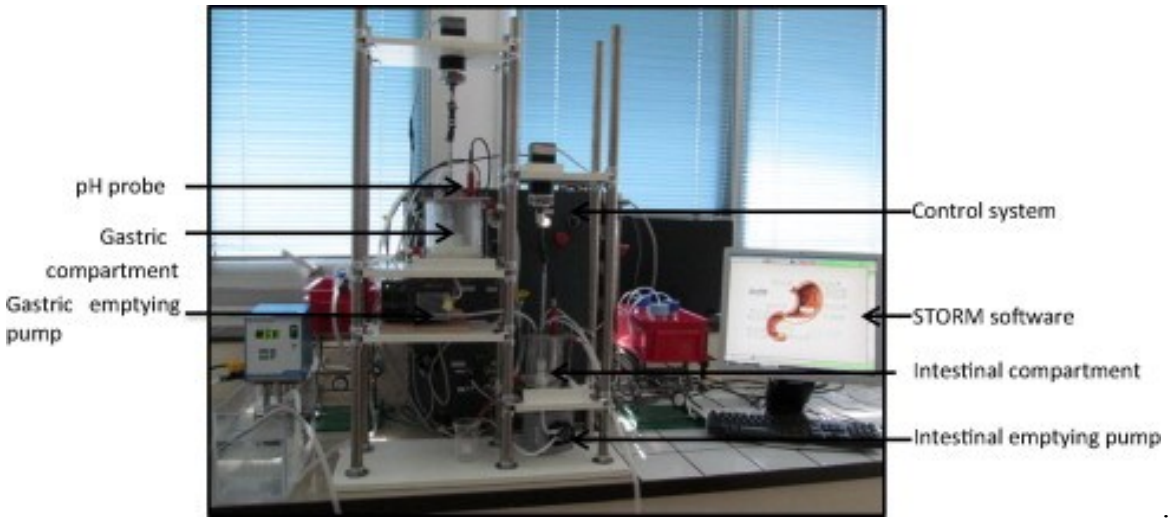


Figure 2.15 Presentation of the gastro-intestinal dynamic digestion system. Adapted from Ménard et al. (2014), with permission of Elsevier.

The addition of sample, HCl, Na_2CO_3, bile and enzyme is controlled by peristaltic pumps. Once the contents of the gastric compartment are ready for digestion in the duodenum, the sample passes through a Teflon membrane to simulate the sieving process of *in vivo* digestion. DIDGI® system is controlled by a computer with StoRM software that allows monitoring the conditions (Ménard et al. 2014).

Lastly, the multicompartmental computer controlled dynamic model named TNO Gastro-Intestinal Model (TIM-1), invented by Minekus et al. (1995) is a very complete model that simulates *in vivo* gastrointestinal digestion (Figure 2.16). It consists of four consecutive compartments that simulate the stomach and three parts of the small intestine, the duodenum, jejunum and ileum. It was designed with the purpose of mimicking gastrointestinal transit, peristaltic movements, pH, gastric and intestinal secretions, and bile salt concentrations. In addition, it was invented to determine the bioaccessibility of macronutrients, measured by the absorption of glucose, amino acids and fatty acids from the duodenum through a dialysis system or filtration membrane. The model is equipped with peristaltic valves that enable the transit of chyme between compartments, pH electrodes, and temperature and pressure sensors (Minekus 2015).

Diverse *in vitro* gastrointestinal models have been developed worldwide, in order to understand and predict *in vivo* food digestion and the effect of food components on nutrition and health, in a relatively simple and economic manner. However, none of the previously mentioned advanced *in vitro* models fully mimic *in vivo* conditions. Efforts must be made in optimizing the actual models in order to 1) resemble mastication, 2) improve the accuracy of peristaltic movements or stomach contractions, 3) resemble digestion in different types of population (i.e. age, gender, health condition etc.) and 4) be validated with *in vivo* data.

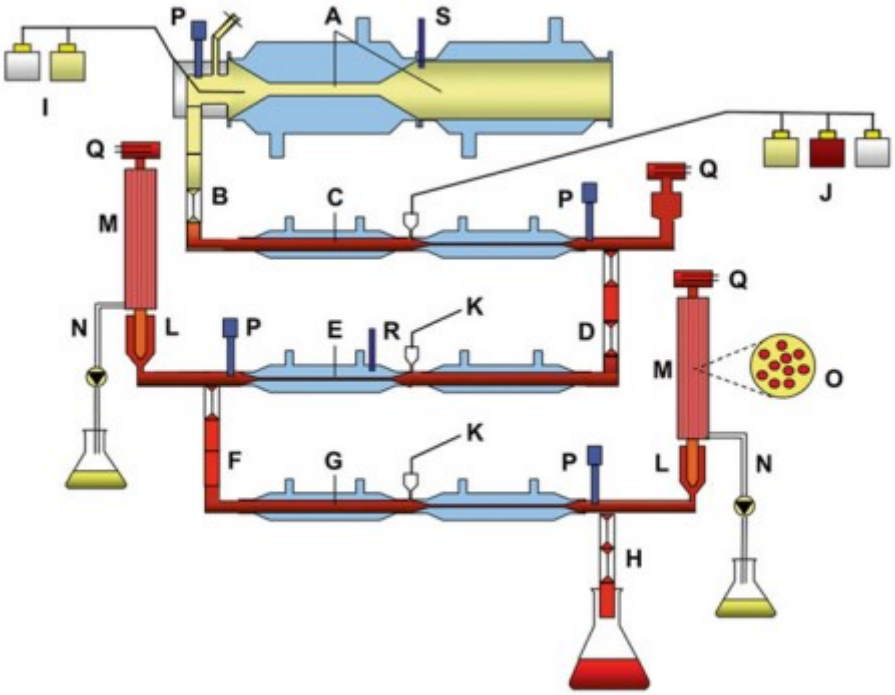


Figure 2.16 Schematic presentation of TIM-1, equipped with filters to study the bio-accessibility of lipids. A. gastric compartment; B. pyloric sphincter; C. duodenal compartment; D. peristaltic valve; E. jejunal compartment; F. peristaltic valve; G. ileal compartment; H. ileal-cecal valve; I. gastric secretion; J. duodenal secretion; K. bicarbonate secretion; L. pre-filter; M. filtration system; N. filtrate with bio-accessible fraction; O. hollow fiber system (cross section); P. pH electrodes; Q. level sensors; R. temperature sensors; S. pressure sensor. Adapted from Minekus (2015), open access article.

2.6 STRATEGIES TO FORMULATE LOW GLYCEMIC INDEX AND LOW GLYCEMIC LOAD FOOD PRODUCTS

In an attempt to clarify how digestible carbohydrates such as starch and related compounds impact blood glucose levels, the glycemic index concept (GI) was introduced by Jenkins et al. (1981). However, since this concept was controversial and criticized for its usefulness and consistency, Salmerón et al. (1997) introduced the term glycemic load (GL) as a complementary concept. In order to clarify and discuss the controversies about these measuring tools, a consensus meeting with international experts on carbohydrate research was held in Rome in 2015 (Augustin et al. 2015). The definitions of the terms glycemic response, glycemic index

(GI) and glycemic load (GL) were clarified as follows. Glycemic response is the change of blood glucose concentration after ingestion of a meal containing carbohydrates. Glycemic index is the glycemic response to 50 g or in some cases, 25 g, of available carbohydrate contained in a portion of food divided by the glycemic response of 50 g (or 25 g) of a reference carbohydrate, usually glucose solution or white bread. Thus, the GI can be both a standardized and a relative glycemic response, based on the same amount of available carbohydrate and the chosen reference food, respectively. Low glycemic foods are considered to be digested, absorbed and metabolized slowly and have a GI of ≤ 55 on the glucose scale. In contrast, high glycemic foods are digested, absorbed and metabolized quickly and have a GI of ≥70. The GI concept was developed for foods with a representative amount of carbohydrates and, therefore, it is not appropriate to clinically measure GI of complete meals that contain other macronutrients such as fat or protein. The GL is the product of the GI and the amount of available carbohydrate contained in a serving size (GL = GI x available carbohydrate/serving size) (Augustin et al. 2015). In general, the consumption of low GI and low GL foods is associated with the reduction of postprandial glucose responses, which mitigate the risk of type 2 diabetes (Barclay et al. 2008; Livesey et al. 2013; Ludwig 2002; Salmerón et al. 1997) and dyslipidemia (Jenkins et al. 1987).

Although the reduction in carbohydrate intake as a percentage of energy would be one of the main dietary approaches to reducing postprandial glycemia, other strategies, as discussed below, have been proposed to formulate low glycemic foods for heathy individuals as well as for patients with metabolic diseases such as fatty liver, insulin resistance and diabetes.

2.6.1 Resistant starch

Foods with high resistant starch content may be linked to be low glycemic and may have the potential to be used in food formulations for diabetic patients (Björck and Asp 1994). Among

the strategies to increase the amount of RS1 in foods, particle size plays a crucial role. The addition of intact or minimally processed grains such as dehulled whole kernels of cereals, pulses, steel-cut grains, coarsely ground seeds, durum wheat and pseudocereals such as quinoa in food formulations increases the amount of RS1. The cell wall and protein matrix in pulses and cereal grains physically entrap starch and restrict accessibility of the amylase enzyme toward starch. Also, such entrapment limits water absorption by starch granules, reducing the susceptibility to gelatinization (Birt et al. 2013).

The amount of RS2 is influenced by processing conditions such as the extent of cooking and heat treatment. For example, native B-type and C-type polymorph starches i.e. potato and green banana that are highly resistant to enzymatic hydrolysis lose their crystallinity after processing at high temperatures (Birt et al. 2013). The complete loss of crystallinity in starch granules is related to an increase in the rate of amylolysis, and thus it results in starchy foods with higher glycemic responses. Therefore, a low degree of gelatinization will be required to preserve the highly ordered starch structure. For example, gentle roasting rather than extensive steaming before cereal flaking could better protect the starch crystallinity in the final product (Björck et al. 2000). Hydrothermal treatments such as annealing (ANN) and high moisture treatment (HMT) have been used to modify the starch structure without gelatinization, with the purpose to improve the crystalline order within the granule and increase RS2 content. ANN is a hydrothermal treatment achieved at moisture contents of 40% or higher. This treatment consists of maintaining the starch granules at temperatures below the gelatinization point in order to preserve or increase the crystalline structure. On the other hand, HMT is performed at moisture levels below 35% with temperatures even above the gelatinization temperature of the starch. Partial acid hydrolysis before ANN or HMT could also improve the yield of RS2 by facilitating

the arrangement of molecules (Thompson 2000). In addition, high amylose starches from engineered crops produced by mutation of the amylose-extender gene and the gene encoding starch branching enzyme I (Regina et al. 2006; Jiang et al. 2010; Li et al. 2008) have been used as ingredients in foods as sources of RS2. High amylose starches have shown positive effects in the control of postprandial glycemia due to their enzymatic resistance toward hydrolysis and the very high temperature required for gelatinization. Granfeldt et al. (1995) demonstrated that maize bread (arepas) made from high-amylose corn flour (70% amylose), elicited favorable low glucose and insulin responses in healthy subjects when compared to bread made with regular flour (25% amylose). Health Canada has accepted resistant corn starch or high amylose corn starch and resistant wheat starch or modified wheat starch as novel fibers that increase sensitivity to insulin and provide energy for the generation of metabolites (Health Canada 2017)

A strategy to increase starch crystallinity as RS3 is to generate retrogradation of gelatinized starches. Retrogradation can be induced by repeated cycles of autoclaving and cooling, and the yield of RS3 obtained is dependent on the amylose content, processing temperature and water content (Berry 1986; Sievert and Pomeranz 1989). Native starches high in amylose are recommended for the preparation of RS3 in order to obtain high yield because they have the tendency to form double helices at low temperatures (4-5°C) (Birt et al. 2013). Double helices of retrograded starches are not compatible with the binding site of amylase enzymes; therefore, they resist enzymatic hydrolysis (Birt et al. 2013). Amylose molecules must have a minimum degree of polymerization of 10, with an optimum of 100, glucose units to form double helices (Gidley et al. 1995). However, typical lengths of amylopectin molecules that are present in the granule range from 20-40 and are far from 100, and may negatively affect the amylose retrogradation yield due to their branched structure (Eerlingen et al. 1994). Therefore, in order to

generate RS3 from amylopectin, debranching enzymes such as pullulanase can be used (Berry 1986). Temperature cycles from 4°C to 100-134°C in excess water appear to be favorable conditions to obtain stable RS3 (Eerlingen et al. 1993; Sievert and Pomeranz 1989). After 3 to 20 autoclaving-cooling cycles, high-amylose starches generate RS, in yields from 30%-40% that resist the action of amylolytic enzymes and are thermally stable with melting temperatures between 120°C-165°C (Gruchala and Pomeranz 1993; Sievert and Pomeranz 1989). Enzyme or acid hydrolysis of high amylose starches before or after gelatinization also has been proposed to allow better polymer mobility for molecular reorganization and enhance the amount of RS3 (Thompson 2000).

RS4 is starch that has been chemically modified by crosslinking or by adding chemical derivatives that change its structure and increase resistance to enzymatic hydrolysis. Highly cross-linked starches have a lower capacity to swell under thermal processing conditions and remain ungelatinized, generating resistance to digestive enzymes. Starches treated with chemical derivatives generate new structures that do not fit into the binding sites of the amylolytic enzymes (Birt et al. 2013).

Amylose and long-branched amylopectin chains in the presence of lipids and fatty alcohols can form complexes that prevent cleavage by amylase enzymes and restrict the swelling of starch granules; the complexes are known as RS5 (Jane et al. 1997; Seneviratne and Biliaderis 1991). Hasjim et al. (2010) studied the effect of complexing high-amylose maize starch VII (HA7), previously debranched with isoamylase, with palmitic acid on the RS content and postprandial and insulin responses. The RS content of the treated samples (52.7%) was significantly higher compared to the HA7 control (35.4%), which indicated higher formation of retrograded starch and starch-lipid complexes. Postprandial and insulin responses were

significantly lower when consuming bread formulated with 60% of the treated starch compared to white bread. Furthermore, Ai et al. (2013) showed that the presence of corn oil, soy lecithin, palmitic acid, stearic acid, oleic acid and linoleic acid resulted in significant reductions in the enzymatic hydrolysis of normal corn starch, tapioca starch and high amylose corn starch. Thus, starch-lipid complexes could be used as a strategy to formulate foods with reduced glycemic index that could alleviate health problems such as fatty liver, insulin resistance, diabetes and obesity.

In general, foods formulated with RS have shown better effects on postprandial glucose responses when RS is added to replace a percentage of rapidly digestible starch, rather than being added as an extra portion of the ingredients (Wong and Louie 2016). Thus, the content of available carbohydrate in a food is the main trigger of glucose release despite the presence of RS. This could suggest that RS does not act as a barrier that can protect the more digestible starch from enzymatic attack. Despite the potential health benefits attributed to the inclusion of RS in the diet, additional research is necessary to have a deeper understanding of the metabolic effects and the mechanisms behind them.

Commercially, resistant starch products are derived mainly from high-amylose corn starch, such as Hi-maize® whole grain corn flour (RS1 and RS2), Hi-maize®260 corn starch (RS2) and Novelose®240 (RS2) in which the starch granules are thermally modified, i.e. heat - moisture treated, Novelose®330 (RS3), PROMITOR™ (RS3) and Amylo-maize N-400 (RS3), which are retrograded resistant starches, and CLEARAM® (RS4), which is chemically modified (Fuentes-Zaragoza et al. 2010; Stewart et al. 2010; Watson et al. 2014). Resistant starch is considered a potential plant-based ingredient that has health benefits when consumed in a minimum dose of 6 g/meal (Alexander 2012; Birt et al. 2013). However, its inclusion into baked

products has been challenging in terms of sensory and quality properties such as texture, gluten network formation, cohesiveness and chewiness, among others (Birt et al. 2013). Nevertheless, when compared to traditional dietary fibers, RS possesses physicochemical characteristics such as water binding/hydration, swelling and gelling that make it suitable for diverse applications (Fuentes-Zaragoza et al. 2010)

2.6.2 Highly viscous soluble fibers

Enzymatic digestibility of starch and glycemic response can be affected by the presence of polysaccharide based gums and soluble fibers that increase the matrix viscosity.

Galactomannans are storage polysaccharides of plant seeds, mainly from the *Leguminosae* family, that are formed by a β-(1-4)-D-mannan backbone with branches of α-(1-6)-D-galactose. There are four common sources of seed galactomannans that differ in the mannose to galactose ratio: locust bean (1.0:3.9–4.0), guar (1.0:1.6–1.8), tara (1.0:3.0) and fenugreek (1.0:1.0–1.1). Galactomannans have the capacity to hydrate easily due to the presence of many hydroxyl groups that allows them to bind water and to create very viscous solutions at low concentrations (Prajapati et al. 2013).When consumed as part of food formulations, i.e. thickening, binding and stabilizing agents, galactomannans increase the viscosity of digesta in the gut lumen, delay transit time, and create a physical barrier that reduces the rate of enzymatic hydrolysis (Jenkins et al. 1978). In addition, they may reduce the transport of amylolytic products (i.e. maltose, α-limit dextrins) through mucosa and their absorption, thus, lowering the postprandial glucose response. As well, galactomannans in starchy foods may impede the complete swelling and gelatinization of starch granules as a result of a competition for water hydration. Therefore, ungelatinized granules will be more resistant to hydrolysis by amylolytic enzymes, and may generate lower postprandial glucose responses (Singh et al. 2010).

β-Glucan is a polysaccharide consisting of D-glucose monomers linked by β-(1-3) and β-(1-4) linkages and is mainly found in the internal aleurone and subaleurone cell walls of oat (up to 7%, w/w) and barley (up to 15%, w/w). β-Glucan can be concentrated or isolated by dry and wet milling technologies (El Khoury et al. 2012). After extraction, the purity of β-glucan can vary from concentrates (8-30%) to isolates (95%). β-Glucan is a soluble fiber that increases the viscosity of the contents of the stomach and the intestine, and hypothetically delays gastric emptying and slows glucose absorption in the intestinal wall (Braaten et al. 1991; Edwards et al. 1988; Wood et al. 1994; Pins and Kaur 2006). Health Canada (2017) has accepted the use of β-glucan concentrate as a novel dietary fiber to reduce postprandial blood glucose levels. It must be obtained chemically from oat grains using aqueous-alcohol-alkaline or aqueous-alcohol-enzymatic processes followed by ethanol precipitation, and must have a molecular weight higher than 250 kDa. The efficacy of β-glucan in the reduction of postprandial glucose and insulin levels depends on the viscosity, which is influenced by the concentration of the fiber and its solubility and molecular weight (2,000,000-3,000,000 g/mol) (Pins and Kaur 2006; Wood 2002; Wood et al. 2000). Factors such as processing and storage temperature, processing technique, interaction with other ingredients and the presence of enzymes (β-glucanases) in the food matrix, may influence β-glucan depolymerisation, affecting its rheological properties and its subsequent physiological activity (Regand et al. 2009). Despite the strong evidence that β-glucan has an effect on postprandial glycemic responses, conflicting results have been noted when incorporating this fiber into different food matrices. Negative results were obtained when including β-glucan in solid foods (Biorklund et al. 2005; Holm et al. 1992; Panahi et al. 2014), whereas its inclusion in liquid foods has shown positive outcomes (Biorklund et al. 2005; Panahi et al. 2007). Therefore, a better understanding of the physicochemical properties of β-glucan is

needed to successfully incorporate this dietary fiber into diverse food formulations while guaranteeing the physiological health effects.

Pectins are structurally complex polysaccharides found in the cell walls and intercellular layers of plant cells. Pectins are typically isolated from citrus, tomato, sugar beet and apple fruits and their chemical properties, such as solubility and viscosity, differ from one plant source to another. The chemical structure of primary cell wall pectin contains mainly 1,4-linked, α-D galactosyluronic acid (GalpA) units (Ridley et al. 2001). Pectins have high water holding capacity and form gels. Similar to other soluble fibers, the beneficial physiological properties of pectin are linked to its viscosity and fermentability. Viscosity is associated with influencing the blood glucose concentration, increasing gastric emptying and reducing the intestinal transit time. Fermentability is related to the production of short chain fatty acids or the influence in laxation (Dikeman and Fahey 2006). In spite of this, the valuable properties of pectins for the control of blood glucose concentration in diabetic people have been contradictory (Gardner et al. 1984; Jenkins et al. 1976). However, studies have shown that the consumption of pectin on a daily basis decreases significantly the blood glucose concentration and insulin levels in healthy participants (Jenkins et al. 1977; Jenkins et al. 1976).

2.6.3 Hydrolysed proteins

Among the strategies to reduce postprandial glycemia *in vivo*, hydrolysed proteins from animal and plant sources have played an important role. Protein hydrolysates from animal sources such as casein, egg and whey obtained by enzymatic hydrolysis have been included in the diets of type 2 diabetic patients in the form of beverages (Claessens et al. 2009; Manders et al. 2009). However, whey protein hydrolysate has been shown to have the highest impact on glycemic control. Whey protein hydrolysate stimulates endogenous insulin secretion,

accelerating glucose disposal and thus mitigating the postprandial increment of blood glucose concentration in healthy and diabetic patients (Esteves et al. 2011). Specifically, the presence of branch-chain amino acids in whey protein hydrolysates, in particular leucine, has been identified as the insulin secretagogue in *in vivo* studies and probably an enhancer of satiety as a result of extra-hepatic metabolism (Diepvens et al. 2008; Frid et al. 2005; Van Loon 2007; Nilsson et al. 2004; Petersen et al. 2009). In fact, the consumption of casein protein hydrolysates with added free leucine demonstrated efficacy in reducing hyperglycemia in type 2 diabetes patients (Manders et al. 2009; Manders et al. 2005) when compared to consumption of casein hydrolysate alone (Manders et al. 2009). Nevertheless, the use of free amino acids has been questioned and requires human studies to provide a margin of tolerability and safety (Maher 1994; Roberts 2016).

The effect of plant protein hydrolysates (soy, gluten, rice and pea) on postprandial glucagon and insulin responses and consequently blood glucose concentration also has been studied, showing positive effects (Claessens et al. 2009; Diepvens et al. 2008). Interestingly, pea protein hydrolysate has shown comparable results with whey protein hydrolysate in increasing plasma insulin and reducing plasma glucose responses (Calbet and MacLean 2002b). However, studies show that the consumption of shakes with pea protein hydrolysate elicits stronger satiety effects in terms of hunger and desire to eat compared to whey protein and milk protein without hydrolysis (Diepvens et al. 2008). Pea protein contains leucine in its structure, which is more physically exposed after hydrolysis and results in a faster increase in plasma concentration, potentiating its insulinotropic effect. The proposed mechanism behind the augmentation of insulin after consumption of leucine-containing proteins is that leucine activates glutamate dehydrogenase activity in pancreatic β-cells, increasing the activity of the tricarboxylic acid

cycle and oxygen intake of the pancreatic β-cells. This process subsequently increases insulin production (Van Loon et al. 2000).

CHAPTER 3. STARCH DIGESTIBILITY PROFILING OF PRESSURE-COOKED SPLIT FIELD PEA SEED, FLOUR AND STARCH[1]

3.1. INTRODUCTION

Pulses such as field pea, lentil, chickpea and fava bean belong to the legume family and are excellent sources of protein, fiber and complex carbohydrates. The utilization of whole pulses, flour and fractionated functional components in food formulation has gained momentum worldwide, due to increased consumer demand for natural, nutritious and sustainable ingredients. Canada is a leader in pulse production and one of the major exporters of pea and lentil worldwide (Agriculture and Agri-Food Canada 2015). The Canadian production area of pulses increased from 5.3 to 10.5 million acres between 2011 and 2016. Factors such as higher international demand and better access to markets as well as higher profitability, and advantages of legume/pulse cultivation on soil fertility, have influenced the shift in pulse production compared to wheat, barley, etc. since 1981 (Statistics Canada 2017).

Pulse starches have been recognized for their low digestion rate and, therefore, low blood glucose response, and have been shown to have influence on enhanced satiety, decreasing food intake and regulating body weight (Clark and Duncan 2017). As the chronic diseases such as overweight, obesity, insulin resistance, diabetes and cardiovascular disease are on a rising trend in the last decades (Fryar et al. 2012; Carroll et al. 2015; World Health Organization 2016; Centers for Disease Control and Prevention 2015), more emphasis is given towards inclusion of pulses in the diet as a strategy to prevent or manage chronic ailments (World Health Organization 2016; Ramdath et al. 2016). In addition, the Food and Agriculture Organization of

[1] A version of this chapter will be submitted to *Cereal Chemistry Journal.*

the United Nations declared 2016 as the International Year of Pulses based on the nutritional, agricultural, economical and environmental importance of these crops (FAO 2016).

Glycemic index (GI) is a clinical measurement of the change in blood glucose concentration determined as the area under the blood glucose response curve (AUC) in response to consuming a test food and a reference food (glucose or white bread), containing a fixed amount of available carbohydrate by the same person on a different day (Jenkins et al. 2002; Jenkins et al. 1981). GI is expressed on a scale from 0 to 100, where values lower than 55 and higher than 70 refer to foods that produce low and high glucose release, respectively, when compared to the glucose reference (Atkinson et al. 2008). Gycemic load (GL), a complementary concept, represents the product of GI and the amount of available carbohydrate in a given amount of food (GL = GI x available carbohydrate/given amount of food) (Salmerón et al. 1997). A serving size of a food with GL $\geq$ 20 is considered high, whereas a GL $\leq$ 10 is considered low (Harvard Medical School 2015). Englyst et al (1992) classified starch in foods as rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) using an *in vitro* model where starch hydrolysis was carried out by the addition of pancreatin and amyloglucosidase. The values obtained within the first 20 minutes (RDS) and between 20 and 120 minutes (SDS) of starch hydrolysis for the analyzed foods, mimicked the rate of *in vivo* starch digestion. Information collected internationally on GI and GL values of different foods confirm that pulses remain classified as low GI foods (Atkinson et al. 2008). Low GI foods are linked to healthy diets that could help to prevent or manage chronic diseases such as type-2 diabetes, heart disease and obesity. In contrast, high GI foods increase blood glucose levels faster and trigger excessive insulin secretion that blocks gluconeogenesis and lipolysis, and thus generates fat storage and inflammatory reactions (Jenkins et al. 2012; Ludwig 2002).

Most of the pulse starch digestibility characterization work reported to date has been done with native/uncooked starches (Hoover and Sosulski 1985; Hyun-Jung et al. 2008; Hoover and Sosulski 1991). In the literature, a slower rate of native starch digestibility (i.e. lower RDS and GI) in pulses when compared to cereal grains such as wheat, oat, barley, etc. has been attributed primarily to their higher amylose content (30-65%), stronger amylose chain interactions leading to higher crystallinity (Hoover and Zhou 2003), smooth starch granule surface and absence of pores (Hoover and Sosulski 1985; Hoover and Sosulski 1991) and the presence of the C-type crystal polymorph (Colonna et al. 1981; Hoover and Sosulski 1985). However, this does not truly reflect on the human food consumption perspective, where starch mostly exists in the cooked/gelatinized form.

A preliminary experiment carried out in our lab that compared the digestibility profiles (i.e. RDS contents) of isolated/purified pea and wheat starches cooked/gelatinized under the same conditions (i.e. 100^{o}C boiling water bath, 30 min cooking time, 1:3 starch:water ratio, freeze drying and grinding to ensure comparable particle size) has shown no significant differences with respect to their RDS content. Also, it was noted that the literature lacks information regarding comparison of the digestibility profiles of pulse starch when present as part of the whole seed (i.e. starch remains entrapped within the tissue structure), flour (i.e. starch is exposed) or after isolation/purification from the whole seed/flour, in order to understand the effect of non-starch grain components on starch digestibility. The objective of the present study was to understand the effect of non-starch seed constituents in pea, especially the endogenous protein as the second most abundant component of pulses, on the starch digestibility profile.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Split yellow pea seeds were purshased in a local store and wheat starch was obtained from Agridient Inc. (Farmington Hills, MI, USA). Reagents and enzymes for starch digestibility determination were purchased from Sigma-Aldrich (Oakville, ON, Canada). Analytical kits for the determination of total starch and phytates were purchased from Megazyme (Megazyme International Ireland Ltd, Wicklow, Ireland). All other chemicals and solvents were of ACS certified grade. Samples were cooked in a pressure cooker (Fresco, Model PC55A/PC90A) and dried in a freeze-drier (VirTis model 50-SRC, Gardiner, NY, USA).

3.2.2 Compositional analysis

Moisture content was determined by AACC approved method 44-15.02 (AACC International, 2010). Protein content was determined by the combustion method with a nitrogen analyzer (FP-428; Leco Corp., St. Joseph, MI, USA) and using a nitrogen-to-protein conversion factor of 6.25. Megazyme analytical kits were used to determine total starch according to AOAC Method 996.11 (AOAC International, 2005). Phytates determination was according to the K-PHYT method using an analytical kit from Megazyme. Lipid quantification was performed by overnight extraction in hexane at room temperature followed by gravimetric measurement. Phosphorus was determined using molybdenum blue method (Whistler et al. 1964). Fiber was determined using the total dietary fiber assay kit obtained from Megazyme and total amylose content was obtained following the protocol suggested by Hoover and Ratnayake (2005).

3.2.3 Preparation of cooked yellow pea seeds, flour and starches

The experimental design is presented in Figure 3.1. Treatments consisted of yellow split pea seeds, yellow pea flour, and mixtures of starches (yellow pea and wheat) with cellulose. Isolated wheat starch plus cellulose was included as a reference. The pea flour was obtained by grinding the split pea seeds in a Retsch mill (Model ZM 200, Haan, Germany) using a ring sieve with an aperture size of 0.25 mm.

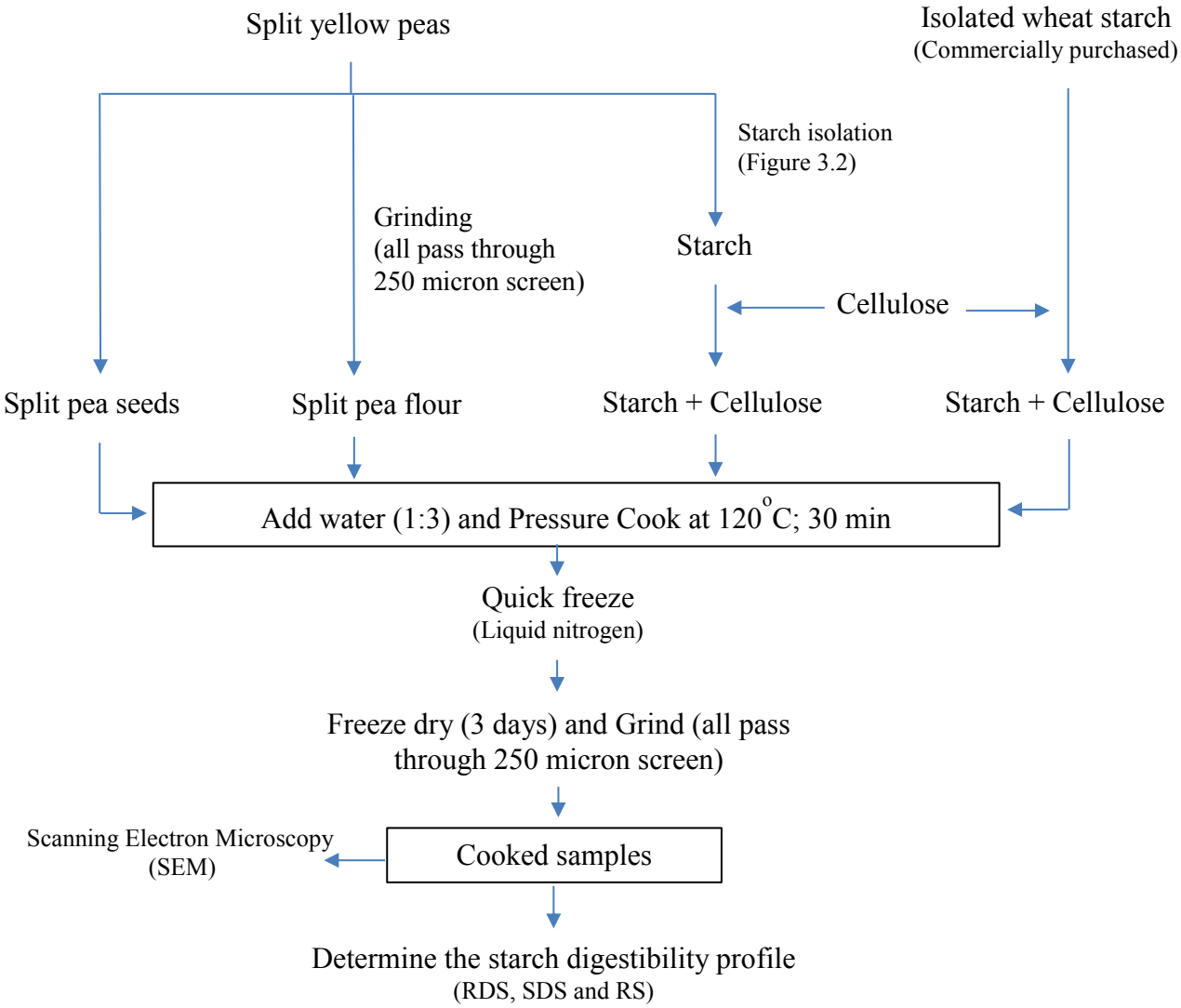


Figure 3.1 Experimental design

Yellow pea starch was isolated from flour according to the protocol presented in Figure 3.2 as follows: Yellow pea flour was mixed with distilled water (1:5, w/v) in a beaker and stirred for 60 min. The slurry was sieved through a 75 μm screen and centrifuged (1000 *g* for 15 min). The supernatant was discarded and the residue was washed twice with a solution of 0.5% NaCl (1:4, w/v), stirred for 30 min and centrifuged (1000 *g* for 15 min). Then, the residue was washed four times with water (1:10, w/v), stirred for 10 min and centrifuged (1000 *g* for 15 min). The residue obtained was further washed four times with 50% ethanol (1:5, w/v), stirred for 10 min, centrifuged (1000 *g* for 15 min) and dried at room temperature (20^{o}C). The total starch content of pea flour, pea starch, and wheat starch was determined for further calculations. The starch contents of the isolated yellow pea starch (96.3% db) and the commercially obtained wheat starch (97.3% db) were much higher than the starch content of the ground yellow pea seeds (47.9% db). Therefore, an inert filler (microcrystalline cellulose) was used in order to adjust the starch content in pea and wheat starches to the same starch concentration found in pea seeds, for comparison purposes. Microcrystalline cellulose was selected because its melting temperature (260-270°C) is well above the cooking temperature used in this study (120°C) and it is not hydrolysed by digestive enzymes.

Yellow split pea seeds, pea flour and the mixtures of pea and wheat starches with cellulose were pressure cooked (120°C, 30 min, 15 psi) with water (1:3, w/v). In order to avoid the effect of pea starch retrogradation on *in vitro* digestibility, all samples along with the cooking water were immediately frozen with liquid nitrogen and then freeze dried, ground and packed air-tight for further analysis. All samples were prepared in duplicate.

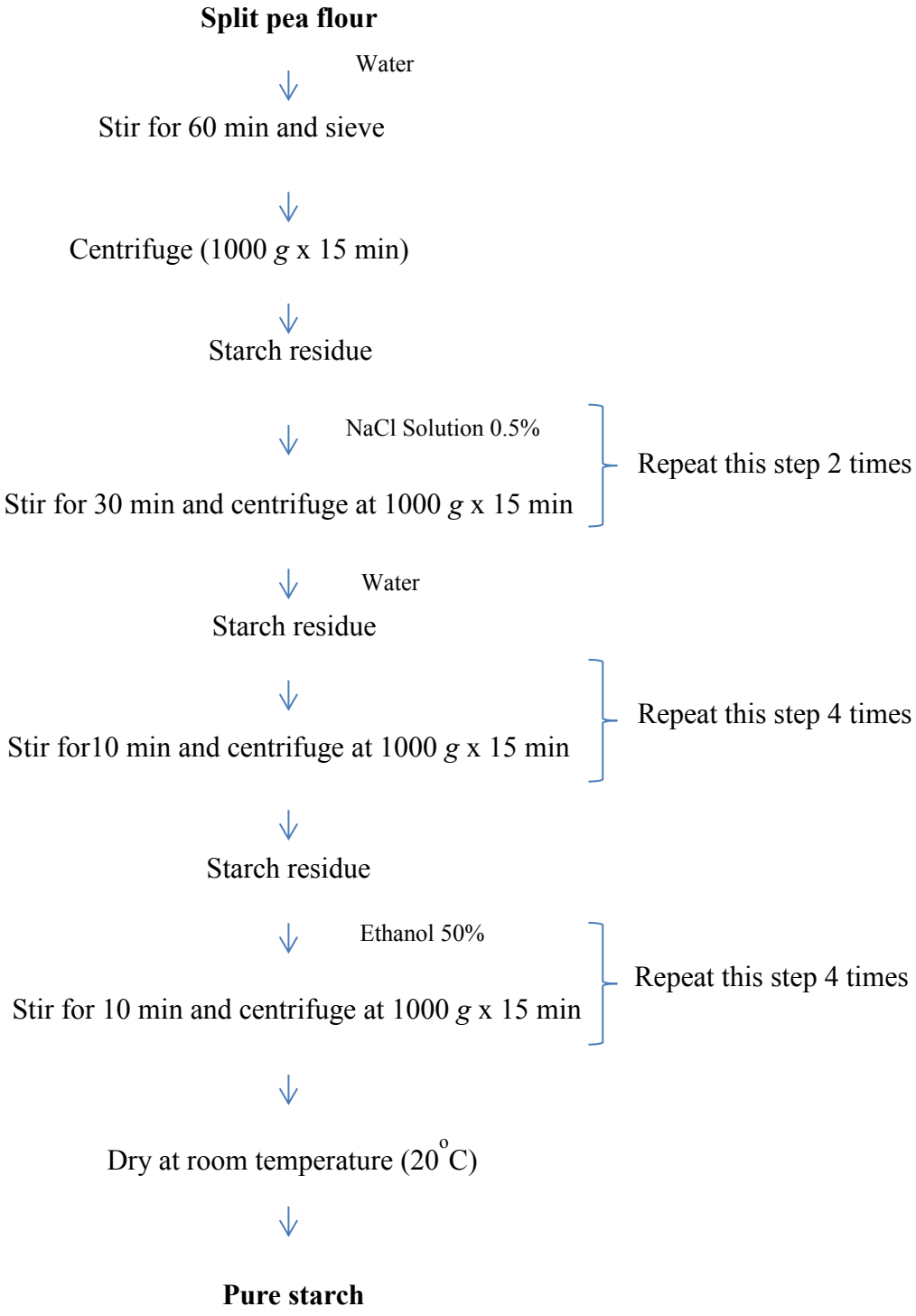


Figure 3.2 The protocol for yellow pea starch extraction

3.2.4 Determination of RDS, SDS and RS contents

The starch digestibility profile of cooked samples was determined according to Englyst et al. (1992) with some modifications. Briefly, ~100 mg of each sample (pressure cooked/freeze dried/ground split pea seeds, pea flour, pea starch + cellulose and wheat starch + cellulose) were weighed, vortexed with 4 mL of pancreatic alpha-amylase (30 U/mL) containing 3 U/mL of amyloglucosidase (AMG), and then incubated for 20 min (RDS) or 120 min (SDS) at 37°C with continuous shaking (200 strokes/min). Ethanol (99% v/v; 4.0 mL) was added and vigorously stirred in a vortex. One mL of sample was withdrawn in an Eppendorf tube and centrifuged (1500g for10 min).

The total soluble starch/sugar in the supernatant was determined by using the Megazyme method as described below. The supernatant/solution (0.5 mL) was incubated with 6 mL of 100 mM sodium acetate buffer (pH 4.5) and 0.1 mL of amyloglucosidase solution for 30 min at 50°C. The glucose content of the solution was then determined by adding 10 μL of sample to a corning 96-well microplate, followed by 150 μL of glucose oxidase/peroxidase (GOPOD) reagent and continued incubation for 20 min at 50°C. The absorbance of the samples was measured at 510 nm against a blank and three glucose standards, upon the development of pink color. The starch analysis controls contained the same sample mixture and reagents without the enzymes. The glucose content of the samples, including the starch analysis controls was calculated according to instructions provided in the Megazyme total starch determination method. RDS and SDS contents were determined by subtracting the glucose content of the experiment control from that of the samples. The glucose released in the first 20 min represented the RDS fraction. The SDS fraction was calculated by subtracting the RDS fraction from the

value obtained after 2 h of digestion. The RS fraction was calculated by difference as 100 - (RDS+SDS). All samples were analysed in triplicate.

3.2.5 Differential scanning calorimetry (DSC)

The thermal characteristics (gelatinization transition temperatures and the enthalpy of gelatinization) of yellow pea starch in split pea seeds, flour and a mixture of starch+cellulose were determined by using a differential scanning calorimeter DSC Q100 (TA Instruments-Waters, New Castle, DE, USA). A mixture of wheat starch + cellulose also was included as a reference in this experiment. Cellulose was added in order to equal the starch contents of purified starches to that of pea seeds and flour (47.9%). The sample (one piece of a split pea seed, flour and mixtures of pea or wheat starch plus cellulose) was weighed into a stainless steel DSC pan, which was then hermetically sealed and equilibrated at room temperature (20^{o}C) overnight before loading into the DSC cell. The sample to water ratio was 1: 3. The addition of sample into the stainless steel DSC pan was based on calculations in order to have at least 3.0 mg of starch. Indium was used as a calibration standard, and a sealed empty stainless steel pan was used as a reference. Each sample was heated from 20 to 150°C at 5°C/min. The gelatinization temperature parameters (onset, T_o; peak, T_p; and conclusion, T_c) and transition enthalpy (ΔH) were calculated with thermal analysis software (version 4.5A, Universal Analysis 2000, TA Instruments-Waters, New Castle, USA). Gelatinization temperature range ($T_c - T_o$) was calculated as the temperature difference between T_c and T_o. All samples were analysed in duplicate.

3.2.6 Scanning electron microscopy (SEM)

Morphological characterization of samples was carried out by scanning electron microscopy (SEM). Samples were mounted on circular aluminum stubs with double-sided sticky

tape and then coated with 12 nm of carbon, examined at 200x, 500x or 1000x magnification, and photographed in a scanning electron microscope (Zeiss Sigma 300 FESEM) at an accelerating voltage of 5 kV.

3.2.7 Statistical analysis

One-way analysis of variance (one way ANOVA) was performed using the General Linear Model (GLM) procedure of SAS Statistical Software (SAS Institute Inc., Cary, NC, USA, 2012). The difference among means was determined using Tukey's multiple comparison test ($P<0.05$). Treatments shown in Figure 3.1 were performed in duplicates.

3.3 RESULTS AND DISCUSSION

3.3.1 Proximate composition of split yellow pea seeds, starch and wheat starch

The chemical composition of split yellow pea seeds (ground flour), purified pea starch and commercial wheat starch is shown in Table 3.1. The starch content of split pea seeds was ~ 48.0%. The purity of pea and wheat starches was 96.3 and 97.3%, respectively. The protein content of split pea seeds was ~25.0%. Purified pea and wheat starches had traces of protein, 0.12 and 0.23%, respectively. The lipid content of split pea seeds (2.9%) was higher than those of pea starch (0.61%) and wheat starch (0.52%). The fiber contents in split pea seeds, pea starch and wheat starch were 21.5%, 1.0% and 0.5%, respectively. Trace amounts of phosphorous and phytates were present among samples. Pea starch had higher total amylose content (42.5%) compared to wheat starch (25.5%).

Table 3.1 Proximate composition (% dry basis) of the raw materials

Component (%)	Split yellow pea seed	Yellow pea starch	Wheat starch
Starch	47.9±0.89	96.3±1.1	97.3 ± 1.21
Protein	24.7±0.13	0.12±0.0	0.23±0.04
Lipid	2.9±0.04	0.61±0.03	0.52±0.04
Fiber	21.5±0.65	1.0±0.3	0.5 ± 0.01
Phosphorous	0.5±0.04	0.007±0.0	0.06±0.02
Phytates	0.88±0.01	0.22±0.1	0.39±0.01

The values represent the mean ± standard deviation of triplicates.

3.3.2 *In vitro* starch digestibility profile of pea seeds, flour, and starches

The *in vitro* starch digestibility profile (RDS, SDS and RS contents) of pressure cooked split pea seeds and flour, as well as isolated pea and wheat starches, are presented in Table 3.2. The RDS content of cooked split pea seeds (80.3%) was in agreement with Ring et al. (1988), and was significantly lower than that of the flour sample (84.1%). In addition, the RDS contents of both split pea seeds (80.3%) and flour (84.1%) samples were significantly lower than those of isolated cooked starches (pea, 88.3% ~ wheat, 88.6%). No significant difference in RDS content was observed between isolated and cooked pea (88.3%) and wheat starches (88.6%). Similarly, Faulks and Bailey (1990) found no significant difference in starch digestibility after 30 min of hydrolysis with porcine pancreatic α-amylase of smooth pea starch (90%) and wheat starch (90%), previously boiled for 2 h in excess water. The SDS contents of split pea seeds and flour were similar (12.2% and 13.2%, respectively), but significantly higher when compared to isolated starches (pea, 6.4% and wheat, 9.9%). The SDS contents of isolated and cooked pea and wheat starches were significantly different. The RS content (100 – [RDS+SDS]) was significantly higher in split pea seeds (7.5%) followed by pea starch isolate (5.3%), pea flour (2.7%) and wheat starch isolate (1.5%).

Table 3.2 Amylase digestibility profile of starch in yellow peas compared to that of isolated wheat starch processed according to Figure 3.1.

Sample	% RDS	% SDS	% RS
Pea – Split seed	80.3 ± 0.57 c	12.2 ± 0.16 a	7.5 ±0.02 a
Pea – Flour	84.1 ± 0.22 b	13.2 ± 0.22 a	2.7 ± 0.01c
Pea – Isolated starch + cellulose	88.3 ± 0.65 a	6.4 ± 0.65 c	5.3 ± 0.01 b
Wheat – isolated starch + cellulose	88.6 ± 0.02 a	9.9 ± 0.13 b	1.5 ± 0.01 d

Values are mean ± standard deviation of two replicates. The statistical analysis was performed by Tukey's HSD test and means in a column bearing the same letters are not significantly different ($p \geq 0.05$).

Grinding of split peas to fine particles before cooking resulted in significantly higher starch hydrolysis when compared to the cooked intact split pea seeds. The lower RDS content of cooked split seeds compared to flour could be due to the difference in physical form and surface area available for enzymatic digestibility. Milling of split pea seeds before cooking generates a mechanical disruption of the structure, increasing the surface area that enhances starch gelatinization and further enzyme hydrolysis. Similarly, *in vivo* studies on healthy subjects demonstrated that consumption of ground-cooked white and brown rice meals potentiated the increment of postprandial glucose and insulin responses compared to the whole-cooked counterparts (O'Dea et al. 1980). The data showed that cooked purified starches, regardless of the source, are digested similarly. In terms of crystallinity, it has been proposed that amylopectin double helixes in starch are uniquely arranged to form crystal structures named A, B or C types that differ with plant origin. A and B types differ in the geometry and packing density, as well as the amount of bound water within the crystals. The crystalline structures of cereal and tuber starches have been found to be A-type and B-type, respectively. Pulse starches exhibit a characteristic C-type diffraction pattern, which is a mixture of both A and B types in varying amounts (Davydova et al. 1995; Gernat et al. 1990; Hoover and Sosulski 1985). Most of the studies to date comparing digestibility of native starches, have reported C-type to be digested more slowly than A-type and B-type starches. The reduced bioavailability of native pulse

starches is attributed in part to the presence of B-type crystals within the granules that have been related to a higher resistance to amylolysis (Ring et al. 1988; Ratnayake et al. 2001). Indeed, research has suggested that the low digestibility linked to raw B-type starches is due to a greater crystalline structure content (Gallant et al. 1992). However, processes that involve high temperature and excess water, i.e. gelatinization, eliminate starch crystallinity and increase availability of starch molecules to enzyme digestion (Asp and Björck 1992; Björck et al. 1984a; 1984b). Thus, B-type starches (potato) that are highly resistant to amylolysis in native form, exhibit a lower gelatinization point and higher digestibility after cooking. Therefore, although it has been hypothesized that the low digestibility of native pea starches is linked to their C-type crystallinity, it is not applicable to gelatinized starches.

3.3.3 Scanning electron microscopy (SEM)

SEM micrographs of cooked split pea seeds (ground), pea flour and isolated starches are presented in Figure 3.3. Cooked split pea seeds showed starch particles that remained intact for the most part with a high integrity of the cotyledon tissue structure (Figure 3.3 A-B). In contrast, images of cooked flour exhibited a highly opened/disrupted structure of the tissue and components (Figure 3.3 C-D). Isolated/purified and cooked pea and wheat starches showed complete damage of the native granule structure (Figure 3.3 E-F, G-H, respectively). The restricted availability of starch in cooked split pea seeds is attributed to a possible protection of the starch granules by protein matrix and the cotyledon cell walls. This finding is in agreement with previous reports (Tovar et al. 1990; Tovar et al. 1991) where authors described similar microscopic observations for red kidney beans and lentils, cooked in boiling water for 70 min, at a seed to water ratio of 1:3 (w/v), freeze dried and milled. In general, these microscopic observations support the findings related to the lower RDS content of cooked split pea seeds

(80.3%) compared to cooked pea flour (84.1%) and cooked isolated pea (88.3%) and wheat (88.6%) starches (Table 3.2).

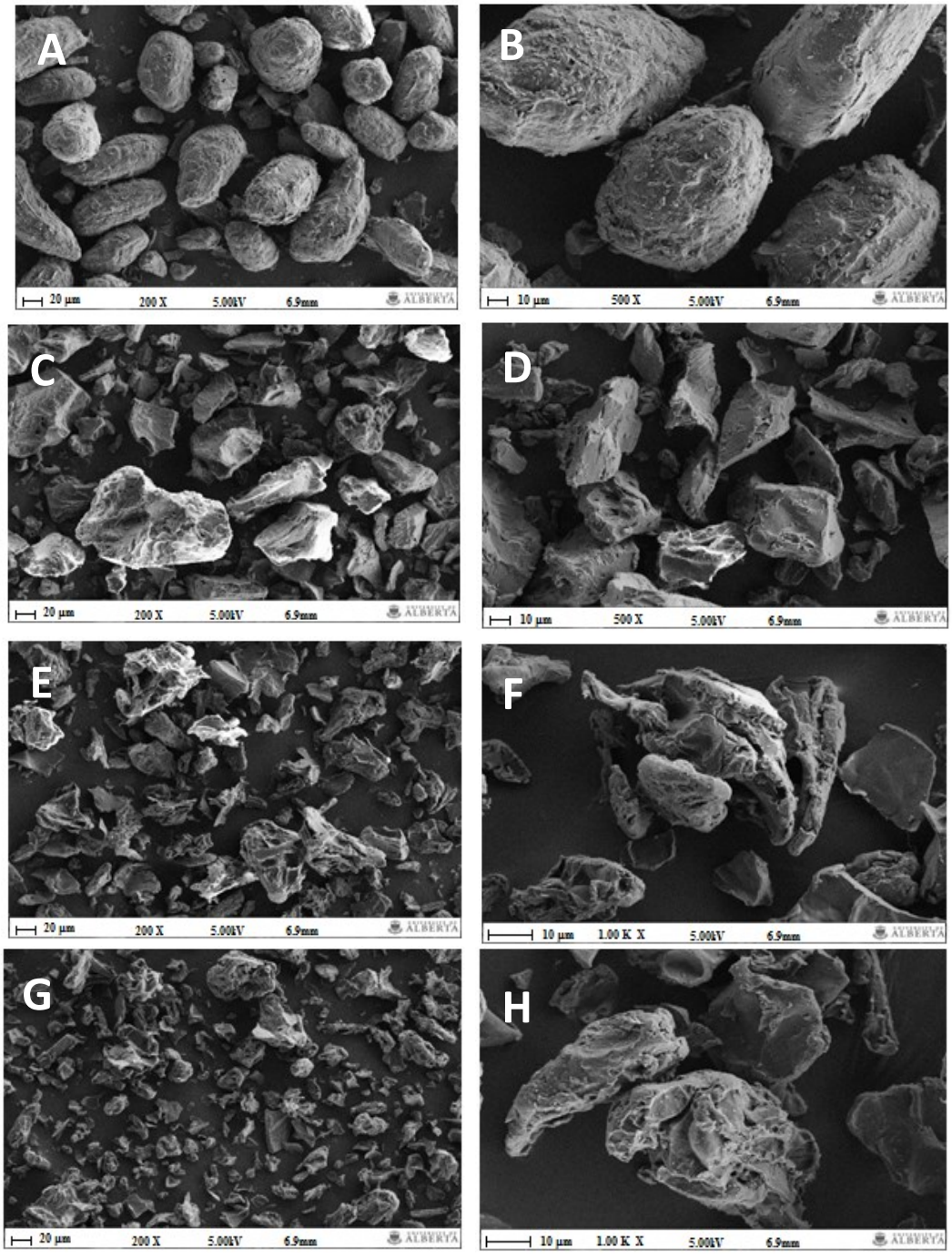


Figure 3. 3 Scanning electron micrographs of pressure cooked, freeze dried and ground samples of split pea (A & B), pea flour (C & D), pea starch + cellulose (E & F) and wheat starch + cellulose (G & H).

3.3.4 Thermal properties

The gelatinization temperatures (onset, T_o; peak, T_p; and conclusion, T_c), gelatinization temperature range ($T_c - T_o$), and gelatinization enthalpy (ΔH) of native starch in yellow split pea seeds and flour, as well as those of isolated pea and wheat starches, are presented in Table 3.3 and Figure 3.4. The endotherms for the split seed samples were considerably different compared to that of the flour and isolated pea or wheat starches. In other words, two distinct endothermic peaks were observed in the split pea seed sample as opposed to a single peak in the other samples (Figure 3.4). This behaviour could be explained due to the presence of intact cell walls in the split seed compared to the flour that act as a barrier and protect starch granules toward hydration and gelatinization. The first endotherm peak could be attributed to the disorganization of starch crystallites in starch granules that were more easily accessible and closer to the surface of the grain, whereas the second peak could be explained as the gelatinization of the starch granules that were more protected or located in interior of the particle.

Table 3.3 Thermal characteristics of yellow pea starches in split pea seed, flour, and isolated starch compared to that of isolated wheat starch.

Sample	Peak #	T_o (°C)	T_p (°C)	T_c (°C)	$T_c - T_o$	ΔH (J/g)
Split pea seed (1 small piece)	1	63.4 ± 0.8a	70.3 ± 0.1a	76.2 ± 0.8a	12.7 ± 1.5b	4.2 ± 0.0d
	2	79.7 ± 0.3	88.9 ± 1.3	97.7 ± 2.8	18.0 ± 2.4	2.5 ± 0.0
Pea flour	1	60.2 ± 0.8b	67.9 ± 0.6a	76.6 ± 0.0a	16.4 ± 0.7ab	10.8 ± 0.3c
Pea starch + cellulose	1	56.8 ± 0.0c	63.6 ± 1.0b	74.4 ± 1.6 a	17.5 ± 1.6a	17.9 ± 1.1a
Wheat starch + cellulose	1	56.1 ± 0.3c	61.9 ± 0.1b	69.6 ±0.0 b	13.5 ± 0.3ab	13.9 ± 0.1b

Values are mean ± standard deviation of two replicates. The statistical analysis was performed by Tukey's HSD test and means in a column bearing the same letters are not significantly different ($p \geq 0.05$). T_o = onset temperature; T_p = peak temperature; T_c = conclusion temperature; $\mathbf{T_c - T_o}$ = gelatinization temperature range and ΔH = transition enthalpy

Split pea seeds showed the highest onset temperature (63.4°C) compared to all the samples. The onset temperature for pea flour (60.2°C) was significantly lower than that of split pea seed (63.4°C) but significantly higher than the isolated pea (56.8°C) and wheat (56.1°C) starches.

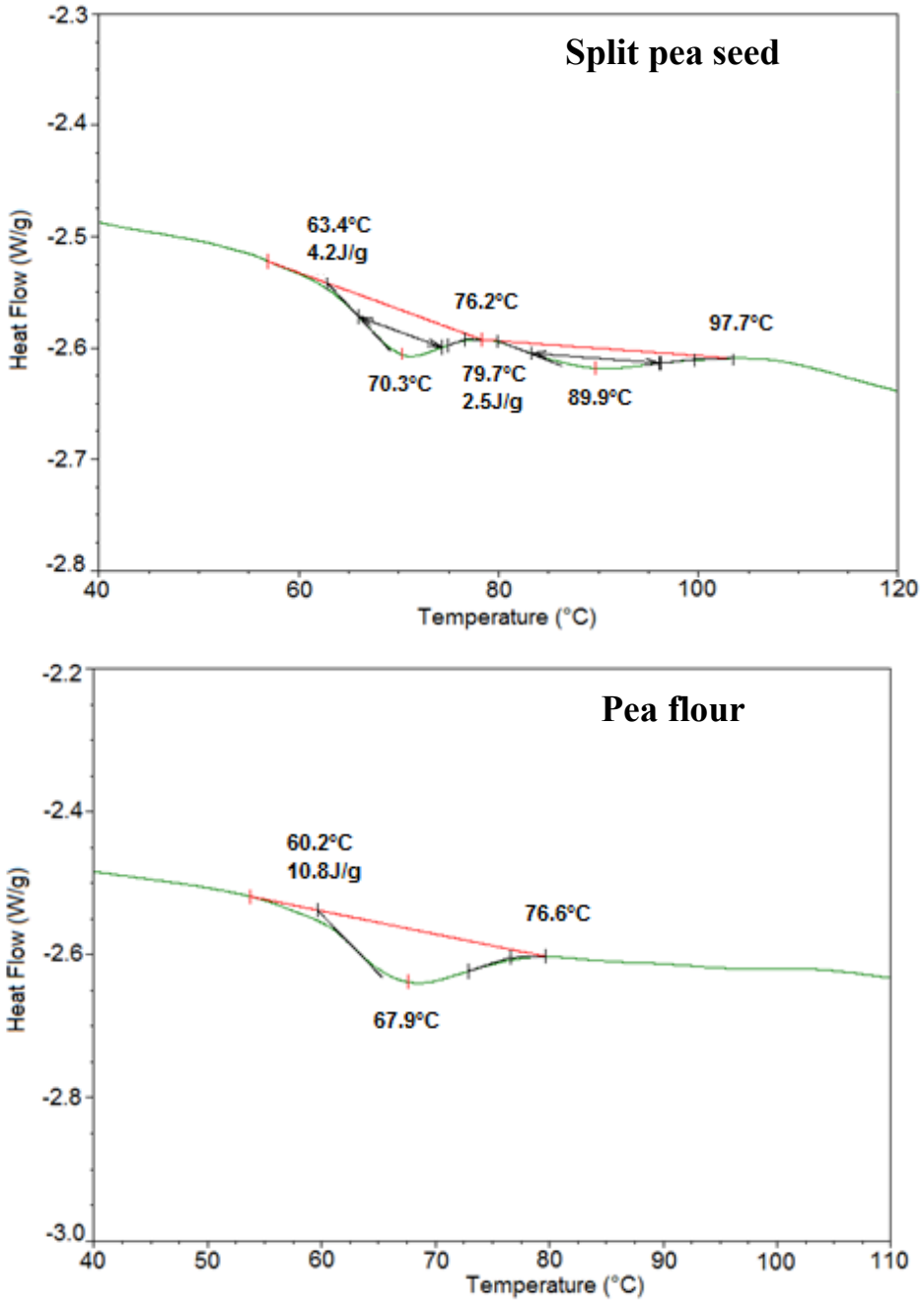
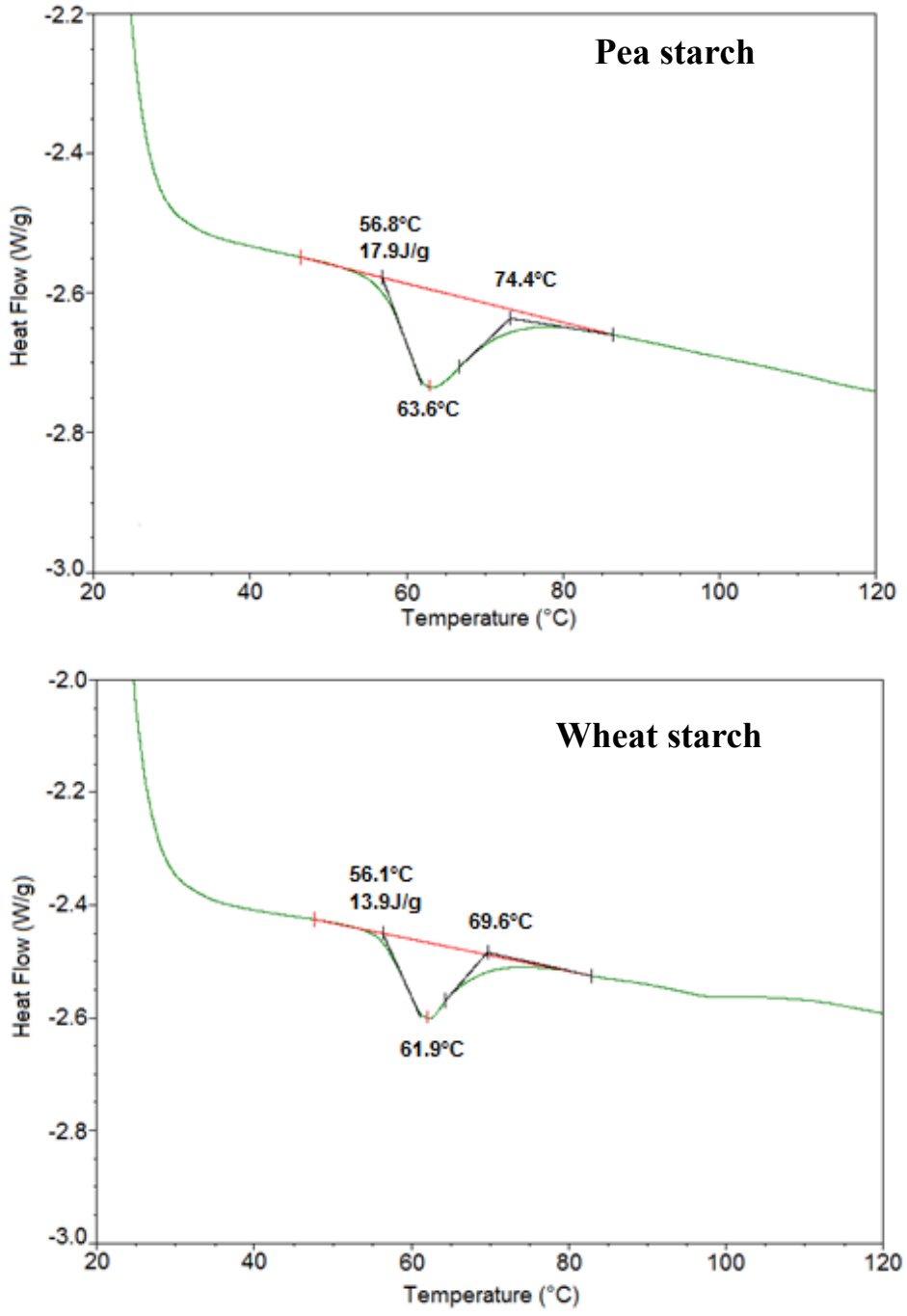


Figure 3.4 DSC thermograms of split pea seeds, pea flour, pea starch and wheat starch.

Split pea seed (70.3°C) and pea flour (67.9°C) exhibited higher peak temperatures of gelatinization compared to the other samples. There was no significant difference in the onset and peak temperatures between isolated pea (56.8°C, 63.6°C) and wheat starches (56.1°C, 61.9°C).



Cont´ Figure 3.4 DSC thermograms of split pea seeds, pea flour, pea starch and wheat starch.

Conclusion temperatures of split pea seed (76.2°C), pea flour (76.6°C) and isolated pea starch (74.4°C) did not show significant differences and were significantly higher compared to that of isolated wheat starch (69.6°C). In general, gelatinization in split pea seed and flour samples occurred at higher temperatures compared to isolated pea and wheat starches. This means that starch granules in split pea seed and flour were more resistant to hydrate, swell and gelatinize. Higher protein content in these samples, compared to isolated starches could have an influence through a physical competition for water in protein gelation against starch gelatinization. Polar amino acids present in pea seeds and flour, such as asparagine, glutamine, lysine, arginine, serine and threonine, have an affinity for water molecules, increasing the absorption and retention capacity. Furthermore, the greater amount of protein in split pea seed and flour samples could potentiate protein-starch interactions, which could decrease starch swelling (Liu et al. 2007).

The gelatinization enthalpies for split seeds and flour samples were significantly lower (4.2, and 10.8 J/g) compared to the isolated starches (17.9 and 13.9 J/g), which could be explained as a net thermal effect that occurs in pea seeds and flour when two opposite processes happen simultaneously. First, there is an endothermic reaction at onset temperature (starch melting), and then at the same time there is an exothermic reaction (crystallization of amylose-lipid complexes) (Biliaderis et al. 1986). The significantly higher enthalpies of gelatinization of pea and wheat starches could be related to the higher crystallinity of the samples (Elgadir et al. 2009).

3.4 CONCLUSION

The RDS contents of purified pea and wheat starches, after pressure cooking, showing no significant difference indicated that differences in the native starch crystal polymorphs (pea, C-

type and wheat, A-type) had no relevance to starch digestibility immediately after gelatinization (i.e. crystal melting). The observed two endothermic peaks (DSC) and the intact starch granules (SEM) in the pressure cooked split pea seeds may be responsible for their lowest RDS and highest SDS and RS contents. This suggested that particle size, cooking temperature and time may be used as the means to manipulate the RDS content (i.e. GI) of cooked whole pulses. The RDS content of pressure cooked split whole seed flour, being significantly lower than that of similarly processed purified pea starch, suggested the possible influence of non-starch grain components, possibly the protein, on starch digestibility. Further research is warranted to study the mechanisms by which protein influences *in vitro* starch digestibility in pulses.

CHAPTER 4. PLANT PROTEINS MITIGATE *IN VITRO* WHEAT STARCH DIGESTIBILITY[2]

4.1 INTRODUCTION

Refined wheat flour, or "white" flour, is a staple food ingredient around the world. The rapidly digestible nature of cooked wheat starch negatively impacts human health, because it can decrease glucose tolerance leading to obesity and other complications. Consumption of foods low in rapidly digestible starch (RDS) and high in slowly digestible starch (SDS) and resistant starch (RS) has many human health benefits (Barros et al. 2012; Dartois et al. 2010; Shi et al. 2014). SDS and RS are characterized by lowering the glycemic index (GI) after food intake. GI is a clinical measurement of the change in blood glucose concentration in response to consuming digestible carbohydrates (Jenkins et al. 2002). Foods high in rapidly digestible starch (RDS) content show a higher glycemic index and increase the glucose and insulin levels after consumption. Foods with a high content of SDS are digested gradually but completely in the small intestine between 20 and 120 minutes or longer, and thus stabilize the blood glucose level (Englyst and Hudson 1996). RS is not hydrolysed by α-amylase and amyloglucosidase enzymes in the small intestine, and enters the large intestine where it is fermented by colonic microflora producing short chain fatty acids (acetate, propionate and butyrate), reducing colonic pH, glucose, and cholesterol blood level (Sajilata et al. 2006), and improving hindgut immunity.

Starch digestibility is affected by extrinsic factors and mechanisms of resistance to amylolysis, based on which starch has been classified into five categories (Sajilata et al. 2006): a) RS1, physically entrapped by tissue structures and cell components such as protein; b) RS2, highly associated crystalline structures of native starch, especially those formed between the

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shorter branches of amylopectin; c) RS3, highly associated crystalline structures of native starch, especially those formed between amylose molecules; d) RS4, transglycosidated and chemically substituted or cross-linked starch; and e) RS5, V-amylose crystalline units formed by the tight association of amylose-lipid complexes. Besides the above mentioned factors, the presence of other components such as protein has been found to play an important role in the mitigation of starch digestibility and the subsequent glycemic response. Clinical studies on healthy and diabetic subjects have revealed that proteins from pulses, cereal grains and their hydrolysates may be able to reduce blood glucose concentration and enhance insulin response. Studies on healthy subjects concluded that consumption of products without gluten may result in elevated blood glucose level (Jenkins et al. 1987). Likewise, one study demonstrated that the ingestion of a tomato soup containing isolated yellow pea protein (20 g) before a pizza meal can decrease the blood glucose level to 5.95 mM compared with 6.23 mM for the control group (Smith et al. 2012). In another study, the consumption of carbohydrates and wheat protein hydrolysates combined with a mixture of amino acids (leucine and phenylalanine) could stimulate the production and activity of insulin to a greater extent than carbohydrates alone (Van Loon et al. 2000). Protein hydrolysates may be more potent than intact protein in inhibiting starch digestion, as protein hydrolysates from pea, wheat, rice and soybean generate faster hormonal responses of insulin and glucagon in healthy participants than with the intact protein alone (Claessens et al. 2009). The insulin response in plasma appears to be related to the amino acid content from protein hydrolysates, especially leucine, isoleucine, phenylalanine, valine, and arginine (Calbet and MacLean 2002).

In accordance with the findings on the beneficial effects of plant proteins *in vivo*, *in vitro* studies on cereal grains (wheat, corn, kodo millet, sorghum) and oil seeds (soy) have also shown that starch digestibility may be affected by the presence of protein (Berti et al. 2004; Colonna et

al. 1990; Guerrieri et al. 1997; Jenkins et al. 1987; Rooney and Pflugfelder 1986; Ryan and Brewer 2007; Singh et al. 2010). Proteins from wheat are likely to form a resilient gluten network or sheet-like structures that entrap starch (RS1), reducing the access of digestive enzymes (Fleming 1978; Venugopal 2011). Furthermore, protein hydrolysates obtained from enzymatic hydrolysis may interact with starch (Lian et al. 2013).

Celiac disease due to intolerance to gluten affects millions of people worldwide, with the food industry responding by the rapid development of gluten-free food products. However, gluten-free products made with mixtures of potato, tapioca and rice starches enhance starch digestion (Berti et al. 2004) due to the absence of proteins. This triggers our research interest in studying the potential application of employing exogenous proteins from plants to slow the rate of starch digestion in gluten-free foods. Since current *in vitro* studies have been largely focused on starch-protein interactions that occur naturally in plants (Hesso et al. 2015; Jamilah et al. 2009), the effects of exogenous proteins and their hydrolysates on starch digestion via different methods of cooking remain unclear. The objective of this study was to investigate the effect of isolated plant proteins from wheat, corn, soybean, pea and rice in their native, heat-denatured and enzymatically hydrolysed states on the susceptibility of wheat starch to *in vitro* hydrolysis by porcine pancreatic α-amylase.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Protein isolates (corn and soy) and concentrates (wheat, pea and rice) as well as purified wheat starch were obtained from Agridient Inc (Farmington Hills, MI, USA). Analytical kits for the determination of total starch, β-glucan, phytates and starch digestibility were purchased from Megazyme (Megazyme International Ireland, Wicklow, Ireland). Protease from *Aspergillus*

oryzae (*p*=1.27g/mL) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution were obtained from Sigma-Aldrich (Oakville, ON, Canada). Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Mississauga, ON, Canada). All other chemicals and solvents were of ACS certified grade. Samples were cooked in a water bath (Model BS-11, Jeio Tech Inc., Korea) and in a pressure cooker (Fresco, Model PC55A/PC90A). Samples were dried in a freeze-drier (VirTis model 50-SRC, Gardiner, NY, USA). Centrifugation was carried out using Accuspin 400 (Fisher Scientific, Pittsburgh, PA, USA) and Beckman J2-21 (Beckman Instruments Inc., Palo Alto, CA, USA) centrifuge.

4.2.2 Compositional analysis

Protein content was determined by combustion with a nitrogen analyzer (Model FP-428, Leco Corp., St. Joseph, MI, USA), and estimated by multiplying the determined nitrogen content by a nitrogen-to-protein conversion factor (6.25). Total starch and β-glucan were determined with kits from Megazyme according to AOAC Methods 996.11 (AOAC International, 2005) and 995.16 (AOAC International 2000), respectively. Phytates were determined according to the K-PHYT method using a kit from Megazyme. Lipids were extracted overnight in hexane at room temperature (20°C), followed by gravimetric analysis. Phosphorus was determined using the molybdenum blue method (Whistler et al. 1964). Total phenolics were evaluated according to the Folin-Ciocalteu spectrophotometric method and moisture content was determined by approved method 44-15.02 of AACC International (2010).

4.2.3 Preparation of wheat starch and protein mixtures

The process of preparation of wheat starch and protein mixtures is illustrated in Figure 4.1. Wheat starch and protein blends consisted of starch (70%) and protein (12%) at a fixed

proportion by weight. These percentages were set in order to resemble the typical composition of bread-making flour (Goesaert et al. 2005; Shewry 2009).

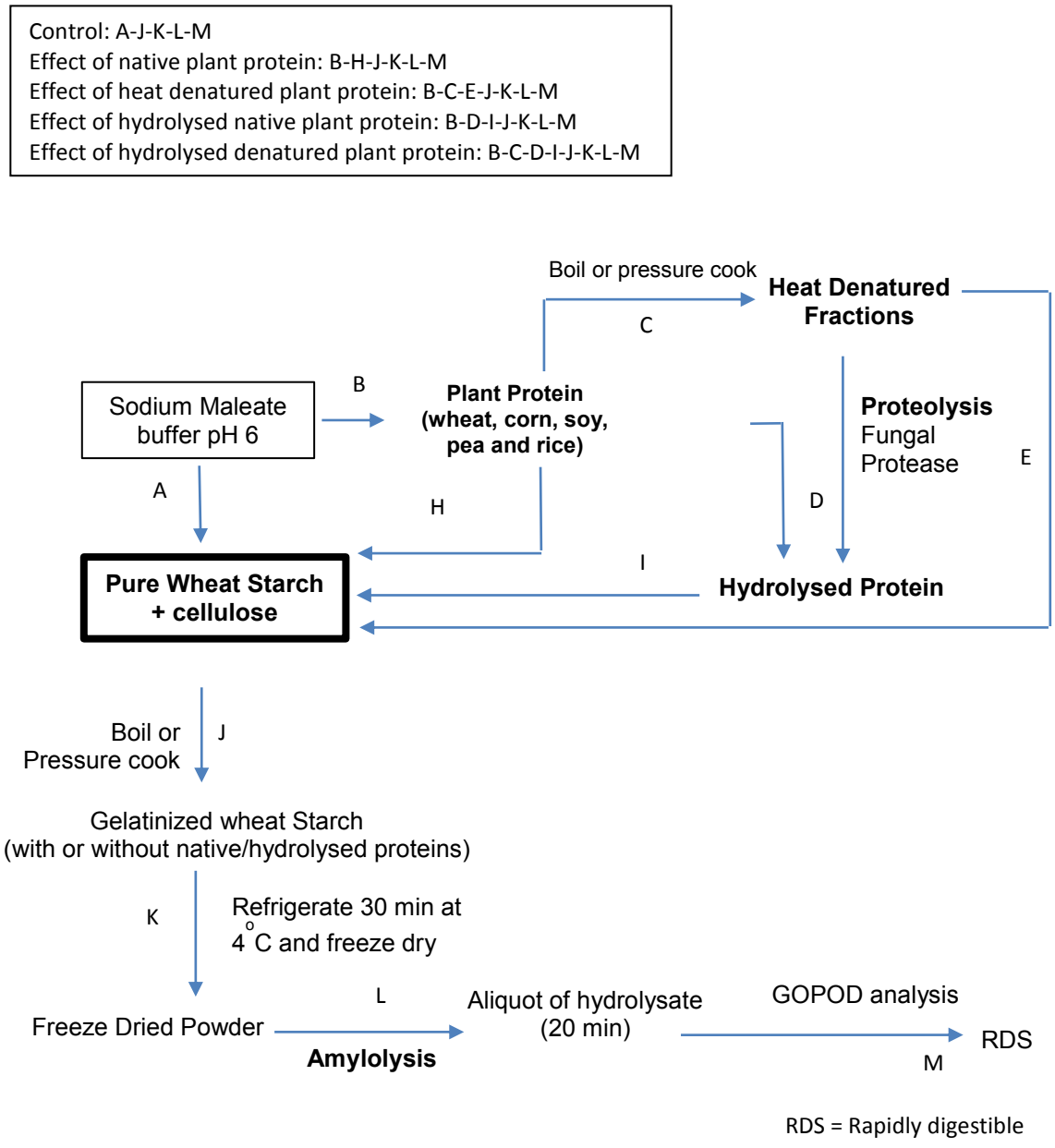

Figure 4.1 The flow chart of the study investigating the effects of purified plant protein on the RDS content of wheat starch.

Since the purity of the protein isolates/concentrates used in this study was not 100%, an inert filler (microcrystalline cellulose) was used to precisely adjust the starch and protein concentrations to 70% and 12%, respectively, in all of the blends. Microcrystalline cellulose was selected because it is very unlikely to interact/bind with any of the components present in the mixtures and also it is not hydrolysed by the digestive enzymes. In addition, its melting temperature (260-270^{o}C) is well above the cooking temperatures used in this study.

A mixture of native wheat starch and cellulose was used as the control (starch concentration 70%, dry basis). Purified plant proteins in four different forms: 1) native, 2) denatured, 3) hydrolysed, and 4) denatured-hydrolysed were mixed and cooked with purified wheat starch containing cellulose (WSC). Commercial protein isolates or concentrates without any further modification are referred throughout this chapter as "native protein". However, the real native nature of these proteins may have been affected during commercial isolation processing from seeds/grains. Specifically, each native protein was mixed with WSC in 15 mL sodium maleate buffer (pH 6). Protein denaturation was performed by dispersing each purified plant protein in sodium maleate buffer, followed by boiling (95ºC, 30 min) or pressure cooking (120ºC, 30 min, 15 psi). Protein hydrolysis was carried out using a fungal protease from *Aspergillus oryzae* (4% net protein basis) for 120 min at 50ºC. Denatured-hydrolysed proteins were obtained by following the same denaturation and hydrolysis conditions as mentioned above. Plant proteins in their four different forms were then mixed with WSC. Each "starch-protein-cellulose" mixture was vortexed and cooked by boiling or pressure cooking using the same conditions as mentioned above. After cooking, the mixture was cooled, freeze-dried and packaged air-tight for further analysis.

4.2.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Pea and soybean proteins were dispersed in SDS-PAGE running buffer (250 mM Tris base, 1920 mM Glycine and 0.1% SDS) at 2 mg/mL, then diluted with a mixture of 10% β-mercaptoethanol (prepared in 2×Laemmli sample buffer) at a ratio of 1:1 (v/v). The samples were then heated at 95°C for 5 min in an Eppendorf thermomixer dry block heating and cooling shaker (Eppendorf Canada, Mississauga, ON), and cooled down to room temperature prior to SDS-PAGE analysis. Rice protein was dispersed in SDS-PAGE running buffer (250 mM Tris base, 1920 mM Glycine and 0.1% SDS) at 50 mg/mL, then diluted with a mixture of 10% β-mercaptoethonal and 8 M urea (prepared in 2×Laemmli sample buffer) at a ratio of 1:1 (v/v). The samples were heated at 95°C for 5 min in an Eppendorf thermomixer dry block heating and cooling shaker (Eppendorf Canada, Mississauga, ON), and centrifuged with a mini centrifuge (Fisher Scientific, Ottawa, ON, Canada) at 13,000 rpm for 15 min. Supernatants (15 μL for pea and soybean, 20 μL for rice) were then loaded on a Tris-HCL 4-20% gradient gel (Bio-Rad, Ontario, Canada), run at 150 V for approximately 35 min in a Mini-Protean II electrophoresis cell (Bio-Rad, Ontario, Canada). The gel was stained with Coomassie brilliant blue for 1 h, followed by de-staining with 30% methanol and 10% acetic acid. Images of gel were analyzed with AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA, USA).

4.2.5 Degree of hydrolysis (DH)

The DH of five plant proteins (wheat, corn, rice, pea and soybean) was determined according to the Adler-Nissen (1979) method. Protein acidic hydrolysis was performed according to the Simpson method (Simpson et al. 1976) with some modifications. From each original protein, 0.5 mg were mixed with 200 μL of 4 M methanesulfonic acid in a glass sample tube (6 mm × 50 mm). The glass sample tubes were then placed inside the reaction vial and connected to

the Pico-Tag station (Eldex Laboratories, Napa, CA, USA). The hydrolysis was performed at 115°C for 24 h. Samples were prepared in duplicates. The reaction mixture was then neutralized by adding 200 µL of 4 M NaOH and kept at 4°C until use.

4.2.6 Determination of RDS content

RDS content was determined according to the Englyst et al. (1992) method with modifications. Approximately 100 mg of each sample mixture (starch + protein + cellulose) was weighed, vortexed with 4 mL of pancreatic α-amylase (10 mg/mL) containing 3 U/mL of amyloglucosidase (AMG), and then incubated for 20 min at 37°C with continuous shaking. Ethanol (99% v/v; 4.0 mL) was then added, vortexed and centrifuged (1500 *g* for 10 min). The supernatant was collected. The precipitate was washed twice with 8 mL of 50% ethanol. The supernatant obtained from incubation with pancreatic α-amylase/amyloglucosidase and the subsequent washings were adjusted to 100 mL with 100 mM sodium acetate buffer (pH 4.5). The total soluble starch/sugar in the supernatant was determined by the Megazyme method. The supernatant/solution (0.1 mL) was incubated with 10 µL of dilute AMG solution for 20 min at 50°C. The glucose content of the solution was then determined by adding 3.0 mL of glucose oxidase/peroxidase (GOPOD) reagent and incubation was continued for another 20 min at 50°C. The absorbance of the samples upon the development of a pink color was measured at 510 nm. The experimental control contained the same sample mixture and reagents without the enzymes. All the samples were prepared in triplicates. RDS content was determined by subtracting the glucose content of the control from that of the sample.

4.2.7 Differential scanning calorimetry (DSC)

The thermal properties of wheat starch in the presence of plant proteins were determined with a DSC Q100 (TA Instruments-Waters, New Castle, DE, USA). Each sample contained

purified plant protein in denatured or hydrolysed form, mixed with WSC, except for the control which contained only WSC. The final contents of starch and protein were 70% and 12%, respectively. The sample to water ratio was 1:3 w/v. The sample was weighed into a stainless steel DSC pan, which was then hermetically sealed and equilibrated at ambient temperature overnight before loading into the DSC cell. All samples were prepared in duplicate. Indium was used as a calibration standard, and a sealed empty stainless steel pan was used as a reference. Each sample was heated from 20 to 100°C at 5°C/min. The gelatinization temperature parameters (onset, T_o; peak, T_p; and conclusion, T_c) and endothermic enthalpy (ΔH) were calculated with thermal analysis software (version 4.5A, Universal Analysis 2000, TA Instruments-Waters, New Castle, USA).

4.2.8 Fluorescence labeling and confocal laser scanning microscopy (CLSM)

Fluorescence labeling of starch and phosphorus-associated molecules were performed using a double-staining technique (Li et al. 2014). In brief, 20-30 mg of each "starch-protein-cellulose" mixture were stained in 25 µL of freshly prepared APTS (20 mM 8-amino-1,3,6-pyrenetrisulfonic acid in 15% acetic acid) and 25 µL of 1 M sodium cyanoborohydride at 30°C for 15 h, followed by washing 5 times with deionized water and staining with 0.5 mL Pro-Q Diamond solution at room temperature for 1 h. After repeated washings (× 5) with deionized water, the stained "starch-protein-cellulose" mixture was suspended in 0.5 mL 50% glycerol, where 10 µL was taken and dropped into a glass-bottom culture dish (MatTek Corporation, Ashland, MA, USA). This 10 µL sample was then mixed with 0.1 mL deionized water, and visualized under a CLSM (Zeiss LSM 710, Carl Zeiss MicroImaging, Jena, Germany) equipped with a 40X 1.3 oil objective lens. The excitation wavelengths for APTS and Pro-Q Diamond

stains were at 488 and 561 nm, respectively, with an emission light interval of 490-560 nm. Images were obtained and analyzed with ZEN 2011 software (Carl Zeiss MicroImaging).

4.2.9 Statistical analysis

Treatments shown in figure 4.1 were prepared in duplicates. One-way analysis of variance (one way ANOVA) was performed using the General Linear Model (GLM) procedure of SAS Statistical Software (SAS Institute Inc., Cary, NC, USA, 2012). The difference among means was determined using Tukey's multiple comparison test ($P<0.05$).

4.3 RESULTS AND DISCUSSION

4.3.1 Proximate composition of purified wheat starch and plant proteins

Compositional analysis of purified wheat starch and plant proteins obtained from commercial sources is presented in Table 4.1. The purity of wheat starch was ~98%, while the purity of plant proteins ranged between 51-95%. Corn and soy proteins showed the highest purity (more than 90%) whereas rice protein possessed the lowest (50.99%). The lipid contents of pea (5.64%) and rice (5.14%) proteins were higher than those of wheat, corn and soy proteins. Soy protein showed the highest percentage of β-glucan (0.64%), while pea protein featured the highest amount of phosphorus (1.14%). There were minor differences in phytate and total phenolic contents among the proteins studied.

Table 4.1 Compositional analysis results of raw wheat starch and purified proteins from different plant sources (db) [1].

Parameter (%)	Starch	Proteins				
	Wheat	Wheat	Corn	Soy	Pea	Rice
Starch	97.93±1.36a	9.95±0.03c	0.63±0.03e	0±0.00e	3.48±0.07d	29.26±0.23b
Protein	2.93±0.02e	85.68±0.26b	94.76±0.08a	93.75±0.07a	82.49±0.04c	50.99±0.66d
Lipid	0.33±0.28b	1.89±0.11b	0.93±0.48b	0.45±0.34b	5.64±0.64a	5.14±0.31a
Beta-glucan	0.08±0.01b	0.11±0.00b	0.08±0.00b	0.64±0.01a	0.03±0.01c	0±0.00c
Phosphorous	0.06±0.02d	0.38±0.01c	1.05±0.01ab	1.09±0.04ab	1.14±0.04a	1.01±0.02b
Phytates	0.39±0.01c	0.48±0.01bc	0.64±0.01a	0.65±0.01a	0.61±0.08ab	0.64±0.04a
Total phenolics	0±0.00b	0.13±0.01ab	0.18±0.03a	0.11±0.07ab	0.14±0.00a	0.11±0.02ab

[1] Values are mean ± standard deviation of three replicates. The statistical analysis was performed by Tukey's HSD test and means in a row bearing the same letters are not significantly different ($p \geq 0.05$). Db= dry base.

4.3.2 SDS-PAGE analysis of selected plant proteins

SDS-PAGE analysis of purified plant proteins from pea, soybean and rice is presented in Figure 4.2. Proteins in four different forms (native, denatured, hydrolysed, and denatured and hydrolysed) were cooked by pressure cooking or boiling to resemble the conditions used in the RDS content test. In general, protein hydrolysates exhibited increased numbers of smaller molecular weight markers (10-25 kDa), and reduced numbers of larger molecular weight markers (50-150 kDa) compared to native proteins, indicating the release of smaller peptides after digestion with *Aspergillus Oryzae*. Protein denaturation by pressure cooking or boiling, prior to protein hydrolysis, generally improved the extent of hydrolysis. Specifically, boiled and hydrolysed soybean protein showed a 20 kDa molecular weight marker that was not present in its pressure-cooked counterpart, suggesting that the method of cooking may influence the extent of hydrolysis.

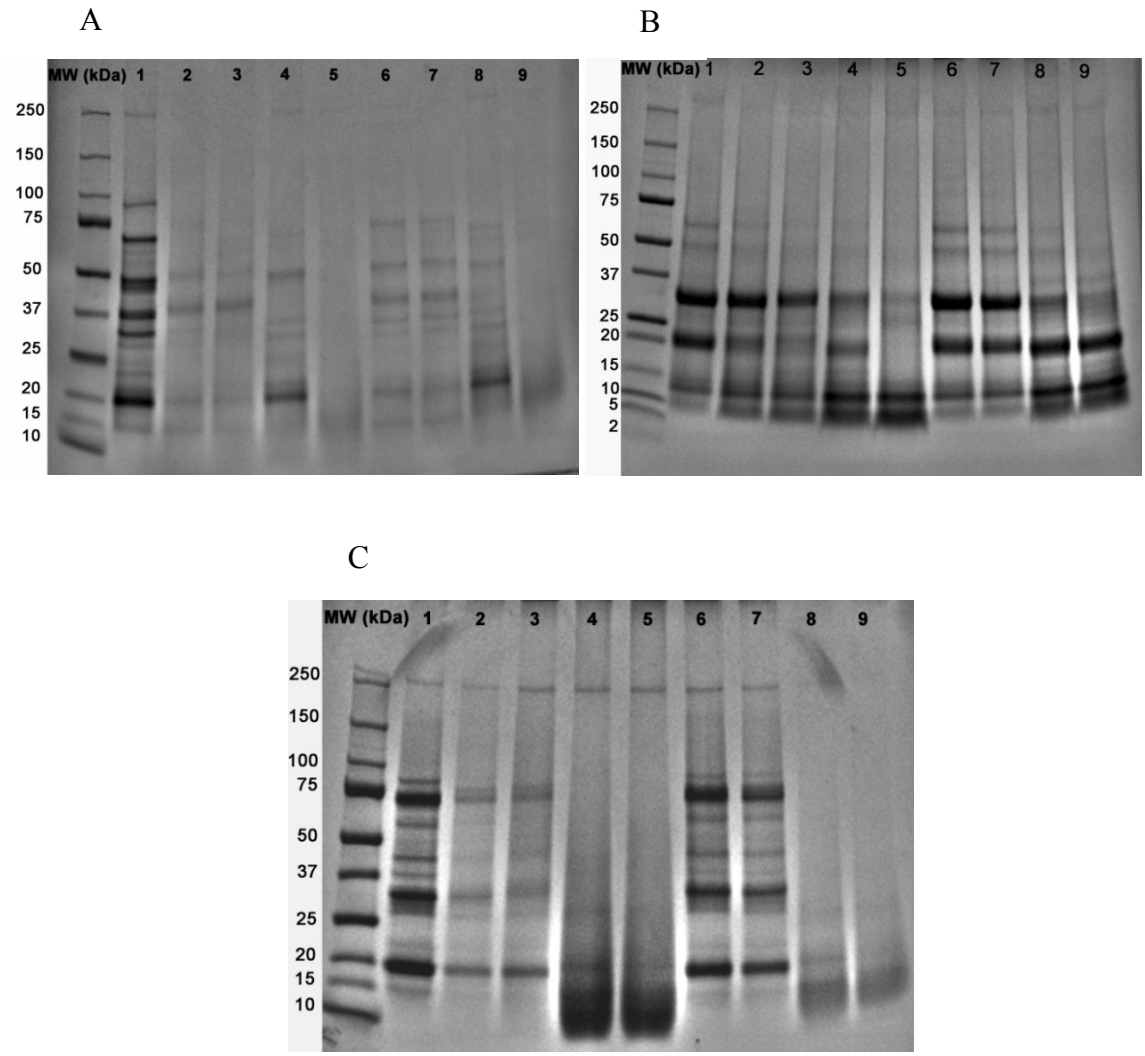

Figure 4.2 SDS-PAGE analysis of selected plant proteins. (A) Pea, (B) Rice, (C) Soybean. The first left lane represents a molecular weight marker and lane 1 represents native protein without cooking. Lanes 2-5 represents pressure-cooked native protein, denatured protein, hydrolysed protein and denatured and hydrolysed protein, respectively. Lanes 6-9 represents boiled native protein, denatured protein, hydrolysed protein and denatured and hydrolysed protein, respectively.

4.3.3 Degree of hydrolysis

The DH of purified plant proteins from wheat, corn, rice, pea and soybean is shown in Table 4.2. DH measures the percentage of peptide bonds cleaved in hydrolysis using 2,4,6-trinitrobenzenesulfonic acid (TNBS). Soybean protein hydrolysates showed the highest DH values, followed by corn, pea, wheat and rice. DH could be affected by protein solubility, purity and hydrolysis conditions.

Table 4.2 Degree of hydrolysis of plant proteins after *Aspergillus oryzae* digestion [1].

Plant proteins	Hydrolysed only (%)	Denatured and hydrolysed Pressure cooking (%)	Denatured and hydrolysed Boiling (%)
Wheat	14.12	13.72	15.80
Corn	46.10	40.97	39.81
Soybean	64.12	77.82	78.47
Pea	7.90	18.77	18.77
Rice	3.54	7.36	5.33

[1] Values are mean of two replicates.

4.3.4 Effects of plant proteins on RDS content – *in vitro* study

The *in vitro* digestibility of wheat starch was characterized by its RDS content. The effect of purified plant proteins in different forms (native, denatured, hydrolysed, and denatured and hydrolysed) on the RDS content of wheat starch is summarized in Table 4.3. The cooked wheat starch (without plant protein) had average RDS contents of 88.7% (pressure cooking) and 86.01% (boiling), respectively. Native plant proteins showed no significant effect on the RDS content, except rice protein, which reduced RDS content from 88.7% to 80.5% after pressure cooking. Denatured or hydrolysed proteins from pea, rice, soybean and wheat significantly reduced the RDS content when subjected to either pressure cooking or boiling (except denatured

pea protein), whereas denatured or hydrolysed proteins from corn significantly reduced the RDS content only via pressure cooking.

Table 4.3 Effects of purified plant proteins on the rapidly digestible starch (RDS) content of wheat starch determined by *in vitro* assay[1].

Mixtures (wheat starch + protein + cellulose) [2]	**Cooking method**	
	Pressure cooking	Boiling
	%RDS	%RDS
Wheat starch + cellulose (Control)	88.7± 0.1a	86.01 ± 0.65ab
Wheat starch + wheat protein + cellulose		
Native	86.5 ± 0.36ab	86.8 ± 0.85a
Denatured	80.01 ± 2.51cdefgh	78.9 ± 0.24efg
Hydrolysed	79.0 ± 1.15defgh	80.1 ± 1.91def
Denatured and hydrolysed	81.9 ± 1.74bcdef	80.8 ± 0.16cdef
Wheat starch + corn protein + cellulose		
Native	85.7 ± 0.95abc	83.7 ± 0.97abcd
Denatured	78.3 ± 0.44efgh	84.9 ± 1.31abc
Hydrolysed	81.0 ± 0.32bcdefg	84.0 ± 0.68abcd
Denatured and hydrolysed	79.6 ± 0.22defgh	83.0 ± 0.58abcde
Wheat starch + soybean protein + cellulose		
Native	84.7 ± 0.17abcd	82.1 ± 0bcde
Denatured	77.8 ± 1.13efgh	75.45 ± 1.56g
Hydrolysed	76.28 ± 0.1fgh	81.7 ± 0.81bcde
Denatured and hydrolysed	82.74 ± 0.1bcde	81.2 ± 0.34cdef
Wheat starch + pea protein + cellulose		
Native	87.0 ± 0.28ab	83.4 ± 0.07abcde
Denatured	74.9 ± 0.67h	82.6 ± 0.23abcde
Hydrolysed	75.5 ± 0.35gh	80.4 ± 0.73cdef
Denatured and hydrolysed	82.9 ± 0.01abcde	76.7 ± 0.47fg
Wheat starch + rice protein + cellulose		
Native	80.5 ± 0.58cdefgh	84.1 ± 0.02abcd
Denatured	76.35 ± 0.48fgh	80.4 ± 0.69cdef
Hydrolysed	77.1 ± 0.03gh	79.0 ± 0.27efg
Denatured and hydrolysed	80.17 ± 0.42cdefgh	76.8 ± 0.3fg

[1] The purified plant proteins were used in their native, denatured or hydrolysed forms and were mixed and cooked with wheat starch and cellulose by pressure cooking or boiling. Values are mean ± standard deviation of three replicates. The statistical analysis was performed by Tukey's HSD test and means in a column bearing the same letters are not significantly different ($p \geq 0.05$).

[2] The amount of wheat starch and purified plant proteins added to the mixture were specifically calculated based on the compositional analysis of each individual protein. The final composition in the mixture was 70% starch, 12% protein, except for the control which did not contain protein.

For each plant protein, there was no significant difference between the denatured and hydrolysed form, except for soybean (boiling). The findings also showed that protein denaturation prior to protein hydrolysis significantly reduced the RDS content, as in the case of rice, soybean and wheat (pressure cooking or boiling), corn (pressure cooking), and pea (boiling).The RDS content determination revealed that unlike soybean, pea and rice protein isolates, denatured or hydrolysed corn protein isolates only showed significant effect on the RDS content via pressure cooking (but not boiling), implying that soybean, pea and rice protein isolates may exert a more prominent effect in a real home-cooking environment. Therefore, pea, soybean and rice proteins were selected for future analysis.

The impact of protein-starch interaction occurring naturally in cereal grains on starch digestibility has been reported (Singh et al. 2010). Protein may form a matrix surrounding starch granules that acts as a barrier to starch digestibility. This study has indicated that exogenous proteins may have similar effects. The reduced RDS content of purified wheat starch may be associated with mitigated enzymatic access due to starch-protein interaction. Ryan and Brewer (2007) reported that the production of glucose was significantly lowered when gliadin was present, compared to starch alone or together with bovine serum albumen. Gliadin appeared to be more efficient than gluten or high molecular weight glutenin subunits (HMW-GS) in impeding amyloglucosidase action. This indicates that protein-starch interaction is dependent on the molecular configuration of proteins, as gliadin is more flexible and may be more conveniently adapted to binding with starch (Guerrieri et al. 1997).

4.3.5 Thermal characteristics of wheat starch in the presence of plant proteins

The effects of purified plant proteins on the thermal properties of wheat starch were studied by DSC (Table 4.4). Soy, rice and pea protein in their native, denatured (by pressure

cooking), and hydrolysed forms were selected for DSC study as they showed the most prominent effects on RDS content. The addition of native plant proteins showed no significant effect on wheat starch gelatinization, whereas proteins from all three plants in either denatured or hydrolysed form significantly increased T_o and T_p. Denatured soybean protein exhibited the most prominent effect, as it significantly increased T_o, T_p, T_c and ΔH of wheat starch gelatinization.

Table 4.4 Thermal characteristics of wheat starch with addition of purified plant proteins [1].

Mixtures (wheat starch + protein + cellulose)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
Wheat starch + cellulose (Control)	56.33 ± 0.33	62.41 ± 0.54	69.69 ± 0.91	11.69± 0.24
Wheat starch + soybean protein + cellulose				
Native	57.47 ± 0.13	63.52 ± 0.01	70.22 ± 0.26	11.86 ± 0.08
Denatured (pressure cooked)	60.51 ± 0.37*	65.94 ± 0.20*	73.34 ± 1.10*	13.64 ± 0.09*
Hydrolysed	60.52 ± 0.28*	66.79 ± 0.06*	72.71 ± 0.10	11.87 ± 0.23
Wheat starch + pea protein + cellulose				
Native	57.30 ± 0.23	63.58 ± 0.09	70.11 ± 1.02	11.55 ± 0.03
Denatured (pressure cooked)	59.96 ± 0.23*	65.65 ± 0.36*	72.36 ± 0.58	14.98 ± 0.83*
Hydrolysed	60.99 ± 0.20*	66.92 ± 0.12*	72.89 ± 0.66	11.75 ± 0.27
Wheat starch + rice protein + cellulose				
Native	57.72 ± 0.22	64.02 ± 0.16	70.10 ± 0.71	10.57 ± 0.47
Denatured (pressure cooked)	60.41 ± 0.38*	65.75 ± 1.28*	71.91 ± 0.75	12.36± 0.34
Hydrolysed	60.84 ± 1.32*	66.30 ± 1.14*	72.98 ± 2.23*	12.44 ± 0.31

[1] Values are mean ± standard deviation of two replicates. The statistical analysis was performed by Dunnett's test and means followed by an asterisk (*) in each column are significantly different when compared to control ($P<0.05$). T_o = onset temperature; T_p = peak temperature; T_c = conclusion temperature; and ΔH = endothermic enthalpy

The addition of denatured or hydrolysed plant proteins (pea, rice and soybean) significantly affected the thermal properties associated with wheat starch gelatinization. This significant increase in T_o and T_p in the presence of plant proteins is attributed to protein-starch interaction, which restricts starch hydration and swelling, an initial step of starch gelatinization. Heat denaturation and enzymatic hydrolysis not only enhance the surface hydrophobicity of protein, but also alter its water-holding capacity, which ultimately influence water availability and starch hydration (Panyam and Kilara 1996).

4.3.6 Confocal laser scanning microscopy (CLSM) of wheat starch in the presence of plant proteins

CLSM images of starch-protein mixtures are presented in Figure 4.3. The mixtures were stained with both APTS (green) and Pro-Q Diamond stain (red). Basically, wheat starch was labeled by APTS (8-amino-1,3,6-pyrenetrisulfonic acid) (green color), whereas phosphorous-associated protein and lipids were labeled by Pro-Q Diamond stain (red color). The yellowish green area in the overlay images indicated the formation of complexes between starch and protein or lipids.

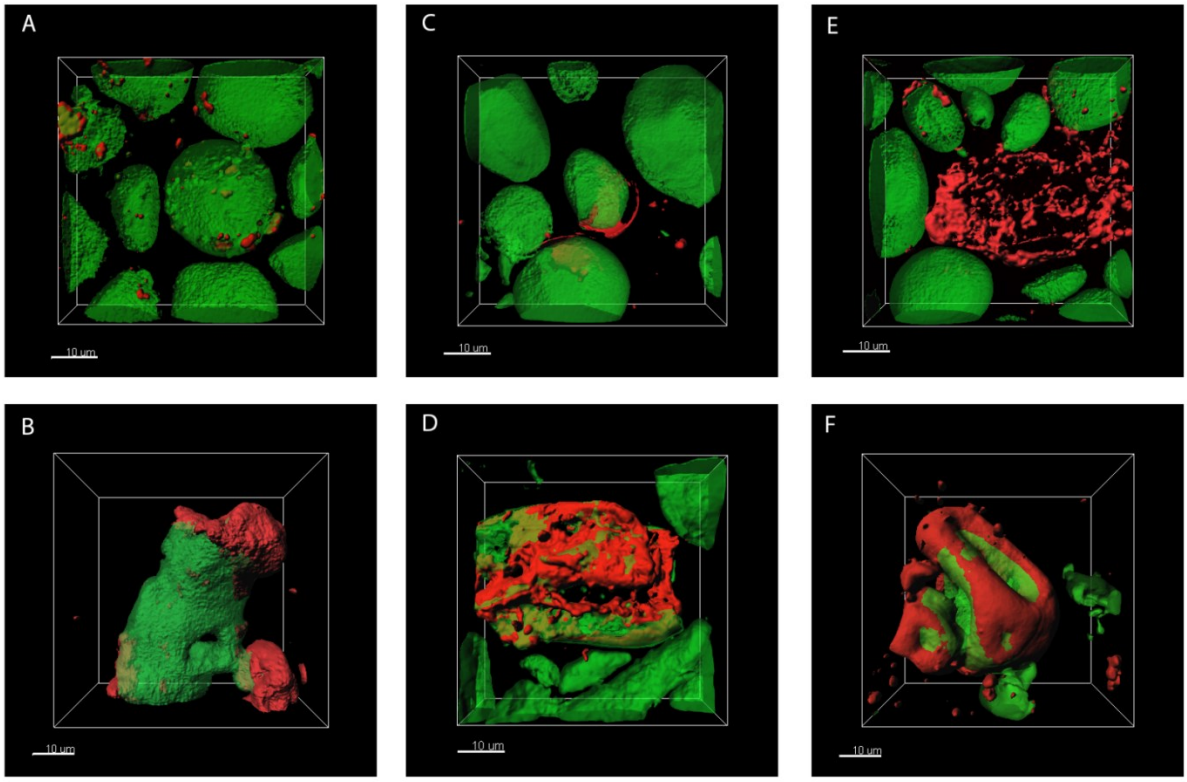

Figure 4.3 Confocal laser scanning micrographs of wheat starch in the presence of purified plant proteins. Wheat starch and cellulose were mixed with (A) pea protein, no cooking, (B) denatured pea protein, pressure cooked, (C) rice protein, no cooking (D) hydrolysed rice protein, pressure cooked, (E) soybean protein, no cooking, (F) hydrolysed soybean protein, pressure cooked. Images are overlays of staining by APTS and Pro-Q stains.

The labeled components can be easily distinguished from the background. Wheat starch in "native starch + native protein" mixture exhibited intact structure, and the majority of its surface was free of non-starch components. In case of any possible embedded protein or lipids, the green color was set to be ~ 50% transparent. The overlay images revealed that for the "native starch + native protein" mixture, barely any non-starch components were buried inside the starch granules. In contrast, enzymatic hydrolysis and heat denaturation substantially enhanced protein-starch interaction. The images showed that protein may interact with starch in two ways: 1) forming a layer of coating on the surface of starch, and 2) being embedded within the interior of starch granules.

CLSM provided an alternative way of characterizing protein-starch interaction in this study. Uncooked wheat starch and plant protein generally had loose association with each other, which may be partially due to vortexing and shaking that induced mild protein denaturation by exerting shear stress. The protein-starch association was substantially strengthened by protein denaturation/hydrolysis and cooking (pressure cooking or boiling). This agrees with the RDS content determination and DSC study, suggesting that protein-starch interaction results in forming a coating or encapsulation of the starch granule, impeding amyloglucosidase action. The CLSM images also showed that protein may be embedded within the interior of starch granule, partially owing to the disintegration of the starch granule during cooking, which allows protein to penetrate into the internal compartment of the granule.

In the last few years, the variety of attractive forces associated with protein-carbohydrate interactions has been intensively investigated. Hydrogen bonding is the most prominent hydrophilic interaction owing to the presence of abundant -OH groups in the carbohydrate (starch). These hydroxyl groups contact not only the side chains of polar residues of protein, including aspartic acid, glutamic acid, asparagine, glutamine, arginine and serine, but also the

backbone amino and carbonyl groups (Fernández-Alonso et al. 2012). Research regarding the non-covalent protein-carbohydrate interaction has revealed carbohydrate-aromatic binding in many carbohydrate-protein complexes (Asensio et al. 2012). The architecture of the binding site is dependent on many factors, including the numbers and relative location of aromatic residues, and the orientation of adjacent C-H bonds present in the carbohydrate. The binding may adopt different geometric shapes, and take place in different manners, as the interaction is strictly dependent on carbohydrate configuration. Systematic studies employing X-ray, fluorescence, NMR and other techniques have confirmed the essential role of the aromatic ring in the stacking (Chavez et al. 2005; Muraki 2002). The affinity of the interaction is determined by the nature of the aromatic residues, and an increase in the size of the aromatic ring dramatically improves the binding (Chavez et al. 2005; Muraki 2002). Despite all these findings, further research is necessary to reveal more details of the interactions (Asensio et al. 2012, Fernández-Alonso et al. 2012).

This study has shown that protein denaturation by pressure cooking or boiling, and protein hydrolysis by *Aspergillus oryzae* decreased the RDS content of wheat starch. During protein denaturation, the hydrophobic groups (e.g. methionine and cysteine), which are normally concealed in the protein interior core are then exposed. This may facilitate non-polar interaction between starch and protein. In addition, purified wheat starch contains proteins strongly associated with starch granules. These proteins are located either inside or near the surface of the starch granules (Baldwin 1995; Lowy et al. 1981; Rayas et al. 1995; Russell et al. 1987; Ryan and Brewer 2007; Skerritt et al. 1990). The surface proteins may be involved in mediating the binding of exogenous proteins to the starch surface (Ryan and Brewer 2007). Heating promotes protein aggregation and induces the formation of inter-protein disulfide bonds, which may contribute to "bonding" the surface proteins and exogenous proteins into a matrix surrounding

the starch granules, against enzymatic cleavage and water infusion (Cabra et al. 2006). This protein network may be further supported by hydrophobic interactions (via Van der Waals forces), as heating potentiates the exposure of hydrophobic amino acids buried within the interior of granule-bound starch proteins. Meanwhile, heating causes swelling of starch granules, enlarging the starch granule surface and increasing the leaching of starch molecules for binding with proteins (Eliasson and Tjerneld 1990). A schematic diagram depicting the potential interaction between starch and denatured protein is presented in Figure 4.4.

In this study, a commercial protease from *Aspergillus oryzae* with both endoprotease and exopeptidase activities was used to hydrolyze the plant proteins. *Aspergillus oryzae* is a generally recognized as safe (GRAS). As a neutral protease, the property of reaching optimal activity at neutral pH range further extends its application in the food processing industry to reduce bitterness of protein hydrolysates by hydrolyzing hydrophobic amino acid bonds (Sandhy et al. 2005). This study showed that the hydrolysis of proteins (wheat, corn, pea, rice and soybean) by *Aspergillus oryzae* enzymes significantly decreased the RDS content of wheat starch. This is attributed to partial enzymatic hydrolysis, which often results in an increased number of ionizable groups and the exposure of hydrophobic groups. In proteins, some hydrophobic groups are not exposed due to details of the protein secondary and tertiary structures. Enzymatic hydrolysis generates polypeptides with shorter amino acid sequences, limiting protein folding and making the total protein hydrophobicity more like the combined hydrophobicity of its constituent amino acid residues (Panyam and Kilara 1996).

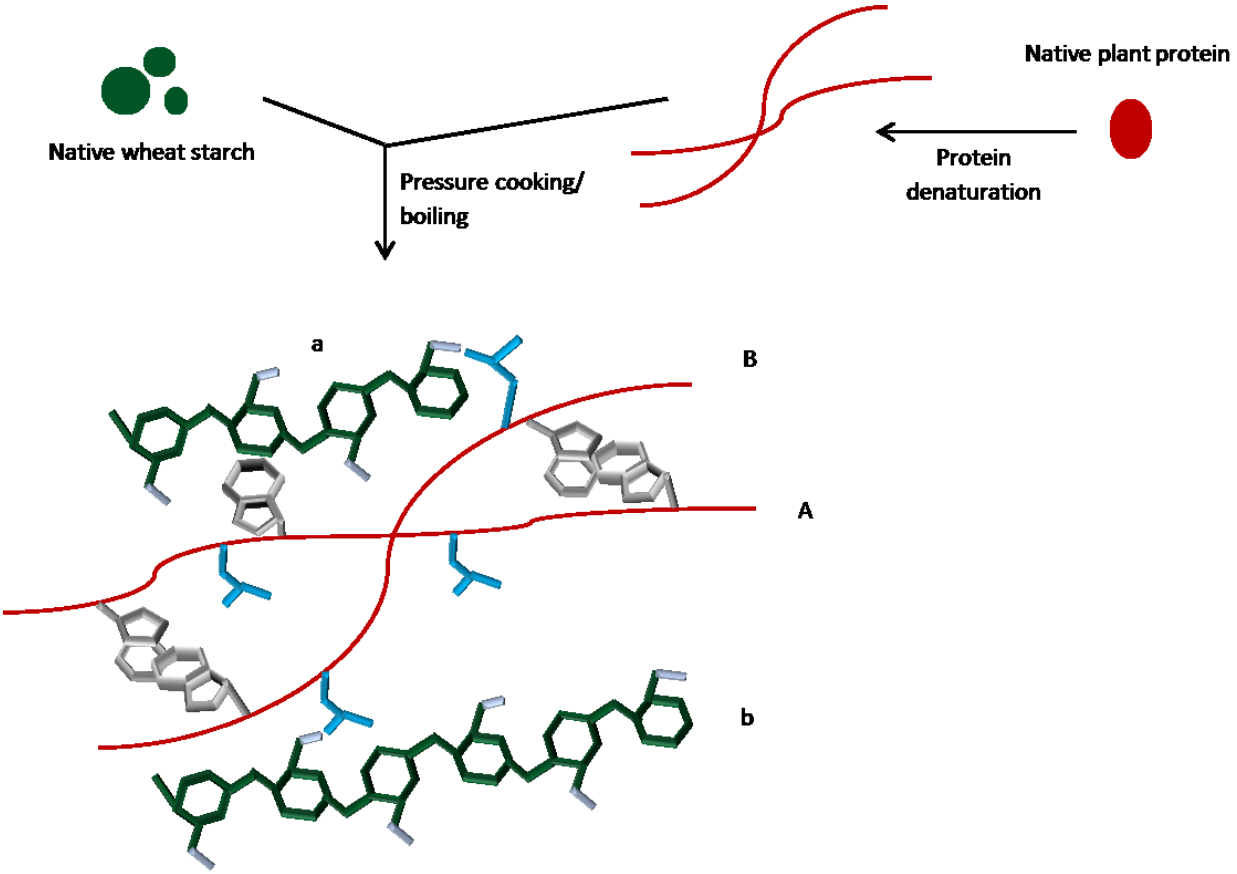

Figure 4.4 Schematic diagram of proposed protein-starch interaction after protein denaturation. Plant protein (red) is transformed into polypeptide chains (red) via heat denaturation, which interact with wheat starch chains (green). Polypeptide chains A and B present phenylalanine residues (gray) and aspartic acid residues (blue) as binding sites, which form non-polar and polar interactions, respectively with starch chains a and b (green). Polypeptide chains A and B interact through tryptophan residues (i.e. hydrophobic interaction), facilitating the coating effect of denatured protein on the surface of gelatinized starch matrix. Also, polypeptide chain (B) interact with starch chains (a and b) through aspartic acid residues (blue), connecting both starch chains. This may happen when protein is embedded within the interior of gelatinized starch matrix.

4.4 CONCLUSION

This study evaluated the potential effects of purified plant proteins from pulses, cereals and oilseeds on the RDS content of wheat starch, which was characterized by its *in vitro* amylase digestibility. The key findings of this study were that: a) the addition of plant proteins (pea, rice and soybean) in denatured and/or hydrolysed form significantly reduces the RDS content of a "starch + protein" mixture via cooking, and b) protein denaturation and protein hydrolysis substantially enhance starch-protein interaction. The reduced RDS content is associated with protein-starch interactions. Proteins effectively coat cooked the starch granules/mass, and thus block enzymatic access during starch digestion. The 3-D CLSM images provided new evidence on the interaction between wheat starch and exogenous proteins. Also, the study with *Aspergillus oryzae protease* suggests that protein hydrolysis may further mitigate starch digestion by enhancing protein-starch interactions. This is the first study on the effects of protease hydrolysis of exogenous proteins on wheat starch digestion. It is the intention that these findings may contribute to the creation of gluten-free food products with a low glycemic index.

CHAPTER 5. HYDROLYSED PEA PROTEINS REDUCE *IN VITRO* DIGESTIBILITY OF STARCH IN WHEAT FLOUR EXTRUDATES[3]

5.1 INTRODUCTION

In the last five decades, a number of events relating food intake to health issues have influenced the consumer. For example, a reduction in total fat and an increase in carbohydrates recommended for a healthy diet were linked to an increase in obesity, diabetes and coronary heart disease rates across the population (Fryar et al. 2012; Carroll et al. 2015; World Health Organization 2016; Centers for Disease Control and Prevention 2015). In addition, the consumption of gluten-containing foods (US Food and Drug Administration 2014) is associated with celiac disease characterized by inflammation and damage of the intestinal mucosa and immunogenic responses (Ciccocioppo et al. 2005). Although celiac disease rates are between 0.6 to 1.0% of the population worldwide (Fasano and Catassi 2012), the number of people consuming gluten-free foods is continuously increasing, due mainly to the perception that these are healthier products with weight loss benefits (Gaesser and Angadi 2012). Paradoxically, gluten-free diets, except those formulated with whole ancient grains and pulses, have been shown to contain greater amounts of starches and flours with lower contents of fibre, B-vitamins and iron (Thompson et al. 2005). These nutrient deficiencies may in turn increase body weight (Gaesser and Angadi 2012) and induce different metabolic responses such as increased glucose release and increased insulin levels. Patients with celiac disease are associated with a high incidence of type I diabetes. Thus, gluten-free foods formulated with high glycemic flours (e.g. corn, potato, cassava and rice) could worsen the control of diabetes and also potentiate the risk of insulin resistance, obesity and cardiovascular disease (Cronin and Shanahan 1997). Therefore,

[3] A version of this chapter was published in *Food Hydrocolloids*, 2018, 79:117-126.

there is a need to develop ingredients that enable foods to be formulated to alleviate these aforementioned health issues.

There are different strategies in developing low glycemic food products. For example, the addition of resistant starch (RS) (Englyst et al. 1992) such as RS1 made by increasing the milled particle size, RS2 by minimal food processing, RS3 by including flours rich in amylose, RS4 by starch chemical modification, and RS5 by adding amylose lipid complexes in the formulations. Another way would be to include soluble fibers such as barley β-glucan to impart positive effects due to its viscosity and specific molecular weight (Wood 2007). Besides these strategies, the inclusion of plant proteins also may show promising benefits, since like soluble fibre, they have the potential to lower starch digestibility. Plant proteins from sources such as pulses have many other positive attributes that address consumer preferences. They have a well-balanced amino acid composition and bioavailability (Roy et al. 2010) and they are obtained from agricultural crops that could solve nutritional, environmental and food security concerns worldwide (Zentner et al. 2001; United Nations 2013).

A number of *in vivo* studies have demonstrated that the inclusion of proteins in the diet from pulses, grains and oilseeds could generate faster hormonal responses (insulin and glucagon) and suppress blood glucose levels (Claessens et al. 2007; Claessens et al. 2009; Smith et al. 2012). Specifically, isolated pea proteins and their hydrolysates have become potential food ingredients because of their related health benefits such as decreased blood glucose levels when combined with carbohydrates (Smith et al. 2012). *In vitro* studies on cereal grains and oilseeds also support the hypothesis that starch digestibility may be reduced by the presence of proteins (Berti et al. 2004; Jenkins et al. 1987; Ryan and Brewer 2007). Cooked wheat starch, blended with native, heat-denatured and protease-hydrolysed pea proteins, followed by *in vitro* amylase

digestion has demonstrated that protein denaturation and protein hydrolysis enhances the starch-protein interaction (Chapter 4). The increased starch-protein interaction observed was suggested to be the reason for the efficient suppression of starch digestion in this system. However, more precise mechanisms behind such an effect have not yet been clarified, and it remains to be tested whether these effects remain in a more complex and physiologically relevant system. Realistically, before clinical trials, *in vitro* digestibility tests using an authentic food matrix that simulates the human gastrointestinal tract should be evaluated.

The dynamic gastric model (DGM) in combination with the static duodenal digestion model (SDM) is a novel *in vitro* technique that simulates physio-chemical and mechanical conditions found in human gastrointestinal digestion in a rather realistic, time-dependent manner (Thuenemann et al. 2015). This system controls *in silico* variables similar to a human stomach such as enzyme addition, changes in pH, mixing, shearing and retention time (Pitino et al. 2010; Vardakou et al. 2011b). The instrument can be fed a variety of 'meals' and deliver samples from the antrum to the "duodenum" in the same manner and at the same rate as observed *in vivo* (Vardakou et al. 2011a). The DGM was programmed using data obtained from echo-planar imaging studies (Marciani et al. 2001a; Marciani et al. 2001b; Marciani et al. 2008) and from published information presenting physiological ranges for the rate of secretion of gastric solutions (Lentner and Wink 1981). Studies have clearly shown that gastric emptying profiles measured by DGM were in agreement with those assessed by gamma scintigraphy *in vivo* studies in humans (Vardakou et al. 2011a). The objective of this study was to test if the inhibitory effects of amylolytic activity on wheat starch imparted by pea protein observed by simple static digestion trials, could be verified using a more complex and realistic system based on an

extruded snack matrix, prepared with blends of wheat flour and native or protease-hydrolysed pea proteins at 12% dry basis (db) digested by a combined DGM and SDM system.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Pea protein isolate was obtained from Nutri-Pea Limited (Portage la Prairie, MB, Canada) and wheat flour was purchased at a local store. Protease from *Aspergillus oryzae* (p= 1.27g/mL), reagents and enzymes for starch digestibility determination were purchased from Sigma-Aldrich (Oakville, ON, Canada). Analytical kits for determination of total starch, β-glucan and phytates were purchased from Megazyme (Megazyme International Ireland Ltd, Wicklow, Ireland). All other chemicals and solvents were of ACS certified grade.

5.2.2 Compositional analysis

Protein content was determined by combustion with a nitrogen analyzer (FP-428; Leco Corp., St. Joseph, MI) and using a nitrogen-to-protein conversion factor of 6.25. Megazyme analytical kits were used to determine total starch and β-glucan according to AOAC Methods 996.11 and 995.16, respectively. Phytate determination was according to the K-PHYT method using an analytical kit from Megazyme. Lipid quantification was performed by overnight extraction in hexane, at room temperature (20°C) followed by gravimetric measurements. Phosphorus and total phenolics were determined using the molybdenum blue method (Whistler et al. 1964) and Folin-Ciocalteu spectrophotometric methods, respectively.

5.2.3 Preparation of pea proteins and extruded mixtures with wheat flour

Pea protein hydrolysis was carried out according to the procedure specified in Chapter 4 (section 4.2.3), using a fungal protease from *Aspergillus oryzae* (4% net protein basis) for 120

min at 50ºC in sodium maleate buffer (pH 6) at a protein buffer ratio 1:6 (w/v). After hydrolysis, proteins were immediately frozen with liquid nitrogen and freeze dried. The protein recovery was 100%. Commercial pea protein isolate without any further modification is referenced throughout this chapter as "native protein". Wheat flour was mixed with native (treatment 1) and hydrolysed (treatment 2) pea proteins, respectively, at 12% protein (w/w) concentration. Wheat flour only was used as a control. The samples were mixed thoroughly and packaged air-tight for storage prior to extrusion. In the earlier studies (Chapter 3 and 4), the effect of pressure cooking on starch amylolysis was evaluated. However, extrusion cooking was selected in this study because this is a popular technology, especially used in the breakfast cereal and snack food processing industries. The extrusion conditions although selected to represent high temperature and pressure as similar to pressure cooking, it is noteworthy that extrusion processing significantly differ from pressure cooking in terms of the amount of moisture used (i.e. low moisture levels ranging from 20-50% used in extrusion processing) as well as the extent of shear applied during processing.

The extrusion process was performed on a laboratory-scale, co-rotating intermeshing twin-screw extruder (Process 11, Thermo Fisher Scientific, Karlsruhe, Germany). The barrel diameter and its length-to-diameter ratio (L/D) were 11 mm and 40:1, respectively. The extruder barrel was fitted with a circular 3 mm die nozzle. The extruder was powered by a 1.5 kW motor and the screw speed was kept constant at 350 rpm. The extruder had 7 internal and 1 external heating zones, and the temperature profile was set as follows: zone 1-7, 25-25-25-50-60-100-130°C, and die zone, 140°C. A high-shear screw configuration was employed. The raw material was metered into the extruder by a gravimetric, twin-screw feeder (MT-S, MiniTwin, Brabender Technologie, Duisburg, Germany) at a speed of 0.8 kg/h. Water was added into the extruder by a

peristaltic pump (Fillmaster Type 421, Delta Scientific Medical, Store Heddinge, Denmark) at a speed of 7.5 mL/min, resulting in a feed moisture content of 36%.

5.2.4 Sample collection and determination of process responses

Data from the extrusion process (torque, power, melt temperature, actual feed rate, screw speed and die pressure) was collected every 5 s by a data logging software (V3.2, Thermo Fisher Scientific, Copenhagen, Denmark). Samples were collected 3 min after the feed rate, water flow, temperature and screw speed had reached a steady-state. The samples were cooled to room temperature, oven dried (30°C) overnight, milled (99% pass through 500 microns), packed in polyethylene bags and stored at 5°C until further analysis. The specific mechanical energy (SME) was calculated (Eq. 1) from the rated screw speed (1000 rpm), actual screw speed, % motor torque, motor power rating (1.5 kW), and mass flow rate (Gogoi et al. 1996) and expressed in Wh/kg.

$$SME = \frac{actual\ screw\ speed\ (rpm)}{rated\ screw\ speed\ (rpm)} * \frac{\%\ motor\ torque}{100} * \frac{motor\ power\ rating\ (kW)}{feed\ rate\ \left(\frac{kg}{h}\right)} \quad (Equation\ 1)$$

5.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Pea protein (2 mg/mL) was dispersed in SDS-PAGE running buffer (250 mM Tris base, 1920 Mm Glycine and 0.1% SDS). Forty µL of dispersed sample was mixed with 10 µL of Laemmlii buffer 5X + dithiotheitol, heated at 95°C for 5 min in an Eppendorf thermomixer (Eppendorf, Copenhagen, Denmark), and cooled to room temperature prior to SDS-PAGE analysis. Fifteen µL of supernatant and 10 µL of pre-stained protein standards (10-250 kDa) (Bio-Rad, Copenhagen, Denmark) were then loaded on a Biorad criterion TGX 12% stain free gel (Bio-Rad, Copenhagen, Denmark) and run at 150V for approximately 30 min in a Mini-

Protean II electrophoresis cell (Bio-Rad, Copenhagen, Denmark). Images of gels were analysed with image Lab software (Bio-Rad, Copenhagen, Denmark).

5.2.6 Simulated *in vitro* human digestion

A simulated *in vitro* human digestion was carried out in the "dynamic gastric model (DGM)" (Figure 5.1) and a static duodenal model (SDM) (Fig. 5.1) at Bioneer-FARMA, Copenhagen, Denmark. The protocol for gastrointestinal digestion simulation was performed according to Klindt-Toldam et al. (2016) with modifications as described below.

The Dynamic Gastric Model (DGM): Test samples comprised 100 g of pulverized extruded flour or pulverized extruded pea protein-flour blends mixed with 190 mL water and 50 mL human saliva. Upon addition of water and saliva, the samples were mixed with a spoon for 1 min to ensure complete hydration and mimic mastication. In this experiment, a mincer was not used because the product is a hydrated powder, which is already in a masticated form. Once the mixing was completed, samples were transferred to the flexible fundus/main body, which contained 20 mL priming gastric solution. Gastric digestion was started, the pH of the digesta was computer-sensed by means of a glass pH electrode (Metrohm, 178 mm microelectrode) placed inside the fundus, and the temperature of the system was maintained at 37°C. Addition of gastric acid solution and gastric enzyme solution was computer-controlled and added at a maximum rate of 1.5 mL/min, as the gastric pH and volume changed (see Table 5.1 for compositions). The amounts of gastric acid solution added were 48.07 ± 20.48 mL (control), 37.70 ± 27.42 mL (treatment 1) and 57.97 ± 5.73 mL (treatment 2). The amounts of gastric enzyme solutions added were 25.83 ± 1.10 mL (control), 24.83 ± 2.18 mL (treatment 1) and 27.23 ± 0.40 mL (treatment 2).

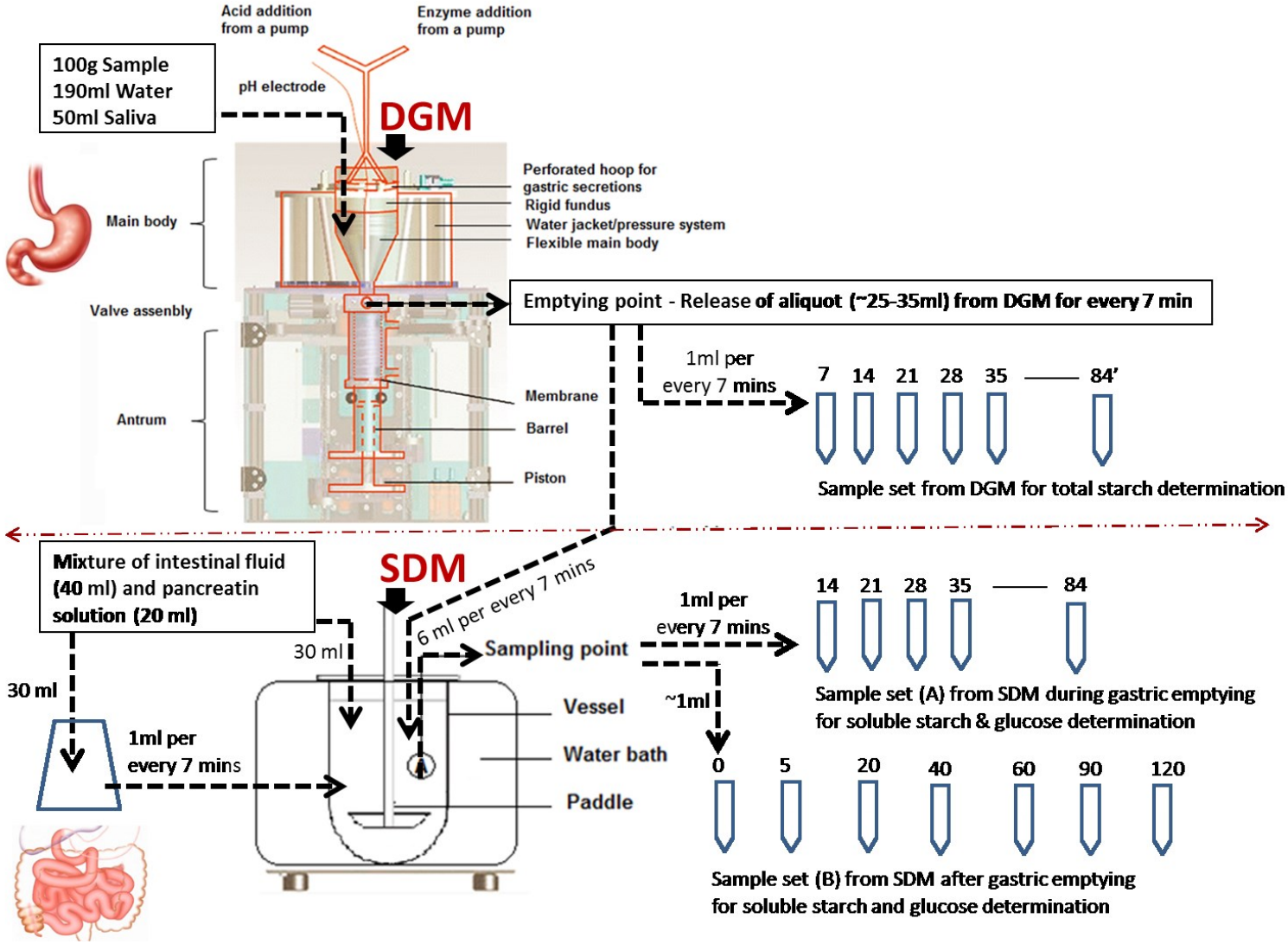

Figure 5.1 Schematic representation of the combined DGM and USP II paddle dissolution apparatus (SDM) with sampling protocol indicated. Modified from Vardakou et al. (2011).

The total residence time of the samples moving from the main body into the antrum, allowing reflux and mixing was approximately 84 min. Ejections of digesta (25-35 mL) from the valve assembly were collected approximately every 7 min.

Sample weight and pH were immediately recorded and aliquots (2 mL) were taken in falcon tubes containing 10 mL absolute ethanol to inhibit gastric enzyme activity. These samples were used to determine total starch content for further calculations.

Static duodenal digestion model (SDM): Intestinal fluid (20 mL) and pancreatin solution (10 mL) (See Table 5.1 for compositions) were placed in a USP II paddle dissolution apparatus (100 mL container), i.e. duodenum (Figure 5.1), and agitated (100 rpm) at 37°C. The pH was adjusted to 6.5 using 1M NaOH. An aliquot (6 mL) of each gastric sample was added to the duodenal dissolution vessel as they were ejected from DGM, for a total addition of 72 mL. An aliquot (1 mL) was taken prior to adding each DGM sample to the dissolution vessel (except for the first ejection) (Figure 5.1, sample set A), and at 0, 5, 20, 40, 60, 90 and 120 min after adding the last 6 mL from DGM (Figure 5.1, sample set B). After withdrawing each sample, 1 mL of intestinal fluid was added in order to keep constant the volume in the dissolution vessel. All the aliquots were collected in Falcon tubes containing 5 mL of absolute ethanol to inhibit enzyme activity. Samples were vortexed and centrifuged (1500 *g* for 10 min) and the supernatant was collected. The precipitate was washed twice with 5 mL of 50% ethanol. Total solubilized starch and free glucose in the supernatant was determined by using the Megazyme method with modifications.

Table 5.1 Constituents of solutions used in the DSM and SDM

	Gastric solution		Gastric priming solution		Gastric enzyme solution	Gastric acid solution		Intestinal fluid		Standard pancreatin solution	
Reagent	NaCl	3.389 g	NaCl	3.289 g	200 mL gastric sln	NaCl	3.4 g	NaCl	2944 mg		
	KCl	2.237 g	KCl	2.237 g		KCl	2.2 g	Porcine bile extract	2490 mg	Pancreatin	14 g
	NaH_2PO_4	104 mg	NaH_2PO_4	104 g		NaH_2PO_4	0.1 g	Lecithin	680 mg	MilliQ water	15 mL
	0.5M $CaCl_2$	1 g	0.5M $CaCl_2$	1 g		0.5 M $CaCl_2$	1 g	Trisma	160 mg		
			12M HCl (37%)	0.8 mL		12 M HCl (37%)	20 mL	MilliQ water	50 mL		
Enzyme					Pepsin 400 mg						
					Lipase 80 mg						
					Phospholipid 16 mg						
Water	Up to 1 L		Up to 1 L		Up to 1 L	Up to 1 L					

5.2.7 Fourier-Transform Infrared (FTIR) spectroscopy

In order to avoid confounding effects with the moisture content present in the samples in the region of 3000 cm^{-1} to 3600 cm^{-1}, extruded samples as well as native and hydrolysed pea proteins were placed overnight in an Isotemp vacuum oven model 282 A at 30°C and 0.38 in Hg (Fisher Scientific, Pittsburgh, PA, USA). The samples were stored in a desiccator until FTIR analysis. The absorbance measurements were performed on an Alpha Bruker FTIR instrument (Bruker Optics Ltd, London, ON, Canada) equipped with an Attenuated Total Reflectance (ATR) device with a single reflection diamond crystal. IR spectra were recorded in the range from 4000-400 cm^{-1} with a spectral resolution of 4 cm^{-1}. The milled sample was squeezed against the crystal surface with a concave needle compressor. Each spectrum represents the average of 32 scans rationed against the background (64 scans measured on the surrounding air). Data analysis was carried out in Opus (v. 7.0). All data were subjected to multiplicative scatter correction (MSC).

5.2.8 Statistical analysis

One-way analysis of variance (one way ANOVA) was performed using the General Linear Model (GLM) procedure of SAS Statistical Software (SAS Institute Inc., Cary, NC, USA, 2012). Significant difference among means was determined using Tukey's multiple comparison test ($P<0.05$). The extrusion process for each treatment and the control was performed in three replicates. All digestibility experiments (DGM and SDM) were performed in three replicates.

5.3 RESULTS AND DISCUSSION

5.3.1 Proximate composition of pea protein and wheat flour

The contents of starch and protein for wheat flour were 69% and 12.6%, respectively (Table 5.2) and for the pea protein, 3.5% starch and 82% protein. Minor components constituted

lipids (1.3% in wheat flour and 3.0% in pea protein) and β-glucan (0.4% in wheat flour and 0.03% in pea protein). Phosphorus content and total phenolics presented similar results for the two samples. The content of phytates was greater for pea protein (0.61%) compared to wheat flour (0.44%). The percentage of ash for wheat flour was 1.8% whereas for pea protein it was 4.0%.

Table 5.2 Composition (%, db) of raw material wheat flour and pea protein isolate

Component	Wheat flour	Pea protein isolate
Starch	69±0.00	3.48±0.07
Protein	12.64±0.03	82.00±0.04
Lipid	1.34±0.34	3.0±0.34
Beta-glucan	0.45±0.01	0.03±0.01
Phosphorous	1.14±0.04	1.1±0.02
Phytates	0.44±0.01	0.61±0.08
Total phenolics	0.13±0.00	0.14±0.00
Total dietary fiber	4.19 ± 0.11	0.0 ±0.00
Ash	1.8±0.1	4.0±0.02

5.3.2 Protease hydrolysis of pea protein isolate and protein profiling

SDS-PAGE analysis of native and protease-hydrolysed pea protein isolate samples (Figure 5.2) showed that the pea protein-hydrolysed with *Aspergillus oryzae* protease exhibited an increased number of smaller polypeptides, especially in the range <15 kDa. A general "smear" of the hydrolysed protein preparation demonstrated the multitude of hydrolytic events. This was followed by a reduced number of the larger native polypeptides ranging from 50-250 kDa with the exception of two large approximately 120 and 200 kD proteins (Figure 5.2), which were partly resistant to hydrolysis.

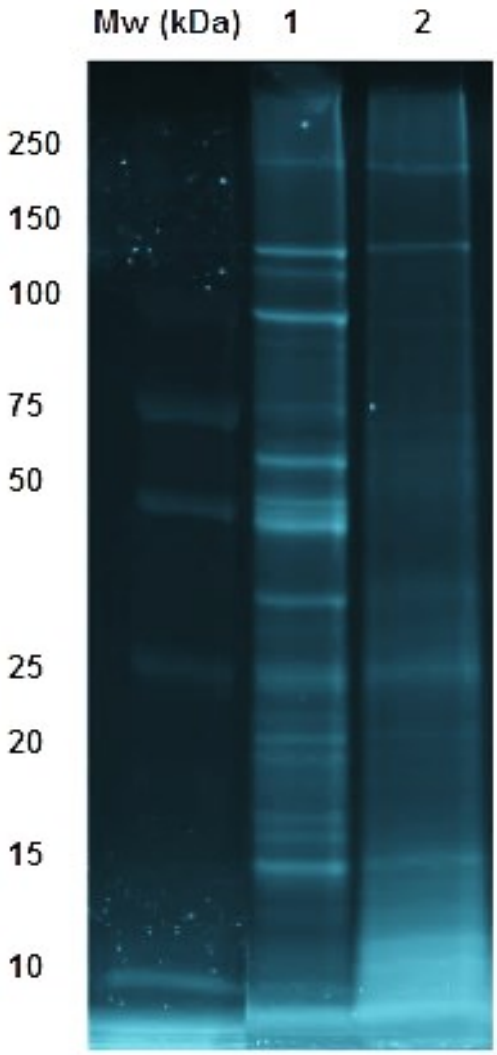

Figure 5.2 SDS-PAGE of pea protein preparations. Mw: molecular weight marker; 1: native pea protein; 2: enzyme hydrolysed pea protein

5.3.3 Extrusion responses of mixtures of flour and native or hydrolysed pea protein

The temperature of the melt when exiting the extruder was approximately 126°C, the pressure drop over the die was 1-2 bar, the torque exerted on the screw drive shaft was 6-7%, and the average SME was calculated to be 43 Wh/kg during the processing time. The extruded samples were brown and noodle like with a rough surface (Figure 5.3)

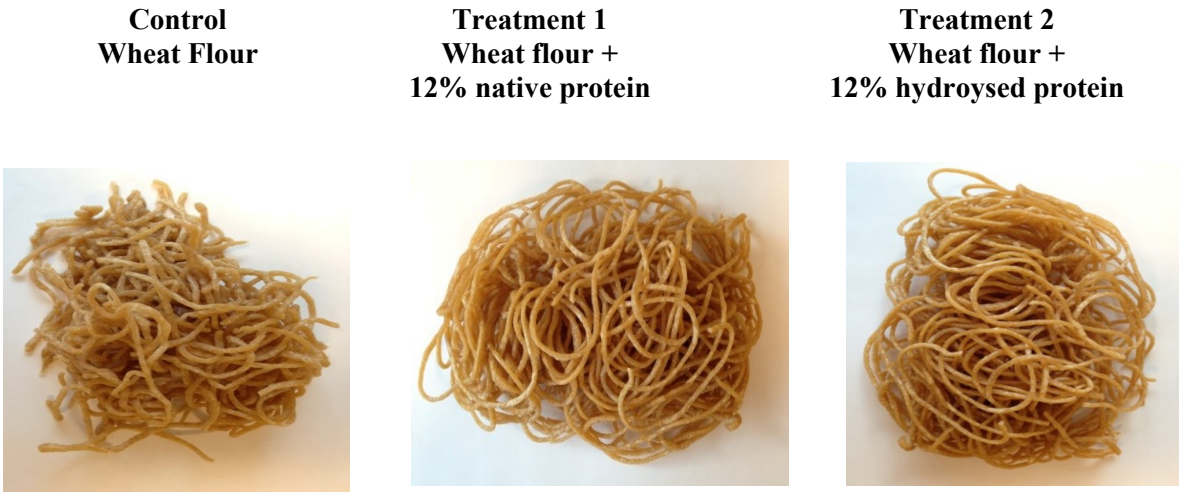

Figure 5.3 Extrudates of wheat flour and their blends with native or hydrolysed pea protein.

5.3.4 Effect of pea proteins on wheat flour amylolysis

The digestibility of wheat starch in the extruded samples, with and without native or hydrolysed-pea protein, was evaluated by an *in vitro* gastro-intestinal digestion system. Sample collection was performed in two stages as follows: Stage 1, from the vessel/duodenum during gradual emptying of the contents from the "antrum/stomach" at 7 min intervals (Figure 5.1 - sample set A); and Stage 2, from the "duodenum" at selected intervals, 0, 5, 20, 40, 60, 90 and 120 min, starting just after complete emptying of the stomach contents (Figure 5.1 – sample set B). Each sample was analysed for ethanol soluble sugars and free glucose contents.

The amounts of ethanol soluble sugars (expressed as glucose equivalents) and free glucose released from the samples during stage 1 are presented in Table 5.3 The content of ethanol soluble sugars released for the control (extruded wheat flour) was from 47.4% to 84.3%, whereas for treatment 1 (wheat flour with native pea protein) and treatment 2 (wheat flour with hydrolysed pea protein), was from 45.2% to 67.2% and 35.4% to 49.8%, respectively.

Table 5.3. Effect of native and protease-hydrolysed pea proteins on wheat starch digestion during gradual emptying of contents from DGM-fundus (i.e. stomach) to SDM-vessel (i.e. duodenum), where aliquots were collected from SDM-vessel between 14-84 minutes (i.e. Figure 5.1 – Sample set A) [1].

(min)	Control (wheat flour) [2]	Treatment 1 (wheat flour + 12% native pea protein) [3]	Treatment 2 (Wheat flour + 12% hydrolysed pea protein) [3]
	%	%	%
14	47.4 ± 1.8a (6.8 ± 2.2a)	45.2 ± 5.7a (5.2 ± 1.8a)	35.4 ± 2.1a (6.4 ± 1.1a)
21	54.3 ± 3.3a (5.4 ± 0.5a)	51.7 ± 0.6a (5.9 ± 1.4a)	47.7 ± 1.6a (5.6 ± 1.4a)
28	57.1 ± 1.3a (7.9 ± 1.9a)	56.8 ± 10.0a (6.5 ± 0.0b)	51.7 ± 1.9a (5.6 ± 0.6c)
35	57.3 ± 3.6a (8.0 ± 1.1a)	56.7 ± 0.5a (6.2 ± 0.4ab)	50.3 ± 2.3a (5.4 ±0.2b)
42	68.2 ± 2.3a (8.5 ± 0.7a)	63.5 ± 2.0a (7.0 ± 0.7a)	50.2 ±3.2b (5.9 ± 1.3a)
49	69.1 ± 1.4a (9.4 ±1.2a)	66.3 ±4.5a (7.8 ± 1.2a)	49.5 ± 2.4b (4.6 ± 0.1a)
56	72 ± 3.0a (8.7 ± 1.0a)	66.2 ± 3.7a (8.4 ± 0.0a)	49.9 ± 2.4b (5.2 ±0.2b)
63	72.1 ± 8.5a (9.9 ± 1.2a)	66.3 ± 1.0a (8.1 ± 0.3b)	49.9 ± 3.2a (5.3 ± 0.2c)
70	79.0 ± 3.2a (9.6 ± 0.8a)	65.8 ± 3.3b (8.1 ± 0.1b)	49.9 ± 1.4c (5.4 ± 0.1c)
77	82.2 ± 3.7a (9.5 ± 1.4a)	66.8 ±0.7b (7.4 ± 0.5a)	49.9 ± 1.8c (5.0 ± 0.1b)
84	84.3 ± 0.1a (10.1 ± 0.1a)	67.2 ± 3.2a (8.1 ± 0.0a)	49.8 ± 3.6b (4.5 ± 0.4b)

[1] Data represents the release of ethanol soluble sugars (expressed in terms of glucose equivalents) and free glucose in parenthesis. Values are mean ± standard deviation of two replicates. The statistical analysis was performed by Tukey's HSD test and means in a row bearing the same letters are not significantly different ($p \geq 0.05$).

[2] The final composition in the mixture was 69% starch and 12.6% protein

[3] The final composition of treatment 1 and 2 was 61.1% starch and 20.9% protein

The addition of hydrolysed pea protein to extruded wheat flour (treatment 2) significantly reduced the release of ethanol soluble sugars at 42, 49, 56 and 84 min of digestion, compared to control and treatment 1. Treatment 2 showed the lowest ethanol soluble sugars content at 70 min and 77 min. The free glucose content for the control ranged between 6.8% and 10.1%, whereas for treatment 1 and treatment 2, it was 5.2%-8.1% and 6.4%-4.5%, respectively. Inclusion of hydrolysed pea protein significantly reduced the release of free glucose at 28, 35, 56, 63, 70, 77 and 84 min compared to the control.

For stage 2, the release of ethanol soluble sugars from control samples ranged from 74.8% - 96.7%, whereas treatment 1 and treatment 2 presented a range between 71.0% - 82.3% and 56.3% - 77.9%, respectively (Table 5.4). The addition of hydrolysed pea protein to wheat flour caused a significant reduction of ethanol soluble sugars content at times 0, 5, 20 and 40 min (after complete emptying from the "stomach") when compared to the control.

In vivo studies have shown that endogenous proteins can have an inhibitory effect on wheat starch amylolysis (Jenkins et al. 1987). Proteins are found on the surface of starch granules, and may act as a physical barrier to digestion (Svihus et al. 2005), decreasing the degree of starch hydrolysis and sterically blocking enzyme action on the granule (Ryan and Brewer 2007). The outcome of the present study suggests that exogenous pea protein added to extruded wheat flour may have similar effects. In this particular case, samples were subjected to extrusion processing that combined high heat and large shear under low moisture conditions. Studies have shown exogenous protein-starch interactions under similar conditions (Allen et al. 2007; Fernández-Gutiérrez et al. 2004, Matthey and Hanna 1997). However, the extent of this interaction and its consequent impact on starch digestibility, especially on the dynamics of soluble starch and glucose release, were not reported.

Table 5.3 Effect of native and protease-hydrolysed pea proteins on wheat starch digestion in the SDM-vessel (i.e. duodenum), where aliquots were collected between 85-210 minutes (i.e. Figure 5.1 – Sample set B) from SDM-vessel after quantitative emptying of the contents from DGM-antrum[1]

Duodenal digestion after complete emptying of DGM (min)	Total time of digestion (min)	Control (wheat flour) [2]	Treatment 1 (wheat flour + 12% native pea protein) [3]	Treatment 2 (Wheat flour + 12% hydrolysed pea protein) [3]
		%	%	%
0	85	74.8 ± 4.2a (9.3 ± 1.4a)	71.0 ±3.1a (8.4 ± 0.4a)	56.3 ± 1.9b (6.1 ± 0.2b)
5	90	81.7 ± 0.5a (10.2 ± 1.9a)	77.2 ± 2.4a (8.8 ± 0.6ab)	63.3 ± 0.0b (6.4 ± 0.2b)
20	110	82.7 ± 2.4a (11.2 ±0.9a)	78.2 ± 5.3ab (11.5 ± 1.9a)	71.5 ± 2.1b (7.5 ± 0.1a)
40	130	84.2 ± 0.7a (13.7 ± 3.6a)	82.3 ± 4.2a (12.0 ± 1.3a)	72.1 ± 2.3b (8.7 ±0.2a)
60	150	83.8 ± 0.5a (15.9 ± 0.0a)	84.2 ± 2.7a (14.0 ± 3.2a)	73.7 ± 3.1a (9.5 ± 0.0a)
90	180	93.6 ± 0.2a (18.9 ± 0.7a)	84.0 ±5.9a (15.7 ± 4.6a)	75.1 ± 1.5a (10.6 ± 0.0a)
120	210	96.7 ± 3.6a (20.6 ± 0.1a)	82.3 ± 6.6a (16.9 ± 5.0a)	77.9 ± 0.3a (13.8 ± 0.1a)

1 Data represents the release of ethanol soluble sugars (expressed in terms of glucose equivalents) and free glucose in parenthesis. Values are mean ± standard deviation of two replicates. The statistical analysis was performed by Tukey's HSD test and means in a row bearing the same letters are not significantly different ($p \geq 0.05$).

2 The final composition in the mixture was 69% starch and 12% protein

[3] The final composition of treatment 1 and 2 was 60.8% starch and 20.6% protein

Polypeptide chain unfolding during heat and pressure processing may expose hydrophobic groups normally existing in the interior of a protein, which then could improve the protein's binding interaction potential (Ryan and Brewer 2007). Gluten contains a number of hydrophobic amino acids such as proline, leucine, phenylalanine, valine and isoleucine (Rombouts et al. 2009; Woychik et al. 1961). These amino acids could form non-polar interactions with hydrophobic amino acids from pea protein, e.g. leucine, phenylalanine, glycine, alanine and valine, when treated at higher temperatures (Wang and Daun 2004). In addition, surface proteins of starch that are hydrophobic in nature may be involved in attracting and binding exogenous proteins to the starch surface (Ryan and Brewer 2007).

According to Moore and Carter (1974), heat-treated proteins could either aggregate and covalently bind to carbohydrate (starch) or dissociate into protein subunits that can rearrange to form a large aggregate, physically entrapping starch. Thus, electrostatic interactions and hydrogen bonding would retain the carbohydrate within the protein matrix favoured by protein aggregation, making it less accessible to amylolysis.

In addition, the molecular configuration of exogenous proteins, e.g. molecular size, also could be responsible for the efficiency of the protein-starch granule interaction. Monomers, e.g. gliadins, are more flexible and may bind better to starch (Guerrieri et al. 1997). In the present study, electrophoretic analysis of protease-hydrolysed pea protein (Fig 5.2), showed an increased number of smaller molecular weight protein fractions (<15kDa), compared to native proteins, indicating the release of smaller polypeptides after enzymatic hydrolysis. As a result, an increased number of ionizable groups is more likely to interact with starch, primarily through hydrogen bonding.

5.3.5 FTIR spectroscopy of wheat flour in the presence of isolated pea proteins

The nature of the intermolecular interactions between wheat starch and proteins in the extruded samples was analysed using FTIR. Specific differences were found between the extruded wheat flour (control) and blends (treatments) (Table 5.5, Figure 5.4). Broad bands were identified at 3271cm^{-1} with the greatest intensity for treatment 2, followed by treatment 1 and the control (Figure 5.4A). This band is attributed to the stretching vibration of hydrogen bonding (Bernardino-Nicanor et al. 2016; Ogunmolasuyi et al. 2016), which is regarded as the most important intermolecular interaction that determines the properties of a starch matrix (Lu et al. 2016). These bonds can be present as free or bound hydroxyl groups and are observed above 3000 cm^{-1} of the infrared spectra (Lu et al. 2016). Bound hydroxyl groups could be formed by the interaction of -OH groups in starch with polar residues of pea protein, including glutamic acid, aspartic acid, serine and threonine, as well as by binding to backbone amino and carbonyl groups (Fernández-Alonso et al. 2012).

Table 5.4 FTIR band assignments for different bond stretching

Wavelength (cm^{-1})	Band assignment
3271	O-H stretching (Intramolecular hydrogen bonding) (Ogunmolasuyi et al. 2016)
2923	C-H stretching (Kizil et al. 2002)
1630	C=O stretching vibrations of amide groups - Amide I (Li et al. 2006)
1520	N-H deformation - Amide II (Li et al. 2006)
1450	C-H deformation (protein) (Soares et al. 2005)
1230	C-N stretching and vibrational band of N-H (Soares et al. 2005)
1148-1077	vibrations of C-O bond of C-OH from starch (Zullo and Iannace 2009)
1077	COH bending (starch) (van Soest et al. 1995)
994	O-H (Intramolecular hydrogen bonding) (van Soest et al. 1995)
930	Skeletal mode vibrations of α-1,4 glycosidic linkage (C-O-C) (Kizil et al. 2002)

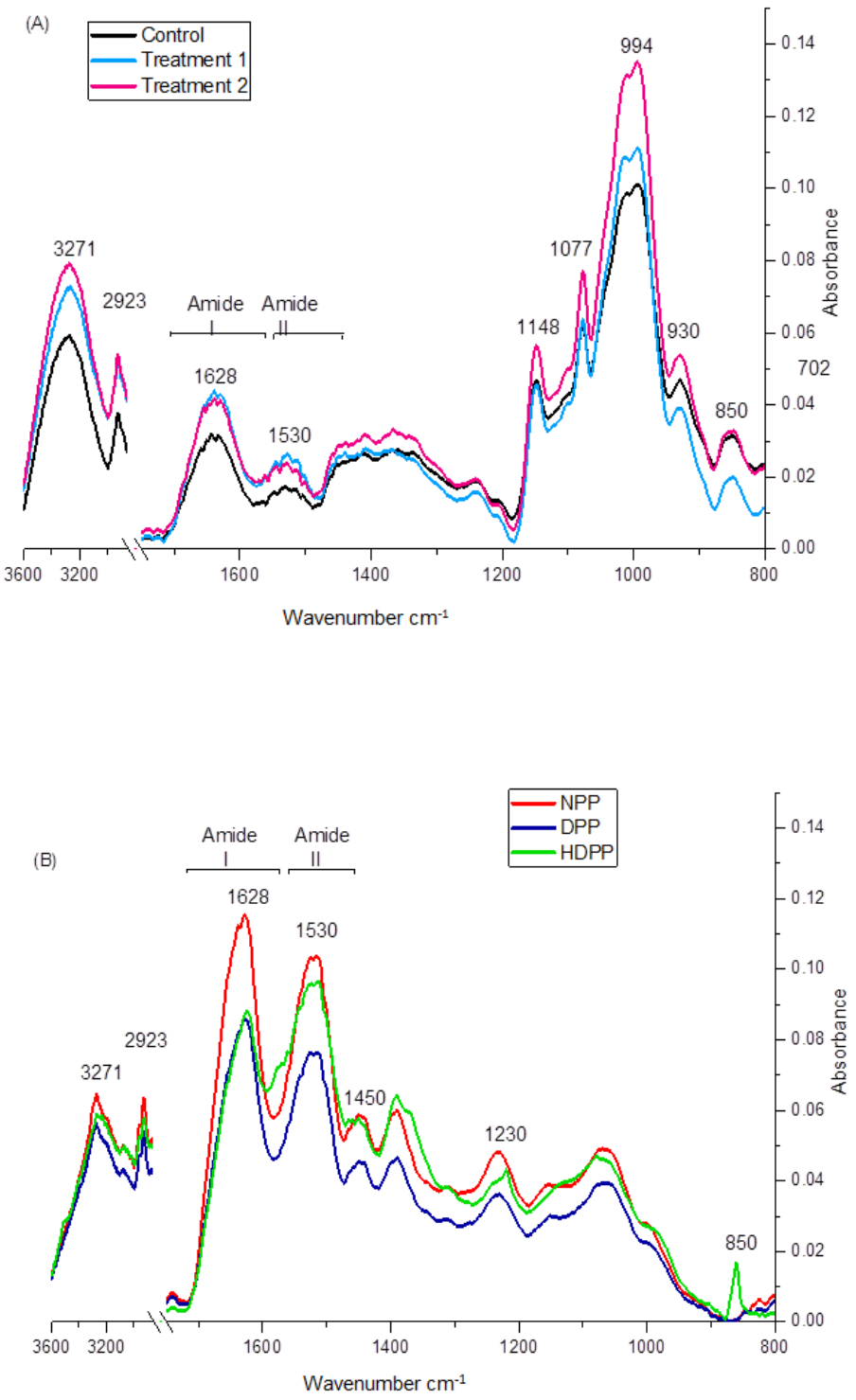

Figure 5.4 Fourier transformed infrared (FTIR) spectra of extruded samples and protein in different forms. (A) Wheat flour (control), wheat flour + native protein (treatment 1), wheat flour +hydrolysed protein (treatment 2); and (B) native pea protein (NPP), heat-denatured pea protein (DPP) and hydrolysed heat-denatured pea protein (HDPP).

The hydrolysed pea protein potentially will have a greater number of free carboxylic groups, increasing the ability to bind to starch, compared to native protein where the peptide bonds are intact. The band at 2923 cm^{-1} was assigned to the C-H stretching region of starch (Kizil et al. 2002). Treatments 1 and 2 showed a higher intensity band compared to the control.

Typical protein bands can be clearly recognized at wavenumbers of 1630 cm^{-1} related to C=O stretching vibrations of amide groups, 1520 cm^{-1} to deformation of N-H, 1450 cm^{-1} to C-H deformation, and 1230 cm^{-1} to C-N stretching and the vibrational band of N-H (Soares et al. 2005) (Fig 5.4B). These bands are assigned to specific protein components, namely amide I (1580-1720 cm^{-1}), amide II (1480-1580 cm^{-1}) (Li et al. 2006) and amide III (1200-1350 cm^{-1}) (Cai and Singh 2004), respectively. In the present study, native protein exhibited bands with higher intensities, followed by hydrolysed and denatured pea protein. Peaks at the same wavenumber corresponding to the treatments and control (Fig 5.4A) showed lower intensities compared to pure protein in its different forms (Fig 5.4B). Treatment 1 and 2 presented bands with higher intensities compared to the control. This may be attributed to the greater protein contents of the treatment samples.

Bands corresponding to the fingerprint of starch (Fig 5.4A) were observed between 800 cm^{-1} and 1200 cm^{-1}. Bands at 1148 cm^{-1} and 1077 cm^{-1} are characteristic peaks of starch molecules that possibly interact with other components through hydrogen bondings (Zullo and Iannace 2009). In this study, treatment 2 showed bands with higher intensities at these wavenumbers, which suggests greater vibrations of the C-O bond of C-OH from starch (Zullo and Iannace 2009), as a result of greater hydrogen bonding formation with hydrolysed protein. The band at 994 cm^{-1}, which is prominent for treatment 2, followed by treatment 1 and the control, shows greater intramolecular hydrogen bonding of the hydroxyl group at C-6 (Van Soest

et al. 1995), potentially with polar residues and backbone amino and carbonyl groups of the hydrolysed pea protein. Bands at 930 cm^{-1} were attributed to the skeletal mode vibrations of α-1,4 glyosidic linkages (C-O-C) (Kizil et al. 2002). There were no new bands detected in spectra of the control, treatment 1 and treatment 2, which shows that there were no covalent interactions between starch and protein. The above results confirm that the increase in hydrogen bonding was positively related to the amount of protein added as well as the form of the protein (native vs hydrolysed).

5.4 CONCLUSION

The potential effects of native and protease-hydrolysed pea proteins on the wheat starch amylolysis profile were studied in an extruded snack matrix formulated with wheat flour, using a combined *in vitro* dynamic gastric and static duodenal digestion system. Hydrolysed, but not native, pea protein was found to suppress the *in vitro* digestibility rate of starch in wheat flour. The amount of soluble starch and glucose released from the system when adding hydrolysed pea protein was significantly reduced at early time points of digestion, compared to both native pea protein and the control (wheat flour). The observed effect is suggested to be a result of the high number of low molecular weight proteins and peptides (<15 kDa). These may bind better with the starch granule due to increased interaction surface area of these polypeptides, including more exposed polar and charged residues in the backbone amino and carbonyl groups. Extensive intermolecular hydrogen bonding of the hydrolysed protein and wheat starch was supported by FTIR analysis. This study sets the foundation for further optimization of plant protein systems to reduce starch digestion in low glycemic foods.

CHAPTER 6. GENERAL CONCLUSIONS

6.1 SUMMARY AND SIGNIFICANCE OF RESEARCH

In the past three decades, clinical research in the field of food and nutrition has better established the link between the human diet and certain chronic diseases such as diabetes, obesity and cardiovascular disease. Rapid changes in lifestyles and increased consumption of processed diets with refined food ingredients such as white wheat flour, refined corn sugar, etc., have been attributed to the increase in chronic diseases around the world. The effect of digestible carbohydrates on human health, and the interventions to modify their impact on the aforementioned diseases have been studied. The rapidly digestible nature of cooked wheat starch potentiates a fast blood glucose release and, therefore, an increase in insulin secretion that generates unhealthy responses, i.e. inflammation and insulin resistance. In addition, the increasing trend in the consumption of starchy gluten-free foods, not only by people suffering from celiac disease but also by those that wrongly choose these food products as healthier alternatives, have exacerbated these health problems. Therefore, the importance of daily consumption of food products containing slowly digestible starch (SDS) and resistant starch (RS) has become evident, since they have been shown to protect humans against chronic diseases such as type II diabetes. Glycemic response is a measurement of the change in blood glucose concentration after consuming digestible carbohydrates. SDS and RS impart a lower glycemic response after food intake. The inclusion of resistant starches has been proposed as a strategy to reduce the glycemic index of foods. Specifically, resistant starches have been added as: RS1 in the form of whole grains or coarse particles, allowing the physical protection of starch granules against enzymatic hydrolysis; RS2 as minimally cooked starches, which are partially gelatinized, lowering their digestibility; RS3 as retrograded high amylose starches, which totally resist

digestion; RS4 as chemically modified starches, where the chemicals interfere with the action of amylolytic enzyme; and lastly, RS5 as lipid complexed amylose V-crystals, that resist amylolysis. Another strategy to reduce starch digestibility is the consumption of soluble viscous fibers such as β-glucan, which increase the viscosity of the digesta acting as a barrier against enzymatic cleavage and mucosal absorption. Besides the aforementioned approaches, the consumption of pulse seeds and their inclusion in processed foods has gained attention due to their lower starch digestibility and glycemic response compared to cereal grains and tubers. However, it remains unclear if the lower digestibility of cooked pulse seeds is mainly attributed to intrinsic factors of starch itself or rather to the presence of non-starch components such as proteins. Furthermore, little research has been done to determine if the addition of exogenous plant proteins to foods containing wheat starch could mitigate *in vitro* starch digestibility.

The present thesis research addressed some of the gaps in understanding the effect of endogenous pea proteins, as well as the effect of other selected exogenous plant proteins, on *in vitro* digestibility of pea and wheat starches, respectively. The effect of particle size of the pea seeds in the cooking process, i.e. flour vs whole grain, and the nature of the exogenous protein, i.e. native, heat-denatured or hydrolysed, on *in vitro* starch digestibility were studied.

In Chapter 3, this research demonstrated that endogenous pea protein and its interaction with starch during pressure cooking may play a role in lowering *in vitro* pea starch digestibility, due to: 1) the RDS content of pressure cooked grains and flour was significantly lower compared to that of isolated pea starch; and 2) no significant difference in RDS content was found between pressure cooked isolated pea and wheat starches, which indicates a similar melting pattern of C-type and A-type crystals, respectively. Differential scanning calorimetry (DSC) and scanning electron microscopy (SEM) experiments indicated that the particle size, i.e. split pea seeds vs pea

flour, influenced starch gelatinization during pressure cooking. Pressure cooked split pea seeds showed more intact (less gelatinized) starch particles compared to pea flour, which was reflected in the lower starch digestibility.

In Chapter 4, the effect of exogenous native, heat-denatured, protease-hydrolysed and heat-denatured-protease hydrolysed plant proteins from wheat, corn, soy, pea and rice on *in vitro* digestibility of isolated wheat starch was investigated. Native proteins (except rice) showed no significant effect on the RDS content of protein-starch mixtures. Denatured and/or hydrolysed plant proteins significantly reduced the RDS content, while this effect could be influenced by the cooking method and protein origin. Confocal laser scanning microscopy (CLSM) and DSC analyses of pressure cooked mixtures of denatured as well as hydrolysed pea, soy and rice proteins with starch suggested that protein denaturation or protease hydrolysis promotes starch-protein interaction, and thus restricts starch hydration and enzymatic cleavage.

Following the in depth investigation, as discussed above, of the effect of exogenous plant proteins on wheat starch digestibility, field pea protein was further investigated using a dynamic *in vitro* digestion method and a static duodenal model. The study determined the efficacy of the addition of native and hydrolysed pea proteins on lowering the digestibility of starch in wheat flour extrudates (Chapter 5). Native pea protein did not influence the release of ethanol soluble sugars and glucose after complete gastric emptying into the duodenal vessel. However, the addition of hydrolysed pea protein significantly reduced starch amylolysis at the first 40 min of digestion. No inhibitory effect was observed at longer digestion times. Fourier-Transform Infrared (FTIR) analysis of the extruded samples clearly indicated enhanced starch-protein interactions, where the magnitude of interaction was found to be greatest in the blend with hydrolysed pea protein through hydrogen bonding. To the best of the author's knowledge, this is

the first study using dynamic gastric model (DGM) to determine the effect of exogenous protein on the digestibility of starch in extruded wheat flour.

Overall, this thesis research suggests the possibility of using a pulse-protein-based strategy to formulate low-glycemic food products for which the demand is growing quickly. A number of approaches, as discussed earlier, are used by the food industry to formulate low-glycemic foods. However, the addition of pulse proteins to food formulations would show double benefit by also enhancing the nutritive value and the amino acid balance of the formulated foods.

6.2 RECOMMENDATIONS FOR FUTURE WORK

This research sets the foundation for the development of a pulse protein ingredient that could be used to decrease starch digestibility in wheat-starch-containing foods. The *in vitro* methods used in this study, suggested that pea protein, in its fungal-protease-hydrolysed form, has great potential in lowering amylolysis of starch in wheat foods made under high pressure processing, such as pressure cooking and extrusion. However, a precise correlation between the *in vitro* digestibility profile (i.e. RDS, SDS and RS) and *in vivo* glycemic response and glycemic index is still needed. Although different *in vitro* techniques have been suggested to predict *in vivo* glycemic response (Bornet et al.1989) and glycemic index (Jenkins et al. 1982; Goñi et al.1997) in healthy participants, lack of clarity still remains in this prediction process. Therefore, it is crucial to find a standardized *in vitro* method that provides a complete kinetic analysis of *in vivo* starch digestion in wheat flour based products. Clinical studies could further explore the efficacy of this protein hydrolysate in lowering blood glucose responses, when added to food formulations containing wheat flour. Further *in vitro* studies are needed to determine the effect of fungal-protease-hydrolysed proteins isolated from different pulse sources such as lentil, fava

bean, red bean, and chickpea on reducing wheat starch amylolysis. Moreover, the effectiveness of using hydrolysed pulse proteins produced by using proteases from other bacterial and plant origins.

As one of the current trends is the increased consumption of gluten-free foods that are mostly formulated with highly digestible starches, research is warranted in order to determine the influence of hydrolysed pulse proteins on the *in vitro* amylolysis of corn, potato, rice and tapioca starches. Another valuable study would be to investigate the *in vitro* digestion of wheat starch, in the presence and absence of hydrolysed pulse proteins, individually by procine pancreatic alpha-amylase and brush boarder carbohydrases. Characterizing the progression of starch hydrolysis at frequent time intervals by probing the nature of digestible carbohydrates (i.e. glucose, maltose, maltotriose, limit dextrins) released, using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex), may shed further light on the mechanism of action.

References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. Journal of Agricultural and Food Chemistry, 27, 1256-1262.

Allen, K., Carpenter, C., and Walsh, M. K. (2007). Influence of protein level and starch type on an extrusion-expanded whey product. International Journal of Food Science and Technology, 42, 953-960.

Agriculture and Agri-Food Canada. "Canadian Pulses;", http://www.agr.gc.ca/eng/industry-markets-and-trade/buying-canadian-food-products/canadian-pulses/?id=1426000399901. Accessed 30 June 2016.

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

Alcázar-Alay, S., and Meireles, M. (2015). Physicochemical properties, modifications and applications of starches from different botanical sources. Food Science and Technology, 35, 215-236.

Alegría, A., García-Llatas, G., and Cilla, A. (2015). Chapter 1 static digestion models: General introduction. In: The impact of food bio-actives on gut health. Cotter, P., López-Exposito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., and Wichers, H. (Eds.), pp. 338. Springer International Publishing.

Alexander, D. (2012). Postprandial effects of resistant starch corn porridges on blood glucose and satiety responses in non-overweight and overweight adults. PhD Thesis: Iowa State University (USA).

Arakawa, T., Prestrelski, S. J., Kenney, W. C., and Carpenter, J. F. (2001). Factors affecting short-term and long-term stabilities of proteins. Advanced Drug Delivery Reviews, 46, 307-326.

Asensio, J., Ardá, A., Cañada, F., and Jiménez-Barbero, J. (2012). Carbohydrate-aromatic interactions. Accounts of Chemical Research, 46, 946-954.

Ashogbon, A., and Akintayo, E. (2014). Recent trend in the physical and chemical modification of starches from different botanical sources: A review. Starch - Stärke, 66, 41-57.

Asp, N., and Björck, I. 1992. Resistant Starch. Trends in Food Science and Technology, 3, 111-114.

Atkin, N., Cheng, S., Abeysekera, R., and Robards, A. (1999). Localisation of amylose and amylopectin in starch granules using Enzyme-Gold labelling. Starch-Stärke, 51, 163-172.

Atkinson, F., Foster-Powell, K., and Brand-Miller, J. (2008). International Tables of Glycemic Index and Glycemic Load Values: 2008. Diabetes Care, 31, 2281-2283.

Atwell, W., Hood, L., Lineback, D., Varriano-Marston, E., and Zobel, H. (1988). The terminology and methodology associated with basic starch phenomena. Cereal Foods World, 33, 311.

Augustin, L., Kendall, C., Jenkins, D., Willett, W., Astrup, A., Barclay, A., and Poli, A. (2015). Glycemic index, glycemic load and glycemic response: An international scientific consensus summit from the international carbohydrate quality consortium (ICQC). Nutrition, Metabolism, and Cardiovascular Diseases, 25, 795-815.

Baldwin, P. (1995). Studies on the surface chemistry, minor component composition and structure of granular starches. In PhD Thesis: The University of Nottingham (United Kingdom).

Barclay, A., Petocz, P., McMillan-Price, J., Flood, V., Prvan, T., Mitchell, P., and Brand-Miller, J. (2008). Glycemic index, glycemic load, and chronic disease risk - a meta-analysis of observational studies. The American Journal of Clinical Nutrition, 87, 627-637.

Barros, F., Awika, J., and Lloyd, W. R. (2012). Interaction of Tannins and Other Sorghum Phenolic Compounds with Starch and Effects on *in Vitro* Starch Digestibility. Journal of Agricultural and Food Chemistry, 60, 11609-11617.

BeMiller, J. N., and Whistler, R. L. (2009). Starch: Chemistry and technology (2nd edition). London. Academic Press. 718p.

Bender, M., and Kezdy, F. (1965). Mechanism of action of proteolytic enzymes. Annual Review of Biochemistry, 34, 46-76.

Berg, J., Tymoczko, J., and Stryer, L. (2002). Biochemistry (5th edition). New York: W. H. Freeman and Company. 132p.

Bernardino-Nicanor, A., Acosta-García, G., Gemes-Vera, N., Montañez-Soto, J., Vivar-Vera, M., and González-Cruz, L. (2016). Fourier transform infrared and raman spectroscopic study of the effect of the thermal treatment and extraction methods on the characteristics of ayocote bean starches. Journal of Food Science and Technology, 54, 933-943.

Berry, C. S. (1986). Resistant starch: Formation and measurement of starch that survives exhaustive digestion with amylolytic enzymes during the determination of dietary fibre. Journal of Cereal Science, 4, 301-314.

Berti, C., Riso, P., Monti, L., and Porrini, M. (2004). *In vitro* starch digestibility and in vivo glucose response of gluten-free foods and their gluten counterparts. European Journal of Nutrition, 43, 198-204.

Bertoft, E. (2013). On the building block and backbone concepts of amylopectin structure. Cereal Chemistry, 90, 294-311.

Bertoft E. (2015) Fine Structure of Amylopectin. In: Starch metabolism and structure. Nakamura Y. (eds). Springer, Tokyo. 451p.

Bertoft, E. (2017). Understanding starch structure: Recent progress. Agronomy, 7, 1-29.

Bertoft, E., Laohaphatanalert, K., Piyachomkwan, K., and Sriroth, K. (2010). The fine structure of cassava starch amylopectin. part 2: Building block structure of clusters. International Journal of Biological Macromolecules, 47, 325-335.

Bertoft, E., Manelius, R., and Qin, Z. (1993). Studies on the Structure of Pea Starches. Part 1: Initial stages in α-amylolysis of granular smooth pea starch. Starch - Stärke, 45, 215-220.

Bertoft, E., Piyachomkwan, K., Chatakanonda, P., and Sriroth, K. (2008). Internal unit chain composition in amylopectins. Carbohydrate Polymers, 74, 527-543.

Biliaderis, C., Maurice, T., and Vose, J. (1980). Starch gelatinization phenomena studied by differential scanning calorimetry. Journal of Food Science, 45, 1669-1674.

Biliaderis, C., Page, C., and Maurice, T. 1986. On the multiple melting transitions of starch/monoglyceride systems. Food Chemistry, 22,279-295.

Biorklund, M., Van Rees, A., Mensink, R., and Onning, G. (2005). Changes in serum lipids and postprandial glucose and insulin concentrations after consumption of beverages with beta-glucans from oats or barley: A randomised dose-controlled trial. European Journal of Clinical Nutrition, 59, 1272-1281.

Birt, D., Boylston, T., Hendrich, S., Jane, J., Hollis, J., Li, L., and Whitley, E. (2013). Resistant starch: Promise for improving human health. Advances in Nutrition, 4, 587-601.

Björck, I., and Asp, N. (1994). Controlling the nutritional properties of starch in foods — a challenge to the food industry. Trends in Food Science & Technology, 5, 213-218.

Björck, I., Liljeberg, H., and Östman, E. (2000). Low glycaemic-index foods. British Journal of Nutrition, 83, 149-155.

Björck, I., Asp, N., Birkhed, D., and Lundquist, I. 1984a. Effects of processing on availability of starch for digestion *in vitro* and in *vivo*; I Extrusion cooking of wheat flours and starch. Journal of Cereal Science, 2, 91-103

Björck, I., Asp, N., Birkhed, D., Eliasson, A., Sjöberg, L., and Lundquist, I. 1984b. Effects of processing on starch availability *in vitro* and *in vivo*. II. Drum-drying of wheat flour. Journal of Cereal Science, 2, 165-178.

Bornet, F., Fontvieille, A., Rizkalla, S., Colonna, P., Blayo, A., Mercier, C and Slama, G. (1989). Insulin and glycemic responses in healthy humans to native starches processed in different ways:

correlation with in vitro alpha-amylase hydrolysis. The American Journal of Clinical Nutrition, 50, 315-323.

Borovsky, D., Smith, E., Whelan, W., French, D., and Kikumoto, S. (1979). The mechanism of Q-enzyme action and its influence on the structure of amylopectin. Archives of Biochemistry and Biophysics, 198, 627-631.

Braaten, J., Wood, P., Scott, F., Riedel, K., Poste, L., and Collins, M. (1991). Oat gum lowers glucose and insulin after an oral glucose load. The American Journal of Clinical Nutrition, 53, 1425-1430.

Buisson, G., Duee, E., Haser, R., and Payan, F. (1987). Three dimensional structure of porcine pancreatic alpha-amylase at 2.9 A resolution. role of calcium in structure and activity. The EMBO Journal, 6, 3909-3916.

Bull, H., and Breese, K. (1973). Thermal stability of proteins. Archives of Biochemistry and Biophysics, 158, 681-686.

Bulon, A., Colonna, P., Planchot, V., and Ball, S. (1998). Starch granules: Structure and biosynthesis. International Journal of Biological Macromolecules, 23, 85-112.

Burton, P., and Lightowler, H. (2008). The impact of freezing and toasting on the glycaemic response of white bread. European Journal of Clinical Nutrition, 62, 594-599.

Cabra, V., Arreguin, R., Vazquez-Duhalt, R., Farres, A. (2006). Effect of temperature and pH on the secondary structure and processes of oligomerization of 19 kDa alpha-zein. Biochimica et Biophysica Acta, 1764, 1110-1118.

Cai, S., and Singh, B. R. (2004). A distinct utility of the amide III infrared band for secondary structure estimation of aqueous protein solutions using partial least squares methods. Biochemistry, 43, 2541-2549.

Calbet , J., and MacLean, D. (2002). Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. Journal of Nutrition, 132, 2174-2182.

Carroll, M., Navaneelan, T., Bryan, S., and Ogden, C.. Prevalence of obesity among children and adolescents in the United States and Canada. U.S. Department of Health and Human Services.

Centers for Disease Control and Prevention. National Center for Health Statistics (2015). https://www.cdc.gov/nchs/products/databriefs/db211.htm.Accesed 26 January 2017.

Centers for Disease Control and Prevention. Number (in Millions) of People with Diabetes Aged 35 Years Or Older with Self-Reported Heart Disease Or Stroke, United States, 1997-2011. (2015). https://www.cdc.gov/diabetes/statistics/cvd/fig1.htm/.Accessed 26 January 2017

Ciccocioppo, R., Di Sabatino, A., and Corazza, G. R. (2005). The immune recognition of gluten in coeliac disease. Clinical and Experimental Immunology, 140, 408-416.

Chavez, M., Andreu, C., Vidal, P., Aboitiz, N., Freire, F., Groves, P., Asensio, J., Asensio, G., Muraki, M., Canada, F., and Jimenez-Barbero, J. (2005). On the importance of carbohydratearomatic interactions for the molecular recognition of oligosaccharides by proteins: NMR studies of the structure and binding affinity of AcAMP2-like peptides with non-natural naphthyl and fluoroaromatic residues. Chemistry-A European Journal, 11, 7060-7074.

Chen, J., Wang, S., Deng, Z., Zhang, X., Feng, S., Yuan, H., and Tian, J. (2012a). Effects of enzymatic hydrolysis of protein on the pasting properties of different types of wheat flour. Journal of Food Science, 77, 546-550.

Chen, J., Tian, J., Zheng, F., Li, X., Zhao, Y., Gao, X., and Zhang, X. (2012b). Effects of protein hydrolysis on pasting properties of wheat flour. Starch - Stärke, 64, 524-530.

Chibbar, R., Ambigaipalan, P., and Hoover, R. (2010). Molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. Cereal Chemistry, 87, 342-352

Chopra, H., and Panesar, P. (2010). Food chemistry. United Kingdom. Alpha Science International Limited.

Hyun-Jung, C., Liu, Q., Hoover, R., Warkentin, T., and Vandenberg, B. 2008. *In Vitro* starch digestibility, expected glycemic index, and thermal and pasting properties of flours from pea, lentil and chickpea cultivars. Food Chemistry, 111, 316-321.

Claessens, M., Calame, W., Siemensma, A., Van Baak, M., and Saris, W. (2009). The effect of different protein hydrolysate/carbohydrate mixtures on postprandial glucagon and insulin responses in healthy subjects. European Journal of Clinical Nutrition, 63, 48-56.

Claessens, M., Calame, W., Siemensma, A., Saris, W., and Van Baak, M. (2007). The thermogenic and metabolic effects of protein hydrolysate with or without a carbohydrate load in healthy male subjects. Metabolism, 56, 1051-1059.

Claessens, M., Saris, W. H. M., and van Baak, M. A. (2008). Glucagon and insulin responses after ingestion of different amounts of intact and hydrolysed proteins. British Journal of Nutrition, 100, 61-69.

Clark, D., and Pazdernik., N. (2016). Biotechnology (2^{nd} edition). Amsterdam. Elsevier, APCell. 850p.

Clark, S and Alison M. Duncan. (2017). The Role of Pulses in Satiety, Food Intake and Body Weight Management. Journal of Functional Foods, 38, 612-623.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. Trends in Food Science and Technology, 11, 254-262.

Colonna, P., Buléon, A., and Lemarié, F. (1988). Action of bacillus subtilis alpha-amylase on native wheat starch. Biotechnology and Bioengineering, 31, 895-904.

Colonna, P., Buleon, A., and Mercier, C. (1981). Pisum sativum and Vicia faba carbohydrates: structural studies of starches. Journal of Food Science, 46, 88-93.

Colonna, P., Barr, J., Cloarec, D., Bornet, F., Gouilloudt, S., and Galmiche, J. (1990). Enzymic susceptibility of starch from pasta. Journal of Cereal Science, 11, 59-70.

Cooke, D., and Gidley, M. (1992). Loss of crystalline and molecular order during starch gelatinisation: Origin of the enthalpic transition. Carbohydrate Research, 227, 103-112.

Cronin, C., and Shanahan, F. (1997). Insulin-dependent diabetes mellitus and coeliac disease. The Lancet, 349, 1096-1097.

Cummings, J., Beatty, E., Kingman, S., Bingham, S., and Englyst, H. (1996). Digestion and physiological properties of resistant starch in the human large bowel. British Journal of Nutrition, 75, 733-747.

Damodaran, S. (1997). Food proteins and their applications. New York. Dekker. 694p.

Dartois, A., Singh, J., Kaur, L., and Singh, H. (2010). Influence of Guar Gum on the *In Vitro* Starch Digestibility—Rheological and Microstructural Characteristics. Food Biophysics , 5, 149-160.

Davydova, N., Leont'ev, S., Genin, Y., Sasov, A., and Bogracheva, T.(1995). Some Physico-Chemical Properties of Smooth Pea Starches, 27, 109-115.

Diepvens, K., Haberer, D., and Westerterp-Plantenga, M. S. (2008). Different proteins and biopeptides differently affect satiety and anorexigenic/orexigenic hormones in healthy humans. International Journal of Obesity, 32, 510-8.

Dikeman, C., and Fahey, G. (2006). Viscosity as related to dietary fiber: A review. Critical Reviews in Food Science and Nutrition, 46, 649-663.

Dilawari, J., Kamath, P., Batta, R., Mukewar, S., and Raghavan, S. (1981). Reduction of postprandial plasma glucose by bengal gram dal (Cicer arietinum) and rajmah (Phaseolus vulgaris). The American Journal of Clinical Nutrition, 34, 2450-2453.

Dhital, S.,Warren, J., Butterworth, P., Ellis, P and Gidley, M. (2017) Mechanisms of starch digestion by α-amylase—Structural basis for kinetic properties. Critical Reviews in Food Science and Nutrition, 57:5, 875-892

Donner, E., Chung, H., and Liu, Q. (2011). Resistant starches in foods. In: Comprehensive Biotechnology (Second Edition), M. Moo-Young (Ed.), Academic Press, Burlington. 534p.

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

Dreher, M., Dreher, C., and Berry, J. (1984). Starch digestibility of foods: A nutritional perspective. Clinical Reviews in Food Science and Nutrition, 20, 47-71.

Dupont, D., Bordoni, A., Brodkorb, A., Capozzi, F., Cirkovic, T., Corredig, M., and Wickham, M.(2011). An international network for improving health properties of food by sharing our knowledge on the digestive process. Food Digestion, 2, 23-25.

Edwards, C., Johnson, I., and Read, N. (1988). Do viscous polysaccharides slow absorption by inhibiting diffusion or convection? European Journal of Clinical Nutrition, 42, 307-312.

El Khoury, D., Cuda, C., Luhovyy, B., and Anderson, G. (2012). Beta glucan: Health benefits in obesity and metabolic syndrome. Journal of Nutrition and Metabolism, 2012, 1-28.

Elgadir, M., Bakar, J., Zaidul, I., Abdul Rahman, R,. Abbas, K., Hashim, D., and Karim, R. (2009). Thermal behavior of selected starches in presence of other food ingredients studied by differential scanning calorimetery (DSC)-Review. Comprehensive Reviews in Food Science and Food Safety, 8, 195-201.

Eliasson, A., and Tjerneld, E. (1990). Adsorption of wheat proteins on wheat starch granules. Cereal Chemistry, 67, 366-372.

Englyst, H., Kingman, S., and Cummings, J. (1992). Classification and measurement of nutritionally important starch fractions. European Journal of Clinical Nutrition, 46, 33-50.

Englyst, H., and Hudson, J. (1996). The classification and measurement of dietary carbohydrates. Food Chemistry, 57, 15-21.

Fasano, A., and Catassi, C. (2012). Celiac disease. New England Journal of Medicine, 367, 2419-2426.

Fernández-Alonso, M., Diaz, D., Berbis, M., Filipa, M., Canada, J., and Jimenez, J., A. (2012). Protein-carbohydrate interactions studied by NMR: from molecular recognition to drug design. Current Protein Peptide Science, 13, 816-830.

Fernández-Gutiérrez, J., Martín-Martínez, S., Martínez-Bustos, F., and Cruz-Orea, A. (2004). Physicochemical properties of casein-starch interaction obtained by extrusion process. Starch-Stärke, 56, 190-198.

Eerlingen, R., Crombez, M., and Delcour, J. (1993). Enzyme-resistant starch. I. quantitative and qualitative influence of incubation time and temperature of autoclaved starch on resistant starch formation. Cereal Chemistry, 70, 339-344.

Eerlingen, R., Jacobs, H., and Delcour, J. (1994). Enzyme-resistant starch. V. effect of retrogradation of waxy maize starch on enzyme susceptibilityandnbsp; Cereal Chemistry, 71, 351-355.

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., and Portmann, R. (2016). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. Food Research International, 88, 217-225.

Eliasson, A. (1986). On the effects of surface active agents on the gelatinization of starch — a calorimetric investigation. Carbohydrate Polymers, 6, 463-476.

Englyst, H., and Cummings, J. (1987). Digestion of polysaccharides of potato in the small intestine of man. The American Journal of Clinical Nutrition, 45, 423-431.

Englyst, H., Wiggins, H., and Cummings, J. (1982). Determination of the non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates. Analyst, 17, 37-318.

Evans, A., and Thompson, D. (2004). Resistance to α-amylase digestion in four native high-amylose maize starches. Cereal Chemistry, 81, 31-37.

Ezeogu, L., Duodu, K., Emmambux, M., and Taylor, J. (2008). Influence of cooking conditions on the protein matrix of sorghum and maize endosperm flours. Cereal Chemistry, 85, 397-402.

Esteves, F., Pinheiro, A., and Alfenas, R. (2011). Impact of different protein sources in the glycemic and insulinemic responses. Nutricion Hospitalaria, 26, 669-676.

Fannon,JE., Hauber, RJ and BeMiller, JN. (1992). Surface pores of starch granulesandnbsp; Cereal Chemistry, 69

FAO. Fact Sheets. International Year of Pulses." http://www.fao.org/pulses-2016/communications-toolkit/fact-sheets/en/. Accesed 5 July 2017.

"FAO. International year of pulses, http://www.fao.org/pulses-2016/about/en/. Accessed 27 September 2017

Faulks, R., and Bailey, A. (1990). Digestion of cooked starches from different food sources by porcine α-amylase. Food Chemistry, 36, 191-203.

Fersht, A. (2006). Structure and mechanism in protein science (7th edition). New York. Freeman. 631p.

Fleming, S. (1978). Microscopic evaluation of bread fortified with concentrated plant proteins. Cereal Chemistry, 53, 373-382.

Frayn, K. 2010. Metabolic Regulation: A Human Perspective. (3rd edition). Oxford. Wiley Blackwell. 384p.

Fredriksson, H., Silverio, J., Andersson, R., Eliasson, A., and Man, P. (1998). The influence of amylose and amylopectin characteristics on gelatinization and retrogradation properties of different starches. Carbohydrate Polymers, 35, 119-134.

French, D. (1972). Fine structure of starch and its relationship to the organization of starch granules. Journal of the Japanese Society of Starch Science, 19, 8-25.

Frid, A., Nilsson, M., Holst, J., and Björck, I. (2005). Effect of whey on blood glucose and insulin responses to composite breakfast and lunch meals in type 2 diabetic subjects. The American Journal of Clinical Nutrition, 82, 69.

Fryar, C., Carroll, M., and Ogden, C. Prevalence of overweight, obesity, and extreme obesity among adults: United states, trends 1960–1962 through 2009–2010. Hyattsville, MD: National Center for Health Statistics (2012). http://www.stevesaenz.com/uploads/1/0/6/4/10642571/prevalence_of_overweight_obesity_and_extreme_obesity_among_adults.pdf. Accessed 9 August 2017.

Fuentes-Zaragoza, E., Riquelme-Navarrete, M., Sánchez-Zapata, E., and Pérez-Álvarez, J. (2010). Resistant starch as functional ingredient: A review. Food Research International, 43, 931-942.

Gaesser, G., and Angadi, S. (2012). Gluten-free diet: Imprudent dietary advice for the general population? Journal of the Academy of Nutrition and Dietetics, 112, 1330-1333.

Gallant, D., Bouchet, B., Buléon, A., and Pérez, S. (1992). Physical characteristics of starch granules and susceptibility to enzymatic degradation. European Journal of Clinical Nutrition, 46, 3-16.

Gallant, D., Bouchet, B., and Baldwin, P. (1997). Microscopy of starch: Evidence of a new level of granule organization. Carbohydrate Polymers, 32(3), 177-191.

Gardner, D., Schwartz, L., Krista, M., and Merimee, T. (1984). Dietary pectin and glycemic control in diabetes. Diabetes Care, 7, 143-146.

Gernat, C., Radosta, S., Damaschun, G., and Schierbaum, F. (1990). Supramolecular structure of legume starches revealed by X-Ray scattering. Starch - Stärke, 42, 175-178.

Gidley, M., Cooke, D., Darke, A., Hoffmann, R., Russell, A., and Greenwell, P. (1995). Molecular order and structure in enzyme-resistant retrograded starch. Carbohydrate Polymers, 28, 23-31.

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

Goesaert, H., Brijs, K., Veraverbeke, W., Courtin, C., Gebruers, K., and Delcour, J. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. Trends in Food Science Technology, 16, 12-30.

Gogoi, B., Oswalt, A., and Choudhury, G. (1996). Reverse screw element (s) and feed composition effects during Twin-Screw extrusion of rice flour and fish muscle blends. Journal of Food Science, 61, 590-595.

Gomand, S., Lamberts, L., Derde, L., Goesaert, H., Vandeputte, G., Goderis, B., and Delcour, J. (2010). Structural properties and gelatinisation characteristics of potato and cassava starches and mutants thereof. Food Hydrocolloids, 24, 307-317.

Granfeldt, Y., Drews, A., and Björck, I. (1995). Arepas made from high amylose corn flour produce favorably low glucose and insulin responses in healthy humans. The Journal of Nutrition, 125, 459-465.

Grard, C., Barron, C., Colonna, P., and Planchot, V. (2001). Amylose determination in genetically modified starches. Carbohydrate Polymers, 44, 19-27.

Gruchala, L and Pomeranz Y. (1993). Resistant starch: Studies using differential scanning calorimetry. Cereal Chemistry, 70, 163-170.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., and Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. Trends in Biotechnology, 30, 591-600.

Guerrieri, N., Eynard, L., Lavelli, V., and Cerletti, P. (1997). Interactions of protein and starch studied through amyloglucosidase action. Cereal Chemistry, 74, 846-850.

Hamada, J. S. (2000). Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. Journal of Food Science, 65, 305-310.

Hanson, E. A., and Katz, J. R. (1934). Abhandlungen zur Physikalischen Chemie der stärke und der Brotbereitung. Zeitschrift Fr Physikalische Chemie, 169, 135-142.

Haralampu, S. G. (2000). Resistant starch—a review of the physical properties and biological impact of RS3. Carbohydrate Polymers, 41, 285-292.

Harvard Medical School. "Glycemic Index and Glycemic Load for 100+ Foods." https://www.health.harvard.edu/diseases-and-conditions/glycemic-index-and-glycemic-load-for-100-foods. Accesed 10 August 2017.

Hasjim, J., Lee, S., Hendrich, S., Setiawan, S., Ai, Y., and Jane, J. (2010). Characterization of a novel resistant-starch and its effects on postprandial plasma-glucose and insulin responses. Cereal Chemistry, 87, 262.

Health Canada. (2017). List of dietary fibres reviewed and accepted by health canada's food directorate. https://www.canada.ca/en/health-canada/services/publications/food-nutrition/list-reviewed-accepted-dietary-fibres.html. Accesed 20 October 2017.

Hesso, N., Marti, A., Le-Bail, P., Loisel, C., Chevallier, S., Le-Bail, A., and Seetharaman, K. (2015). Conformational chages of polymers in model batter systems. Food Hydrocolloids, 51, 101-107.

Hizukuri, S. (1986). Polymodal distribution of the chain lengths of amylopectins, and its significance. Carbohydrate Research, 147, 342-347.

Hollar, C., Parris, N., Hsieh, A., and Cockley, K. (1995). Factors affecting the denaturation and aggregation of whey proteins in heated whey protein concentrate mixtures. Journal of Dairy Science, 78, 260-267.

Holm, J., Björck, I., Ostrowska, S., Eliasson, A., Asp, N., Larsson, K., and Lundquist, I. (1983). Digestibility of amylose-lipid complexes in-vitro and in-vivo. Starch - Stärke, 35, 294-297.

Holm, J., Koellreutter, B., and Würsch, P. (1992). Influence of sterilization, drying and oat bran enrichment of pasta on glucose and insulin responses in healthy subjects and on the rate and extent of *in vitro* starch digestion. European Journal of Clinical Nutrition, 46, 629-640.

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

Hoover, R. (2001). Composition, molecular structure, and physicochemical properties of tuber and root starches: A review. Carbohydrate Polymers, 45, 253-267.

Hoover, R., Hughes, T., Chung, H., and Liu, Q. (2010). Composition, molecular structure, properties, and modification of pulse starches: A review. Food Research International, 43, 399-413.

Hoover, R., and Ratnayake, W. (2002). Starch characteristics of black bean, chick pea, lentil, navy bean and pinto bean cultivars grown in Canada. Food Chemistry, 78, 489-498

Hoover, R. and Ratnayake. W. (2001). Determination of total amylose content of starch. Current Protocols in Food Analytical Chemistry. John Wiley and Sons Ltd.

Hoover, R. and Sosulski. F. (1985). Studies on the functional characteristics and digestibility of starches from Phaseolus Vulgaris biotypes. Starch – Stärke, 37, 181-191.

Hoover, R., and Sosulski, F. W. (1991). Composition, structure, functionality, and chemical modification of legume starches: A review. Canadian Journal of Physiology and Pharmacology, 69, 79-92.

Hoover, R., and Zhou, Y. (2003). *In vitro* and in vivo hydrolysis of legume starches by α-amylase and resistant starch formation in legumes—a review. Carbohydrate Polymers, 54, 401-417.

Hoseney, R. C. (1994). Principles of cereal science and technology (2nd edition). St. Paul. American Assoc. of Cereal Chemists. 378p.

Hur, S., Lim, B., Decker, E., and McClements, D. (2011). *In vitro* human digestion models for food applications. Food Chemistry, 125, 1-12.

Imberty, A., Chanzy, H., Pérez, S., Bulon, A., and Tran, V. (1988). The double-helical nature of the crystalline part of A-starch. Journal of Molecular Biology, 201, 365-378.

Imberty, A., and Perez, S. (1988). A revisit to the three-dimensional structure of b-type starch. Biopolymers, 27(8), 1205-1221.

INRA. (2011). Cost infogest. http://www.cost-infogest.eu/. Accesed 18 October 2017.

Jacobs, H., Eerlingen R., Clauwaert, W., and Delcour, J. (1995). Influence of annealing on the pasting properties of starches from varying botanical sources. Cereal Chemistry, 72, 480–487.

James, J., and Lee, B. (1997). Glucoamylases: Microbial sources, industrial applications and molecular biology - a review. Journal of Food Biochemistry, 21, 1-52.

Jamilah, B., Mohamed, A., Abbas, K., Rahman, R., Karim, R., and Hashim, D. (2009). Protein-starch interaction and their effect on thermal and rheological characteristics of a food system: a review. Journal of Food, Agricule and Environment, 7, 169-174.

Jane, J., Chen, Y., Lee, L., McPherson, F., Wong, K., Radosavljevic, M., and Kasemsuwan, T. (1999). Effects of amylopectin branch chain length and amylose content on the gelatinization and pasting properties of starch. Cereal Chemistry, 76, 629-637.

Jane, J., Wong, K., and McPherson, A. (1997). Branch-structure difference in starches of A- and B-type X-ray patterns revealed by their naegeli dextrins. Carbohydrate Research, 300, 219-227.

Jenkins, D., Ghafari, H., Wolever, T.,Taylor, R.,Jenkins, A., Barker, H., Fielden, H., and Bowling, A. (1982). Relationship between rate of digestion of foods and post-prandial glycaemia. Diabetologia, 22, 450-455.

Jenkins, D., Kendall, C., Augustin, L., Franceschi, S., Hamidi, M., Marchie, A., Jenkins, A., and Axelsen, M. (2002). Glycemic index: overview of implications in health and disease. American Journal of Clinnical Nutrition, 76, 266-273.

Jenkins D., Leeds A., Gassull M., Cochet, B., and Alberti, G. (1977). Decrease in postprandial insulin and glucose concentrations by guar and pectin. Annals of Internal Medicine, 86, 20-23.

Jenkins, D., Leeds, A., Wolever, T., Goff, D., George, K., Alberti, M., and Hockaday, R. (1976). Unabsorbable carbohydrates and diabetes: Decreased post-prandial hyperglycæmia. The Lancet, 308, 172-174.

Jenkins, D., Thomas., M. Wolever, A., Leeds, R., Gassull, M., Haisman, P., Dilawari, J., and Alberti, K. (1978). Dietary fibres, fibre analogues, and glucose tolerance: Importance of viscosity. The British Medical Journal, 1, 1392-1394.

Jenkins, D., Thorne , M., Wolever , T., Jenkins, A., Rao, A., and Thompson, L. (1987). The effect of starch-protein interaction in wheat on the glycemic response and rate of *in vitro* digestion. American Journal of Clinical Nutrition, 45, 946-951.

Jenkins, D., Wolever, T., Kalmusky, J., Guidici, S., Giordano, C., Patten, R., and Buckley, G. (1987). Low-glycemic index diet in hyperlipidemia: Use of traditional starchy foods. The American Journal of Clinical Nutrition, 46, 66-71.

Jenkins, D., Wolever, T., Taylor, R., Barker, H., and Fielden, H. (1980). Exceptionally low blood glucose response to dried beans: Comparison with other carbohydrate foods. British Medical Journal, 281, 578-580.

Jenkins, D., Wolever, T., Taylor, R., Barker, H., Fielden, H., Baldwin, J. M., and Goff, D. (1981). Glycemic index of foods: A physiological basis for carbohydrate exchange. The American Journal of Clinical Nutrition, 34, 362-366.

Jenkins, P. J. (1994). X-ray and neutron scattering studies of starch granule structure. PhD thesis. University of Cambridge (England).

Jiang, H., Campbell, M., Blanco, M., and Jane, J. (2010). Characterization of maize amylose-extender (ae) mutant starches: Part II. structures and properties of starch residues remaining after enzymatic hydrolysis at boiling-water temperature. Carbohydrate Polymers, 80, 1-12.

Juliano, B. (1971). A simplified assay for milled-rice amylose. Cereal Science Today, 16, 334-360.

Kannan, A., Hettiarachchy, N., and MArshall, M. (2012). Food Proteins–Peptides: Chemistry and structure. Food proteins and peptides: Chemistry, functionality, interactions, and commercialization (1st edition). London. CRC Press.

Keeling, P. L., and Myers, A. M. (2010). Biochemistry and genetics of starch synthesis. Annual Review of Food Science and Technology, 1, 271-303.

Kilara, A. (1985). Enzyme-modified protein food ingredients. Process Biochemistry, 20, 149-158.

Kim, H., and Huber, K. (2008). Channels within soft wheat starch A- and B-type granules. Journal of Cereal Science, 48, 159-172.

Kizil, R., Irudayaraj, J., and Seetharaman, K. (2002). Characterization of irradiated starches by using FT-raman and FTIR spectroscopy. Journal of Agricultural and Food Chemistry, 50, 3912-3918.

Klindt-Toldam, S., Larsen, S., Saaby, L., Olsen, L., Svenstrup, G., Müllertz, A., Knøchel, S., Heimdal, H. Nielsen, D., Zielińska, D. (2016). Survival of Lactobacillus acidophilus NCFM® and Bifidobacterium lactis HN019 encapsulated in chocolate during *in vitro* simulated passage of the upper gastrointestinal tract. LWT - Food Science and Technology, 74, 404-410.

Klucinec, J., and Thompson, D. (1998). Fractionation of high-amylose maize starches by differential alcohol precipitation and chromatography of the fractions. Cereal Chemistry, 75, 887-896.

Koehler P., Wieser H. (2013) Chemistry of Cereal Grains. In: Gobbetti M., Gänzle M. (eds) Handbook on Sourdough Biotechnology. Springer, Boston, MA. 298p.

Kong, F., and Singh, R. (2010). A human gastric simulator (HGS) to study food digestion in human stomach. Journal of Food Science, 75, 627-635.

Kopf-Bolanz, K., Schwander, F., Gijs, M., Vergères, G., Portmann, R., and Egger, L. (2012). Validation of an *in vitro* digestive system for studying macronutrient decomposition in humans. The Journal of Nutrition, 142(2), 245-250.

Koroteeva, D., Kiseleva, V., Krivandin, A., Shatalova, O., Błaszczak, W., Bertoft, E., and Yuryev, V. (2007). Structural and thermodynamic properties of rice starches with different genetic background: Part 2. Defectiveness of different supramolecular structures in starch granules. International Journal of Biological Macromolecules, 41, 534-547.

Larner, J., Illingworth, B., Cori, G., and Cori, C. (1952). Structure of glycogens and amylopectins II. analysis by stepwise enzymatic degradation. Journal of Biological Chemistry, 199, 641-651.

Lauro, M., Forssell, P., Suortti, M., Hulleman, S., and Poutanen, K. (1999). Alpha-amylolysis of large barley starch granules. Cereal Chemistry, 76, 925-930.

Lauro, M., Suortti, T., Autio, K., Linko, P., and Poutanen, K. (1993). Accessibility of barley starch granules to α-amylase during different phases of gelatinization. Journal of Cereal Science, 17, 125-136.

Leach, H., and Schoch, T. (1961). Structure of the starch granule. II. Action of various amylases on granular starches. Cereal Chemistry, 38, 34-46.

Lee, E., Mercier, C., and Whelan, W. (1968). A method for the investigation of the fine structure of amylopeclin. Archives of Biochemistry and Biophysics, 125, 1028-1030.

Lelievre, J., and Mitchell, J. (1975). A Pulsed NMR Study of Some Aspects of Starch Gelatinization. Starch - Stärke, 27, 113-115.

Lentner, C., and Wink, A. (1981). Units of measurement, body fluids, composition of the body, nutrition. (8th edition). Switzerland. Novartis. 298p.

Lian, X., Zhu, W.,Wen, Y., Li, L., & Zhao, X. (2013). Effects of soy protein hydrolysates on maize starch retrogradation studied by IR spectra and ESI-MS analysis. International Journal of Biological Macromolecules, 59, 143-150.

Li, J., Vasanthan, T., Gao, J., Naguleswaran, S., Zijlstra, R.T., and Bressler, D.C. (2014). Resistant starch excaped from ethanol production: evidence from confocal laser scanning microscopy of distiller's dired grains with solubles (DDGS). Cereal Chemistry, 91, 130-138.

Li, L., Jiang, H., Campbell, M., Blanco, M., and Jane, J. (2008). Characterization of maize amylose-extender (ae) mutant starches. part I: Relationship between resistant starch contents and molecular structures. Carbohydrate Polymers, 74, 396-404.

Li, W., Dobraszczyk, B., Dias, A., and Gil, A. (2006). Polymer conformation structure of wheat proteins and gluten subfractions revealed by ATR-FTIR. Cereal Chemistry, 83, 407-410.

Liu, Q., Gu, Z., Donner, E., Tetlow, I., and Emes, M. (2007). Investigation of digestibility *in vitro* and physicochemical properties of A- and B-Type starch from soft and hard wheat flour. Cereal Chemistry, 84, 15-21.

Livesey, G., Taylor, R., Livesey, H., and Liu, S. (2013). Is there a dose-response relation of dietary glycemic load to risk of type 2 diabetes? meta-analysis of prospective cohort studies. The American Journal of Clinical Nutrition, 97, 584-596.

Lowy, G., Sargeant , J., and Schofield, J. (1981). Wheat starch granule protein: the isolation and characterisation of a salt-extractable protein from starch granules. Journal of the Science of Food and Agriculture, 32, 371-377.

Lu, Z., Donner, E., Yada, R., and Liu, Q. (2016). Physicochemical properties and *in vitro* starch digestibility of potato starch/protein blends. Carbohydrate Polymers, 154, 214-222.

Ludwig, D. (2002). The glycemic index: Physiological mechanisms relating to obesity, diabetes, and cardiovascular disease. Jama, 287, 2414-2423.

Lund, D., and Lorenz, K. (1984). Influence of time, temperature, moisture, ingredients, and processing conditions on starch gelatinization. Critical Reviews in Food Science and Nutrition, 20, 249-273.

Maher, T. (1994). Safety concerns regarding supplemental amino acids: Results of a study. Institute of Medicine (US) Committee on Military Nutrition Research; Marriott BM, editor. Washington National Academies Press.

Mahmoud, M. I. (1994). Physicochemical and functional properties of protein hydrolysates in nutritional products. Food Technology, 48, 89-113.

Mainville, I., Arcand, Y., and Farnworth, E. (2005). A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. International Journal of Food Microbiology, 99, 287-296.

Majeed, T., Wani, I., and Hussain, P. (2017). Effect of dual modification of sonication and γ-irradiation on physicochemical and functional properties of lentil (lens culinaris L.) starch. Internationa Jounal of Biologycal Macromolecules, 101, 358-365.

Rao, M., Tanksale, A., Ghatge, M., and Deshpande, V. (1998). Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews, 62, 597-635.

Manders, R., Wagenmakers, A., Koopman, R., Zorenc, A., Menheere, P., Wyse, D., and Van Loon, L. (2005). Co-ingestion of a protein hydrolysate and amino acid mixture with carbohydrate improves plasma glucose disposal in patients with type 2 diabetes. American Journal of Clinical Nutrition, 82, 76-83.

Manders, R., Praet, S., Vikström, M., Saris, W., and van Loon, L. (2009). Protein hydrolysate co-ingestion does not modulate 24 h glycemic control in long-standing type 2 diabetes patients. European Journal of Clinical Nutrition, 63, 121-126.

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

Manners, D., and Matheson, N. K. (1981). The fine structure of amylopectin. Carbohydrate Research, 90, 99-110.

Marciani, L., Faulks, R., Wickham, M. S., Bush, D., Pick, B., Wright, J., Cox, E.F., Fillery-Travis, A.,Gowland, P., and Spiller, R. (2008). Effect of intra-gastric acid stability of fat emulsions on gastric emptying, plasma lipid profile and postprandial satiety. British Journal of Nutrition, 101, 919-928.

Marciani, L., Gowland, P., Fillery-Travis, A., Manoj, P., Wright, J., Smith, A., Young, P., Moore, R.., and Spiller, R. (2001a). Assessment of antral grinding of a model solid meal with echo-planar imaging. American Journal of Physiology-Gastrointestinal and Liver Physiology, 280, 844-849.

Marciani, L., Gowland, P., Spiller, R, Manoj, P., Moore, R., Young, P., and Fillery-Travis, A. (2001b). Effect of meal viscosity and nutrients on satiety, intra-gastric dilution, and emptying assessed by MRI. American Journal of Physiology-Gastrointestinal and Liver Physiology, 280, 1227-1233.

Marshall, W., and Chrastil, J. (1992). Interaction of food proteins with starch. In B. J. F. Hudson (Ed.), Biochemestry of food proteins .Elsevier. 419p.

Matheson, N., and Caldwell, R. (2008). Modeling of α (1–4) chain arrangements in α (1–4)(1–6) glucans: The action and outcome of β-amylase and pseudomonas stutzeri amylase on an α (1–4)(1–6) glucan model. Carbohydrate Polymers, 72, 625-637.

Matthey, F., and Hanna, M. (1997). Physical and functional properties of twin-screw extruded whey protein concentrate–corn starch blends. LWT-Food Science and Technology, 30, 359-366.

McCleary, B., and Monaghan, D. (2002). Measurement of resistant starch. Journal of AOAC International, 85, 665-675.

McCrory, M., Hamaker, B., Lovejoy, J., and Eichelsdoerfer, P. (2010). Pulse consumption, satiety, and weight management. Advances in Nutrition, 1, 17-30.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., and Picque, D. (2014). Validation of a new *in vitro* dynamic system to simulate infant digestion. Food Chemistry, 145, 1039-1045.

Meyer, K., and Bernfeld, P. (1940). Recherches sur l´amidon V. l´amylopectine. Helvetica, 23, 865-875.

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

Minekus, M. (2015). The TNO gastro-intestinal model (TIM). In Cotter, P., López-Expósito, I., Kleiveland, C.,Lea, T., Mackie, A., Requena, T., Swiatecka, D., Wichers, H. (Ed.). Springer International Publishing. 338p.

Minekus, M., Marteaul, P., Havenaarl, R., and Huis, J. (1995). A multicompartmental dynamic computercontrolled model simulating the stomach and small intestine, 23, 197-209.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., and Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food - an international consensus. Food and Function, 5, 1113-1124.

Mojica, L., Dia, V., and Mejia, E. (2015). Soy proteins. In Z. Ustunol (Ed.), Applied food protein chemistry. Chichester, UK: John Wiley and Sons, Ltd. 528p.

Monteiro Desouza, P., Deassis Bittencourt, M., Canielles Caprara, C., Defreitas, M., Coppini Dealmeida,R., Silveira, D., and Oliveira Magalhães, P. (2015). A biotechnology perspective of fungal proteases. Brazilian Journal of Microbiology, 46, 337-346.

Moore, W., and Carter, J. (1974). Protein-carbohydrate interactions at elevated temperatures. Journal of Texture Studies, 5, 77-88.

Morita, T., Hayashi, J., Motoi, H., Yagishita, T., Takeya, K., Sugiyama, K., and Kiriyama, S. (2005). *In vitro* and in vivo digestibility of recrystallized amylose and its application for low glycemic foods. Journal of Food Science, 70, 179-185.

Muraki, M. (2002). The importance of CH/ π interactions to the function of carbohydrate binding proteins. Protein and Peptide Letters, 9, 195-209.

Naguleswaran, S., Li, J., Vasanthan, T., and Bressler, D. (2011). Distribution of granule channels, protein, and phospholipid in triticale and corn starches as revealed by confocal laser scanning microscopy. Cereal Chemistry, 88, 87-94.

Nelson, D., and Cox, M. (2017). Lehninger principles of biochemistry (7th edition). New York, NY. Freeman. 1328p.

Nielsen, P., Petersen, D., and Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. Journal of Food Science, 66, 642-646.

Nikuni, Z. (1978). Studies on starch granules. Starch-Stärke, 30, 105-111.

Nilsson, M., Stenberg, M., Frid, A., Holst, J., and Björck, I. (2004). Glycemia and insulinemia in healthy subjects after lactose-equivalent meals of milk and other food proteins: The role of plasma amino acids and incretins. The American Journal of Clinical Nutrition, 80, 1246.

Ogunmolasuyi, A., Egwim, E., Adewoyin, M., and Awoyinka, O. (2016). A comparative study of functional and structural properties of starch extracted from dioscorea rotundata and colocasia esculenta. Starch-Stärke, 68, 771–777.

Ohtani, T., Yoshino, T., Hagiwara, S., and Maekawa, T. (2000). High-resolution imaging of starch granule structure using atomic force microscopy. Starch-Stärke, 52, 150-153.

O'Dea, K., Nestel, P., and Antonoff, L. 1980. Physical factors influencing postprandial glucose and insulin responses to starch. The American Journal of Clinical Nutrition, 33, 760-765.

Osborne, T. B. (1924). The vegetable proteins (2nd edition.). London. Longmans, Green. 154p.

Owus-Ansah, F., Van de Voort, F., and Stanley, D. (1982). Determination of starch gelatinization by X-ray diffractometry. Cereal Chemistry, 59, 167-171.

Panahi, S., Ezatagha, A., Jovanovski, E., Jenkins, A., Temelli, F., Vasanthan, T., and Vuksan, V. (2014). Glycemic effect of oat and barley beta-glucan when incorporated into a snack bar: A dose escalation study. Journal of the American College of Nutrition, 33, 442-449.

Panahi, S., Ezatagha, A., Temelli, F., Vasanthan, T., and Vuksan, V. (2007). Beta-glucan from two sources of oat concentrates affects postprandial glycemia in relation to the level of viscosity. Journal of the American College of Nutrition, 26, 639-644.

Panyam, D., and Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. Trends in Food Science and Technology, 7, 120-125.

Parada, J., and Aguilera, J. (2009). *In vitro* digestibility and glycemic response of potato starch is related to granule size and degree of gelatinization. Journal of Food Science, 74, 34-38.

Parada, J., and Aguilera, J. (2012). Effect of native crystalline structure of isolated potato starch on gelatinization behavior and consequently on glycemic response. Food Research International, 45, 238-243.

Pazur, J. H., and Kleppe, K. (1962). The hydrolysis of alpha-D-glucosides by amyloglucosidase from aspergillus niger. The Journal of Biological Chemistry, 237, 1002-1006.

Peat, S., Whelan, W.J., and Thomas, G.J. (1952). Evidence of multiple branching in waxy maize starch. Journal of the Chemical Society (Resumed), 4536-4538.

Perera, C., and Hoover, R. (1999). Influence of hydroxypropylation on retrogradation properties of native, defatted and heat-moisture treated potato starches, 64, 361-375.

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

Petersen, B. L., Ward, L. S., Bastian, E. D., Jenkins, A. L., Campbell, J., and Vuksan, V. (2009). A whey protein supplement decreases post-prandial glycemia. Nutrition Journal, 8, 1-5.

Pins, J J and Kaur, H. (2006). A review of the effects of barley beta-glucan on cardiovascular and diabetic risk. Cereal Foods World, 51, 1-11.

Pitino, I., Randazzo, C. L., Mandalari, G., Curto, A. L., Faulks, R. M., Le Marc, Y., and Wickham, M. (2010). Survival of lactobacillus rhamnosus strains in the upper gastrointestinal tract. Food Microbiology, 27, 1121-1127.

Popov, D., Bulon, A., Burghammer, M., Chanzy, H., Montesanti, N., Putaux, J., and Riekel, C. (2009). Crystal structure of A-amylose: A revisit from synchrotron microdiffraction analysis of single crystals. Macromolecules, 42, 1167-1174.

Power, O., Hallihan, A., and Jakeman, P. (2009). Human insulinotropic response to oral ingestion of native and hydrolysed whey protein. Amino acids, 37, 333-339.

Prajapati, V., Jani, G., Moradiya, N., Randeria, N., Nagar, B., Naikwadi, N., and Variya, B. (2013). Galactomannan: A versatile biodegradable seed polysaccharide. International Journal of Biological Macromolecules, 60, 83-92.

Raja, R., Anusheela, V., Agasimani, S., Jaiswal, S., Thiruvengadam, V., Chibbar, R., and Ram, S. (2017). Validation and applicability of single kernel-based cut grain dip method for amylose determination in rice. Food Analytical Methods, 10, 442-448.

Ramdath, D., Simone, R., and Alison, M., and Duncan. A. 2016. The Role of Pulses in the Dietary Management of Diabetes, 40, 355-363.

Ramulu, P., and Rao, P. (1997). Effect of processing on dietary fiber content of cereals and pulses. Plant Foods for Human Nutrition (Dordrecht, Netherlands), 50, 249-257.

Ratnayake, W.S., Hoover, R., Shahidi, F., Parera, C. and Jane, J. 2001. Composition, molecular structure, and physicochemical properties of starches from four field pea (Pisum sativum L.) cultivars. Food Chemistry, 74, 189-202.

Rayas, P., Robinson, S., and Freeman, T. (1995). Situ location of a starch granule protein in Durum wheat endosperm by immunocytochemistry. Cereal Chemistry, 72, 269-279.

Regand, A., Tosh, S., Wolever, T., and Wood, P. (2009). Physicochemical properties of β-glucan in differently processed oat foods influence glycemic response. Journal of Agricultural and Food Chemistry, 57, 8831-8838.

Regina, A., Bird, A., Topping, D., Bowden, S., Freeman, J., Barsby, T., and Morell, M. (2006). High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proceedings of the National Academy of Sciences of the United States of America, 103, 3546-3551.

Ribotta, P., Colombo, A., and Rosell, C. (2011). Enzymatic modifications of pea protein and its application in protein–cassava and corn starch gels. Food Hydrocolloids, 27, 185-190.

Ridley, B., O'Neill, M., and Mohnen, D. (2001). Pectins: Structure, biosynthesis, and oligogalacturonide-related signaling, 57, 929-967.

Ring, S., Colonna, P., I'Anson, K., Kalichevsky, M., Miles, M., Morris, V., and Orford, P. (1987). The gelation and crystallisation of amylopectin. Carbohydrate Research, 162, 277-293.

Ring, S., Gee, J., Whittam, M., Orford, P., and Johnson, I. (1988). Resistant starch: Its chemical form in foodstuffs and effect on digestibility *in vitro*. Food Chemistry, 28, 97-109.

Roberts, A. (2016). The safety and regulatory process for amino acids in europe and the united states. The Journal of Nutrition, 146, 2635-2642.

Robin, J. P. (1974). Lintnerized starches. gel filtration and enzymatic studies of insoluble residues from prolonged acid treatment of potato starch. Cereal Chemistry, 51, 389-406.

Robyt, J., and French, D. (1967). Multiple attack hypothesis of α-amylase action: Action of porcine pancreatic, human salivary, and *aspergillus oryzae* α-amylases, 122, 8-16.

Rombouts, I., Lamberts, L., Celus, I., Lagrain, B., Brijs, K., and Delcour, J. A. (2009). Wheat gluten amino acid composition analysis by high-performance anion-exchange chromatography with integrated pulsed amperometric detection. Journal of Chromatography A, 1216, 5557-5562.

Rooney, L., and Pflugfelder, R. (1986). Factors affecting starch digestibility with special emphasis on sorghum and corn. Journal of Animal Science, 63, 1607-1623.

Roy, F., Boye, J., and Simpson, B. (2010). Bioactive proteins and peptides in pulse crops: Pea, chickpea and lentil. Food Research International, 43, 432-442.

Russell, P., Gough, B., Greenwell, P., Fowle, A., and Munro, H. (1987). A study by ESCA of the surface of native and chlorine treated wheat starch granules: the effects of various surface treatments. Journal of Cereal Science, 5, 83-100.

Ryan, K., and Brewer, M. (2007). In situ examination of starch granule-soy protein and wheat protein interactions. Food Chemistry, 104, 619-629.

Sajilata, M., Singhal, R., and Kulkarni, P. (2006). Resistant Starch: a review. Comprehensive Reviews in Food Science and Food Safety, 5, 1-17.

Salmerón, J., Manson, J., Stampfer, M., Colditz, G., Wing, A., and Willett, W. (1997). Dietary fiber, glycemic load, and risk of non—insulin-dependent diabetes mellitus in women. Jama, 277, 472-477.

Sandhya, C., Sumantha, A., Szakacs, G., & Pandey, A. (2005). Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solidstate fermentation. Process Biochemistry, 40, 2689- 2694.

Sarmadi, B., and Ismail, A. (2010). Antioxidative peptides from food proteins: A review. Peptides, 31, 1949-1956.

Savelkoul, F., Poel, V., and Tamminga, S. (1992). The presence and inactivation of trypsin inhibitors, tannins, lectins and amylase inhibitors in legume seeds during germination. A review. Plant Foods for Human Nutrition, 42, 71-85.

Schirmer, M., Höchstötter, A., Jekle, M., Arendt, E., and Becker, T. (2013). Physicochemical and morphological characterization of different starches with variable amylose/amylopectin ratio, Food Hydrocolloids, 32, 52-63.

Schoch, T. J. (1942). Fractionation of starch by selective precipitation with butanol. Journal of the American Chemical Society, 64, 2957-2961.

Seetharaman, K., and Bertoft, E. (2013). Perspectives on the history of research on starch part V: On the conceptualization of amylopectin structure. Starch-Stärke, 65, 1-7.

Seneviratne, H., and Biliaderis, C. (1991). Action of α-amylases on amylose-lipid complex superstructures. Journal of Cereal Science, 13, 129-143.

Shetty, R., Lineback, D., Seib, P. (1974). Determining the degree of starch gelatinization. Cereal Chemistry, 51, 364-375.

Shevkani, K., Singh, N., Kaur, A., and Rana, J. (2015). Structural and functional characterization of kidney bean and field pea protein isolates: A comparative study. Food Hydrocolloids, 43, 679-689.

Shewry, P. (2009). Wheat. Journal of Experimental Botany, 60, 1537-1553.

Shewry, P., Hawkesford, M., Piironen, V., Lampi, A., Gebruers, K., Boros, D., and Ward, J. (2013). Natural variation in grain composition of wheat and related cereals. Journal of Agricultural and Food Chemistry, 61, 8295-8303.

Shi , M., Zhanga, Z., Yua, S., Wang , K., Gilbert, R., and Gao, Q. (2014). Pea starch (Pisum sativum L.) with slow digestion property produced using β-amylase and transglucosidase. Food Chemistry, 164, 317–323.

Sievert, D., and Pomeranz, Y. (1989). Enzyme-resistant starch. I. characterization and evaluation by enzymatic, thermoanalytical, and microscopic methods. Cereal Chemistry, 66, 342-347.

Simpson, R., Neuberger, M., and Liu, T. (1976). Complete amino acid analysis of proteins form a single hydrolysate. Journal of Biological Chemistry, 251, 1936-1940.

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

Skerritt, J., Frend, A., Robson, L., and Greenwell, P. (1990). Immunological homologies between wheat gluten and starch granule proteins. Journal of Cereal Science, 12, 123-136.

Smith, A., Denyer, K., and Martin, C. (1997). The synthesis of the starch granule. Annual Review of Plant Biology, 48, 67-87.

Smith, C., Mollard, R., Luhovyy, B., and Anderson, H. (2012). The effect of yellow pea protein and fibre on short-term food intake, subjective appetite and glycaemic response in healthy young men. British Journal of Nutrition, 108, 74-80.

Soares, R., Scremin, F., and Soldi, V. (2005). Thermal stability of biodegradable films based on soy protein and corn starch. Macromolecular Symposia, 229, 258-265.

Srichuwong, S., and Jane, J. (2007). Physicochemical properties of starch affected by molecular composition and structures: A review. Food Science and Biotechnology, 16, 663-674.

Srinivasan, D., Kirk, P., and Fennema, O. (2007). Food chemistry (4^{th} edition). New York. Dekker. 1069p.

Statistics Canada. "Seeding Decisions Harvest Opportunities for Canadian Farmers." http://www.statcan.gc.ca/pub/95-640-x/2016001/article/14813-eng.htm. Accesed 30 October 2017.

Stevens, D., and Elton,G. (1971). Thermal properties of the starch/water system. I. measurement of heat of gelatinization by differential scanning calorimetry. Stärke, 23, 8-11.

Stewart, M., Nikhanj, S., Timm, D., Thomas, W., and Slavin, J. (2010). Evaluation of the effect of four fibers on laxation, gastrointestinal tolerance and serum markers in healthy humans. Annals of Nutrition and Metabolism, 56, 91-98.

Sujka, M. and Jamroz, J. (2007). Starch granule porosity and its changes by means of amylolysisandnbsp; International Agrophysics, 21, 107-113.

Sumantha, A., Larroche, C., and Pandey, A. (2006). Microbiology and industrial biotechnology of food-grade proteases: A perspective. Food Technology and Biotechnology, 44, 211-220.

Surget, A., and Barron, C. (2005). Histologie du grain de blé Industries des céréales. INRA. 3-7.

Svihus, B., Uhlen, A., and Harstad, O. (2005). Effect of starch granule structure, associated components and processing on nutritive value of cereal starch: A review. Animal Feed Science and Technology, 122, 303-320.

Takeda, Y., Hizukuri, S., and Juliano, B. (1989). Structures and amounts of branched molecules in rice amyloses. Carbohydrate Research, 186, 163-166.

Takeda, Y., Hizukuri, S., Takeda, C., and Suzuki, A. (1987). Structures of branched molecules of amyloses of various origins, and molar fractions of branched and unbranched molecules. Carbohydrate Research, 165, 139-145.

Takeda, Y., Maruta, N., and Hizukuri, S. (1992). Examination of the structure of amylose by tritium labelling of the reducing terminal. Carbohydrate Research, 227, 113-120.

Tang, H., Mitsunaga, T., and Kawamura, Y. (2006). Molecular arrangement in blocklets and starch granule architecture. Carbohydrate Polymers, 63, 555-560.

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

Tester, R., Qi, X., and Karkalas, J. (2006). Hydrolysis of native starches with amylases. Animal Feed Science and Technology, 10, 39-45.

Tharanathan, R. (2002). Food-derived carbohydrates - structural complexity and functional diversity. Critical Reviews in Biotechnology, 22, 65-84.

Thompson, D. B. (2000). Strategies for the manufacture of resistant starch. Trends in Food Science and Technology, 11, 245-253.

Thompson, T., Dennis, M., Higgins, L., Lee, A., and Sharrett, M. (2005). Gluten-free diet survey: Are americans with coeliac disease consuming recommended amounts of fibre, iron, calcium and grain foods? Journal of Human Nutrition and Dietetics, 18, 163-169.

Thuenemann, E., Mandalari, G., Rich, G., and Faulks, R. (2015). Dynamic gastric model (DGM). In Verhoeckx, K., Cotter, P., López-Expósito, I.., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., and Wichers, H. (Eds.), The impact of food bioactives on health. New York. Springer. 338p.

Tovar, J, Bjoerck, I., and Asp, N. (1990). Analytical and Nutritional Implications of Limited Enzymic Availability of Starch in Cooked Red Kidney Beans." Journal of Agricultural and Food Chemistry, 38, 488-493.

Tovar, J., Björck, I., and Asp, N. (1992). Incomplete digestion of legume starches in rats: A study of precooked flours containing retrograded and physically inaccessible starch fractions. The Journal of Nutrition, 122, 1500-1507.

Tovar, J., De Francisco, A., Bjorck, I., and Asp, N. (1991). Relationship between Microstructure and *in Vitro* Digestibility of Starch in Precooked Leguminous Seed Flours. Food Structure, 10, 19-26.

United Nations. Resolution adopted by the general assembly on 20 december 2013, 68/231. International year of pulses 2016. A/RES/68/231. (2013). http://www.un.org/en/ga/search/view_doc.asp?symbol=A/RES/68/231. Accessed 8August 2017.

US Food and Drug Administration. Questions and Answers: Gluten-Free Food Labeling Final Rule. (2014). https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm362880.htm. Accessed 8August 2017.

Ustunol, Z. (2015). Amino acids, peptides, and proteins. Applied food protein chemistry. John Wiley and Sons, Ltd. 528p.

Van der Maarel, M., Van der Veen, B., Uitdehaag, J., Leemhuis, H., and Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family, Journal of Biotechnology, 94, 137-155.

Van Loon, L. (2007). Amino acids as pharmaco-nutrients for the treatment of type 2 diabetes. Immunology. Endocrine and Metabolic Agents in Medicinal Chemistry, 7, 39-48.

Van Loon, L., Saris, W., Verhagen, H., and Wagenmakers, A. (2000). Plasma insulin responses after ingestion of different amino acid or protein mixtures with carbohydrate. American Journal of Clinical Nutrition, 72, 96-105.

Van Soest, J., Tournois, H., de Wit, D., and Vliegenthart, J. (1995). Short-range structure in (partially) crystalline potato starch determined with attenuated total reflectance fourier-transform IR spectroscopy. Carbohydrate Research, 279, 201-214.

Van Soest, J., and Vliegenthart, J. (1997). Crystallinity in starch plastics: Consequences for material properties, 15, 208-213.

Vandeputte, G., Vermeylen, R., Geeroms, J., and Delcour, J. (2003). Rice starches. I. structural aspects provide insight into crystallinity characteristics and gelatinisation behaviour of granular starch. Journal of Cereal Science, 38, 43-52.

Vardakou, M., Mercuri, A., Naylor, T. A., Rizzo, D., Butler, J. M., Connolly, P. C., Wickham, M.S.J., Faulks, R. M. (2011a). Predicting the human in vivo performance of different oral capsule shell types using a novel *in vitro* dynamic gastric model. International Journal of Pharmaceutics,149, 192-199.

Vardakou, M., Mercuri, A., Barker, S. A., Craig, D. Q., Faulks, R. M., and Wickham, M. S. (2011b). Achieving antral grinding forces in biorelevant *in vitro* models: Comparing the USP dissolution apparatus II and the dynamic gastric model with human in vivo data. Aaps Pharmscitech, 12, 620-626.

Venugopal, V. (2011). Marine Polysaccharides: Food Applications. Boca Raton. CRC Press. 396p.

Versantvoort, C., Oomen, A., Van de Kamp, E., Rompelberg, C., and Sips, A. (2005). Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. Food and Chemical Toxicology, 43, 31-40.

Vishwanatha, K., Rao, A., and Singh, S. (2010). Acid protease production by solid-state fermentation using *aspergillus oryzae* MTCC 5341: Optimization of process parameters. Journal of Industrial Microbiology and Biotechnology, 37, 129-138.

Waigh, T., Kato, K., Donald, A., Gidley, M., Clarke, C., and Riekel, C. (2000). Side-chain liquid-crystalline model for starch. Starch-Stärke, 52, 450-460.

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

Wang, N., and Daun, J. K. The chemical composition and nutritive value of canadian pulses. Canadian Grain Commission Report. (2004). http://www.pulsecanada.com/uploads/c4/91/c491f652f0cf53390d9a5b86aa63aeea/The-Chemical-Composition-and-Nutritive-Value-of-Canadian-Pulses.pdf. Accessed 7 August 2017.

Wang, T., Wang, L., Wang, R., and Chen, Z. (2016). Effects of freeze-milling on the physicochemical properties of rice protein isolates. LWT - Food Science and Technology, 65, 832-839.

Waterschoot, J., Gomand, S. V., Fierens, E., and Delcour, J. A. (2015). Production, structure, physicochemical and functional properties of maize, cassava, wheat, potato and rice starches. Starch-Stärke, 67, 14-29.

Watson, S. A. (1964). Determination of starch gelatinization temperature. In Whistler, R. L., Smith, R. J., and Wolfrim, M. L. (Ed.), Methods in carbohydrate chemistry. New York. Academic Press. 572p.

Watson, R. R., Preedy, V. R., and Zibadi, S. (2014). Wheat and rice in disease prevention and health. US. Academic Press. 576p.

Wickham, M., Faulks, R., Mann, J., and Mandalari, G. (2012). The design, operation, and application of a dynamic gastric model. Dissolution Technologies, 19, 15-22.

Wickham, M., Faulks, R., and Mills, C. (2009). *In vitro* digestion methods for assessing the effect of food structure on allergen breakdown. Molecular Nutrition and Food Research, 53, 952-958.

Willis, L., Lessard, P., and Sinskey, A. (2001). Amino acids. In Brenner, S., and Miller, J. (Eds.), Encyclopedia of genetics. New York. Academic Press. 4368p.

Whistler, R., Smith , R., BeMiller, J., and Wolfrom, M. (1964). Methods in carbohydrate chemistry. New York: Academic Press. 394p.

Wong, T., and Louie, J. (2016). The relationship between resistant starch and glycemic control: A review on current evidence and possible mechanisms. Starch - Stärke, 69, 1-9.

Wood, P. (2002). Relationships between solution properties of cereal b-glucans and physiological effects—a review. Trends in Food Science and Technology, 13, 313–320.

Wood, P. (2007). Cereal β-glucans in diet and health. Journal of Cereal Science, 46, 230-238.

Wood, P., Beer, M., and Butler, G. (2000). Evaluation of role of concentration and molecular weight of oat β-glucan in determining effect of viscosity on plasma glucose and insulin following an oral glucose load. British Journal of Nutrition, 84, 19-23.

Wood, J., Braaten, P., Scott, J., Riedel, F., Wolynetz, K., and Collins, M. (1994). Effect of dose and modification of viscous properties of oat gum on plasma glucose and insulin following an oral glucose load. British Journal of Nutrition, 72, 731-743.

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

World Health Organization. Global report on diabetes. (2016). http://apps.who.int/iris/bitstream/10665/204871/1/9789241565257_eng.pdf. Accessed 28 July 2017.

World Health Organization. "Obesity and Overweight.", http://www.who.int/mediacentre/factsheets/fs311/en/. Accesed 28 July 2017.

Woychik, J. H., Boundy, J. A., and Dimler, R. J. (1961). Wheat gluten proteins, amino acid composition of proteins in wheat gluten. Journal of Agricultural and Food Chemistry, 9, 307-310.

Zentner, R. P., Campbell, C. A., Biederbeck, V. O., Miller, P. R., Selles, F., and Fernandez, M. R. (2001). In search of a sustainable cropping system for the semiarid Canadian prairies. Journal of Sustainable Agriculture, 18, 117-136.

Tacer-Caba, Z., Nilufer-Erdil, D., and Ai, Y. (2015). Chemical composition of cereals and their products. In P. C. K. Cheung, and B. M. Mehta (Eds.), Handbook of food chemistry . Berlin. Springer-Verlag. 1173p.

Zheng, G., Han, H. L., and Bhatty, R. S. (1999). Functional properties of cross-linked and hydroxypropylated waxy hull-less barley starches. Cereal Chemistry, 76, 182-188.

Zia-ud-Din, X, H., and Fei, P. (2017). Physical and chemical modification of starches: A review. Critical Reviews in Food Science and Nutrition, 57, 2691-2705.

Zobel, H. (1988). Molecules to granules: a comprehensive starch review. Starch - Stärke, 40, 44-50.

Zullo, R., and Iannace, S. (2009). The effects of different starch sources and plasticizers on film blowing of thermoplastic starch: Correlation among process, elongational properties and macromolecular structure. Carbohydrate Polymers, 77, 376-383.