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**A BIOCHEMICAL APPROACH TO STUDY WHEAT PRODUCTS
DIGESTIBILITY
AND WHEAT ALLERGENS DEGRADATION**

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To my Lovely Sister

Lucia,

To my precious Family

ABSTRACT

This thesis is focused on wheat digestibility, particularly referring to proteins, starch and fibre nutrients, which can play a crucial role in several fields as in food allergic reactions, glycemic index assessment and in the fibre degradation.

Wheat is one of the most important crops in the world, used to make a vast range of food products, such as bread, pasta and cakes. Allergic reactions to food, including wheat products, are becoming more common in Western countries affecting up to 2% of the adult population and 6% of children (Sotkovsky et al., 2008). The stability of proteins to the gastro-intestinal digestion process after heat treatment may contribute to their allergenic potential. It is thought that resistance to digestion plays an important role in the development of sensitisation, and it is postulated that resistance to gastric conditions (low pH and pepsinolysis) may have a significant impact on the solubility and allergenic potential of food proteins. Indeed, both intact proteins and digested polypeptides have the potential to elicit an allergic reaction. In order to better understand how chemical and biochemical changes induced by food processing (such as cooking) affect digestibility of wheat flour proteins, one dimensional (1D) and two-dimensional electrophoresis (2-DE) digestion maps of heated and unheated flour have been produced. Experiments were carried out using an *in vitro* static system with an additional 'chewing' step including human salivary amylase, in order to study oral degradation of wheat flour proteins. Afterwards, an *in vitro* dynamic model of digestion has been performed to gain a more reliable comprehension on protein degradation.

Besides, as known, starch is the main constituent in cereal food and it influences the rate and the extent of digestion and, consequently, its nutritional and health properties. Foods having a low glycemic index have been suggested from FAO/WHO organisations (1998) since it promotes the slow release of glucose in the body. This work involved also the study of three types of pasta which were subjected to *in vitro* and *in vivo* digestions in order to assess their glycemic index. Finally, another crucial nutrient component in wheat is represented by arabinoxylans (AX) which are hemicellulose belonging to the fibre group. This fibre has been described as resistant to gastrointestinal digestion, since it is usually

degraded in the colon tract, by microbiota. However *in vitro* simulated digestions of arabinoxylans, extracted from wheat dough, were performed, and the effect on arabinose substitution level was investigated.

Results mainly showed a reduced protein degradation in heated flour, suggesting that heat treatment can modify protein susceptibility to digestion. Results concerning the glycemic index study gave evidence that the wheat genotype can affect the rate of starch hydrolysis due to some nutrients, as proteins, which may vary among wheat varieties.

Finally, concerning the arabinoxylans digestions, interesting findings showed that enzymes and surfactants and either the acid gastric pH, were able to affect AX region IR spectra.

ABSTRACT (Italian Version)

Il lavoro sviluppato in questa tesi riguarda la digeribilità dei nutritivi del frumento, con particolare riferimento a proteine, amido e fibra. La digestione dei nutritivi gioca un ruolo chiave nelle reazioni allergiche agli alimenti, nella valutazione dell'indice glicemico e infine nella degradazione della fibra.

Il frumento è una tra le più importanti produzioni cerealicole mondiali e viene utilizzato per la produzione di svariati prodotti: pane, pasta, dolci.

Le reazioni allergiche agli alimenti, incluse quelle indotte dal frumento, stanno diventando sempre più diffuse nei paesi occidentali, colpendo circa il 2% degli adulti e il 6% dei bambini (Sotkovsky et al., 2008). Tra i fattori che contribuiscono al potenziale allergenico di un alimento si deve considerare la stabilità delle proteine in seguito al processo digestivo e di cottura. Infatti è noto che la resistenza degli allergeni al trattamento di digestione è tra i parametri coinvolti nello sviluppo della sensitizzazione individuale, e le condizioni ambientali gastriche possono influenzare la solubilità di potenziali allergeni.

È altresì noto che proteine o polipeptidi non digeriti hanno la potenzialità di scaturire una reazione allergica. In questo lavoro è stato studiato l'effetto del trattamento termico e della digestione, in farine di frumento cotta e non, attraverso l'analisi di profili proteici 1D e di mappe bidimensionali 2D.

Questi esperimenti sono stati effettuati utilizzando un sistema di digestione statico simulando la fase orale, gastrica e intestinale. Inoltre, successivamente, è stato messo a punto un sistema di digestione dinamico, per lo studio della degradazione delle proteine in farina cotta.

Inoltre, è noto che l'amido è il principale componente dei prodotti cerealicoli e ne influenza le proprietà salutistiche nutrizionali. Alimenti con basso indice glicemico sono stati suggeriti dalle organizzazioni FAO/WHO.

Questo lavoro ha coinvolto l'utilizzo di tre varietà di pasta, ove, esperimenti di digeribilità dell'amido *in vitro* e *in vivo*, hanno permesso di fornire una valutazione del loro indice glicemico.

Infine, componenti della fibra in farine di frumento sono stati studiati attraverso la messa a punto di un sistema di digestione in vitro, per valutare l'effetto dell'ambiente gastro-intestinale sugli arabinoxilani estratti da impasti di farina. I risultati mostrano come il trattamento termico della farina di frumento modifichi la degradazione degli allergeni durante la digestione gastro-intestinale in vitro. Inoltre, lo studio delle tre varietà di pasta dimostra differenze significative nei valori di indice glicemico, imputabili al genotipo. Infine dalla digestione degli arabinoxilani, i risultati riportano sorprendentemente un effetto della digestione gastrica e intestinale, sulla parziale modificazione di queste fibre.

AIMS OF THE WORK

Wheat is one of the most important crops in the world since it is used to produce a vast range of products as bread, pasta, cakes, which are mainly present in the daily diet.

Thus, taking into account the importance of wheat in people diet, wheat nutrients digestibility have been studied using *in vitro* models of digestion and a proteomic approach. This work is divided in three main parts. The first part was focused on wheat proteins degradation during *in vitro* digestion, performed by using a biochemical model and a dynamic gastric model. This aimed to gain a better understanding on proteins degradation and their resistance after the simulated human digestion, which plays an important role in food allergic reactions.

Moreover, one of the main tasks in the food allergy research is to assess whether food processing could affect protein allergenicity and individual sensitisation after food ingestion; this is why the work aimed also to compare heated and unheated wheat flour and their behaviour during simulated gastrointestinal digestion.

Secondly, this thesis has been focused on starch digestibility which is related to the glycemic index of starchy foods. I aimed to compare *in vitro* and *in vivo* digestion studies using different varieties of pasta, and to assess their glycemic index, as this is an important issue to highlight wheat products with a low impact on human glycemic response. Finally the wheat arabinoxylan fraction was studied, in order to achieve a better understanding on its behaviour during the transit through the gastrointestinal tract. *In vitro* digestion were performed using a “gel layer” of arabinoxylans extracted from wheat dough, and the effect of the different enzymes and chemicals included in the digestion mix, was studied.

The main part of this work has been done at the “Institute of Food Research” placed in Norwich (UK) and funded by the “Healthgrain Project” (project code: 41889000E) and by the BBSRC competitive strategic grant to IFR.

NOTE FOR THE READER:

The figures, tables, graphs, describing the obtained results, have been located in the “appendix” sections.

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CHAPTER 1

INTRODUCTION



1.1 THE GASTROINTESTINAL TRACT: GENERAL FUNCTIONS AND STRUCTURE

By definition, preparation of the food for absorption is called “digestion” whereas “absorption” involves uptake of the resulting molecules through the epithelium into the bloodstream (Hinsberger et al. 2004).

The digestive system includes the GI tract and the accessory organs of digestion, including the salivary glands, liver gallbladder and exocrine pancreas (Wickham and Reed 2009). The GI tract is simultaneously a transport system, an absorptive organ, a regulator of food intake, fluids and electrolyte balance, and it has multiple interactions with other organ and system. The gut-brain axis, together with the enteric nervous system and intestinal hormones, play a major role in controlling motility, digestion and appetite. Moreover the GI tract also has a critical role in immune surveillance via gut-associated lymphoid tissue (GALT) found throughout the tract (Huether 2006).

The primary function of the gastrointestinal tract (GIT) is to transform ingested food, consisting of carbohydrates, proteins, fat and macronutrients, into smaller components (Johansson et al. 1972). The first step of this process is the mechanical act of chewing that reduces the size and mixes the food. As a consequence of eating, several enzymes-containing fluids are secreted into the GIT and these aid hydrolysis of proteins, fats and carbohydrates.

Digestion occurs by mechanical and chemical processes. Mechanical digestion includes chewing, swallowing and peristalsis (the method by which food moves through the entire gut) (Wickham and Reed 2009). Chemical digestion is the enzymatic breakdown of food in the mouth, stomach and small intestine. After partially digestion food and fluids exit the stomach and enter the small intestine, where the enzymes secreted by the liver and exocrine pancreas break it down into absorbable monosaccharides, amino acids and fatty acids. These nutrients pass through the small intestine wall into blood and lymphatic vessels and are transported to the liver for storage or further processing (Huether 2006). Gastrointestinal functions are regulated by hormones as well as by the autonomic nervous system (ANS).

The autonomic nervous system (ANS) includes nerves that are both extrinsic and intrinsic to the gastrointestinal tract (Johnson 2007).

Enteroendocrine hormones are secreted mainly by the stomach and duodenum. As ingested food and fluids move through the GI tract these hormones, such as gastrin, secretin, cholecystokinin (CCK) and gastric inhibitory peptide (GIP), are released to either stimulate or inhibit peristalsis and to cause secretion of substances that facilitate digestion (Sherwood 2005). For instance, gastrin stimulates gastric juice production and smooth muscle contraction in the stomach, small intestine, large intestine and controls the pyloric sphincter. Cholecystokinin also facilitates digestion by relaxing the hepatopancreatic ampulla, which allows bile and pancreatic juice to flow into the duodenum. Secretin is released in the duodenum and small intestine and leads to production of bicarbonate, which neutralizes the acidity of chyme, bile production by the liver and inhibits gastric motility to slow digestion and gastric emptying.

The GI tract is composed of the oral cavity, pharynx, esophagus, stomach, small intestine, large intestine, rectum, anal canal and anus. The four distinct concentric layers of the gut wall are: the mucosa, the submucosa, the muscularis externa and either the adventitia or the serosa (Huether et al. 2006).

The mucosa, the innermost layer of the gut wall, lines the entire GI tract and consists of epithelium, lamina propria and muscularis mucosa. The mucosal epithelium is differentiated along the gastrointestinal tract; tissue specialization correlates with the regional function of the tract. At the upper and lower ends of the GI tract (the mouth, esophagus and anal canal) the mucosal epithelium is protective and composed of stratified squamous epithelial cells. On the other hand, the mucosal epithelium in the stomach, small intestine and colon are composed of glandular epithelial cells. The cells in these regions secrete mucous, enzymes and other substances that either protect the mucosa or aid in digestion (Johnson 2007).

The lamina propria and the muscularis mucosa lie outside the mucosal epithelium. The lamina propria consists primarily of connective tissue, as well as blood and lymphatic vessels that deliver nutrients to the mucosal epithelium, transport

hormones secreted by the endocrine epithelium and absorb end products of digestion from the lumen of the GI tract. In addition the lamina propria is infiltrated with lymphocytes and lymphnodes, collectively known as the GALT, that protect the GI tract wall from resident bacterial flora and ingested pathogens (Wickham and Reed 2009). The tonsils, the appendix and the Peyer's patches in the ileum are aggregates of lymph nodes that comprise GALT in the GI tract and are part of the larger mucosal associated lymphoid tissue (MALT) that constitutes 50% of the body total immunity and 70% of antibody production (Kang et al. 2007).

The third sub-layer of the mucosa, the muscularis mucosa, is a thin layer of smooth muscle that is the boundary between the mucosa and the submucosa.(Kang et al. 2007).

The submucosa is a connective tissue layer that lies outside of and supports the mucosa. The submucosa contains blood vessels, lymphatic vessels, submucosal glands and Meissner's plexus (a nerve network that influences the smooth muscle of the muscularis) (Johnson 2007). The next outer layer of the gut wall is the muscularis externa.

In the mouth, the pharynx and the upper esophagus, the muscularis externa is composed of striated muscle cells that aid swallowing. In the rest of the GI tract the muscularis externa has two smooth muscle layers: an inner circular layer and an outer longitudinal layer. Auerbach's plexus lies between the two layers and this nerve network coordinates contractions of the layers resulting in rhythmic peristalsis (Johnson 2007).

The outermost layer of the gut wall that lies far from the lumen is either the adventitia or serosa. This layer is composed of simple squamous epithelial cells.

DIGESTIVE ORGANS

Food is prepared for digestion by chewing and mixing saliva that contains water, mucus, electrolytes and enzymes to facilitate mechanical breakdown of food and begin carbohydrate digestion. Partially digested food then passes from the pharynx into the esophagus and then to the stomach, small intestine, large intestine, during which digestion is affected by other organs, hormones and nervous system effects (Wickham and Reed 2009).

STOMACH

The J-shaped stomach lies below the diaphragm and has three divisions: the fundus, the body and the antrum (Huether et al. 2006). The stomach is a temporary storage area that mixes food with water and gastric juices to break food down physically and chemically and to control the transit of the chyme into the small intestine via regulation of the pyloric sphincter (Alcama 1996). Special cells located throughout the gastric mucosa produce various substances. Among these cells, parietal cells produce hydrochloric acid which denatures proteins and kills bacteria and other substances for the absorption of vitamin B12. Chief cells secrete pepsinogen, enzyme precursor that is converted to pepsin (Huether et al. 2006).

SMALL INTESTINE

The duodenum, jejunum and ileum constitute the small intestine, which is approximately 731 cm long. The duodenum begins at the pyloric valve and joins the jejunum at the ligament of Trietz. The small intestine ends at the ileocecal valve, which controls the flow of digested material from the ileum into the large intestines. Blood is supplied to the small intestine by the gastroduodenal and superior mesenteric arteries, whereas blood containing digestive products is transported from the small intestine to the liver via the hepatic portal circulation (Huether et al. 2006).

Digestion and the majority of nutrients absorption occur in the small intestine (Alcama 1996, Johnson 2007). After chyme leaves the stomach, intestinal and

pancreatic enzymes as well as bile salts enter the duodenum via pancreatic ducts and the bile duct to continue digestion in the duodenum (Alcamao 1996, Johnson 2007). Mucosal folds (plicae) slow the passage of chyme, thereby increasing the time for digestion and absorption. Intestinal villi and microvilli increase the luminal surface and the intestinal absorption of nutrients. Each villus has an artery, a vein and a lymphatic channel that absorbs and transports nutrients. The surface of each columnar epithelial cell contains tiny projections called microvilli that are covered with glycocalyx. Microvilli create a mucosal surface called the brush border, which is where digestive enzymes act. Stem cells arise in the crypts of Lieberkuhn, which lie at the base of each villus and differentiate into enterocytes, the functional cells of the small intestine. The entire epithelial population is replaced every 4 to 7 days.

LARGE INTESTINE

The large intestine, made up of the cecum, the appendix, the colon, the rectum and the anal canal, is approximately 122-152 cm long. Chyme passes from the ileum into the cecum and then into the colon. The four sections of the colon are the ascending, transverse, descending and sigmoid colons. The mucosa of the large intestine consists of columnar epithelial cells and mucus secreting goblet cells that allow absorption of fluids and electrolytes, as well as they provide mucosal lubrication. The main function of the colon is to reabsorb water and by the time the digestive mass reaches the sigmoid colon, it becomes solid (Wickham and Reed 2009).

ACCESSORY ORGANS OF DIGESTION

The liver, the gallbladder and the exocrine pancreas secrete substances that are delivered to the duodenum and are necessary for the digestion of chyme. The liver produces bile, necessary for digestion and absorption of fats, which is stored in the gallbladder when food is not being digested. The exocrine pancreas produce enzymes necessary for carbohydrates, proteins and lipids digestion, as well as the pancreatic juice, an alkaline fluid that neutralizes acidic chyme leaving the stomach, and allows pancreatic enzymatic reactions (Huether et al., 2006).

1.1.1 THE GASTRIC DIGESTION

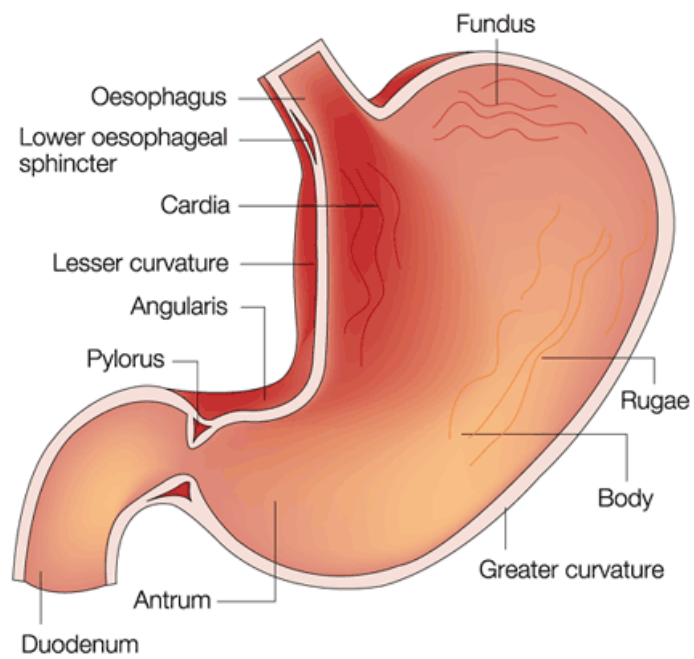
A phenomenon extensively studied in the last few years is gastric accommodation which, differently from adaptive relaxation, is directly correlated with food entering the stomach. Accommodation of the stomach to a meal consist in the relaxation of the proximal stomach, providing the meal with a reservoir and enabling volume increase without a rise in pressure (Cuomo et al. 2004). The effects of the interaction of food with the stomach not only depend on volume but also on the rate at which food is ingested, or on its nutrient content (Cuomo et al. 2004).

Stomach is divided into three main parts, fundus, body and antrum (figure 1.2) where digestion takes place, therefore in these compartments food is differently processed (table 1.1).

In the stomach, protein digestion occurs as one of the main process which will be better explained in the next section.

FILLING	Fundus receptive relaxation induced by swallowing. Fundus gastric accommodation to a meal.
CHURNING	Propulsion of material towards the closed pylorus. Grinding of material in the antrum. Retropulsion of material back to the proximal stomach to begin the cycle again.
EMPTYING	Increased peristaltic contractions. Pyloric sphincter relaxation. Effect of duodenal receptors. (for example fatty acids slow gastric emptying.

Table 1.1: Gastric motor activity after food ingestion. (Cuomo et al. 2004).



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Figure 1.2: the main compartments of the stomach (Richard M. Peek, Martin J. Blaser. 2002. Nature Reviews cancer 2: 28-37.

1.1.2 DIGESTION OF DIETARY PROTEIN

THE GASTRIC PHASE

The first step in protein digestion takes place in the stomach. After a relatively quick passage through the esophagus, proteins contained in the macerated food bolus enter the gastric lumen. Here the stomach is distended by the entering food, resulting in increased gastric secretion (Jensen-Jarolim et al. 2006). Absorbed from the blood stream, gastrin triggers hydrochloric acid production in the parietal cells, and, to a lesser extent, digestive enzyme secretion by the chief cells of the gastric glands (Schubert 2007). In the stomach, the chyme is not only exposed to hydrochloric acid, mucins and inorganic salts but also to different pepsins, the major gastric proteases (Etherington et al. 1970). These proteinases are produced and secreted into the gastric lumen as inactive proenzymes called zymogens or pepsinogens (Tang et al. 1987). At low pH levels, the acid amino acid residues in the active enzyme moieties undergo protonation. The electrostatic interactions between the N-terminal prosegment and the active pepsin are disrupted, which initiates a conformational change in both the prosegment and the active enzyme portion. Thus the removal of the prosegment results in conversion into the enzymatically active form of pepsin (Khan et al. 1998). At this stage the substrate binding cleft, with the two active-site aspartates, is accessible for binding to protein chains and thus protein cleavage can take place (figure 1.3).

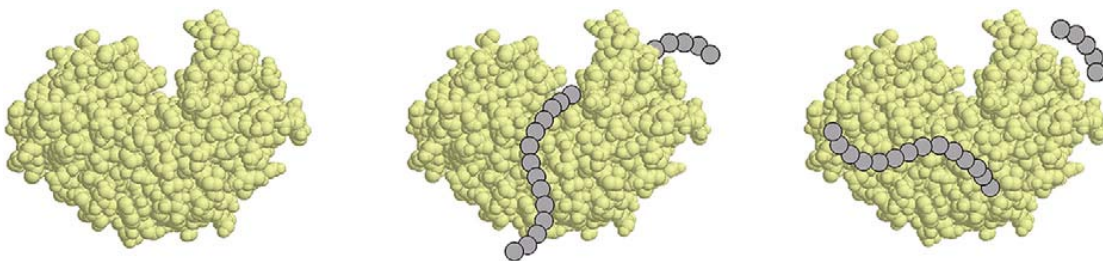


Figure 1.3: Physiologic gastric protein digestion by pepsin. The figure was created with the program Protein Explorer 2.411 Beta (available at: <http://proteinexplorer.org>) by using the structural information of porcine pepsin provided by the Research Collaboratory for Structural Bioinformatics Protein. (Jensen-Jarolim et al. 2006).

Whereas at a pH of greater than 5.0 limited pepsin is activated, the rate of active enzyme increases with decreasing gastric pH (McPhie 1972). An acidic milieu is required for the proteolytic activity of pepsin, with an optimum of activity between pH 1.8 and 3.2 (Samloff et al. 1989). Pepsin has a broad specificity against large molecular peptides, preferentially cleaving proteins at phenylalanine, tyrosine and leucine residues (Oka et al. 1970). The release of free aminoacids into the stomach is a necessary factor for the induction of gastric acid, CCK (cholecistokinin) and secretin secretion and a stimulus to empty the gastric content into the duodenum (Hinsberger et al. 2004). Subsequently, the remaining peptones and polypeptides present in the chyme are released into the small intestine where digestion can continue.

THE DUODENAL PHASE

Nearly one third of the hydrolysis of dietary proteins delivered to the small intestine occurs in the duodenum (Hinsberger et al. 2004). Here they are exposed to a variety of proteases and peptidases produced and secreted by the pancreas, such as trypsin, chymotrypsin, or carboxypeptidases, or to a brush border enzymes of the intestinal mucosa. Endopeptidases, such as trypsin, cleave central peptide links whereas exopeptidases cleave the terminal ones (table 1.4).

The pancreatic proteases, requiring an alkaline pH, catalyse further digestion into small peptides of up to 3 aminoacids in length, which are actively taken up by enterocytes and serve as nutrients for the human body (Erickson et al. 1990). Despite intestinal proteases are unable to cleave proline containing peptides (Hinsberger et al. 2004), they are able to hydrolyse polypeptides into small ones which usually are not reactive with any structure responsible for antigen recognition and presentation and they are therefore immunologically ignored. (York et al. 1999).

The intracellular transport of amino acids, di- and tripeptides depends on specific transport mechanisms that are mostly Na dependent, but which show an overlapping specificity. Part of the transport of peptides occurs together a

hydrogen-peptide co-transporter (Nixon et al. 1970). Another mechanism is the facilitated or passive Na-dependent diffusion of amino acids into the cells.

ENZYME	TYPE	FUNCTION
Trypsin	endopeptidase	Cleaves internal bond within lysin or arginine residues
Chymotrypsin	endopeptidase	Cleaves bonds within aromatic or neutral amino acid residues
Elastase	endopeptidase	Cleaves bonds within aliphatic amino acid residues
Carbopeptidase A	exopeptidase	Cleaves non-alkaline and aromatic residues from oligopeptides
Carbopeptidase B	exopeptidase	Cleaves alkaline residues like lysine or arginine from oligopeptides

Table 1.4: Pancreatic proteases and their functions (Hinsberger et al. 2004).

1.1.3 DIGESTION OF CARBOHYDRATES

THE ORAL DIGESTION

Carbohydrate digestion begins into the mouth. Oral processing is both a physiological process controlled by central nerve system and a physical process modulated by mechanical and structural properties of the food (Chen, 2009). Saliva plays a fundamental role in food oral processing and in maintaining oral health. Saliva is a complex heterogeneous clear fluid consisting of roughly 98% water and 2% organic and inorganic substances, including electrolytes, mucus, glycoproteins, proteins, antibacterial compounds and enzymes (Chen, 2009). The natural pH of saliva is fairly neutral ranging between 5.6 and 7.6 with an average of 6.75 (Jenkins, 1978). Saliva has the following physical functionalities and biological benefits: maintaining tooth integrity, providing antibacterial activity, helping lubrication and protection, food buffering and enhancing taste and digestion (Humphrey et al., 2001). As a seromucous coating, saliva lubricates and protects oral tissues, acting as a barrier against irritants. It is this lubrication effect which smoothens food movement inside the mouth and minimizes any irritation to soft oral tissue (Chen et al., 2009). Saliva will respond to food intake and provides buffering effect. It was indicated that the pH of saliva rises during the first 5 minutes after the intake of most foods and falls to its minimum of around 6.1 approximately 15 minutes after food consumption. Afterwards the pH gradually returns to its resting pH between 6 and 7. The pH variation, during and after food consumption, is believed to give protection to oral tissues and in particular to teeth (Humphrey et al., 2001). Saliva could also interact with food components leading to structure formation or structure breakdown. The presence of amylase, a major component of saliva, plays an important role in an early breakdown of starch components. The interaction of amylase enzyme with starch ingredients produces almost an immediate effect on hydrolysis and thus making the food much easily mixable and digestible in the stomach (Chen, 2009). Some authors (Hoebler et al., 2000) found that during a short period of oral processing about 50% of bread and 25% of pasta starch was hydrolysed and transformed into smaller molecules. They

concluded that the starch hydrolysis began in the mouth and the different rate of starch hydrolysis was caused by the structural differences of the solid foods. Salivary amylase is most active at its optimum pH of 7.4; it works to full function inside the mouth, but become inactivated in the stomach because of the gastric acid (Chen, 2009). However, it is also worth to note that, even though enzyme interaction begins almost immediately after food ingestion, its contribution to starch full breakdown is relatively insignificant. Most of starch digestion results from pancreatic amylase, rather than from salivary amylase.

THE INTESTINAL DIGESTION

The intestinal digestion of dietary carbohydrates occurs in two distinctive phases: a luminal and a mucosal phase. About 75% of all carbohydrates are absorbed in the proximal 70 cm of the small intestine (Hinseberger et al., 2004).

Luminal phase

When the ingested food reaches the duodenum, pancreatic α -amylases start the intraluminal hydrolysis of carbohydrates. These amylases breakdown α -1-4-linkages thereby leaving α -1-6-linkages intact (Hinseberger et al., 2004).

Starch consists of approximately 15% amylose, composed of α -1-4-linked glucose molecules and 85% amylopectin, a branched chain molecule with α -1-4- and α -1-6-glycosidic linkages between glucose molecules. Amylopectin is only partially digested. The endproducts of α -1-4-hydrolysis of the ingested oligo and polysaccharides are α -limit-dextrin, maltriose, maltose, sucrose and lactose, which than need to undergo breakdown during the mucosal phase of carbohydrate digestion.

Mucosal phase

In the villous cells, the mucosal surface of small intestinal enterocytes contains enzymes for the hydrolysis of residual sugars and α -1-6-linked carbohydrate molecules (Dahlqvist et al., 1963). These enzymes are called brush border membrane hydrolases, disaccharidases or oligosaccharidases and are

predominantly found in the duodenum and jejunum. Brush border enzymes are composed of a single β -galactosidase and three α -glucosidases with sucrase having a double role (table 1.5). There is no single maltase enzyme and all three α -glucosidases hydrolyse the starch oligosaccharides. Sucrase and isomaltase are linked together and form a dimer, but the two enzyme activities are independent of each other. Dietary intake, pancreatic enzyme activity and gastrointestinal tract diseases regulate these enzymes. Once the carbohydrates are reduced to monosaccharides, they are taken up into the enterocytes. Glucose transporters facilitate monosaccharide absorption (Hinseberger et al., 2004).

	ENZYME	FUNCTION
β -galactosidase	Lactase	Hydrolysing lactose to glucose and galactose
α -glucosidases	Sucrase	Hydrolysing maltose and maltotriose of starch
	Sucrase	Hydrolysing sucrose to glucose and fructose
	Isomaltase	Cleaving α -1-4 and α -1-6 links in oligosaccharides
	Glucoamylase	Cleaving α -1-4 links from straight-chain oligosaccharides

Table 1.5: Function of brush border enzymes (Hinseberger et al., 2004).

1.1.4 DIGESTION OF LIPIDS

Digestion of lipids starts in the mouth and stomach where an emulsion is made out of ingested food (Hinseberger et al., 2004). By this mechanical process, the dietary lipids are released from interacting proteins and form an emulsion of smaller particles. In the stomach a small amount of the triglycerides undergo hydrolysis induced by a gastric lipase able to function in an acid environment. Once the acidic gastric content reaches the duodenum, secretin is released from the duodenal mucosa into the portal circulation. This stimulates the pancreas to produce and secrete bicarbonate into the duodenum to create a pH-neutral environment, which maximizes the activity of pancreatic lipase and co-lipase. As soon as fatty acids and amino acids reach the duodenum and jejunum, CCK is released into the portal circulation and stimulates the pancreas to release triglyceride lipase and co-lipase, which is required to facilitate the lipase to access triglycerides within emulsified particles. Both enzymes act at the surface of the particles and hydrolyse triglycerides to monoglycerides and fatty acids (Lowe, 1994). The last step in fat digestion is the formation of mixed micelles by the interaction of fatty acids and monoglycerides with bile salts (Carey, 1983).

The liposoluble vitamins D, E and K especially require this micelle formation for absorption. A portion of the triglycerides and fatty acid may form vesicles after digestion with pancreatic lipase before being transported to the intestinal mucosa (Hernell et al., 1990). After lipid digestion, micelles and fatty acids are taken up by passive diffusion, facilitated diffusion and active transport through the enterocyte membrane. In the cytosol they bind to a fatty-acid-binding protein, which transports them from the cell membrane to the smooth endoplasmic reticulum (ER). Thereby a concentration gradient is maintained and the uptake of fatty acids into the cell is facilitated. In the smooth ER, fatty acids and monoglycerides are transformed into triglycerides and phospholipids, whereas fat-soluble vitamins are bound to apolipoproteins produced in the rough ER and form chylomicrons. After processing, triglycerides, phospholipids and fat-soluble vitamins are transferred to the Golgi apparatus and incorporated into secretory vesicles to be released by exocytosis into the extracellular space. From the lamina propria, they move to the

underlying lymph lacteals. Direct uptake into the bloodstream is not possible because the molecules are too big to pass through capillary fenestration spaces. Lipid uptake distends the lymph lacteals; gaps between the endothelial cells are formed and allow uptake of chylomicrons into the lymphatic system, which delivers them to the systemic circulation (Hinseberger et al., 2004).

1.2 FOOD ALLERGY

Despite being considered a pleasure by most persons, food intake might also represent a health hazard in situations of altered metabolism or if food proteins are recognised as potentially harmful by the immune system (Untersmayr et al., 2008). Food allergy is now recognized as a worldwide problem in westernized nations, and like other atopic disorders, it appears to be on the increase (Sampson, 2004). Even though population studies indicate that more than 20% of all patients believe themselves to be allergic to food, the true prevalence of this disorders ranges between 3% and 4% in the general population (Sicherer et al., 2001). Adverse food reactions (food hypersensitivities) include any abnormal reaction resulting from the ingestion of food and might be the results of food intolerance (nonallergic food hypersensitivities) or food hypersensitivities/allergy (food allergy) (Sampson et al., 1997). Food intolerances are adverse responses caused by some unique physiologic characteristic of the host, such as metabolic disorders. Food allergic disorders can be broadly divided into those that are mediated by IgE antibodies and those that are not. Disorders with acute onset of symptoms after ingestion are usually mediated by IgE, which arm tissue mast cells and blood basophils, to result in a state termed sensitisation (Sicherer et al., 2001). After re-exposure, food proteins bind to the IgE molecules specific for them and trigger the release of mediators, such as histamine, that cause the symptoms (figure 1.6). Another group of food hypersensitivity disorders are subacute or chronic, and are mediated mainly by T cells (Sampson et al., 2000).

A third group of chronic disorders attributed to food allergy are variably associated with measurable IgE (IgE associated or cell mediated disorders). IgE dependant food-allergic reactions affect one or more target organs: the skin (urticaria, angio-oedema), respiratory tract (rhinitis, asthma), gastrointestinal tract (pain, emesis, diarrhoea), and cardiovascular system (anaphylactic shock) (Sicherer, 2002).

Toxic reactions might mimic food hypersensitivities and typically are due to factors inherent in a food, such as toxic contaminants (eg. histamine in scombroid fish poisoning) or pharmacological substances within the food (eg. tyramine in aged

cheeses) which can affect most healthy individuals when given in appropriate doses.

1.2.1 FOOD ALLERGENS

Any food has the potential to trigger an allergic reaction, but a few foods account for most food allergies. In fact, about 90% of food allergies are triggered by one of these 8 foods: eggs, milk, wheat, soy, peanuts, nuts, fish, shellfish. Potent food allergens are usually water soluble glycoproteins with molecular weights of 10-60 kDa that are stable at low pH. Cooking can reduce the allergenicity of certain food proteins, as shown with fruits and vegetables in oral allergy syndrome and with cooked versus raw or undercooked egg and fish (Urisu A., 1997). In other cases, heating can increase the allergenicity of certain proteins through the induction of covalent modifications that led to new antigens or improved stability (Simonato et al., 2001). In peanuts, for example, the roasting process produces end-products with greater resistance to digestion and heightened allergenicity compared with those produced by frying or boiling (Maleky et al., 2000).

1.2.2 WHEAT ALLERGY

Wheat is one of the major cereals grains belonging to the grass family Poaceae (Graminaceae) and is a staple food item in most diets.

Moreover, wheat is among the six major foods that account for hypersensitivity reactions in children (James et al., 1997). Although major allergenic proteins from egg, milk and peanuts have been implicated in specific humoral and cell-mediated immune responses in human beings, much less evidence is available regarding wheat allergens.

Wheat flour proteins are classified in four solubility groups: 1. the water soluble albumins (15 % of the total protein content), 2. the salt soluble proteins (5%), 3. the water/salt insoluble monomeric gliadins (soluble in alcohol/water mixtures, about 40% of the total protein) and polymeric glutenins (soluble in diluted acetic

acid, 40 % of the total protein) which are made up of high and low molecular weight subunits (HMW-GS and LMW-GS) linked together by disulphide (SS) bonds. While most of the components of the soluble protein fraction have a physiological functions (enzymes, enzyme inhibitors), gliadins and glutenin, that are also known as prolamins and constitute the gluten proteins, are the storage proteins of the seed. A more recent classification that is based on the molecular characteristics rather than on solubility, divides the wheat storage proteins into high molecular weight prolamins (corresponding to HMW-GS, around 100 kDa), sulphur-poor prolamins, corresponding to ω -gliadins (around 60 kDa) and sulphur-rich prolamins, comprising LMW-GS plus α , β , γ -gliadins (31-45 kDa).

Wheat proteins belonging to both the soluble and insoluble fractions can act as allergens in sensitised individuals (De Zorzi et al., 2007).

Hypersensitivity reactions have been noted in cases of wheat flour, both by inhalation or ingestion (Baldo et al., 1980). "Baker's asthma", a common disease among workers with occupational exposure to flour, is an allergy caused by its inhalation.

Most prominent allergens for baker's asthma have been related to the salt – soluble fraction of wheat (Weiss et al., 1997). Gomez et al. (1990) identified and characterized several 12-16 kDa salt-soluble proteins, members of the α -amylase/trypsin inhibitor family, as major allergens associated with baker's asthma. Hypersensitivity reactions after wheat ingestion have been reported by several authors (Sutton R. et al., 1982) and include cereal-dependant exercise induced anaphylaxis (Varjonen et al. 1997) and various cutaneous symptoms associated with atopic dermatitis (Varjonen et al. 2000).

Both patients with wheat allergic atopic dermatitis and those suffering from cereal-dependant exercise induced anaphylaxis have been found to have IgE antibodies against gliadins and it has been suggested that peptides deriving from these proteins are important allergens in those patients (Varjonen et al., 1997). Moreover, Sotkovsky et al. (2008) reported β -amylases and serpins as potential allergens involved in wheat food allergy.

1.2.3 THE GUT BARRIER

Food allergy represents an abnormal response of the mucosal immune system to antigens delivered through the oral route. (Sampson, 2004). During human evolution a sophisticated safety system developed to simultaneously allow immune defence against harmless substances such as food. The gastrointestinal mucosal barrier is a complex structure that provides an enormous surface area for processing and absorbing ingested food and discharging waste products (Mayer, 2003). This barrier uses both physicochemical and cellular factors to prevent the penetration of foreign antigens. The physical barrier, consisting of intestinal epithelial cells joined together by apical and basolateral tight junctions and mucus produced by specialized epithelial cells, such as goblets cells, prevents antigen penetration (Deplancke et al., 2001). Moreover, lumen and brush border enzymes, bile salts and very acid pH, all help to destroy pathogens and render antigens nonimmunogenic. Innate (natural killer cells, polymorphonuclear leukocytes, macrophages, epithelial cells) and adaptive immune responses (intraepithelial and lamina propria lymphocytes, Peyer's patches, secretory IgA, cytokines) provide an active barrier to foreign antigens (Sampson, 2004).

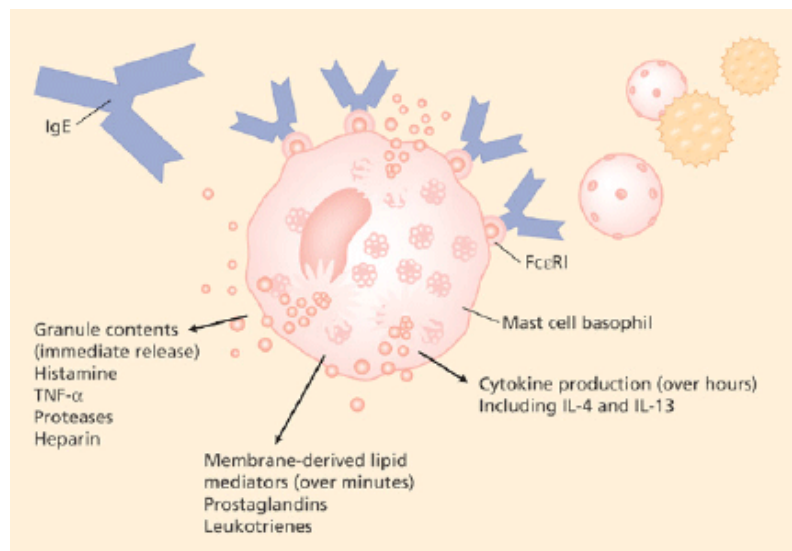


Figure 1.6: Classical allergic reactions occur when allergens bind and crosslink allergen specific IgE antibodies present on the cell membranes of mast cell and basophils, triggering the release of numerous mediators of inflammation. Source: enhs.umn.edu/current/5100/asthma/allergen.gif

1.2.4 THE IMMUNE MECHANISM: THE ORAL TOLERANCE

Food hypersensitivities are born out of a malfunction of the normal immune responses to dietary proteins. Healthy gastrointestinal immune responses need mechanisms by which to recognise but ignore harmless dietary antigens and commensal bacteria and to simultaneously identify and protect against harmful pathogens (Sicherer, 2002). The process by which the gastrointestinal immune system avoids attacking harmless antigens is termed oral tolerance (Strobel et al., 1998). These mechanisms have been investigated mostly in animal models and the induction of Y-cell, deletion of reactive T-cells and generation of suppressor T cells are implicated. Concomitantly, active immunity, such as the production of IgA, reduces infections. To co-ordinate these events, soluble antigens must be differentiated from particulate antigens, which are more likely to be pathogenic.

An important role in this process is accomplished, in part, by differential processing of luminal contents (Sicherer, 2002). The follicle-associated epithelium (M cells) overlying Peyer's patch is thought to be mainly responsible for sampling particulate antigens where macrophages and regulatory T cells induce the IgA responses (Mayer L. et al., 2001). Soluble antigens are sampled by intestinal epithelial cells and immune processing by antigen presenting cells and intraepithelial and lamina propria lymphocytes, results in tolerance.

Although luminal contents meet various physiological barriers at the intestinal wall (epithelial cells, glycocalyx and enzymes) a small portion of ingested antigens penetrates the gut barrier and has the potential to stimulate systemic immune responses. (Sicherer, 2002).

Feeding of new antigens to healthy adults results in T-cell tolerance but antibody responses (IgG) proceed and are apparently physiological (Husby, 2000). The gastrointestinal cytokine milieu includes transforming growth factor β , which induces T-cell suppression, promotes B-cell switching to IgA production and preserves epithelial barrier function (Planchon et al., 1999). Additional cytokines, representing non-allergic T-helper-cell profiles, Th1-type (interferon-gamma and interleukin-2) and Th2 (interleukin-4), take part in these processes. It is possible

that alterations in the immunological response (among Th1, Th2 and abrogation of tolerance) result in diseases (Sicherer, 2002).

When T helper type 2 responses dominate, food hypersensitivity associated with IgE mediated mechanisms can ensue. The most remarkable conundrum is that food-specific IgE is often present (sensitization) without clinical reactivity, although the concentration of specific IgE correlates positively with risk of reactions (Sampson et al., 1997). Several other variables seem to influence the clinical response. Target organ reactivity is one such variable; patients with asthma are more likely than those without to have respiratory reactions during anaphylaxis (Sicherer et al., 2001). Food absorption can also affect responses. Increased absorption might explain why infants, who have leakier gut barriers than older people, frequently have food allergy. In experimental models, sensitization (IgE) itself enhances transepithelial transport in the intestine (Yu et al., 2001). Alcohol, aspirin and exercise are important co-factors in food-induced anaphylaxis, probably through facilitation of increased absorption (Romano et al., 2001).

The immune mechanisms underlying disorders that are not dependant on IgE have been partly elucidated. For example the skin might be targeted in food responsive atopic dermatitis by food –specific T cells that express the skin homing molecule cutaneous lymphocyte antigen (Reekers et al., 1999).

In some cases of isolated gastrointestinal allergy without measurable serum IgE, localised gut IgE is thought to mediate the response (Lin et al., 2002).

In the last several years, there has been increased interest in the role of the commensal gut flora in shaping the mucosal immune response. It is estimated that there are 10^{12} to 10^{14} bacteria per gram of colonic tissue, suggesting that there are more bacteria in the colon than cells in the body (Mayer, 2003).

Gut flora is largely established in the first 24 hours after birth, it is relatively stable throughout life and is dependant on maternal flora, genetics and the local environment. Recent study feeding lactating mothers and their offspring with *lactobacillus rhamnosus*, suggests that probiotics might be of benefit in preventing atopic dermatitis (Kalliomaki et al., 2003), but whether they will be useful for preventing food allergy remains to be demonstrated (Sampson, 2004).

1.2.5 THE ROLE OF DIGESTION ON FOOD ALLERGY ASSESSMENT

The digestive process is likely to play a major role in the development of allergic sensitization, as well as in the clinical severity of food allergy symptoms since exposure of the immune system to proteins is required to initiate an allergic response (Thomas et al., 2007). Resistance of proteins to pepsin digestion has been proposed as a marker for potential allergenicity because it does appear to be a characteristic shared by many food allergens (Taylor et al., 1996).

Thus, resistance to pepsin digestion using simulated gastric fluid (SGF) (Board of Trustees, 1995) has been included as one of the relevant criteria for the allergenicity assessment of novel proteins (FAO/WHO 2001).

In fact, it is possible that the large stable proteolytic fragments generated during digestion have the potential to bind IgE and play a role in sensitization.

Studies (van Beresteijn et al., 1995) found the minimum molecular mass required for whey peptides necessary to elicit an immunological response which was between 3000 and 5000 Da. Likewise, Huby et al. (2000) stated that an allergen must contain at least two IgE binding sites or epitopes, each of which with a minimum of 15 amino acid residues long, in order to make possible the antibody binding.

Moreover, the relative abundance of the allergen in the food should be another factor to be considered together with its structural stability since abundance may influence the dose of allergen that survives gastrointestinal digestion (Mills et al., 2004).

Additionally, some proteins that are very stable to pepsin digestion have not been reported as allergens, such as zein from corn or concanavalin (Fu et al., 2002). This stresses that, in addition to digestive stability, proteins should have the ability to stimulate the immune system in order to sensitise individuals and/or elicit an allergic reaction.

The immunologic or clinical outcome after the consumption of a digestion-sensitive dietary protein depends, to a certain degree, on the gastric digestive capacity.

If the food protein is exposed to gastric enzymes during transit, protein cleavage takes place, inducing either oral tolerance or immune ignorance toward the

ingested food protein. However, if proteins persist during the gastric transit because of impaired digestion, such as during acid-suppression treatment, IgE-mediated food allergy can be induced (Untersmayr et al., 2008). Gastric digestion might also influence the extent of reactivity in already sensitized patients. Physiologic gastric proteolysis substantially decreases the allergenic capacity of ingested food proteins, whereas severe allergic reactions at much lower amounts of ingested food proteins could occur if digestion is impaired (Figure 1.7).

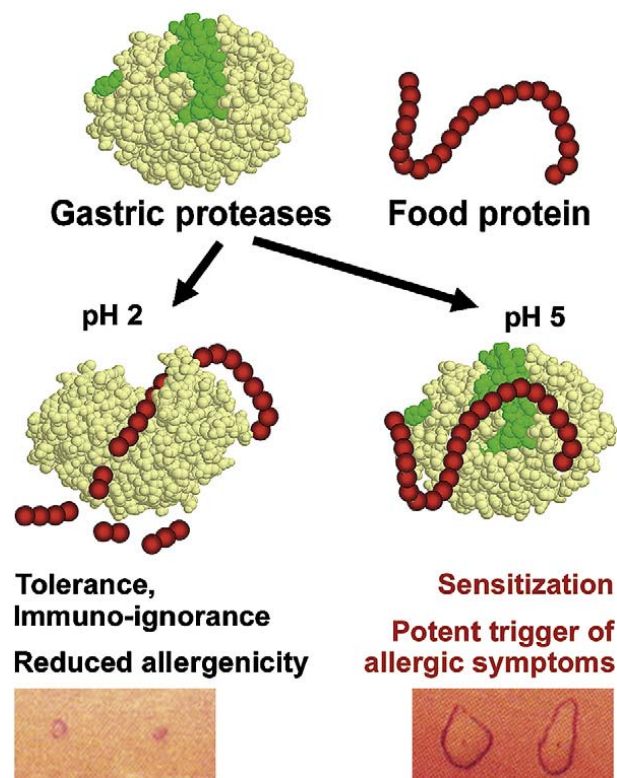


Figure 1.7: The gate-keeping function of the stomach in the sensitization and effector phase of food allergy (Untersmayr et al., 2008).

1.2.6 PARAMETERS AFFECTING THE PEPSINOLYSIS

pH

It is noteworthy that the secretory capacity of the stomach changes physiologically throughout a lifetime, influencing gastric protein digestion.

Depending to some extent on the substrate, optimum pepsin activation can take place at a relatively broad pH range between 1.2 and 3.5 (Jensen-Jarolim et al., 2006). The buffering effect of foods can influence the pH found in the stomach of healthy adult humans. Although, after ingestion of a meal it is common that the pH in the stomach drops to pH 2.0/2.5 (Armand et al., 1994) it can increase up to values around 3 after food intake (Fordtran et al., 1966). Also, the pH in the stomach can be reduced to as low as pH 1.5 when the individual fasts and the stomach is empty (Fallingborg et al., 1989).

Early studies indicated that in newborns the intragastric pH ranges from 6.0 to 8.0 (Avery et al., 1966), which is followed by a burst of acid secretion leading to adult gastric pH levels (pH 1.0-3.0) 24 to 48 hours after birth. After these first days of life, the gastric acid production remains low during the next months, and adult pH levels in the stomach are not reached until the average age of 2 years (Euler et al., 1977). It is well established that gastric acid secretion decreases with age, resulting in low gastric acidity in more than 50% of all patients aged 60 years and older (Davies et al., 1930). It has been reported that low gastric acid output is associated with pathologies like atrophic gastritis, celiac disease, diabetes mellitus, rheumatoid arthritis, and Sjögren syndrome (de Witte et al., 1979). On the other hand, increase of the gastric pH is the therapeutic goal in patients with dyspepsia, such as gastritis, ulcer, erosions, and reflux symptoms. Approximately 25% to 54% of the adult population in Western countries is affected by dyspeptic disorders per year (Talley et al., 1992).

Moreover, gastroesophageal reflux (ie, the presence of gastric fluid proximal to the stomach) is one of the most prevalent problems affecting the gastrointestinal tract in infancy (Heikkinen et al., 1996) being today treated with long-term acid suppression by proton pump inhibitors (PPIs) or H₂-receptor blockers.

Despite large differences in mechanisms of action between the currently available drug subclasses of antacids, sucralfate, H₂-receptor blockers, and PPIs, all these pharmaceuticals effectively suppress gastric acidity and therefore substantially increase intraluminal pH levels (Aihara et al., 2003).

PEPSIN TO PROTEIN RATIO

It is clear that the apparent susceptibility of allergens to proteolysis is strongly dependent on the pepsin to allergen ratio (Mills et al., 2004). It has been estimated that pepsin secretion in adults is between 20 and 30 kUnits of enzyme activity/24 h at 37°C (Documenta Geigy 1973) and from the activity of commercially available pepsin preparations used in digestion assays, this can be estimated to be equivalent to around 10 mg of pepsin secreted/24 h; however, a typical adult dietary intake of protein could be estimated around 75 g/24 h (Mills et al., 2004).

THE FOOD MATRIX EFFECT

Protein hydration status, protease inhibitors and other matrix effects may also contribute to the ability of a protein to reach the sites of active immune sampling in the gastrointestinal mucosa and, thus, be an influence on the potential allergenicity (Teuber, 2002).

A study by Grimshaw et al. (2003) showed that the food matrix has a critical impact on allergen availability. In this study, three peanut-allergic subjects consumed larger doses of allergen in a vehicle with a high fat content in double-blind placebo-controlled food challenges (DBPCFC) and they did not experience the oral “early warning” symptoms previous to the manifestation of more severe symptoms.

This fact was attributed to allergenic epitopes that are concealed by the relatively high-fat food matrix, and are detected only after digestion of the fat. This could mean that allergens contained in a high-fat food matrix are released, and thus absorbed, more slowly than if they were in a lower-fat matrix. In addition, these

authors also observed that the presence of fat significantly inhibited the *in vitro* allergen detection.

On the other hand, the susceptibility of some allergens to proteolysis has been reported to be altered as a result of processing and interactions between allergens and other food ingredients, particularly lipids or polysaccharides.

The susceptibility of β -lactoglobulin to proteolysis by trypsin and chymotrypsin was apparently retarded by polysaccharides such as pectins, gum arabic and xylan probably due to the existence of non-specific interactions between molecular species in protein/polysaccharide mixtures. A significant reduction of the *in vitro* gastrointestinal digestibility of the peanut protein isolate was also observed in presence of gum arabic and xylan. The non-specific interactions between peanut protein isolate and these polysaccharides influenced the nature of the peptides released and produced a reduction of antibodies binding with digestion products (Mouécoucou et al., 2004).

Interactions between nutrients might affect food allergenicity. Indeed, the ability of proteins to associate with cell membranes and other types of lipid structures formed in foods has been recently described as a property that may play an important role in promoting allergenicity of food proteins (Breiteneder et al., 2005). Such associations may either occur naturally in the food, because of processing, or in the GI tract because of the digestive process (Mills et al., 2004).

Burnett et al. (2002) showed a range of protein allergens, such as milk allergens β -lactoglobulin, β -casein, BSA, 2S albumins from Brazil nuts and sunflower seeds, that were adsorbed to model stomach emulsions, providing a further means of resisting the pepsin digestion, whilst all allergens tested were desorbed with the addition of bile salts when the duodenal environment was mimicked. These authors suggested that the desorbed protein could be denatured and bound to surfactants (possibly associated with the mixed micelles present in the duodenum), further impairing the duodenal digestion.

FOOD PROCESSING

Food processing induces several physical, chemical and biochemical changes that are known to potentially impact the allergenic potential of proteins. Certain methods of food processing may enhance, reduce, or eliminate the allergenic potential of a food. This impact is affected by the nature of the food process used (i.e. heat, pH, enzyme, presence of water, type of gaseous atmosphere, concentration of proteins, chaotropes, etc.) and the time and intensity of the process (Wal, 2003). Processing may destroy existing epitopes on a protein or may generate new ones (neoallergen formation) as a result of change in protein conformation.

The formation of neoallergens after processing may partially explain why some people can tolerate unprocessed food. Conformational epitopes are typically expected to be more susceptible to processing induced destruction than the linear epitope on the same allergen (Sathe et al., 2005). Alteration of the conformation of the heat-labile proteins, and thus loss of conformational epitope(s), may explain the reduction or abolition of allergenic potential for some of these proteins (Thomas et al., 2007) (Figure 1.8).

In general, heat-treatments have been found to significantly reduce the IgE reactivity of well known allergens, most likely as the result of unfolding mechanisms (Besler et al., 2001). Baking, cooking, roasting, grilling, drying, pasteurization, as well as sterilization, denaturation (by unfolding and/or aggregation) and reaction with other molecules from the food matrix during thermal treatment, could result in a modification to the allergenic potential. Aggregation mechanisms may alter IgE binding by inducing the formation of intermolecular disulfide bonds.

Despite the presence of an allergen in a food, how it behaves in a food matrix may also impact its allergenicity. Therefore, the presence of other compounds, such as lipids and sugars, is also important. The Maillard reaction between free amino acids and aldehyde or ketone groups of sugars is one of the main chemical reactions that may affect the allergenicity of food proteins by inducing the

formation of aggregates which bind more effectively than unmodified allergens, and are also more resistant to gastric digestion (Thomas et al., 2007).

Wheat flour proteins have been shown to undergo heat-induced aggregation that results in a reduce digestibility (Hansen et al., 1976). this suggest that, after baking products have been ingested, at least a fraction of the original flours proteins can reach the intestine in the form of relatively large cross-linked structure (Simonato et al., 2001).

Finally, structural changes induced by food processing, such as thermal treatment, might change the intestinal transport properties of some allergens as recently shown for the native and heat denatured β -lactoglobulin, where the native protein was more abundantly transported in both M cells and enterocytes and was also degraded less during the transport (Ritkönen et al., 2006).

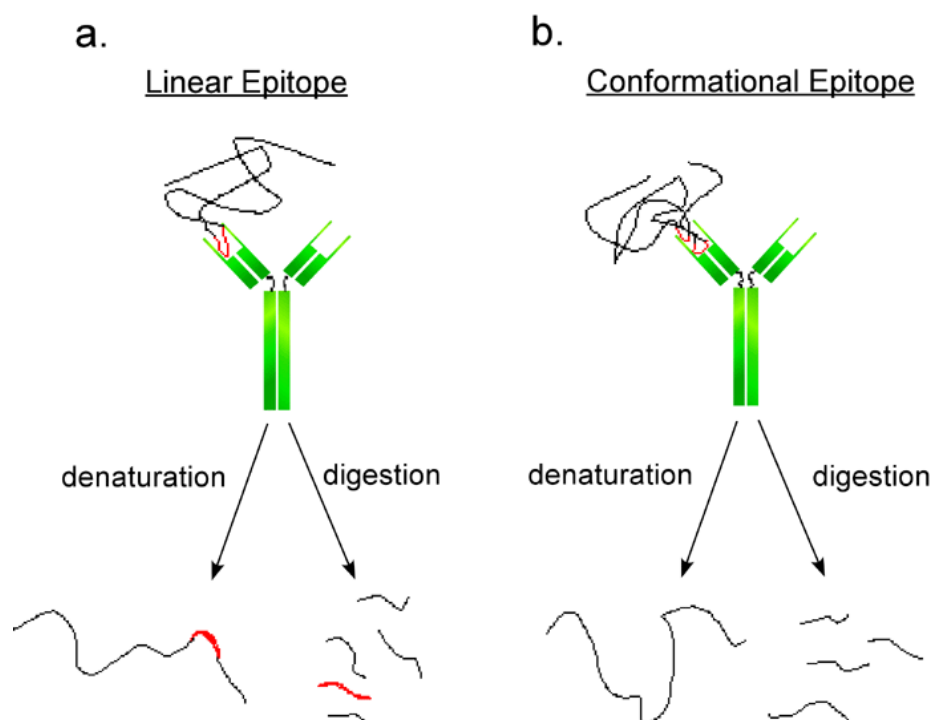


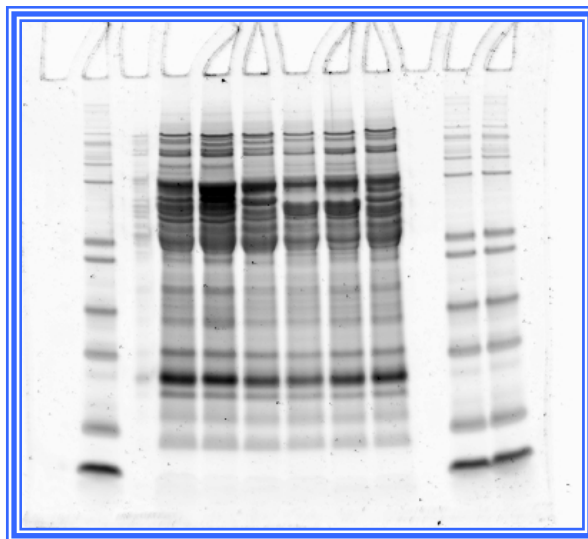
Figure 1.8: Schematic representation of two antibodies interacting with linear and conformational epitopes:

a. linear epitopes are short and continuous. After denaturation the linear epitopes may still be able to bind the antibody.

b. conformational epitopes are domains of proteins composed of specific regions of proteins chains. After denaturation the discontinuous epitope can no longer bind the antibody.

CHAPTER 2

ORAL-GASTRO-INTESTINAL DEGRADATION OF WHEAT PROTEINS USING AN IN VITRO MODEL OF HUMAN DIGESTION



2.1 INTRODUCTION

The human body uses a complex system to breakdown foods in order to extract the nutrients required for the maintenance of health. When food is ingested it is first crushed and sheared by chewing in the mouth, where it is mixed with saliva; a mixture containing salts, minerals, carbohydrates, anti-microbial compounds and enzymes. Salivary amylase is one of the most important enzymes present in the saliva, which is primarily involved in the oral processing of starches in foods, yielding glucose and maltose, but its activity in human is not exactly defined. After ingestion, food is subjected to gastric processing for a variable period of time during which the pH of the contents of the stomach, may fall to as low as pH 2.0, causing further breakdown of the food by acid hydrolysis.

The physiological conditions of gastrointestinal protein digestion have been well-defined. The gastric pH is between 1.5 and 2.0 during fasting, and it can increase up to pH 7.0 after ingestion of a meal due to the diluting and buffering effect of the food components (Charman et al., 1997). The pH is therefore likely to change over time with the digestion profile. Proteins are cleaved in the stomach by the endopeptidase pepsin with rather broad substrate specificity and an optimal pH in the range of 1-2 (Ganapathy and Leibach, 1999). In the duodenum, endopeptidases such as trypsin, chymotrypsin and elastase, and carboxypeptidase A and B continue the splicing of the polypeptide chain at a more alkaline pH with an optimal activity in the range of 7-8 (Charman et al., 1997). At the brush border membrane, the oligopeptides are further cleaved by amino-, di-, and endo-peptidases, resulting in a mixture of amino acids and small peptides, which can be absorbed by the enterocytes. The half-emptying time for the stomach is 0.5-3 h for fed conditions, while the residence time for the duodenum is 2 h. As the partially digested material enters the small intestine, the pH is neutralised and subjected to jejunal and ileal conditions, on its passage to the large intestine. During all of these phases, the food is mixed with enzymes and surfactants. Indeed, the bolus produced in the mouth is afterward subjected to proteases (pepsin), lipases and phospholipids in the stomach. Amylases,

proteases (trypsin and chymotrypsin), lipases (pancreatic lipase, colipase, phospholipases and cholesterol esterase), phospholipids and detergents (bile salts) act in the duodenal compartment. Pancreatic amylases (in the duodenum) act on carbohydrates, leading the breakdown of α -1-4-linkages thereby leaving α -1-6-linkages intact (Hinseberger et al. 2004). The endproducts of α -1-4-hydrolysis of the ingested oligo- and polysaccharides are α -limit-dextrin, maltriose, maltose, sucrose and lactose, which then need to undergo breakdown during the mucosal phase of carbohydrate digestion. Consequently, brush border enzymes, composed of a single β -galactosidase and three α -glucosidases, hydrolyse the remaining oligosaccharides by reducing them to monosaccharides which are taken up into the enterocytes.

As mentioned above, proteins undergo pepsinolysis in the stomach but some proteins may be resistant to digestion and persist processing. Food proteins are able to act as antigens in the human body, thus causing adverse reactions in sensitized individuals. In fact, proteins are released from food at all stages of the digestion process, and their degradation is dependent on their solubility and the accessibility of digestive secretions. Allergic reactions to the ingestion of wheat, can be divided into two main groups: (1) proteins responsible of WDEIA (Wheat Dependant Exercise Induced Anaphylaxis), associated with the ω_5 -gliadin which cause severe reactions including anaphylaxis, (2) all the other allergic reactions related to a vast range of wheat proteins. Symptoms, associated with the second group, include atopic dermatitis, urticaria and anaphylaxis. Despite the fact that that ω_5 -gliadin is responsible for the WDEIA reaction, most of the other symptoms are not clearly linked to a specific protein. Indeed, several proteins have been already detected as allergens which might elicit an allergic reaction after food ingestion. These include α -amylase inhibitors, serpins, wheat germ agglutinin, β -amylases, thioredoxin (Tatham and Shewry, 2008).

In order to sensitise an individual, food allergens must possess certain structural and biological attributes that preserve it from the denaturation and degradation conditions prevalent in the gastrointestinal tract. To achieve a better understanding

in the food allergy field it is important to assess the protein resistance to proteolysis.

Other factors may affect the gastric-intestinal digestion: food processing will influence digestion, for example, do baked foods undergo the same level of digestion as the natural food matrix, and does heating result in the resistance of certain proteins to proteolysis?

Moreover, studies focused on the oral digestion mentioned that the bolus formation incorporates two simultaneous oral processes: food comminution and lubrication (Chen 2009). They recommended that size reduction is crucial for bolus formation. Small particles size makes it possible for the tongue to press against the palate and pack them tightly together. At the same time, saliva gradually fills the gaps between food particles and increases their viscous cohesion. It is thought that the simulation of the oral breakdown of food is one of the most difficult tasks to build up, because of the complexity of the mouth and the several factors affecting the chewing step.

Therefore, this chapter was firstly focused on *in vitro* oral simulation and afterwards, oral-gastro-duodenal digestion profiles of proteins, from heated and unheated bread making flour (c.v. Hereward), were produced, in order to establish which proteins were resistant to digestion and if these effects were due to processing. This was done by further developing an existing *in vitro* biochemical model of digestion for foods (Mandalari et al., 2008); a static system which mimics the biochemical conditions found in the mouth, stomach and duodenum. Protein digestion profiles were followed by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1-D SDS PAGE).

2.2 THE *IN VITRO* BIOCHEMICAL MODEL OF DIGESTION FOR WHEAT FLOUR

2.2.1 PRINCIPLES OF *IN VITRO* DIGESTION

In view of the variety of physiological factors and structural food components which may afford protection to food proteins in the digestive environment, the assessment of the digestive stability of food allergens should involve simulating the environment of the human digestive system. *In vitro* digestion models should consider three main stages:

1. Processing in the mouth: this includes the mechanical breakdown of food particles and the enzymatic hydrolysis of starch due to the α -amylase present in the saliva.
2. Processing in the stomach: proteases and lipases activate degradation of proteins and lipids respectively. Moreover, the peristalsis motion helps the food ingested to go from the fundus through the antrum and phospholipids secreted by the gastric mucosa might have an effect on pepsinolysis (Moreno et al., 2007).
3. Processing in the duodenum: proteases, amylases, lipases, are involved in the further hydrolysis of food nutrients which is enhanced by the presence of biosurfactants.

Static models of digestion are systems which do not mimic the physical processes that occur *in vivo* (Wickham M. et al., 2009). Many of these models are quite simple because they involve homogenisation of the food, acidification with hydrochloric acid, addition of gastric enzymes followed by a varying delay simulating gastric residence time, neutralisation with sodium carbonate or hydroxide and addition of pancreatic enzymes and bile salts to perform the duodenal phase.

Several *in vitro* static models of digestion have been developed for the evaluation of protein digestion, their major limitations being that the digestion products are not removed during incubation, and they may have a potential inhibitory effect on enzyme activities (Thomas et al., 2007; Moreno et al., 2005; Fu et al 2002). Also

the key GI physical processes, including the temporal nature of gastric and duodenal processing, structure of food, pattern of mixing, particle size reduction and shear, all of affecting the digestion rate, are ignored. Therefore *in vitro* methods, which closely simulate transit through the upper GI tract, are needed to establish any losses in the gastric and small intestinal environment and ultimately the large bowel.

Static models differ from physical models, which are more complicated systems. A biochemical model and a physical model of digestion have been used for the work described in this thesis. The two systems are quite different since the physical model takes into account parameters described above, such as shear rate and dilution effects, enabling a better simulation of the human gastric digestion process. Ultimately, this method will contribute towards improving the understanding of how food structure can affect protein digestion.

The ratio of enzymes, surfactants and substrate used in static models of digestion are usually estimated on the basis of the physiological amount, but it is difficult to simulate exactly the ratios that occur *in vivo*. For example, pepsin secretions in adults have been estimated between 20-30 KU of enzyme activity in 24 hours at 37°C (Wickham et al. 2009). From the activity of commercially available pepsin preparations used in digestion assays, this would be the equivalent of around 10 mg pepsin secreted in 24 hours. Despite the difficulty in mimicking the digestion process with *in vitro* systems, resistance of proteins to pepsinolysis has become a crucial point in the approaches used for assessing the allergenic potential of novel proteins. In fact it is already known (Thomas et al. 2007) that peptides require a molecular weight greater than 3 kDa in order to stimulate an immune response and large stable fragments, as well as intact proteins, as the potential to act as sensitizers.

The use of models of digestion to evaluate the resistance of food protein to hydrolysis is a key point for assessing food allergenicity. Another important factor to take into account in food allergy studies is the structure of food and the kind of treatment that allow unheated foods to become edible.

2.2.2 MATERIALS

Bread making flour (*Triticum aestivum*, c.v. Hereward) was supplied by the ECF P6 project; Health Grain (Food-CT-2005-514008). All chemicals and reagents were of analytical grade and were supplied by Sigma (Poole, Dorset, UK) unless otherwise stated. Human salivary amylase (Type IX-A, lyophilized powder, 210 U mg⁻¹ solid), pepsin (3.260 U mg⁻¹ protein), pancreatic lipase (15000 U mg⁻¹ solid), trypsin type IX-S (13700 U mg⁻¹ solid), chymotrypsin type II (57.24 U mg⁻¹ solid), α -amylase from porcine pancreas DFP treated (1325 U mg⁻¹ protein), Bowmann-Birk trypsin-chymotrypsin inhibitor from *Glycine max*, sodium thaurocholate hydrate, sodium glycodeoxycolate and cholesterol powder were supplied by Sigma (Poole, Dorset, UK). Egg L α - phosphatidylcholine (PC, lecithin grade 1, 99 % purity) was obtained from Lipid Products (South Nutfield, Surrey, UK). Co-lipase from porcine pancreas (70000 U mg⁻¹ solid) was supplied by Roche (West Sussex, UK).

2.2.3 METHODS

2.2.3.1 Sample preparation

A heated flour paste was prepared by mixing 5 g of flour with 21 g of distilled water (Figure 2.1 A) to form a paste. The paste was subsequently heated at 100°C for 10 min (Figure 2. 1 B) in order to achieve full gelatinisation of the starch. Afterwards the heated material was cooled down and an unheated paste was prepared. An amount of paste (Figure 2. 1 C), containing 20 mg of protein, was subjected to *in vitro* digestion following the method described below.

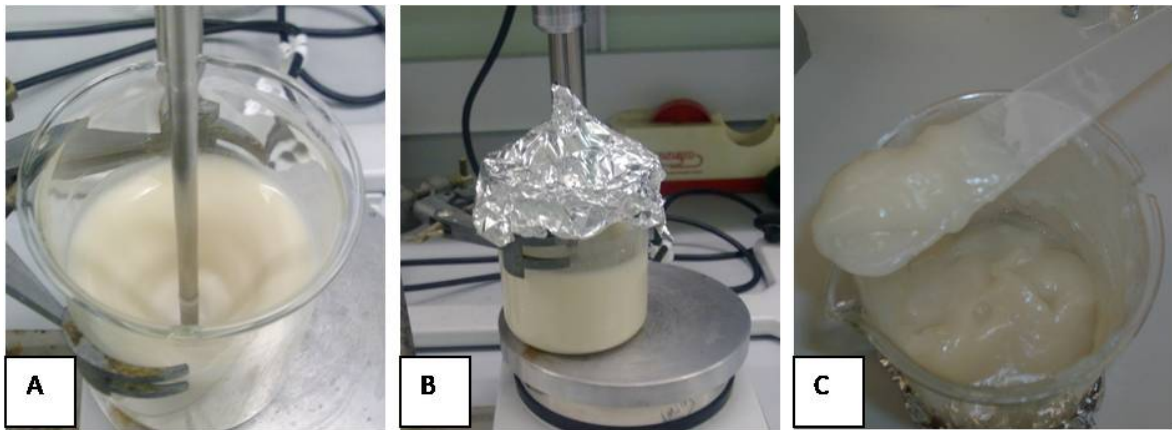


Figure 2.1 Preparation of heated flour. A: flour and water were mixed with a stirrer; B: the mixture was heated on a hotplate covered with foil; C: the obtained heated paste.

2.2.3.2 *In vitro* digestion

Before performing the *in vitro* digestion, the following solutions were prepared.

Simulated Salivary Fluid (SSF): SSF was prepared with 0.15 M NaCl and 3 mM $\text{CO}(\text{NH}_2)_2$, adjusting the pH at 6.9 with HCl or NaOH. The solution was filtered through a 0.2 μm filter (Millipore).

Simulated Gastric Fluid (SGF): SGF was prepared with 0.15 M NaCl adjusting the pH at 2.5. The solution was filtered through a 0.2 μm filter (Millipore) and stored at 4°C until use.

Simulated Duodenal Fluid (SDF): SDF was made of 0.15 M NaCl adjusting the pH at 6.5 and filtering it through a 0.2 μm filter (Millipore).

Gastric lecithin solution: A standard stock solution of egg lecithin (grade 1, Lipid Products) was prepared dissolving one vial of lecithin in chloroform to obtain a total volume of 10 mls and a final concentration of 63.5 mM. Once prepared, the stock solution was stored in the freezer at -18°C.

The day before the lecithin was required, 94 μl of the lecithin stock solution (63.5 mM) was added to 50 ml round-bottomed flasks. The sample was dried down using a rotary evaporator (Buchi, Rotavapor-R, Buchi Labortechnik AG, Flawil, Switzerland) until the solvent was removed and subsequently was placed in a vacuum oven (Model S40403, Townson & Mercher Ltd., Runcorn, UK) (sparged

three times with nitrogen, room temperature) and dried down under vacuum overnight. On the same day as the digestions were performed, Simulated Gastric Fluid (SGF) solution was placed was incubated at 37°C, 170rpm for approximately 20 min, to bring to temperature. The flask containing the dry lecithin was removed from the vacuum oven and 12.175 g of the SGF was weighed into the flask, in order to obtain a final lecithin concentration of 0.127 mM. Approximately 5 (3 mm) glass beads were added to the sample and was subsequently incubated at 37°C for 30 min (setting 170 rpm). The lecithin solution was sonicated (Branson digital sonifier, model 250, Branson Ultrasonics Corporation, Conneticut, USA) until clear to the eye (40% power on a 3 pulse cycle; for a total of 4.5 minutes) while being cooled in ice. This step was performed to reduce the crude lecithin solution into a solution of single shelled liposomes. After sonication, the sample was filtered through a Nalgene 0.22 µm nylon syringe filter to remove any titanium deposited by the sonicator.

Hepatic mix: 512 µl of 63.5 mM standard stock solution of egg lecithin plus 1500 µl of 10 mM cholesterol solution, were dried together in a round-bottomed flask, using a rotary evaporator, which was kept overnight in the vacuum oven. Before the sonication step, 12.5 mM of sodium thaurocholate hydrate and 12.5 mM of sodium glycodeoxycolate were added and the flask was incubated until the solution cajnaged from cloudy to clear, if this did not happen, the solution was sonicated as previously described.

2.2.3.2.a *In vitro* Oral and Gastric Digestion

Simulated *In vitro* digestion experiments were performed on both unheated and heated wheat flour. Samples were digested by following a modified method based on that described by Mandalari et al. (2008) and by Aura et al. (1999). *In vitro* digestion was usually performed in one bottle on the whole sample but this proved to be problematic with the flour samples that formed an in-homogenous mixture when done this way. Therefore, to overcome this, *in vitro* digestion was performed

in individual bottles, each one corresponding to one sampling time point of gastric digestion. To perform the oral digestion, 20 g of heated and unheated flour pastes were mixed with 4 ml of SSF (pH 6.9) and 2 U ml^{-1} (Aura et al. 1999) of Human salivary amylase (Sigma, Dorset, UK) and were mixed for 5 min in an orbital shaking incubator at $37\text{ }^{\circ}\text{C}$, 170 rpm. Afterwards, the pastes were divided into amounts containing 20 mg protein (estimate); weighed into individual Bijoux bottles (Sterilin, UK). Gastric digestion was performed sequentially, for both unheated and heated oral digesta samples. Salivary amylase activity was halted by decreasing the pH to 2.5 followed by addition of $1400\text{ }\mu\text{l}$ simulated gastric fluid (SGF, pH 2.5), $550\text{ }\mu\text{l}$ 0.1 mM lecithin solution (representing physiological PC conditions) and pepsin (163 U mg^{-1} protein), according to an approximately physiological ratio of enzyme/substrate (1:20, w/w). The bottle, corresponding to time 0, was stopped immediately after addition of the pepsin and was mixed well with $300\text{ }\mu\text{l}$ 1 M ammonium bicarbonate to halt pepsinolysis (0 time point). The other samples were incubated at 37°C , 170 rpm until they were stopped at the following time points: 1, 2, 5, 10, 20, 30, 60 and 120 min of incubation. Collected samples from the gastric phase were stored at $-20\text{ }^{\circ}\text{C}$ while all the remaining samples were adjusted to pH 7.0, in order to stop pepsin activity and stored on ice, ready for the duodenal phase.

2.2.3.2.b *In vitro* Duodenal Digestion

The pH of the gastric digesta for both heated and unheated flour pastes was adjusted to pH 6.5 and $60\text{ }\mu\text{l}$ of 0.5 M BIS-TRIS buffer pH 6.5 plus $340\text{ }\mu\text{l}$ hepatic mix were added. Digestion was carried out by addition of the following solutions: pancreatic lipase solution (91.6 U/sample), pancreatic α -amylase DFP treated (25 U ml^{-1}), co-lipase (0.0088 mg/sample), trypsin at an approximate ratio of enzyme/substrate 1:100 w/w (0.05 mg/sample corresponding to 34.25 U mg^{-1} protein), chymotrypsin at an approximate ratio of enzyme/substrate 1:400 w/w (0.2 mg/sample corresponding to 0.5 U mg^{-1} protein). A time 0 sample of digesta was taken immediately after addition of trypsin and chymotrypsin and all samples were

incubated at 37°C, 170 rpm, until they reached their sampling time points. Proteolysis was stopped after 1, 2, 5, 10, 20, 30, 60 and 120 min of incubation, by addition of two-fold excess of trypsin-chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix. Samples were stored at -20 °C until required for further analysis.

2.3 ONE-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (1-D PAGE)

2.3.1 PRINCIPLES OF 1D-SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) is a reliable method available for protein separations in complex mixture. This technique, which is based on the mobility of charged solutes in an applied electrical field, is influenced not only by charge, but also by voltage, distance between the electrodes, size and shape of the molecule, temperature and time. The electrophoretic separation and high resolution of proteins and peptides on polyacrylamide gels is achieved by using a stacking gel, which concentrates proteins, even from dilute solutions. This process significantly improves the resolution of the subsequent separation by shrinking the original sample into very thin, highly concentrated starting zones so that all of the protein molecules begin the electrophoretic separation at the same point.

Electrophoresis is performed using a detergent, SDS (sodium dodecyl sulphate), in order to dissociate proteins into individual chains and separate them according to their molecular weight. Denaturation and binding of the anionic detergent SDS to proteins and peptides generally results in a relatively uniform negative charge because most proteins bind similar amounts of SDS and become highly negatively charged by the addition of strongly acidic sulfonic acid groups. Therefore, possible changes in electrophoretic behaviour of different tertiary protein structures are negated since complete unfolding of proteins occurs by SDS binding.

In polyacrylamide gel electrophoresis,, proteins, charged negatively by the binding of the anionic detergent SDS (sodium dodecyl sulfate), can be separated within a matrix of polyacrylamide gel in an electric field according to their molecular weights.

Polyacrylamide is formed by the polymerization of the monomer molecule-acrylamide crosslinked by N,N'-methylene-bis-acrylamide (BIS). Free radicals generated by ammonium persulfate (APS) and a catalyst acting as an oxygen

scavenger (-N,N,N',N'-tetramethylethylene diamine [TEMED]) are required to start the polymerization since acrylamide and BIS are nonreactive by themselves or when mixed together without APS and TEMED..

The distinct advantage of acrylamide gel systems is that the initial concentrations, of acrylamide and BIS, control the hardness and degree of crosslinking of the gel. The hardness of a gel controls the friction between macromolecules (proteins) as they move through the gel in an electric field, and therefore it affects the resolution of the components to be separated. Hard gels (12-20% acrylamide) retard the migration of large molecules more than they do small ones. In certain cases, high concentration acrylamide gels are so tight that they exclude large molecules from entering the gel but allow the migration and resolution of low molecular weight components of a complex mixture. Alternatively, in a loose gel (4-8% acrylamide), high molecular weight molecules migrate much farther down the gel and, in some instances, can move right out of the matrix

In addition, the mobility of polypeptides in SDS-PAGE gel systems is proportional to their molecular weights. This property makes it possible to measure the molecular weight of an unknown protein.

SDS-PAGE can be carry out using non-reduced or reduced samples. In fact, denaturation of proteins is performed by heating them in a buffer containing a soluble thiol reducing agent (2-mercaptoethanol; dithiothreitol) and SDS. Mercaptoethanol reduces all disulfide bonds of cysteine residues to free sulfhydryl groups, and heating in SDS disrupts all intra- and intermolecular protein interactions. This treatment yields individual polypeptide chains which carry an excess negative charge induced by the binding of the detergent, and an identical charge:mass ratio.

Gels resulting from the SDS-PAGE need to be fixed and stained in order to visualize the protein bands. The fixing step is necessary to immobilize the separated proteins in the gel and to remove any non-protein components which might interfere with subsequent staining. Depending on gel thickness, the gel is

submersed in the fixative solution for one hour at least, but usually overnight, with gentle shaking.

Widely used fixatives are either 20% (w/v) trichloroacetic acid (TCA), or methanolic (or ethanolic) solutions of acetic acid (e.g. methanol / distilled water / acetic acid 45/45/10). A disadvantage of the latter procedure is that low molecular weight polypeptides may not be adequately fixed (Görg et al., 1988).

The most used staining reagents are: Coomassie Brilliant Blue R250, silver stain and SYPRO[®] Ruby (Hetty C. van den Broeck et al. 2008). The Coomassie and the silver stain are the most widely used for the visualization of protein separated by SDS-PAGE. Coomassie is an organic dye that complexes with basic amino acids such as arginine, lysine, histidine and tyrosine. Conventional Coomassie staining is able to detect as little as 30-100 ng of protein. Silver stain is more sensitive but it gives less reproducible results because of the staining-end-point which is not easy to determine. SYPRO[®] Ruby is perhaps the most sensitive fluorescent staining technique in common use for detecting polyacrylamide gel resolved protein, which is able to detect 1-2 ng protein/band (Simpson R.J., 2003). To detect protein bands, a fluorescence scanner is required. The excitation maximum and emission maximum for SYPRO[®] Ruby are 280 nm and 450/610 nm, respectively. SYPRO[®] Ruby dye interacts strongly with lysine, arginine and histidine residues.

2.3.2 MATERIALS

MES buffer (10x), NuPAGE LDS (4x) sample buffer, Mark 12™ molecular weight markers, NuPAGE 10% Bis-tris PAGE gels and Sypro Ruby Protein Stain® were supplied by Invitrogen (Chalfont St Giles, UK). All other chemicals and reagents were of analytical grade and were supplied by Sigma (Poole, Dorset, UK) unless otherwise stated.

2.3.3 METHODS

Oral, gastric and duodenal digesta samples of unheated and heated flour were processed to obtain a total, soluble and insoluble protein extraction. Each of the fractions was separated as follow.

2.3.3.1 Total protein extraction

500 µl of undigested and digested sample of both unheated and heated flour, were mixed with an equal amount of NuPAGE LDS (4x) sample Buffer diluted to 1x (Invitrogen, UK) and subsequently incubated at 100 °C for 10 min. Samples were cooled down and centrifuged at 10,000 x g for 10 min. Supernatants, containing the protein extract were collected and retained for the SDS-PAGE analysis.

2.3.3.2 Soluble and insoluble protein extraction

1000 µl of undigested and digested sample of both unheated and cooked flour were centrifuged at 10,000 x g for 10 min. Supernatants were collected for SDS-PAGE while pellets were further processed. Pellets, containing the insoluble protein fraction were added to 500 µl of NuPAGE LDS sample Buffer 1x and incubated at 100 °C for 10 min. The supernatants were retained and used to perform SDS-PAGE analysis.

2.3.3.3 SDS-PAGE analysis

Prior to electrophoresis, 65 μl of protein extract was added to 25 μl of NuPAGE LDS sample Buffer 4x and 10 μl of 0.5 M DL-DTT (Sigma, Poole, Dorset, UK). The samples were whirli-mixed and heated at 70°C for 10 min and were left to cool to room temperature.

SDS-PAGE was performed using 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK) according to the manufacturer's instructions. 10 μl of each sample was loaded into the gel wells and 7 μl of unstained molecular weight markers were also loaded. Molecular weight markers (Invitrogen, UK) comprised the following mix of proteins: insulin α -chain (M_r 2500 Da), insulin β -chain (M_r 3500 Da), aprotinin (M_r 6000 Da), lysozyme (M_r 14400 Da), trypsin inhibitor (M_r 21500 Da), carbonic anhydrase (M_r 31000 Da), lactate dehydrogenase (M_r 36500 Da), glutamic dehydrogenase (M_r 55400 Da), BSA (M_r 66300 Da), phosphorylase B (M_r 97400 Da), β -galactosidase (M_r 116300 Da), myosin (M_r 200000 Da). Gels were run for 35 minutes. Gels were fixed using 40% (v/v) methanol and 10% (v/v) trichloroacetic acid, stained with Sypro Ruby[®] protein stain (Invitrogen, UK) destained using methanol 10% (v/v), 6% (v/v) acid trichloroacetic and imaged at 100 μm resolution using the Pharox FX Plus Imager (Excitation: 532nm; Emission: 605nm) (BioRad, UK).

2.4 RESULTS AND DISCUSSION

Wheat is a typical example of a food which cannot be consumed without any type of thermal processing such as cooking and baking. Protein digestibility can be strongly affected by thermal processing, which induces modifications of the physicochemical and immunological characteristics of the potential allergens of foods (Davis et al., 1998). *In vitro* digestion studies performed using the meal as eaten are an important tool to achieve a better understanding of a food allergen's degradation. Recently, authors have been focused on digestion experiments involving real food (Simonato et al., 2001; Pasini et al., 2001; De Zorzi et al., 2006; Petitot et al., 2009) by using *in vitro* chemical models of digestion as well as simulating the oral phase (Petitot et al., 2009). However, the models only incorporated proteases and no other physiologically relevant components such as bio-surfactants and bile salts.

My experiments were performed using a biochemical model of digestion modified from Mandalari et al. (2008) and Aura et al. (1999). This *in vitro* digestion system involved the use of proteases, amylases, bio-surfactants and bile salts. It has been shown that surfactants, like phosphatidylcholine, that are secreted by the gastric mucosa, might alter the kinetic of protein breakdown during *in vitro* gastric and duodenal digestion (Moreno et al., 2005, Mandalari et al., 2009).

My experiments were carried out by using unheated and heated flour. In order to obtain samples at different time points during *in vitro* digestion we firstly tried to digest flour in a single bottle by taking samples during pepsinolysis, however we could not achieve reproducible results especially with the heated sample, as the gelatinised starch made the sample in-homogenously dispersed into the gastric fluid (Figure 2.1, 2.2, 2.3 appendix). Then, we performed the same experiment but divided the flour pastes into individual Bijoux bottles so that we could achieve reproducibility. Digestion experiments were firstly carried out with unheated flour studying protein degradation during oral, gastric and intestinal conditions. Samples were separated by 1-D electrophoresis and resulting gels were stained with SYPRO[®] Ruby stain. This dye is an end-point stain with a high sensitivity. It is

reported to detect lysine, arginine as well as histidine: it is an easy one step staining of protein without long destaining steps and it is compatible with MS. A disadvantage of SYPRO ruby is the speckled background staining (Hetty C. van den Broeck et al., 2008). Oral digestion was performed using human salivary amylase according to Aura et al. (1999). We did not see changes in protein digestion peptides after performing the oral digestion as expected, because the salivary amylase acts on the starch rather than proteins. There was clear evidence that most of the protein degradation has been achieved during the gastric phase rather than the duodenal one (figure 2.4). Regarding the insoluble protein fraction (pellet) it was noticeable that after 5 min of gastric pepsinolysis a vast group of proteins, belonging to the prolamin family, seemed to disappear quickly (figure 2.6). The same behaviour has been seen for the heated flour (figure 2.9). The soluble protein profile of heated flour, showed a strong band around 12 kDa which seemed to be resistant to gastric digestion (figure 2.8). Indeed, these proteins that may belong to the alpha amylase inhibitors family seemed to be more resistant to digestion in the heated flour, where they were still well noticeable after 20 min of gastric pepsinolysis (figure 2.8).

When observing the total extracted proteins it was evident that a group of proteins resolved between the range of 36 and 55 kDa, which may include high molecular weight and low molecular weight glutenin subunits, disappeared slower in the heated flour rather than in the unheated flour. It has been thought that processing of wheat, such as bread making and boiling pasta, might modify proteins and alter their resistance to protein digestion. However, the data from this study did not show any clear or consistent differences in protein digestion profiles using 1 D SDS-PAGE to detect qualitative changes. Therefore it was hypothesised that the cooking procedures might slow digestion of proteins, probably because of interactions with proteins and the gelatinised starch.

Protein profiles, corresponding from duodenal digestion, were not well defined and hindered understanding how wheat proteins are degraded in the intestinal tract. This was due to the presence of Bowman Birk Inhibitors (soybean trypsin inhibitor)

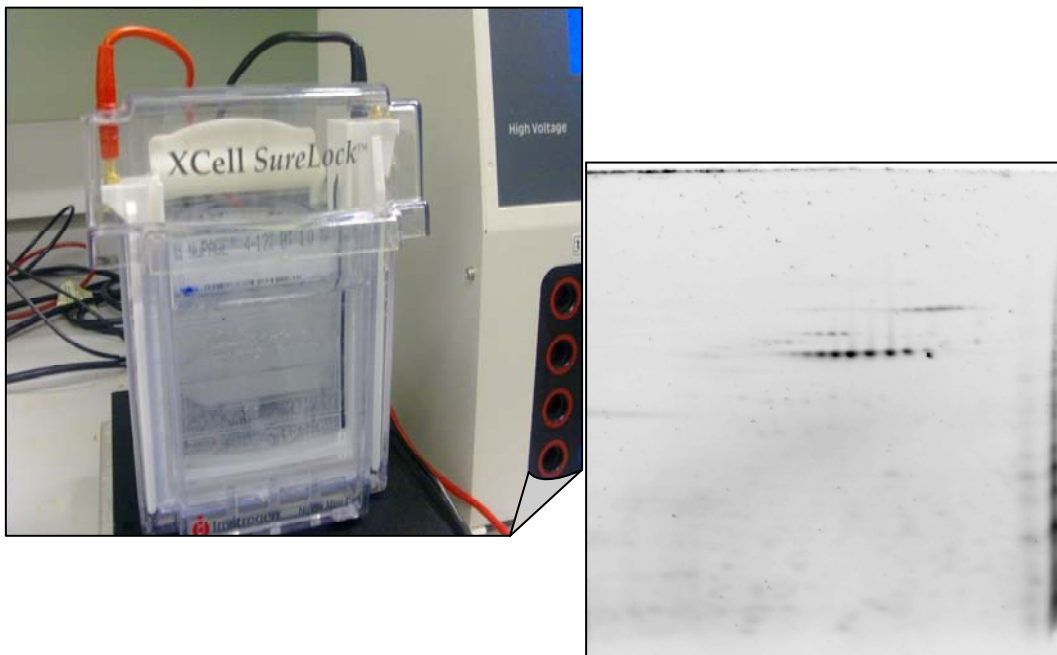
and digestive enzymes bands, which were well detected on the gels and interfered with the resolution of protein bands from the digested samples (figure 2.4-2.9, lines 13-24). This limitation suggested that the use of 1-D PAGE was not sensitive enough to define physical changes to proteins and their relative abundance. On the basis of these ideas the next step, for the evaluation of wheat protein degradation during digestion, the use of a more sensitive method to separate proteins was employed. In the next chapter 2-DE has been used to obtain protein profile maps of unheated and heated flour upon oral and gastric digestion, separating individual proteins according to isoelectric point (pI) and their molecular mass (M_r).

CHAPTER 3

SIMULATED GASTROINTESTINAL DEGRADATION OF WHEAT FLOUR

ALLERGENS:

A proteomic study



3.1 INTRODUCTION

In the first part of this work protein profiles, obtained from the oral, gastric and duodenal digestion, have been separated and compared by using 1D SDS-PAGE. However to gain a better identification of changes in protein patterns due to the cooking process or to the gastric pepsinolysis, a proteomic approach has been involved in this second part.

Separation of peptides and proteins represents a key element in proteomics analysis. By far the two most popular methods for protein separation are two-dimensional polyacrilamide gel electrophoresis (2D-PAGE) and liquid chromatography (LC) (Monaci et al., 2009).

Proteomics is a branch of science focused on proteins. The term “proteome”, coined in 1994 as the equivalent concept of “genome”, is used to describe the complete set of protein that is expressed by the entire genome.

Comparison of 2-DE spot patterns from different samples to generate quantitative protein expression profiles allows the identification of potential biomarkers that are characteristic of a specific pathological or physiological state of a cell or tissue. Thus, proteomic techniques are used to answer questions relevant to a variety of biological processes, including differentiation and development, aging, cancer and allergy (Dietz et al., 2009).

Moreover, proteomics is a promising approach to identify proteins in food matrix and to assess changes in protein profile due to the technological processing.

Indeed, in the food science field, proteomics is widely used to assess the technological, nutritional and safety quality. For instance, bioactive compounds can be screened in order to identify the so called “functional food” (Carbonaro et al., 2004). In the meat science field, proteomics has been used to identify proteins that may be involved in meat quality alteration. Moreover, the proteome map of muscle mouse as been employed as a reference to compare meat from different species (Roncada et al., 2002).

Furthermore, proteomic has shown a powerful tool in cereal science, since, using a proteomic approach, wheat proteins responsible of physiological and

technological functions and involved in breadmaking quality, have been characterised (Amiour et al., 2002).

Finally, advance proteomic has been applied in food allergy studies (Sander et al., 2001; Akagawa et al., 2007; Sotkovsky et al., 2008), in order to identify potential allergens involved in both adverse respiratory and food allergic reactions.

Moreover, the identification of four sesame seed allergens, carried out by using proteomic techniques, was a useful finding which allowed the production of recombinant allergens that may be use in immunotherapy (Beyer et al., 2002).

Proteomic techniques used 2D gel electrophoresis and mass spectrometry to separate and identify proteins, and, moreover, sophisticated informatics approach to analyse the obtained data.

Nowadays proteomic involved different kinds of studies which may be divided in 3 main groups (Simpson, 2003):

1. proteomic analysis: it is focused on identification and characterisation of proteins, including their post-translation modifications, for example phosphorylation and glycosylation; mass spectrometry identification is the main medium used;
2. expression proteomics: it is used to investigate about the global profile of expressed proteins by comparing 2D gel profiles but also using novel techniques such as liquid-based isoelectric focusing (IEF) or ion-exchange chromatography/reversed-phase high-performance liquid chromatography (RP-HPLC). The protein identification is subsequently performed by using spectrometry techniques. These methods are also complemented by DNA-based array methods;
3. cell-mapping proteomics: it is focused on protein-protein interaction studies and intracellular signalling which are determined by the identification of protein complexes obtained by affinity purification and protein identification by mass spectrometry.

The workflow of a standard proteomic experiment includes most of these steps: the sampling preparation, the protein extraction or fractioning or purification, the protein separation, the mass spec analysis and identification, and the statistical analysis of data.

Separation of protein by 2D-E is by far the predominant technology and it is continuously being improved in the areas of separation of hydrophobic proteins, gel staining, image capture and analysis.

Moreover there are second generation techniques which can improve protein resolution and give a better reproducibility. For instance, it is the case of protein labelling techniques prior to gel separation (Jorin Novo et al., 2009). These pre-labelling dyes are widely used in a variety of applications also known as 2-D DIGE approaches.

Mass spectrometry (MS) is a widely applied technique, used for mass determination and protein identification and represents one of the major techniques involved in proteomic studies.

Classical MS techniques are based on quadrupole and ion trap mass analysers which are able to produce a peptide mass fingerprint (PMF); moreover, in the past few years the development of Orbitrap and other techniques, that are able to give more accurate fragment mass fingerprint (FMF), opened new possibilities in proteome analysis.

Second generation systems in MS analysis include, for instance, the use of a hybrid ion trap Fourier transform mass spectrometer which should give highly accurate mass determinations (Jorin Novo et al., 2009).

Mass spectrometry, performed after digestion of proteins, allows their identification, since it provides peptide mass fingerprints (PMFs) which are mass spectra of the peptide mixtures, resulting from the previous digestion carried out usually by trypsin.

In order to figure out the peptide sequence by using peptide mass fingerprints, database search engines, are currently the method of choice for annotating mass spectra with peptide sequences. The mainly used databases are MASCOT, SEQUEST and TANDEM which use mass spectrometry data to identify proteins from primary sequence databases.

These databases search algorithms calculate, for every peptide spectrum match (PSM), a score that reflects the quality of the cross-correlation between the experimental and the computed theoretical peptide spectrum. The scored PSMs are ranked, and typically, only the best matches for each spectrum are reported. Sometimes PMF data are not sufficient for significant protein identifications, or the peptide or protein searched is not present in the database.

This may happen especially for those organisms which genome has not been completely sequenced, as wheat.

Indeed, among the three major crops, rice, maize and wheat, only the rice and maize genomes have already been completely sequenced. This is because wheat has a very large genome that is 35 times larger than rice (Mustafa et al., 2004), thus only several hundred genes out of the probable 120,000 coding sequences that are presumed present in the wheat genome have been mapped to chromosomes (Moore et al., 2007).

However, the use of *Brachypodium* genome, an ancestral wheat specie which has been entirely sequenced, might be used to search matching peptide sequences. Therefore, not really often wheat proteins could be identify by using *Brachypodium* genome, thus requiring new tools for the identification.

Indeed, in recent years, new molecular marker based genetic maps, microarrays and libraries of sequence as ESTs improved protein identification (Moore et al., 2007).

ESTs (Expressed Sequence Tags) are small pieces of DNA sequence (usually 200 to 500 nucleotides long) that are generated by sequencing either one or both ends of an expressed gene. In this approach, a partial amino acid sequence, known as the sequence tag, is combined with the mass of the peptide to search relevant databases. Whether peptide mass fingerprints searched against the database do not match any sequence data, ESTs libraries can be used to identify those proteins. The advantage to this method is that it is fast and, if enough amino acid sequence is obtained, can be a very specific method for protein identification. However, this search method also depends upon a complete and well-annotated sequence database.

Other proteomic techniques, such as liquid chromatography (LC) which allows the identification of proteins but without involving 2D-E protein separation, are currently used. Some of the emerging second generation techniques include the ICAT techniques (Isotope coded affinity tagging), that allows a better protein quantification and identification (Kvasnička 2003).

Wheat proteome has been investigated by several researchers, since the identification of proteins, especially those with technological properties, has been one of the main purposes which allowed a better understanding on factors regulating wheat products quality.

Different authors give a contribution on the identification of wheat proteins. Indeed, soluble proteins from dough were studied by Salt et al. (2005). This study aimed to identify proteins by coupling 2-DE and MALDI-ToF (matrix-assisted laser desorption/ ionization time of flight) analyses. The main groups of proteins found by these authors were protective proteins like those belong to the α -amylase inhibitors family, tritin and serpins, and metabolic proteins as β -amylases.

Puroindolines, which are important proteins associated with grain texture were described by Amiuor et al. (2002).

As already known, most of the wheat proteins belong to the storage proteins, as gliadins and glutenins with high and low molecular weight, which form, when hydrated, a viscoelastic network called "gluten".

Some authors identified wheat gliadins using MS/MS (Mamone et al., 2005). They found α, ω, γ - gliadins which showed highly conserved sequences and a complex heterogeneity nature.

Moreover, high molecular weight glutenin subunits (HMW-GS) were studied recently (Zhang et al., 2008) by using proteomic techniques which coupled high performance liquid chromatography (HPLC) and MALDI-ToF mass spectrometry, thus finally identified 16 major HMW glutenins from wheat flour.

Proteomic studies have also been used during the last few years to identify wheat allergens, since 2D-E, followed by immunoblotting and subsequently amino acid

sequencing, allowed the identification of proteins potentially involved in adverse reactions to wheat.

Respiratory allergies as baker's asthma were widely studied rather than food allergy. Indeed, it is well known that the salt-soluble proteins of wheat are mainly involved in baker's asthma. Studies, applying proteomic techniques, were performed by Weiss et al. (1997) who detected the N-terminal amino acid sequencing of reactive proteins, thus identifying albumins and globulins as potential allergens involved in baker's asthma.

Sander et al. (2001) also performed a proteomic study and mentioned the glyceraldehyde-3-phosphate dehydrogenase, the triosephosphate isomerase and serpin proteins, found by using mass spectrometry, as allergens involved in respiratory allergy to wheat.

The application of proteomic strategies, to identify food allergens, has been recently exploited by some authors that identified wheat allergens by mapping proteins extracted from wheat flour (Sotkovsky P. et al., 2008, Akagawa M. et al., 2007).

Wheat food allergy has been studied by performing 2D-E followed by mass spectrometry. Akagawa et al. (2007) showed IgE binding to γ -gliadin and LMW subunits using sera from patients suffering of wheat food allergy. Moreover, the same authors found a dimeric α -amylase inhibitor and serpins as potential allergens involved.

Recently, advanced strategy, to study food allergy, involved the identification of proteins, separated by 2D-E, which firstly underwent *in vitro* pepsin digestion. Sotkovsky et al. (2008) identified, following these techniques, 19 potential wheat allergens, including α -amylase inhibitors, serpins, β -amylases, and 27K proteins belonging to the soluble wheat fraction.

In the previous section (chapter 2), digestion profiles of heated and unheated flour were obtained using 1-D SDS PAGE, but the number of bands visible on the gels did not correspond to the high number of individual protein spots observed on 2-D gels for previous studies (Sotkovsky P. et al., 2008; Akagawa M. et al., 2007).

Thus, this 3rd chapter has been focused on the identification of proteins, of heated and unheated wheat flour (c.v. Hereward), resistant to simulated gastric digestion, and 2D-E maps were performed.

A proteomic approach was used to effectively separate individual proteins and to obtain a clearer representation of the changes to proteins during *In vitro* digestion, and to provide protein identification using MALDI-MS and MASCOT.

3.2 TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (2D-PAGE)

3.2.1 PRINCIPLES OF THE METHOD

2D-PAGE is a multi-dimensional system coupling isoelectric focussing (IEF: the first dimension) and SDS-PAGE (the second dimension), and enables the separation of complex mixture of proteins according to their isoelectric point (pI) and their molecular weight (M_r). Usually spot identification is performed on the resulting 2D gels in order to characterized separated proteins. Briefly, the major steps of the separation and characterization of protein with proteomics techniques include: (1) sample preparation, protein solubilisation and quantification; (2) protein separation by 2-DE (IEF plus SDS-PAGE); (3) protein staining (Coomassie, silver stain or SYPRO[®] Ruby); (4) image analysis (which allows comparison between gels by matching spots and enables spot quantification) and (5) protein identification (mass spectrometry methods produce peptide mass fingerprints, PMFs, or fragment mass fingerprints FMFs).

3.2.1.1 Sample preparation

To achieve a high resolution of 2-DE gel proteins, the sample has to be denatured, disaggregated, reduced and solubilised, to obtain a complete disruption of molecular interactions. Sample preparation should be as simple as possible to increase reproducibility, and protein modifications during sample preparation must be minimized, because they might results in artefact spots on 2D gels.

Interference compounds: one of the problems encountered during 2-DE experiments is the presence of interfering compounds such as salts, lipids, and polysaccharides. However they should be removed before starting the IEF.

Salts that should be remove if their concentration is too high (>100mM) do not interfere through their binding to proteins but they interfere directly with the IEF process. Briefly, salts migrate through the pH gradient, generating Joule heat, and they accumulate at both ends of the electrophoresis support. This accumulation

creates very high conductivity zones. Because of the high conductivity the voltage drop and thus the electric field is very low in these zone and proteins cannot focused but they appear as streaks. Salts increases the conductivity of the IEF gel, thereby prolonging the time required to reach the steady-state. In extreme cases IEF may stop due to salts front. Salt removal can be achieved by dialysis or by using precipitation methods (with TCA or cold acetone).

Furthermore, a new recent kit (2-D clean-up kit, Amersham, Biosciences, UK) that allows desalting and cleaning of samples has been recently developed. The 2-D clean-up kit provides a method for selectively precipitating protein for 2D electrophoresis analysis without interference from detergents, chaotropes and other common reagents used to solubilise proteins. Protein recovery is generally above 90%. Another method regards the dilution of sample below a critical salt concentration (Gorg et al., 2004).

Lipids are interference compounds that may interact and complex with membrane proteins. This reduces their solubility and can affect both the pI and the molecular weight. Lipids form complexes with detergents, reducing the effectiveness of the detergent as a protein solubilizing agent.

Lipids can be removed with precipitation with acetone or by using strongly denaturing conditions and detergents.

Polysaccharides and nucleic acid can interact with carrier ampholytes and proteins and give raise to streaky 2-D patterns.

Moreover these macromolecules may also increase the viscosity of the solutions and obstruct the pores of the polyacrilamide gels. Unless present at low concentrations, polysaccharides and nucleic acid should be removed.

A common method is precipitation of proteins with acetone or TCA/acetone or ultracentrifugation to remove high-molecular-weight polysaccharides.

3.2.1.2 Protein extraction

The solubilisation of proteins in two-dimensional electrophoresis has to achieve several parallel goals:

1. breaking macromolecular interactions in order to yield separate polypeptide chains. This includes breaking disulphide bonds and disrupting all non-covalent interactions.
2. preventing any artefactual modifications of polypeptides in the solubilisation medium.
3. keeping proteins in solution during the two-dimensional electrophoresis process.

Sample solubilisation is usually carried out into a buffer containing chaotropes (urea and thiourea), non ionic or zwitterionic detergents (CHAPS) and reducing agents (DTT).

Urea is quite efficient in disrupting hydrogen bonds leading to protein unfolding and denaturation. Anyway urea is not ideal for the solubilization of all protein classes, membranes or other highly hydrophobic proteins.

Improvement in the solubilization of hydrophobic proteins has come with the use of thiourea which is better suited for breaking hydrophobic interactions, but its usefulness is somewhat limited due to its poor solubility in water. However, it is more soluble in concentrated urea solutions.

Currently the best solution for solubilization of hydrophobic proteins is a combination of 5-7 M urea and 2 M thiourea, in conjunction with appropriate detergents. The major problem associated with urea in aqueous solutions is that urea exists in equilibrium with ammonium isocyanate, which can react with the α -amino groups of the N-terminus and the ϵ -amino groups of lysine residues, thereby forming artefacts such as blocking the N-terminus and introducing charge heterogeneities (altered pI). To prevent this carbamylation reaction, temperatures above 37°C have to be avoided under all circumstances.

Detergents are utilised to prevent hydrophobic interactions between the hydrophobic protein domains to avoid loss of proteins due to aggregation and precipitation. SDS is a very effective protein solubilizer, but because it is charged

and forms complexes with proteins, it cannot be used as the sole detergent for solubilizing samples for 2-D electrophoresis. Frequently SDS is replaced with a non-ionic or zwitterionic detergent, that are currently favoured for protein solubilization. Within the zwitterionic detergents, CHAPS and sulfobetaines are the most used (Gorg A. et al., 2004).

Reduction and prevention of re-oxidation of disulphide bonds is also a critical step of the sample preparation procedure.

Reducing agents are necessary for cleavage of intra- and intermolecular disulphide bonds to achieve complete protein unfolding. The most commonly used reductants are DTT or DTE which are applied at concentrations ranging from 20 to 100 mM. Unfortunately these agents are weak acids with pK values between 8.5 and 9, which means that they will ionize at basic pH, and therefore, they run short in the alkaline gel area due to the migration to the anode during IEF.

Carrier ampholytes or IPG buffer (up to 2% v/v) can be included in the sample solution. They enhance protein solubility by minimizing protein aggregation due to charge-charge interactions.

A sample solubilised into a solution containing these reagents should be mixed at room temperature for at least 1 hour, for full denaturation and solubilisation prior to centrifugation and subsequent sample application. Heating of the sample in the presence of detergent can aid solubilization, but should only be done prior to the addition of urea, as heating in the presence of urea can introduce protein charge modifications.

3.2.1.3 The first dimension: the isoelectric focusing (IEF)

Proteins are amphoteric molecules that carry a positive, negative or zero net charge, depending on the pH of their surroundings. The Isoelectric point (pI) of a protein is the pH value of the protein's surroundings at which the protein has a zero net charge. At pH values above its pI, a protein carries a net negative charge, and at pH values below the pI the protein carries a net positive charge. IEF takes advantage of this phenomenon. By placing proteins in a pH gradient (Figure 3.1 A) within an electric field, the proteins will migrate to the pH where they have no net

charge, at their pI (Figure 3.1 A and B). Proteins with a positive net charge will migrate toward the cathode through the pH gradient, becoming progressively less positively charged, until it reaches its pI. A negatively charged protein will move toward the anode, progressively becoming less negatively charged, until it reaches its pI. Once there, if a protein drifts from its pI, it gains a net charge and will then migrate back to its pI.

The original IEF method depended on carrier ampholyte-generated pH gradients in polyacrylamide tube gels. Carrier ampholytes are small amphoteric molecules, which have high buffering capacities near their pIs. When a voltage is applied across a mixture of these molecules, the carrier ampholytes align themselves according to their pIs. Tube gels can be prepared in the laboratory but they are complex and errors can occur during preparation, therefore commercial preparations of precast gel strips incorporating immobilized pH gradients, are now commonly used.

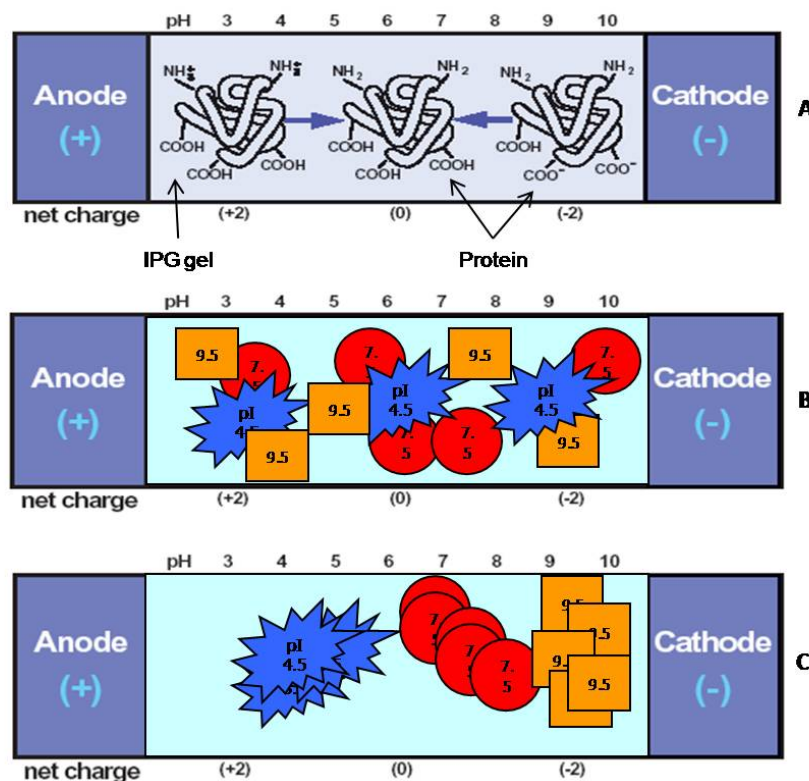


Figure 3.1. Stages of isoelectric focussing: (A) schematic of IEF gel strip containing an immobilised pH gradient (pH 3-10) in a gel matrix (proteins are absorbed into the gel during rehydration); (B) position of proteins with different pI's prior to IEF and (C) position of proteins after IEF, immobilised at their pI.

3.2.1.4 The second dimension: SDS-PAGE

After IEF, the focussed IPG strips (Immobiline Polyacrilamide Gel strips) must be equilibrated prior to performing the second dimension. The equilibration step is normally carried out in two steps; reduction and alkalisation. In the first equilibration step DTT is added to ensure that any reformed disulphide bridges are reduced and during the second equilibration step, iodoacetamide covalently modifies the reduced sulfhydryls and remove the excess of DTT. The second dimension, PAGE, allows proteins to be separated on the basis of their molecular weight and the technique is performed using polyacrilamide gels containing the anionic detergent SDS, which denatures proteins by wrapping around the polypeptide backbone and the bound SDS gives the protein a net negative charge per unit mass. In addition SDS disrupts hydrogen bonds, blocks hydrophobic interactions and partially unfolds the protein, thus eliminating secondary and tertiary structures. Proteins can be completely unfolded by reacting with reducing agents such as DTT.

3.2.1.5 Analysis of 2D gels

2D gels are complex maps, which can display thousands of proteins on a single gel, and are best analysed using specialised image analysis software. In this way it is possible to detect and quantify even faint spots and quantitatively compare 2D images with each other. Currently there are several major commercially available 2D analysis software system as Z3 (Compugen), Melanie III (Bio-Rad), PDQuest (Bio-Rad). These all, follow the same general step for analysis of the gels. First the image of the gel must be displayed in the program. Once the file is opened the image may need to be edited so that it is in the correct orientation and extraneous area are removed from the image. To visualised faint spots it is possible to increase the contrast of the image by using the transform or adjust contrast feature in these programs. Changing the contrast only affects how the image is displayed on the screen and does not alter the underlying data used for spot detection and quantification. Once the gel images of interest are open in the desired format, the

next step is spot detection and this is the most critical phase of the analysis. Spot detection is usually automated. Pairs matching is done in the match tool mode in PDQuest. When comparing 2D gel images, there is often some variation in spot intensity between gels that might not due to differential protein expression. This variation can be caused by differences in sample preparation, loading, staining and imaging between gels. The process of compensating for this background variation is called normalization.

Once spot have been picked and matched, and the data values have been normalized, programs offer a variety of ways of analyzing the data. Spots may be displayed that have only certain characteristics, for instance, matched spots found in at least 90% of all members of a gel.

Once specific groups of spots have been selected for analysis, the changes in spot characteristics between individual gels can be visually displayed in histograms. The software generates also reports of statistics (mean, standard deviation, variation, etc.).

3.2.2 MATERIALS: REAGENTS AND ENZYMES

Urea, thiourea, CHAPS, DTT, were purchased from Sigma (Poole, Dorset, UK). 2D Clean-up Kit™, 2D Quant-Kit™, Immobiline DryStrip™ and DryStrip Cover Oil™ were supplied by GE Healthcare (Buckinghamshire, UK). Pre-cast 4-12 % Bis Tris gradient gels, MES buffer, Sypro Ruby® protein stain, Mark 12™ molecular weight markers, cover oil, were purchased by Invitrogen (Paisley, UK).

3.2.3 METHODS: 2D-E

3.2.3.1 2D-E: Sample preparation

Digested flour samples were centrifuged at 10,000 x g for 10 min, using a bench-top centrifuge (Jouan – supplier address), in order to separate the soluble fraction (SF) from the insoluble fraction (pellet). SF was treated using a 2D Clean-up Kit™ to remove interfering compounds such as lipids and salts. The SF and pellet proteins were solubilised in re-hydration buffer containing 7M urea, 2M thiourea, 2 % (w/v) CHAPS, 1 % (w/v) DTT and 0.5 % IPG-buffer pH 3-11 NL.

3.2.3.2 2D-E : Protein quantification

Several of the reagents used in the sample preparation (e.g. detergents, reductants, chaotropes, carrier ampholytes) are incompatible with many protein assays such as the Bradford Assay. To overcome this the protein concentration of samples was determined by the 2D Quant-Kit following the manufacturer instructions. The assay is based on the specific binding of copper ions to protein. The cupric ions bind to the polypeptides backbones of any protein present. The kit initially precipitates the proteins and leaves interfering substances in solution. The precipitated proteins are then resuspended in a copper containing solution and unbound copper is measured with a colorimetric agent. The colour density is inversely related to the protein concentration, which can be accurately estimated by comparison to a standard curve. The assay has a linear response to protein in

the range 0-50 µg and is independent of amino acids composition, since does not depend on reaction with protein side group.

The protein assay was performed by using a sample as prepared in section 3.2.3.1. An amount of sample, which contained around 0.5-50 ng of protein estimated, was processed.

Samples underwent two main steps with a precipitant and a co-precipitant solution, which allowed the removal of interference compound (especially urea and thiourea).

Afterwards, a copper solution and a colour reagent were added and the absorbance was read at 480 nm. The protein concentration was assessed on the basis of a standard curve of BSA (Bovine Serum Albumin).

3.2.3.3 2D-E: the isoelectric focusing (IEF)

IPG strips (7 cm, pH ranges of 4-7, 6-11 or 3-11NL) were re-hydrated in a levelled re-swelling tray (GE Healthcare, Buckinghamshire, UK), using 125 µl re-hydration buffer [7M urea, 2M thiourea, 2 % (w/v) CHAPS, 1 % (w/v) DTT and 0.5 % of relevant IPG-buffer] containing 20 µg of protein per individual IPG strip. The buffer containing the sample was dispensed into the wells of the re-swelling tray (Figure 3.2), ensuring that bubbles were kept to a minimum. IPG strips were layered gel-side down, on top of the sample and any air bubbles were removed by gently applying pressure, using a pipette tip. The strips were covered with oil, by filling the entire well of the tray and the IPG strips were re-hydrated for 16 h at a constant temperature of 20 °C; if the temperature fell below 20 °C the urea would crystallise and precipitate; and if the temperature was over 20 °C then the proteins may have undergone carbamylation in the presence of uric acid formed when urea is heated.

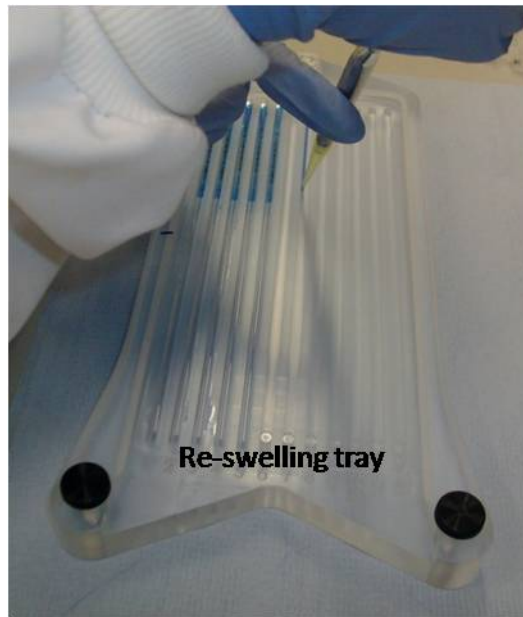


Figure 3.2: Strip re-hydration prior to IEF

After re-hydration, excess oil was drained away from the swollen IPG strips (to prevent any non-absorbed material being focussed during IEF) and were transferred to 7 cm ceramic strip holders, ensuring that the acidic end of the gel strip (labelled with + symbol on the strip) was in contact with the anode electrode and that the basic end of the gel strip was in contact with the cathode electrode (labelled with – symbol). 250 μ l of cover oil was dispensed over the strips (enough to prevent the strip drying out and overheating during IEF) and the ceramic strip holders were placed onto the 20°C temperature controlled peltier of an Ettan Dalt IPGPhor isoelectric focussing unit (GE Healthcare, Buckinghamshire, UK) (Figure 3.3). IEF was performed for a total of 6.4 kVh at 20°C (2 h: 45 min at 50 μ A per strip). Once focussed, the strips were drained of any excess oil and stored at -80 °C until required.



Figure 3.3. Ettan-Dalt IPGPhor Isoelectric focussing unit

3.2.3.4 2D-E: the SDS -PAGE

Prior to running the second dimension IPG strips were removed from the freezer, allowed to thaw at RT for 5 min and were rinsed with Millipore water. The strips were then equilibrated in 5 ml Tris-acetate buffer (0.122 M Tris-acetate, 0.5% w/v SDS, 6 M urea, 3% w/v glycerol, 0.01% w/v Bromophenol blue) containing 52 mM DTT (reducing step). The strips were placed on a flatbed shaker and incubated at RT for 15-30 min. Afterwards the strips were transferred into equilibration buffer containing 0.14 M iodoacetamide and were incubated, in the dark (iodoacetamide is light sensitive), RT, for a further 15-30 min (Alkylation step).

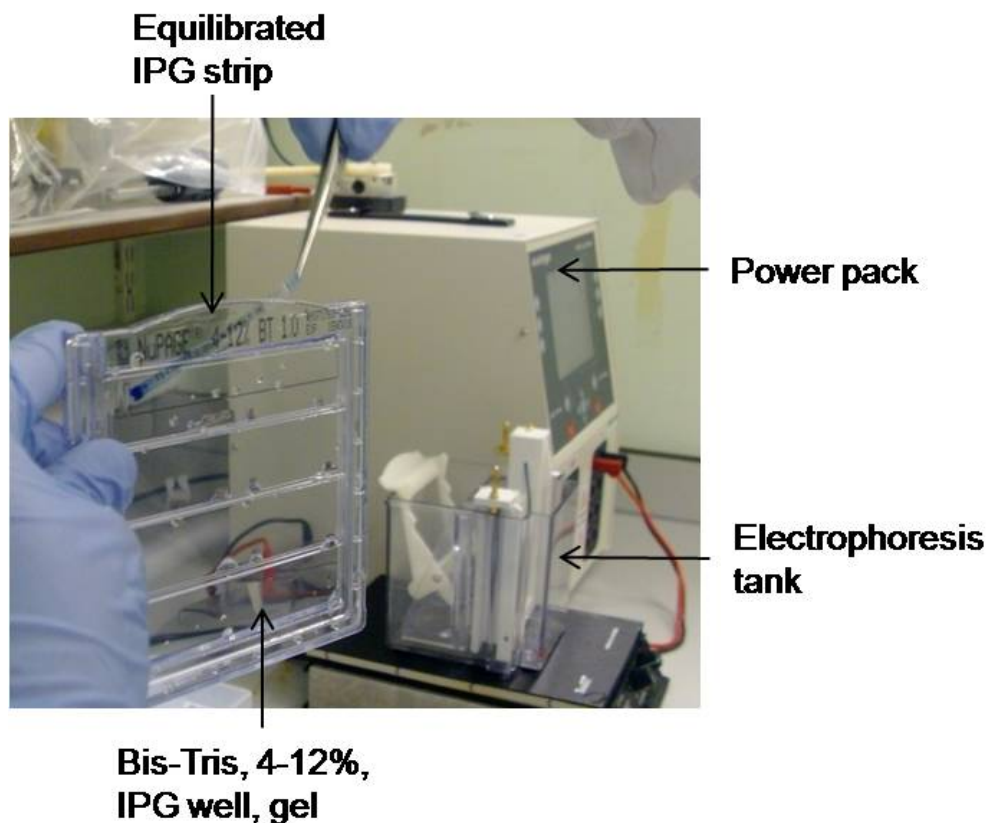


Figure 3.4. Transferring IPG strip to the 2nd dimension

IPG strips were then transferred to 7 cm pre-cast, 4-12 % Bis Tris gradient gels (Figure 3.4), with an IPG well and marker well (Invitrogen, UK). Molecular weight markers (Mark 12TM, Invitrogen, UK) were added to the marker well (7 μ l) and strips were subjected to electrophoresis using NuPage MES-SDS running buffer system under reducing conditions at a constant Voltage of 200 V for 40 min.

Resulting gels were fixed overnight in a solution containing 50% v/v methanol, 10% w/v TCA (used to fix wheat proteins which tend to wash out during fixing when acetic acid is used). Afterwards gels were stained with light-sensitive SYPRO Ruby[®] protein stain for a minimum of 90 min, in the dark (gel container was wrapped in aluminium foil) and were transferred into destain solution (10% v/v methanol, 6% w/v TCA) for a minimum of 90 min. The stained gels were imaged at 100 μ m resolution using the PharoX FX Plus Imager (Excitation: 532nm; Emission: 605nm) (BioRad, UK).

3.2.3.5 Image analysis

The undigested sample (both soluble or insoluble, heated or unheated) was matched with the oral digesta and subsequently with the 5, 30 and 60 min of gastric digestion samples, using Proteomweaver® software v3.0 (Definiens, UK). The image analysis allowed visual comparison between the digestion profiles and highlighted the protein changes between the heated and unheated flour during digestion.

3.3 MASS SPECTROMETRY

3.3.1 PRINCIPLES OF THE METHOD

One of the most used techniques for identification of proteins is mass spectrometry (MS), which is a very sensitive method and required small amounts of samples. Recent advances in mass spectrometry also allow the investigation of PTMs (post translation modification, including phosphorylation and glycosylation). MS technique is based on the finding that a set of peptide masses obtained by MS analysis of a protein digested, provides a characteristic mass fingerprint of that protein. The protein is then identified by comparison of the experimental mass fingerprint with theoretical peptide masses generated *in silico* using protein and nucleotide sequence database.

MS is used for both mass determination and protein identification. After removal of the protein spot, the protein is digested with a protease (usually trypsin) and the digested peptides are then analyzed. A mass spectrometer separates proteins according to their mass to charge (m/z) ratio.

The molecule is ionized and the ion is propelled into a mass analyzer by an electric field that resolves each ion according to its m/z *ratio*. The detector passes the information to the computer for analysis. Ionisation methods frequently used are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) because they cause little or no fragmentation of the molecule during the ionization and desorption process.

Ionization by MALDI involves a protein suspended or dissolved in a crystalline structure (the matrix) of small organic UV-absorbing molecules (such as 2,5-dihydroxybenzoic acid or α -cyano-4-hydroxycinnamic acid).

The analyte is spotted, along with the matrix, on a metal plate and crystals are formed by evaporation. The crystal absorbs energy at the same wavelength of the laser that is used to ionize the protein.

The laser energy strikes the matrix to cause rapid excitation of the matrix and subsequent the passage of matrix and analyte ions into the gas phase.

The principal ion detected using threshold laser intensity for MALDI is a single charged ion. The ionized protein is accelerated by an electrostatic field and expelled into a flight tube. The analyzer is often a time of flight analyzer (TOF). This is based on the principle that when accelerated by application of a constant voltage, the velocity with which an ion reaches the detector is determined by its mass.

Because MALDI is able to tolerate small amounts of contaminants, sample preparation is easier compared with other mass spectrometry techniques like electrospray ionization (ESI).

Proteolytic digestion of gel separated proteins into peptides followed by mass analysis of the peptides provides a peptide mass fingerprint. The composition of the peptide mass ions thus identified can be searched on Internet database, such as the ExPASy Molecular Biology Server located at the Swiss Institute of Bioinformatics.

A more detailed MS analysis can be performed when necessary to gain peptide sequence information. In this case, the peptides can be sprayed into a tandem mass spectrometer (MS/MS) that has the ability to resolve peptides, isolate one peptide and dissociate it into amino or carboxy terminal containing fragments. Although is more complex than the peptide mass approach, the sequence information is more specific for the identification of a protein than a list of peptide masses. The data can be used to search protein sequence database and also nucleotide database.

3.3.2 MATERIALS

Trypsin Gold Mass Spectrometry Grade was from Promega (Madison USA).

3.3.3 METHODS: PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

3.3.3.1 Spot excision

Protein spots were excised using HT Analyzer software (Genomic Solutions, Huntingdon, UK) and the excised gel pieces were placed into modified 96-well microtitre plates (Genomic Solutions). Subsequently these were stored at 2°C overnight prior to trypsin digestion, which was performed manually.

The gel plugs were initially treated with 200mM ammonium bicarbonate in 50% acetonitrile to increase the pH to approximately pH 8, to give suitable conditions for Trypsin digestion to occur. The gel pieces were then shrunk in acetonitrile and then air dried for 10 mins before trypsin digestion was performed at 37 °C for 3 h, by adding 5µl containing 50 ng of sequenced grade porcine Trypsin (Promega, Southampton, UK) per sample; digestion was stopped and peptides extracted by addition of 5µl of 5% v/v formic acid. Samples were frozen and stored at -70 °C until required for MALDI-ToF.



Figure 3.5: Robotic spot cutter

3.3.3.2 MALDI-ToF

The acidified digests, were automatically spotted onto pre-spotted anchor chip plastic MALDI target plates (PAC384 target plates, Bruker Daltonics Ltd, Coventry, UK) using a MALDI-AutoPrep (MAP II) spotting robot (Bruker Daltonics Ltd). The PAC384 plates contains 384 pre-prepared spots of matrix (α -cyano-4-hydroxycinnamic acid) and 96 spots of a peptide calibrant mixture. Onto the matrix only spots, 0.4 μ L of each sample was applied.

MS analysis of the samples was carried out on an Ultraflex MALDI-ToF/ToF mass spectrometer (Bruker Daltonics Ltd). A 200Hz nitrogen laser was used to desorb/ionise the matrix/analyte material, and ions were detected in positive ion reflectron mode. All spectra were acquired automatically using the Bruker fuzzy logic algorithm.

Successful spectral packets for each sample were summed, calibrated, annotated and background subtracted using FlexAnalysis 3.0, the results of which were then automatically set up for searching against the SPTrEMBL sequence database for Viridiplantae (Green Plants) using the pre-assigned Biotoools method which submitted the searches to the on offline version of the Mascot 2.1 search engine (Matrix Science Ltd, London).

The search parameters were as follows: (1) tryptic digest was assumed to have a maximum number of 1 missed cleavage, (2) peptide masses were stated to be non-isotopic: (3) methionine residues were assumed to be partially oxidised, (4) the carbamidomethylation of cysteine residues was included, (5) the mass tolerance was kept at 50 ppm.

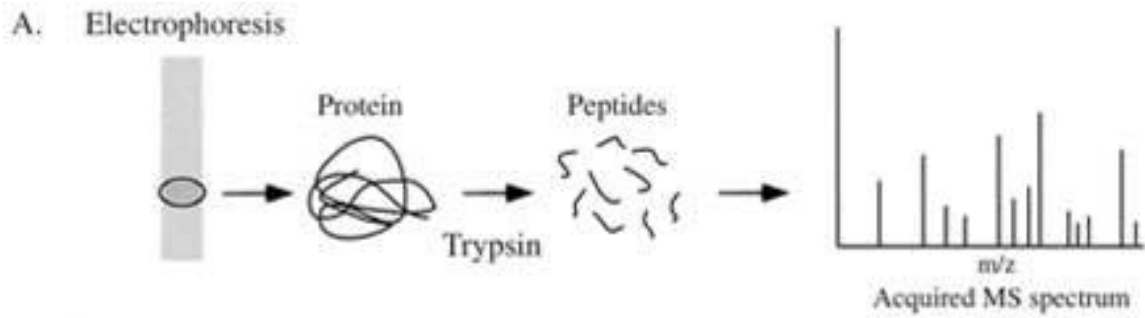


Figure 3.6: Traditional proteomics experiment (from Graves and Haystead, 2002): (A) Electrophoresis to obtain proteins which are excised from the gel and digested using Trypsin. The resulting peptide fragments are analysed by MS and a spectrum is obtain providing mass information.

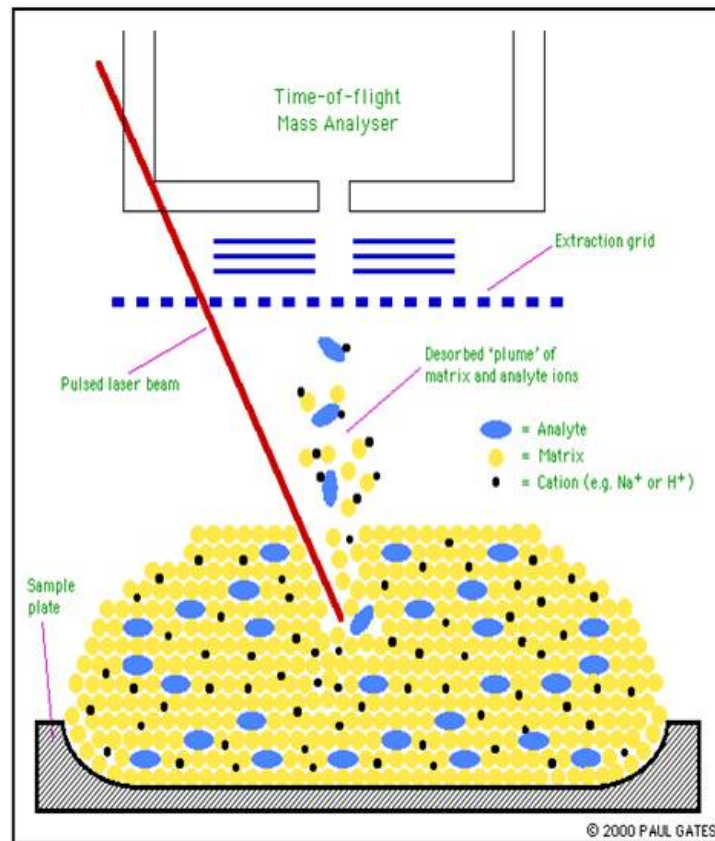
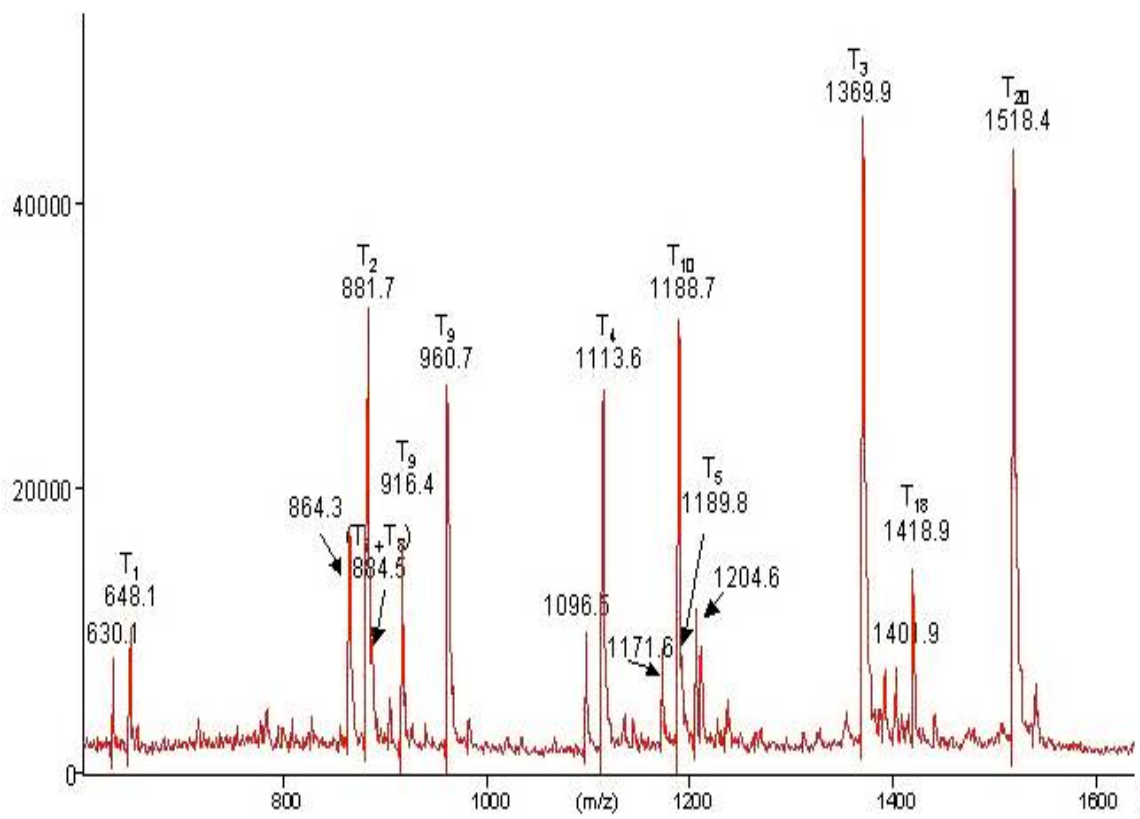


Figure 3.7: MALDI-ToF



Maldi ToF mass spectrum of the tryptic digest of a high molecular weight (85.000) glutenin (DY10)

Figure 3.8: MALDI ToF spectrum of the tryptic digest of a high molecular weight glutenin.

3.4 RESULTS AND DISCUSSION

The aim of this work was to monitor the protein digestion profiles of heated and unheated wheat flour. Attention was focused initially on the oral and gastric phases of digestion, which were simulated using a biochemical model of digestion (Mandalari et al., 2009) with sampling at three time points to follow pepsinolysis. 2D-E combined with image analysis was used to compare protein profiles and to identify changes in protein patterns during digestion.

Protein maps were performed using 7 cm strips. Advantages to use these mini gels were:

1. Reproducibility of resulting gel using 7 cm pre-cast gels;
2. Possibility to perform a large number of 2D maps in a short time;
3. Possibility to obtain good resolution gels also by loading a low amount of protein (40-10 µg).

On the other way, it has been tested that the use of narrow pH range strips that cover the whole separation pH range 3-11, cannot give a well-defined separation of wheat protein, particularly referred to basic proteins (prolamins).

For this reason, soluble and insoluble proteins extracted in rehydration buffer and subsequently treated with a “clean-up” Kit to remove interference compounds, have been separated at two different pH range: 4-7 and 6-11.

This approach allowed a better separation and resolution of wheat proteins in both unheated and heated materials.

3.4.1 UNHEATED AND HEATED FLOUR: DIFFERENCES OF THE STARTING MATERIALS

Firstly 2D-E maps of heated and unheated undigested flour were obtained. These showed that most of the proteins, especially those belonging to the prolamin family, were insoluble and remained clearly visible in the pellet maps. In figure 3.3B and 3.4B it was possible to identify gliadins and glutenins by reference to other annotated maps (D’Ovidio et al. 2004). Gliadins at Mr 30000 – 80000 kDa, high molecular weight glutenin subunits (HMW-GS) at 65000 – 90000 kDa and low

molecular weight glutenin subunits (LMW-GS) in the Mr 30000-60000 kDa range, could be defined. Focusing on the insoluble fraction (figure 3.3B and 3.4B), prolamins were better solubilised in the unheated sample compared to the heated one. It might be that after the heating treatment prolamin proteins were more soluble in simulated gastrointestinal fluid. It was also evident that, after the heating treatment, pellet proteins were less extracted in the thiourea, urea, CHAPS, DTT buffer.

Regarding the insoluble proteins separated in the pH range 6-11 (figure 3.3B and 3.4B), it was also possible to notice differences in spots abundance between the raw and the cooked flour. Indeed, some proteins around 14 kDa, which were belonging to the α -amylase inhibitors (AAI) protein family, were more evident in the heated sample.

It might be possible that the cooking treatment caused changes that altered the solubility properties of this matrix. AAI proteins (around 14 kDa) seemed to become less soluble after the heating treatment (figure 3.3B, 3.4B) even if they were still abundantly present in the soluble fraction (figure 3.1, 3.2).

It is well known that soluble proteins belonging to the α -amylase inhibitor family are responsible for respiratory allergy, which affects especially bakers, because of the high amount of flour they daily handle (Baur et al., 1998). People suffering of baker's asthma are usually not allergic to wheat after ingestion. This means that the cooking treatment and the digestion process might modify the immunological properties of wheat proteins.

Differences have been shown in proteins of unheated and heated flour undigested (figure 3.3A, 3.4A), separated at pH 4-7, due to the heating process.

Indeed, proteins, that have been subsequently identify as starch synthase, (around 60 kDa) seemed to disappear or to be faintly present in figure 3.4A.

Thus, to carry out experiments involving digestion models, it is necessary to process the food as it is eaten, because proteins that might be resistant to digestion in the raw sample may be destroyed by the heating treatment (before performing digestion). This will be argued later.

3.4.2 THE EFFECT OF THE SIMULATED ORAL DIGESTION ON PROTEIN

As already known (Neyraud et al., 2009), the oral digestion is characterized by the mechanical breakdown of the food ingested and the enzymatic activity of the α -amylase present in the saliva. Starch is the main nutrient that might be hydrolysed and partially digested into the mouth.

Focusing on 2D-E maps (figure 3.5 and 3.6), we did not identify any clear or important differences between starting material and after simulated oral processing in either the unheated or the heated flour (soluble and insoluble phases). This was not unexpected since no proteases are present in the salivary fluid.

Even if there were no changes in protein profiles due to the oral digestion, this first step has to be performed because it might affect the rate of digestion rather than the single protein degradation.

3.4.3 PROTEIN DEGRADATION DURING GASTRIC DIGESTION

The *in vitro* gastric digestion was performed by using a biochemical model in order to underline changes in protein profile. Therefore, it has been decided to map proteins that underwent pepsinolysis for 5, 30 and 60 min, using a two-dimensional approach.

Digested proteins have been centrifuged prior to perform the extraction, in order to obtain pellet and supernatant fractions. In this way, it has been possible to focus in both insoluble and soluble proteins.

It were not identified significant changes in protein profile due to the oral process, consequently more extensive changes in protein profiles were detected after simulated gastric digestion.

Looking to the protein profile maps separated at pH range 4-7 (figure 3.7) it was clearly defined that some proteins were still abundantly present after 60 min of gastric digestion in the insoluble phase. Anyway, these detected proteins were differently expressed comparing the heated flour (figure 3.7D) with the unheated material (figure 3.7H).

Spot proteins around 40 kDa, belonging to the serpins family, disappeared quickly in the raw flour (figure 3.7 A-D) while they were resistant to proteolysis in the cooked sample (3.7 E-H).

Serpins are chymotrypsin inhibitors (Hejgaard et al., 2002) and they are members of a superfamily of proteins involved in regulation of complex physiological process in mammals (Rosenkrands et al., 1994).

Serpins, in contrast to the well-characterized low molecular weight protein inhibitors, undergo reversible and irreversible conformational changes which regulate their activity. Thus, proteolytic cleavage in the reactive site loop results in a protein with new biochemical properties, including increased stability against degradation by proteinases and denaturation at high temperature (Rosenkrands et al., 1994).

On the contrary, spot proteins, subsequently identified as starch syntase (around 60 kDa), were abundantly persistent in the unheated sample digested up till 60 min (figure 3.7 D). These spots seemed to disappear after few minutes of proteolysis in the processed flour (3.7 F-H).

The overlaid images confirmed these observations. Indeed, focusing the attention on the overlaid image (figure 3.13) of unheated flour digested 5 min versus the same digested 60 min it was easily understandable that starch syntase proteins were not modified during the gastric digestion. The overlaid image showed black spots that correspond to proteins (starch synthase) present equally in both the two gel matched.

Moreover, regarding the image analysis of the unheated flour digested 60 min matched with the heated one digested at the same time (figure 3.20), it appeared that there were no black spots, the two protein patterns did not match each others and orange and blue coloured spots were clearly marked. This meant that the two samples (unheated and heated) digested in the same way, were consistently different. Thus, there were significant demonstrations that the cooking process affected wheat proteins and also their proteolysis.

Insoluble proteins separated at pH range 4-7 (figure 3.7) showed others interesting notations: looking at low molecular weight spots, it was evident a group of proteins around 14 kDa manifested faintly in the unheated flour. These spots corresponded

to the α -amylase inhibitor family (AAI) and seemed to become more resistant to proteolysis in cooked wheat products.

In relation to the importance of α -amylase inhibitor family (AAI) in food allergy, at the moment, there are contrasting hypothesis able to explain the role of these allergens in food adverse reactions. Indeed, it is well known that AAI are allergens involved basically in respiratory allergy, especially in baker's asthma, an allergic disease caused by the inhalation of flour particles.

Moreover, some authors investigated the involvement of AAI in allergic reactions occurring after wheat ingestion. James et al. (1997) demonstrated that wheat AAI are relevant allergens in patients experiencing hypersensitivity reactions after the ingestion of wheat proteins. Other studies carried out by Simonato et al. (2001) found that the 16 kDa allergen is recognized by IgE of individuals with food allergy to wheat, but this occurred only when the unheated flour proteins were tested. It might be possible that the thermal treatment is able to modify protein structure and their immunological properties, thus eliminating the allergenic activity.

Both the two different results need to be more investigated, but surely it is clearly evident that there is a slower proteolysis of AAI in processed wheat (heated) rather than in the raw material.

By separating proteins at pH range 6-11, it was possible to gain a good resolution of prolamin proteins. Prolamins are divided into gliadins and glutenins and, when they are mixed with water, they form a viscoelastic matrix called "gluten".

Looking to the protein maps at pH range 6-11, (figure 3.9) obtained from the insoluble fraction and comparing the heated (3.9 E-H) and the unheated flour (3.9 A-D), a different rate of digestion appeared evident, which was slower for the heated sample.

Thus, results showed that the cooking procedure might affect digestion, probably due to:

1. wheat proteins may become more resistant to proteolysis after heating procedure; as described by Hansen et al. (1975) peptide bonds of heat aggregated protein were shown to be less accessible to pepsin attack than those of unheated protein, thus altering the substrate availability for the enzymes;

2. after heating a starchy food, like bread or pasta, starch changes its conformation becoming gelatinized during a process that is called “gelatinization” which starts when starch is brought up to 50-60° C. This allows starch granules to lose their original structure, creating a viscous matrix which has got important technological, biochemical and nutritional features. From a nutritional point of view, the viscous gelatinised starch might be able to slow down digestion, because the proteases cannot easily join the substrate, since the starch matrix is not completely broken down.

These two hypotheses can explain the different resulted protein profile maps.

Focusing on the three time points of digestion, 5, 30 and 60 min, and matching gels by using image analysis software, it was possible to gain a more comprehensible investigation. Indeed, figure 3.15 showed that after 5 minutes of unheated flour gastric digestion, a number of blue spots (around 30 kDa), corresponding to new peptides derived from the gastric digestion, appeared on the gel and cannot match the undigested sample gel.

On the contrary, the overlaid image obtained by matching the two gels of the cooked flour (figure 3.16) did not show blue spots, but most of the black spots indicated that pepsin did not act on proteins during the first five minutes of hydrolysis.

Moreover, looking to the overlay image of cooked flour digested 5 and 30 min (figure 3.17), it was noticeable that proteins, especially those belonging to the prolamin superfamily, remained almost intact during 30 minutes of pepsinolysis.

After 30 minutes of digestion, prolamins (gluten proteins) of the heated flour underwent pepsinolysis and this change was well detected looking the overlaid image (figure 3.19).

Maps developed at pH range 6-11 (figure 3.9) could highlight not only the vast prolamin proteins group, but it was also well evident a cluster of proteins at low molecular weight, around 6.5 pI, which were strongly evident during the whole digestion process. These spot proteins were belonging to the α -amylase inhibitors family and confirmed their resistance to digestion after the thermal treatment.

Regarding proteins of the soluble phase, separated at pH range 6-11 (figure 3.10), there was proof of the hypothesis explained above. Indeed, in the unheated flour the supernatants (figure 3.10 B-D) showed a lot of new peptides, around 30 kDa, which derived from the proteolysis and went through the soluble phase. Contrary, in the cooked flour, where digestion slowed down, it was not highlighted this group of new spots but there were more evidence of proteins belonging to the AAI, abundantly present in both insoluble and soluble fractions.

3.4.4 IDENTIFICATION OF PROTEINS RESISTANT TO IN VITRO GASTRIC DIGESTION, BY USING MASS SPECTROMETRY (MALDI-TOF)

Mass spectrometry has become the technique of choice for identification of proteins from excised 2-D gel spots. Wheat proteins, unheated and heated, were first separated with two dimensional SDS-PAGE, according to their isoelectric point and molecular weight, under denaturing conditions. Consequently, a total of 75 spots were picked from 4 gels:

- 21 gel plugs were from the heated flour sample, digested 30 min, (insoluble proteins fraction), separated at pH range 4-7 (gel 3A, figure 3.24);
- 33 gel plugs were from the heated flour sample, digested 30 min, (insoluble proteins fraction), separated at pH range 6-11 (gel 3B, figure 3.25);
- 8 gel plugs were from the heated flour sample, digested 30 min, soluble protein extracted, separated at pH range 4-7 (gel 3C, figure 3.26);
- 13 gel plugs were from the unheated flour sample, digested 30 min, insoluble protein extracted, separated at pH range 4-7 (gel 3D, figure 3.27).

Proteins were identified by searching *Triticum*, *Oriza sativa* and *Brachypodium* protein sequences from Swiss-Prot and using MALDI-ToF PMFs. Using this approach 41 spots have been identified (table 3.6 below). Most of the 35 spots not identified were proteins from the cooked flour that might not be identified because of the modifications due to the cooking process.

Identity	Gel / Spot number	Sequence/ Accession number	PMF MOWSE score	Sequence coverage %	Peptides matched	pI Calculated / observed	M _r Calculated / observed
Prolamin storage proteins							
High molecular weight glutenin, fragment	3B/1	<i>T.aestivum</i> B8YPU3	76	35	6	8.9/	17.5/
High molecular weight glutenin, fragment	3B/2	<i>T.aestivum</i> B8YPU3	79	35	6	8.9/	17.5/
Seed maturation protein PM39	3B/5	<i>Glycine max</i> Bradi3g30320.1	64	19	11	5.2/	61.8/
Chloroform / methanol proteins							
CM 17	3A/18	<i>T. aestivum</i> Q41540	51	29	4	5.7/	16.5/
CM16 subunit	3A/19	<i>T. aestivum s. macha</i> B9VR13	82	35	6	5.3/	16.2/
CM3	3B/31	<i>T. aestivum</i> P17314	68	48	6	7.4/	18.8/
CM1	3B/32	<i>T. aestivum</i> C7C4X0	66	60	6	6.7/	13.6/
α-amylase inhibitors							
Monomeric α-amylase inhibitor	3A/20	<i>T. monococcum</i> A4ZIT6	109	56	8	7.4/	13.6/
Dimeric α-amylase inhibitor	3C1(a)	<i>T. turgidum</i> A4GFN8	67	67	6	6.5/	13.7/
Monomeric α-amylase inhibitor	3C2(b)	<i>T. turgidum</i> A4ZIT6	86	56	7	6.2/	13.7/
Serpin							
Serpin 2	3A/10	<i>T. aestivum</i> COLF31	84	22	9	5.1/	43.5/
Serpin-Z2B,	3A/11	<i>T. aestivum</i> P93692	132	44	13	5.18/	43/
Serpin 1	3A/13	<i>T. aestivum</i> COLF30	75	26	9	5.4/	43.2/
Serpin 3	3A/14	<i>T. aestivum</i> COLF32	107	31	13	5.5/	43.2/
Serpin-Z2A	3A/15	<i>T. aestivum</i> Q9ST57	145	47	15	5.4/	43.3/
Serpin-Z1A	3A/16	<i>T. aestivum</i> Q41593	146	33	14	5.6/	43.2/
Serpin-Z1C	3A/17	<i>T. aestivum</i> Q9ST58	104	19	9	5.6/	42.9/
Tritin	3B/26	<i>T. aestivum</i> Q07810	55	20	5	9.7/	29.5/
Triticin	3B/28	<i>T. aestivum</i> B2CGM5	53	10	5	6.4/	65.2/

Antioxidant and heat shock proteins							
Secretory peroxidase	3B/10	<i>Oriza sativa</i> Q0DRN6	67	30	8	8.9/	43.8/
Peroxidase 1	3B/12*	Q8LK23	59	15	7	8.1/	39.2/
23.1kDa heat-shock protein	3B/19*	<i>T.monococcum</i> A5A8U5	51	37	5	5.4/	23.2/
Metabolic proteins							
Beta amylase, fragment	3A/5	<i>T. aestivum</i> Q7X9M2	66	27	7	8.6/	31/
Beta amylase, fragment	3A/6	<i>T. aestivum</i> Q7X9M2	71	24	7	8.6/	31.1/
Beta amylase, fragment	3A/7	<i>T. aestivum</i> Q7X9M2	87	40	10	8.6/	31.1/
Beta amylase, fragment	3A/8	<i>T.aestivum</i> Q7X9M2	66	31	8	8.6/	31.1/
Beta amylase, fragment	3A/9	<i>T. aestivum</i> Q7X9M2	62	28	7	8.6/	31.1/
ATP binding protein	3B/9	<i>Zea Mais</i> Bradi2g24390.1	58	24	8	7.6/	54.7/
ATP binding protein	3B/7	<i>Zea Mais</i> Bradi2g24390.1	61	25	8	7.6/	54.7/
Limit dextrinase type starch debranching enzyme	3B/11*	<i>T. aestivum</i> A1YUM8	50	12	7	5.5/	106/
Granule-bound starch synthase	3D/1	<i>T.aestivum</i> Q8LLD5	114	22	12	6.59/	66/
Starch synthase (GBSSI)	3D/2	<i>T. turgidum</i> Q9SLS8	207	38	22	7.49/	67/
Granule-bound starch synthase, fragment	3D/3	<i>T.aestivum</i> Q8W2G8	188	44	20	5.6/	59.5/
Starch synthase (GBSSI)	3D/4	<i>T. turgidum</i> Q9SXX4	167	36	20	7.11/	67/
Starch synthase (GBSSI)	3D/5	<i>T. turgidum</i> Q9SLS8	178	39	20	7.5/	67/
Starch synthase	3D/6	<i>T. turgidum</i> Q9SLS8	182	43	24	7.5/	67/

(GBSSI)							
Granule-bound starch syntase	3D/7	<i>T.aestivum</i> Q9SYU0	176	41	22	8.4/	64/
Starch synthase 1	3D/8	<i>T. aestivum</i> Q43654	82	14	9	5.8/	71.5/
Starch synthase II-B	3D/9	<i>T. aestivum</i> Q9LEE2	75	10	8	6.27/	87/
Starch synthase	3D/10	<i>T. aestivum</i> O24398	77	17	8	6.15/	56/
Starch synthase II-D	3D/12	<i>T. aestivum</i> Q2WGB1	66	8	7	6.14/	87/

Table 3.6: Protein identified from unheated and heated flour digested (spot identification can be visualised in figure 3.24, 3.25, 3.26, 3.27 Appendix).

(*) peptide tolerance increased to 50 ppm and 2 miss cleavage.

(a) A4GFN8 is the dimeric- α -amylase inhibitor and matches another similar peptide Q5UHH6.

(b) A4ZIT6 is the monomeric- α -amylase inhibitor and matches another similar peptide A4ZIU3.

Looking to the table above, it was understandable that most of the spot identified after gastric digestion belong to 4 major proteins family: serpins, β -amylases, α -amylases inhibitor, prolamins, which have been highlighted as potential allergens in wheat (Sotkovsky et al., 2008, Akagawa et al., 2007). The mains group of proteins identified has been described below.

B-AMYLASES

β -amylases are metabolic proteins which have been found in wheat-dough liquor (Salt et al., 2005). β -amylases are enzymes involved in mobilising glucose from the seed starch reserve during germination.

Looking to the proteins of heated flour digested 30 min, separated at pH 4-7 (figure 3.24, gel no. 3A), there were identified five spots belonging to the β -amylases (3A/5, 6, 7, 8, 9).

These proteins showed on the gel a Mr around 66 and pl around 5.5, but the theoretical Mr and pl calculated from the PMF data did not matched the pl

observed. Furthermore it was noticeable, from the MASCOT identification, that these proteins were fragments and thus might explain why the theoretical Mr was 33 and the pI shifted toward 8.6.

Despite it has been found a fragmentation of β -amylase probably due to the gastric process, it was recognized the resistance of these proteins after digestion only in the heated flour. In the unheated flour, β -amylase disappeared within 30 min of gastric digestion.

This means that the heating process modified the proteins conformation or the substrate availability for the digestive enzymes.

Looking to the profile maps (fig. 3.7), it was evident that these β -amylases were less abundant after 60 min of digestion but still present on the gel. It means that they might be digested during the duodenal phase or that they remain undigested thus acting as potential allergens in sensitized people. These β -amylases, identified with the accession number Q7X9M2, were recognized also in a previous work (Sotkovsky et al., 2008), performed with proteins extracted from the salt soluble phase of wheat raw flour.

Carrying on IgE binding analysis with sera of patients suffering of wheat food allergy, some authors (Sotkovsky et al., 2008) found that β -amylases might be potentially allergenic proteins. Indeed, they did not performed the experiments with heated flour, thus they could not identify β -amylases in the sample hydrolysed with pepsin.

In contrast, in this work, it has been showed not only the key role of the gastric process but also the importance to perform experiments with the sample in the form that "it is eaten", in order to assess potentially allergenic proteins.

SERPINS

Serpins are proteins that often act as inhibitors of serine proteinases. They are the main protein components in chicken eggs, the third most abundant protein family in the blood, part of the host defence mechanism of insects and present in hyperthermophilic bacteria, nematodes and even plants.

The serpin architecture, comprising nine α -helices and three β -sheet, is highly conserved among family members. (Huntington, 2006). Serpins were previously identified by different authors: Salt et al. (2005) found serpins in the dough liquor, Sotkovsky et al. (2008) and Sander et al. (2001) identified serpins in the salt-soluble fraction of wheat and finally Akagawa et al. (2007) in a total protein extraction.

In this work were identified 7 different spots belonging to the serpin family, all detected in the insoluble phase of the heated flour sample digested 30 min. Looking to figure 3.7, serpins were resistant to the gastric digestion only if the sample was heated, (3.7, H) comparing with the unheated flour in which serpins were nearly completely hydrolysed in 30 min of digestion (3.7, C).

As well as discovered for the β -amylases, serpins were quickly hydrolysed during the *in vitro* gastric digestion in the unheated flour and this meant that the heating process was able to modify serpins and their behaviour upon digestion.

Moreover, it was possible to match some of the serpins identified here, with others already found, using the literature. For instance, serpin-Z2B (3A/11) and serpin-Z1C (3A/17) have been identified also by Akagawa et al. (2007) who recognised furthermore IgE binding on these serpins by using human sera from wheat food allergy patients.

Up till now, there has been a lack of information regarding serpin proteins involved in wheat food allergy. At the moment, few studies have shown the importance of serpins in respiratory allergy such as baker's asthma (Sander et al., 2001).

THE PROLAMIN SUPERFAMILY

The prolamin superfamily is composed of seed proteins which can be broadly divided into two types: the low-molecular-mass sulphur rich seed proteins and the high-molecular-mass prolamins. The first group includes 2S storage albumins from dicotyledonous seeds, inhibitors of α -amylase and trypsin, puroindolines, grain softness proteins from cereal seeds and non specific-lipid transfer proteins.

α-AMYLASE INHIBITORS (AAIs)

The presence in wheat flour of water soluble inhibitors of α -amylase has been well known and documented. It has been also estimated that AAIs account for up to two thirds of all albumins. Most of the AAIs are inactive against endogenous enzymes of wheat and correspond to a class of proteins initially defined on their selective extraction in chloroform-methanol mixtures as “CM proteins” (Salcedo et al. 1978).

This group includes three inhibitors that were initially defined as 0.19, 0.28 and 0.53 on the basis of their relative electrophoretic mobility at alkaline pH. AAIs have subunit mass ranging from about 12,000 to 16,000 and exist in monomeric, dimeric and tetrameric forms.

Although they are not active against the endogenous α -amylase of wheat, the CM proteins are active against enzymes from other organism, including human salivary and pancreatic α -amylases. However, they vary substantially in their inhibitory spectra; for instance, the dimeric inhibitor is highly active against the human salivary α -amylase, while the monomeric form shows less activity and the tetrameric form little activity (Salcedo et al. 2004).

Most subunits of the AAIs have been found to react with IgE thus causing an allergic reaction that is specifically called baker’s asthma. Moreover, some studies found that AAIs might be involved also in wheat food allergy (James et al., 1999).

In this work, 5 different AAIs have been identified from the heated flour upon 30 min gastric digestion. The CM17 AAI (3A/18) and the CM 16 AAI (33A/19) have been recognized also by Sotkovsky et al. (2008). These authors found IgE binding with sera from patients suffering of wheat allergy.

Moreover, the mass spectrometry revealed that, even if the flour was heated, some proteins belonging to the AAIs were resistant to digestion. This may indicate their potential allergenicity after ingestion.

Nakayama et al. (2000) found the CM3 AAIs, as a relevant allergen in patients experiencing hypersensitivity reactions after ingestion. The CM3 has been identified in this work as a protein resistant to gastric digestion.

THE PROLAMIN PROTEINS

The prolamins themselves form the second major type of protein classified in the superfamily. Prolamins are storage proteins of cereal seeds and are characterized by their insolubility in water but their solubility in alcohol water mixture. Prolamins are particularly rich in proline and glutamine and many of them are complex multi-domain proteins with one or more domains comprising repeated sequences based on the re-iteration of one or more short peptide motifs (Shewry et al. 2002).

In this work, spot proteins, apparently belonging to the prolamin group, were excised from a 2D gel corresponding to the heated flour sample digested 30 min. Two high molecular weight glutenin subunits were identified (table 3.6 above) by using common MALDI-ToF techniques and MASCOT.

Most of the proteins which were supposed to belong to the prolamins could not be identified. It is well known that, by using MS analysis, proteins are identified by comparison of the experimental mass fingerprint with theoretical peptide masses generated *in silico* using protein and nucleotide sequence databases. Moreover, MS may not allow the identification of proteins that are extensively post translational modified (PTM), since the peptides generated from these proteins might not match with the unmodified protein in the database.

Regarding the poorly identification of prolamin proteins, it might be possible that the cooking process modified proteins conformation, thus creating PTM proteins which were not identified using common MS techniques.

In this case it may be useful to obtain amino acid sequence information using MS/MS or other techniques that are able to produce a fragmentation of the peptides thus generating a fragment mass fingerprinting (FMF).

Moreover, it might be possible that the tryptic digestion, performed before MS analysis, which acts on lysine and arginine residues, was not completely efficient.

Indeed, in recent studies focused on wheat gliadins (Mamone et al., 2005), mass spectrometry was performed using excised peptides digested with chymotrypsin instead of the commonly used trypsin, in order to provide an efficient gliadin spot digestion with a great number of medium size peptides, better suited for a mass spectrometry identification.

TRITICIN

Triticin, also called 11S globulins, belong to the major storage protein fractions reported in seeds of oats and rice by Shotwell (1999) and it is located in the starchy endosperm cells.

Triticin comprises four subunits called A (52 kDa), D (58 kDa), α (23 kDa) and δ (22 kDa), which are linked by disulfide bonds to form dimers ($A\alpha$, $D\delta$) tetramers ($A\alpha A\alpha$) and higher oligomers.

Early studies (Robert et al., 1985) showed that triticin subunit, around 60 kDa, appeared to be replaced by bands of 40 and 20 kDa in the presence of reducing agents. These properties are typical of 11S globulin subunits that are post translationally processed to give 40 and 20 kDa chains which remain associated by a single disulfide bond (Casey, 1999).

In the cooked flour digested 30 min, it has been identified a protein corresponding to the triticin with an observed molecular weight around 22 kDa and pI of 6.4. Even if the theoretical Mr estimated was 65.5 (table 3.6 above), the one observed was lower because of the reducing process performed before the IEF.

TRITIN

Tritin has been already identified in the aqueous phase of the dough (Salt et al., 2005). Tritin is a protein synthesis inhibitor which is an RNA N-glycosidase that was recognized also in the insoluble fraction of the cooked flour digested 30 min. Tritin showed a high pI of 9.6 and a Mr around 36 kDa and was resistant to the heating and to the gastric digestion process.

PUROINDOLINES

Puroindolines (also called friabilin) are starch granule associated proteins that contain five disulfide bridges.

These are amphiphilic proteins (soluble in non-ionic detergent) that include puroindolines associated to grain texture. Puroindolines (PIN) are formed by two isoforms: PIN-a and PIN-b. Both PIN-a and PIN-b are composed of several spots, with some differences being observed between varieties (Skylas et al., 2005). These differences were assumed to be genetically determined because they were not observed for samples of the same genotype, grown in different environments. In this study, a protein with pI around 9 and Mr of 15 kDa has been identified as a puroindoline-a. Despite the low score of the mass spectrometry, this protein was easily recognized as belonging to the puroindolines group because of the characteristic high pI and low Mr.

The identified puroindoline-a seemed to be resistant to digestion in both heated and unheated flour.

STARCH SYNTASE

Looking to the raw flour profiles, it has been noticeable that some spots separated in the pH range 4-7 with a Mr around 60 kDa were resistant to the gastric digestion while in the cooked sample they were hydrolysed quickly since they have been susceptible to digestive enzymes.

Using MS, these spots (figure 3.27) were identified as starch synthase GBSSI (figure 3.27, spots 2,4,5,6) and starch synthase (figure 3.27, spots 1,3,7,9,10,12). GBSSI has been designated as granule-bound starch synthase I and it has been detected in both pericarp and endosperm starch granules. On the contrary, GBSSII was distinguished only in the pericarp.

Both the isoforms are in the pericarp since this enzyme is required for amylose synthesis but they have slightly different pI and Mr. In fact, GBSSI has been detected at Mr 61 kDa and GBSSII at 59 kDa (Nakamura et al., 1998).

Studies showed that these enzymes seemed to be strongly bound to the starch granules and they could only be released after heat-induced swelling occurred (Nakamura et al., 1998).

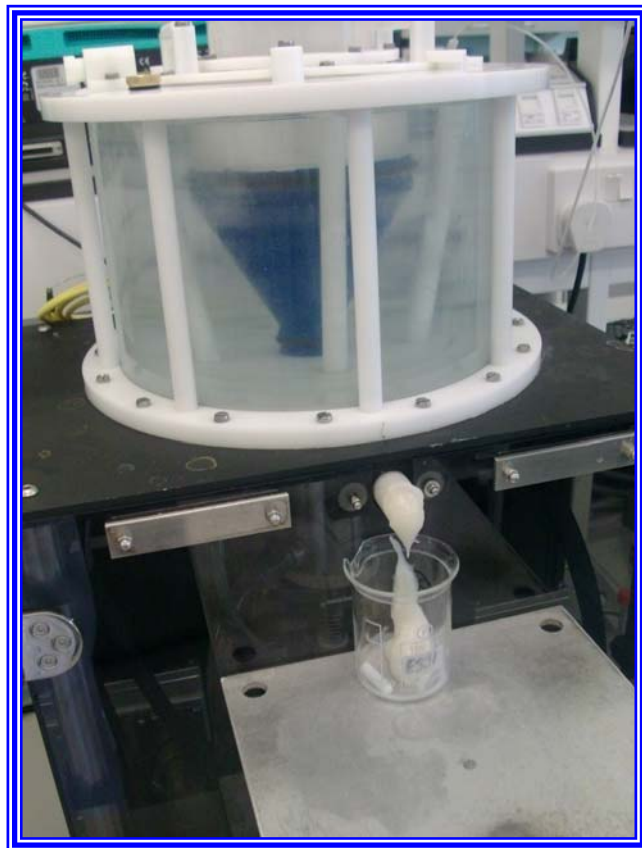
Moreover, it was also found that starch synthases may probably be entrapped into the granules during starch formation (Martin et al., 1995).

These mentioned studies might explain the different behaviour when the gastric digestion, of unheated and heated flour, was performed. Starch synthase proteins were resistant to digestion only in the unheated sample, probably because they were hidden between starch granules, thus appearing unavailable as substrate for the digestive enzymes.

In the heated flour, where starch was gelatinized, starch synthase proteins could be released after heat-induced swelling and became available for the pepsin attack which acted quickly by hydrolysing starch synthase proteins.

CHAPTER 4

ORAL AND GASTRIC PROCESSING OF HEATED WHEAT FLOUR USING A DYNAMIC GASTRIC MODEL OF DIGESTION



4.1 INTRODUCTION

Static methods of *in vitro* digestion, also called biochemical models, usually involve the homogenisation of the food, acidification with hydrochloric acid, addition of gastric enzymes followed by a varying delay simulating gastric residence time. Afterwards, the neutralisation with sodium bicarbonate or hydroxide create the conditions close to those present in the duodenum, and the addition of pancreatic enzymes and biosurfactants such as phosphatidylcholine and bile salts complete the simulation of the intestinal tract environment. Digestion is normally carried out in an orbital shaking incubator set at 37°C with a motion of 170 rpm, to promote homogenous mixing of the sample being digested and the simulated digestive secretions.

Static models are useful for digestion studies involving purified proteins, nutrients or simple food structures, and moreover they are supposed to be ideal for assessments of isolated allergenic proteins (Wickham M. et al., 2009).

However, the methods mentioned above do not take into account several factors which could play an important role on the digestibility of proteins; indeed physical conditions such as meal viscosity, particle size reduction, the unstirred layer of the meal, creation of colloid phases, shear rate, and dilution of the meal in the stomach are all important parameters which are not considered when dealing with a static (biochemical) model of *in vitro* digestion.

The oral phase provides the first step in the digestion process, since food degradation results from the simultaneous actions of the mechanical grinding and the enzymatic hydrolysis (Hoebler et al., 2000). During mastication food is broken down into small particles and lubrication of food by saliva allows the salivary amylase to reach the available starch.

The literature has already reported that salivary amylase influences carbohydrate digestion and absorption (Hoebler et al., 2000), even if previous studies (Mandel, 1987) mentioned that the enzymatic degradation of starch, during the oral phase, should be considered insignificant, because of the short time spent by food in the mouth.

However, until now, the research has not been focused on the effect of oral digestion upon the subsequent degradation of food proteins in the gastric compartment.

The physiological conditions of gastrointestinal digestion are well known. The gastric pH is ca. 2 during fasting, but can increase to pH7 after ingestion of a meal due to the diluting and buffering effect of the food components (Ganapathy and Leibach, 1999). The dynamics of GI digestion have a major role in the profile of peptides produced. For example, factors such as pH will affect both the kinetics and specificity of enzymes, and inclusion of biological surfactants, e.g. phosphatidylcholine (PC) and bile, will affect the susceptibility of proteins to gastrointestinal digestion (Mandalari et al., 2009). Investigation of the digestion and production of potentially absorbable biopetides has been limited due to the inability to obtain *in vivo* samples from the upper GI tract.

Moreno et al. (2007) described several parameters that affect digestibility and allergenicity of food proteins; including, the buffering effect of food ingredients, the mechanical breakdown of food tissue, peristalsis, gastric emptying, intestinal transit and finally the permeability and absorption through the intestinal mucosa.

Among all the parameters quoted, the matrix breakdown, the viscosity and the dilution of the meal in the stomach are prominent factors affecting digestion of starchy food such as porridge, bread or pasta, which are accounted for physical models of digestion.

Thus, the physical form of the food which interacts with the stomach contents is one of the major factors affecting the digestion process. Moreover, some studies showed that the calorie content and meal viscosity have an additive effect in delaying gastric emptying and increasing the sense of satiety during meal consumption. (Marciani et al., 2000). Contrary to the traditional idea of rapid and complete homogenization of a meal, gastric contents seem to be rather poorly mixed (Marciani et al., 2000). Studies performed using Echo-planar magnetic resonance imaging (EPI), which is a non-invasive analysis performed in humans, showed that, (figure 4.1 below) long after ingestion the meal remained heterogeneous, with gastric secretion only poorly penetrating the centre of the

food bolus (Marciani et al., 2000). This is important because there are several enzymes (including amylase and lipase) that are inactivated at low pH but are able to continue working for perhaps an hour in the centre of the bolus.

Static models usually rapidly expose foods to highly acidic environments. Results shown in figure 4.1 (below) suggested that during *in vivo* digestion, the centre of the meal bolus is not diluted by secretions for an appreciable time. Moreover, the volume of secretions present in the digesta increased with high meal viscosity, thus the more viscous the meal the higher total volume of secretion is produced.

A number of dynamic *in vitro* digestion models have been previously developed, ranging from a Simulator of the Entire Human Gastrointestinal Model Ecosystem (SHIME) (Molly et al, 1993), to a dynamic computer-controlled upper gastrointestinal tract model (TNO, Organisation for Applied Scientific Research, The Netherlands). The SHIME model is a multi-compartmental simulator equipped with six reactors representing the stomach, duodenum/jejunum, ileum, caecum, ascending colon, transverse and descending colon.

The TNO model consists of four chambers simulating the stomach, small intestine, jejunum and ileum. It should be noted that all *in vitro* digestion models have their disadvantages, particularly replicating a chewed meal, inhomogeneous mixing in the fundus, shear and mixing conditions in the antrum and the temporal delivery of gastric digesta to the duodenum.

This chapter was firstly focused on finding a reproducible method of *in vitro* oral simulation, afterwards, two different gastric digestions of heated bread-making flour, using a simulated oral phase coupled with a dynamic model of gastric digestion (DGM), were performed, and protein digestion profiles were monitored by 1-D and 2-D SDS-PAGE, in order to establish differences in protein digestion patterns.

The Dynamic Gastric Model used here can not only process a food or meal 'as eaten' but also replicates the additions, residence time, mixing and shear experienced by the digesta in the upper part of the stomach and the antrum.

This would allow us to gain a better understanding of wheat flour protein degradation using a physiologically relevant model of digestion and to design a process for *in vitro* digestions of other starchy foods.

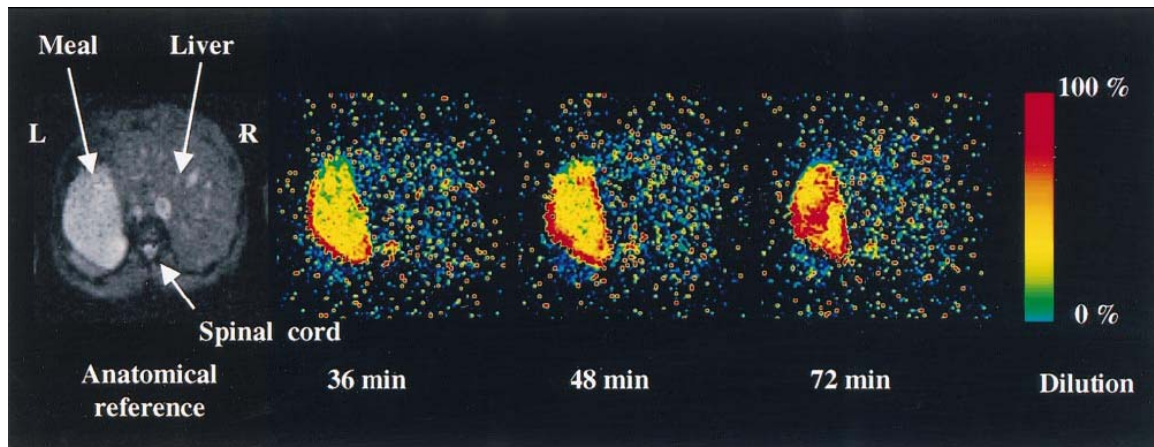


Figure 4.1: Colour-coded dilution maps acquired at different times after a volunteer ingested 500 ml of viscous locust bean gum meal. A transverse EPI image is also shown as an anatomic road map (L, left; R, right). Gastric secretion made the outerboundaries of the viscous meal more diluted (in red) at an early time, whereas the inner bolus remained viscous (in green) for longer. As time progressed, the viscous meals appeared more diluted and mixed. The liquid meals were homogeneously diluted with time (Marciani et al. 2000).

4.2 THE DYNAMIC GASTRIC MODEL (DGM)

4.2.1: PRINCIPLES OF THE SYSTEM

The “Dynamic Gastric Model (DGM) is a sophisticated physical model, mimicking the human digestive process in the stomach. The DGM is located at the Institute of Food Research (Norwich, UK), and this complex system was designed by Dr. Richard Faulks and Dr. Martin Wickham (IFR) and was built by PBL, the Plant Bioscience Limited, based at John Innes Centre (Norwich, UK).

The DGM was developed to study the influence of structure, hydration, mixing, shear, transport and delivery of meals, within the gastrointestinal tract and represents the physical embodiment of over 10 years of studies designed to understand and then simulate the human gastric process. It retains the advantages of *in vitro* models (e.g. simplicity, low cost, fast throughput, ability to screen numerous variables, lack of ethical issues, etc.) but at the same time is a complete departure from current practice by combining the biochemical environment with a real time simulation of the complex physical forces involved in gastric digestion.

In order to achieve a good explanation on the behaviour of the meal into the stomach compartment, Echo-Planar Magnetic Resonance Imaging (EPI) has been used to evaluate the gastric processing time and conditions of meals, in human volunteers (Richard Faulks, personal communication).

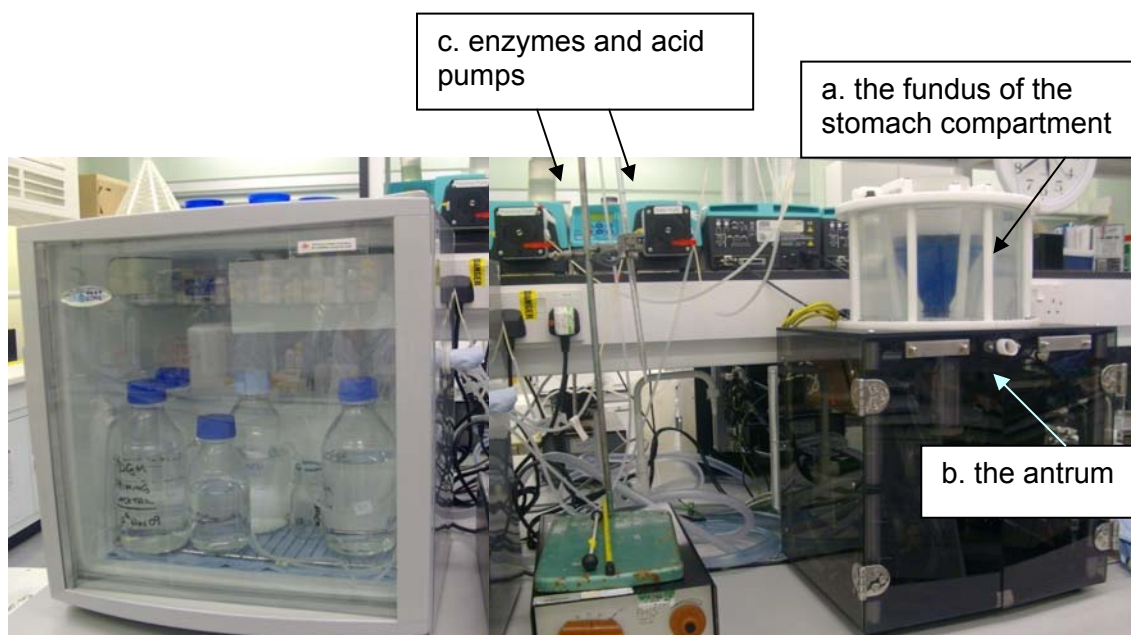


Figure 4.2: the Dynamic Gastric Model.

The DGM (figure 4.2) is composed of two main parts: the fundus and the antrum. The fundus is the main body of the stomach that is represented as a conical compartment (blue-coloured, figure 4.2 above), made from polyurethane, which is able to shrink and expand, mimicking the slow peristaltic massage of the stomach. The process is computer-controlled, where the residence time of the meal in the fundus is calculated on the basis of the meal calorie content with *in silico* models. The fundus compartment is surrounded by a heated bath of water to maintain the temperature at 37°C, mimicking human body temperature conditions. The antrum is the lower part of the stomach, where the food is subjected to increased shear inducing a mechanical breakdown of the food structure. Digested food is collected at the antrum for further analysis such as duodenal digestion (static model only). Other important components include the pumps which are connected to the system, continually providing enzymes and acids. All of the DGM processes are controlled by specialised software that permits monitoring of all parts in real-time.

4.2.2 MATERIALS

4.2.2.1 Chemicals and enzymes

Human salivary amylase (Type IX-A, lyophilized powder, 210 U mg⁻¹ solid), was supplied by Sigma (Poole, Dorset, UK).

The specific concentrations of acids, salts and enzymes can not be published, since they are property of Plant Biosciences Ltd.

The concentrations used for the Dynamic Model are mid Reference Range and they are added at physiological rates. (Letner C., 1981, Geigy Scientific Tables).

4.2.2.2 Preparation of heated flour paste

Heated flour paste was prepared using the method described in chapter 2 (section 2.2.3.1), using a larger amount of flour (cv. Hereward). 42 g of flour was mixed with 168 g of water and the mixture was heated by continuously stirring at 100°C for 10 min.

4.2.3 METHODS

4.2.3.1 The Simulated Oral Digestion

Oral digestion was carried out using a heated flour paste. Two different salivary amylase concentrations (Sigma) were used for the heated flour meal: a low amount (3U/ml) and high (115U/ml) of enzyme, based on data from Aura et al. (1999) and Neyraud et al. (2009). The saliva flow rate was estimated on the basis of the time required for the oral chewing step, which was assessed by performing previous *in vivo* chewing test with a volunteer. In order to simulate the oral breakdown of food, a whisk was used, for heated flour, before adding the commercial salivary amylase. Afterwards, samples were placed in an orbital shaking incubator for 5 min and they subsequently underwent the gastric phase in the DGM.

4.2.3.2 The *in vitro* Gastric Dynamic Model

The heated flour that underwent the oral phase of digestion was processed in the DGM (figure 4.2 above), adding 20 ml of residual gastric solution. Digestion was carried out for a total of 30 min (based on the calorific content of the paste), and samples were collected every 5 min.

4.2.3.3 1D and 2D SDS-PAGE

Samples were processed as described in chapter 2.3.3.2, 2.3.3.3 and 3.2.3.1

4.2.3.4 Viscosity Measurements

Viscosity measurements were obtained using the flour heated paste, the same sample treated with simulated salivary fluid (SSF) and with SSF plus 115U/ml commercial salivary amylase, in order to follow the oral breakdown of the matrix during *in vitro* oral digestion

The AR2000 rheometer, (TA Instruments, Crawley, UK) was used to measure the viscosity and samples were tested using a cone and plate attachment, at a temperature of 37C.

4.3 RESULTS AND DISCUSSION

4.3.1 THE BIOCHEMICAL MODEL OF ORAL DIGESTION

A model of oral digestion was developed using heated flour. Initially 3U of amylase per ml of saliva was used according to the method described by Aura et al. (1999). This amount of salivary amylase was first used for digestion studies using the biochemical model and then was taken through to use in the DGM, however it was shown that this amount of amylase did not have any significant effect upon starch degradation in the wheat flour paste *in vitro*, when compared to paste that was chewed *in vivo*. Therefore a second model was produced. The formulation was modified based on the findings of Neyraud et al. (2009) where, the units of salivary amylase were calculated on the basis of saliva flow rate, knowing the chewing time of the meal. These new findings reported that the average amount of salivary amylase secreted in humans was around 115 U/ml of saliva. The daily output of saliva is estimated at 500-1500 ml and the average saliva flow rate is estimated to be 1ml/min, in stimulated individuals (Lentner C., 1981). The amount of salivary fluid required to form a food bolus was determined by performing an *in vivo* oral phase using a volunteer. The chewing time for 200 g heated flour paste was estimated at approximately 8 min, and finally it was possible to simulate the oral processing by using the estimations found with the *in vivo* experiment. Simply, the amount of salivary amylase to add to the model was calculated by multiplying the average salivary amylase (115 U) (Neyraud et al., 2009) and the chewing time (8 min), considering the saliva flow rate as 1ml/min. Finally, heated flour was mixed with a whisk (figure 4.3 C) to simulate the mastication process (food breakdown). Subsequently, 920 U of commercial salivary amylase from human saliva (Sigma, Dorset, UK) was added to heated flour paste.

Salivary amylase has been shown to consistently affect the food structure of starchy food. In fact the initial disruption of the physical structure (during the mastication) increased the available surface to amylase, and subsequently the

breakdown of some glycosidic bonds by the salivary amylase led to a loss of viscosity of gelatinized starch (figure 4.3 B and D).

The rheology measurements confirmed that after adding the 116U/ml of salivary amylase, the viscosity dropped down due to the effect of the enzyme on the hydrolysis of the starch network (figure 4.11 appendix)

Oral and gastric digestion was successfully performed for heated flour paste and the effect of the low and high salivary amylase upon wheat protein degradation, has been studied and described in the next section.

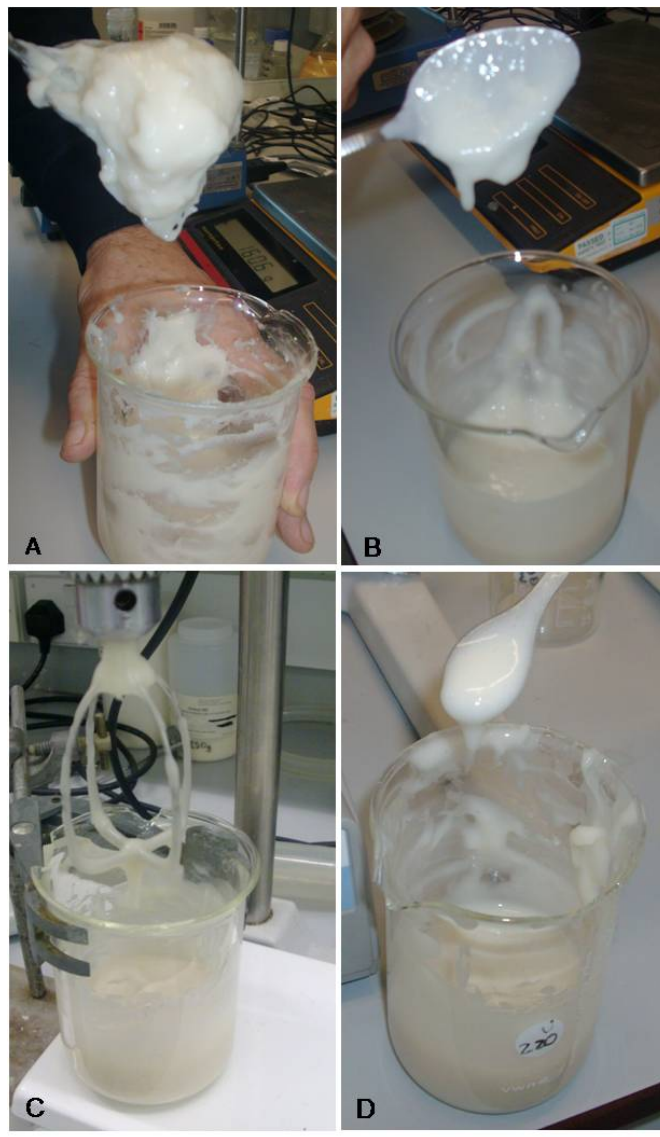


Figure 4.3: A. heated flour paste before chewing; B. heated flour paste chewed by a volunteer. C. heated flour paste mixed with a whisk to simulate mastication and D. subsequent addition of 920 U of salivary amylase and incubated 5 min at 37°C.

4.3.2 DOES SALIVARY AMYLASE AFFECT PROTEIN DEGRADATION DURING IN VITRO DYNAMIC DIGESTION?

Heated flour paste was initially processed with salivary amylase in two different ways: (1) with a low amount of enzyme (3U/ml: experiment A) and (2) with a higher enzyme concentration (115 U/ml: experiment B). The two oral digesta were apparently different; in fact the macroscopic structure revealed changing in viscosity due to the mechanical breakdown of the viscous network and to the action of the salivary amylase on starch, as described above. The consistency of the paste orally digested with 3U/ml salivary amylase appeared to be inhomogeneous and viscous (Figure 4.3 A (above)), and the consistency of the paste orally digested with 115 U/ml salivary amylase was shown to be more fluid and homogenous ((Figure 4.3 D).

Subsequently, the two different pastes behaved differently, when subjected to the dynamic gastric digestion with DGM.

The two DGM experiments showed different trends regarding the volume of acid solution and enzymes released by the pumps. Indeed, looking at graphs 4.5 and 4.7 (appendix) it was clearly noticeable that in the first case (low amount of salivary amylase) the amount of acid titrated into the fundus was 1.8 ml and in the second run (higher amount of salivary amylase) was around 16 ml. This happened because the first *in vitro* digestion (performed with the low salivary amylase) had a very viscous chewed starting material which did not permit the pH meter to detect the exact pH of the digesta, thus slowing the rate of the acid solution added. It was also observed that the pH of samples collected at the antrum increased gradually up to pH 4.8, limiting the pepsin action (table 4.9). In contrast, the gastric digestion performed using a higher amylase concentration, showed a gradual increase of addition of the acid during the process, and the pH of all of the samples collected, was around 2.5 (table 4.10).

From this data we can conclude that the amount of salivary amylase used in oral digestion played an important role in the production of a bolus that was suitable to allow effective processing in the gastric phase using the DGM. Indeed, the slow

shear rate applied by the model had a minimal effect on breaking down the starch-protein matrix of the orally digested pastes, however proteases (pepsin) were employed to perform protein hydrolysis and to break down the protein matrix formed when the flour was hydrated with water to form a paste and then heated to fully gelatinise the starch. Degradation of this protein network would result in breakdown of the food matrix, resulting in a much less viscous system. However, the viscosity of the bolus in the gastric compartment will affect the accessibility of gastric secretions reaching the protein. Indeed, the salivary amylase will probably still be active in the centre of the bolus where the conditions will not be at low pH. Therefore the protein digestion profiles of heated flour paste, orally digested with high and low amounts of salivary amylase were assessed to see if the viscosity of the food matrix effected protein hydrolysis during *in vitro* gastric digestion.

After oral and gastric digestion, protein digestion profiles were evaluated, comparing digested products collected from heated flour pastes subjected to oral digestion using the two levels of salivary amylase (3U/ml and 115U/ml). Soluble and insoluble proteins were separated by centrifugation, to obtain a supernatant (soluble phase) and pellet (insoluble phase).

Protein profiles obtained from 1-D SDS-PAGE did show different results in regard to protein degradation when comparing the digesta from heated flour paste using the different amounts of salivary amylase.

Figure 4.12 showed the insoluble protein profile patterns related to the first DGM (flour paste with low salivary amylase).

It was clearly noticeable that there were no differences between the undigested materials and the chewed one (lines 1,2). Moreover, surprisingly it has been found that during 30 min of gastric digestion proteins seemed to be not attacked by pepsin (lines 3-8).

In fact it was evident a group of proteins within 35 kDa and 60 kDa, belonging to the storage proteins, which were highly packed and persistent up till 30 min of *in vitro* digestion.

Moreover, the HMW-GS, between 60 and 120 kDa, visualised in the undigested material (figure 4.12, line 1), did not disappear and they were not degraded during the 30 min gastric digestion (Figure 4.12 lines 3-8).

Furthermore, a band around 12 kDa, belonging to the α -amylase inhibitors, was persistent during the whole gastric digestion.

Soluble proteins from experiment A (low salivary amylase) were also separated with 1D SDS-PAGE (Figure 4.12, lines 9-16). Protein bands between 6 kDa and 120 kDa were detected during the whole digestion and neither the HMW proteins seemed to undergo digestion.

Afterwards, protein patterns of digested samples derived from experiment B (DGM using a paste processed with a higher amount of salivary amylase) were obtained (figure 4.13).

The insoluble protein profiles (lines 1-8) appeared strongly different compared to the ones obtained from the experiment A (figure 4.12, lines 1-8).

Firstly, the undigested sample and the chewed (lines 1,2) did not report any differences in protein bands, result which was expected since the salivary amylase, even if presented in a higher amount, did not act on protein hydrolysis.

However, the 1-D SDS-PAGE showed that during the gastric digestion, protein profiles underwent many changes. In fact, the insoluble protein profiles (figure 4.13, lines 1-8) enhanced the group of protein bands between 35 and 60 kDa which decreased in intensity within 20 min of digestion, and after 30 min it appeared weakly visible. Moreover, a protein band around 12 kDa behaved as described in experiments A.

Soluble proteins, derived from the experiment B, were also separated using 1D SDS-PAGE (figure 4.13, lines 9-16). It was possible to highlight three protein bands between 30 and 20 kDa which appeared after 5 min of gastric digestion, that were still markedly visible at the end of the gastric digestion. The strong band around 12 kDa appeared to be resistant to digestion.

Thus, these experiments underlined that proteins separated from the digestion products of experiment A (Figure 4.12, insoluble proteins lines 1-8, soluble lines 9-16) did not show any clear changes due to the pepsinolysis, in fact the samples appeared resistant to digestion for the entire gastric residence time of 30 min. In

contrast, gastric digestion performed using orally digested starting material, containing a physiologically relevant salivary amylase concentration (115U/ml), showed distinct degradation patterns (Figure 4.13, insoluble proteins lines 1-8, soluble lines 9-16) during 30 min gastric digestion.

As shown in chapter 2 and 3, the 1-D PAGE allows the detection of protein bands and their disappearance over the time but there are other advanced techniques with a higher resolving power, as 2D-PAGE which allows hundreds of protein to be separated.

Thus, based on the evidence provided by 1-D PAGE, 2-D PAGE was performed using the insoluble fractions from oral and gastric samples from heated flour paste, digested in the oral phase using a physiologically relevant salivary amylase concentration (115U/ml). This analysis was performed in order to achieve a better resolution of wheat proteins after *in vitro* digestion.

Insoluble wheat proteins were separated at pH range 6-11. This allowed the separation of the storage proteins which previously seemed to be slowly digested in the cooked flour (chapter 3) and the α -amylase inhibitors which were already detected in the 2D maps produced using the biochemical model.

Moreover, three time points of gastric digestion were picked up and explored

Actually, the undigested heated flour (control), the chewed material (corresponding to the heated flour process with the salivary amylase) and samples corresponding to 5, 15 and 30 min of gastric digestion were mapped in 2D gels. Looking to figure 4.14, it was evident that after 5 min of gastric processing (C) new peptides around 30 kDa appeared because of the degradation of high molecular weight proteins. Within 30 min of gastric digestion there were still some proteins belonging to the prolamin family, which were not completely degraded.

Moreover, as already described in chapter 3, AAI (alpha amylase inhibitors) seemed to be resistant to proteolysis and were not degraded during the gastric processing.

Thus, it is possible to mention that salivary amylase affected not only carbohydrate digestion (as reported in the literature) but also protein degradation during digestion. This might happen because of changes in the structure of starchy food

during the oral phase thus consequently affecting the availability of protein for the pepsin attack.

It might be called “an indirect effect” of the oral digestion on the gastric one. The interaction of amylase enzyme with starch ingredients produces almost an immediate effect on hydrolysis, and thus making the food much easily mixable and digestible in the stomach.

This finding is important for analytical improvements, to perform digestions which are able to simulate the food behaviour into human body, but also for a nutritional point of view.

Indeed, it is well known that digestion starts into the mouth but it is quite unexplored the effect of a proper oral mastication and bolus formation on protein and allergens digestion.

Meals, food, that are well destroyed into the mouth might easily released nutrients like proteins and starch which consequently may be exposed and available to digestive enzymes.

The structure of the food matrix can have a great impact on the digestibility of proteins and the further elicitation of allergic reactions, and may affect the kinetics of allergen release, potentiating the severity of allergic reactions.

Coupling the simulation of the oral digestion with an appropriate mixing at each stage of digestion using a dynamic model, it was possible to obtain a more reliable *in vitro* system which permitted to give a better explanation on the parameters that might affect protein digestibility and allergenicity of starchy food.

Oral digestion is a short step in the overall digestive process as compared with the period of gastric and intestinal digestion, and has therefore often been neglected in some investigations regarding starch or protein *in vitro* digestion.

This work finally conclude that the simultaneous processes of oral digestion involving mastication, saliva lubrication, mixing, and hydrolysis by salivary amylase has to be considerate significantly when performing *in vitro* gastric digestion of starchy food.

Thus, the oral digestion, which affected the structure viscosity, has been extremely significant for the subsequently gastric model which showed to work adequately if the starting material was properly “chewed”.

CHAPTER 5

STARCH DIGESTION:

In vitro and *in vivo* digestibility
of durum wheat pasta varieties



5.1 INTRODUCTION

Pasta is a widely consumed product, especially in Italy and it is one of the main foods on the basis of the Mediterranean diet.

Pasta is obtained after kneading semolina and water, extruding and drying. The most noticeable recent innovation is the application of high temperature in the drying process.

In Italy, the product called “pasta” has to be produced with durum wheat (*Triticum turgidum ssp. Durum*) only. All the other products, like pasta produced by using different wheat varieties rather than *Triticum turgidum ssp. Durum*, need a proper label, describing the specie or the variety of wheat used.

Moreover, in these last few years, an interest towards the use of ancient wheat varieties (spelt) has led to resurgence in its cultivation (Bonafaccia et al., 2000).

Nowadays, more spelt-based products are available including flour, bread, breakfast cereals, pasta and biscuits. It seems that these ancient cereals have valuable nutritional and physiological properties which could help promoting the consumption of these novel products (Marques et al., 2007). However, few scientific studies concerning the benefits of spelt products have been done (Bonafaccia et al., 2000, Marques et al., 2007, Vicentini et al., 2007, El-Sayed et al., 2008).

Indeed, spelt was found to induce cellular mechanism implicated in the pathogenesis of celiac disease, like the common durum wheat, but *Triticum turgidum ssp. monococcum and ssp. dicoccum* wheat species had shown very few toxic effects (Vicentini et al., 2007).

Cereal foods like pasta are mainly composed of starch and proteins. Starch is the main constituent and it provides the majority of the energy in the diets. Thus nutritional and health properties of starch would be of great interest to consumers and food processors. Starch can be found in various physical and chemical forms in foods, which influence the rate and the extent of digestion and consequently its nutritional and health properties. The FAO/WHO expert consultation on carbohydrates in human nutrition recommends the consumption of foods that

possess low glycemic index or food which slow down digestion, for example the ones that promote a slow release of glucose in the body (FAO/WHO 1998).

Carbohydrate foods that exhibit a lower rate and extent of starch digestion would be preferable due to their physiological functions and impact on health, including reduction of the glycemic and insulinemic responses, hypocholesterolemic effects and protective effects against colon-rectal cancer (El-Sayed et al., 2008).

The glycemic response and consequently the insulin demand appear to be closely related to the enzymatic susceptibility of starch. In particular food composition and processing affect carbohydrates availability and cause different glycemic responses (Berti et al., 2004).

Indeed, among cereals products, pasta appears to possess unique nutritional features in that the starch is slowly digested and absorbed in the small intestine, hence promoting a low plasma glucose response (Petitot et al., 2009).

The low glycaemic Index (GI) of pasta is generally attributed to its compact structure but other numerous factors have been suggested to explain the different rates of starch degradation. The surface area accessible to digestive enzymes, the encapsulation of starch granules by fibre and proteins, the physical structure of starch such as its degree of gelatinization and its amylose:amylopectin ratio, may affect starch digestion. Some works carried out studies regarding the effect of the drying temperature on *in vitro* protein and starch digestibility in pasta (De Zorzi et al., 2007, Petitot et al., 2009).

The work carried out in this chapter was designed to evaluate the extent of starch digestion using an *in vitro* static model, and the glycemic response with an *in vivo* study testing three different types of pasta. These pastas involved in the study were made with three varieties of wheat: the common *Triticum turgidum ssp. durum* and two ancient varieties, *Triticum turgidum ssp. polonicum* (also called Kamut) and a *Triticum turgidum ssp. dicoccum* (Spelt). The main aim was to assess differences in starch digestion and glycemic Index due to the wheat genotype, in order to rediscover and promote two of the ancient wheats cultivated in the past. Moreover, this study was performed to gain a better understanding about the phenomena that affect starch digestion, in order to improve the nutritional quality of cereals food, like pasta.

5.2 MATERIALS AND METHODS

5.2.1 PASTA SAMPLE PREPARATION

The varieties of pasta used for the experiments were:

A. *Triticum turgidum ssp. durum* (common wheat);

B. *Triticum turgidum ssp. polonicum* (Kamut);

C. *Triticum turgidum ssp. dicoccum* (spelt).

Pasta samples were from Ecor S.p.a (Treviso, Italy). The company made the three pasta samples using the same processing procedure, by drying them at temperature that reached 90 °C. Moreover they all were made as “spaghetti shape”.

In order to perform *in vitro* and *in vivo* experiments, pasta was cooked into boiling water for 12 min, as suggested by the producers.

Subsequently pasta was immediately processed as described in section 5.2.3.

5.2.2 CHEMICALS AND ENZYMES

The “Resistant starch kit” which enclosed the enzymes used for the *in vitro* experiments was provided by Megazyme (Milan, Italy).

The glucometer and the strips “Accu-chek, Aviva”, used for the *in vivo* measurements of the glucose blood level, were supplied by Roche Diagnostics (Monza, Milan, Italy).

All the other chemicals and reagents were of analytical grade.

5.2.3 QUALITATIVE AND QUANTITATIVE PROTEIN DETERMINATION IN PASTA SAMPLES

Protein quantification has been performed in pasta samples using Kjeldhal method described in the official method of analysis AOAC 17th ED. 2000 976.05.

Proteins have been also separated using 1D SDS-PAGE as described in section 2.3.3.

Prior to load samples (20 μ l) into the polyacrilamide gel, a total protein extraction from each pasta sample boiled, dried and subsequently grinded has been done. Thus, 100 mg of sample was extracted with 4 ml of extraction buffer containing 25 mM Tris-HCl, 6M urea, 2.5% (p/v) SDS, 2.5% (v/v) 2-mercaptoethanol and glycerol 10% (p/v). Afterwards, samples were centrifuged at 10000 *g* for 20 min and the supernatant was collected and stored at -20°C.

The resulting gels were stained with Comassie blue staining solution.

5.2.4 IN VITRO STARCH DIGESTION

In vitro starch digestion of pasta samples was performed by using the reagents enclosed into the Kit supplied by Megazyme. Thus, the pancreatic amylase (E.C. 3.2.1.1), the amyloglucosidase (AMG) (E.C. 3.2.1.3) and the reagent for glucose determination (GOPOD), were used.

Starch digestion was performed by slightly modifying the method of Englyst et al. (1992).

In order to obtain a kinetics of starch digestion over the time, 6 time point of digestion (0, 20, 60, 120, 180, 240 min of hydrolysis) have been chosen and the experiments were performed in individual bottles, because of the heterogeneity of the matrix which did not permit to process the sample in one big bottle and sampling during the time.

Briefly, pasta samples were boiled in boiling water and subsequently minced with a mincer in order to simulate the oral food breakdown. Afterwards, 500 mg of samples were weighted out into different individual bottles, each one corresponding to one point of starch digestion. Subsequently, 4 ml of a solution containing pancreatic amylase (10 mg/ml) and amyloglucosidase (300 U/ml) were added to each sample which were quickly incubated into a water bath at 37°C until the time required.

To stop hydrolysis samples were mixed with a volume of ethanol, and subsequently, they were centrifuged at 1500 *g* for 10 min, in order to obtain the supernatant and the pellet fractions.

Supernatants collected were treated with a volume of 100mM Sodium acetate pH 4.5 plus 10 µl of AMG 300 U/ml and incubated at 50°C for 20 min, which allowed the AMG to hydrolyse maltose, sucrose or other sugars produced from the degradation of the starch into glucose molecules.

The glucose content, of each digested product, was detected with a spectrophotometric assay using GOPOD reagent, by incubating samples at 50°C for 20 min prior to absorbance detection.

In order to quantify the soluble glucose released during digestion, over the time, the absorbance value was multiplied x 0.9.

Triplicates for each pasta sample have been performed.

5.2.5 IN VIVO GLYCEMIC RESPONSE

In vivo glycemic responses, of the three types of pasta, were assessed by including 10 healthy volunteers (three males and seven females) in the study.

The age of the subjects ranged from 24 to 34 years old and their body mass index (BMI=kg/m²) was from 55 to 85 kg.

Subjects gave informed written consent, before experiments and a standard protocol, accepted by FAO/WHO (1998), was used to assess the glycemic responses.

Briefly, subjects attended 4 morning sections having fasted over night. They were fed with a portion of white bread (test food) or common wheat pasta, spelt pasta and kamut pasta, containing 50 gr of carbohydrates, determined using AOAC.996.11 method. During the meal assumption volunteers could drink a glass of water corresponding to 140 ml.

Blood samples were taken from subjects before (time 0) and 15, 30, 45, 60, 90, 120 min after starting the meal.

The glycaemia was measured with a strip gluco-meter based on glucose dehydrogenase mediated oxidation of glucose (Roche, Italy).

The area under the curve was calculated, after plotting the glucose values, by applying the following formula:

$$\mathbf{AUC= \Delta t [(t_2-t_1) + (t_3-t_1) + (t_4-t_1) + 3/2 (t_5-t_1) + 2 (t_6-t_1) + (t_7-t_1)]}$$

Where:

AUC= Area Under Curve;

t₁, is the glucose value detected at time point 0;

t₂, is the glucose value detected at time point 15;

t₃, is the glucose value detected at time point 30;

t₄, is the glucose value detected at time point 45;

t₅, is the glucose value detected at time point 60;

t₆, is the glucose value detected at time point 90;

t₇, is the glucose value detected at time point 120.

The glycemic Index (GI) of each pasta sample tested was calculated by applying this second formula:

$$\mathbf{GI= (AUC\ test\ food / AUC\ reference\ food) \times 100}$$

Where:

Test food: is the pasta sample examined;

Reference food: is the white bread, used as a control sample.

Statistical analyses were performed by using the Wilcxon rank test.

5.3 RESULTS AND DISCUSSION

5.3.1 IN VITRO STARCH DIGESTION

The rate and the extent of starch digestion vary in foods depending on several intrinsic and extrinsic factors, including physical form of food and food composition (El-Sayed ,2008).

Starchy foods such as wheat products, having the same amount of starch, may not have similar rates and extents of digestion and accordingly different glycemic and insulinemic responses.

The protein and starch content of the three cooked pasta samples was measured. As shown in table 5.2, the protein content of kamut pasta (15%) was higher than the one observed in spelt and in common durum wheat products, which was estimated around 12%. Starch behaved differently, since it was less abundant in kamut and spelt pasta rather than the modern common wheat sample.

Indeed, the amount of starch was 71%, 73% and 80% respectively for spelt, kamut and common wheat pasta.

The protein content was different among the three samples but protein profile patterns (figure 5.1) were somewhat similar among the pasta samples evaluated. This was not unexpected since all the three varieties investigated belong to the *Triticum turgidum* family.

Focusing on figure 5.1, it was possible to notice some more intensive protein bands, around 30 and 45 kDa, in the sample corresponding to Kamut pasta. Indeed, it has been described above that this ancient wheat is characterised by a higher amount in protein.

Subsequently, the work aimed to explore starch digestibility, thus, the kinetics of starch digestion was firstly measured in samples as eaten, by using an *in vitro* method of digestion.

The rate and the extent to which the starch is digested *in vivo* are influenced by the particulate structures present in the food when eaten. Thus, to measure the

rate and the extent of digestion *in vitro*, sample should be analysed as eaten, without extensive milling or other preparative treatment.

Moreover, it was possible to calculate three starch nutritional fractions which helped to gain a better understanding about starch digestion in starchy extruded foods like pasta.

Finally, an *in vivo* study was performed on a group of healthy volunteers and the glycaemic index was assessed.

Concerning the *in vitro* digestion, figure 5.3 displayed the kinetics of hydrolysis. The three trends showed similar behaviours during the first 20 min of digestion but afterwards, pasta made with kamut seemed to release the starch slowly.

Actually, starch digestion is slowed in the small intestine if the physical form of the food hinders access of pancreatic amylase. This usually occurs if starch is contained within whole or partly disrupted plant structures such as grain or seeds, if rigid cell walls inhibit swelling and dispersion of starch or if starch is very densely packed in food such as spaghetti (Englyst et al., 1992).

This study reported also that the rate of starch digestion in spelt and common wheat pasta was similar (figure 5.3). Spelt pasta was made following the standard procedures used for both common wheat and Kamut pasta but the starting flour contained also the bran.

It has already known that fibre is one of the factors that might slow down starch digestion but surprisingly, in this work, *in vitro* measurements revealed a different behaviour. This is why further experiments were focused on *in vivo* analysis.

The values obtained from the *in vitro* experiments were used to estimate three important starch fractions:

1. RDS: it is called “rapidly digestible starch” and it is referred to the starch released within 20 min of digestion;
2. SDS: it is the “slowly digestible starch” that is the fraction digested within 120 min and it is calculated by difference between SDS and RDS.
3. RS is the “resistant starch” that is not digested within 120 min of starch hydrolysis, but it may be fermented by probiotic microorganisms placed into the colon.

Thus, RDS, SDS and RS fraction were calculated, for all the three samples examined, and plot in a graph (figure 5.4).

The graph highlighted that RDS is almost similar among the types of pasta.

Kamut and spelt pasta showed a slightly slower amount of RDS, which played around 37% compared with the common durum wheat, which reached 40% of starch released within 20 min.

The presence of slowly digestible starch fraction (SDS) in higher quantity in spelt and, subsequently, in common wheat pasta has been detected (figure 5.4).

Finally, the RS fraction showed that Kamut pasta seemed to be less digestible, because the starch remained undigested is higher, compared with the other samples.

The lower value of SDS in Kamut pasta and the higher amount of RS are related to the kinetics of digestion which was slower rather than spelt and the common wheat samples. This behaviour may be due to interactions between starch and proteins, which might render the starch less readily available for digestion.

As described before, Kamut pasta showed a higher amount in protein which may give an explanation about the rate of starch digestion. Indeed, the protein network, that entraps the starch, might be more packed, due to the higher amount in protein and the drying temperature conditions used in pasta manufacture, thus rendering the starch less available to the enzyme attack.

A study carried out by Bonafaccia et al. (2000) showed that spelt cultivars compared to common wheat had a higher content of some nutrients, such as protein and soluble fibre, implicated in the drop of the glycemic index.

5.3.2 IN VIVO GLYCEMIC RESPONSE

The glycemic index (GI) ranks foods on how they affect the blood glucose levels. This index measures how much blood glucose increases in the two or three hours after eating.

Glycemic index assessment was proposed in 1981 by Jenkins for classifying starchy food on the basis of their glycemic response.

GI is calculated as the area under the post-prandial plasma glucose response curve of a test meal, compared with a reference food equivalent to 50 g available carbohydrates.

Marques et al. described that the GI depends more on glucose metabolism than glucose absorption, thus the last carbohydrate intake (the evening before the *in vivo* test) or the fasting glycemia, may affect GI.

Figure 5.5 reported the glucose levels detected during the *in vivo* experiments. They showed that mean values, corresponding to the fasting glycemia (time 0), were almost similar in all the three *in vivo* experiments performed (88 mg/dl glucose, with a standard deviation of 8 or 9).

Indeed, the protocol gave to the volunteers, before the experiment, posted that subjects involved in this study should not eat any carbohydrate-based meal the night before the test.

Looking to the values in table 5.5, it was easily understandable that the variation in glucose measurements was quite evident between volunteers.

Therefore, measurements performed in human subjects are quite often variable, especially when dealing with a small group of people. Indeed, the number of volunteers, that was defined also in other *in vivo* studies (Marques et al., 2007, Berti et al., 2004), was around 7-10 subjects.

In order to better visualise the data of table 5.5, the means of glucose measurements obtained by 10 subjects, for each kind of pasta, were plotted (figure 5.6), thus obtaining a glycemic response curve.

Thus, the three trends were clearly defined and they showed some differences between samples.

As reported above, looking the *in vitro* kinetics of digestion, (figure 5.3) the first part of the *in vivo* process did not reveal differences between the three tested meals; within 15 min no significant differences, in glucose response, have been detected (figure 5.6). However, after 15 min from the assumption of the meal, the glucose level reached the maximum peak only if spelt and kamut pasta were ingested. Pasta made with common wheat showed the shoulder after 30 min from ingestion.

The glucose response seemed to drop down quickly, after 15 min, especially when kamut pasta was tested, and, afterwards, it reached the baseline glucose level within the 120 min.

Pasta made with spelt wheat was expected to show interesting behaviours, but it did not.

Theoretically, the glycemic response of carbohydrate may increase following the removal of gluten, as the gluten protein network surrounds the starch granules so not allowing amylase to easily access the granule and inhibiting the rate of starch hydrolysis in the lumen of the small intestine (Berti et al., 2004).

Finally, the glucose measurements were used to calculate the glycemic Index.

The GI values found are listed below:

Durum wheat (*T.t. ssp. durum*) pasta: 54

Spelt wheat (*T.t. ssp. Dicoccum*) pasta: 56.5

Kamut pasta (*T.t. ssp. Polonicum*) pasta: 44

Subsequently, by performing the statistical analysis using Wilcoxon rank test, it has been found a significant difference (*p value* <0.05) between kamut and spelt pasta.

As showed in figure 5.8, the glycemic index of kamut pasta is clearly slower than spelt pasta.

Besides, kamut pasta had a lower glycemic index rather than the common durum wheat (*p value* = 0.054) and it was statistically significant at $p=0.01$.

Surely, more in vivo experiments should be perform in order to give a more statistically sustainable result.

Among the different factors known to affect the rate and extent of starch digestion and therefore, the glycaemic response to starchy foods, the botanical origin and the level of processing play an important role.

Pasta manufacture is itself a process that affects the glycemic index by producing a highly packed structure that renders the starch less available for digestion, thus, macromolecular interactions in pasta, that are responsible for the pasta texture, are also responsible for the low glycemic index properties of this food group.

Moreover, the origin of the wheat used to make pasta can affect starch digestibility and the glycemic response, so, it should be account as one of the main factors affecting the rate of digestion.

In conclusion, results indicated that kamut pasta displayed a lower glycemic index compared to the common pasta produced with modern durum wheat (*Triticum durum*) and to spelt pasta, made with *Triticum dicoccum*.

The hypothesis highlighted in this study, concerning the involvement of protein-starch interactions, may explain this founding.

Thus, the synergic effect of pasta processing, which involved high drying temperature, and the protein content in kamut flour, might affect the rate of starch hydrolysis and subsequently the glycemic index.

These studies underline also that the *in vitro* method of digestion gave reproducible results, which were comparable to those obtained by performing *in vivo* experiments.

Further investigations will focus on pasta manufactured by using low and medium drying temperature conditions.

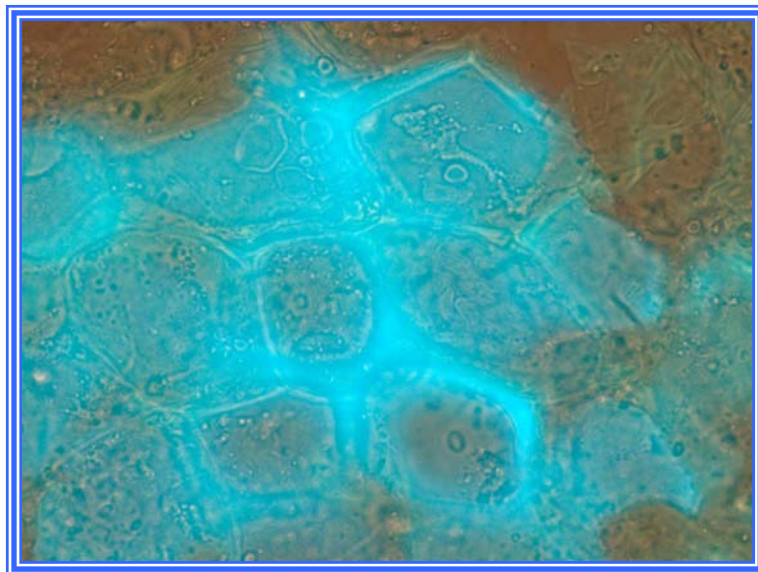
Moreover, more experiments focused on the microscopic features of pasta structure should be performed, in order to gain a better understanding and to give a more reliable explanation.

CHAPTER 6

ARABINOXYLANS IN WHEAT

(*Triticum aestivum*):

Exploring variation in arabinose substitution within cell-wall arabinoxylans for differing cultivars, and an investigation of their *in vitro* digestibility



6.1 INTRODUCTION

Cereal grains provide an important source of dietary fibre as non-starch polysaccharides (NSP). NSP's can be differentiated into cellulose, pectins, and hemicellulose, which have $\beta(1-4)$ -linked backbones of xylose, mannose or glucose.

Arabinoxylan (AX) is one of the predominant hemicelluloses in cereals (Hopkins et al. 2003), and is the primary component of the cell walls in the starchy endosperm of most cereals. In wheat, about 14-15% of the endosperm cell walls comprises protein with the remaining 75% being NSP's, of which about 70% is AX, 20% (1-3)(1-4)- β -D-glucan, 7% β -glucomannan and 2% cellulose (Bacic and Stone 1980). Cereal AX consists of backbone chains of $\beta(1-4)$ -linked D-xylopyranosyl residues to which α -L-arabinofuranose units are linked as side chains. Although most of these side chains are monomeric, a small proportion of oligomeric side chains consisting of two or more arabinosyl residues, linked via $\alpha(1-2)$, $\alpha(1-3)$, and $\alpha(1-5)$ bonds, have been reported for some AX (Hopkins et al. 2003). In addition, ferulic acid residues are also covalently linked through ester linkages to C(O)-5 of some of the arabinose residues, as 5,5', 8-O-4' and 8,5' dimers (Adams et al. 2003).

In general AX from rice consists of more highly-branched xylan backbones than AX from wheat, rye and barley which may also contain galactose and glucuronic acid moieties in addition to the pentose sugars (Hopkins et al. 2003).

NSP's, like AX, from cereal cell walls can resist digestion and absorption in the upper gastrointestinal tract and enter the colon, where they undergo fermentation by the colonic microbiota. The consequences of fermentation by the gut microbiota include increased fecal bulk through bacterial proliferation and the formation of microbial metabolites, for example, short chain fatty acids (SCFA) and gas (Hopkins et al. 2003).

Dietary fibre has been shown to improve health and give benefits to the human body. The more soluble, viscous and fermentable fibre sources are, the more they may reduce the glycemic index, the insulin sensitivity and the cholesterol absorption of a meal. Fibre also dilutes the energy density of the diet and prolongs intestinal digestive processes, which in turn aid the control of the satiety (Hughes et al. 2007). The benefits of AX even stretch to increased natural killer-cell activity by increased IL-2 and INF- γ production (Hughes et al. 2007).

The structural features of arabinoxylans vary depending on their genotypic or cellular origin. These variations include the ratio of arabinose to xylose residues (Ara/Xyl), the relative amount and sequence of mono-, di-, and unsubstituted xylopyranosyl (Xylp) residues, as well as the presence of other substituents (ferulic acid or other carbohydrate residues).

The ratio of Ara/Xyl residues indicates the degree of branching in the polymer chain, but it is only a rough indicator of the fine molecular structure which is better described by the substitution pattern of the xylan backbone (Izydorczyk et al. 2008).

In cereals the Ara/Xyl ratio may vary from 0.3 to 1.1 (Izydorczyk et al. 2008). In wheat the degree of branching depends on the cell-wall type and large variations may occur even within a single cell-wall type; in fact AX in the outer pericarp, scutelum and embryonic axis are relatively highly substituted with arabinose (Ara/Xyl>1), whereas those of the aleurone are poorly substituted (Ara/Xyl<0.5) (Barron et al. 2006).

The amount and the structure of AX polymers show large differences according to grain tissue but also between grain cultivars (cv's) (Saulnier et al. 2007). The structure of the AX present in the endosperm cell wall is genetically controlled (Ordaz-Ortiz and Saulnier 2005), with about 35% of the total being classified as water soluble/extractable (WE-AX) and the remainder water insoluble/unextractable (WU-AX) (Mares and Stone 1973). The structural features

of WE-AX are well documented but less information is available for WU-AX. However, there is no clear evidence for a relationship between water-solubility or extractibility of AX and their branching patterns with arabinose.

In the aleurone layer, which is associated with the bran fraction, AX exhibits specific features; firstly, the aleurone AX are not WE-AX and they have a lower Ara/Xyl ratio compared to the starchy endosperm AX. Moreover, aleurone AX are heavily esterified compared with those of the starchy endosperm and ferulic acid and dehydrodiferulic acids represent about 3.2% and 0.45% of the WU-AX respectively (Antoine et al. 2003). Furthermore some *p*-coumaric acid and acetyl groups, which are probably esterified to the xylose backbone of AX are also detected in the aleurone cell walls (Rhodes et al. 2002).

Recently developed sample preparation methods have allowed the application of FT-IR (Fourier-Transform Infrared) spectroscopic imaging methods to the analysis of wheat endosperm cell wall composition, allowing the spatial distribution of structural components to be determined without the limitations of conventional chemical analysis (Toole et al. 2007). The advantages of these methods, are that they determine the composition of endosperm cell walls *in situ* and with minimal modification during preparation. Studies carried out over two successive seasons on differing cv's showed that the structure of the AX in the endosperm cell walls changes from a highly-branched form to a less-branched form, starting in the outer regions of the grain. Furthermore, the rate of restructuring during development was faster when the plants were grown at a higher temperature with restricted water availability, with differences in the rate of restructuring occurring between the cv's. Further analyses using Raman and ¹H NMR spectroscopy (Toole et al. 2009), and immunochemical techniques (Guillon et al. 2004, Phillippe et al. 2006) also support this theory, suggesting that AX is either delivered to the cell wall in a highly substituted form and is remodelled through the action of arabinoxylan arabinofuranohydrolases or arabinofuranosidases, or that low-level substituted AX are incorporated into the wall late in cell wall development.

AX display viscosity building properties due to their high Mr and locally stiff semiflexible coil conformation (Izydorczyk et al. 2008). The viscosity generating properties of AX have been attributed to a slowing down in the rate of gastric emptying and a reduction in small intestine mobility, thus lowering postprandial glucose and insulin responses in humans (Lu et al. 2000).

Cereal AX are known to form covalently stabilised gels through the formation of cross-links between feruloyl units of neighboring AX chains by oxidative coupling reactions. The gelation capacity and the strength of gels depend on the concentration of the AX, their Mr and ferulic acid content (Izydorczyk et al. 2008). Assumptions have been made that AX, as a dietary fibre, does not undergo degradation during gastric and duodenal digestion, but that they are degraded by the enzymes of the bacterial flora residing in the large intestine (Glitsko et al. 1999). However, Zhang et al. (2003) found that under acidic conditions similar to those of the human stomach, up to 10% of the L-arabinose was released from the hemicellulose of corn hull, larch wood and banana peel.

This chapter is focused on the effect of the digestive process on wheat endosperm cell walls in order to identify how different cell-wall structures (highly-branched (HB-AX) versus low-branched (LB-AX) may respond to digestion. A new, high-throughput, method has been developed whereby intact endosperm cell walls may be extracted from a wheat flour dough. The extracted endosperm cell-wall 'gel' was then subjected to *in vitro* simulated gastro-duodenal digestion. FT-IR spectroscopic imaging was used to examine changes in AX structure and composition at various stages during the digestion process. Light Microscopy was also used in order to visualise these changes. These initial digestion studies have provided extremely promising results. Differences in the endosperm cell walls, due to modification by the GI tract environment (pH, biosurfactants, hydrolases) following gastric and duodenal digestion, have been identified for both the protein and the AX components of the endosperm cell walls, suggesting that they may play a far more significant role in the digestion of dietary fibre than previously thought.

6.2 FT-IR SPECTROSCOPY AND MICROSCOPY

6.2.1 PRINCIPLES OF FT-IR (Fourier-Transform Infrared)

Fourier Transform Infrared (FT-IR) Spectroscopy bases its functionality on the principle that almost all molecules absorb infrared light. Only the monatomic and homopolar diatomic molecules do not absorb infrared light. In a molecule the differences in charges in the electronic fields of its atoms produce the dipolar moment of the molecule. Molecules with a dipolar moment allow infrared photons to interact with the molecule causing excitation to higher vibrational states.

Most FT-IR spectrometers use a Michelson interferometer to spread a sample with infrared light, and measure the intensity of the spectrum which is not absorbed by the sample. FT-IR spectroscopy is a multiplexing technique, where all optical frequencies from the source are observed simultaneously over a period of time known as the scan time.

The spectrometer measures the intensity of a specially encoded infrared beam after it has passed through a sample. The resulting signal is called an interferogram and contains intensity information about all frequencies present in the infrared beam. This information can be extracted by switching this signal from a time domain digital signal to a frequency domain digital signal, which is accomplished by applying a Fourier Transform to the interferogram and producing what is called a “single beam spectrum”. Another characteristic of this signal is that it is a statistically stationary signal, so the higher the number of scans of which the signal is composed, the better the estimate that can be extracted from the data.

This technique provides a way to identify a molecular type (qualitative analysis) and the amount of this molecule in the sample (quantitative analysis). Since each type of molecule only absorbs at certain frequencies, it provides a unique absorption spectral pattern, or fingerprint, through the entire infrared light spectrum.

An absorption spectrum is calculated by performing the logarithmic ratio between a sample single beam spectrum (sample) and a background single beam spectrum (background). The background is a single beam spectrum that measures the intensity of the infrared light reaching the detector without any sample. The frequencies of infrared light radiation that are absorbed and the strength of these absorption bands are determined by the sample's chemical makeup. It is important to note that the single beam background spectrum is used as a point of reference to determine the absorption of a sample inside the sample compartment.

The use of an FT-IR spectrometer requires continuous purging with dry nitrogen. This purging provides a stable environment for the spectrometer and reduces interfering impurities inside the spectrometer.

For this work, spectroscopic images were produced using a Stingray Mercury-Cadmium-Telluride (MCT), focal plane array (FPA) detector, which allows hundreds of spectra to be collected in a grid, for areas as small as $5 \times 5 \mu\text{m}$. It allows an area of sample to be spectroscopically mapped, producing thousands of spectra in a relatively short time. Each pixel in an image represents an individual spectrum, and the intensity of the colour of each pixel represents the intensity of the spectral absorbance. The image produced is essentially a hyperspectral data cube, where each X, Y, element or pixel is associated with a spectrum. From these spectra, information about the chemical composition and spatial distribution within a sample may be obtained by using multivariate statistics.

6.2.2. MATERIALS

6.2.2.1 The extraction of arabinoxylan cell wall fragments from wheat flour

Dough was prepared by mixing wheat flour with water using a mini mixer (Fig. 6.1A) that was able to produce 5 g of dough per run. In order to obtain sufficient AX to perform *in vitro* digestion experiments, 20 small doughs were collected for each sample. Doughs (approximately 16 g) were weighed into 10 polycarbonate ultracentrifuge tubes with screw-on caps (Beckman, UK) (Fig. 6.1B) and ultracentrifuged in a fixed angle rotor at 200,000 *g* for 30 min at 25° C.

The separated dough consisted of 5 main layers (Fig. 6.1C):

1. a creamy lipid pellicole, at the top;
2. a straw-coloured viscous material called “dough liquor” (Salt et al. 2006), which contained the soluble proteins and carbohydrates (including WE-AX);
3. a thin opaque ‘gel-like’ layer, which was mainly composed of AX cell-wall fragments;
4. a viscoelastic layer containing the gluten protein network;
5. a deposit of starch at the bottom of the tube.

After carefully removing the lipid pellicole and the dough liquor, the AX ‘gel-layer’ was scraped from each tube and collected (Fig. 6.1D).

The AX was extracted in this way for flour from the following wheat cv’s:

Hereward, Soisson, Yumai-34, Mannital, San Pastore and Claire (provided by the EC FP6 project HEALTHGRAIN (Food-CT-2005-514008)).

Plus a series of cv’s grown under both cool/wet and hot/dry conditions: Rialto ‘03, Spark ‘03, Spark ‘04, SR5 ‘04, SR7 ‘04, Hereward ‘05 (provided by the BBSRC funded Ex-Gen project (218/EGA17713)).

The AX gels were then analysed using FT-IR ATR and the resulting spectra compared using Principal Components Analysis (PCA).

Cv Hereward was then used to prepare AX samples for an *in vitro* Biochemical digestion.

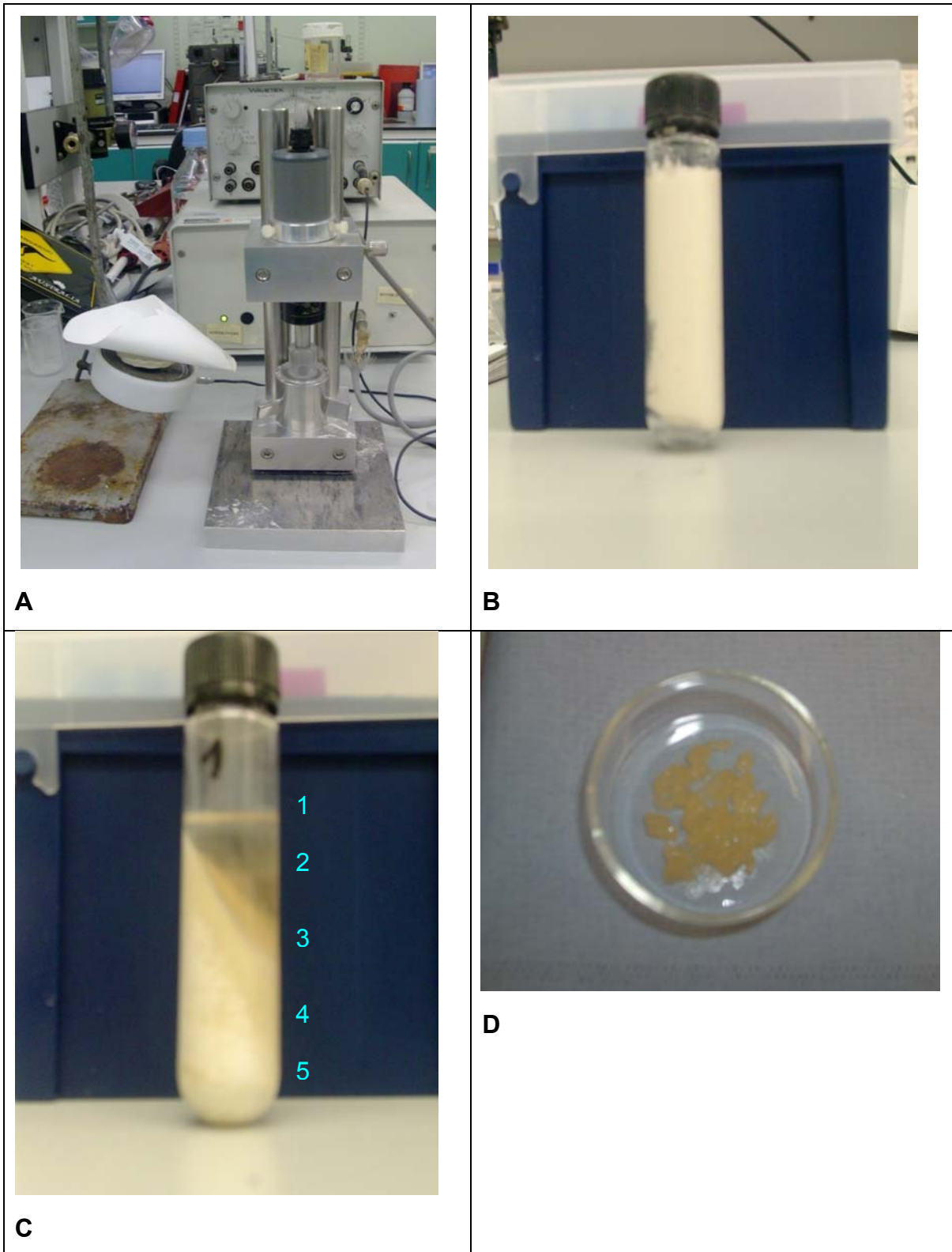


Figure 6.1: preparation of the AX 'gel-layer' from wheat flour dough. A: mini mixer; B: dough in a polycarbonate bottle before centrifugation; C: dough after centrifugation; D: the AX 'gel-layer'.

6.2.3a METHODS: FT-IR SPECTROSCOPY

NB: This part of the laboratory work was carried out by Dr Geraldine A Toole, using the digested samples produced by Elisa Selvatico.

6.2.3.1 FT-IR ATR (Attenuated Total Reflectance)

FT-IR ATR spectra were measured, between 700-4000 cm^{-1} , using a Digilab FTS 6000 spectrometer (Digilab, Cambridge, MA, US), by placing a small spatula of each digesta sample onto the diamond crystal ($1.5 \times 1.5 \text{ mm}^2$) of a Golden Gate single reflection ATR accessory (Specac, UK). The spectral resolution was 2 cm^{-1} , and 128 scans were co-added for each spectrum. The empty crystal was used as a background and the spectrum for water was measured and subtracted from each sample spectrum. PCA was then applied to all spectra measured.

6.2.3.2 FT-IR SPECTROSCOPIC IMAGING

A spatula of each digesta was dispersed in approximately twice as much water, then a drop of this mixture was placed onto a barium fluoride (BaF_2) disc (13 mm diameter \times 1 mm thick) (Crystran Ltd., Dorset, UK) and allowed to dry at room temperature. Eight selected regions of the endosperm cell-wall digesta on each BaF_2 disc were imaged using the Digilab Stingray Imaging spectrometer (Digilab, Cambridge, MA, US). This consists of a FTS-6000 step-scanning FT-IR spectrometer coupled to a UMA 600 microscope, equipped with a camera and a Stingray focal plane array (FPA) detector. The detector is a liquid-nitrogen-cooled 128×128 MCT (Mercury-Cadmium-Telluride) FPA. Using a 15 \times objective and full microscope aperture, the spatial area imaged in a single spectral collection is $640 \times 640 \mu\text{m}$: therefore, each spectral pixel corresponds to an area of $5 \times 5 \mu\text{m}$. A total of 16384 interferograms (equal to the number of pixels in each image) were collected before Fourier transformation, at a spectral resolution of 8 cm^{-1} , between 800 cm^{-1} and 4000 cm^{-1} wavenumbers. All data were collected using Win-IR software version 4.0 (Digilab).

The FPA detector was calibrated, prior to data acquisition, to compensate for non-uniform illumination of the sample. For each sample, a background single-beam spectrum was collected for a clean region of the barium fluoride disc. A visible image was then captured by the camera for each selected region (not shown), followed by 128 scans per image pixel (co-added to increase the signal-to-noise ratio). The sample single-beam spectra for each image pixel were divided by the corresponding pixel in the background single-beam image, with a fixed zero-filling factor (equal to 2), to enable all images to be compared statistically. All spectra were finally truncated to 1800-900 cm^{-1} . Eight spectral images were collected for each sample/ BaF_2 disc.

All data analysis was performed using the image analysis software ENVI 4.0 (Research Systems Inc, Boulder, CO, US). In order to avoid the influence of variation in sample thickness, and therefore spectral intensity, all spectra were baseline corrected and normalised. This was done by converting all spectra to transmittance units, correcting the baseline to 1 (using the continuum-removal function), returning the spectra to absorbance units, and finally conducting an area normalisation to the maximum peak at 1041 cm^{-1} (dividing by the height of the band then multiplying by a factor).

Each spectral image consisted of 16384 spectra, which were averaged to provide 1 spectrum per image, therefore providing 8 spectra for each digesta sample. This enabled the heterogeneity within each sample to be determined. PCA was applied to all spectra for each stage of digestion (**A**, **B** and **C**) and for each digestion criteria (**1-5**).

6.2.3b METHODS: MICROSCOPY

The cell-wall material extracted from dough and from digested products was examined by light microscopy to provide information about its physical structure.

A drop of cell-wall sample in water was placed on a glass microscope slide and covered with a glass cover slip. The cell walls were examined under bright field lighting using an Olympus BX60 (Olympus, Japan). Images were recorded using ProgRes[®] Capture Pro 2.1 software (Jenoptik, Germany).

To visualise the ferulic component of the cell walls before and after digestion, a drop of 1% NaOH was added to the sample before the cover slip was applied. Under these alkaline conditions, cell walls with ferulic links fluoresce green when examined using the UV filter cube (U-MWU, exciter filter BP330-385, barrier filter BA420) of the microscope

6.3 IN VITRO BIOCHEMICAL MODEL OF DIGESTION

6.3.2 MATERIALS: CHEMICALS AND REAGENTS

The chemicals, enzymes and solutions used to perform the *in vitro* gastric-duodenal digestion, were described in chapter 2, section 2.3.2.

The Protease Fluorescent Detection Kit, used to test the protease activity of the commercial enzymes used during the digestion procedure, was supplied by Sigma (Poole, Dorset, UK).

6.3.3 METHODS

6.3.3.1 Protease assays

Protease assays were performed before carrying out the *in vitro* digestion experiments, in order to analyse the enzymes for any proteolytic activity (for example, contamination with proteases or lipases).

The Protease Fluorescent Detection Kit provided a sensitive method for detecting the presence of protease activity, by using casein labelled with fluorescein isothiocyanate (FITC) as the substrate.

Protease activity results in the cleavage of the FITC-labelled casein substrate into smaller fragments, which do not precipitate under acid conditions. After incubation of the protease sample and the substrate, the reaction was acidified with the addition of trichloroacetic acid. The mixture was then centrifuged with the undigested substrate forming a pellet and the acid soluble fragments remaining in solution. The supernatant was neutralised and the fluorescence of the FITC-labelled substrate was measured using a fluorimeter (excitation wavelength 485 nm, emission wavelength 535 nm) (LS 55 Luminescence Spectrometer, Perkin Elmer, UK).

6.3.3.2 Digestion procedure

In vitro digestions were performed following the method described in chapter 2, section 2.2.3, with the following modifications.

In order to assess if individual digestive conditions, enzymes or surfactants have any effect on the composition or structure of the cell-wall AX, the experiments were carried out by differentiating the various steps of the digestion process as described below.

250 mg of AX-gel layer was weighed into three bottles:

- A.** 2 hours of incubation/digestion (corresponding to 2 h of gastric digestion);
- B.** 5 hours (2 h plus 3 h) of incubation/digestion (corresponding to 2 h of gastric digestion followed by 3 h of duodenal digestion);
- C.** 21 hours (2 h plus 3 h plus 16 h) of incubation/digestion (corresponding to 2 h of gastric digestion followed by 19 h of duodenal digestion).

Four individual digestions were carried out, followed by a combined 'full' digestion:

1). A simple acid incubation was performed at pH 2.5 using Simulated Gastric Fluid (SGF) for stage **A**, followed by an increase in the pH to 6.5 to simulate the intestinal conditions for stages **B** and **C**.

2). The effect of amylolytic enzymes was assessed by using salivary amylase for 5 min at pH 6.8 and subsequently incubated at pH 2.5 for stage **A**, followed by an increase in the pH to 6.5 to simulate the intestinal conditions with pancreatic amylase only for stages **B** and **C**. The enzyme concentrations used have already been reported in chapter 2, section 2.3.3.

3). On the basis of the *in vitro* digestion method built for the flour experiments (chapter 2), pepsin, trypsin and chymotrypsin were assessed, using a value of 10% protein in the cell walls, to calculate the amounts of proteases to add to the

system. This was carried out again at pH 2.5 for stage **A**, followed by an increase in the pH to 6.5 to simulate the intestinal conditions for stages **B** and **C**.

4). The effect of lecithin and bile salts (for the concentrations added, see chapter 2, section 2.3.3) was analysed by carrying out the incubation again at pH 2.5 for stage **A**, followed by an increase in the pH to 6.5 to simulate the intestinal conditions for stages **B** and **C**.

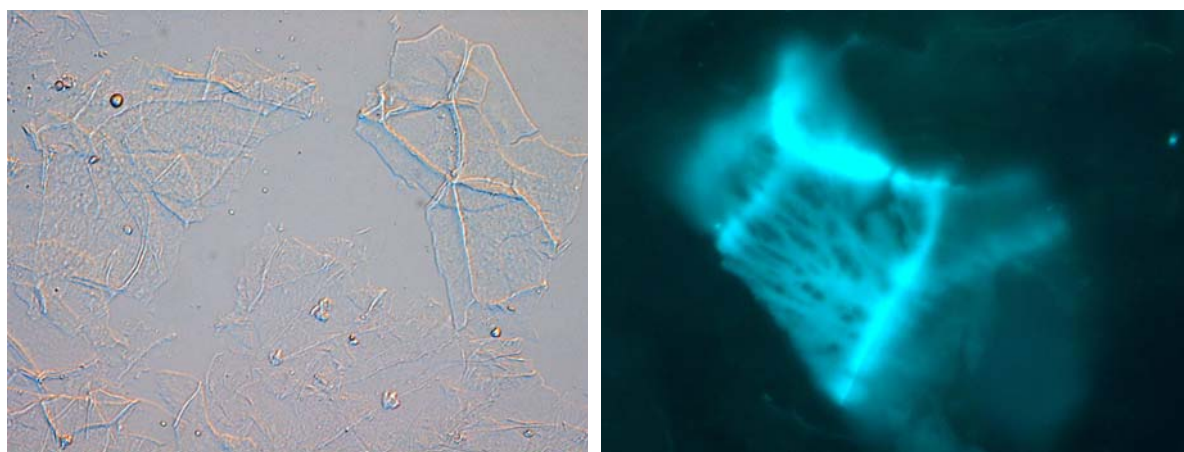
5). Finally, the AX-gel layer was subjected to a 'full' biochemical *in vitro* digestion performed with the addition of; salivary amylase (oral step), pepsin and lecithin (gastric phase), trypsin, chymotrypsin, hepatic mix and pancreatic amylase to simulate the duodenal environment.

At each stage (**A**, **B** and **C**) of the digestion process, the reactions were terminated as described in section 2.3.3, and subsequently centrifuged at 3000 g for 5 min in order to separate the supernatant from the solid pellet, containing respectively the soluble and the insoluble digesta. Fractions were analysed using microscopy and FT-IR spectroscopic imaging.

6.4 RESULTS AND DISCUSSION

6.4.1 THE EXTRACTION OF ARABINOXYLAN CELL-WALL FRAGMENTS FROM WHEAT FLOUR

The new, high-throughput, method that was developed in order to extract intact endosperm cell walls from wheat flour dough was extremely successful. The different components of the flour dough (lipids, dough liquor, AX cell walls, gluten proteins and starch) were separated into clear layers during ultra-centrifugation. The AX 'gel-layer' (Fig. 6.1C, layer 3) consisted almost entirely of endosperm cell-wall fragments, which could be clearly seen using light microscopy (Fig. 6.2a). By using a UV filter (Fig. 6.2b), increased levels of phenolic compounds can be identified at the junctions between adjoining cells. A 'mesh-like' structure can also be seen across the rest of the cell wall, indicating a structural component to the wall that is brought about by the ferulic acid residues that are covalently linked to some of the arabinose residues.



a).

b).

Fig. 6.2 a). Light microscope, and b). UV image, for the extracted 'gel-layer' (layer 3 in figure 6.1C) clearly showing the endosperm cell-wall fragments.

FT-IR ATR spectra taken for the 'gel-layer' (Fig. 6.3) showed spectral features identical to those for AX cell walls (Toole et al. 2007), confirming that layer 3 consists almost entirely of intact endosperm cell-wall fragments. The relative

height of the peak at 1075 cm^{-1} is an indication of the levels of arabinose substitution (the higher the peak, the higher the level of substitution).

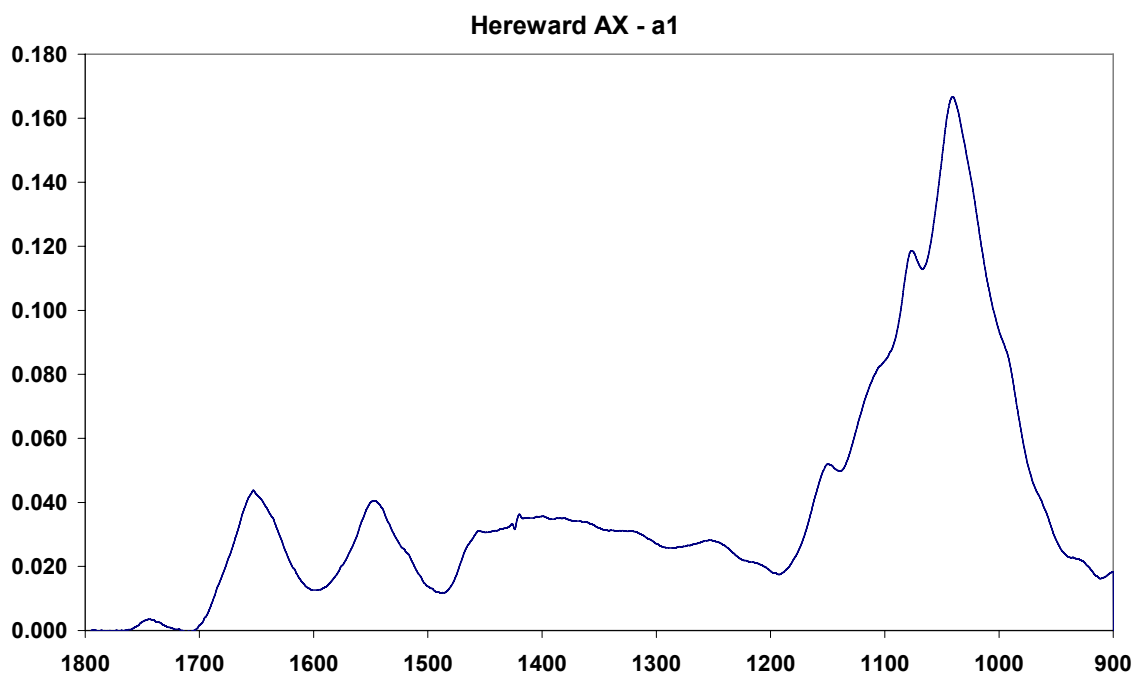


Fig. 6.3. Typical spectrum obtained for the AX 'gel-layer' using the FT-IR ATR method.

6.4.2 DIFFERENCES IN AX COMPOSITION BETWEEN CV'S AND DIFFERING GROWING CONDITIONS

A PCA plot showing the differences in the spectra for the AX 'gel-layer' extracted from wheat varieties grown under cool/wet and hot/dry conditions are shown in Figure 6.4 (appendix 6). The differences identified are comparable to those obtained by spectroscopic imaging of the intact endosperm cell-wall network (Toole et al. 2007), Raman spectroscopic analysis and ^1H NMR analysis (Toole et al. 2009), whereby the effect of hot/dry growing conditions was to lower the level of arabinose substitution in the endosperm cell-wall AX.

Figure 6.5 (appendix 6) shows the PCA plot for the spectra obtained from the flour of six wheat cv's with differing levels of arabinose substitution in the AX in their

endosperm cell walls, as identified during previous work (Toole et al. yet to be published) (San Pastore and Manital having more HB-AX, Claire and Yumai-34 having more LB-AX, and Soissons and Hereward having a mixture of the two). Clear differences can be seen between the cv's, and total separation was evident between the cv's Soissons, Claire and Yumai-34 (which is known to have very high levels of total AX).

6.4.3 PROTEASE ACTIVITY OF COMMERCIAL ENZYMES

To assess the effect of different enzymes and conditions existing in the GI tract on endosperm cell-wall material, it was decided that the chemicals involved in the *in vitro* digestion process would be assessed individually by performing each digestion process separately.

Thus, the cell-wall material extracted from flour dough was subjected to *in vitro* digestion using 5 different procedures: 1. altering only the pH conditions to assess the effect of hydrolysis alone, 2. Altered pH with the addition of amylases, 3. Altered pH with the addition of proteases, 4. altered pH with the addition of surfactants, and 5. A 'full' digestion combining an altered pH with the addition of amylases, proteases and surfactants.

For this reason, it was necessary to evaluate the purity of the commercial enzymes used.

It was found that commercial pancreatic amylase extracted from porcine pancreas (Sigma) was not totally pure and was contaminated with unknown proteases. By performing the protease assay (figure 6.13) it was found that the pancreatic amylase commonly included in *in vitro* digestion models, also contained a proteolytic activity.

Figure 6.13 shows that the salivary amylase has no proteolytic activity since the fluorescent units which have been detected, were equal to the blank sample performed with water. A trypsin sample was included as a positive control.

Afterwards, realising that the porcine pancreatic amylase could not be used in experimental models of digestion, a treated pancreatic amylase was tested. This second, commercially available, amylase (Sigma) was treated with the chemical inhibitors (diisopropylfluorophosphate, DFP) which inhibit proteases thus improving the purity of the amylase enzyme. The pancreatic amylase DFP treated was tested before being used in digestion systems, to evaluate its purity. As shown in figure 6.14 its protease activity was not detected in the novel amylase. It was therefore used to perform *in vitro* digestions.

6.4.4 THE EFFECT OF DIGESTION ON CELL WALL COMPONENTS

Up till now several studies have been carried out to gain a better understanding of dietary fibre degradation by microbiota in the intestinal environment (Hopkins et al. 2003, Glitso et al. 1999), although few authors focused their attention on the effect of the GI conditions on cell wall components (Zhang et al., 2003).

This chapter aimed to clarify whether arabinoxylans or proteins, which are the main constituents of the cell walls, might be subjected to any changes during *in vitro* simulated gastric and duodenal digestion.

Figure 6.7 shows the PCA plots for both the AX and the protein regions of the spectra separately, for the spectra obtained for the digesta at each stage of the digestion process, for each of the separate digestion procedures. Figure 6.8 shows those for the full digestion, and Fig. 6.9 shows a plot combining all of them.

Part of this work focused on the effect of the acid stomach conditions (pH around 2-2.5), by incubating the “gel layer” extracted from dough, with SGF (Simulated Gastric Fluid) and subsequently with SIF (Simulated Intestinal Fluid) at pH 6.5.

Focusing on figures 6.7(1A) and 6.7(1B) it was clearly noticeable that the untreated sample (black spots) differed from those that had undergone the acid incubation (red spots) and from those which correspond to samples processed using intestinal fluid (blue and green spots).

Proteins of the cell wall were detected, as well as the AX, using FT-IR spectroscopy and, looking to the PCA analysis of the spectra collected, they seemed to behave differently when subjected to acidic conditions. It was noticeable that there was a differing effect of the SGF on protein (figure 6.7(1B) red spots), compared to samples treated with SGF but subsequently incubated with SIF at pH 6.5 (red and blue spots). Thus, changing pH conditions, by neutralising the acid environment generated to simulate the stomach, might affect protein solubility, which had differing FT-IR spectra. However, it seemed that the exposure time at pH 6.5 did not affect protein solubility since, blue spots (corresponding to 3 h incubation) and green spots (corresponding to 19 h incubation) were not separated using PCA analysis (figure 6.7). Indeed, low pH conditions might cause denaturation of proteins, thus altering the FT-IR spectra.

Even though hemicelluloses, like AX, were not digested during the gastro intestinal transit, studies involving the effect of gastric acidity on AX degradation reported different results (Zhang et al. 2003). These authors assumed that under low pH conditions, AX, which can have several arabinofuranosyl units attached to the xylose backbone, should release L-arabinonose, thus affecting their degradation. In this work, FT-IR, performed by Dr G. Toole, confirmed that changes in AX might be due to the acidic condition of the stomach, which is known to denature protein.

The release of L-arabinose, which is poorly absorbed in the intestine, causes a lowering in insulin and triacylglycerol concentration (Zhang et al. 2003), which can indirectly affect the glucose response (glycemia) Moreover, the modification of AX structure, due to the hydrolysis of side chains when exposed to stomach acidity, might affect the subsequent degradation of AX by intestinal microbiota. In fact, studies previously carried out (Glitsø et al., 1997) highlighted that the structural characteristics of AX, may influence their colonic degradation, and the degree of arabinose side chain substitution appeared to influence the enzymatic degradation of these polymers by bifidobacteria (Hopkins et al., 2003).

Figure 6.7(2A) shows the effect on AX by the inclusion of amylases in the digestion mixture. The literature reports several works (Hughes et al., 2007, Hopkins et al., 2003) in which they confirmed that the dietary fibre, as hemicellulose, AX, or glucans, is degraded by intestinal microbiota, which have the ability to produce specific enzymes to act on these polymers. In fact, studies performed in human volunteers (Hughes et al., 2007) described that *bifidobacterium* were able to produce arabinofuranohydrolase, an enzyme which acts on AX degradation by removing side chains from the doubly substituted xylose subunits. Moreover other microbiota belonging to the *Bacteroides*, seemed to produce endoxylanases which act on AX by releasing xylooligosaccharides (Hopkins et al., 2003). It is well known that amylases themselves do not act on fibre digestion but they hydrolyse starch, especially during the intestinal phase.

Focusing the attention on figure 6.7(2A), it was evident that the incubation with amylases for 19 h induced changes in the AX region, but no significant differences were assumed to exist comparing the pepsin digestion and the 3 h duodenal digestion. This was not expected since amylases did not contain xylanase activity, therefore, it may be that the 2 h incubation of AX at low pH followed by a long incubation with amylases, has a certain effect on the arabinose substitution of AX. Figure 6.7(2B) showed some differences in the protein region of the spectra, indicating small changes during the digestion process.

As already mentioned, plant cell walls are mainly composed of polysaccharides but they also contain a small amount of protein, around 10% (Jamet et al., 2008). Models of cell-wall structure describe the arrangements of their components into dense networks of polysaccharides and protein. Three types of cell wall proteins (CWPs) can be distinguished on the basis of their interactions with cell wall components: 1. loosely bound CWPs have few or no interactions with cell wall polysaccharides and thus move freely in the intercellular space; 2. CWPs weakly bound to the matrix, by Van der Waals interactions, hydrogen bonds, and hydrophobic and ionic interactions; 3. CWPs which are strongly bound to cell wall components.

Looking at figure 6.7(3A) and (3B) it appeared that the use of proteases in the digestion mixture affected both the AX and protein regions of the spectra. In particular, focusing on proteins (figure 6.7(3B)) it seemed that especially after 16 h of intestinal digestion with trypsin and chymotrypsin, the protein spectra changed, while the 2 h pepsinolysis (red spots) and the 3 h intestinal (blue spots) gave smaller differences for both the protein region and the AX region. It might be that these proteins are strictly bound to cell wall components, and that they need several steps prior to being released.

Then, attention was brought towards the effect of bile salts on AX and cell wall protein behaviour. As described, the gastrointestinal environment contains a complex of many different secretions and bile salts are the major biosurfactants in the small intestine.

It is reported that certain dietary fibre, as mainly plant cell walls, have the ability to bind hydrophobic compounds which are commonly found in the western diet and which can be mutagenic (Ryden et al., 1996). This binding is one of the mechanisms by which fibre has been proposed to protect against colorectal cancer (Harris et al., 1993). Findings, discovered that bile salts and mucins, present in the human gastrointestinal tract, help to maintain some environmental mutagens in solution, and reduced its absorption to low concentrations (Ferguson et al., 1990).

This work also focused on the effect of bile salts. Figure 6.7(4A), (4B) showed that biosurfactants involved in human digestion may also act on AX and protein in the cell walls. In fact both of the plots described a good separation between sample not treated (black spots) and those which were subjected to gastric and intestinal digestion. We could not differentiate whether the lecithin presented in the gastric phase (red spots) or the lecithin plus bile salts introduced in the intestinal simulation (spot blue and green) gave a different effect. However, the hypothesis

that biosurfactants break down the cell walls and remove the intrinsic proteins thus selectively solubilising certain AX, may explain the differences found.

Finally the gel layer obtained from dough was subjected to a full digestion which included salivary amylase, proteases of the stomach (pepsin) and intestinal compartment (trypsin and chymotrypsin), amylases which act during the duodenal phase, lecithin and bile salts released during the passage of the bolus along the gastrointestinal tract. Figure 6.8 provides PCA plots of the AX and protein region, following a full digestion. It was possible to confirm that all of the components included in the biochemical model of digestion may affect AX, by realising L-arabinose or due to changes in solubility of certain types of AX (figure 6.8(1A)). In fact the black spots (representing untreated samples) were well separated from all the others (treated).

As expected, cell wall proteins (CWP) seemed to behave differently, depending on the type of treatment performed. Indeed, looking at figure 6.8(1B) it was clear that there is a major effect of intestinal digestion (green and blue spots) rather than gastric digestion (red spots). It could be that the intestinal proteases (trypsin and chymotrypsin), included in the digestion mixture, predominantly acted on CWPs rather than the gastric pepsin. Moreover, samples undergoing the 3 h intestinal digestion, seemed to behave differently compared to the 19 h digestion. Thus, digestion time also affected the release of cell wall proteins and, due to the long time CWPs need to be released, we could suppose that these CWPs were strongly bound to cell wall components.

Finally, looking at figure 6.9A the PCA analysis includes all of the parameters investigated. The plot corresponding to the AX region spectra showed, as mentioned above, that digestion performed with the whole enzymes and chemicals (black spots) affected the AX spectral region, and it might be that one of the principal components of digestion, playing a key role, are the biosurfactants (blue spots, which are well separated in the plot between untreated and treated). The plot in figure 6.9B, corresponding to the protein region, reflected what we

previously described: the gastrointestinal digestion affected cell-wall proteins, and the main constituents, which act on their degradation appeared to be the proteases and the bile salts.

Samples digested using the full digestion procedure were analysed microscopically (figure 6.10, 6.11, 6.12). The images confirmed that the main structure, of the AX, was still intact after performing the *in vitro* gastric and duodenal digestion, therefore FT-IR analysis allowed the investigation on the level of arabinose substitution, by measuring the relative height of the peak at 1075 cm^{-1} to give an indication of any changes occurring during the digestions, which seemed to be significant by comparing the PCA plots.

In conclusion, even though the dietary fibre was not greatly modified during the passage through the gastrointestinal tract, these findings reported that the digestion process can affect AX, cell wall proteins, and might play a significant role on the further colonic degradation performed by microbiota.

CONCLUSIONS

During the last few years, hypersensitivity reactions to wheat proteins have increased but little information is available about allergens that cause wheat food allergy and how they may be modified by cooking. Studies have found that water soluble cereal albumins can trigger respiratory diseases such as Baker's asthma (James et al. 1997), although the less soluble gliadin and glutenin fractions are also considered responsible for food allergy and wheat dependant exercise induced anaphylaxis (WDEIA). It is thought that the digestion process can play an important role in assessing the allergenic potential of proteins (Thomas et al. 2007). Del Castillo et al. (2004) evaluated the allergenicity, using IgE immunoblotting, of wheat proteins following pepsin digestion, and discovered a water soluble allergen around 35 kDa that was involved in wheat allergy reactions after ingestion. Simonato et al. (2001) studied the effect of both *in vitro* digestion and baking conditions on wheat allergens. It was shown that the thermal treatment of products was an important factor affecting their allergenic activity in individuals suffering from wheat food allergy, as they found that thermally induced protein aggregation prevents complete proteolytic degradation of allergens.

Recently, proteomic strategies have been used to identify allergens, exploiting the high resolution of proteins mixture achieved using 2-DE. Sotkovsky et al. (2008) studied salt-soluble wheat allergens treated with the gastric protease pepsin, in order to characterise proteins resistant to gastric proteolysis. They identified IgE binding proteins, resistant to pepsin hydrolysis, corresponding to the 0.19 dimeric α -amylase inhibitor, peroxidase 1 and the glyceraldehyde-3-phosphate dehydrogenase.

The aim of the first part of this PhD thesis was to study an *in vitro* method of digestion including different enzymes and surfactants. Simulated gastric digestion was performed initially and, subsequently, experiments involved duodenal *in vitro* digestion.

Moreover, this work was focused to gain a more detailed understanding of the behaviour of wheat flour proteins during *in vitro* gastric digestion before and after

cooking, using 2-DE protein maps and image analysis to follow the rate of protein degradation.

2D-E has been a useful tool to map different protein profiles and proteomic strategies allowed the identification of proteins that are resistant to digestion, in both raw and cooked flour samples.

Results confirmed that the heat treatment induces several changes on protein susceptibility to digestion. Heat treatment of flour may affect protein digestibility due to aggregation, peptide formation and amino acid destruction (Hansen et al. 1976). The authors (Hansen et al. 1976) found that protein changes, due to processing, might alter the substrate for digestive enzymes. Peptide bonds of heat aggregated proteins were shown to be less accessible to pepsin attack than those of unheated proteins and it might be possible that protein-starch interactions were involved (Hansen et al. 1976).

The MALDI-ToF mass spectrometry allowed the identification of more than 40 protein spots belonging to both raw and cooked flour sample digested.

Thus, proteins resistant to *in vitro* gastric digestion have been characterised, and some of them might be potentially allergenic.

Proteins of cooked flour that seemed to be resistant to 30 min of gastric digestion were: Serpins, β -amylases, α -amylases inhibithors, puroindoline-a, triticin, tritin, peroxidase 1, and proteins belonging to the prolamin family.

Besides, this study showed that is significantly important to perform *in vitro* digestion using the meal as it is eaten, since it has been found that proteins as starch synthase, resistant to digestion in the raw flour, did not appear in the cooked sample, because of the heating process which might affect proteins structure and subsequently their degradation during digestion.

Moreover, the food matrix can also affect protein degradation during gastric and duodenal conditions and, consequently, the allergenic potential.

Thus, in order to simulate the physical conditions of the stomach, such as the meal viscosity, the particle size reduction, the unstirred layer of the meal, the creation of colloid phases, the share rate and the dilution of the meal, cooked flour has been

digested by using a dynamic model of digestion of the “Model Gut Platform” placed at the Institute of Food Research in Norwich.

During this step, experiments involved also an oral digestion study, which was performed by using two different salivary amylase concentrations.

Indeed, it is known that the salivary amylase activity in the mouth is extremely variable and several factors affect saliva secretion.

Therefore, recent finding (Neyraud et al. 2009) showed that the salivary amylase activity (measured in stimulated subjects) was higher (average of 115U/ml) than previously thought.

In this work, salivary amylase has been shown to affect consistently the food structure of starchy food. Indeed, the initial disruption of the physical structure (during the mastication) increased the available surface to amylase and, subsequently, the breakdown of some glycosidic bonds by the salivary amylase led to a loss of viscosity of gelatinized starch that rendered proteins more available to digestive enzymes.

The second part of the project was focused on starch digestibility of different varieties of pasta (spaghetti) which were made by using the same manufacturing procedures.

This work aimed to assess the kinetics of starch digestion and the glycemic index with an *in vitro* model and *in vivo* glucose response experiments.

Nowadays, the introduction of new foods will expand the range of choices, providing foods which may be selected also for their glycemic index.

The intake of carbohydrates with high glycemic index increases blood glucose, insulin, and triglyceride concentration (Wolever et al. 1991).

This is why foods with a low glycemic index are becoming more requested and the research in this field should support this kind of study, in order to gain a better understanding of the interactions between food components, and to produce novel functional foods.

For instance, recent studies (Marangoni et al. 2008) investigated the effect of fibre enriched food on *in vivo* glycemic response. These finding reported that the

addition of a mix of fibre composed of inulin, guar gum, glucomannan and wheat fiber, to the flour used to prepare biscuits, reduced significantly their glycemic index.

Others scientists (Guerra-Matias et al. 2005), tested grains of different botanical origin, like amaranth, in order to improve the glycemic index of amaranth-based foods.

Studies, focused on glycemic index assessment, were performed in many starchy foods, such as bread, pasta, biscuits, cakes but also beans (Osorio-Diaz et al. 2002) or potato (Tahvonen et al. 2006).

Results reported in this thesis highlighted that, even if pasta is a food which has a low glycemic index, it is still possible to improve it, seeking for varieties that may own these characteristics. Focusing on Kamut wheat, it has been showed that the protein content might play an important role on decreasing the rate of starch digestion in products with a high packed structure, like pasta.

In conclusion, further studies, focused on starch digestion and glycemic index, should involve pasta products made with high protein content flour and processed by using different drying temperatures.

Moreover, in order to investigate protein degradation in simulated gastrointestinal conditions, *in vitro* digestion studies, involving cereal staple foods, should be performed using the dynamic model of digestion, and the allergenic potential of the resistant proteins and peptides, should be assessed using different procedures like immunoblotting, ELISA and flow cytometry.

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Finally, thanks God for all the happy moments I could enjoy during my 3 years PhD.

APPENDIX CHAPTER 2

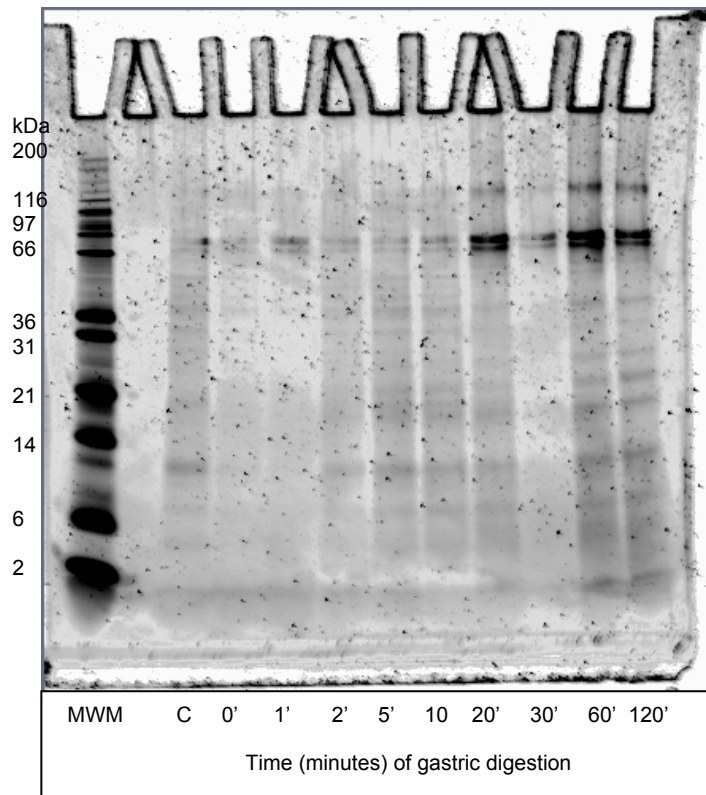


Figure 2.1: 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK). Total protein fraction of cooked flour gastric digestion, performed with sampling. MWM: molecular weight marker: C: control sample (undigested), 0, 1, 2, 5, 10, 20, 30, 60, 120: minutes of gastric digestion.

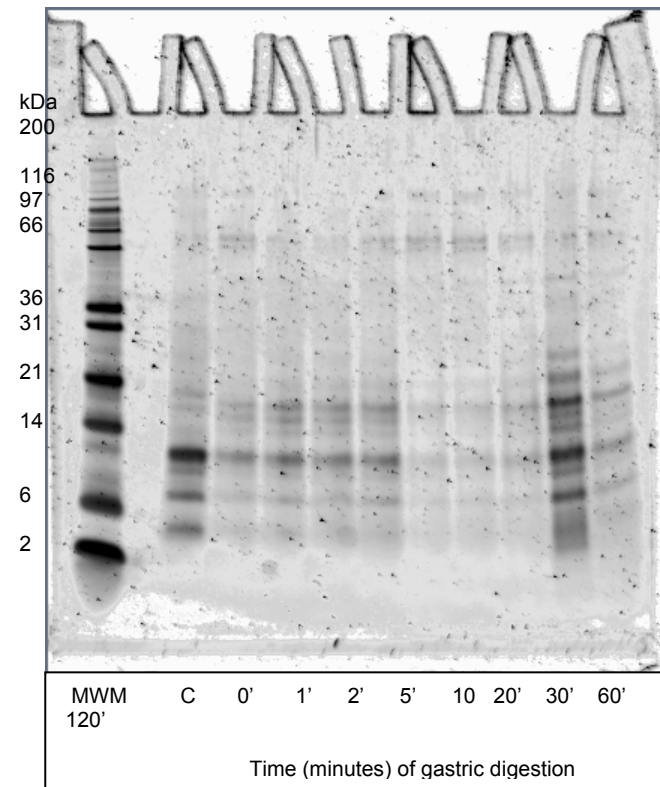


Figure 2.2: 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK). Soluble protein fraction cooked flour gastric digestion performed with sampling. MWM: molecular weight marker: C: control sample (undigested), 0, 1, 2, 5, 10, 20, 30, 60, 120: minutes of gastric digestion.

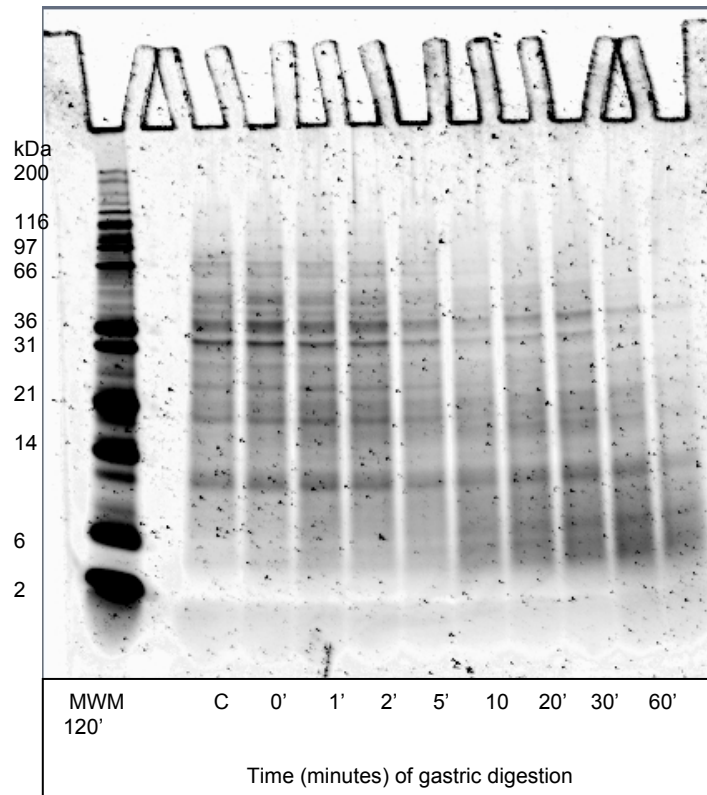


Figure 2.3: 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK). Pellet protein fraction of cooked flour gastric digestion performed with sampling. MWM: molecular weight markers: C: control sample (undigested), 0, 1, 2, 5, 10, 20, 30, 60, 120: minutes of gastric digestion.

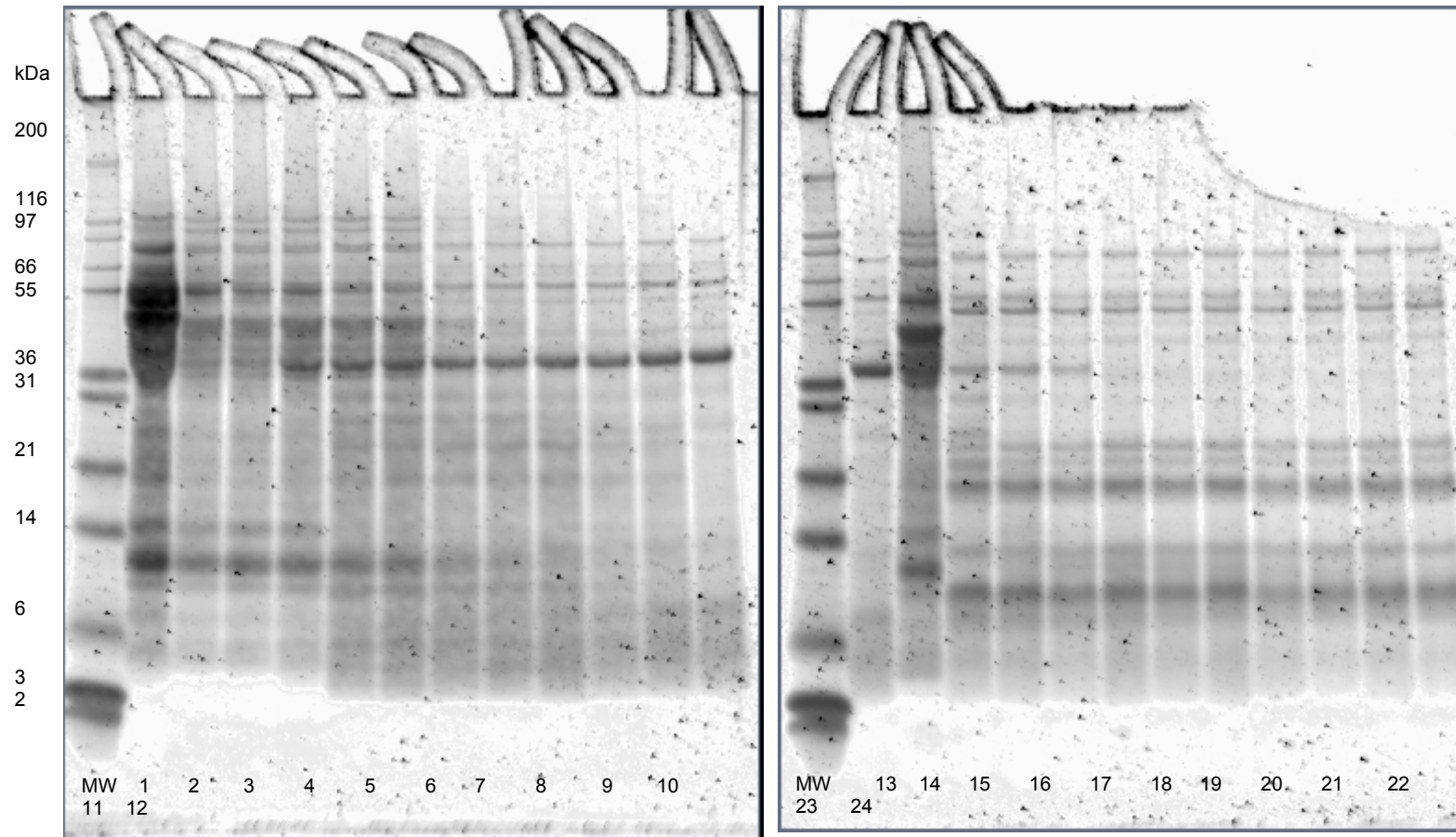


Figure 2.4 : 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK)
Total protein patterns of raw flour digestion performed using individual bijoux bottles: MW: molecular weight markers: 1: control sample (undigested), 2: chew material, 3: gastric control, 4-12: time points of gastric digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min. of pepsin hydrolysis). 13: 120 min. gastric digesta, 14: control flour undigested, 15-24: time points of duodenal digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min). The 36 kDa band presents in wells 4-12 corresponds to pepsin.

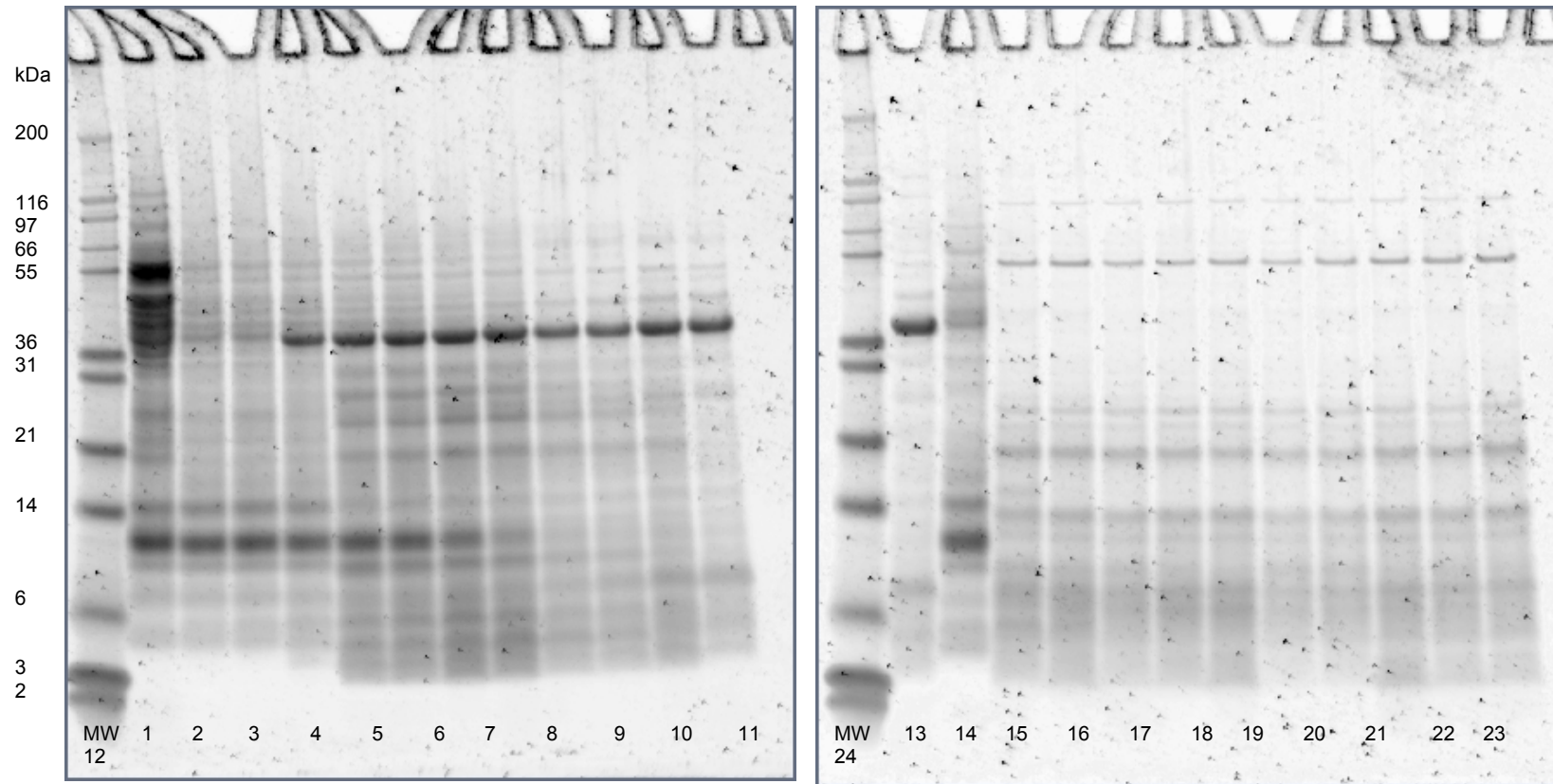


Figure 2.5: 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK). Soluble protein patterns of raw flour digestion performed using individual bijoux bottles: MW: molecular weight markers 1: control sample (undigested), 2: chew material, 3 gastric control, 4-12: time points of gastric digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min. of pepsin hydrolysis). 13: 120 min. gastric digesta, 14: control flour undigested, 15-24: time points of duodenal digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min). The 36 kDa band presents in wells 4-12 corresponds to pepsin.

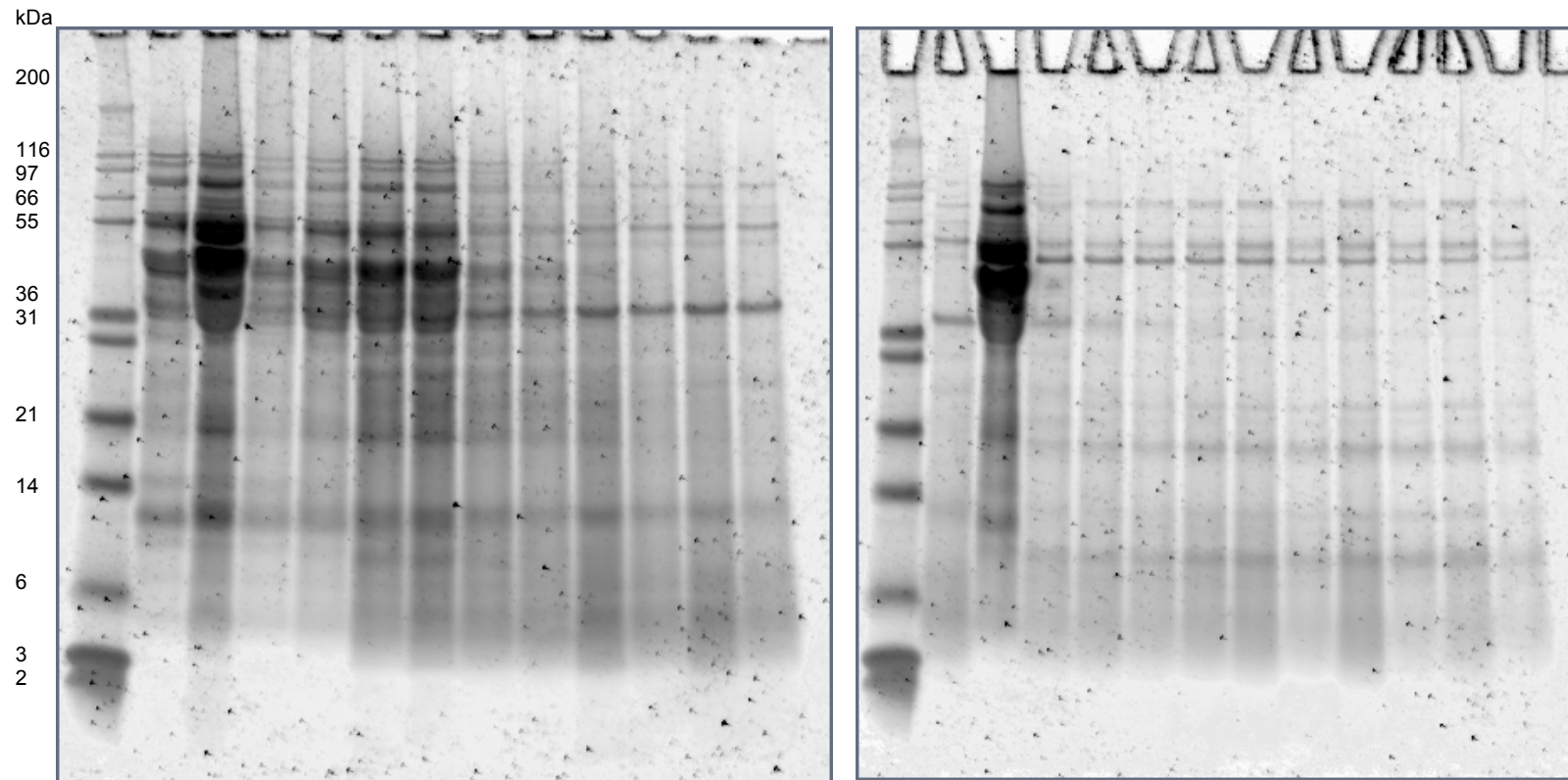


Figure 2.6 : 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK). Pellet protein patterns of raw flour digestion performed using individual bijoux bottles: MW: molecular weight markers: 1: control sample (undigested), 2: chew material 3 gastric control, 4-12: time points of gastric digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min. of pepsin hydrolysis). 13: 120 min. gastric digesta, 14: control flour undigested, 15-24: time points of duodenal digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min). The 36 kDa band presents in wells 4-12 corresponds to pepsin.

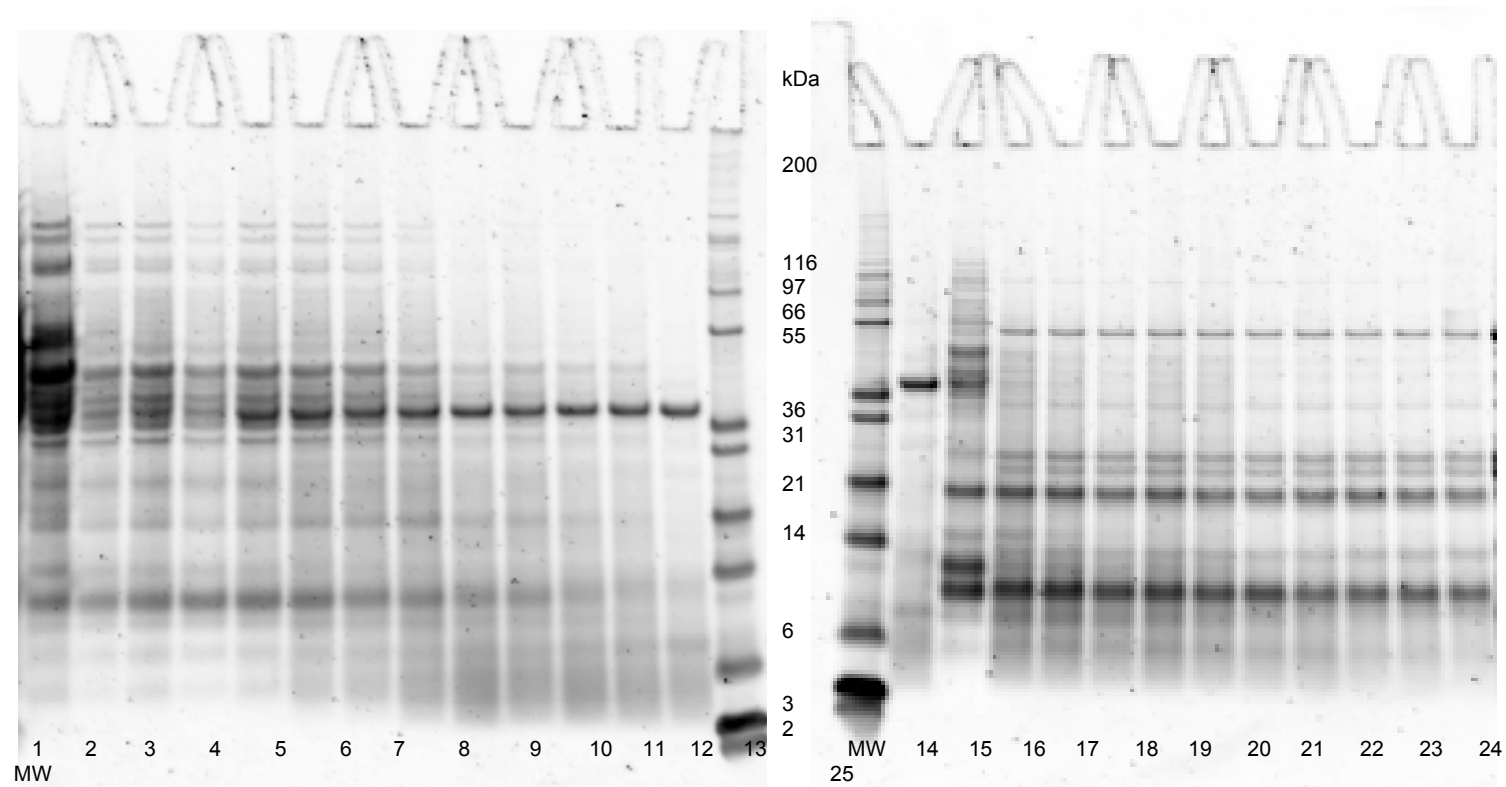


Figure 2.7 : 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, USA)
Total protein patterns of cooked flour digestion performed using individual bijoux bottles: MW: molecular weight markers: 1 raw flour undigested, 2: control sample (undigested), 3: chew material, 4: gastric control, 5-13: time points of gastric digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min. of pepsin hydrolysis). 14: 120 min. gastric digesta, 15: control flour undigested, 16 : duodenal control, 17-24: time points of duodenal digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min). The 36 kDa band presents in wells 4-12 corresponds to pepsin.

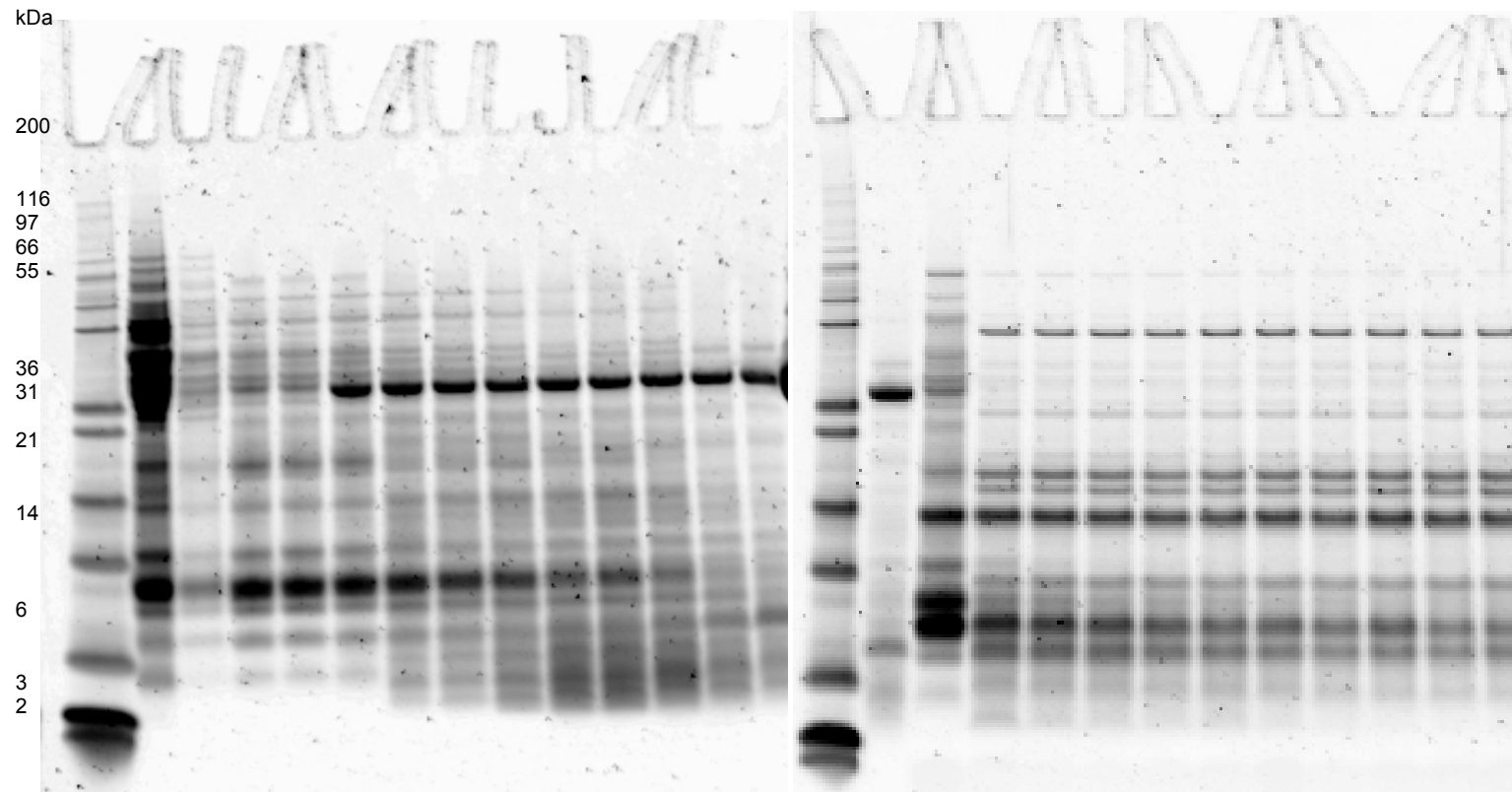


Figure 2.8: 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK) Soluble protein patterns of cooked flour digestion performed using individual bijoux bottles: MW: molecular weight markers: 1 raw flour undigested, 2: control sample (undigested), 3: chew material, 4: gastric control, 5-13: time points of gastric digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min. of pepsin hydrolysis). 14: 120 min. gastric digesta, 15: control flour undigested, 16: duodenal control, 17-24: time points of duodenal digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min). The 36 kDa band, presents in wells 4-12, corresponds to pepsin.

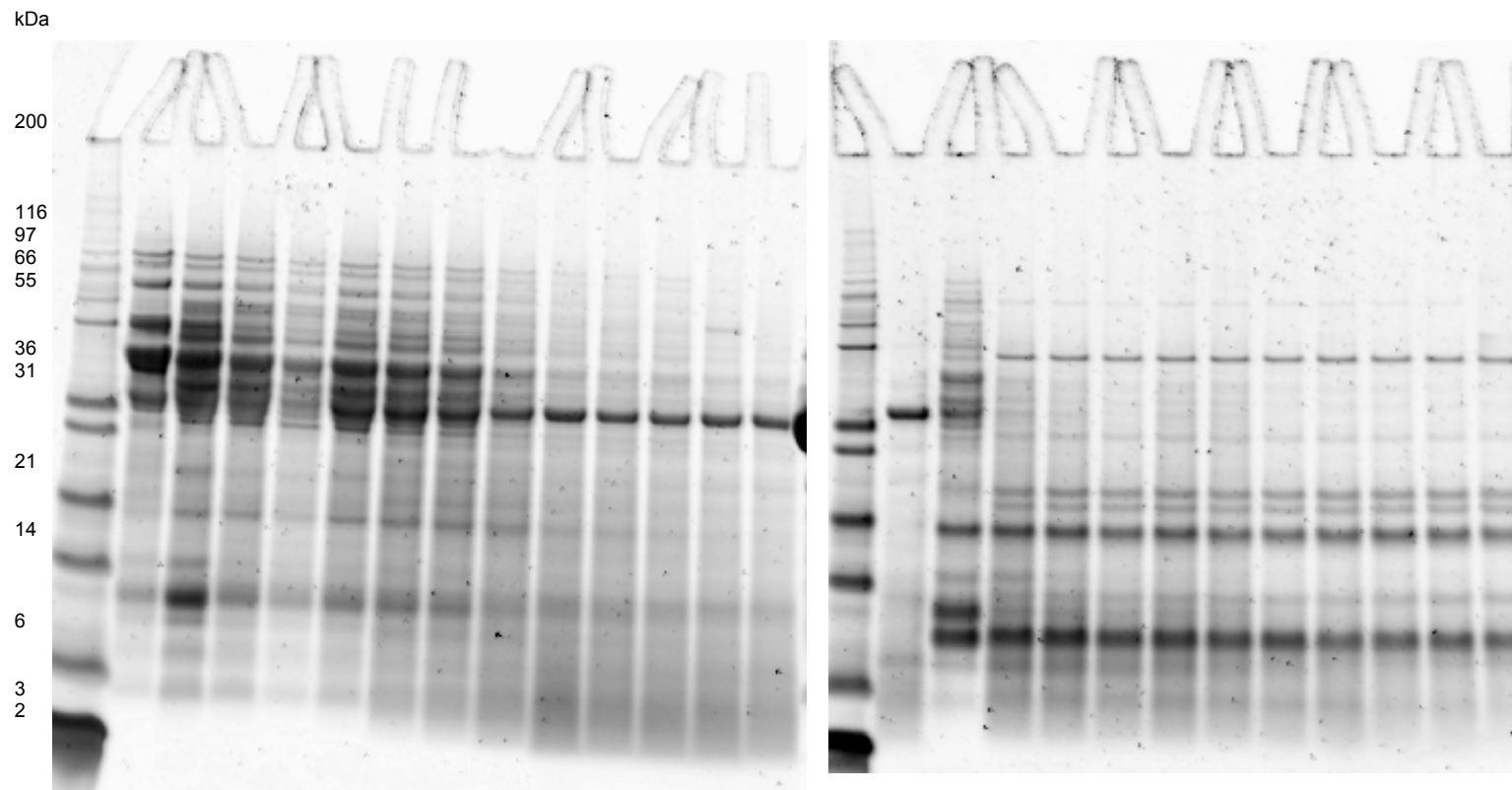


Figure 2.9: 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK)
Insoluble protein patterns of cooked flour digestion performed using individual bijoux bottles: MW: molecular weight markers: 1 raw flour undigested, 2: control sample (undigested), 3 : chew material, 4: gastric control, 5-13: time points of gastric digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min. of pepsin hydrolysis). 14: 120 min. gastric digesta, 15: control flour undigested, 16: duodenal control, 17-24: time points of duodenal digestion (0, 1, 2, 5, 10, 20, 30, 60, 120 min). The 36 kDa band, presents in wells 4-12, corresponds to pepsin.

APPENDIX

CHAPTER 3

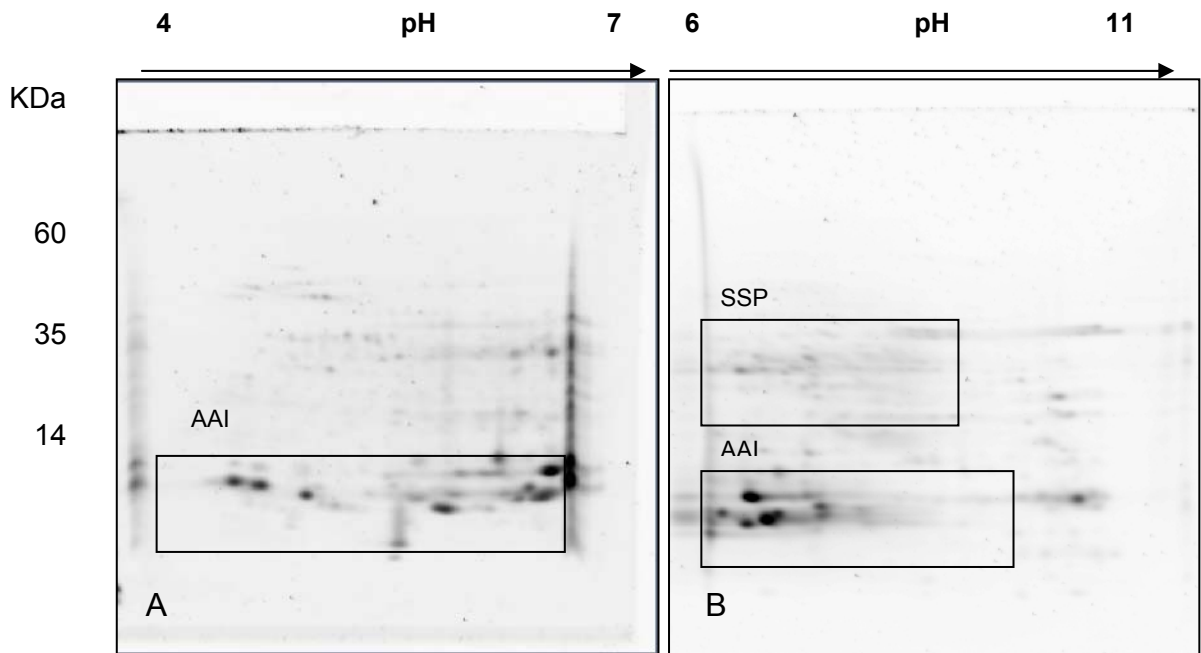


Figure 3.1: 2-DE gel images, pre-cast 4-12 % Bis Tris gradient gels (Invitrogen UK). Unheated flour undigested (supernatant, soluble fraction) separated at 2 pH ranges A: pH 4-7 and B: pH 6-11.

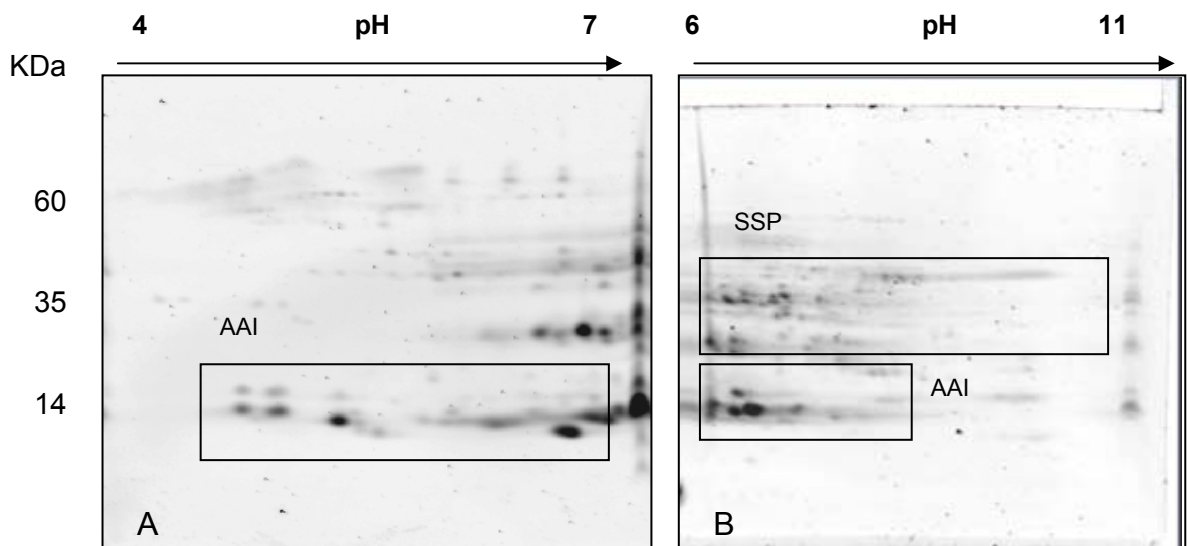


Figure 3.2: 2-DE gel images, pre-cast 4-12 % Bis Tris gradient gels. (Invitrogen UK). Heated flour undigested (supernatant, soluble fraction) separated at 2 pH ranges A: pH 4-7 and B: pH 6-11.

AAI: α -amylase inhibitors, SSP: seed storage proteins.

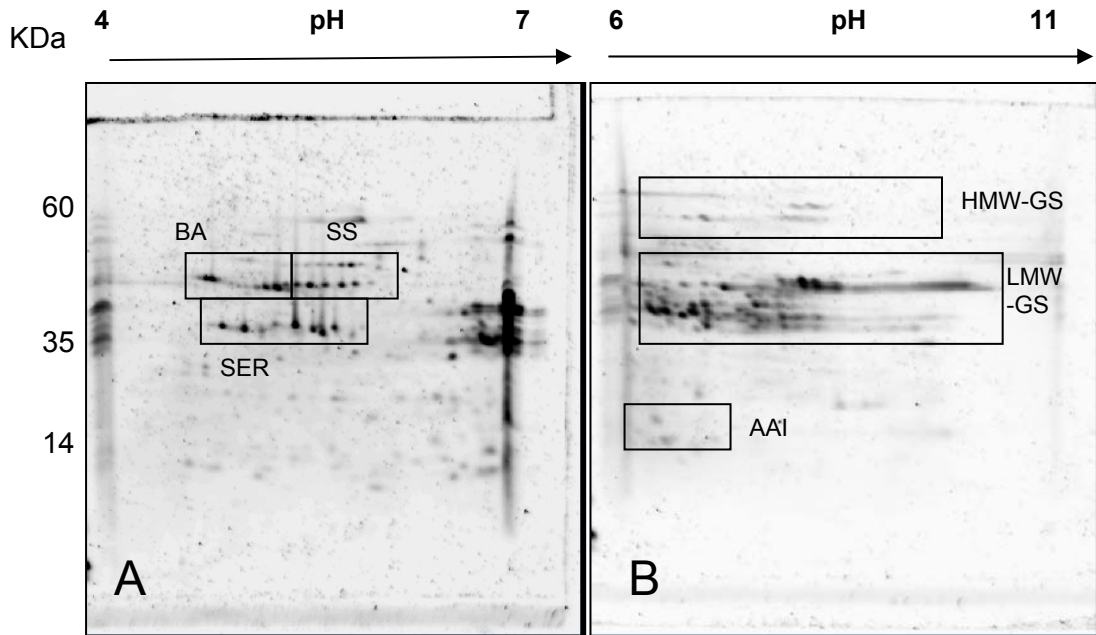


Figure 3.3: 2-DE gel images, pre-cast 4-12 % Bis Tris gradient gels. Unheated flour undigested (pellet, insoluble fraction) separated at 2 pH ranges A: pH 4-7 and B: pH 6-11.

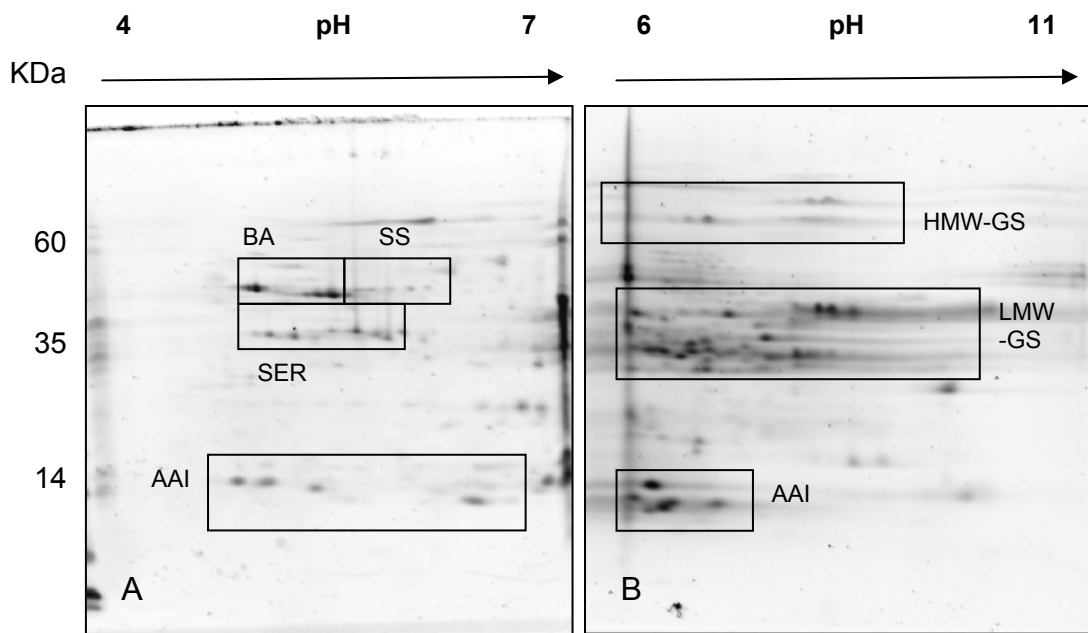


Figure 3.4: 2-DE gel images, pre-cast 4-12 % Bis Tris gradient gels. Heated flour undigested (pellet, insoluble fraction) separated at 2 pH ranges A: pH 4-7 and B: pH 6-11.

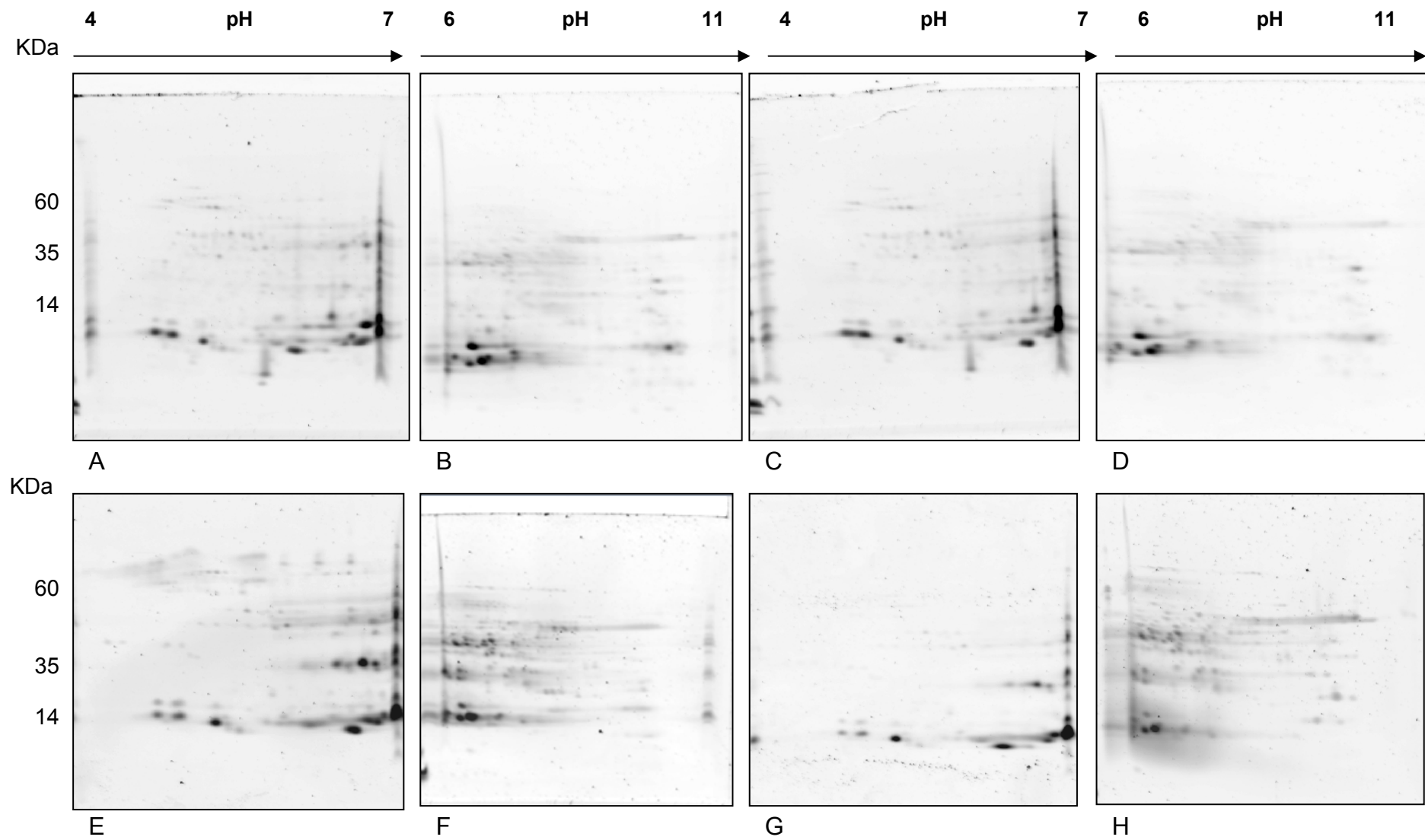


Figure 3.5: 2-DE gel images of soluble proteins (supernatant), separated at 2 pH ranges (4-7 and 6-11), pre-cast 4-12 % Bis Tris gradient gels. (Invitrogen UK). A-B: unheated flour undigested sample, C- D unheated flour chewed sample. E-F heated flour undigested sample, G-H heated flour chewed sample.

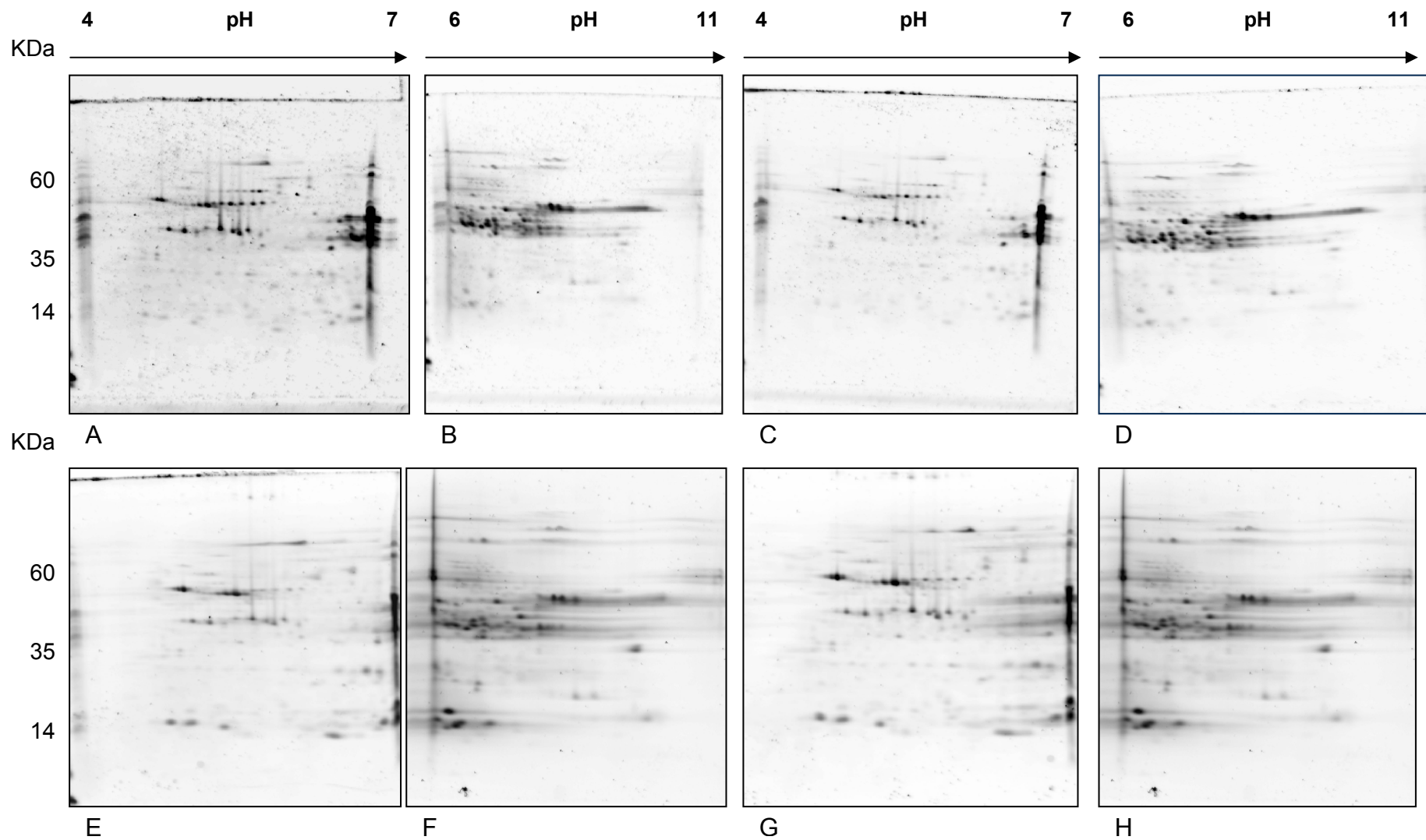


Figure 3.6: 2-DE gel images of insoluble proteins (pellet), separated at 2 pH ranges (4-7 and 6-11), pre-cast 4-12 % Bis Tris gradient gels. A-B: unheated flour undigested sample, C- D unheated flour chewed sample. E-F heated flour undigested sample, G- H heated flour chewed sample.

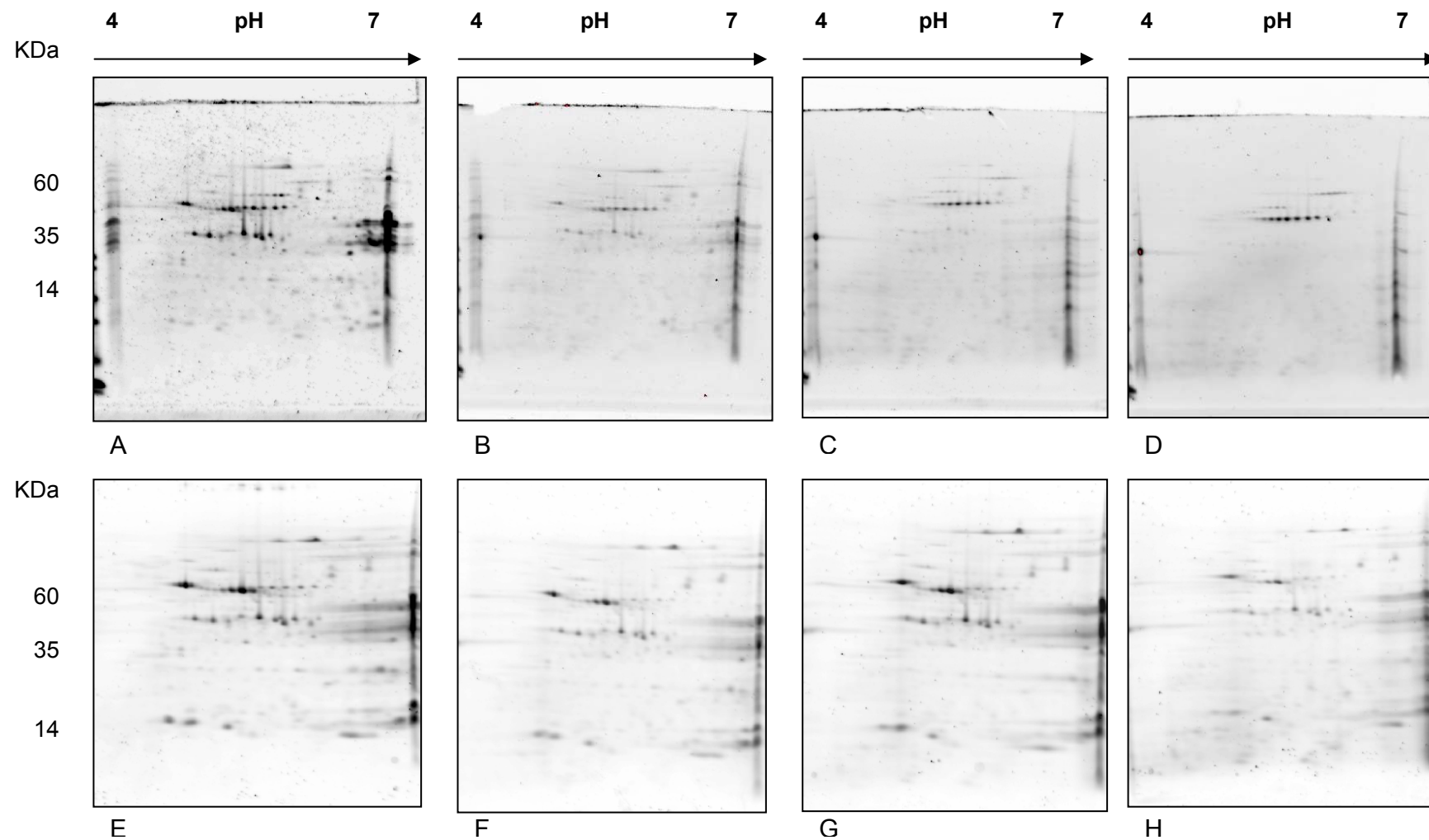


Figure 3.7: 2-DE gel images of insoluble proteins (pellet), separated at pH range 4-7, pre-cast 4-12 % Bis Tris gradient gels. A-D: unheated flour: A undigested sample, B, C, D correspond to 5, 30, 60 min of gastric digestion. E- H heated flour: E undigested sample, F, G, H correspond to 5, 30, 60 min of gastric digestion.

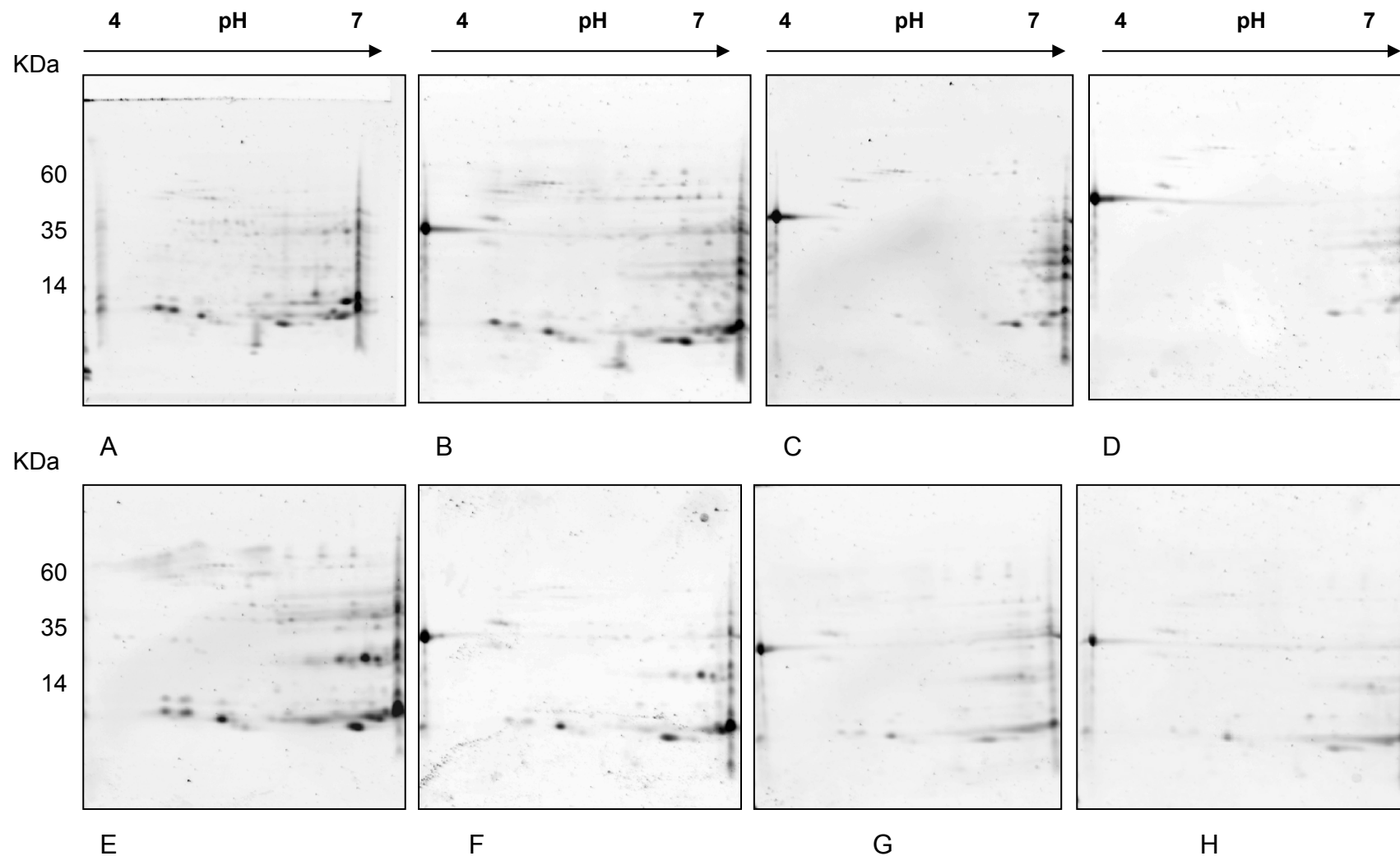


Figure 3.8: 2-DE gel images of soluble proteins (supernatant), separated at pH ranges 4-7, pre-cast 4-12 % Bis Tris gradient gels. A-D unheated flour: A undigested sample, B, C, D correspond to 5, 30, 60 min of gastric digestion. E-H heated flour: E undigested sample, F, G, H correspond to 5, 30, 60 min of gastric digestion.

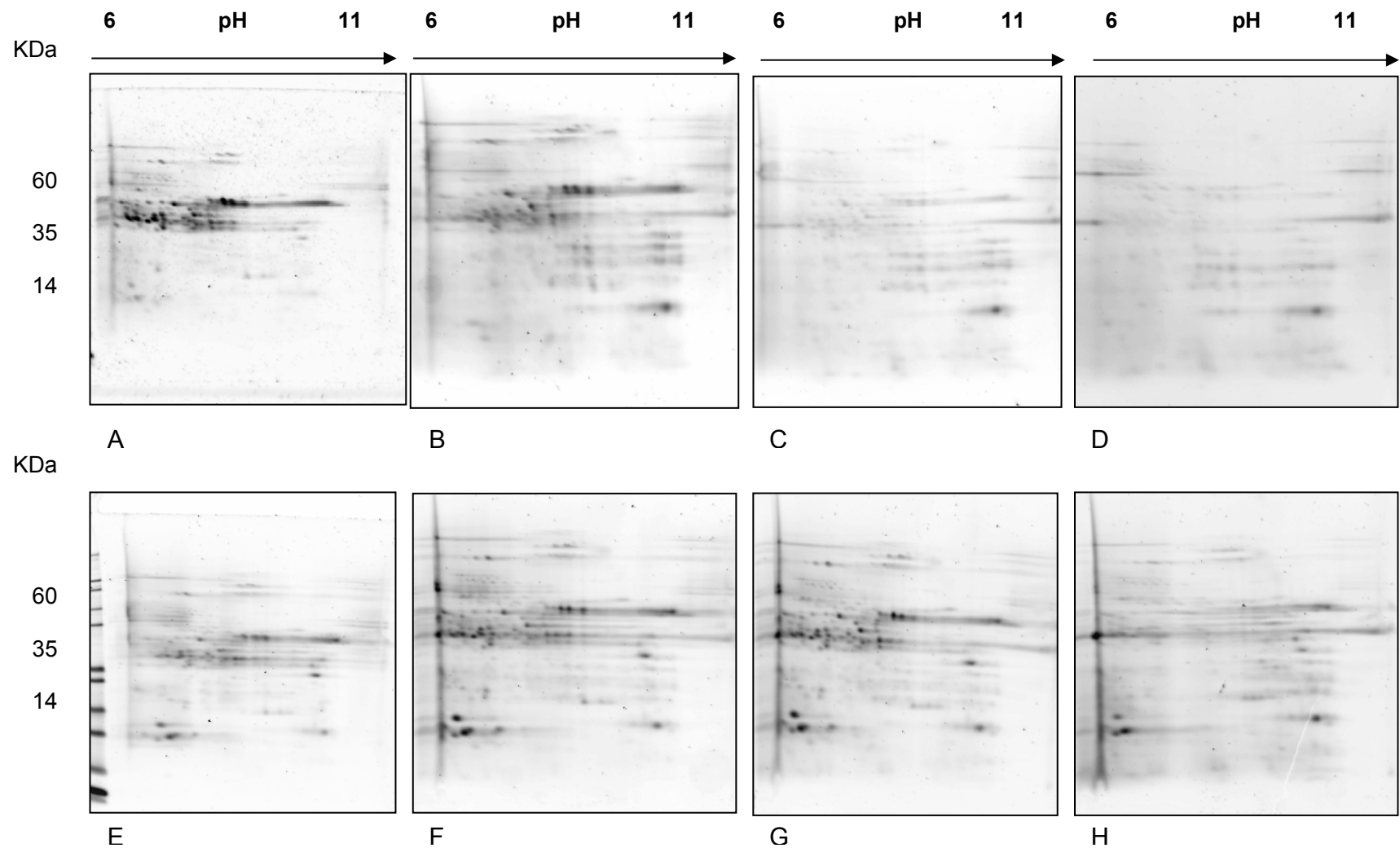


Figure 3.9: 2-DE gel images of insoluble proteins (pellet), separated at pH range 6-11, pre-cast 4-12 % Bis Tris gradient gels. A-D unheated flour: A undigested sample, B, C, D correspond to 5, 30, 60 min of gastric digestion. E- H heated flour: E undigested sample, F, G, H correspond to 5, 30, 60 min of gastric digestion.

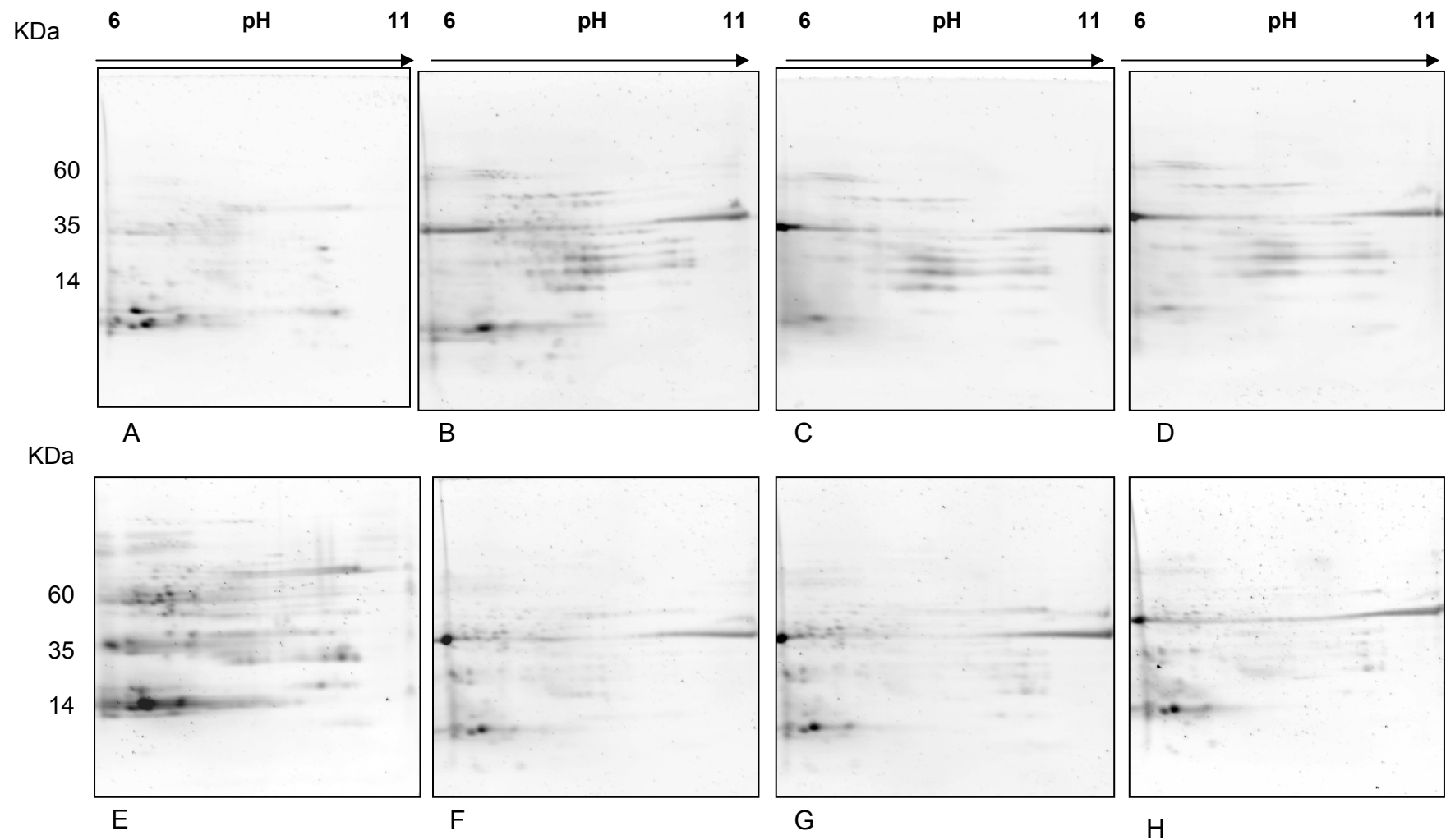


Figure 3.10: 2-DE gel images of soluble proteins (supernatant), separated at pH range 6-11, pre-cast 4-12 % Bis Tris gradient gels. A-D unheated flour: A undigested sample, B, C, D correspond to 5, 30, 60 min of gastric digestion. E- H heated flour: E undigested sample, F, G, H correspond to 5, 30, 60 min of gastric digestion.

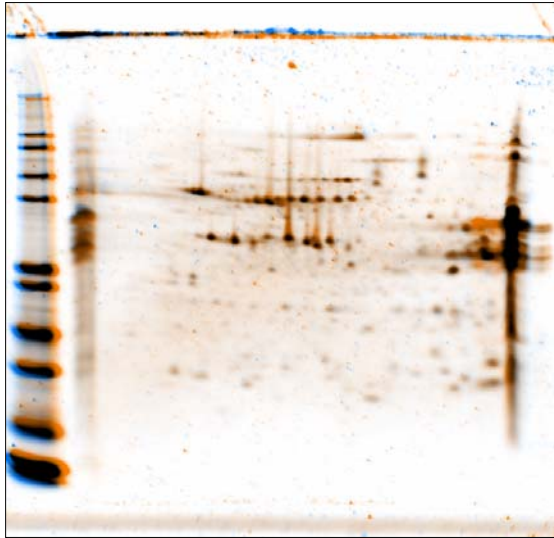


Figure 3.11: overlaid 2D image (insoluble proteins separated at pH range 4-7) of undigested unheated flour (orange spots) vs chewed unheated flour (blue spots). Black spots represent the presence of the same proteins in undigested and

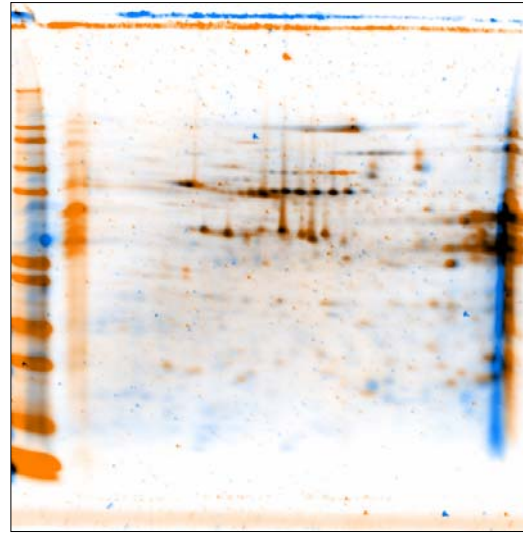


Figure 3.12: overlaid 2D image (insoluble proteins, separated at pH range 4-7) of undigested unheated flour (orange spots) vs 5 min gastric digestion (blue spots). Black spots represent the presence of the same proteins in

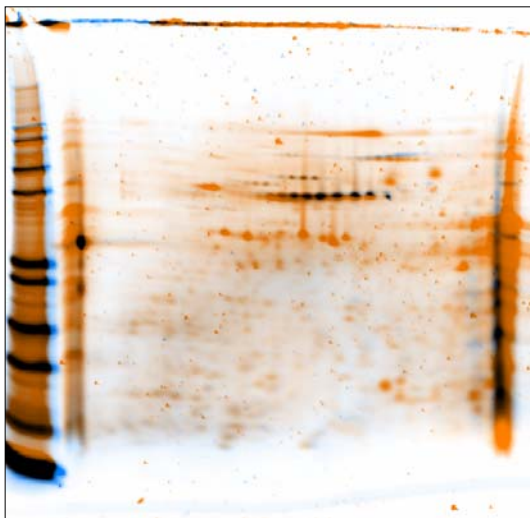


Figure 3.13: overlaid 2D image (unheated flour, insoluble proteins, separated at pH range 4-7) of 5 min gastric digesta (orange spots) vs 60 min gastric digesta (blue spots). Black spots represent the presence of the same proteins in digesta samples.

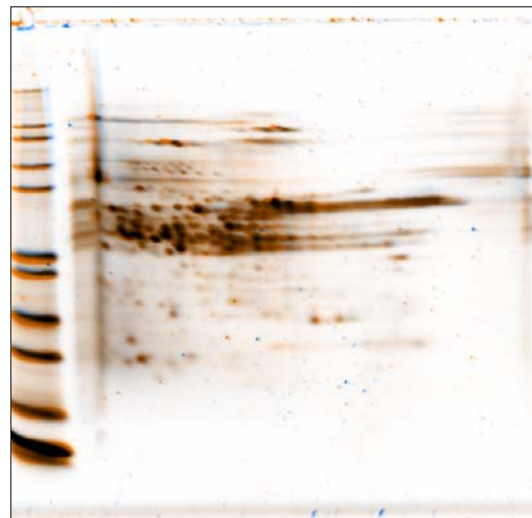


Figure 3.14: overlaid 2D image (unheated flour, insoluble proteins, separated at pH range 6-11) of undigested sample (orange spots) vs chewed sample (blue spots). Black spots represent the presence of the same proteins in both samples.

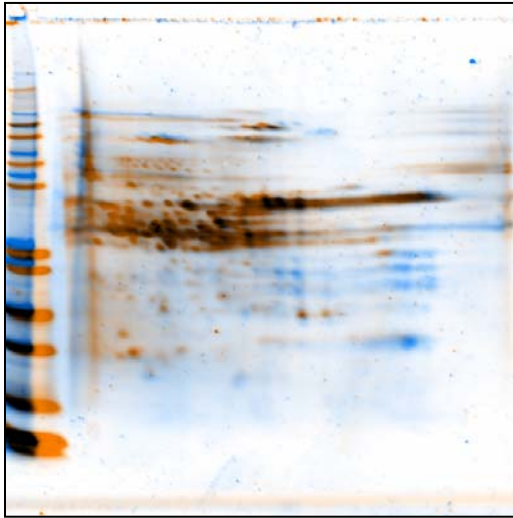


Figure 3.15: overlaid 2D image (unheated flour, insoluble proteins, separated at pH range 6-11) of undigested sample (orange spots) vs 5 min gastric digesta (blue spots). Black spots represent the presence of the same proteins in both samples.

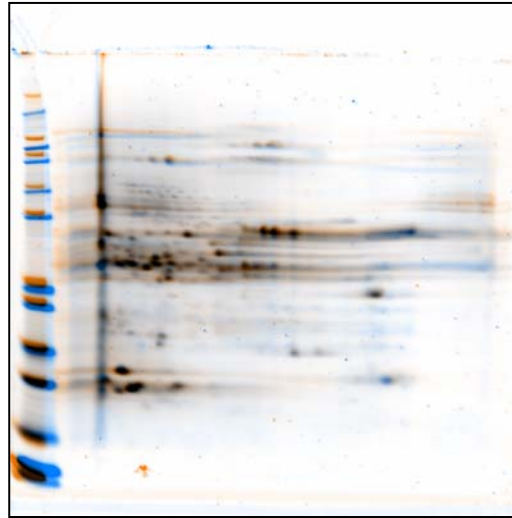


Figure 3.16: overlaid 2D image (heated flour, insoluble proteins, separated at pH range 6-11) of chewed sample (orange spots) vs 5 min gastric digesta (blue spots). Black spots represent the presence of the same proteins in both samples.

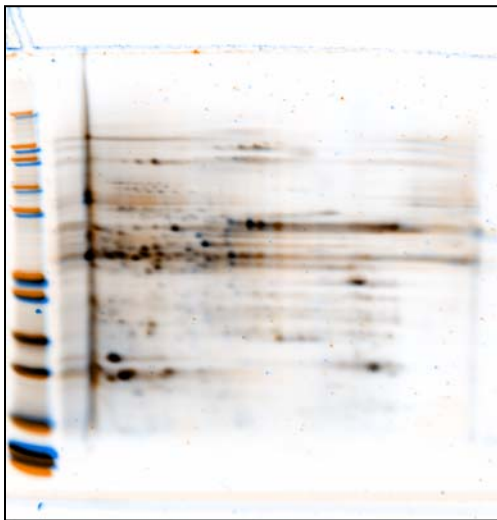


Figure 3.17: overlaid 2D image (heated flour, insoluble proteins, separated at pH range 6-11) of 5 min gastric digesta (orange spots) vs 30 min gastric digesta (blue spots). Black spots represent the presence of the same proteins in both samples.

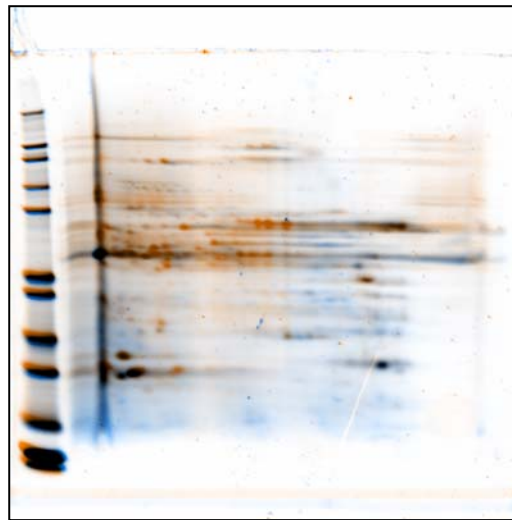


Figure 3.18: overlaid 2D image (heated flour, insoluble proteins, separated at pH range 6-11) of 5 min gastric digesta (orange spots) vs 60 min gastric digesta (blue spots). Black spots represent the presence of the same proteins in both samples.

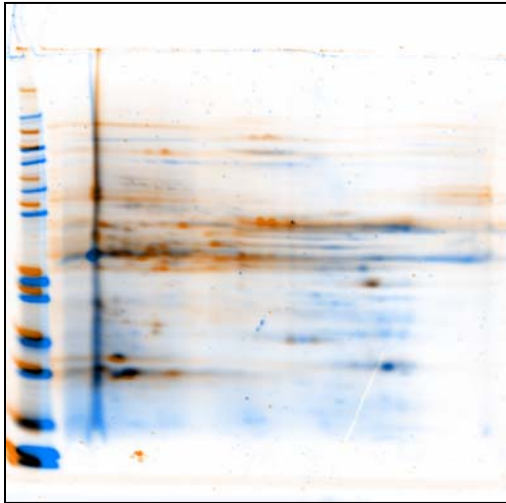


Figure 3.19: overlaid 2D image (heated flour, insoluble proteins, separated at pH range 6-11) of chewed sample (orange spots) vs 60 min gastric digesta (blue spots). Black spots represent the presence of the same proteins in both samples

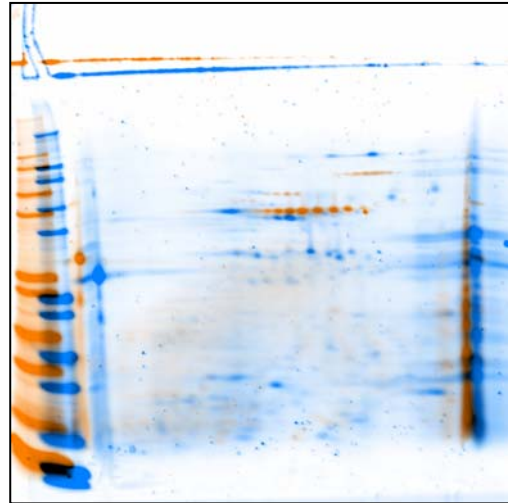


Figure 3.20: overlaid 2D image (insoluble proteins, separated at pH range 4-7) of unheated flour 60 min gastric digestion (orange spots) vs cooked flour 60 min gastric digestion (blue spots).

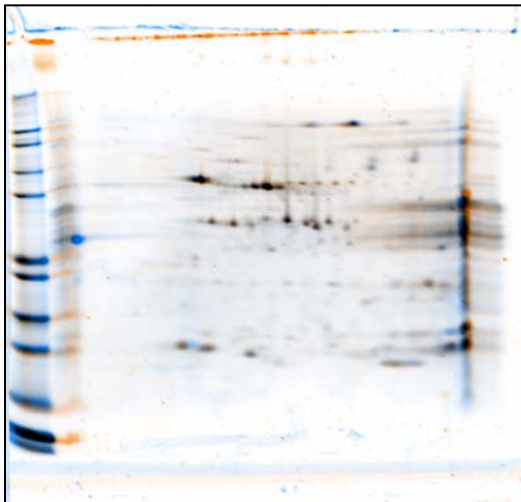


Figure 3.21: overlaid 2D image (heated flour, insoluble proteins, separated at pH range 4-7) of chewed sample (orange spots) vs 5 min gastric digesta (blue spots).

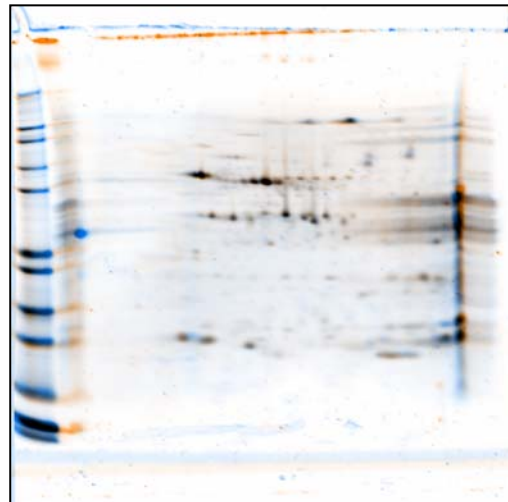


Figure 3.22: overlaid 2D image (heated flour, insoluble proteins, separated at pH range 4-7) of chewed sample (orange spots) vs 30 min gastric digesta (blue spots). Black spots represent the presence of the same proteins in both samples.

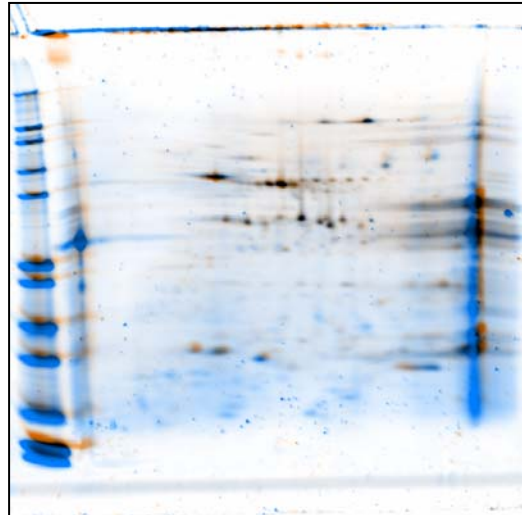
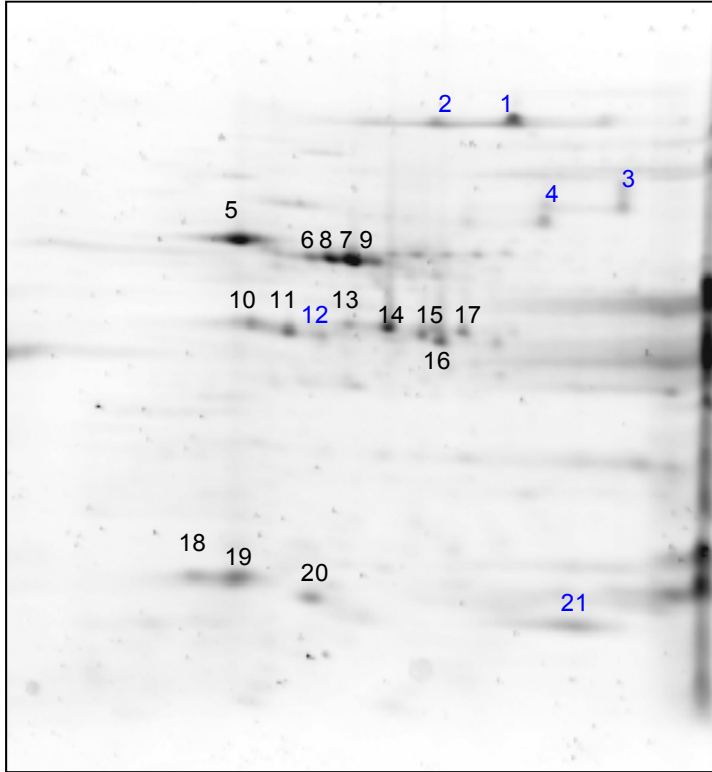
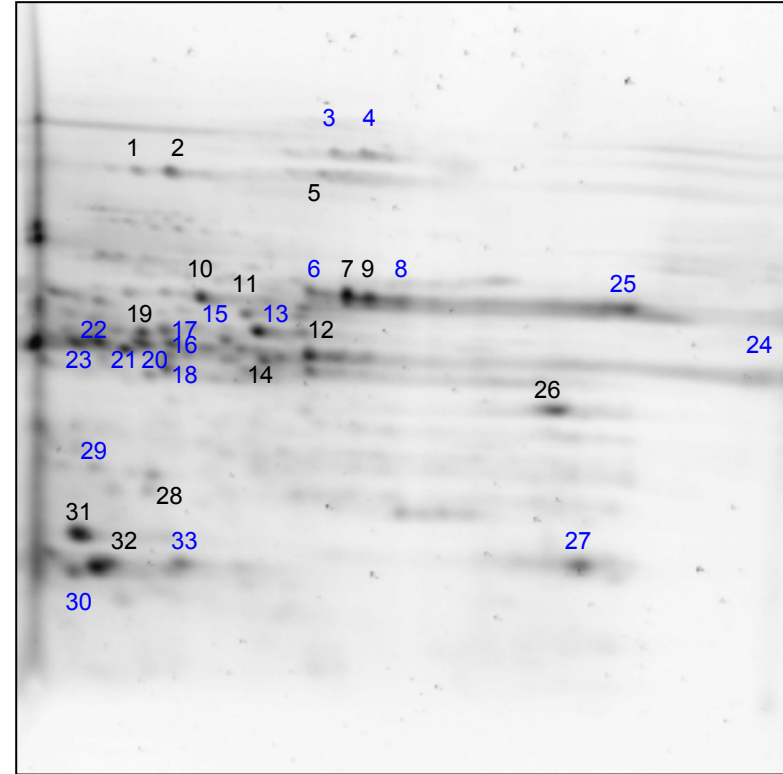


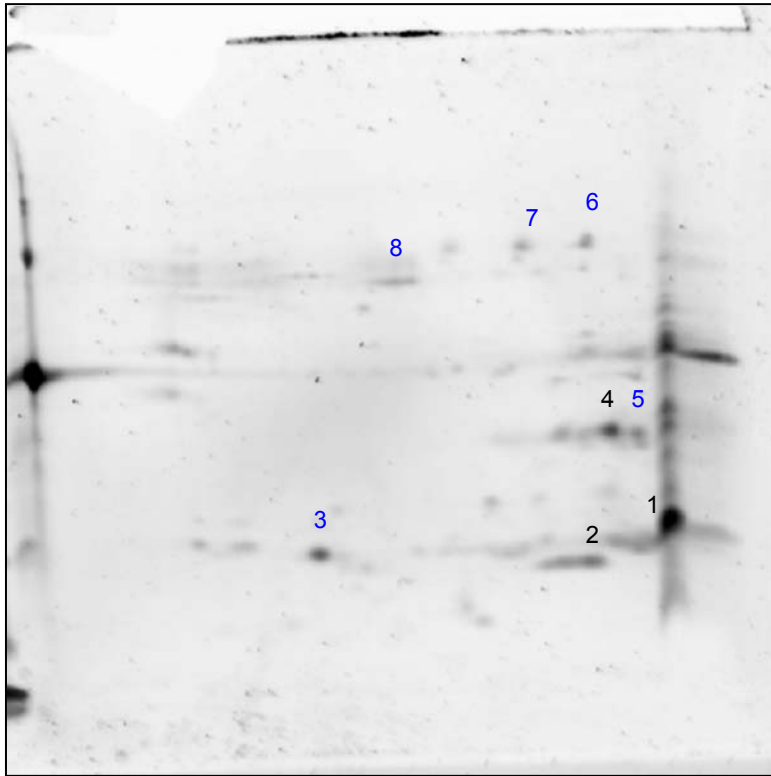
Figure 3.23: overlaid 2D image (heated flour, insoluble proteins, separated at pH range 4-7) of chewed sample (orange spots) vs 60 min gastric digesta (blue spots). Black spots represent the presence of the same proteins in both samples.



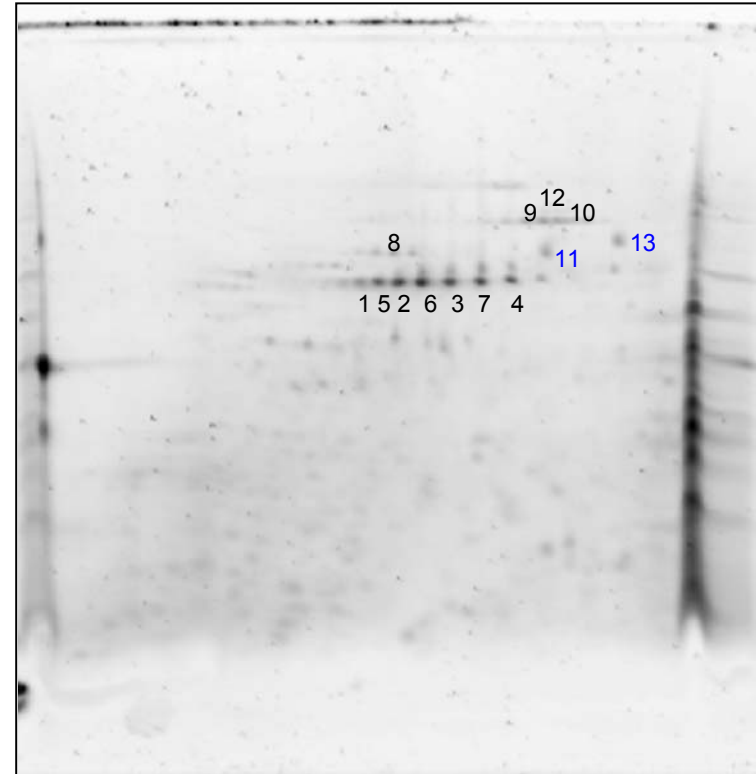
3.24: 2-DE gel no. 3A, insoluble proteins of heated flour, separated at pH range 4-7. Spots picked and processed with the MALDI-ToF. The black-labelled spots represent spot identified with mass spectrometry, the blue one were not identified.



3.25: 2-DE gel no. 3B. Insoluble proteins of heated flour, 30 min digested, separated at pH range 6-11. Spots picked and processed with the MALDI-ToF. The black-labelled spots represent spot identified with mass spectrometry, the blue one were not identified.



3.26: 2-DE gel no. 3C, soluble proteins of heated flour, separated at pH range 4-7. Spots picked and processed with the MALDI-ToF. The black-labelled spots represent spot identified with mass spectrometry, the blue one were not identified.



3.27: 2-DE gel no. 3D, insoluble proteins of unheated flour, separated at pH range 4-7. Spots picked and processed with the MALDI-ToF. The black-labelled spots represent spot identified with mass spectrometry, the blue one were not identified.

APPENDIX

CHAPTER 4

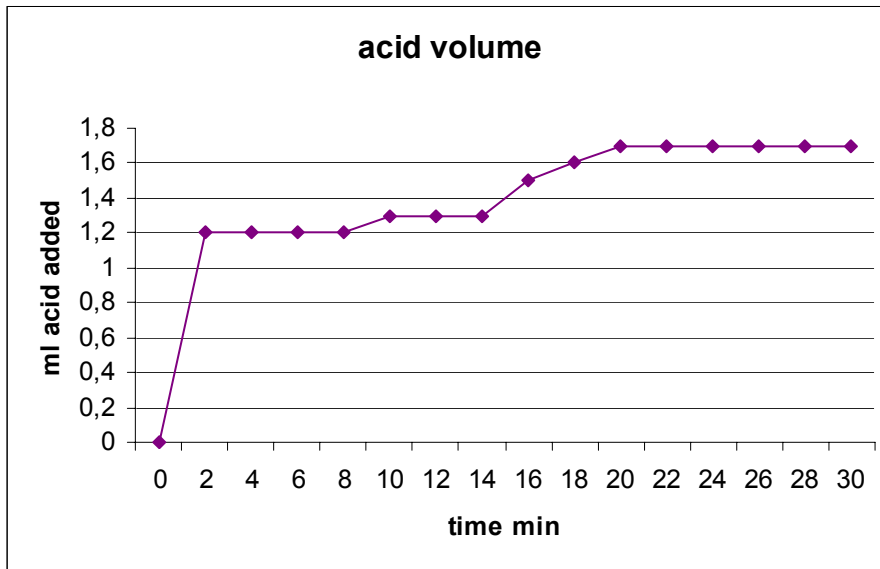


Figure 4.5: trend of the acid released during the first *in vitro* digestion process

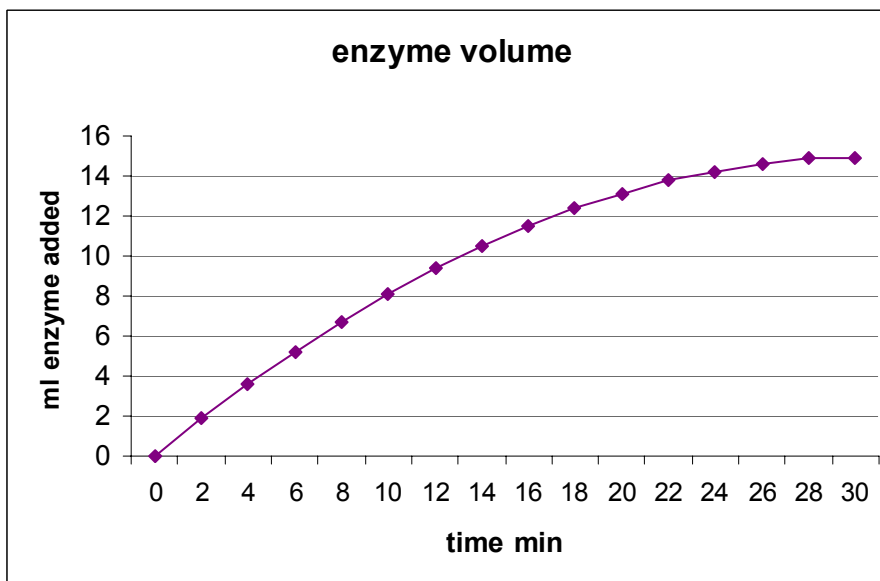


Figure 4.6: trend of the enzymes solution released during the first *in vitro* digestion process.

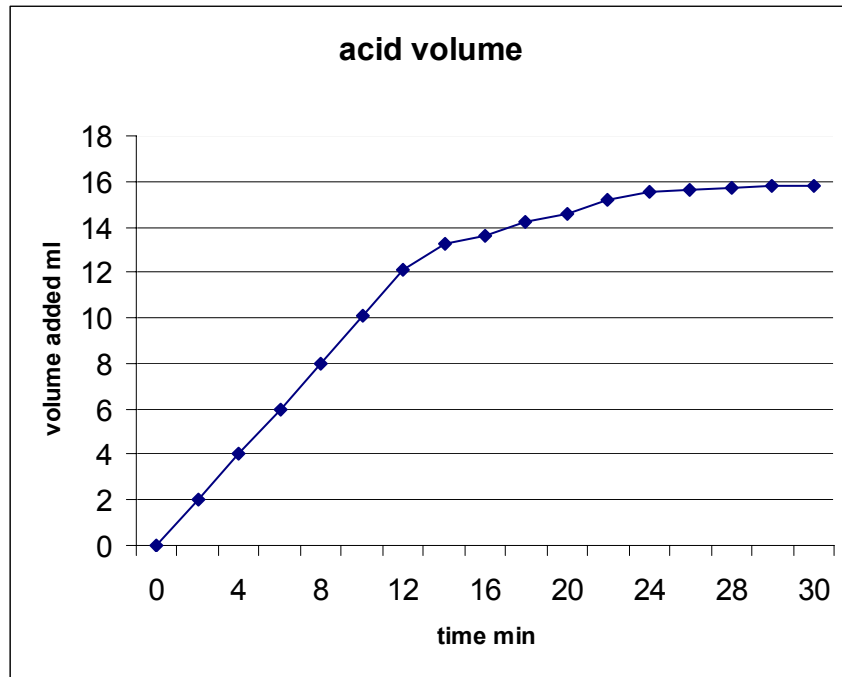


Figure 4.7: trend of the enzymes solution released during the second *in vitro* dynamic digestion.

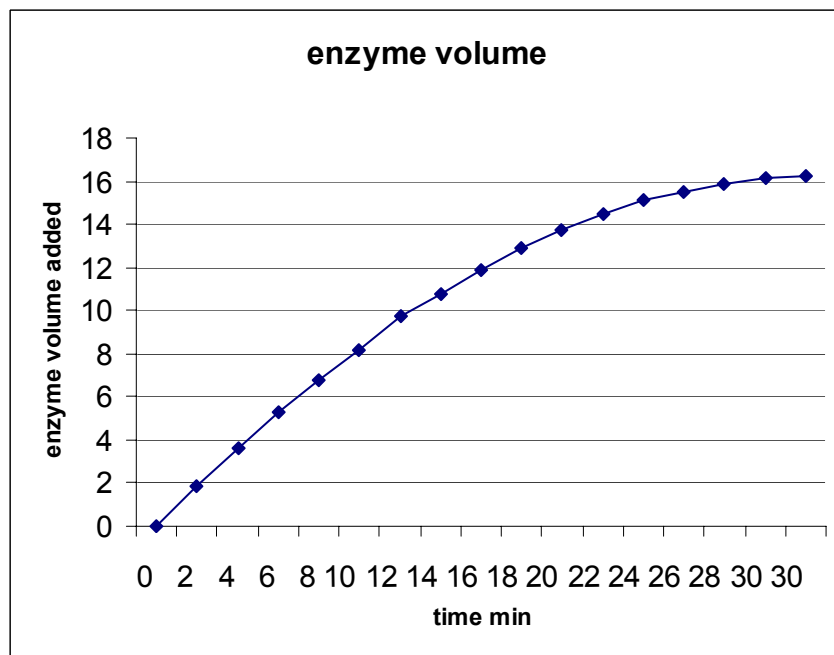


Figure 4.8: trend of the enzymes solution released during the second *in vitro* dynamic digestion process.

Sample	Min of gastric digestion	Amount (g) collected	pH
1	5	11,8	1,61
2	10	39,75	2,14
3	15	46,9	3,14
4	20	28,39	4,5
5	25	29,6	4,58
6	30	27,93	4,8

Figure 4.9: parameters of digestion collected during the first Model Gut run.

sample	Min of gastric digestion	Amount (g) collected	pH
1	5	17.9 (15.9)	1.59
2	10	25	2.19
3	15	39.8 (38.3)	2.34
4	20	13.3	2.49
5	25	18.5	2.50
6	30	19.4	2.57

Figure 4.10: parameters of digestion collected during the second Model Gut run.

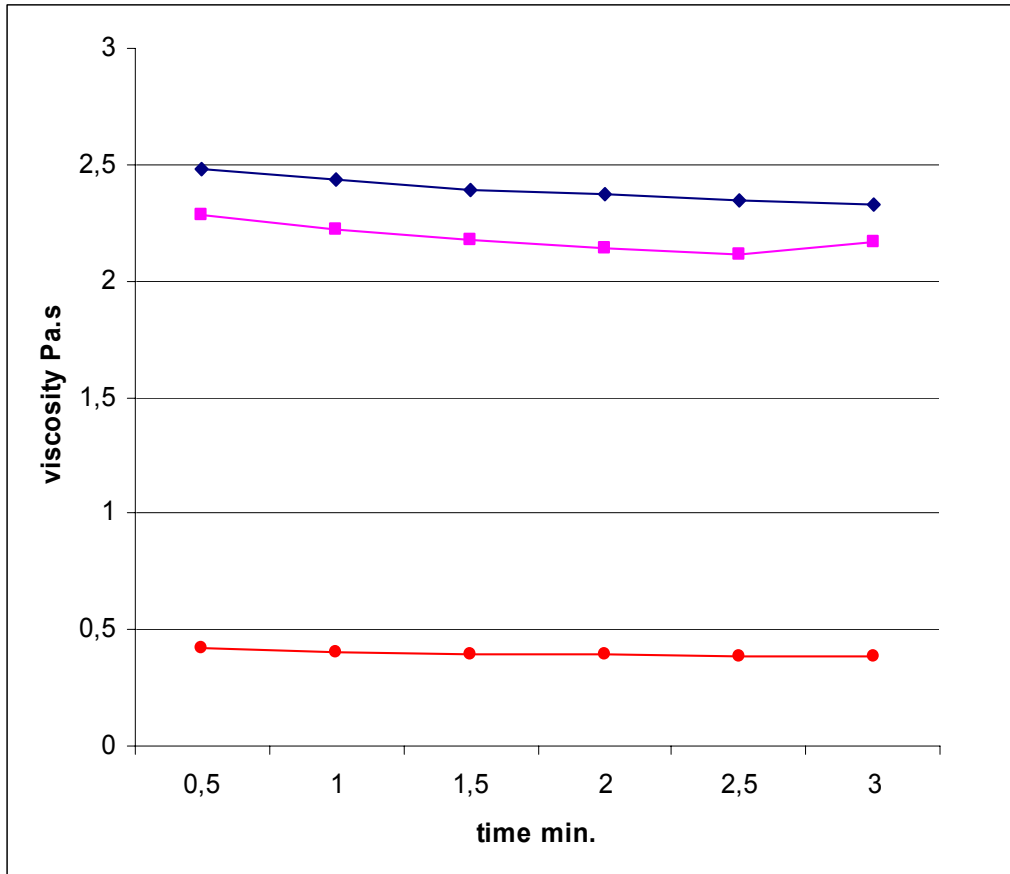


Figure 4.11: viscosity measurements of cooked flour (blue line); cooked flour plus salivary fluid (pink line); cooked flour plus salivary fluid and salivary amylase (red line).

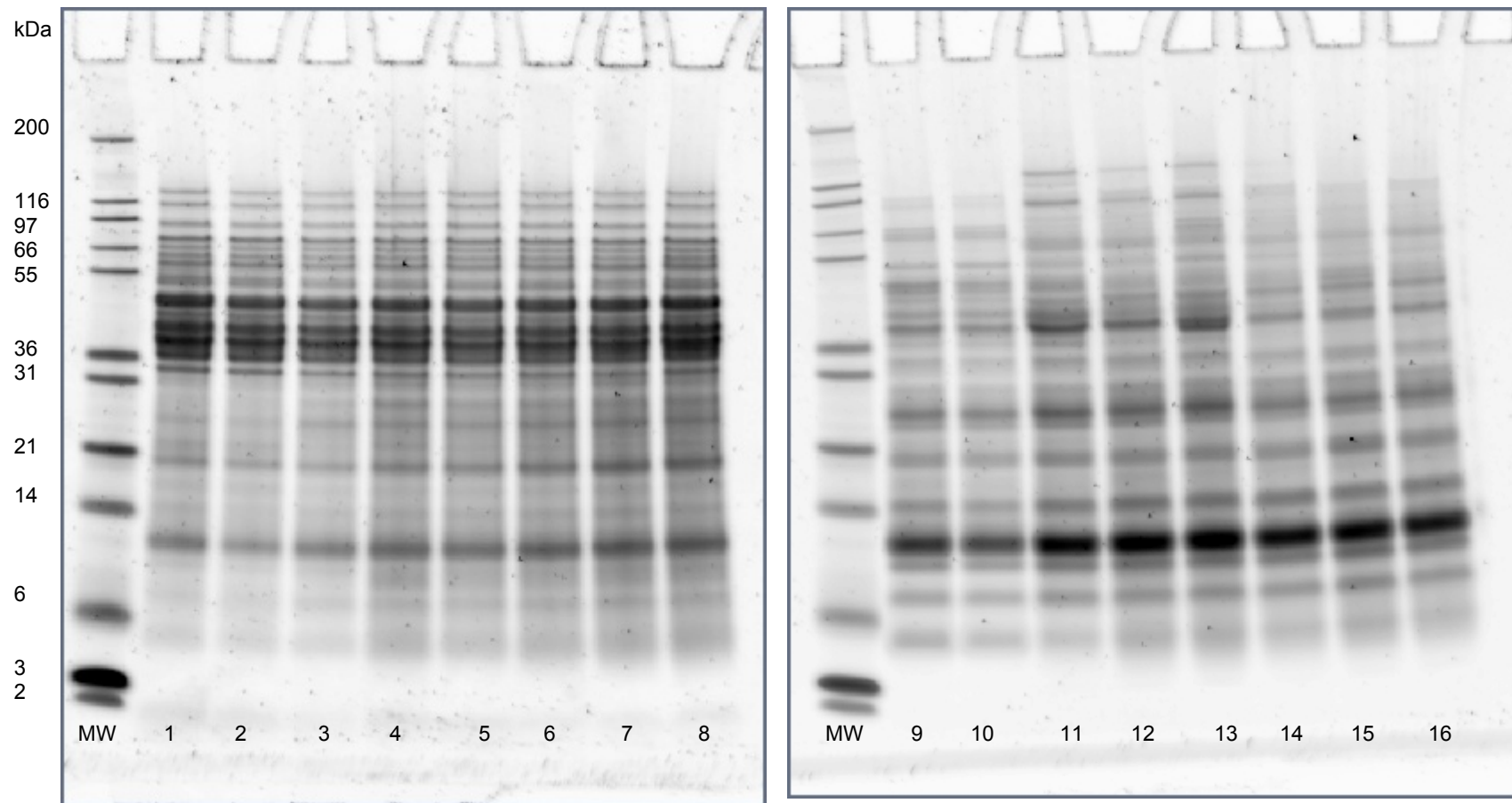


Figure 4.12 : 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK). pellet and supernatant protein fractions of heated flour (first experiment) digested by using the dynamic model of digestion. MW: molecular weight markers: (1-8 pellet protein) 1: control sample (undigested), 2: chew material, 3-8: time points of gastric digestion (5, 10, 15, 20, 25, 30 min. of pepsin hydrolysis). (9-16 supernatant proteins) 9: control sample (undigested), 10: chew material, 11-16: time points of gastric digestion (5, 10, 15, 20, 25, 30 min. of pepsin hydrolysis).

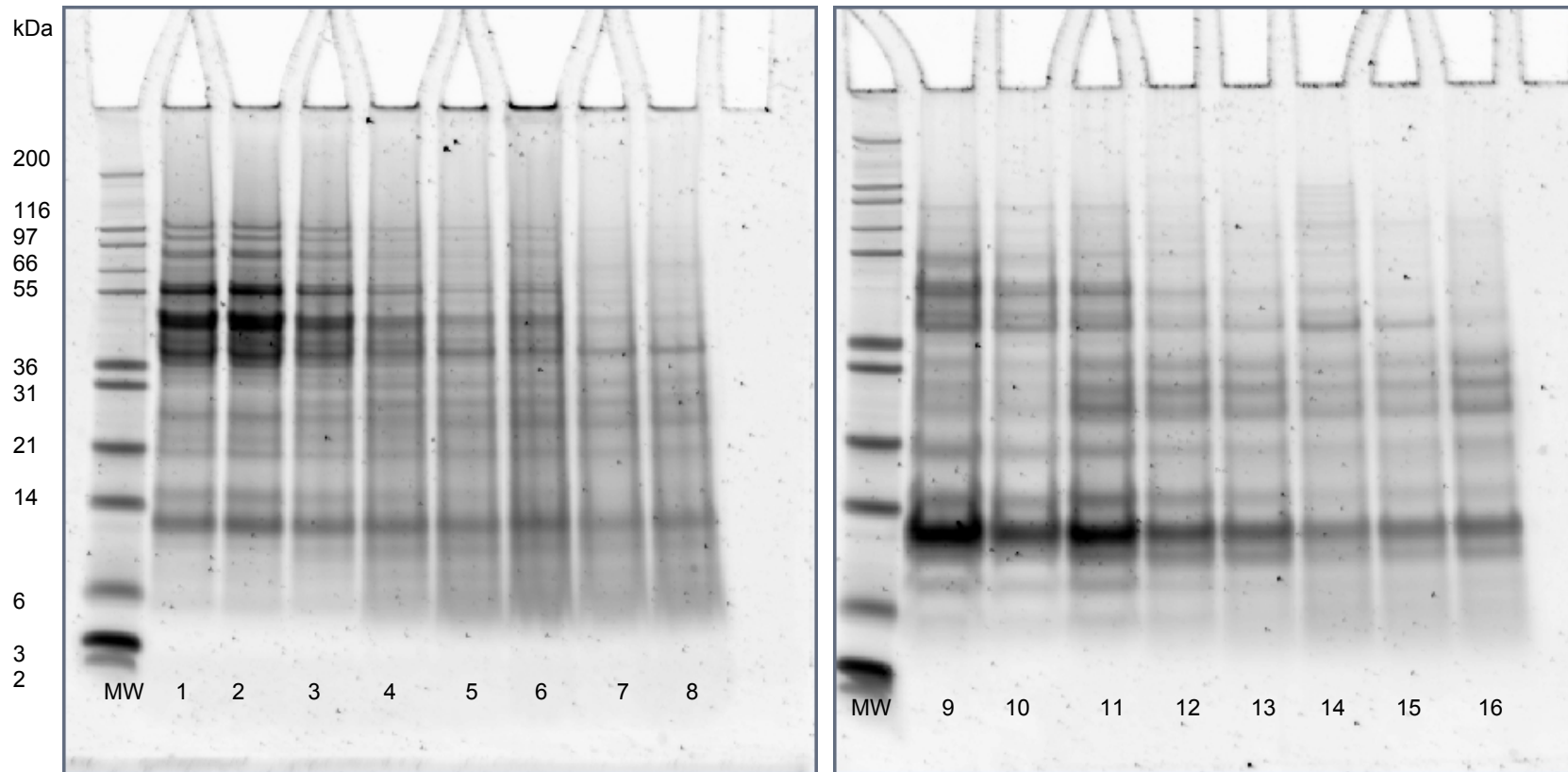


Figure 4.13: 1D SDS-PAGE, 10% polyacrylamide NuPAGE Novex Bis-Tris precast mini gels (Invitrogen, UK).

Pellet and supernatant protein fractions of heated flour (second experiment) digested by using the dynamic model of digestion. MW: molecular weight markers: (1-8 pellet protein) 1: control sample (undigested), 2: chew material, 3-8: time points of gastric digestion (5, 10, 15, 20, 25, 30 min. of pepsin hydrolysis). (9-16 supernatant proteins) 9: control sample (undigested), 10: chew material, 11-16: time points of gastric digestion (5, 10, 15, 20, 25, 30 min. of pepsin hydrolysis).

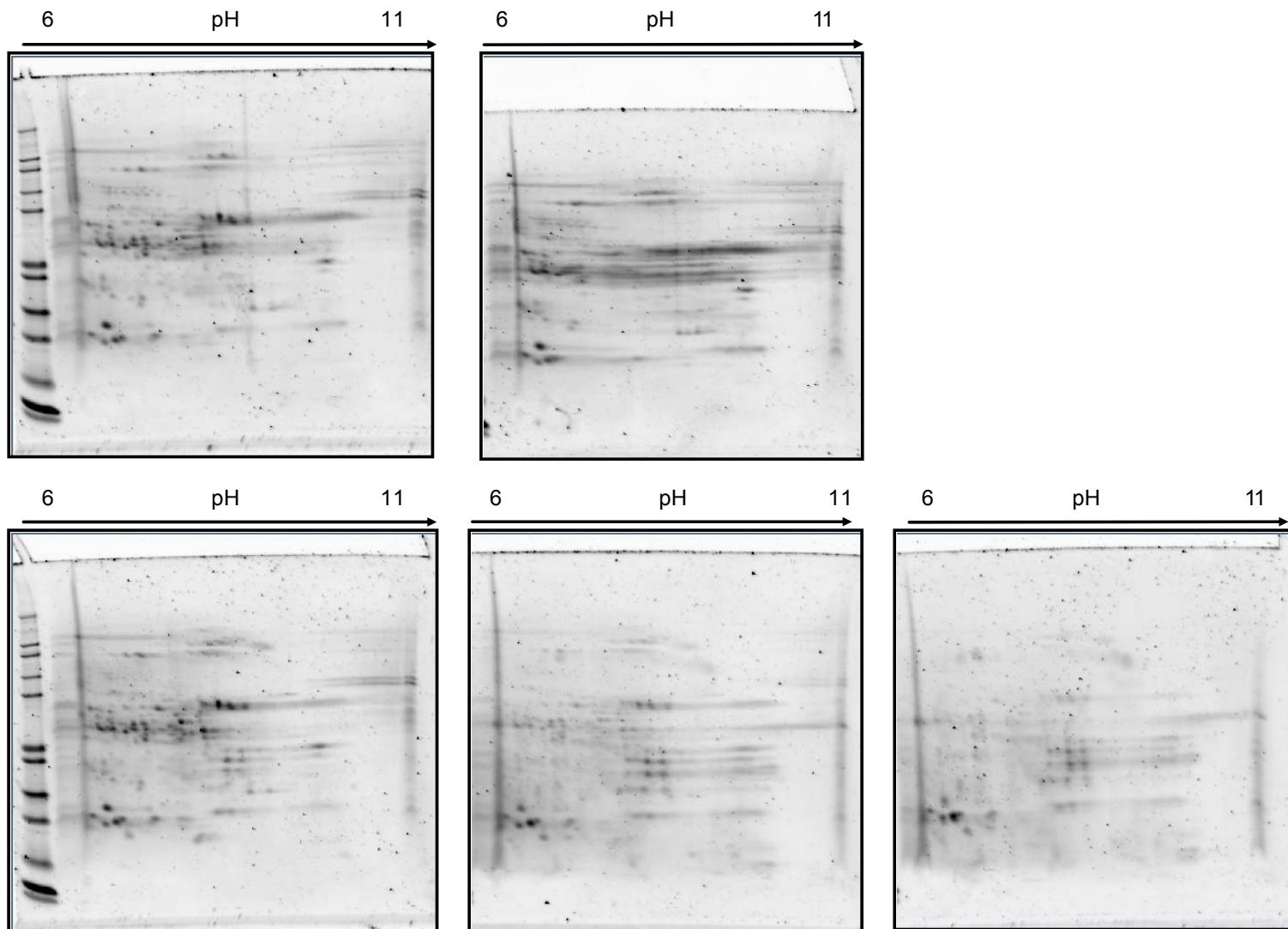


Figure 4.14: 2-DE gel images, pre-cast 4-12 % Bis Tris gradient gels (Invitrogen UK). Insoluble proteins (pellet), of heated flour (second experiment) digested by using the dynamic model of digestion, separated at pH range 6-11. A. undigested sample, B. chew material, C, D, E correspond to 5, 15, 30 min of gastric digestion.

APPENDIX

CHAPTER 5

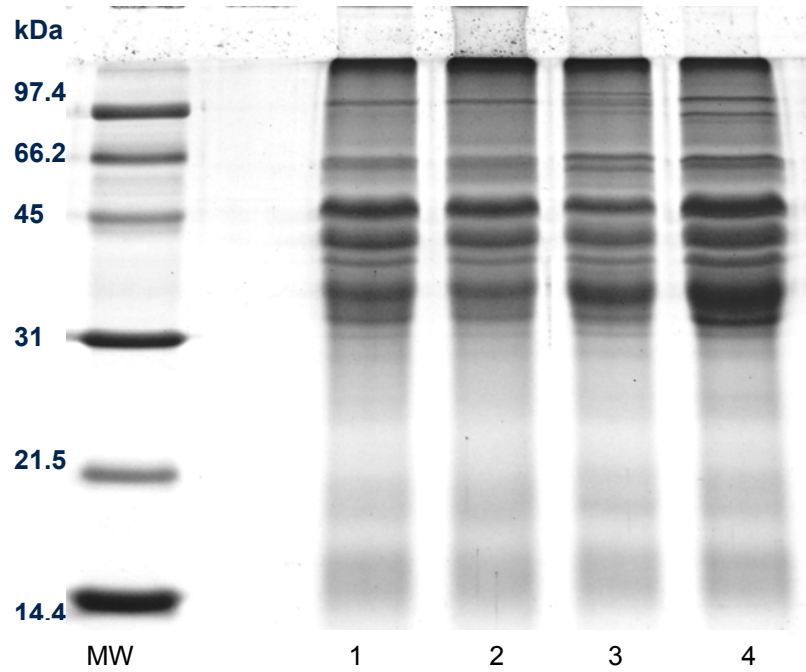


Figure 5.1: 1D SDS-PAGE of total protein extracted from cooked pasta. 1-2 durum wheat pasta; 3 spelt pasta; 4 kamut pasta.

Samples	DM %	Ash %	Protein%	Starch %
<i>Kamut®</i>	95,23	0,73	15,02	73,88
Spelta	95,43	1,00	11,70	71,10
<i>Common wheat</i>	94,71	0,64	12,20	80,25

Figure 5.2: chemical composition of the three pasta samples.

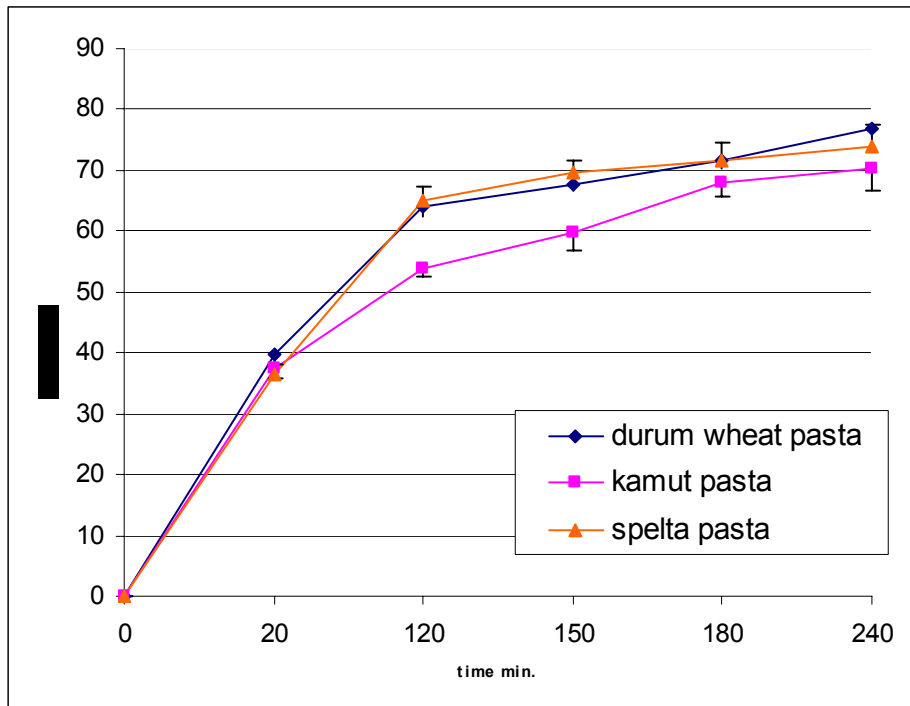


Figure 5.3: kinetics of *in vitro* starch digestion of pasta made with the common durum wheat (blue line), pasta made with spelt (orange line) and pasta made with kamut pasta (pink line). Mean of triplicate \pm SD.

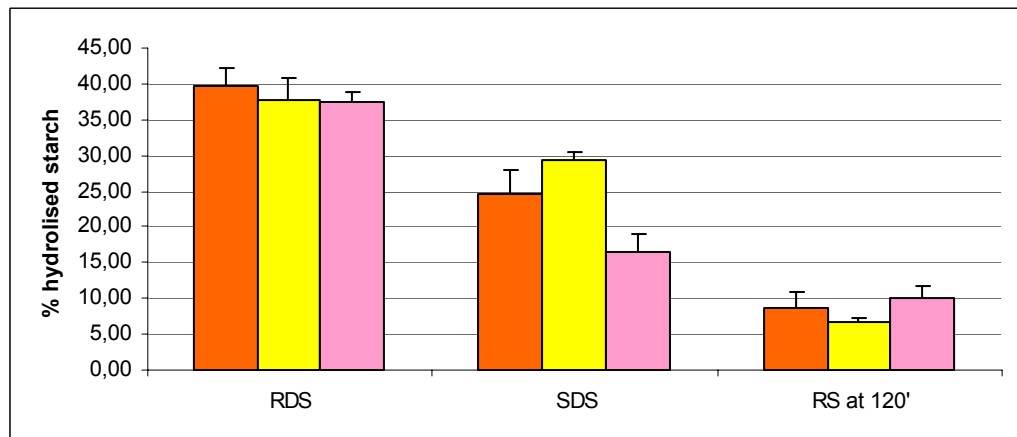


Figure 5.4: Nutritional starch fraction; RDS: rapidly digestible starch, SDS slowly digestible starch, RS: resistant starch. Mean \pm SD.
 Orange: durum wheat pasta
 Yellow: Spelta pasta
 Pink: kamut pasta.

A. Durum wheat pasta

VOLUNTEER	TIME	0	15'	30'	45'	60'	90'	120'
1		78	85	92	87	80	85	88
2		81	88	116	90	88	85	87
3		96	107	123	122	93	102	99
4		94	117	141	122	100	91	102
5		97	99	99	104	103	104	102
6		99	128	96	112	116	109	98
7		84	123	122	111	118	110	102
8		87	136	122	101	99	95	105
9		76	106	93	82	94	83	92
10		87	96	106	102	97	97	93
MEAN		88	109	111	103	99	96	97
SD		8	17	16	14	12	10	6

B. Kamut pasta

VOLUNTEER	TIME	0	15'	30'	45'	60'	90'	120'
1		82	96	98	88	71	91	90
2		84	99	89	89	82	105	91
3		97	118	98	102	103	95	107
4		99	103	107	97	121	94	102
5		96	121	101	98	100	97	95
6		82	103	94	101	97	90	94
7		97	108	122	100	106	117	98
8		81	99	89	82	85	77	73
9		77	106	81	97	89	86	84
10	/	/	/	/	/	/	/	/
MEAN		88	106	98	95	95	95	93
SD		9	9	12	7	15	11	10

C. Spelt pasta

VOLUNTEER	TIME	0	15'	30'	45'	60'	90'	120'
1		82	100	95	96	90	92	88
2		79	100	99	95	84	99	85
3		99	124	112	96	95	100	101
4		101	143	128	122	120	88	93
5		95	117	111	94	101	103	99
6		89	107	86	95	98	99	107
7		80	114	117	108	113	101	99
8		93	117	116	102	105	103	108
9		82	87	91	89	93	84	83
10		81	85	102	102	97	106	96
MEAN		88	109	106	100	100	98	96
SD		8	18	13	9	11	7	9

Figure 5.5: values of glucose (mg/g) detected during the in vivo experiments used to obtain the glycemic response curve reported in figure 6.4.

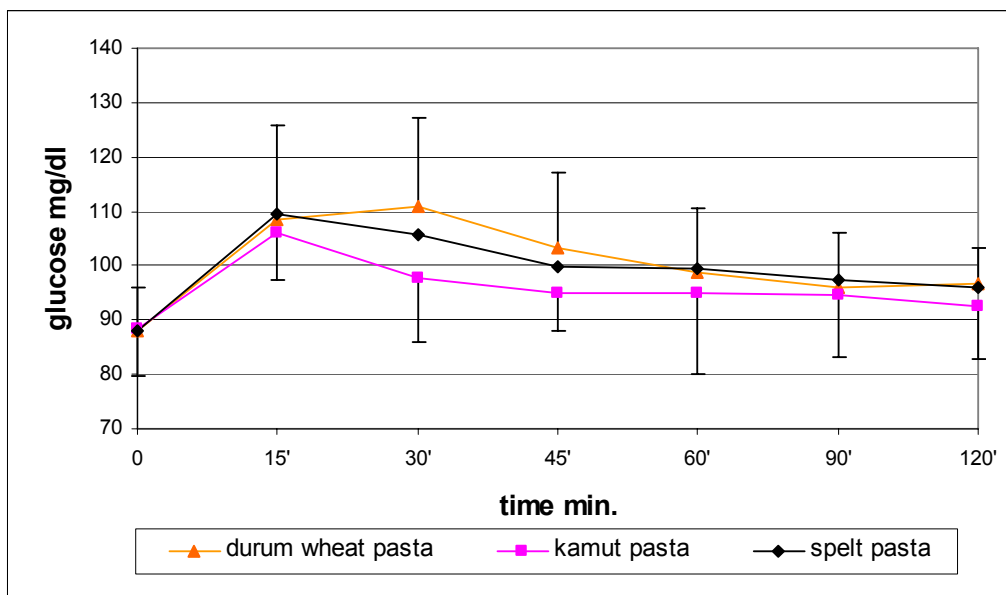
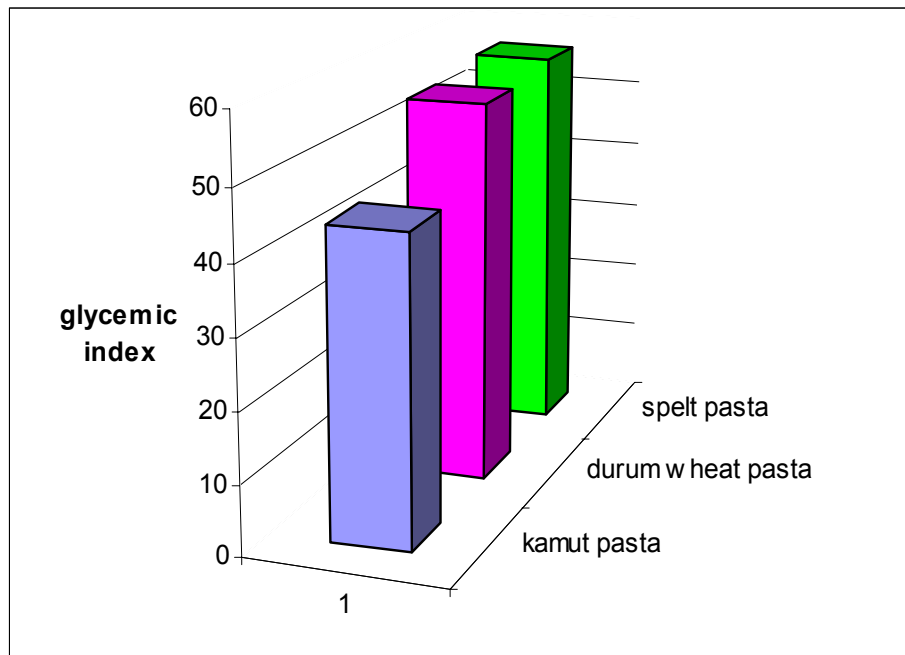


Figure 5.6: *in vivo* glycemic response of the three types of pasta examined. Mean ± SD.

VOLUNTEER	DURUM IG	KAMUT IG	SPELT IG
1	40,9	39,5	60,0
2	70,9	69,5	103,7
3	66,0	40,4	35,1
4	51,2	45,0	58,4
5	54,5	67,1	95,0
6	46,2	21,5	39,8
7	50,6	32,2	37,5
8	58,6	17,8	22,6
MEAN	54,9	41,6	56,5
SD	10,0	18,9	29,2

5.7: glycemic index values, calculated with the mathematical method, for each volunteer.



5.8: Average glycemic index calculated for the three types of pasta examined.

APPENDIX

CHAPTER 6

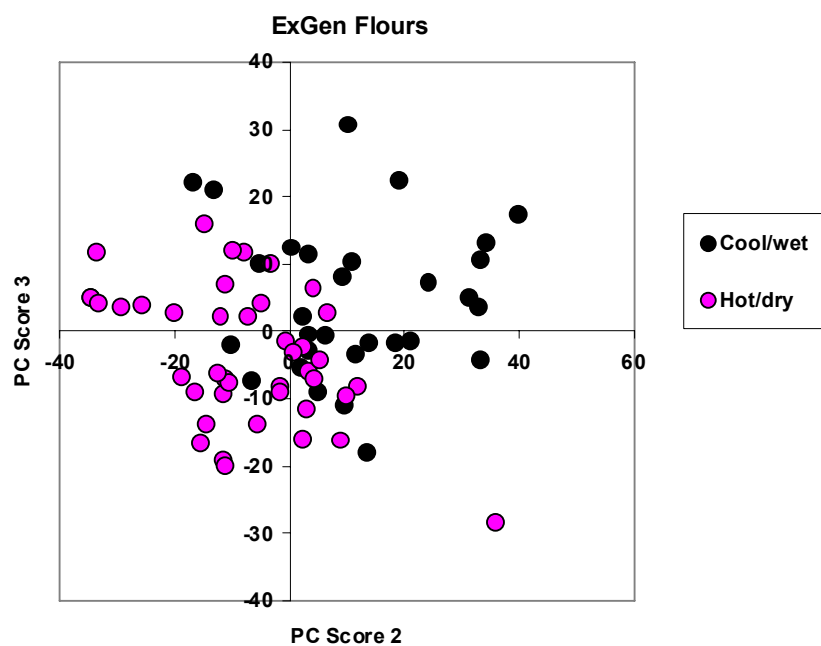


Figure 6.5: PCA plot for spectra collected for the AX 'gel-layer' extracted from dough made from the flour of wheat grown under two differing growing conditions.

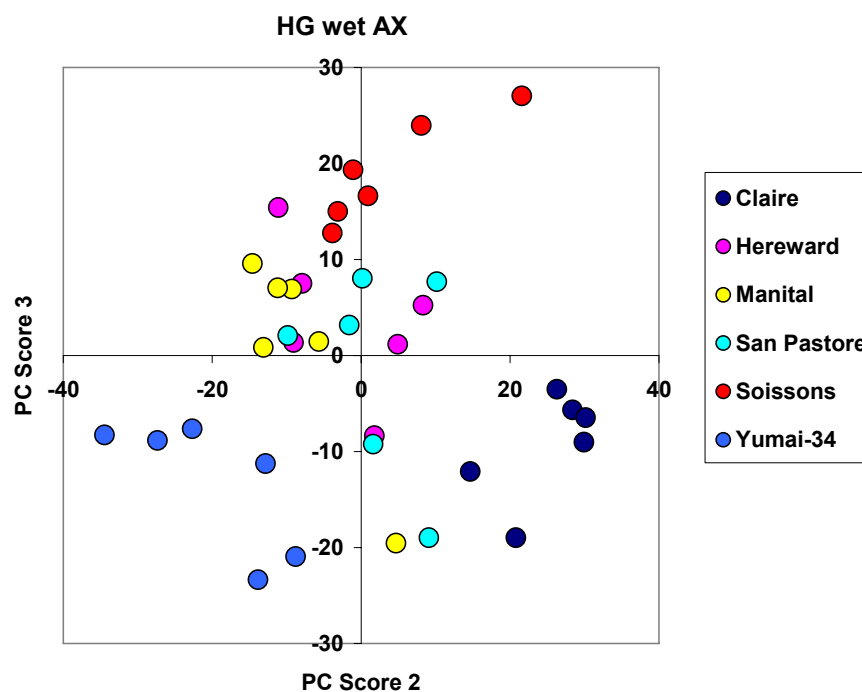
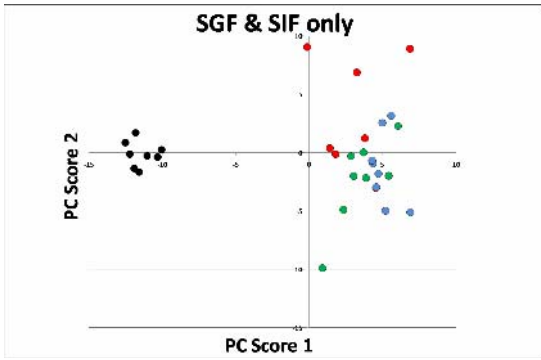
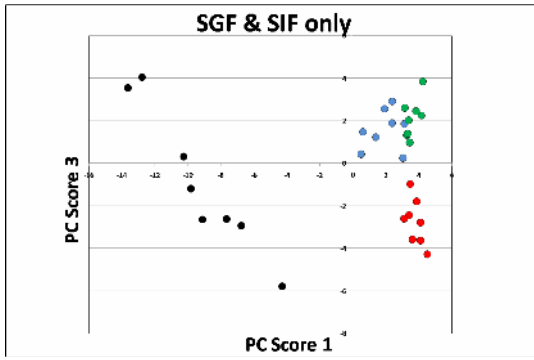


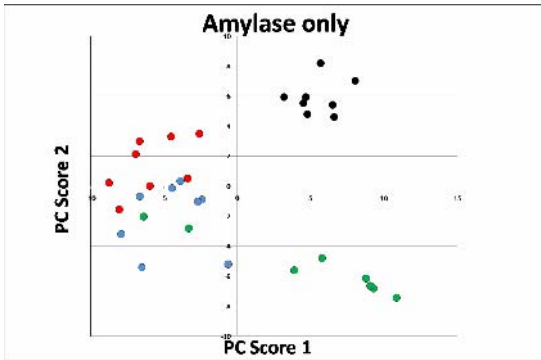
Figure 6.6: PCA plot for spectra collected for the AX 'gel-layer' extracted from dough made from the flour of six wheat cv's with differing levels of arabinose substitution in the AX in their endosperm cell walls.



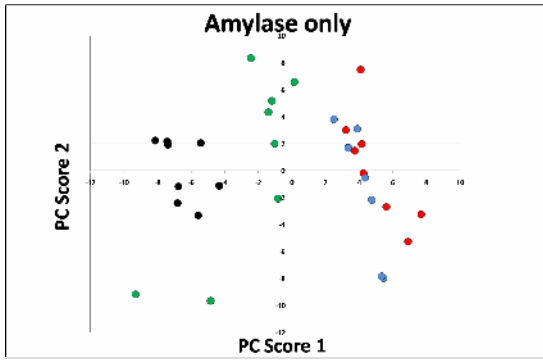
1A.



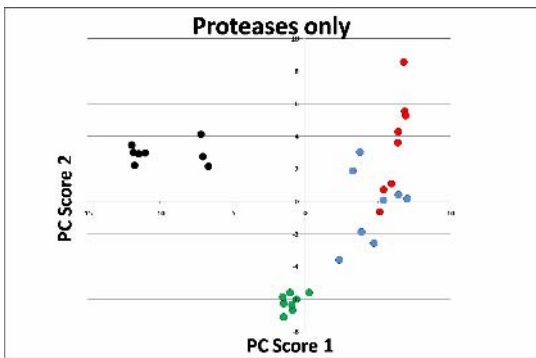
1B.



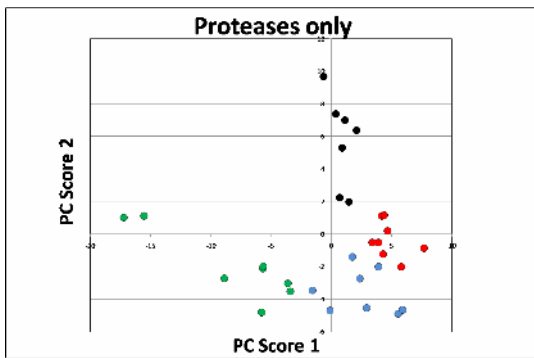
2A.



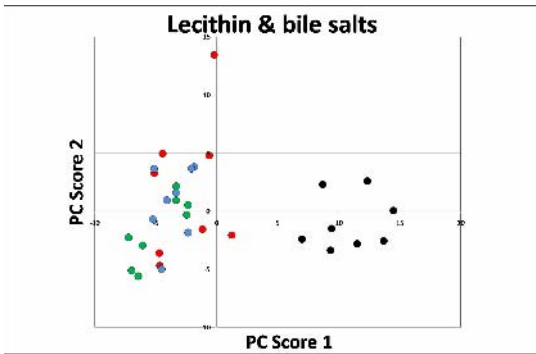
2B.



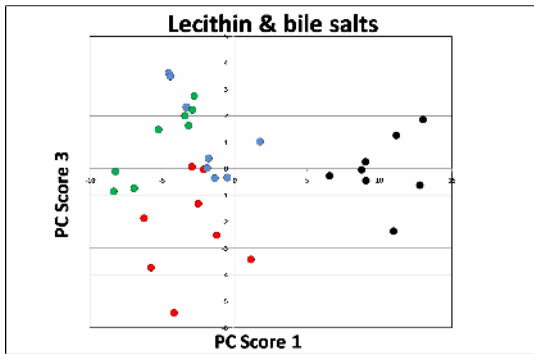
3A.



3B.



4A.



4B.

Figure 6.7: PCA plots for the FT-IR spectra for the digesta at each stage, for each individual digestion (black spots: undigested sample, red spots: 2 h gastric, blue spots: 3 h duodenal, green: 19 h).

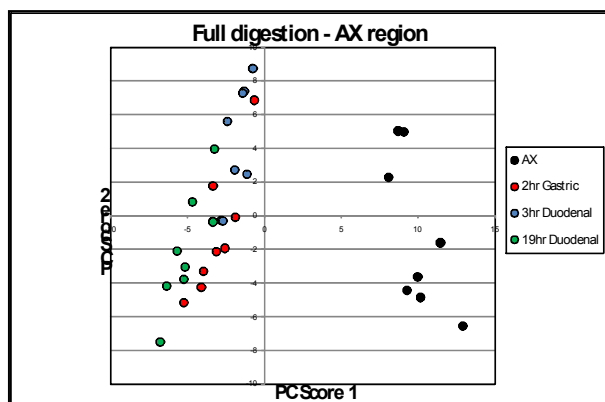


Figure 6.8: PCA plots for the FT-IR spectra for digesta at each stage, for the full digestion.

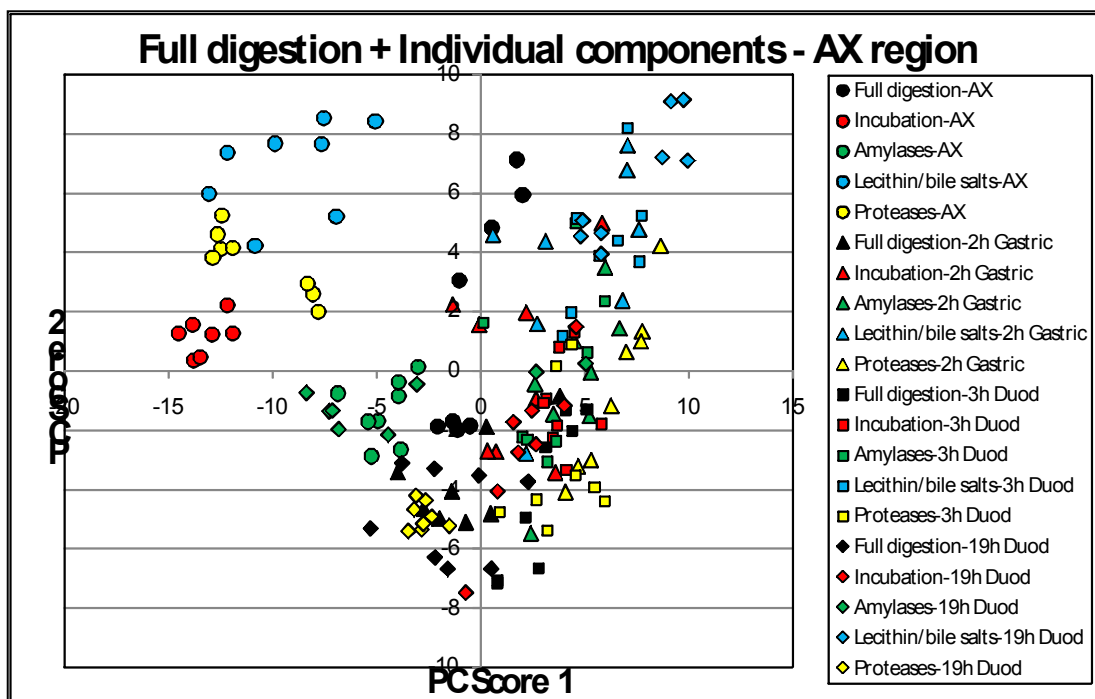


Figure 6.9A: PCA plot for the AX region of the FT-IR spectra for digesta at each stage, for all digestions.

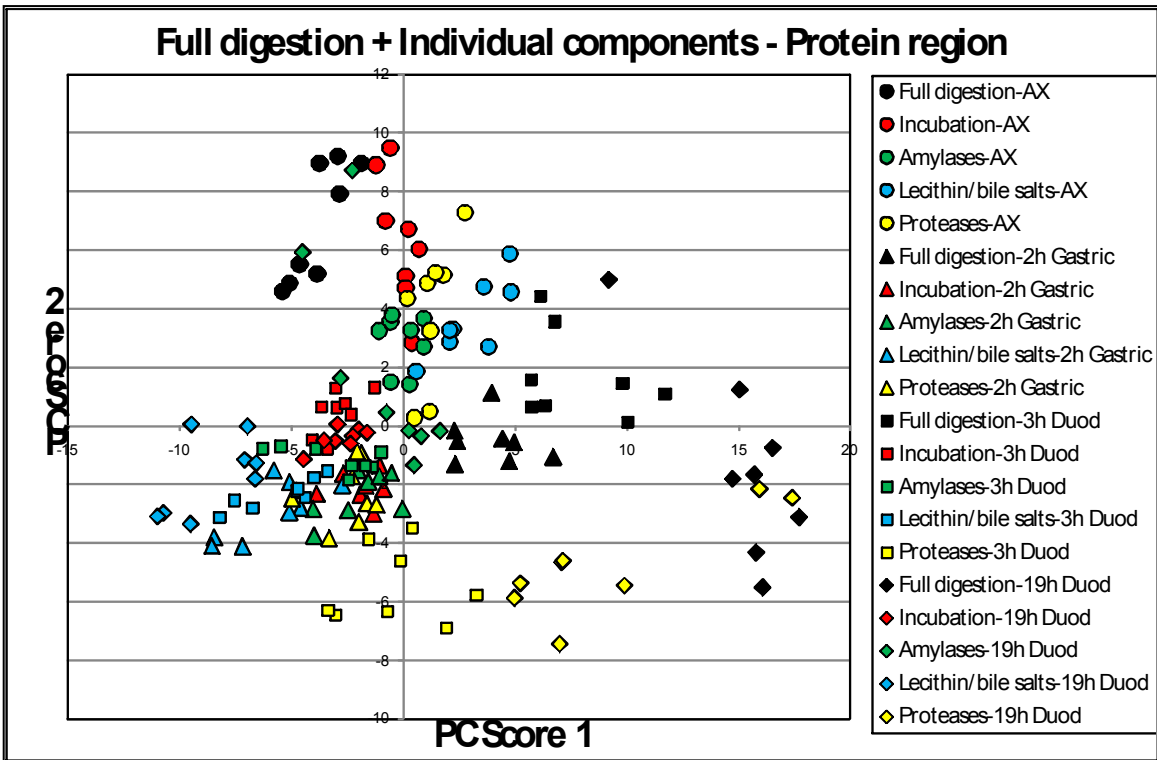


Figure 6.9B: PCA plot for the protein region of the FT-IR spectra for the digesta at each stage, for all digestions.

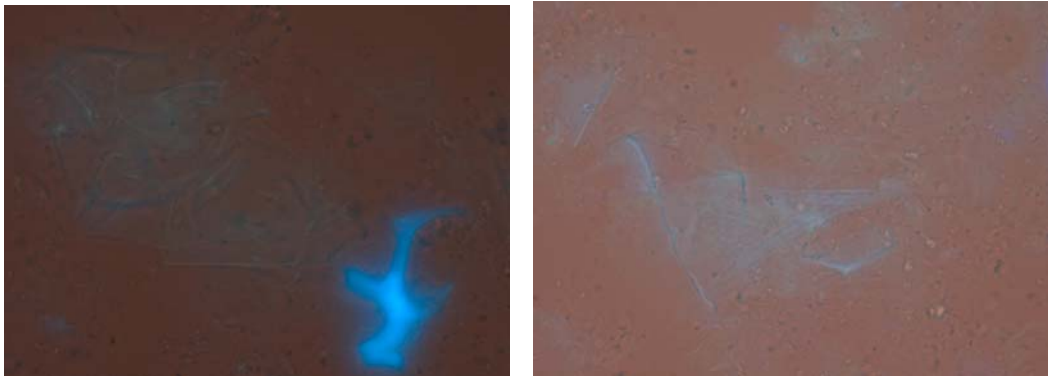


Figure 6.10: microscope images of cell-wall layer.

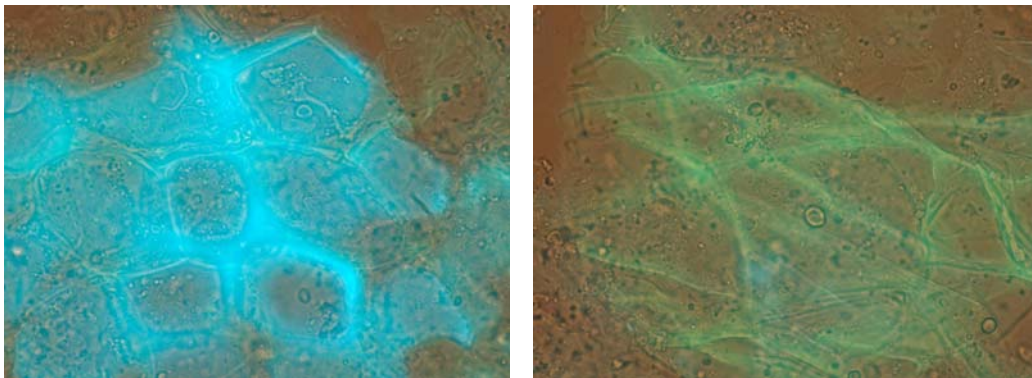


Figure 6.11: microscope images of the cell-wall layer following digestion for 2 hours under gastric conditions.

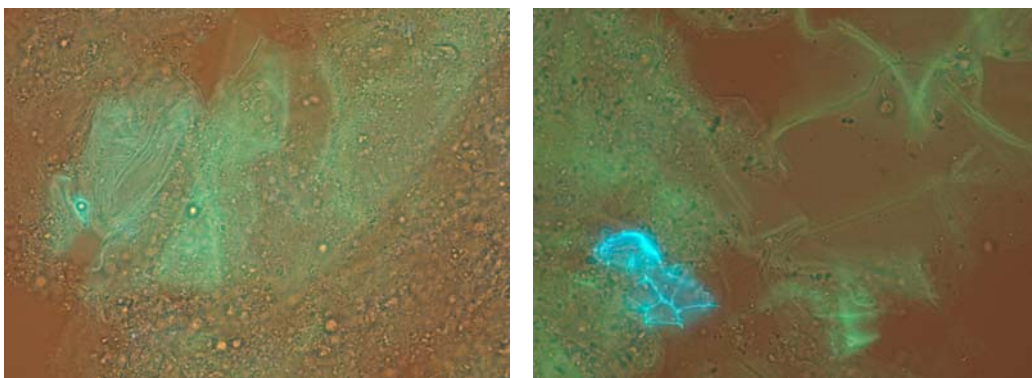


Figure 6.12: microscope images of the cell-wall layer following digestion for 2 hours under gastric conditions plus 16 hours in duodenal conditions.

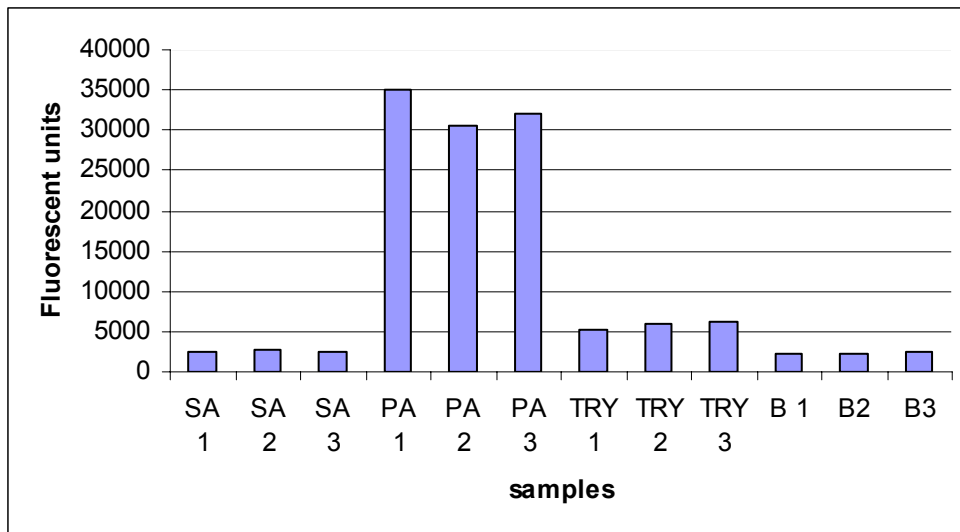


Figure 6.13: protease activity of: SA (salivary amylase), PA (pancreatic amylase), TRY (trypsin), B (blank). The assay was performed in triplicate.

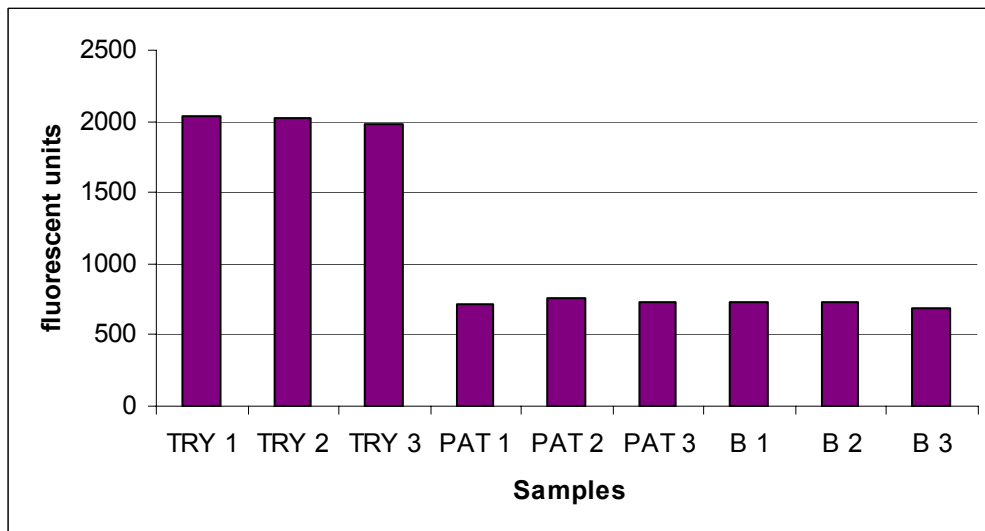


Figure 6.14: protease activity of: PAT (pancreatic amylase DFP treated), TRY (trypsin), B (blank). The assay was performed in triplicate.

