PLANT PROTEINS: FROM PROCESSING TO DIGESTION

Andrea Rivera del Rio

Propositions

1. Digestion is the one process step that all foods are subjected to, yet the one we understand the least.
   (this thesis)
2. Though we generally identify proteins by their origin (soybean, pea, lupin), their digestive behaviour is just as much determined by their fractionation and post-fractionation processes.
   (this thesis)
3. In science, an expert has the know-how but also the know-how-not.
4. Doing research is like starting on a path already traced by someone else, and then extending it towards new destinations.
5. The '*nose to tail*' concept should be applied to the evaluation of plant-based foods as it is not truly sustainable to discard parts of a pulse to obtain only one fraction.
6. Food culture is the ultimate form of oral tradition.
7. In moments of peril, such as during a pandemic, the needs of the individual tend to prevail over those of the collective.

Propositions belonging to the thesis, entitled

Plant proteins: from processing to digestion

Andrea Rivera del Rio

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Thesis

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Contents

List of abbreviations

A	acidified BSA
AA	amino acid
AHP	BSA pepsin-hydrolysate
AnH	acidified, non-hydrolysed BSA
AnHP	acidified, non-hydrolysed BSA in inactive pepsin
BCA	bicinchoninic acid
BSA	bovine serum albumin
DF	diafiltration
DH	degree of hydrolysis
DIAAS	digestible indispensable amino acid score
FTIR	Fourier-transformed infrared spectroscopy
FU	functional unit
GE	gastric emptying
HPSEC	high performance size exclusion chromatography
IAA	indispensable amino acid
ITC	isothermal titration calorimetry
LCA	life cycle assessment
MF	microfiltration
nAnH	non-acidified, non-hydrolysed BSA
NF	nanofiltration
NSPF	non-soluble protein fraction
OPA	*o*-phthalaldehyde
PC	protein concentrate
PDCAAS	protein digestibility-corrected amino acid score
PI	protein isolate
PPI	pea protein isolate
PRF	protein-rich fraction
SPF	soluble protein fraction
SPI	soybean protein isolate
TIA	trypsin inhibitory activity
TIU	trypsin inhibitory units
UF	ultrafiltration
WPI	whey protein isolate

CHAPTER ONE

Introduction and thesis outline

It is well established that a shift towards healthier diets, i.e., those that favour plant over animal based foods, among other characteristics, can not only improve human health but also reduce the environmental impact of the current food production systems (Ruini et al., 2015; Willett et al., 2019). One of the ways that this transition can be facilitated is by the introduction of plant-based alternatives to meat, egg and dairy into the market. These products often rely on the use of protein-rich ingredients for their favourable techno-functionalities.

1.1 Why study the digestibility of plant proteins?

Incomplete protein digestion and absorption in the upper gastrointestinal tract from an otherwise healthy diet, would not only fail to meet nutrient requirements but it could also lead to the formation of harmful compounds in the large intestine. Dietary protein that is not degraded and absorbed in the small intestine is virtually lost for protein anabolism (Darragh et al., 1994). Colonic microbiota will instead hydrolyse the undigested proteins and peptides, and metabolise the resulting amino acids. Some of the resulting metabolites can be harmful to colonic health (Davila et al., 2013; Portune et al., 2016).

Plant proteins have the potential to provide all indispensable amino acids (Gorissen et al., 2018), but these proteins have to be digestible and their amino acids bioavailable (FAO, 2011). In broad terms, the digestibility of plant proteins is governed by the physical and structural exposure of cleavage sites to digestive enzymes, as well as the presence of antinutritional factors. Cell walls in whole pulses enclose the protein bodies and physically limit the contact between enzymes and their substrates (Zahir et al., 2018). Furthermore, the cleavage sites in proteins that are folded into a compact structure may be buried in the matrix (Dupont & Nau, 2019). Antinutritional factors such as trypsin and chymotrypsin inhibitors, polyphenols, phytates and lectins impair the utilization of nutrients by binding to dietary micronutrients, target enzymes or the gastrointestinal

mucosa (Sarwar Gilani et al., 2012). Furthermore, prolonged exposure to protease inhibitors can have harmful effects on gut health (Kårlund et al., 2021). At the same time, some antinutritional factors have shown beneficial health effects (Petroski & Minich, 2020). The processing history of plant protein-rich ingredients can greatly influence the digestibility of plant proteins, by curtailing the cell wall integrity, altering the protein conformation and reducing the activity of some antinutritional factors (Avilés-Gaxiola et al., 2018; Sá et al., 2020a).

1.2 Dynamic aspects of protein digestion

Protein digestion is defined as '*the whole of the physical, chemical, and biochemical processes carried out by living organisms to break down ingested proteins into components that may be easily absorbed and directed into metabolism*' (from the Gene Ontology annotation 0044256, Binns et al. (2009)). These processes involve several intricate neural and endocrine pathways that control many functions, such as motor activity, secretions, transit, local blood flow, satiety, defence mechanisms, among others. As a dynamic process, adequate digestion depends on the interplay of these functions occurring in a timely manner. The following sections describe some of the dynamic events relevant to protein digestion. Secretions, luminal pH, enzymatic activity, gastric emptying and transit are among the dynamic events evolving over time, relevant to protein digestion.

1.2.1 Luminal pH

The intragastric pH is one of the key factors governing protein digestion. Extrinsic factors associated to the properties of the food bolus influence the luminal pH, e.g., the pH of the meal, the buffering capacity of food, particularly of protein (Mennah-Govela et al., 2020) and the physical state of the consumed food (Reynaud et al., 2020). The pH of a meal can influence postprandial metabolic events such as the glycaemic response (Freitas et al., 2021). In the stomach, the secretion of acid and pepsinogen is regulated by hormonal responses (Ferrua et al., 2014; Hunt et al., 2015). Moreover, the meal properties themselves may also influence the secretory response. It has been reported that meals with a higher buffering capacity induce larger amounts of gastric secretion (Bornhorst et al., 2013).

The pH in the stomach not only changes over time but also spatially. Bornhorst et al. (2014) were among the first to clearly demonstrate the pH distribution within the stomach of pigs. The spatial pH heterogeneity was associated to the consistency of the food, as softer foods allow for better intragastric mixing and thus less heterogeneity. Likewise, the relationship between chyme viscosity, mixing efficiency and the kinetics of acidification

was identified in a study with pigs that were fed meals of identical composition but different structure and texture (Nau et al., 2019). Finally, the residence time of the food bolus in the stomach is also related to the propensity to pH reduction within it.

1.2.2 Enzyme activity

The dependency of pepsin activity on the pH has been studied thoroughly (Campos & Sancho, 2003; Luo et al., 2018; Piper & Fenton, 1965). This dependency is also associated to the substrate. Salelles et al. (2021) discarded the notion that pepsin is only active at low pH and highlighted the importance of the initial, lowly acidic, phase of gastric digestion. Given long enough digestion times, similar extents of pepsin-catalysed hydrolysis of casein micelles were achieved in the broad range of pH 1 to 5. Furthermore, the enzyme remained active at up to pH 7, although significantly less than at lower pH. This behaviour was dependent on the substrate, as the degree of hydrolysis of egg white protein was significantly lower at higher pH. The kinetics of pepsin and trypsin-catalysed hydrolysis of proteins has been shown to depend on pH, ionic strength and molecular crowding (Luo et al., 2018; Maximova & Trylska, 2015; Maximova et al., 2018).

1.2.3 Gastric emptying and transit

The transit of chyme, the digestate coming from the stomach towards the small intestine, i.e., gastric emptying, starts almost immediately after a meal is ingested (Val-Laillet et al., 2010). The properties of the components in the gastric content, such as structure, influence the residence time in the stomach and gastric emptying (Barbé et al., 2013; Nau et al., 2019). As is well known, casein is retained in the stomach due to intragastric coagulation, while whey proteins, mostly β-lactoglobulin, are quickly emptied into the duodenum and are therefore, earlier absorbed through the enterocytes (Boirie et al., 1997).

The energy density of the meal also influences the rate of gastric emptying. Camps et al. (2016) found that liquid meals with higher energy density had a longer residence time in the stomach than those with a lower energy density. This enterogastric inhibitory reflex regulates the delivery rate of energy to the duodenum and ultimately, the bloodstream (Hunt et al., 1985; Roman, 1982). When certain receptors on the proximal intestinal mucosa are stimulated by, e.g., osmotic factors or duodenal distension, gastric peristalsis is inhibited, and gastric emptying is slowed down. It has been suggested that longer residence times in the stomach of most of the bolus increases satiety (Mackie et al., 2013).

Similarly, the secretion of pancreatic fluid, the pH adjustments, the transit, the motility and the hydrolysis in the small intestine and the absorption through the enterocytes evolve over time (Sensoy, 2021).

1.3 Digestion models

The great complexity of the digestion process makes it difficult to understand the individual processes at play. *In vitro* modelling involves the creation of a simplified system that only mimics part of the process, thereby reducing the complexity and making assessment of the individual sub-processes more accessible. The understanding that follows from these studies can then by quantified in an *in silico* model, which allows the integration of individual sub-models into a larger one, thereby allowing the study of the interaction between the individual sub-processes. This then allows better comparison to *in vivo* digestion.

1.3.1 *In vitro* models

The field of food digestion is evolving rapidly to address the complexities of foods and the digestive system. As an example, in the course of the study that resulted in this PhD thesis, two updates to the INFOGEST *in vitro* digestion method (Minekus et al., 2014) were published, one static (Brodkorb et al., 2019) and one semi-dynamic (Mulet-Cabero et al., 2020), as well as many additions and adjustments to address specific research questions. The harmonized static *in vitro* digestion method has been validated, comparing gastric and intestinal endpoint protein hydrolysis of milk proteins from an *in vivo* assay (Egger et al., 2017). The content of intact proteins in *in vitro* gastric samples were comparable to that measured in gastric and duodenal *in vivo* samples, while *in vitro* intestinal samples resembled *in vivo* distal jejunal samples.

Even though endpoint determinations from static methods are useful to compare the extent of digestion between different products, no light is shed on the dynamic nature of food digestion. The dynamic aspects can be reproduced to a certain extent with more sophisticated models, such as the Human Gastric Simulator (Kong & Singh, 2010), the Gastric Simulation Model (Li et al., 2019), the Dynamic *In Vitro* Human Stomach system (Peng et al., 2021), the TNO gastrointestinal model (Minekus, 2015), the DIDGI® system (Ménard et al., 2015), or simpler models such as the standardized semi-dynamic method (Mulet-Cabero et al., 2020). One should realize that the disadvantage of more complex models is that they incorporate more aspects and therefore make mechanistic understanding of the phenomena taking place, more difficult. Therefore, both complex models and much simpler models have their utility and typically should be used together.

Similar to the standardised static method, some dynamic models have been validated against *in vivo* observations (Dupont et al., 2019).

1.3.2 *In silico* models

Alongside with dynamic *in vitro* digestion models, the next frontier in simulating food digestion lies in compartmental *in silico* models. *In silico* models of events occurring throughout the oro-gastrointestinal tract continue to be proposed, their scope ranging from food breakdown, secretions, mixing, transit, enzymatic hydrolysis, absorption, and so forth (INFOGEST working group 6, 2021). Computational modelling allows the combination of sub-models into a comprehensive model that can also capture the interaction between different phenomena taking place.

For the luminal pH, the model from van der Sman et al. (2020) simulates the pH changes within a food particle in the gastric environment, accounting for diffusion of relevant gastric juice and food components, in and out of the particle. Meanwhile, Li and Jin (2021) describe the spatial and temporal dynamics of gastric pH considering intragastric mixing by terminal antral contraction of the food bolus and gastric secretions, as well as the gastric emptying of chyme as a function of the energy density. A nutrient feedback mechanism from the rate of absorption of nutrients in the small intestine has been simulated by relating the viscosity of the meal to gastric emptying (Moxon et al., 2017).

Different approaches have been used to describe the enzyme-catalysed hydrolysis of macronutrients considering relevant influencing factors (Le Feunteun, Verkempinck, et al., 2021). For proteins, the most common approach is using the Michaelis-Menten equation, however some stochastic models have been recently proposed as well. The model by Tonda et al. (2017) predicts the products of hydrolysis by endoproteases, such as pepsin, based on the primary structure of the protein and the specificity of the enzyme towards certain positions surrounding the cleavage site. First steps have been taken towards incorporating structural attributes, charge and hydrophobicity into the propensity for pepsin-catalysed hydrolysis of a peptide bond (Suwareh et al., 2021). The affinity of pepsin towards specific peptide bonds was found to be higher in negatively-charged, non-hydrophobic environments surrounding the peptide bond. Conversely, positively charged environments surrounding the cleavage site were preferred for pepsin-catalysed hydrolysis.

Le Feunteun, Al-Razaz, et al. (2021) published a comprehensive review on physiologically based compartmental models. Of note is an extensive compartmental model of digestion in pigs that includes events from the stomach to the large intestine including the action of microbiota (Strathe et al., 2008). This model considers protein,

including endogenous protein, lipids, carbohydrates and fibre. They proposed an option to include the effect of antinutritional factors in feed.

In silico modelling generally helps to better capture the key causal phenomena leading to specific effects and can help quantify the dynamics of the processes. In addition, it can help understand the interaction between different sub-processes, such as mass transfer, pH changes and enzymatic hydrolysis. *In vitro* models are important in obtaining insight in understanding and quantifying the individual sub-process under well-defined conditions.

1.4 Aim and outline of this thesis

While extensive research has focused on studying and maximizing the techno-functionality of plant protein-rich ingredients for new food products, much less is known about how the processing history of these plant proteins and foods influences their digestibility. Furthermore, the relevance of the gastric phase on the overall digestion process has received little attention even though it might have important influence in the overall digestibility of ingredients and foods.

The overall aim of this thesis is therefore to better relate the effects of processing, particularly heating, on the digestibility of plant proteins, which will allow us to make better informed choices when producing sustainable alternatives to animal-based products. Due to the complexity of this task, multiple approaches and methods were employed and proposed. The thesis follows two main axes (Figure 1.1). First, the effect of processing on the digestibility of plant protein rich ingredients is described from previous studies and from *in vitro* assays. Second, different approaches to simulate the dynamic aspects of digestion were implemented to explore the relevance of the gastric phase on protein digestion as a whole.

Chapter 2 reviews the insights from previous studies on the effect on protein digestibility of processing before, during and after fractionation of plant protein-rich ingredients. In Chapter 3, we investigate the effect of heat treatment of full dispersions from soybean and yellow pea protein isolates on the *in vitro* gastric digestion, as well as that of the non-soluble and dispersed fractions. Isothermal titration calorimetry is used in Chapter 4 to quantify the kinetics of pepsin- and trypsin-catalysed hydrolysis of a model protein. In this chapter, the importance of the gastric phase on the gastro-duodenal digestion of protein is assessed by simulating different extents of exposure to the gastric environment prior to trypsin-catalysed hydrolysis.

Continuing along the lines of the relevance of the gastric phase on digestion and plant proteins, the digestion of pea protein was simulated with the standardised semi-dynamic

in vitro method, as presented in Chapter 5. Furthermore, the effect of protein denaturation on digestibility was studied in native and heat-denatured, pea proteins from mild aqueous fractionation, compared to a conventional pea protein isolate that served as a benchmark for denatured protein.

In Chapter 6, the results from Chapter 5 are incorporated to a life-cycle assessment of the production of pea protein-rich ingredients, with digestible protein and digestible indispensable amino acid contents, as functional units. Whey protein isolate served as a reference for a widely used protein of animal origin.

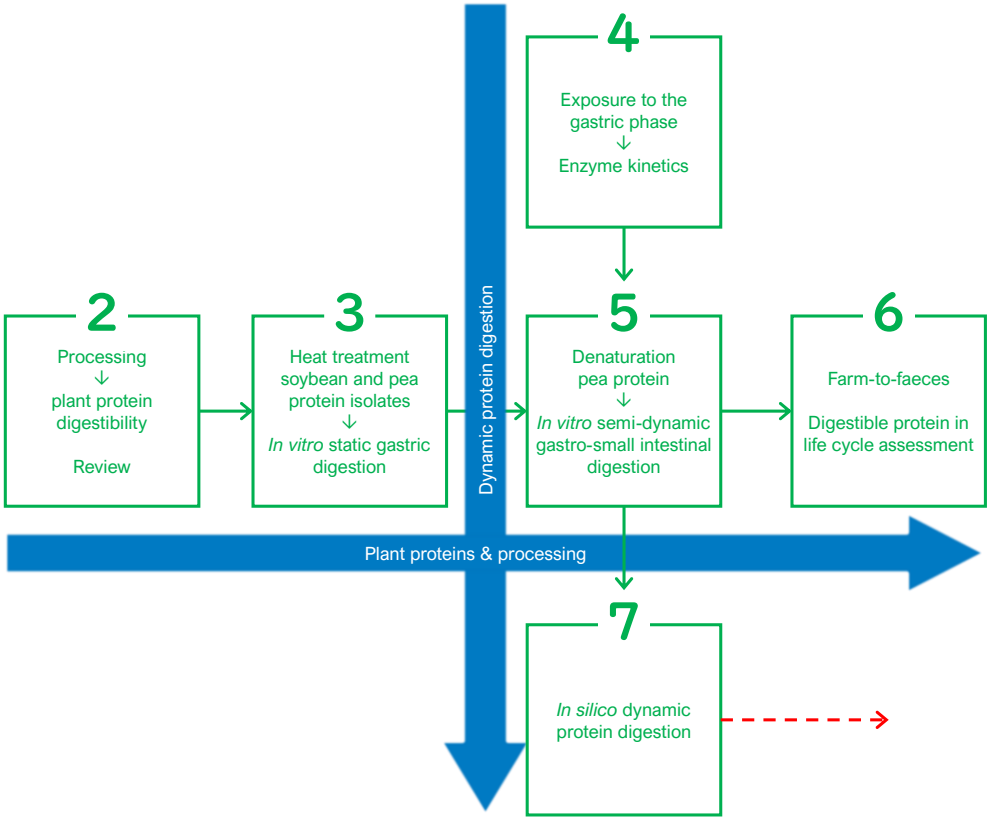

Figure 1.1 Graphical outline of the chapters in this thesis.

An approach to *in silico* modelling of gastro-intestinal digestion is described in Chapter 7. Protein digestion was outlined by hydrolysis as a function of gastric pH and protease concentration, as well as by absorption of amino acids into the bloodstream. Gastric emptying was described as a function of the caloric density of gastric chyme. Further, a lag time to activate antral grinding was incorporated to distinguish the residence time of solid/liquid and blended meals.

Lastly, Chapter 8 includes a general discussion of the results and main findings presented in this thesis. Further applications towards a more widespread use of *in silico* modelling are proposed. The outlook and future challenges to study the digestibility of plant protein ingredients in food products are addressed.

CHAPTER TWO

Effect of fractionation and processing conditions on the digestibility of plant proteins as food ingredients

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Abstract

A shift from animal- to plant-based foods is one of the pathways necessary to make the food production systems more sustainable. Plant protein-rich ingredients are used to produce alternatives to meat, dairy and eggs. Fractionation of ingredients and subsequent processing into food products modify the techno-functional and nutritional properties of proteins. The differences in composition and structure of plant proteins in addition to the wide range of processing steps and conditions can have ambivalent effects on the protein digestibility. The objective of this review is to assess the current knowledge on the effect of processing of plant protein-rich ingredients on their digestibility. We obtained data on various fractionation conditions, processing after fractionation including enzymatic hydrolysis, alkaline treatment, heating, high pressure, fermentation, complexation, extrusion, gelation, as well as oxidation and interactions with other nutrients. We provide an overview of the direction of the effect of some processing steps for different sources. Some studies explored the effect of processing on the presence of antinutritional factors which can be decisive in the fate of proteins in the gastrointestinal tract. A certain degree, and type, of processing can improve protein digestibility, while more extensive processing can be detrimental. We argue that processing, protein bioavailability and the digestibility of plant based-foods must be addressed in combination to truly improve the sustainability of the current food system.

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2.1 Introduction

The current food production system is not sustainable (Rockström et al., 2009). The largest environmental impact can be attributed to the production of animal-based protein (Aiking & de Boer, 2020). One of the measures proposed by Willett et al. (2019) to reduce this negative impact is to lower our consumption of foods of animal origin, and to increase that of plant-based foods. To facilitate this transition, plant-based alternatives to meat, dairy and eggs, are continuously introduced to the market. Nevertheless, the extensive transformation and purification of the ingredients, in addition to the lower protein yield from crop to food product limits the sustainability potential (Smetana et al., 2015; van der Weele et al., 2019).

It is not clear yet whether plant and animal-based proteins can be interchangeable from a nutritional point of view. The dietary requirement of indispensable amino acids (AA) can be satisfied by proteins from various crops (Herreman et al., 2020; Sá et al., 2020b). Antinutritional factors, digestibility and bioavailability must also be considered when assessing the nutritional quality of proteins. The *in vivo* protein digestibility–corrected amino acid score (PDCAAS) (Rutherfurd et al., 2015) and *in vitro* digestibility (Santos-Hernández et al., 2020) of some protein-rich ingredients and whole foods have been reported. Furthermore, the effect of domestic and industrial processing on digestion of proteins from legumes consumed as a whole food or flour, i.e., not as a protein-rich ingredients has been reviewed (Drulyte & Orlien, 2019).

Plant proteins are diverse and most constitute a mixture of various protein units, each with its own properties. For instance, varieties of the same legume species have different globulin to albumin ratios. Globulins have been found to be more susceptible to hydrolysis by digestive enzymes (Liu et al., 2008; Sathe, Iyer, et al., 1982). Moreover, 7S and 11S globulin-rich protein fractions from hemp protein isolate (PI) presented different *in vitro* digestion profiles (Wang et al., 2008). Yang et al. (2016) found that higher proportions of β-7S subunits had a detrimental effect on the *in vitro* digestibility of soybean PI. Protein concentrates (PC) from different cultivars of the same species can present different structural, thermal, techno-functional properties and nutritional value, such as the indispensable AA content and the digestibility, as was found for rice and millet proteins (Mohamed et al., 2009; Singh & Sogi, 2018). Meanwhile, different varieties of lupin and sorghum do differ in composition and structural properties but are digested to a similar extent (Espinosa-Ramírez & Serna-Saldívar, 2016; Vogelsang-O'Dwyer, Bez, et al., 2020). This already suggests that the digestibility of proteins from different plant sources might not be affected in the same way by a given type of processing.

The objective of this article is to review the large body of data on the digestion of protein-rich ingredients and on how processing, before, during or after the extraction of the ingredient, may alter it. We recognize the breadth of protocols used to simulate digestion as well as the methods used to describe or quantify the extent of it (Figure 2.1). As these confounding factors contribute to variations in results, we limited this review to studies that compare some treatment or processing to a control and noted the effect on protein digestibility of a given ingredient.

There is quite some disparity in the number of studies favouring some types of processing over others, as well as some crops over others. Moreover, the wide range of digestion assays make it relatively futile to quantitatively compare results from different studies. We therefore present a narrative review with elements of a systematic one, instead of a full systematic review with meta-analysis.

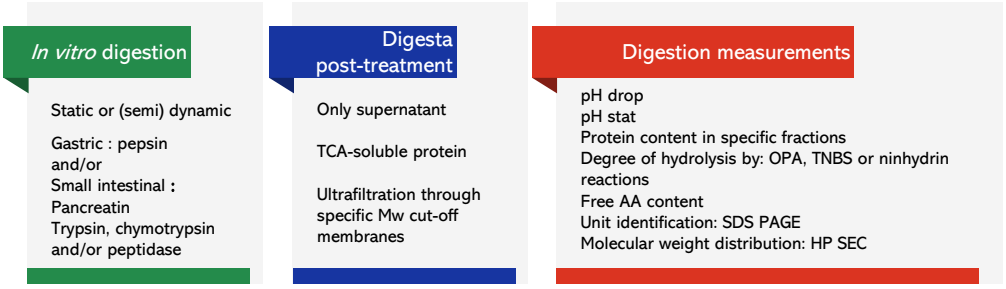

Figure 2.1 Characteristics of *in vitro* assays, treatment of digesta and description or quantification of *digestibility* in the studies reviewed. (AA, amino acid; HP SEC, high performance size exclusion chromatography; Mw, molecular weight; OPA, *o*-phthalaldehyde; SDS PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TNBS, trinitro-benzene-sulfonic acid)

2.2 Method and definitions

Review characteristics: The search query used in Scopus was: *("protein" W/6 digest*) AND "in vitro" AND "human" AND ("gastric" OR "intestinal" OR "gastrointestinal" OR "pepsin" OR "trypsin")*. In PubMed, the MeSH terms for *"Plant proteins, dietary"* and *"Digestion"* were also included. From the results, the works considering some measure of digestibility or protein hydrolysis by digestive enzymes, simulating some physiological condition(s) were included. Studies on whole foods or flours were not considered as these sources have been studied elsewhere. Articles studying feed, e.g., for ruminal digestion, emulsions, animal-sourced foods or proteins, and works dealing with allergenicity or immunoreactivity were excluded.

The term *'protein digestibility'* is used rather ambiguously throughout the reviewed literature. By definition, digestibility is the proportion of an ingested food or nutrient that can be absorbed into the bloodstream or body. However, it is also used to describe

protein degradability, i.e., the proportion of intact protein remaining, the resulting degree of hydrolysis (DH) or the proportion of low molecular weight peptides resulting after the action of digestive enzymes. Other measurements of digestibility are listed in Figure 2.1 and details of the digestion assays and measurements for each of the studies reviewed are listed in the appendix (Table A2.1).

Figure 2.2 presents a scheme of the different processes reviewed. Throughout the text, *'conventional aqueous fractionation'* refers to milling, optional defatting for oil-containing seeds, alkaline extraction, centrifugation, isoelectric precipitation, centrifugation, washing and freeze drying, as it is mostly done in laboratory-setting, or spray drying, more common in commercially available ingredients. Table 2.1 summarizes the effects on digestibility of the more commonly studied processes for different plant sources.

2.3 Ingredient preparation

2.3.1 Pre-fractionation treatment

Most commonly, seeds are milled into a flour or grits prior to alkaline extraction. Soaking seeds at high temperatures, before milling for conventional aqueous fractionation, was shown to improve the *in vitro* digestibility of soybean and cowpea PI. In the work of Wally-Vallim et al. (2014), PI from soybean seeds soaked at 40 °C was more digestible than at 60 °C. The *in vitro* gastric digestibility was improved by longer soaking times for both temperatures. It was argued that at 40 °C, proteins were partially denatured, while at 60 °C the 7S fraction was completely denatured and protein structures had rearranged. Meanwhile, PI from soaked and autoclaved cowpea seeds was more extensively hydrolysed by pepsin-pancreatin than that from raw seeds (Marques et al., 2015).

Some studies explored the effect of germination prior to fractionation of soybean and black bean. A direct relation between the germination time and the extent of hydrolysis achieved by digestive enzymes was observed (Dikshit & Ghadle, 2003; López-Barrios et al., 2016). Concurrently, the trypsin inhibitory activity (TIA) was reduced by germination, associated to protease-catalysed hydrolysis of lectins and trypsin-inhibitors. Aijie et al. (2014) found a similar relation, however, the DH decreased, and the TIA increased for the longest germination times, which they explained by a re-synthesis of trypsin inhibitors by photosynthesis. For black soybean, an inverse relation was observed: the PI produced from non-germinated seeds yielded the largest proportion of low molecular weight peptides (Sefatie et al., 2013). It was hypothesised that these small peptides were used for tissue formation during germination.

Solid state and submerged fermentation of milled lupin with different strains of *Pediococcus* prior to subsequent conventional aqueous fractionation improved the *in*

vitro protein digestibility in the PI compared to the non-fermented control (Bartkiene et al., 2018). At the same time, the fermentation reduced the content of trypsin inhibitors. No clear relation can be drawn between the type of fermentation and digestibility, as many different lupin hybrid lines and strains of *Pediococcus* were studied.

2.3.2 Conventional protein fractionation

After a defatted meal has been obtained, alkaline extraction is the first step in conventional aqueous fractionation. Higher protein purities, at the expense of lower yields, can be obtained with increasingly higher concentrations of a strong alkali, typically NaOH. Alkaline treatment has been associated with the formation of lysinoalanine and AA isomerisation in rice residue PI, reducing the *in vitro* digestibility and absorption in a rat model (Zhang et al., 2019). Protein extracted from defatted lupin meal at acidic pH (pH 2) was more readily and extensively digested than that extracted at neutral or alkaline pH (pH 8.5) conditions, using an *in vitro* digestion assay (Yu et al., 1987). The extraction pH was thought to induce different structural conformations and extents of denaturation. Nevertheless, Ruiz et al. (2016) did not find a significant effect on the *in vitro* gastric digestion of quinoa PI extracted at pH 8 to 11.

Either PC or PI can be obtained from the conventional fractionation process. Commercial PC and PI have been used in *in vivo* rat assays, showing a small variation in PDCAAS, the true or standard ileal digestibility, of soybean ingredients (Hughes et al., 2011; Pedersen et al., 2016). Meanwhile, the *in vitro* gastric digestibility of commercial soybean PI remained unchanged after long-term storage at freezing and high temperatures (Da Silva Pinto et al., 2005).

2.3.3 Alternative protein fractionation strategies

Modifications to the conventional aqueous fractionation process have been proposed to improve the purity, yield or techno-functional properties of the ingredients obtained. Conventionally, alkaline extraction is performed with NaOH, with the pH adjustment for isoelectric precipitation done with HCl. Chamba et al. (2013) proposed the use of alkaline ash from burnt green and purple amaranth and lemon juice as 'natural' alternatives to the more commonly used chemicals to isolate soybean protein from full fat and defatted flour. The PDCAAS was slightly higher for the material extracted with 'natural' chemicals, while no significant difference was observed between the *in vitro* pepsin-pancreatin digestibility of 'natural' and conventional chemicals. The use of conventional chemicals was somewhat more effective at reducing the content of antinutritional factors such as trypsin

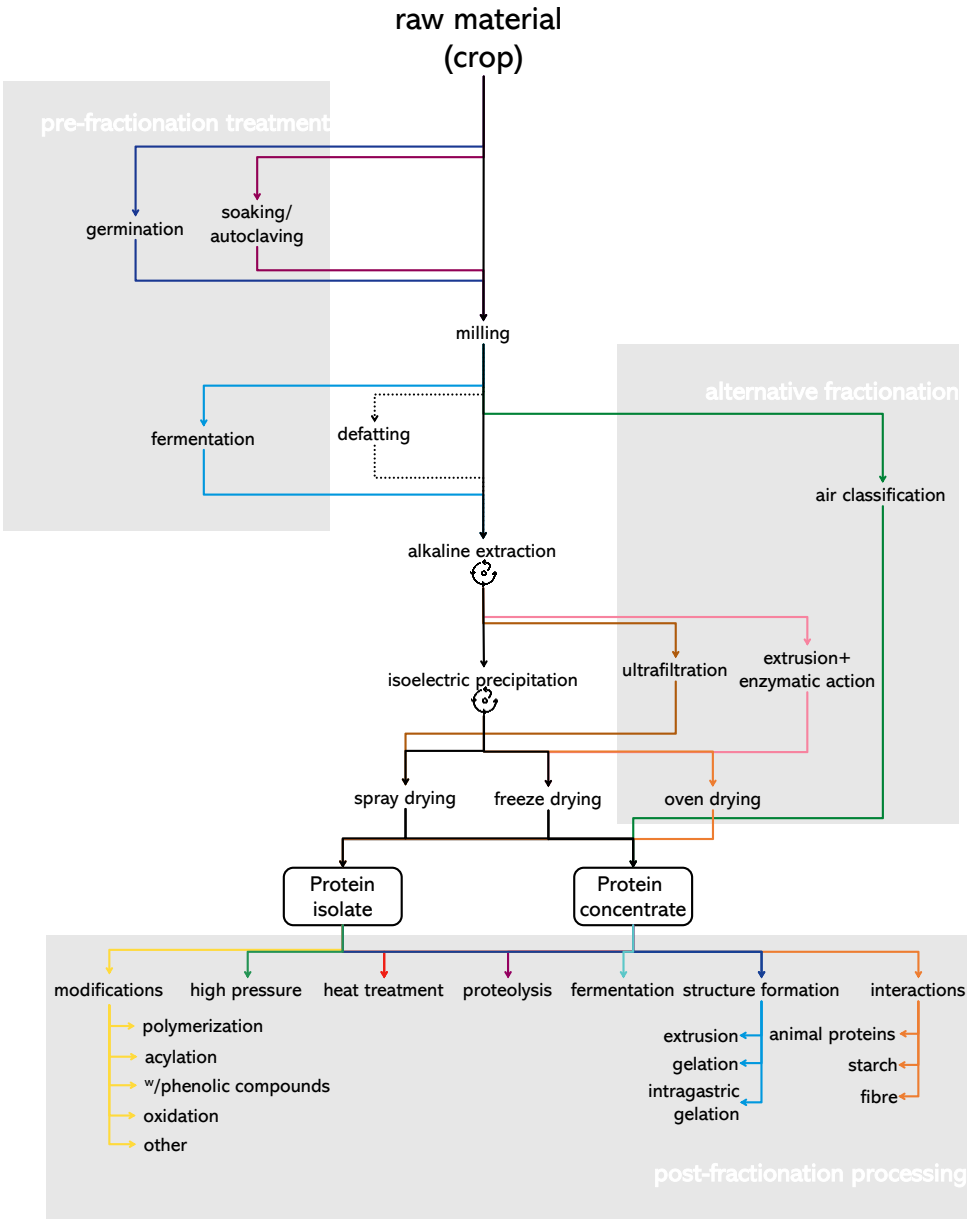

Figure 2.2 Overview of the processing steps before, during and after fractionation of plant proteins from the studies included in this review. Colours indicate the different routes for processing, the conventional route for aqueous fractionation is presented in black, ⟲ represents centrifugation after alkaline extraction and isoelectric precipitation.

inhibitors and phytic acids in PI. Na_2SO_3 has been used to extract proteins and to prevent oxidative darkening of the PI, from lupin and chickpea. The digestibility of Na_2SO_3-extracted lupin PI was higher than the conventionally fractionated ingredient

(Lqari et al., 2002). However, for chickpea PI, the digestibility from both extractions did not differ (Sánchez-Vioque, Clemente, et al., 1999).

Ultrafiltration has been used as an alternative to isoelectric precipitation. The TIA was similarly reduced by either process for soybean PI (Baker & Rackis, 1986). The extent of hydrolysis achieved with pepsin-pancreatin digestion, as well as the reduction of the TIA, was comparable for brown lentil PI separated by ultrafiltration and for conventional isoelectric precipitation (Joehnke et al., 2021).

The effect of different drying methods on the protein digestibility was studied for buckwheat and hempseed PI. Tang (2007) showed that freeze drying, compared to spray drying, produces buckwheat PI that is better digestible by pepsin-trypsin. However, when alkaline extraction was assisted by ultrasonic treatment instead of by just mechanical stirring, freeze- and spray-dried PI were equally digestible. Meanwhile, Lin et al. (2021) compared vacuum oven, oven or freeze drying of hempseed PC. In this study, freeze drying also produced better digestible PC compared to drying at higher temperatures, which was attributed to the formation of poorly digestible Maillard products during oven or vacuum oven drying.

Enzyme-assisted fractionation paired with extrusion has been presented as an environmentally friendly alternative to conventional aqueous fractionation (De Almeida et al., 2014). Oil and protein were simultaneously extracted from soybean flakes that were extruded and treated with a bacterial endoprotease under alkaline conditions to obtain oil-, fibre- and protein-sugar-rich fractions. Extrusion or enzyme action during processing did not alter the pepsin digestibility of the resulting ingredients, though some techno-functional properties were improved. Extrusion and α-amylase-catalysed starch liquefaction were used to concentrate proteins from white sorghum (De Mesa-Stonestreet et al., 2012). While the moisture content in the barrel during extrusion influenced the *in vitro* gastric digestibility, no effect from α-amylase action was observed. Nevertheless, the sorghum PC showed lower digestibility than sorghum flour. This was attributed to re-aggregation during the boiling step that was used for enzyme inactivation.

Air classification is a dry fractionation technique. The digestibility of pea, lentil and fava bean PC obtained from air classification were compared to that of NaCl-extracted PI from aqueous fractionation in a mice-study (Bhatty & Christison, 1984). Overall, the digestibility of the PC was lower than that of the PI, most significantly for pea. Likewise, air-classified fava bean PC was less extensively hydrolysed during pepsin-pancreatin digestion than a PI from isoelectric precipitation and spray drying (Vogelsang-O'Dwyer, Petersen, et al., 2020). Further, the TIA from the initial flour was maintained in the air-

classified ingredient and significantly reduced in the conventionally produced PI. Conversely, air jet-sieved quinoa PC was slightly more extensively hydrolysed by pepsin than a conventional aqueous-fractionated PI (Opazo-Navarrete, Schutyser, et al., 2018). We hypothesize that the protein denaturation achieved through heating during spray drying facilitates the access of digestive enzymes to the cleavage sites within the proteins.

2.4 Post-fractionation processing

Protein ingredients are further processed into finished products. The effects of different protein steps (fermentation, ultrasound treatment, heating, protein modification, among others) have been researched on PI and PC from various crops. Ultrasonic treatment of fava bean PI dispersions slightly reduced the *in vitro* digestibility (Martínez-Velasco et al., 2018).

Fermentation of commercial pea PC with *Lactobacillus plantarum* had a positive effect on the *in vitro* protein digestibility and a reduction of antinutritional factors, phenols, tannins, chymotrypsin and trypsin inhibitors. Nevertheless, the *in vitro* PDCAAS was negatively impacted. This was explained by the catabolism of sulphur-containing AA by the lactic acid bacteria (Çabuk et al., 2018). Similarly, *L. plantarum*-fermented soybean PI released more free AA than the non-fermented control, in a dynamic *in vitro* gastrointestinal digestion assay (Huang et al., 2020). Additionally, protein aggregation was observed in the gastric phase only for the non-fermented PI, as well as a higher proportion of high molecular weight peptides at the beginning of the intestinal phase.

2.4.1 Proteolysis

Protein hydrolysis has mixed effects on protein digestibility. For soybean protein, hydrolysis by immobilized trypsin improved or had no effect on the extent of digestion (Ge & Zhang, 1993). In this study, pre-digested proteins were better digestible under infant gastric condition, simulated by a less acidic pH (pH 4) compared to adult models. Meanwhile, a soybean protein pepsin-hydrolysate was just as digestible as the intact PI, in a different infant model with reduced digestive enzyme concentration, compared to an adult model (Nguyen et al., 2016).

A series of studies investigated the effect of the co-ingestion of soybean PI and dietary actinidin from green kiwifruit extract on the protein digestion. From an *in vitro* pepsin-pancreatin assay, some subunits such as the 11S basic polypeptide showed some effect of the actinidin, however no overall effect on the protein degradability was observed (Kaur et al., 2010). From an *in vivo* rat study, the presence of actinidin in the diet showed no significant effect on the true ileal digestibility of soybean PI (Rutherfurd et al., 2011).

Gastric chyme samples from a subsequent rat study were analysed for their true gastric total protein digestion (Montoya et al., 2014). Interestingly, the presence of actinidin here improved the gastric digestibility of the PI. Meanwhile, actinidin did have a positive effect on the digestibility of zein but had virtually no effect on the digestibility of wheat gluten. These studies highlight the relevance of the type of assay and measure of digestion to assess the effect of processing or modification on plant protein digestibility.

Green lentil PI from conventional aqueous fractionation was hydrolysed with acid protease, actinidin, bromelain and papain, prior to *in vitro* digestion (Aryee & Boye, 2016). Intact proteins proved to be better hydrolysable than the protein hydrolysates. Nevertheless, as a net result, more low molecular weight peptides were produced from the protein hydrolysates than from intact PI.

Hydrolysis positively affected the digestibility of rapeseed and rice bran PI. Fibre and protein from a rapeseed PI that was obtained by membrane processing, were hydrolysed (Fleddermann et al., 2013). The true digestibility of the hydrolysate was higher than the intact PI, as shown by a rat assay. As a result, the PDCAAS of the hydrolysate was also higher, compared to the original ingredient. Similarly, for progressively higher degrees of hydrolysis, a papain-hydrolysate of rice bran PC was more extensively digested than the intact PI by pepsin-pancreatin digestion (Singh et al., 2021).

Chickpea protein hydrolysis did not alter the digestion. Neither alcalase, flavourzyme (Clemente et al., 1999), trypsin, papain nor pepsin (Goertzen et al., 2021) changed the extent of protein digestibility in *in vitro* assays. Nevertheless, the TIA was significantly reduced by the hydrolysis (Clemente et al., 1999).

2.4.2 Heat treatment

The process step most studied in terms of its effect on protein digestibility is heat treatment. Different conditions as well as different crops have been studied with positive, neutral or negative effects of heating on protein digestibility.

It is commonly thought that a certain extent of heat induced-protein denaturation improves the digestibility, while more extensive heat treatment would induce protein aggregation which would, in turn, reduce the digestibility. The work of Tian et al. (2019) demonstrates the relation between heating time and temperature, and the extent of pepsin-catalysed hydrolysis of soybean PI. Dispersions heated at 85 °C for 15 min presented the highest DH, while those heated at 70 or 100 °C were hydrolysed to a significantly lesser extent. In terms of time, PI heated at 85 °C for 20 min showed the highest DH compared to those heated for 10 or 60 min. Overall, all heated samples were more extensively hydrolysed than the unheated control.

Soybean is one of the crops most widely studied in terms of the effect of heat treatment on protein digestibility. Studies have shown improvement but also reduction of protein digestibility as a result of heat treatment. The *in vitro* pepsin-pancreatin digestibility of soybean PI was improved by relatively short heating for 15 min at 95 to 121 °C (Chen et al., 2015; Ren et al., 2018; Zhang et al., 2018). β-conglycinin is known to be less susceptible to pepsin-catalysed hydrolysis than glycinin. Nevertheless, the gastric digestibility of both fractions was improved by heat treatment (Chen et al., 2015). In this study, heating did induce protein aggregation as well as did pepsin during the gastric phase. The TIA of germinated soybean PI was reduced by the heat treatment (Aijie et al., 2014). Conversely, the apparent digestibility of heated, spray-dried and autoclaved pastes of soybean PI, determined in a rat assay, was significantly lower than that of non-autoclaved pastes (Kim & Barbeau, 1991). Besides the heat treatment during drying, these pastes were autoclaved for up to four hours, highlighting that extensive heat treatment, both in time and temperature, has a detrimental effect on protein digestibility.

Heat treatment does not affect the digestibility of different pulse protein ingredients in the same way. Heating at 95 °C for 30 min improved the pepsin-trypsin digestibility of mung bean PI, reduced it for red bean PI, and did not change it for red kidney bean PI (C.-H. Tang et al., 2009). Interestingly, a larger extent of aggregation in heated mung bean PI was reported than in red kidney bean PI. It was suggested that the presence of basic, hydrophobic and uncharged polar AA influences the thermal and structural stability of proteins, and thus the tendency to aggregate when heated. Meanwhile, the *in vitro* digestibility of lupin and winged bean PC was improved by heating in a boiling water bath for up to 30 min (Sathe, Deshpande, et al., 1982a, 1982b). The trypsin and chymotrypsin inhibitory activity of the freeze-dried winged bean PC was inactivated by heat treatment (Sathe, Deshpande, et al., 1982b).

Likewise, the digestibilities of individual protein fractions from different crops are not modified in a similar manner upon heating. Vicilin-like proteins from chickpea and common bean are both resistant to gastric digestion; however, the digestibility of the former was improved by autoclaving, while for the latter, it was reduced (Carbonaro et al., 2005; Tavano & Neves, 2008). Furthermore, chickpea albumin, 11S and total globulin digestibility increased, as a result of heat treatment (Tavano & Neves, 2008). Conversely, native protein fractions from fava bean were better digestible than those that denatured after autoclaving (Carbonaro et al., 2005).

One might expect that preventing heat-induced aggregation would lead to a positive effect on protein digestibility. This was observed for lentil globulins which were

unsusceptible to heat-induced aggregation, given that disulphide interactions were not observed (Neves & Lourenço, 1995). Nevertheless, the negative charge of a protein fraction from common bean made the protein less prone to aggregation and yet less digestible than its unheated, less negatively charged, counterpart (Carbonaro et al., 2005). Based on the effect of heating on the electric charge of proteins and peptides, the latter study suggested that protein electronegativity and hydrophobicity were associated with protein aggregation and digestibility.

Net-zero effects may result from concurring events improving and reducing the DH achieved by digestive enzymes. Commercial soybean and pea PI dispersions heated at 90 and 120 °C for 30 min did not show different DH during *in vitro* gastric digestion compared to their unheated counterparts. Upon close inspection of the soluble and sedimented tailings, we found that heating in fact improved the solubility of the commercial PI, and that the proteins separated into this fraction could be more extensively hydrolysed than those in the sedimented fractions (Rivera del Rio et al., 2020).

Meanwhile, for dry-fractionated ingredients, heat treatment has shown to reduce the gastric digestibility of lupin and quinoa proteins. More small peptides (<3 kDa) were released from the unheated and heated at 60 °C dispersions of air-classified lupin PC than the dispersion heated at 90 °C (Pelgrom et al., 2014). A similar trend was observed for dry fractionated quinoa PC, with unheated and heated at 60 °C dispersions being more extensively hydrolysed than dispersions heated at 90 and 120 °C (Opazo-Navarrete, Schutyser, et al., 2018; Opazo-Navarrete et al., 2019). Similarly, quinoa PI from conventional aqueous fractionation showed lower DH with increasingly higher heating temperatures (Ruiz et al., 2016).

As previously discussed, alkaline heat treatment generally is detrimental for protein digestibility. Heating at higher pH reduced the *in vitro* protein digestibility of globulins from navy bean (Chang & Satterlee, 1981), of soybean PI (Wu et al., 1999) and rapeseed PC (Savoie et al., 1991). These results were confirmed for spray-dried soybean PI by an *in vivo* rat study (Sarwar et al., 1999). For the most part, the limited digestibility can be attributed to the formation of lysinoalanine at high pH (Sarwar et al., 1999; Savoie et al., 1991; Wu et al., 1999).

Thus far we discussed studies on so called moist heating, but the environment during heating does influence the protein digestibility. Sathe, Iyer, et al. (1982) compared dry and moist heating of navy bean PC and PI extracted with Na_2CO_3, as well as water-extracted albumins and NaCl-extracted globulins. The DH achieved with trypsin-α-

Table 2.1 Overview of the effect of different types of processing before, during or after protein fractionation from different crops. 🔴,🟡 negative; 🟢,🟢 positive; or 🔵 neutral effect on protein digestibility. Only processes or ingredients with more than one study reporting on the effect of processing on digestibility were included in this table.

Crop	Pre-fractionation	Fractionation		Processing										Nutrient interactions
	germination	dry fractionation	alkaline treatment	fermentation	enzymatic hydrolysis	heating	high pressure	polymerization	acylation	phenolic compounds	oxidation	gelling	extrusion	starch
soybean	🟢🔴	-	🔴 also post-fractionation	🟢	🔵🟢	🟢🔴	-	🔴	🟢 hydrolysate	🟢🔴	🟢🔴	🔴	🟢	-
black bean	🟢	-	-	-	-	-	-	🔴	-	-	-	-	-	-
chickpea	-	-	-	-	🔵	🟢	-	-	-	-	🔴	-	-	-
fava bean	-	🟡	-	-	-	🔴 legumin	-	-	-	-	-	-	-	-
lentil	-	🔵	-	-	🔴	🟢 globulin	-	-	-	-	-	-	-	-
lupin	-	-	🔴	🟢 pre-fractionation	-	🟡🟢	-	-	-	-	-	-	-	-
maize	-	-	-	-	🟢	-	-	-	-	-	-	-	🟢	-
mung bean	-	-	-	-	-	🟢	-	-	🟢	-	-	-	-	-
navy bean	-	-	🔴	-	-	🟢	-	-	-	-	-	-	-	
quinoa	-	🟢	🔵	-	-	🔴	-	-	-	-	-	-	-	🔴
rapeseed	-	-	🔴	-	🟢	-	-	-	-	-	-	-	-	-
red kidney bean	-	-	-	-	-	🔵	🔴	🟢	🟢	-	-	-	-	-
rice	-	-	🔴	-	🟢	-	-	-	-	-	-	-	-	-
yellow pea	-	🔴	-	🟡🟢	-	🔵	🟢	-	-	🔴	-	🔵	🟢	🔴

chymotrypsin-peptidase was improved more significantly by moist than by dry heating. Similarly, boiling, microwaving, autoclaving, and dry or oven heating improved the digestibility of sweet potato PC (Sun et al., 2012). Autoclaved dispersions presented the highest DH by pepsin-pancreatin digestion, followed by microwave and, lastly, dry heating. The PDCAAS determined in a rat assay was improved for autoclaved PI compared to the unheated ingredient. As previously reported, the TIA was reduced by all types of heat treatments studied.

These observations give a sense of the optimum range of heat treatment to improve the protein digestibility; more heating can negatively impact the digestibility (Table 2.1). The appropriate heat treatment would then depend on the ingredient source, the type of protein fraction, the type and conditions of heating.

2.4.3 High pressure processing

Laguna et al. (2017) conducted a comprehensive study on the effect of heating and high pressure processing at two different pH (3.6 and 6.2) of commercial pea PI on its *in vitro* digestibility. For the most part, high pressure processing improved the gastric digestibility of pea protein. Samples prepared at a higher pH were more digestible than those at pH 3.6. Autoclaving did not alter the protein digestibility at either pH, which shows that the effect of pressure cannot be explained by denaturation, similar to that during heating. High pressure processing followed by a 30 min, 80 °C heat treatment at pH 3.6 reduced the protein digestibility. In contrast, high pressure processed red kidney bean PI presented a significantly lower *in vitro* digestibility by trypsin (Yin et al., 2008). This was attributed to the generally low digestibility of phaseolin, particularly when aggregated. In this case, we may conclude that the protein source, as much as the processing steps, influence the digestibility of proteins.

2.5 Crosslinking, complexation and other modifications

Forming protein complexes with other proteins or other compounds can be an unintended consequence of combining materials in one matrix or can be intentionally induced to achieve certain functions, such as colon-targeted drug delivery (Mariniello et al., 2007) or to confer an added nutritional benefit (Strauch & Lila, 2021).

2.5.1 Transglutaminase-catalysed polymerization

Phaseolin from *Phaseolus vulgaris L.* was cross-linked by microbial transglutaminase (Mariniello et al., 2007). Its isopeptide bonds made phaseolin more resistant to pepsin and trypsin action, especially for pepsin. Similarly, the pepsin-trypsin digestibility was reduced for native and heated crosslinked proteins from soybean PI, while it was

improved by heat treatment alone (Tang et al., 2006). It should be noted that while a single protein source was used in this study, covalent crosslinks were identified between β-conglycinin and acidic subunits of glycinin. In contrast, positive effects on the trypsin-digestibility as a consequence of crosslinking by transglutaminase have been reported in red kidney bean PI (Tang et al., 2008). The digestibility increased in crosslinked protein with longer crosslink reaction times, which was attributed to protein unfolding and denaturation of the vicilin unit.

Limited protein degradation by pepsin-pancreatin was observed for soybean PI polymers and heteropolymers with whey PI or casein, compared to the untreated PI (Li & Damodaran, 2017). Furthermore, soybean PI heteropolymers were more resistant to *in vitro* digestion compared to the whey PI-casein heteropolymer. This was attributed to reduced accessibility for enzymes to the peptide bonds, due to blockage of lysine residues and steric hinderance. Likewise, soybean PI-bovine gelatine composites showed lower pepsin-trypsin digestibility than the PI (Sheng & Zhao, 2013). Trypsin-catalysed hydrolysis, prior to *in vitro* digestion, increased the digestibility slightly but it remained significantly lower for the untreated PI.

Glycation and crosslinking soybean PI with chitosan, or oligo-chitosan with transglutaminase improved the pepsin-trypsin digestibility (Fu & Zhao, 2017; Zhu et al., 2016). Interestingly, the crosslinked soybean PI was more digestible than the untreated PI in both pepsin and pepsin-trypsin digestion assays.

To assess the effect of Maillard reaction products, crosslinked commercial soybean PI was heated with D-ribose or sucrose (Gan et al., 2009). Crosslinking had a negative effect on *in vitro* protein digestibility, particularly at longer transglutaminase incubation times. Overall, sucrose-containing samples were more digestible than ribose-containing samples. AA loss was reported as a consequence of crosslinking, most significantly of lysine.

Therefore, the effect of transglutaminase-catalysed crosslinking on protein digestibility depends on the extent to which cleavage sites become exposed or buried within the structure of the crosslinked protein. Furthermore, AA bioavailability could also decrease as a result of this processing step.

2.5.2 Acylation

Acylation of proteins can result in techno-functionality, such as solubility and emulsifying activity (Yin et al., 2009). Mung bean PI was acylated with succinic and acetic anhydrides (El-Adawy, 2000). The trypsin-pancreatin digestibility was improved by acylation, probably due to protein unfolding. Acetylation was reported to reduce antinutritional

factors (phytic acid, tannins and trypsin inhibitors) to a greater extent than succinylation. Similarly, acetylated and succinylated red kidney bean PI were more digestible by trypsin than their untreated counterpart (Yin et al., 2009). This was attributed to increased protein solubility and protein unfolding.

The improved digestibility due to acylation observed with these ingredients was also reported for a soybean PI hydrolysate (Achouri & Zhang, 2001). The *in vitro* digestibility was significantly higher for succinylated soybean PI hydrolysates compared to the non-succinylated control. The authors also attributed this effect to protein dissociation or unfolding, and an increase in solubility. de Regil and Calderón de la Barca (2004) assessed, the *in vivo* digestibility of a soybean protein hydrolysate enzymatically bound by chymotrypsin to methionine methyl-ester using a rat study. There was no significant difference between the apparent digestibility of modified soybean PI hydrolysate and the control with free methionine. Nevertheless, the protein efficiency ratio was significantly higher for the modified ingredient.

Again, protein unfolding is related to an improvement of its digestibility, as was also observed with thermal denaturation. Moreover, peptides of lower molecular weight and, perhaps as a result, increased solubility would generally result in better digestibility, unless opposed by other cross-effects.

2.5.3 Complexation with phenolic compounds

The digestibility of thermally denatured soybean PI was significantly improved, mostly by pepsin, when complexed with anthocyanins from black rice extract (Zhang et al., 2018). It was suggested that the network formed by the complex promotes enzymatic action is made possible by changes in the secondary structure; again, (partial) unfolding then facilitates the digestion. In a similar manner, soybean PI-curcumin complexes were more extensively hydrolysed than the non-complexed PI, particularly by pepsin, in a sequential pepsin-pancreatin *in vitro* digestion assay (Chen et al., 2015). Heating before complexation did not influence the extent of digestion of the proteins. Furthermore, the typically pepsin-resistant β-conglycinin unit was completely degraded when it was part of the curcumin nanocomplex. Budryn et al. (2015) studied soybean PI-hydroxycinnamic acids complexes, either individual 5-caffeoylquinic acid, caffeic acid or ferulic acid, combined in green coffee extract or encapsulated in β-cyclodextrin. The reduction in average molecular weight after pepsin-(trypsin-chymotrypsin) digestion was greater for the complexes than for the untreated PI. It was suggested that interactions and exposure of hydrophobic AA were responsible for the enhanced digestibility, even though proteases might also interact directly with hydroxycinnamic acids.

In contrast to the positive effects of anthocyanins and hydroxycinnamic acids, protein-polyphenol complexes reduce the digestibility of pea and soybean PI. Nine commercial pea PI with different physical and chemical characteristics were used to form complexes with polyphenols from cranberry pomace (Strauch & Lila, 2021). For some PI, no significant differences were found in the pepsin digestion of non-complexed and complexed proteins, however all complexed isolates were less extensively hydrolysed by pancreatin digestion. Interestingly, the digestion rate was inversely related to the particle size of the PI. Similarly, soybean PI complexed at 70 or 121 °C with polyphenols and flavonoids from black soybean seed coat extract, was less extensively hydrolysed by pepsin-trypsin than the non-complexed ingredient (Ren et al., 2018). Moreover, the DH was further reduced by increasing extract concentrations used to produce the complexes. Extract-enzyme or extract-protein interactions were thought to alter the digestive enzymes' conformation, rendering them inactive for protein hydrolysis. In a rat assay, the true nitrogen digestibility was reduced for soybean PI that was complexed with both chlorogenic acid and quercetin (Rohn et al., 2006). The PDCAAS was significantly reduced for derivatized protein with lysine being the limiting AA.

Yang et al. (2015) proposed a multistep process to produce a fermented soybean milk enriched with isoflavone aglycone. More intact proteins remained after pepsin-trypsin hydrolysis of the soybean PI-isoflavone complex, than of the PI. The isoflavone probably inhibited the protease activity. Nevertheless, heated and fermented soybean PI-isoflavone were more extensively hydrolysed than their unheated or non-fermented counterparts.

Phenolic compounds can modify the conformation not only of the proteins but also of the digestive enzymes. Changes in protein conformation can have a positive or negative effect on protein digestion. The former, if unfolding leads to the exposure of cleavage sites, or the latter, if it leads to steric hinderance surrounding the cleavage sites. Furthermore, phenolic compound could also act as inhibitors when bound to the digestive enzymes.

2.5.4 Protein oxidation

Zhao et al. (2020) found that a certain extent of protein oxidation had a positive effect on the soybean protein gastric digestibility as a result of protein unfolding, particularly for glycinin. However, severe treatments, i.e., by lipoxygenase-catalysed linoleic acid oxidation (Zhao et al., 2020) or by incubation with 2,2′-azobis (2-amidinopropane) dihydrochloride (Chen, Zhao, & Sun, 2013), had a negative impact. In the latter study, the action of the radical-generating compound did not affect the gastric digestion, but it did reduce the DH by pancreatin in the intestinal phase. This effect was directly

influenced by increasing concentrations of the compound in the system. It was shown that oxidation can degrade several AA and induce protein aggregation. Sánchez-Vioque, Vioque, et al. (1999) attributed a reduction in digestibility of chickpea legumin mixed with linolenic acid, to protein oxidation or non-covalent protein-lipid interactions. Meanwhile, no clear relation between carbonyl content, from oxidation products, and extent of hydrolysis in the gastric phase has been observed in thermomechanical processed soybean PC and PI (Duque-Estrada et al., 2019).

2.5.5 Other modifications

Soybean PI incubated with malonaldehyde, a lipid peroxidation product, was subjected to *in vitro* pepsin-pancreatin digestion (Chen, Zhao, Sun, et al., 2013). β subunits of β-conglycinin were somewhat degraded by pepsin but they became more resistant to pancreatin digestion with increasing malonaldehyde concentration. The availability of indispensable and total free AA after digestion decreased in modified soybean PI.
Soybean PI, cottonseed PC and peanut PC formed complexes with glucose or sucrose (Rhee & Rhee, 1981). *In vitro* digestibility was reduced by longer heating times to form the complexes. Protein-glucose complexes were less digestible than the sucrose complexes. Further, available lysine was reduced with heat treatment.
Lastly, soybean PI was incubated with phytase from *Aspergillus niger* to obtain ingredients with different phytate contents (Wang et al., 2014). Phytate content, parallel to TIA, was inversely related to pepsin-pancreatin digestibility.

Repeatedly, we find that any process or modification that would induce a certain degree of unfolding will generally facilitate digestion, but extensive unfolding, leading to aggregation will result in slower or reduced digestion. Furthermore, modification of AA, particularly of lysine, will often lead to their reduced bioavailability. Finally, processes that reduce or inactivate antinutritional factors, such as phytate or protease inhibitors, will also improve or facilitate the digestion of proteins.

2.6 Structure formation

2.6.1 Extrusion and texturization

The *in vitro* digestibility of yellow pea and soybean PC can be improved by extrusion. The barrel temperature and screw speed are positively related to the protein digestibility of air-classified pea PC, while the moisture content has a negative influence on its digestibility (Wang et al., 1999). Soybean PC, maize meal and cassava root starch were mixed and extruded (Omosebi et al., 2018). The samples extruded at the highest temperature, moisture content and screw speed were the most digestible. The TIA,

phytic acid and cyanide contents were reduced by extrusion, however, the tannin content was not reduced. Interestingly, higher temperatures during extrusion led to more digestible proteins, which opposes the observations from moist heat treatments (section 2.4.2), the reason is not fully understood and requires further research.

Duque-Estrada et al. (2019) explored the effect on *in vitro* gastric digestibility of high temperature shearing of soybean protein ingredients, as well as the relevance of structure and size reduction in the digestibility. Sheared samples were cut into small pieces or ground into finer particles. Pepsin-catalysed hydrolysis was faster for unheated dispersions, followed by ground matrices. Cut samples were more slowly and less extensively hydrolysed than the other physical states.

2.6.2 Pre- and intra-gastric gelation

Opazo-Navarrete, Altenburg, et al. (2018) related the mechanical strength and porosity of heat-induced gels of soybean PI and pea PC to their gastric digestibility. No significant differences were observed between gels pre-heated at different temperatures. Soybean protein gels were less extensively hydrolysed than the control consisting of a protein dispersion, unlike pea protein gels that were hydrolysed to a similar extent as the dispersion.

Pressure-induced gels from air-classified lentil and fava bean PC were more digestible than heat-induced gels under *in vitro* gastric conditions (Hall & Moraru, 2021). It was suggested that the network of pressure-induced gels allowed a similar extent of access to pepsin as in concentrated protein dispersions. Meanwhile, both treatments changed the structure of the 55 kDa fractions to be better digestible in the gastric phase. Interestingly, the TIA was more significantly reduced by heating than by pressurization.
Soybean PI coagulates formed with $MgCl_2$ or glucono-δ-lactone were more digestible than gels prepared with transglutaminase (Rui et al., 2016). This was attributed to the covalent iso-peptide bonds formed by transglutaminase that cannot be degraded during *in vitro* gastrointestinal digestion. In contrast, the non-covalent bonds formed during coagulation by $MgCl_2$ or glucono-δ-lactone could be broken during digestion. Soybean PI and glycerol films were prepared with ferulic acid, tannin, corn starch or H_2O_2 at pH 7 to 10 (Ou et al., 2004). The gastric digestibility of the films was significantly lower than that of the PI in a dispersion, except for the films prepared with corn starch, which were digested to a similar extent as the control. Lysine availability also was lower in films. Ferulic acid and tannins were thought to form crosslinks with AA, while H_2O_2 could have oxidized certain AA. Lastly, films formed at pH 9 and 10 were less digestible than at pH<8.5. This was attributed to AA isomerisation and crosslinking at high pH.

In a simulated gastric environment, dispersions of soybean PI and negatively charged polysaccharides (xanthan gum, carrageenan (Hu et al., 2017) or alginate (Huang et al., 2018)) self-assemble into a hydrogel. The pepsin-catalysed hydrolysis of the gels was slower even at low polysaccharide contents, compared to the single PI. Hu et al. (2017) similarly found that soybean PI-carrageenan gels were digested more slowly than those with xanthan gum, due to the more compact and dense gel network in the former.

Generally, structure formation led to a slower and sometimes lower extent of hydrolysis by digestive enzymes compared to liquid dispersions. This is explained by physical hinderance surrounding the cleavage sites. Therefore, looser structures as weaker gels allow for a better digestibility than tighter structures. Further, covalent crosslinking inhibits protein unfolding, while non-covalent bonds can dissociate, especially at lower pH in the stomach, and thus allow for faster digestion. As heat treatment is often required before gelation, antinutritional factors, such as trypsin inhibitors, can also be inactivated.

2.7 Macronutrient interactions

Proteins are almost never processed or consumed on their own. The effect of the interaction of proteins with other macronutrients on protein digestion is not fully understood but there are some general directions suggested.

2.7.1 Animal- and plant-based protein hybrid foods

Reconstituted beverages containing the combination of bovine milk PC and soybean, pea or rice PI showed an improved *in vitro* DH and PDCAAS of blends compared to individual plant proteins (Khalesi & Fitzgerald, 2021). However, this was not observed in solid matrices. Proteins from pea PI, rice protein or lentil flour were enzymatically-bound to beef chuck ground meat using transglutaminase (Baugreet et al., 2019). The cooked restructured beef steaks were digested using the INFOGEST 2.0 model with expectorated boluses. No outstanding differences were observed in the peptide size distribution in the digestates of the samples with different treatments. Lentil-enriched steaks released the highest amounts of free isoleucine, lysine, phenylalanine and valine. Interestingly, protein (re-)aggregation was observed after *in vitro* gastrointestinal digestion.

2.7.2 Starch

Oñate Narciso and Brennan (2018) found a relationship between the amylose content of starch with protein digestion. Pea PI was combined with starch from basmati and glutinous rice, with high and low amylose to amylopectin ratios, respectively. All proteins

from the samples prepared with glutinous rice starch were degraded after pepsin-pancreatin digestion, but the vicilin and legumin acidic subunit from basmati rice starch samples remained after digestion. The authors proposed that the proteins were embedded into the amylose network. Similarly, quinoa protein from aqueous or dry fractionation was combined with starch-rich fractions from dry or mild aqueous fractionation, which after heating showed lower DH from *in vitro* gastric digestion than starch-free, unheated protein dispersions (Opazo-Navarrete, Schutyser, et al., 2018; Opazo-Navarrete et al., 2019). This reduction directly related to the heating temperature and was thus probably associated to starch gelatinization. Therefore, embedding the protein in a gelatinized starch gel does reduce the digestibility, probably due to the inaccessibility of the gel for the enzymes.

2.7.3 Fibre

The DH obtained by pepsin digestion of dry-fractionated quinoa PC was slightly reduced in quinoa fibre-containing unheated and heated dispersions (Opazo-Navarrete et al., 2019). The effect of fibre on quinoa protein gastric digestion was not as significant as for starch. Interestingly, fibre seemed to counter the low hydrolysis induced by starch gelatinization. The fibre does not form a gel that is difficult to penetrate for enzymes but may induce somewhat better mixing due to the higher viscosity.

2.8 Conclusion

Plant proteins have the potential to provide all indispensable amino acids. However, as described at length, processing and plant protein digestibility are strongly related.

Heating and soybean are the process step and crop most researched, reflective of their ubiquity in the production of plant-based food products. Moderate heating may enhance the digestibility by inducing partial unfolding of the proteins, thereby rendering them better accessible for the proteases. However, extensive heating induces aggregation, which makes the cleavage sites less accessible. Similar effects are seen with other types of treatments. Acylation of protein-rich ingredients improved their digestibility, probably also due to partial unfolding. Meanwhile, alkaline treatment, during or after fractionation, consistently reduces the digestibility of different crops, since it strongly changes the structure of the protein and induces AA isomerisation. Again, we see an optimum in the severity of the treatments for digestibility. It is however clear that the exact impact depends on the origins of the proteins.

Ultimately, it is desirable to attain an overarching relationship between the digestibility and the modifications resulting from processing. This review can serve as a guide when considering a certain processing step in the production of plant-based alternatives to animal-sourced products. There are ample opportunities for further research of unexplored processes for promising crops and vice versa, to truly consider the use of plant protein-rich ingredients in food products as a transition pathway to a more sustainable food system.

Appendix

Table A2.1 Summary of digestion assays, sample treatment and measurement from studies investigating the effect of processing on protein digestion.

Crop	Digestion assay gastric	small intestinal	Digestion sample treatment/measurement	Effect	Reference
Pre-fractionation treatments					
	High temperature soaking				
soybean	3 h	N/A	TCA-soluble nitrogen	↑	Wally-Vallim et al. (2014)
cowpea	2 h	2 h pancreatin	supernatant, ultrafiltered protein cut-off 3 kDa TCA-soluble nitrogen defined as DH	↑	Marques et al. (2015)
	Germination				
soybean	2 h	24 h trypsin	TCA-soluble nitrogen	↑	Dikshit and Ghadle (2003)
black bean	1 h	2 h pancreatin	supernatant DH by ninhydrin reaction Samples digested for 24 h considered as 100% DH	↑	López-Barrios et al. (2016)
soybean	N/A	trypsin (time not specified)	DH by OPA reaction	↑↓	Aijie et al. (2014)
black soybean	30 min	30 min pancreatin	supernatant: molecular weight distribution	lower proportion of <0.15 kDa peptides in germinated samples	Sefatie et al. (2013)
	Fermentation				
lupin	N/A	10 min trypsin, α-chymotrypsin and serine-type protease	pH drop	↑	Bartkiene et al. (2018)
Conventional protein fractionation					
soybean	*in vivo* rat assay		PDCAAS	=	Hughes et al. (2011)
soybean	*in vivo* pig assay		Standard ileal digestibility	=	Pedersen et al. (2016)
soybean	3 h	N/A	TCA-soluble nitrogen	=	Da Silva Pinto et al. (2005)
	Alkaline treatment				
rice	2 h and *in vivo* rat assay	2 h trypsin	TCA-soluble nitrogen and absorption	↓	Zhang et al. (2019)
lupin	N/A	up to 28 h trypsin and chymotrypsin	TCA-soluble nitrogen	↓	Yu et al. (1987)
quinoa	6 h	N/A	DH by OPA reaction Size-exclusion chromatography	↓	Ruiz et al. (2016)
Alternative protein fractionation					
	'Alternative' chemicals				
soybean	2 h	24 h pancreatin	TCA-soluble nitrogen	=	Chamba et al. (2013)

lupin	N/A	10 min trypsin, chymotrypsin and peptidase	pH drop	↑	Lqari et al. (2002)
chickpea	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	=	Sánchez-Vioque, Clemente, et al. (1999)
brown lentil	1 h	24 h pancreatin	Free amino groups by TNBS reaction	=	Joehnke et al. (2021)
Alternative drying methods					
hempseed	N/A	10 min trypsin, α-chymotrypsin and protease	pH drop	freeze>oven>vacuum oven drying	Lin et al. (2021)
buckwheat	2 h	2 h trypsin	TCA-soluble nitrogen release and SDS-PAGE	freeze>spray drying	Tang (2007)
Enzyme assisted fractionation					
soybean	not specified	N/A	nitrogen solubility	=	De Almeida et al. (2014)
white sorghum	2 h	N/A	nitrogen solubility	=	De Mesa-Stonestreet et al. (2012)
Air classification					
pea, fava bean and lentil	*in vivo* mouse assay		protein digestibility	↓	Bhatty and Christison (1984)
fava bean	1 h	24 h pancreatin	Free amino groups by TNBS reaction	↓	Vogelsang-O'Dwyer, Petersen, et al. (2020)
quinoa	6 h	N/A	DH by OPA reaction Size-exclusion chromatography	↑	Opazo-Navarrete, Schutyser, et al. (2018)
Post-fractionation processing					
Fermentation					
yellow pea	N/A	10 min trypsin, α-chymotrypsin and protease	pH drop *in vitro* PDCAAS	↑ (lower IVPDCAAS)	Çabuk et al. (2018)
soybean	soft rat gastrointestinal model with dynamic gastric and duodenal (pancreatin) parts		TCA-soluble free-amino acids	↑	Huang et al. (2020)
Ultrasound					
fava bean	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↓	Martínez-Velasco et al. (2018)
Proteolysis					
soybean	30 min	30 min chymotrypsin (non-sequential)	TCA-soluble nitrogen	↑	Ge and Zhang (1993)
soybean	N/A	2 h trypsin and α-chymotrypsin	pH drop and free amino groups by ninhydrin reaction	=	Nguyen et al. (2016)
soybean and zein	30 min	2 h pancreatin	Tricine-SDS PAGE	= soy, ↑ zein	Kaur et al. (2010)
soybean, wheat gluten and zein	*in vivo* rat assay		true ileal digestibility	= soy, wheat gluten, ↑ zein	Rutherfurd et al. (2011)
soybean	*in vivo* rat assay		true gastric total protein digestion	↑	Montoya et al. (2014)

green lentil	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop and size-exclusion chromatography	↓	Aryee and Boye (2016)
rapeseed	*in vivo* rat assay *in vivo* human assay		true digestibility PDCAAS	↑	Fleddermann et al. (2013)
rice bran	2 h	2 h pancreatin	TCA-soluble nitrogen	↑	Singh et al. (2021)
chickpea	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	=	Clemente et al. (1999)
chickpea	N/A	11 min trypsin, α-chymotrypsin and protease	pH drop	=	Goertzen et al. (2021)
	Heat treatment				
soybean	1 h	N/A	DH by OPA reaction	↑↓	Tian et al. (2019)
soybean	1 h	3 h pancreatin	TCA-soluble nitrogen and SDS PAGE	↑	Chen et al. (2015)
soybean	1 h	2 h trypsin	DH by OPA reaction	↑	Ren et al. (2018)
soybean	Infogest 1.0		TCA-soluble nitrogen	↑	Zhang et al. (2018)
soybean	*in vivo* rat assay		Apparent digestibility from faeces	↓	Kim and Barbeau (1991)
lupin	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↑	Sathe, Deshpande, et al. (1982a)
winged bean	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↑	Sathe, Deshpande, et al. (1982b)
red bean, red kidney bean and mung bean	2 h	2 h trypsin	TCA-soluble nitrogen	↓ red bean, = red kidney bean ↑↓ mung bean	C.-H. Tang et al. (2009)
soybean and pea	2 h	N/A	DH by OPA reaction and size-exclusion chromatography	=	Rivera del Rio et al. (2020)
quinoa	6 h	N/A	DH by OPA reaction and size-exclusion chromatography	↓	Opazo-Navarrete et al. (2019)
lupin	30 min	N/A	<3 kDa peptides from size-exclusion chromatography	= ↓	Pelgrom et al. (2014)
navy bean	N/A	24 h trypsin, α-chymotrypsin and peptidase	DH by TNBS reaction	↑	Sathe, Iyer, et al. (1982)
sweet potato	*in vivo* rat assay		PDCAAS	↑ autoclaved> microwaved> dry heated> native	Sun et al. (2012)
lentil	N/A	2 h trypsin and chymotrypsin	DH by TNBS reaction	↑	Neves and Lourenço (1995)
chickpea	2 h	2 h trypsin and chymotrypsin or 10 min trypsin, chymotrypsin and peptidase	DH by TNBS reaction and pH drop	↑	Tavano and Neves (2008)

white common bean and fava bean	*in vivo* rat assay		absorption or protein intestinal digestibility	↓	Carbonaro et al. (2005)
navy bean	not specified		'*in vitro* protein digestibility'	↑↓	Chang and Satterlee (1981)
soybean	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↓	Wu et al. (1999)
soybean and rapeseed	30 min	pancreatin and diffusion through a 1000 MWCO membrane	nitrogen and amino acid release in dialysates	↓	Savoie et al. (1991)
soybean	*in vivo* rat assay		true protein digestibility	↓	Sarwar et al. (1999)
	High pressure processing				
yellow pea	Infogest 1.0		SDS PAGE and pH stat	↑	Laguna et al. (2017)
Modifications					
	Transglutaminase-catalysed polymerization				
red kidney bean	N/A	2 h trypsin	TCA-soluble nitrogen	↓	Yin et al. (2008)
Phaseolus vulgaris L. bean	1 h	1 h trypsin (non-sequential)	SDS PAGE	↓	Mariniello et al. (2007)
soybean	2 h	2 h trypsin	TCA-soluble nitrogen	↓	Tang et al. (2006)
red kidney bean	N/A	2 h trypsin	TCA-soluble nitrogen	↑	Tang et al. (2008)
soybean	1 h	3 h pancreatin	SDS PAGE	↓	Li and Damodaran (2017)
soybean	2 h or 1 h for sequential digestion	1 h trypsin	TCA-soluble nitrogen	↓	Sheng and Zhao (2013)
soybean	time not specified	trypsin (time not specified)	TCA-soluble nitrogen	↑	Fu and Zhao (2017)
soybean	2 h or 1 h for sequential digestion	1 h trypsin	TCA-soluble nitrogen	↑	Zhu et al. (2016)
soybean	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↓	Gan et al. (2009)
	Acylation				
red kidney bean	N/A	2 h trypsin	TCA-soluble nitrogen	↑	Yin et al. (2009)
mung bean	N/A	trypsin and pancreatin (time not specified)	pH stat	↑	El-Adawy (2000)
soybean	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↑	Achouri and Zhang (2001)
soybean	*in vivo* rat assay		protein efficiency ratio	↑ =	de Regil and Calderón de la Barca (2004)
	Complexation with phenolic compounds				
soybean	2 h	24 h trypsin and chymotrypsin	size exclusion chromatography	=	Budryn et al. (2015)

soybean	time not specified	trypsin and chymotrypsin (time not specified)	TCA-soluble nitrogen	↓	Rohn et al. (2006)
	in vivo rat assay		nitrogen efficiency true nitrogen digestibility PDCAAS		
yellow pea	3 h	4 h pancreatin	Free amino groups by TNBS reaction	↓	Strauch and Lila (2021)
soybean	1 h	2 h trypsin	SDS PAGE	↓	Yang et al. (2015)
	Other modifications				
soybean	1 h	2 h pancreatin	SDS PAGE and free amino acids	↓	Chen, Zhao, Sun, et al. (2013)
soybean, cottonseed and peanut	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↓	Rhee and Rhee (1981)
soybean	2 h	2 h pancreatin	TCA-soluble nitrogen	↑	Wang et al. (2014)
	Protein oxidation				
soybean	Infogest 1.0	N/A	DH by OPA reaction	↑↓	Zhao et al. (2020)
soybean	1 h	2 h pancreatin	SDS PAGE	↓	Chen, Zhao and Sun (2013)
soybean	3 h	N/A	DH by OPA reaction	=	Duque-Estrada et al. (2019)
Structure formation					
	Gelling				
soybean and yellow pea	3 h	N/A	DH by OPA reaction and size-exclusion chromatography	↓ soybean = yellow pea	Opazo-Navarrete, Altenburg, et al. (2018)
lentil and fava bean	Infogest 1.0		ultrafiltration through 50 kDa, 30 kDa, and 10 kDa Mw cut-offs SDS PAGE	=	Hall and Moraru (2021)
soybean	1 h	2 h pancreatin	pH stat	↑	Rui et al. (2016)
soybean	2 h	N/A	TCA-soluble nitrogen	↓	Ou et al. (2004)
	Extrusion and texturization				
soybean	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↑	Omosebi et al. (2018)
yellow pea	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↑	Wang et al. (1999)
	Intragastric gelling				
soybean	2 h	N/A	SDS PAGE of aqueous phase	↘	Hu et al. (2017)
soybean	Infogest 1.0	N/A	SDS PAGE	↘	Huang et al. (2018)
Animal-plant hybrid foods					
soybean, rice and yellow pea	1 h	4 h trypsin and chymotrypsin	TCA-soluble nitrogen, amine content by ninhydrin reaction, *in vitro* PDCAAS	↑	Khalesi and Fitzgerald (2021)
yellow pea, rice and lentil	Infogest 2.0		TCA-soluble free amino acids	↓	Baugreet et al. (2019)

Nutrient interactions					
yellow pea	2 h	2 h pancreatin	SDS PAGE	↓	Oñate Narciso and Brennan (2018)
chickpea	N/A	10 min trypsin, α-chymotrypsin and peptidase	pH drop	↓	Sánchez-Vioque, Vioque, et al. (1999)

DH, degree of hydrolysis; N/A, not applicable; OPA, *o*-phthalaldehyde; PDCAAS, protein digestibility-corrected amino acid score; SDS PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TNBS, trinitrobenzenesulfonic acid. The pH drop method normally refers to the method by Hsu et al. (1977).

CHAPTER THREE

Heat-induced changes in microstructure of spray-dried plant protein isolates and its implications on *in vitro* gastric digestion

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Abstract

The quickly expanding field of plant-based food, generally uses protein concentrates or isolates as protein source. It is however not clear to what extent the intensive processing of these raw materials affects their digestibility. We here report on the *in vitro* gastric digestibility of the structures present in unheated and heated dispersions of spray-dried protein isolates of soybean and yellow pea. Unheated dispersions consist primarily of insoluble individual spray-dried particles, agglomerates of these and only a small fraction of soluble protein. Pepsin activity was followed in real-time through microscopic observations, showing the disassociation of agglomerates and inward-breakdown of individual particles, which are otherwise stable at gastric pH and ionic strength. This demonstrates that solubility is not necessarily an incentive for gastric protein digestion.

Heating does not significantly affect the overall digestibility of protein isolate dispersions. Nevertheless, heating disrupts the structure of spray-dried particles, increasing the amount of smaller and better digestible particles that remain suspended after centrifugation. Conversely, heat-induced aggregates remain in the pellet and are up to 50% less digestible than their unheated counterparts. This impaired digestibility is counterbalanced by a reduced proportion of poorly-digestible species in the full system (up to 11% for soybean and 23% for pea).

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3.1 Introduction

Plant-based alternatives to animal-sourced food products are commonly produced from plant protein concentrates or isolates. These raw materials offer higher protein concentrations, extended shelf life and improved overall digestibility, compared to the original plant source (Rutherfurd et al., 2015). During concentration or isolation of proteins from oil seeds and pulses, the cell walls that surround the protein are broken up, thus facilitating the physical access of digestive enzymes to their substrate. Moreover, trypsin inhibitors are inactivated by heat treatment during the fractionation process. While these factors might extrinsically improve protein digestibility, intrinsically, it may be reduced by the conditions during concentration or isolation. During the conventional isolation process, most proteins become irreversibly denatured. Isoelectric precipitation and dehydration by heat promote hydrophobic interactions and polymerization of the protein chains (Wang et al., 2009). Meanwhile, denaturation and aggregation of plant proteins reduce their digestibility (Carbonaro et al., 2012; Ruiz et al., 2016).

Besides reducing digestibility, denaturation generally decreases protein solubility as well (Adebiyi & Aluko, 2011; Taherian et al., 2011). Protein solubility largely determines the techno-functionality of a protein ingredient and its potential use in food products (Kristo & Corredig, 2015). Well solubilized proteins show desirable functionalities such as emulsifying, gelation and water holding capacity. Interestingly, the solubility and other functionalities can sometimes be partially recovered with additional heating of aqueous dispersions of spray dried plant protein isolates (He et al., 2015; Keerati-u-rai et al., 2011), which is often done to access the functionality of plant protein ingredients. Nevertheless, the effect of such additional heat treatment of plant protein isolates on the protein digestibility is unclear.

Little is known about the influence of the microstructure of spray-dried particles in plant protein isolates and its effect on gastric digestibility. Thévenot et al. (2017) have pointed out the lack of information of the digestion process at the microscopic scale. They explored the mechanism behind the disintegration of a gel particle in the gastric environment and described it as a combination of penetration of gastric fluid and degradation of the interior, plus erosion at the surface.

We here report on the effect of heat treatment on the pepsin-digestibility of spray-dried plant protein isolates. Soybean and yellow pea protein isolates were chosen, as they are two of the most widely produced and consumed plant protein ingredients. Furthermore, to the best of our knowledge, the breakdown of spray-dried protein isolates particles has not been previously reported in literature. Thus, our approach combines the use of

microscopic observations and measurements of the degree of pepsin-mediated hydrolysis on suspended and insoluble protein species with the aim of better understanding the influence of solubility and microstructure on digestibility.

3.2 Materials and methods

3.2.1 Materials

Soybean protein isolate (SPI, SUPRO® 500E IP, Solae, USA) and yellow pea protein isolate (PPI, Nutralys S85 fine, Roquette, France) were used. Their nitrogen content was measured in triplicate by Dumas analysis (FlashEA 1112 series, Thermo Scientific Interscience, The Netherlands). A conversion factor of N x 5.71 was used for soybean protein, resulting in a protein content of 83.4% w/w. Likewise, the protein content of the PPI was 75.0% w/w (N x 5.52).

Unless otherwise stated, all other chemicals were purchased from Sigma Aldrich (USA). Milli-Q water (resistivity 18.2 MO, Merck Millipore, France) was used to prepare all samples and reagent solutions.

3.2.2 Sample preparation and characterization

SPI and PPI were used to prepare 5% w/v protein dispersions. The required amount of powder was mixed with MilliQ water at room temperature for 30 min, at 700 rpm and allowed to hydrate overnight. Unheated dispersions were compared with two heat treatments (90 °C and 120 °C), as for previous studies (Opazo-Navarrete, Schutyser, et al., 2018; Opazo-Navarrete et al., 2019; Ruiz et al., 2016). For the first, samples were heated in 2 ml-Eppendorf tubes to 90 °C for 30 min in a Thermomixer (Eppendorf AG, Germany) at 800 rpm. For the second heat treatment, samples were heated to 120 °C for 30 min, in a dry heating block QBT4 (Grant Instruments LTD, UK) and vortexed every 5 min to simulate Thermomixer-heating.

3.2.2.1 Protein identification and estimation of changes in secondary structure

The protein dispersions were characterized with non-reducing and reducing SDS polyacrylamide gel electrophoresis. The sample buffer for the polyacrylamide gel electrophoresis was prepared with 25% w/v glycerol, 2% w/v sodium dodecyl sulphate (SDS), 2% v/v 2-mercaptoethanol (only for reducing conditions), 1% w/v tris-HCl and 0.01% w/v bromophenol blue. The running buffer contained 1.44% w/v glycine, 0.3% w/v N-tris hydroxymethyl methylglycine and 0.1% w/v SDS. Pre-cast mini-protean TGX gels and Bio safe coomassie brilliant blue r-250 staining reagent were used (Bio-Rad Laboratories Inc., USA). Protein samples were mixed 1:1 with the sample buffer to reach a final concentration higher than 0.5 mg/ml. The mix was then heated to 90 °C, for 4 min

at 800 rpm. The cooled mix was centrifuged at 13000 rpm for 3 min. 10 µl of the supernatant were loaded into a lane of the pre-cast gel, as well as a 10 – 250 kDa molecular weight standard. Electrophoresis was carried out at 300 V. The gels were rinsed three times with water, stained and rinsed once more, before scanning for band analysis.

Changes in the secondary structure of the proteins were recorded by Fourier-transformed infrared spectroscopy with attenuated total reflectance (FTIR-ATR), based on the procedures described by Rabotyagova et al. (2010) and Haque et al. (2015). Briefly, an IRPrestige-21 FTIR spectrometer (Shimadzu Corporation Pte. Ltd., Japan) was used in the absorption mode at 4.0 cm^{-1}, with a resolution from 4000 to 400 cm^{-1}. A sample of 10 µl was placed on the liquid retainer and pressed with a flat tip plunger until suitable peaks were obtained. Samples were scanned at room temperature. IRsolution software version 1.10 (Creon Lab Control AG, Shimadzu Corporation Pte. Ltd., Japan) was used to process the recorded data. Protein spectra were smoothed with a 12-point Savitsky-Golay function and the second-derivative spectra were calculated. Absorbance data were selected from 1600 to 1700 cm^{-1} (amide I region). α-Helix (1652 cm^{-1}), anti-parallel β-sheet (1635 cm^{-1}), β-turn (1667 and 1660 cm^{-1}), β-strand (1674 cm^{-1}), β-type turn and bend (1691 cm^{-1}) and random coil (1644 cm^{-1}) structures were assigned to different peaks in the second derivative spectra (Tang & Ma, 2009).

3.2.2.2 Size-based particle separation

Unheated and heated protein dispersions were centrifuged at 2500xg for 30 min. In doing so, large and insoluble particles were separated from their small and suspended counterparts without disrupting their structure and maintaining as much as possible the interstitial water in the pellet.

- Protein quantification

A Pierce™ Bicinchoninic acid protein assay kit (ThermoFisher Scientific Inc., USA) was used to quantify the amount of protein separated into the pellet and the supernatant. Bovine serum albumin (BSA) with a known concentration (ThermoFisher Scientific Inc., USA) was used to prepare a standard curve. Samples were diluted to estimate concentrations within the standard curve of 20 – 2000 µg BSA/ml. The method followed the standard protocol, incubating the reacting samples at 37 °C for 30 min with the prepared reagent. The resulting absorbance of the colorimetric reaction was measured at 562 nm. The protein quantification was conducted in triplicate.

- Particle size distribution

Laser diffraction was used to determine the particle size distribution in the full protein dispersions, and the pellets and supernatants with a Mastersizer 2000 equipped with a wet sample dispersion unit Hydro 2000MU (Malvern Instruments Ltd, UK). Once the sample volume reached an obscuration rate between 10 and 20%, the diluted sample was stirred at 1200 rpm. The measurements were conducted considering a refractive index of 1.45 and 1.33 for the dispersed and continuous phase, respectively. The particle size distribution was reported as volume equivalent sphere diameter. Samples were measured in triplicate.

- Light microscopy

The particle morphology was observed with light microscopy using an Axioscope microscope equipped with a LED lamp and an Axiocam mrc5 camera (Zeiss, Germany). Images were adjusted using Axiovision software (Zeiss, Germany). Protein dispersions, supernatants and pellets were observed as such or re-dispersed in different media: water, NaCl solution at neutral or gastric pH, and SGF. Slides were observed for up to 10 min. We presumed that the composition of the sample in the different media would change significantly for longer time periods despite the protection from the coverslip.

3.2.3 Static *in vitro* gastric digestion

A simulated gastric fluid (SGF) was prepared according to Kong and Singh (2008) with minor modifications. The composition of the SGF was 1 g/l of pepsin from porcine gastric mucosa (561 U/mg, reported by manufacturer) and 8.775 g/l of sodium chloride, dissolved in MilliQ water. A 2 M hydrochloric acid solution was used to adjust pH to 2. The SGF was transferred to jacketed glass vessels connected to a water bath set to a constant temperature of 37 °C and stirred with a magnetic bar at 100 rpm.

In total, nine samples of either soybean or pea protein isolate were digested in individual vessels. For each treatment (unheated, heated at 90 °C and at 120 °C), a full dispersion, its pellet and supernatant were added to SGF to reach an enzyme-to-substrate ratio of 1:2. Samples were collected after 20, 60 and 120 minutes of digestion for further analysis. The digestion was halted by heating the aliquots at 95 °C for 5 min. Digestion assays were conducted in duplicate.

3.2.3.1 Peptide characterization

High Performance Size Exclusion Chromatography (HPSEC) was carried out in an UltiMate 3000 chromatographer (ThermoFisher Scientific Inc., USA) through a dual column system with TSK gel columns G3000SWXL and G2000SWXL for proteins and

peptides. Large particles were separated from the digestion samples by centrifuging at 1000 rpm for 30 seconds. 10 µl of supernatant were injected for each measurement. An aqueous solution of 30% v/v acetonitrile (Actu All Chemicals, The Netherlands) and 0.1% v/v trifluoroacetic acid was used as eluent. Signals were measured with UV detector set at 214 nm.

Data analysis was performed in Chromeleon 7.2 CDS software (ThermoFisher Scientific Inc., USA). The processing step was adjusted to integrate peaks between molecular weight ranges. A calibration curve of molecular weight on a logarithmic scale against elution time was plotted for thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44.3 kDa), α-lactalbumin (14 kDa), aprotinin (6.51 kDa), bacitracin (1.42 kDa) and phenylalanine (165 Da).

3.2.3.2 Degree of hydrolysis

Samples were analysed for their degree of hydrolysis by measuring the colorimetric reaction between o-phthaldialdehyde (OPA) and free amino groups as previously described by Nielsen et al. (2001). The OPA reagent was prepared with final concentrations of 3.81% w/v sodium tetraborate decahydrate and 0.1% w/v SDS, 0.08% w/v o-phthalaldehyde pre-dissolved in 2% v/v ethanol, and 0.088% w/v dithiothreitol. The solution was filtered through a 0.45 µm syringe filter and stored in the dark to avoid light exposure as the reagent is sensitive to light.

A standard curve was prepared by using L-serine (Alfa Aesar, Germany) in a concentration range of 0.5 – 2 mM. The OPA assay was carried out by adding 200 µl of sample (or standard) to 1.5 ml of OPA reagent. After the reaction took place for exactly 3 min, the optical density of the fluorescent product at 340 nm was measured with a spectrophotometer. The measured values were converted to a concentration of 'free amino groups' ([$NH_{2,\ free}$], meqSer-NH_2/l) from the standard curve. [$NH_{2,\ free}$] from digestion samples was corrected by subtracting the contribution of free amino groups from SGF. The hydrolysis equivalent during *in vitro* digestion (*h*, meqSer-NH_2/g protein) was calculated with Equation 3.1.

$$h = \frac{\frac{[NH_{2,\ free}]}{[Protein]} - \beta}{\alpha} \qquad \text{Equation 3.1}$$

In which [Protein] is the protein concentration in the digestion vessel, β accounts for the ε-amino groups and is 0.342 for soybean and 0.4 for pea, α represents the relationship between the colour intensity measured and [$NH_{2,\ free}$], it was considered as 0.97 for soybean and 1 for pea. The degree of hydrolysis (%) is the proportion of cleaved peptide

bonds. It was calculated by dividing the hydrolysis equivalent of each sample (h) by the total peptide bonds (h_{tot}) and multiplying by 100%, where h_{tot} is 7.8 meq/g for soybean and 9.3 meq/g for pea. Equations and parameters were obtained from the work of Adler-Nissen, except for the value of h_{tot} for pea which is not reported in literature and was determined by acid hydrolysis with 6 M HCl at 95 °C for 24 h (Adler-Nissen, 1986).

3.2.4 Statistical analysis

Statistical analysis was performed using SPSS Statistics software (version 25, IBM, USA). One-way analysis of variance and post-hoc Tukey's Honestly Significant Difference were used to compare significant differences between means at a confidence interval of 95% ($p \leq 0.05$).

3.3 Results and discussion

3.3.1 Sample characterization

Reducing SDS PAGE analysis of unheated and heat-treated protein dispersions was used to identify the protein units present in the protein isolates (Figure 3.1). The composition of unheated dispersions is rather similar to that of dispersions heated at 90 °C. The most significant change was observed for dispersions treated at 120 °C. For this treatment, the same bands from other treatments were present however, they appear faded in comparison. We propose that the lower intensity bands observed at 120 °C for both non-reducing and reducing conditions might evidence the formation of heat-induced aggregates unable to be separated into the gel.

Additional evidence of heat-induced aggregation was obtained through particle size distribution. A monodispersed distribution was observed in dispersions without heat treatment and heated at 90 °C (Figure 3.2A and B), whereas dispersions heated at 120 °C showed a poly-dispersed distribution, with both smaller and larger particles, compared to their unheated counterparts.

Furthermore, virtually no change was observed in FTIR spectra and therefore in the secondary structure of proteins in unheated and heated SPI and PPI dispersions (Figure A3.1). This suggests that the proteins in the commercial isolates were already denatured extensively and further heating did not alter the proportions of conformations in the secondary structure of the proteins. It should be noted that both SPI and PPI are a mixture of proteins and that conformational changes within one type of protein can be masked by changes in another.

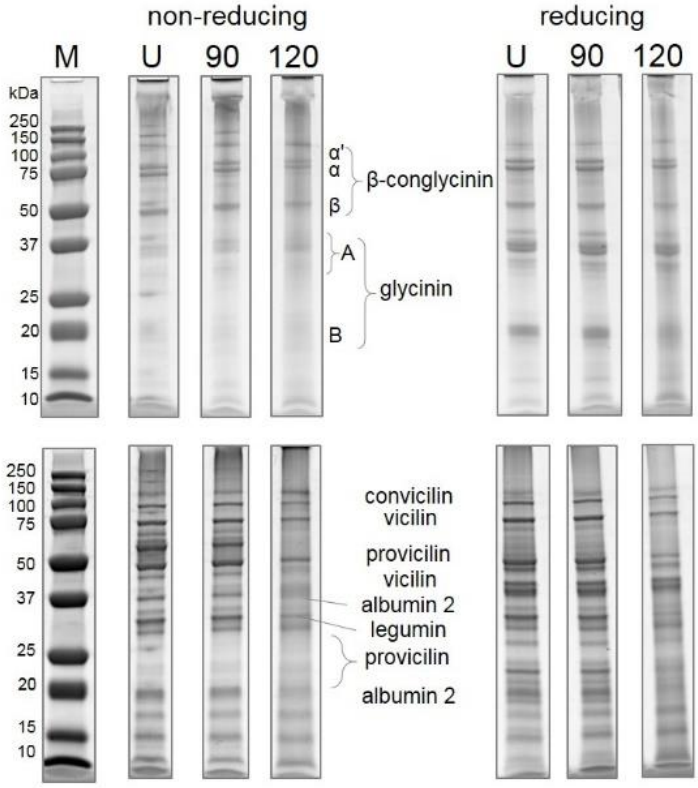

Figure 3.1 Non- reducing and reducing SDS PAGE patterns of untreated and heat treated at 90 and 120 °C 5% w/v protein dispersions of soybean (top) and pea (bottom) protein isolates.

Small and suspended particles were separated from large and insoluble ones by centrifuging the full dispersions. Particle size distribution measurements confirmed that small particles remained in the supernatant and larger particles sedimented to the pellet (Figure 3.2). For unheated dispersions, slightly more matter was separated into the pellet, whereas for heated dispersions more matter remained in the supernatant (Table 3.1). The amount of protein in each fraction was determined with the BCA assay, this was related to the weight distribution and the initial protein content to determine the protein ratio in each fraction. For unheated dispersions of SPI or PPI, close to 80% of the initial protein content ended up in the pellet. For SPI, the pellet of both heated dispersions contained a little over 10% of the protein. For PPI, heating at 90 °C produced a pellet with almost 40% of the initial protein content of the full dispersion, and this fraction decreased for the heat treatment at 120 °C to nearly 20% initial protein. As expected, the content of suspended protein of the unheated dispersions was low but it increased significantly upon heat treatment (He et al., 2015; Keerati-u-rai et al., 2011).

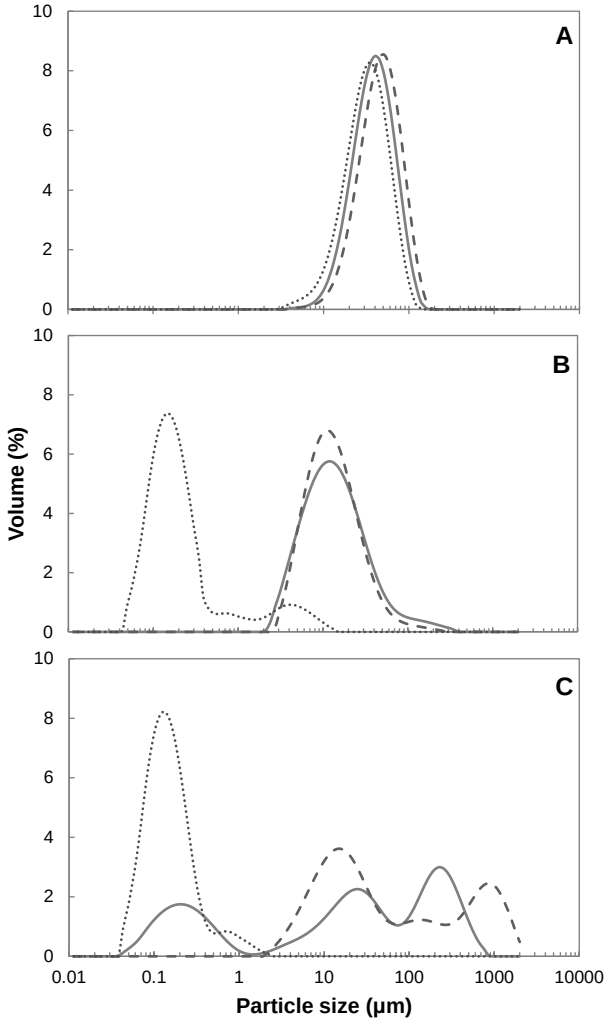

Figure 3.2 Particle size distribution of (A) unheated, (B) heated at 90 °C and (C) at 120 °C for 30 min, 5% w/v pea protein full dispersions (solid line), their pellet (dashed line) and supernatant (dotted line). Average of three consecutive measurements.

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Table 3.1 Weight distribution into pellet and supernatant of unheated and heated, full dispersions of 5% w/v protein from soybean or pea protein isolates (SPI and PPI, respectively). Protein content in both fractions quantified by the bicinchoninic acid method. Protein distribution into pellet and supernatant expressed as a weighted ratio. Standard deviation from triplicate measurements is given between brackets. For SPI and PPI separately, values with the same letter did not differ significantly ($p > 0.05$).

		Mass distribution		Protein content (kg/dm^3)		Protein ratio
Material/ Treatment	Fraction	pellet	supernatant	pellet	supernatant	pellet : supernatant
SPI	unheated	0.54 (0.01)	0.46 (0.00)	0.141 (0.008)	0.045 (0.001)	0.79^{b} : 0.21^{a}
	90 °C	0.09 (0.00)	0.91 (0.00)	0.108 (0.006)	0.085 (0.002)	0.12^{a} : 0.88^{b}
	120 °C	0.11 (0.01)	0.89 (0.01)	0.097 (0.004)	0.090 (0.008)	0.11^{a} : 0.89^{b}
PPI	unheated	0.68 (0.10)	0.32 (0.10)	0.108 (0.007)	0.035 (0.011)	0.87^{c} : 0.13^{a}
	90 °C	0.25 (0.01)	0.74 (0.01)	0.131 (0.011)	0.073 (0.002)	0.38^{ab} : 0.62^{bc}
	120 °C	0.16 (0.01)	0.83 (0.01)	0.119 (0.010)	0.079 (0.004)	0.23^{ab} : 0.77^{c}

3.3.2 Particle microstructure

The characteristic morphology of spray-dried droplets or particles was observed in unheated dispersions (Figure 3.3). Primary droplets in the spray dryer form the smallest particles: these primary particles typically agglomerate into larger clusters. Indeed, we can see the primary particles with their shell-like morphology, and clusters of partially merged primary particles. To assess the stability of these structures, unheated dispersions were re-dispersed in saline solution at pH 7 and saline solution at pH 2, the latter simulating the gastric environment without enzymatic action. None of the new environmental conditions altered the morphology of particles in both SPI and PPI dispersions over a course of up to five minutes.

In a gastric environment that included pepsin, the spray-dried particles were no longer stable, and changes in particle morphology were observed over several minutes. Figure 3.4 shows a particle being detached from an agglomerate and degrading inwardly. By the end of the observation, particles on average had degraded significantly compared to the first micrograph. The stability of the particles in a gastric environment without pepsin (Figure 3.3) allows us to attribute these changes to enzymatic hydrolysis. We propose that the porous structure of the clusters and particles allows pepsin to penetrate and hydrolyse them, relatively homogeneously in an inward motion. As digestion continues, the spray-dried particle structure weakens and ultimately falls apart.

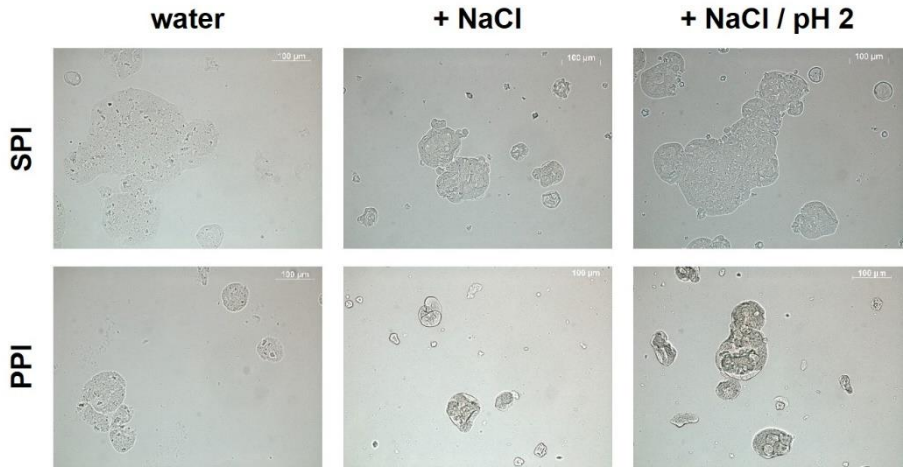

Figure 3.3 Light microscopy observations of unheated soybean or pea protein isolate dispersions re-dispersed in water, NaCl solution and NaCl solution at pH 2.

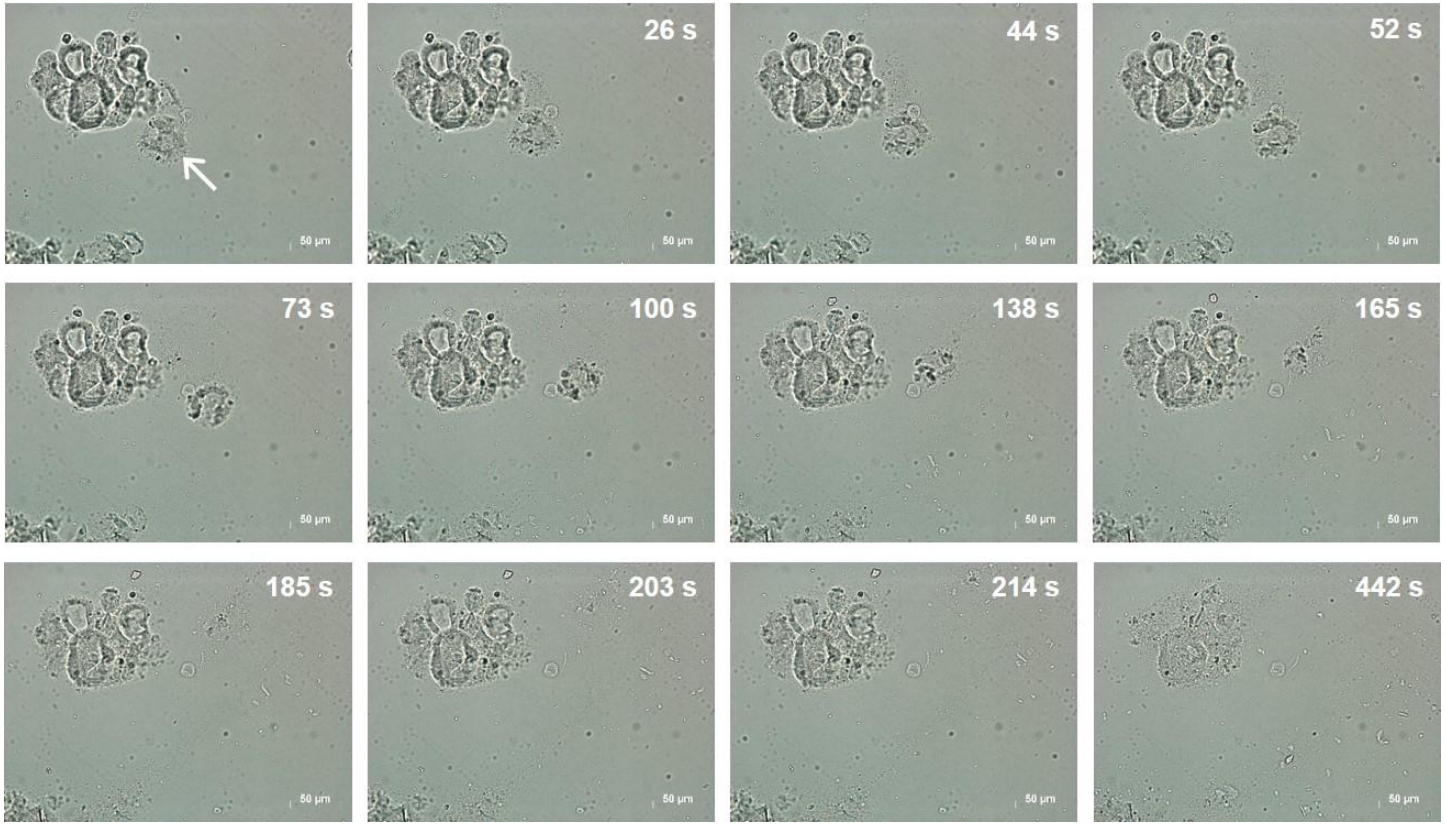

Figure 3.4 Light microscopy observations of the pellet fraction of an unheated 5% w/v soybean protein dispersion, digested in simulated gastric fluid (pepsin + NaCl/pH 2). Arrow included to direct attention to an encapsulate being detached from a cluster and degraded into smaller particles over 4 min. After 7 min the cluster is significantly degraded.

The distinct morphology of the spray-dried particles was mostly lost during heat treatment. Heating a dispersion transformed the spherical spray-dried particles into amorphous patches of different sizes (Figure 3.5A and B). Based on the particle sizes, we assume that the spray-dried particles might have broken down, which released smaller and less dense particles that were found in the supernatant (Figure 3.2A and B). This explains the increase in suspended protein observed upon heat treatment. Additionally, some of the disrupted spray-dried particles might have aggregated leading to the larger particles separated into the pellet: the heat induced aggregates were larger at 120 °C than at 90 °C. Potentially, the smaller aggregates that were formed at 90 °C nucleated into larger clusters at 120 °C.

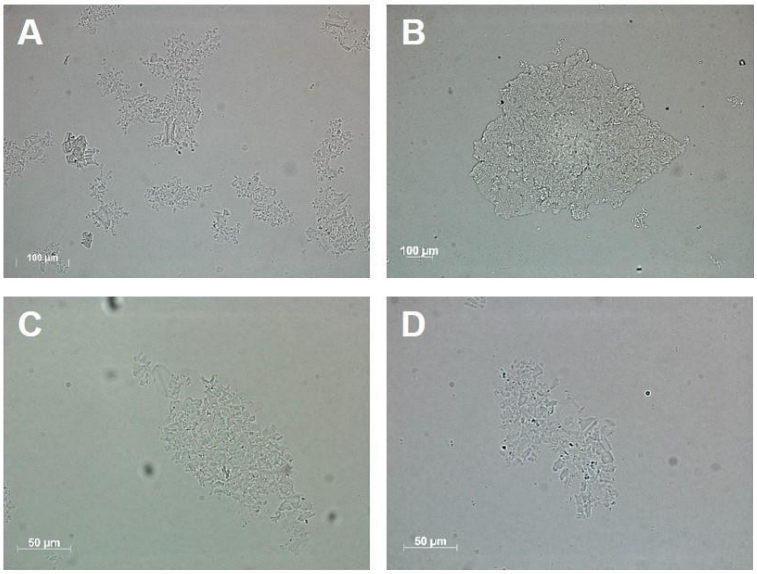

Figure 3.5 Light microscopy observations of particles in soybean protein dispersions heated at (A) 90 °C and (B) 120 °C. Components of the pellet of heated dispersions at (C) 90 °C and (D) 120 °C re-dispersed in simulated gastric fluid (pepsin, NaCl, pH 2) observed over 10 min.

Similarly, to their unheated counterparts, heat-induced aggregates in the pellets from the heat treated dispersions were stable under gastric conditions without pepsin. However, unlike unheated particles, the heated aggregates appeared to be stable in the presence of pepsin (Figure 3.5C and D): particles in SGF did not show any apparent change in morphology or size over ten minutes. Thus, the microstructure of the sedimented particles significantly influences their digestibility by pepsin. For the untreated spray-dried particles, pepsin in the SGF could sufficiently access peptide bonds to erode the particle surface and diffuse inwards. In contrast, the densely packed structure of the heat-

induced aggregates prevented pepsin access and resulted in lower pepsin-digestibility compared to their unheated counterparts.

We have shown that the gastric environment without pepsin does not influence the insoluble protein species separated into the pellet of unheated and heated dispersions. However, gastric pH does seem to alter the suspended proteins in the supernatant. The solid line in Figure 3.6 is the size exclusion chromatogram of the supernatant of an unheated PPI dispersion mixed in water, which yielded a clear solution (insert in Figure 3.6, tube A). However, when this supernatant was put in contact with a simulated gastric medium (NaCl/pH 2 solution), a precipitate or clot was formed (tube B). This reduced the total area under the curve by 40%. This reduction is proportional to the total suspended protein in the gastric medium and was mainly in proteins of high molecular weight, implying that the clot was composed mostly of bigger proteins (Table A3.1). Ye et al. (2016) also observed the formation of a clot during simulated gastric digestion of milk. They identified that thermal treatment of protein affected clot density and the presence or lack of pores throughout the structure. The underlying mechanism of clot formation may differ for the present study mainly because of the type of proteins and their initial denaturation state. Gastric-insoluble particles were also observed by Overduin et al. (2015) during *in vitro* digestion of PPI. It is unclear to what extent clot formation affects the digestibility of proteins in the supernatant. *In vivo*, this could result in delayed gastric emptying, compared to fully soluble proteins in the gastric environment. This was observed by Overduin et al. (2015) as a moderate delay of intestinal bioavailability.

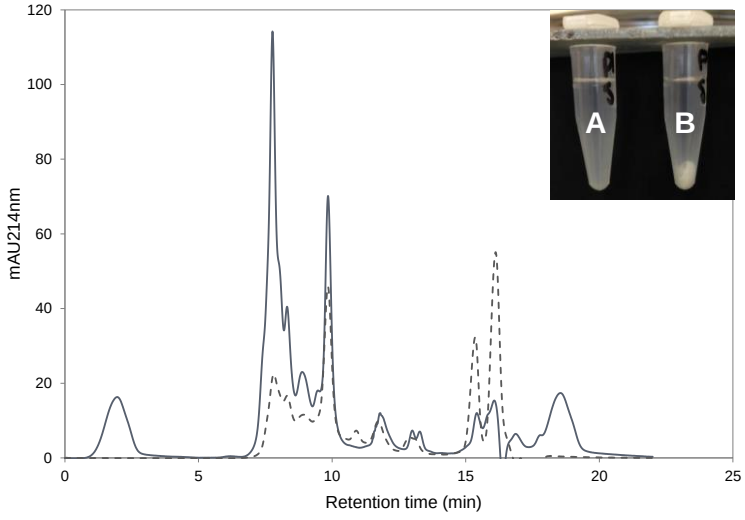

Figure 3.6 Size exclusion chromatograms of undigested full dispersion of pea protein in water (solid line, tube A) and in simulated gastric environment (NaCl solution at pH 2, dashed line, tube B).

3.3.3 Protein digestibility

The purpose of the digestion experiments was to assess the effect of the thermal treatment on the susceptibility of soybean and pea proteins to pepsin hydrolysis. Aliquots from the digestion of full dispersions, their corresponding pellets and supernatants were sampled from the digestion vessels 20, 60 and 120 min after the start of the digestion. Each aliquot was analysed with HPSEC to discern the resulting peptide size distribution, and the OPA method to estimate the DH.

There was only a limited difference between the digestibility, expressed as peptide release (Figure 3.7) and degree of hydrolysis (Figure 3.8A and C), of unheated and heated full dispersions. Digestibility was slightly reduced when heating at 90 °C and then recovered at 120 °C. This finding could imply that thermal processing does not significantly affect the gastric digestibility of full dispersions. Interestingly, heat treatment did change the digestibility of the individual fractions: the pellet and the supernatant.

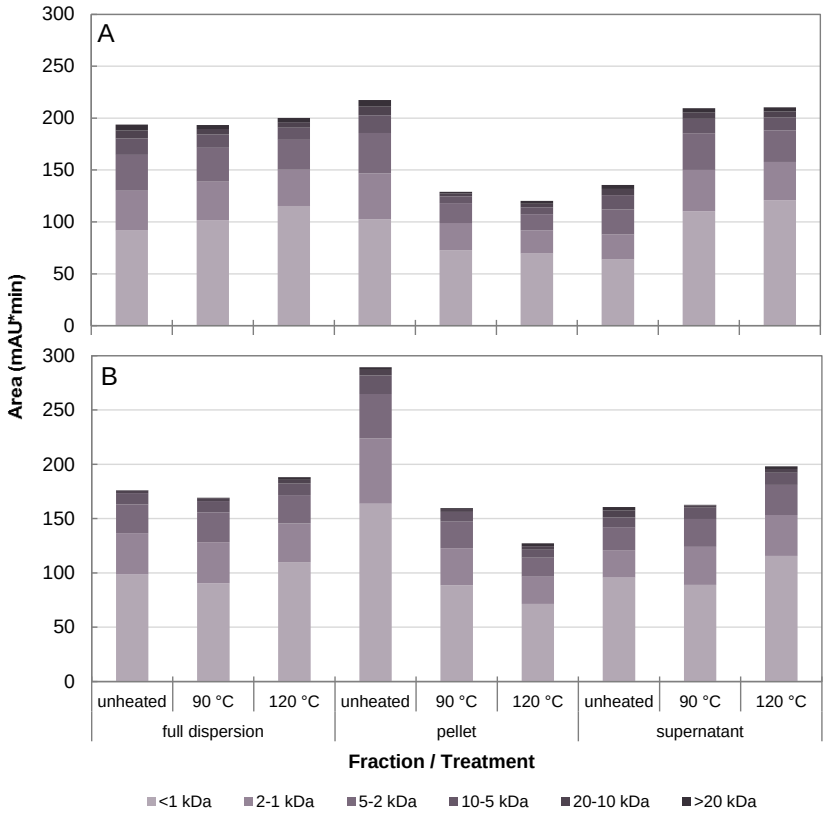

Figure 3.7 Peptide profile integrated from size exclusion chromatograms after 120 min of simulated gastric digestion of heated and unheated (A) soybean and (B) pea protein dispersions and their corresponding pellets and supernatants (1:2 enzyme to substrate ratio).

For both SPI and PPI, the proteins in the pellet of unheated dispersions were better hydrolysed by pepsin than those that remained in the supernatant (Figure 3.8B and D). The opposite relationship was observed in the fractions of heated dispersions. The proteins in the supernatant released a larger amount of peptides and produced a higher degree of hydrolysis compared to the pellet fraction. Regardless of the hydrolysed material, large peptides (>10 kDa) remained even after 120 min of gastric digestion. This suggests that pepsin-resistant peptides were present in both suspended and insoluble fractions.

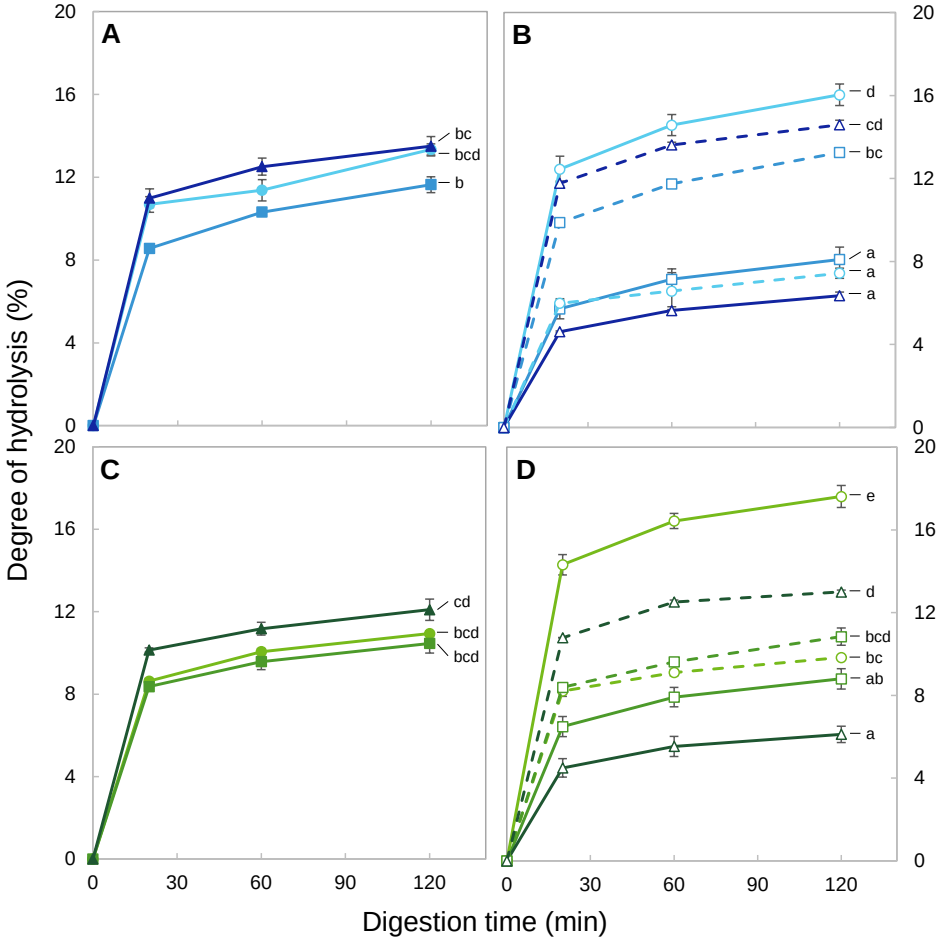

Figure 3.8 Degree of hydrolysis of digestion samples of unheated (●), heated at 90 °C (■) and heated at 120 °C (▲) soybean (A and B) and pea (C and D) protein dispersions and fractions. Full dispersions (filled symbols and solid line), pellets (hollow symbols and solid line) and supernatants (hollow symbols and dashed line). 1:2 pepsin to protein ratio. Error bars represent standard deviation for duplicate determinations. For SPI and PPI separately, values of degree of hydrolysis (120 min) with the same letter did not differ significantly ($p > 0.05$).

Proteins in the ingredients studied were significantly denatured. Limited heating increased the amount of suspended protein, which may render them more suitable for food applications (Kristo & Corredig, 2015). This study has identified that heat treatment of commercial protein isolates does not have an apparent effect on the overall *in vitro* gastric digestibility. However, upon close inspection of the protein species present in unheated and heated dispersions, the contribution of each element changed strongly. In sum, insoluble particles in unheated dispersions were the most digestible, whereas suspended proteins in the heated dispersions contributed the most to the overall digestibility. Heating thus disrupts the spray dried particles resulting in small and suspended particles which were better hydrolysed by pepsin, i.e., the better digestible proteins were re-distributed from the pellet of unheated dispersions into the supernatant of heated dispersions.

Moreover, low digestibility in the gastric phase does not necessarily imply a lower overall digestibility. The mean residence time of the proteins in the pellet might be longer than those in the supernatant. A delayed gastric emptying might result in a longer, sustained input of protein into the duodenum, which, in turn, might even improve protein digestion efficiency. Future work should study the susceptibility of the pepsin-hydrolysates to intestinal digestion.

3.4 Conclusion

Solubility is not necessarily an incentive for protein hydrolysis by pepsin, but rather microstructure is an important determinant of digestibility. While the spray-dried particles and agglomerates sediment into the pellet, their relatively high susceptibility to pepsin action was evidenced by their degree of hydrolysis and by the microscope observations. In contrast, the insoluble particles in heated dispersions are heat-induced aggregates with far lower digestibility.

Appendix

FTIR spectra were used as a measure of changes in secondary structure due to heat treatment. Our results show that the spectrum of unheated SPI dispersion does not change significantly due to heating at 90 or 120 °C (Figure A3.1A and C). Heat-treated pea protein isolate dispersions showed similar spectra as the unheated dispersion except for a smaller peak at a high frequency (1684 cm^{-1}), which is associated to a reduction in β-turns (Carbonaro et al., 2012). These findings suggest that the proteins in the commercial isolates are already denatured extensively. Further heating does not alter the proportions of β-sheets and α-helices in the secondary structure of SPI and PPI in aqueous dispersions.

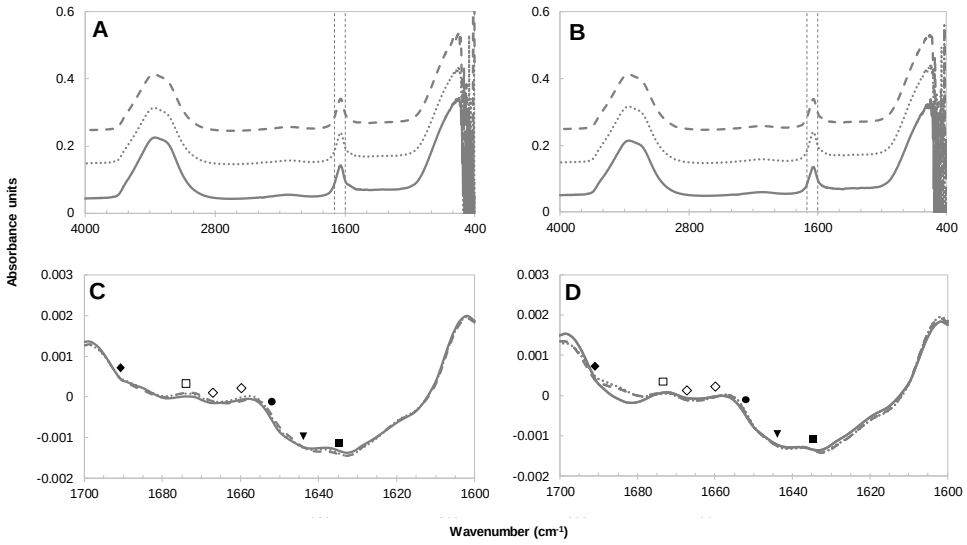

Figure A3.1 Fourier transform infrared stacked spectra of 5% w/v protein dispersions of soybean (A) and pea (B) protein isolates, unheated (solid line) and heat-treated at 90 °C (dotted line) and 120 °C (dashed line) for 30 min. Amide I band analysis by second-derivative spectra of soybean (C) and pea (D) protein isolate. Characteristic positions associated to β-type turn and bend (◆), β-strand (□), β-turn (◇), α-helix (●), random coil (▼) and anti-parallel β-sheets (■) (Tang & Ma, 2009).

Table A3.1 Absolute areas under the curve (AUC) of the chromatograms from HPSEC (mAU*min) of untreated (U) and heat treated 5% w/v pea protein dispersions and their respective supernatants, re-dispersed in water (W) or a NaCl solution at pH 2 (N2, gastric environment) to reach a concentration of 0.2% w/v protein. The total AUC is higher for the supernatants, relative to the full dispersions; however, the relative proportions of peptide sizes were maintained leading to suppose that all that was measured in the full dispersion is the suspended protein i.e., the supernatant. No evident pattern was observed for the peptide size redistribution of protein fractions under gastric environment conditions.

	Treatment/ Medium		Molecular weight range (kDa)							
			1000 -500	500- 200	200- 100	100- 50	50- 10	10 - 1	< 1	Sum
Full dispersion	U	W	0.3	19.1	7	4.6	8.2	2.6	4.4	46.3
		N2	0	7.6	4.4	2.5	7.9	5.7	4.6	32.6
	90 °C	W	3.3	35.3	1.4	3.7	7.6	3.4	3.9	58.5
		N2	0	13.9	5.8	4.1	8.8	6.2	4.3	43.1
	120 °C	W	0	41.6	12.1	4.9	14.5	10.9	6.2	90.1
		N2	0	9.9	3.8	5.5	13	6.9	3.6	42.7
Supernatant	U	W	3.5	51.9	16.1	11.6	23.1	11.4	18.9	136.5
		N2	0	13	8.1	7.9	20.6	9.9	16.5	76
	90 °C	W	3.8	40.3	4.3	1.2	8.4	4.7	4.5	67.2
		N2	1.9	19.4	10.1	2.6	10.8	7	7.9	59.8
	120 °C	W	3.7	38.1	9.2	5	11.3	9.2	5.2	81.6
		N2	0	16.8	5.6	7.4	13.3	7	4.4	54.3

CHAPTER FOUR

Protein acidification and hydrolysis by pepsin ensure efficient trypsin-catalysed hydrolysis

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Abstract

Enzyme-catalysed hydrolysis is important in protein digestion. Protein hydrolysis is initiated by pepsin at low pH in the stomach. However, pepsin action and acidification happen simultaneously to gastric emptying, especially for liquid meals. Therefore, different extents of exposure to the gastric environment change the composition of the chyme that is emptied from the stomach into the small intestine over time. We assessed the susceptibility of a protein to trypsin-catalysed hydrolysis in the small intestine, depending on its pH and hydrolysis history, simulating chyme at different times after the onset of gastric emptying. Isothermal titration calorimetry was used to study the kinetics of pepsin and trypsin-catalysed hydrolysis. Bovine serum albumin (BSA) that was acidified and hydrolysed with pepsin, showed the highest extent and most efficient hydrolysis by trypsin. BSA in the chyme that would be first emptied from the stomach, virtually bypassing gastric acidity and peptic action, reduced trypsin-catalysed hydrolysis by up to 58% compared to the acidified, intact protein, and 77% less than the acidified, pepsin-hydrolysate. The least efficient substrate for trypsin-catalysed hydrolysis was the acidified, intact protein with a specificity constant (k_{cat}/K_m) nearly five times lower than that of the acidified, pepsin-hydrolysate. Our results illustrate the synergy between pepsin and trypsin hydrolysis, and indicate that gastric hydrolysis increases the efficiency of the subsequent trypsin-catalysed hydrolysis of a model protein in the small intestine.

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4.1 Introduction

During food digestion, proteins are hydrolysed into polypeptides and ultimately down to single amino acids, prior to absorption into the bloodstream. The first protease that catalyses the hydrolysis of these intact proteins is pepsin, which is secreted in the stomach. Pepsin is active at pH ranging from 1 to 6, with a maximum activity between 1.5 to 2.5 (Piper & Fenton, 1965). However, this optimum depends on the type and denaturation state of the substrate (Christensen, 1955; Cornish-Bowden & Knowles, 1969). Meanwhile, trypsin, chymotrypsin and elastase are secreted from the pancreas into the small intestine. Of these, trypsin seems to be the endopeptidase with the most significant activity as it has been found in human duodenal aspirates, in higher proportion in mass and activity than chymotrypsin (Goldberg & Wormsley, 1970). Trypsin further hydrolyses proteins and polypeptides into smaller peptides that are better accessible for exopeptidases (Feher, 2012). Pepsin-catalysed hydrolysis happens in parallel with gastric emptying (Gentilcore et al., 2006; Marciani et al., 2012; Pal et al., 2007). Therefore, the composition of chyme that enters the duodenum changes over time, with varying extents of gastric acidification and hydrolysis. This causes pancreatic endopeptidases to interact with different substrates over the course of the postprandial state. As chyme enters the duodenum, its pH is slowly neutralized by the bicarbonate present in the pancreatic juice approaching the pH of optimum activity of intestinal endopeptidases (Feher, 2012; Sipos & Merkel, 1970). An enzyme's affinity and efficiency to catalyse the hydrolysis of intact proteins, compared to hydrolysates, can be better understood by studying its kinetics as a function of the substrate and conditions.

Isothermal titration calorimetry (ITC) is a suitable method to study enzyme kinetics (Todd & Gomez, 2001). Unlike alternative methods, calorimetry allows the direct estimation, in real time, of rates of enzyme-catalysed reactions without the need to label or modify the substrate. Other methods are based on measuring the concentration of substrate or product to derive the rate of catalysis, some requiring separation techniques such as chromatography or electrophoresis after the reaction has taken place. Spectrometric methods rely on fluorogenic or chromogenic substrates. Since virtually all reactions require or release heat, calorimetry can be used to study nearly any reaction. An isothermal titration calorimeter consists of a reference and a sample cell. Usually, the sample cell is loaded with an enzyme solution and a substrate solution is injected into it. The thermal power required to maintain isothermal conditions between the two cells is recorded, being equal to the heat absorbed or released by the system after injection. This heat rate is then proportional to the rate of the reaction (Todd & Gomez, 2001). ITC has been used to study the kinetics of pepsin-catalysed hydrolysis in gastric conditions

(Luo et al., 2018). In the current work, we aim at extending the knowledge of kinetics of digestive enzymes towards trypsin in small intestinal conditions and of the role of the gastric phase in the overall digestion process of proteins. While some studies have assessed the kinetics of trypsin-catalysed hydrolysis, they have done so with proteins or short, synthetic peptides without a varying pH history and without prior hydrolysis by other enzymes (Lobo et al., 1976; Maximova & Trylska, 2015; Olsen, 2006; Todd & Gomez, 2001). We compare the extent and rate of trypsin-catalysed hydrolysis of a protein with varying pH histories and prior peptic hydrolysis. Bovine serum albumin (BSA) was used as a model protein. BSA was acidified at pH 2 and hydrolysed by pepsin action, simulating the longest residence time in the stomach. Shorter residence times were simulated by acidifying and not hydrolysing the protein, and by dissolving the intact protein directly at pH 8.

4.2 Materials and methods

All materials, unless otherwise stated, were purchased from Sigma-Aldrich. All buffers and solutions were prepared with Milli-Q water (resistivity 18.2 MO, Merck Millipore, France).

4.2.1 Sample preparation

4.2.1.1 Enzymes

A stock solution (1 mM) of pepsin from porcine gastric mucosa (4228 U haemoglobin/mg, 145900 U /ml) was prepared in water. A 1 mM trypsin from porcine pancreas (1655 U N-benzoyl-L-arginine ethyl ester/mg, ≤0.1% chymotrypsin, 38560 U/ml) stock solution was prepared in 1 mM HCl. Stock solutions were prepared daily and kept at 4 °C. The pH of the stock solutions was chosen to be well away the optimum enzyme activity pH to avoid excessive autolysis prior to the substrate hydrolysis assays.

4.2.1.2 Substrates

Lyophilised bovine serum albumin (BSA, ≥96%) was used as a substrate under different environments (Figure 4.1).

- Pepsin-catalysed hydrolysis

Acidified BSA (A): BSA was dissolved in a sodium phosphate buffer (100 mM, I = 43 mM, pH 2) to a concentration of 300 µM. Due to the buffering capacity of the protein, pH was adjusted to 2 with phosphoric acid.

- Trypsin-catalysed hydrolysis

Pepsin-hydrolysate (AHP): A 13 µM pepsin solution was prepared by diluting the pepsin stock solution in pH 2 buffer and was incubated at 37 °C for 30 min in a jacketed glass

vessel connected to a water bath. An aliquot of previously prepared 300 µM BSA solution in pH 2 buffer was added to the enzyme solution. The hydrolysis reaction was allowed to proceed to completion for 60 min. A 3 M NaOH solution was used to bring the pH of the pepsin-hydrolysate solution from 2 to 8, resulting in an 8 µM pepsin, 99.6 µM BSA-equivalent solution with an ionic strength of 304 mM.

Acidified, non-hydrolysed BSA with inactive pepsin (AnHP): the composition of the pepsin hydrolysate was reproduced for this solution, except the pepsin stock solution was added after the pH had been adjusted to 8, thus keeping the pepsin presence but not its activity.

Acidified, non-hydrolysed BSA (AnH): The composition was the same as the previous solution, only the stock pepsin solution was substituted with water.

Non-acidified, non-hydrolysed BSA (nAnH): BSA was dissolved in a sodium phosphate buffer (100 mM, I = 290 mM, pH 8) to a concentration of 99.6 µM. At this pH, no buffering capacity was observed.

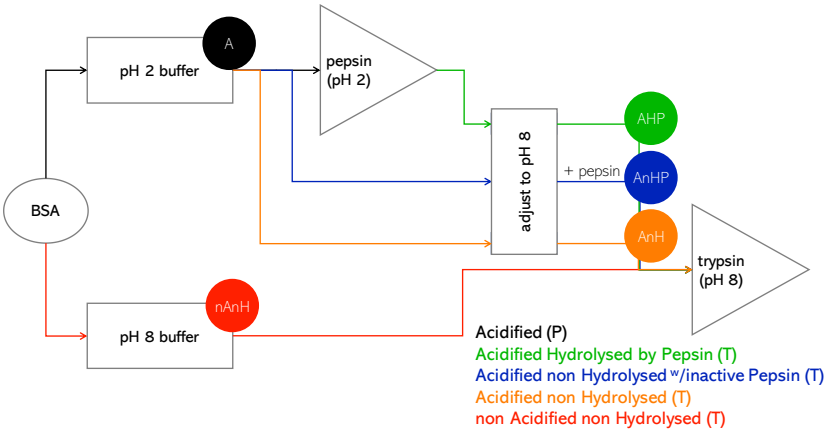

Figure 4.1 Sample preparation of the five substrates for pepsin (BSA acidified, A) and trypsin-catalysed hydrolysis (BSA acidified and hydrolysed by pepsin, AHP, BSA acidified non-hydrolysed, with inactive pepsin, AnHP, BSA acidified non-hydrolysed, AnH, and BSA non-acidified non-hydrolysed, nAnH).

4.2.2 Determination of hydrolysis ratio

Pepsin and trypsin have a preference to catalyse the cleavage of specific peptide bonds within a protein or peptide (Keil, 2012). This means that hydrolysis of all peptide bonds in a protein will be achieved by neither pepsin nor trypsin. Next to the enzyme's specificity and selectivity, the reaction conditions influence both the enzymatic activity and the protein conformation. It has been noted that the true substrates for proteases are peptide bonds and not proteins themselves (Luo et al., 2018; Ye et al., 2014). Hence, we need to measure the real achievable hydrolysis towards the different substrates.

Enzyme stock solutions were diluted in 100 mM sodium phosphate buffer, to 12.8 µM pepsin, at pH 2 and to 2.5 µM trypsin, at pH 8. 925 µl of the enzyme dilutions were incubated in Eppendorf tubes at 37 °C for 30 min, at 300 rpm in a Thermomixer (Eppendorf AG, Germany). At four time intervals of 2000 s, 20 µl of substrate solution was added. The hydrolysis ratio was determined and was defined as the molar concentration of free amino groups after hydrolysis per total protein concentration (Equation 4.1, Luo et al. (2018)). The hydrolysis ratio was determined for each of the four additions of all five substrate-enzyme combinations.

$$\text{Hydrolysis ratio} = \frac{[\text{free } NH_{2\,\text{from hydrolysis}}]}{[\text{protein}_{\text{total}}]} \qquad \text{Equation 4.1}$$

Free amino groups were quantified with the colorimetric reaction between *o*-phthalaldehyde (OPA) and free amino groups, as previously described (Nielsen et al., 2001). Briefly, the OPA reagent was prepared with final concentrations of 3.81% sodium tetraborate decahydrate and 0.1% SDS, 0.08% *o*-phthalaldehyde pre-dissolved in 2% ethanol, and 0.088% dithiothreitol. The solution was filtered through a 0.45 µm syringe filter and stored to avoid light exposure as the reagent is light sensitive. A standard curve was prepared by using L-serine (Alfa Aesar, Germany) in a concentration range of 10 – 200 mg/L. The OPA assay was carried out by adding 200 µL of sample, or standard, to 1.5 mL of OPA reagent. After the reaction took place for exactly 3 minutes, the optical density was measured at 340 nm with a spectrophotometer. Measurements were conducted in duplicate for triplicate hydrolysis samples.

4.2.3 Tryptophan fluorescence

Samples of substrates and products of hydrolysis were diluted with the appropriate buffer to a protein concentration of 0.01wt%. Diluted samples were transferred to a quartz cell with 10.00 mm light path. Fluorescence emission spectra were scanned between 300 and 500 nm, at an excitation wavelength of 294 nm, following the method of Heyn et al. (2019). Measurements were conducted in a RF-6000 spectrofluorometer (Shimadzu Corporation, Japan). The excitation and emission slit widths were set at 5.0 nm, with a scan speed of 200 nm/min. Spectra were measured in duplicate.

4.2.4 Isothermal titration calorimetry

ITC assays were conducted in a power compensation, low volume isothermal titration calorimeter (Affinity ITC, TA Instruments, USA) with a 190 µl gold cell (185 µl active volume, considering the stirring paddle and needle). All ITC assays were conducted at

37 °C and a stirring speed of 75 rpm. Instrumental thermal power was recorded every 5 seconds. The reference cell was filled with degassed, deionized water for all assays.

Substrate solutions were degassed in a vacuum degassing station (TA Instruments, USA) for at least 30 min. Enzyme stock solutions were degassed and appropriate dilutions were made just before the ITC assays into degassed, 100 mM sodium phosphate buffers, at pH 2 for pepsin and at pH 8 for trypsin, to avoid excessive autolysis. The sample cell was filled with enzyme solution and allowed to equilibrate until a stable baseline was measured. Baseline data was collected for 100 s. It was followed by a small injection of 0.5 µl to account for diffusion into the syringe needle. The thermal power was allowed to return to the baseline for at least 250 s. The calorimeter records the required heat for the sum of all heat effects in the reaction cell, and to maintain its temperature equal to that of the reference cell. When injecting substrate into enzyme, the reaction heat is measured as well as the dilution heat. Therefore, a blank assay of substrate solution injected into buffer is needed to account for the dilution heat. For all assays the substrate dilution heat was measured in a blank assay by injecting the substrate into buffer, with the same conditions of injection volume and interval as the reaction assay.

4.2.4.1 Continuous injection assay

For this assay, the enzyme-to-substrate ratio was kept equal to the hydrolysis ratio determination (Section 4.2.2) yet given the capacity of the sample cell of the calorimeter, the amount of both enzyme and substrate solutions was reduced five times. After a baseline equilibration, four aliquots of 4 µl of substrate solution were injected every 2000 s, ensuring that the injection interval was long enough to allow the heat rate to return to the baseline. Injections two to four were made to investigate product inhibition with the different substrates. For pepsin-catalysed hydrolysis, 300 µM BSA solution was injected into 13 µM pepsin solution, both in pH 2 buffer. For trypsin-catalysed hydrolysis, 99.6 µM BSA pepsin-hydrolysate or BSA (AnHP, AnH, nAnH) were injected into 2.5 µM trypsin solution at pH 8.

The total heat of the reaction was obtained by integrating the peaks of each injection with respect to time $\left(\int_{t=0s}^{t=2000s} \frac{dQ(t)}{dt} dt\right)$ and correcting for the dilution heat from the blank assay. The apparent molar enthalpy (ΔH_{app}) was estimated with Equation 4.2, where $[S]_{total}$ is the concentration of substrate in the cell after each injection, and V is the volume of the cell (Todd & Gomez, 2001).

$$\Delta H_{app} = \frac{1}{[S]_{total} V} \int_{t=0s}^{t=2000s} \frac{dQ(t)}{dt} dt \qquad \text{Equation 4.2}$$

4.2.4.2 Multiple injection assay

Unlike the continuous injection assay, the substrate was here injected into the enzyme solution at shorter intervals. For this assay, the interval between injections should be long enough to account for the time constant of the instrument and the reaction; it should also be short enough to measure the reaction rate under steady state conditions and to avoid a significant amount of substrate being consumed. To estimate the kinetic parameters, increasing amounts of substrate were titrated into the enzyme solutions. 15 injections of 3 µl of acidified BSA (300 µM) were titrated every 100 s into a 13 µM pepsin solution in pH 2 buffer. For the trypsin-catalysed hydrolysis assays, 20 injections of 2.5 µl of substrate (AHP, AnHP, AnH, nAnH, 99.6 µM) were titrated every 100 s into the sample cell loaded with a 2.5 µM trypsin solution in pH 8 buffer. The rate of conversion of substrate into product $\left(\frac{d[P]}{dt}\right)$ at each injection was estimated from Equation 4.3, where $\frac{dQ}{dt}$ is the thermal power after each injection (Todd & Gomez, 2001). $\frac{dQ}{dt}$ from each injection was considered as the average of the heat rate, 25 s before the following injection. ΔH_{app} was determined from the first injection of the continuous injection assay.

$$\text{Rate} = \frac{d[P]}{dt} = \frac{1}{V\,\Delta H_{app}} \frac{dQ}{dt} = \frac{k_{cat}[E][S]}{K_m + [S]} \qquad \text{Equation 4.3}$$

4.2.5 Data analysis

Data from the continuous injection assay were integrated with NanoAnalyze (TA Instruments, USA). Data were fit to the Michaelis Menten equation (Equation 4.3) using a least-squares approach with the nonlinear fitting function 'lsqcurvefit' on Matlab R2019b (Mathworks, USA). Statistical analysis was performed using SPSS Statistics software (version 25, IBM, USA). One-way analysis of variance and post-hoc Tukey's Honestly Significant Difference were used to compare significant differences between means at a confidence interval of 95% ($p \leq 0.05$).

4.3 Results and discussion

4.3.1 Hydrolysis ratio

The aforementioned enzyme specificity towards certain peptide bonds (Keil, 2012) means that we need to determine the maximum achievable hydrolysis by one combination of protease and protein, at predetermined conditions (Luo et al., 2018). This maximum achievable hydrolysis was used to convert the concentration of protein to the concentration of cleavage sites. The hydrolysis ratio was the determined for each of the four substrate additions. To obtain only the free amino groups that are formed by

hydrolysis, the terminal and ε-amino groups were determined before hydrolysis (β, Table 4.1). The β values show that for the acidified substrates (A, AnHP and AnH), the exposure of terminal and side chain amino groups was similar (53.8±0.7 to 55.5±0.8). Interestingly, when the substrate was not acidified (nAnH), the exposure of the terminal or side chain amino groups was less than for the acidified substrates (45.1±1.8), suggesting a more compact structure in the former. As expected, β was much higher for pepsin-hydrolysate (101.8±2.0) as more terminal amino groups had been formed.

For the first time interval of the pepsin-catalysed hydrolysis of acidified BSA at pH 2, we determined a maximum hydrolysis ratio of 80.9 mol free amino groups from hydrolysis per mol non-hydrolysed protein (Table 4.1). The hydrolysis ratio after trypsin-catalysed hydrolysis for the pepsin-hydrolysate (AHP, 34.5±3.4) was significantly larger than that of the intact protein (AnHP, AnH and nAnH, 8.1±2.0 to 14.4±1.2). Therefore, more peptide bonds in BSA became susceptible to trypsin-catalysed hydrolysis after the protein had been hydrolysed by pepsin, potentially increasing the overall digestibility of the protein. The pepsin hydrolysate of A and AHP, before trypsin-catalysed hydrolysis, were the same sample, only with different final pH, 2 and 8, respectively. As such, β + hydrolysis ratio$_1$ of A would be similar to β of AHP. Approximately 34 more free amino groups were quantified for β + hydrolysis ratio$_1$ of A than β of AHP. This suggests a pH-induced conformational change that results in a lower exposure of terminal and ε-amino groups or perhaps even aggregation of peptides in the hydrolysate at pH 8.

The pH history of the intact protein also influences the hydrolysis ratio by trypsin. For the previously acidified BSA (AnHP and AnH, 14.4±1.2 and 12.4±1.4), significantly more peptide bonds were cleaved than for the not-acidified protein (nAnH, 8.1±2.0). This can be attributed to the more compact structure of BSA at pH 8, compared to pH 2, suggested by the low β of nAnH. A tighter protein conformation might hinder the access of trypsin to its preferred cleavage sites (Robertson et al., 2016). The slightly higher hydrolysis ratio of AnHP, compared to AnH, might be explained by the trypsin-catalysed hydrolysis of the inactive pepsin in the former. This finding highlights the importance of the gastric pH reaching low enough values to alter the protein conformation for BSA to be better hydrolysed by trypsin in the following stage of food digestion.

One molecule of BSA has 582 peptide bonds. Of those, according to the ExPASy PeptideCutter tool, the maximum potential cleavage sites by pepsin (pH>2) are 147 and by trypsin, 74 cleavage sites (Gasteiger et al., 2005; Keil, 2012). This lower selectivity partly explains the significantly larger hydrolysis ratio observed for pepsin than for trypsin. Nevertheless, the hydrolysis ratio reached by pepsin is 55% of the maximum theoretical

hydrolysis ratio, while for trypsin, it ranges between and 10 and 19% for the intact proteins.

The hydrolysis ratio decreased from the first to the fourth substrate addition in the pepsin-catalysed hydrolysis of acidified BSA assay (from 80.9±3.5 to 66.1±2.7). The opposite was seen for the trypsin-catalysed hydrolysis, where the hydrolysis ratio of subsequent substrate additions increased to an apparent maximum reached at the third addition. The reaction conditions were not the same from the first addition compared to the subsequent additions. In the first step, the substrate solution was added to an enzyme solution. For the subsequent steps, the substrate was added into a solution containing enzyme, peptides and, perhaps, intact proteins. It is uncertain whether the higher hydrolysis ratio of subsequent substrate additions were solely from products of the freshly added substrate or if some uncleaved peptide bonds from the previous additions were also targets for trypsin-catalysed hydrolysis.

The varying hydrolysis ratios for the different substrates and additions make evident that the history of the protein, in terms of pH and hydrolysis, influences the amount of peptide bonds susceptible to *further* hydrolysis, particularly by trypsin.

Table 4.1 Terminal and ε-amino groups (β), and hydrolysis ratio of substrate samples added to a pepsin solution at pH 2 (P) or into a trypsin solution at pH 8 (T), determined by the OPA method. Standard deviation from triplicate measurements is given between brackets. Values with the same letter did not differ significantly ($p > 0.05$, uppercase letters for β-values and lowercase letters for hydrolysis ratios across columns and rows).

	β $\left(\frac{\text{mol free } NH_2 \text{ before hydrolysis}}{\text{mol protein}}\right)$	Hydrolysis ratio $\left(\frac{\text{mol free } NH_2 \text{ after hydrolysis}}{\text{mol protein}} - \beta\right)$			
Substrate	-	addition 1	addition 2	addition 3	addition 4
A (P)	55.5^{B} (0.8)	80.9^{a} (3.5)	74.1^{b} (1.7)	71.2^{bc} (2.7)	66.1^{c} (2.7)
AHP (T)	101.8^{A} (2.0)	34.5^{e} (3.1)	40.9^{d} (4.1)	43.4^{d} (3.9)	44.0^{d} (3.7)
AnHP (T)	54.8^{B} (0.8)	14.4^{gh} (1.2)	23.9^{f} (2.3)	23.9^{f} (1.7)	22.3^{f} (1.7)
AnH (T)	53.8^{B} (0.7)	12.4^{hi} (1.4)	13.2^{hi} (3.4)	21.5^{f} (2.0)	21.9^{f} (1.8)
nAnH (T)	45.1^{C} (1.8)	8.1^{i} (2.0)	15.2^{gh} (2.2)	18.9^{fg} (2.4)	18.9^{fg} (2.9)

BSA non-hydrolysed at pH 2 (A), BSA pepsin-hydrolysate (AHP), acidified, non-hydrolysed with inactive pepsin (AnHP), acidified, non-hydrolysed (AnH) and non-acidified, non-hydrolysed (nAnH). The hydrolysis ratio is the amount of free NH_2 groups after hydrolysis, minus the amount present before hydrolysis (i.e., β), normalized on the amount of protein.

4.3.2 Tryptophan fluorescence

The pH-influenced protein conformation may be a key feature determining the extent of hydrolysis. We measured the tryptophan fluorescence of the different substrates and their hydrolysis products. Tryptophan is an intrinsic fluorophore of proteins. The maximum measured fluorescence intensity and emission wavelength provides information on the tryptophan residues' neighbouring amino acids and accessibility to the solvent (Royer, 2006). For instance, a red-shifted maximum, i.e., at a higher emission wavelength, indicates greater exposure of the tryptophan residues towards a polar solvent, while the maximum intensity is a reflection of the surroundings of the residue and is quenched upon protein unfolding due to solvent effects. The structure of BSA is divided into three domains, each subdivided into two subdomains, A and B. BSA has two tryptophan residues in its structure, Trp-134 and Trp-213. The former is located in the subdomain IB near the surface of BSA, while the latter is closer to the hydrophobic core of the subdomain IIA (Figure 4.2). The response from both residues in the two locations convenes in the recorded fluorescence spectrum. Nevertheless, it has been demonstrated that Trp-134 dominates the intensity over Trp-213 (Viallet et al., 2000).

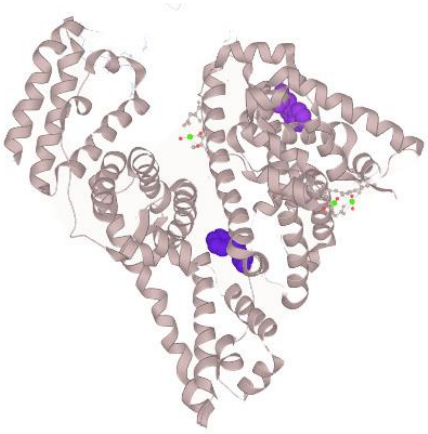

Figure 4.2 Crystal structure of BSA (Majorek et al., 2012). Tryptophan residues represented as surfaces (TRP-134 in purple, TRP-213 in blue; selection made on LiteMol, Sehnal et al. (2017)).

Among the five substrates, BSA dissolved in pH 2 buffer (A) showed the most blue-shifted maximum at 327 nm, i.e., lowest fluorescence emission wavelength at maximum intensity (Figure 4.3, purple filled circle), indicating a more apolar environment as well as quenching of one or both Trp residues. Non-acidified and previously acidified BSA returned at pH 8 (nAnH and AnH(P)) emitted at 336 nm, at comparable maximum intensities, revealing only a slight difference in the direct environment of Trp. Meanwhile, the pepsin-hydrolysate at pH 8 showed the most red-shifted maximum implying the

greatest exposure of Trp residues to the polar environment, including strong quenching effects. This substrate not only differs to the other substrates in conformation, but also in peptide length.

We also measured the fluorescence spectrum of the products of hydrolysis from the first substrate addition assay of the hydrolysis ratio determination (Section 4.3.1). Pepsin catalysed hydrolysis (A_1) resulted in peptides that were red-shifted with a markedly higher fluorescence intensity at maximum compared to the intact protein. This shows a change in the protein conformation that significantly affects the microenvironment surrounding the Trp residues, perhaps by losing the rigidity of the intact protein. Conversely, trypsin catalysed hydrolysis of the pepsin-hydrolysate (AHP_1) resulted in a slight blue-shift from the substrate. Hydrolysis did not always result in a shift in fluorescence emission at maximum intensity. No change in fluorescence emission wavelength was observed for BSA trypsin-hydrolysates ($AnHP_1$, AnH_1 and $nAnH_1$), although in all cases, fluorescence was slightly quenched after hydrolysis (Figure 4.3B). Interestingly, the extent of hydrolysis was reflected in the difference in fluorescence emission and intensity at maximum, between samples before and after hydrolysis.

Our findings agree with previous research which showed a change in the tryptophan fluorescence emission of BSA under acidic and alkaline conditions, from blue to red-shifted (Bhattacharya et al., 2011; Lan et al., 2020). Protonation at low pH increases the repulsion between charged groups which releases the electrostricted water from the hydrophobic pockets (Katchalski et al., 1957; Peters, 1996). As a result, Trp remains embedded in a dry molten globule with an apolar environment (Acharya et al., 2016). In contrast, the deprotonation of amino acids in alkaline conditions leads to a conformational shift resulting in increasing polarity near Trp (El Kadi et al., 2006). Furthermore, researchers who studied the far-UV circular dichroism spectra of BSA proposed that, at high pH, the protein has a tighter structure compared to low pH (Kun et al., 2009). This can also be related to charges and their distribution throughout the protein. At low pH, the strong repulsion between protonated groups, which are distributed uniformly, weakens the intra and inter-domain interactions and provides the largest stability in an expanded protein conformation (Lan et al., 2020; Muzammil et al., 1999). Meanwhile, at pH 8, the net electrical charge is nearly neutral, allowing for a folded structure (Lan et al., 2020). This is supported by the low β of nAnH BSA (Table 4.1).

Tryptophan residues in proteins are sensitive to changes in the local environment (Callis & Liu, 2004). This was observed particularly between the BSA pepsin-hydrolysate at pH 2 and at pH 8 (purple-hollow and green-filled circles in Figure 4.3A). These two samples

have the same protein, hydrolysed by the same enzyme at pH 2. However, the pH of the AHP substrate had been adjusted to 8 to be further hydrolysed by trypsin, avoiding differences in composition of the buffer in which the enzyme is dissolved. We therefore propose an alkalinity-induced aggregation which is supported by the aforementioned discrepancy between β + hydrolysis ratio$_1$ of A and β of AHP (Section 4.3.1).

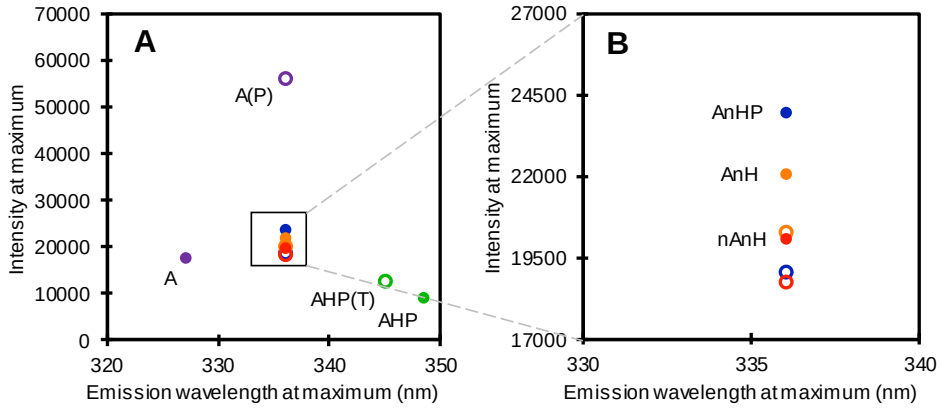

Figure 4.3 Tryptophan fluorescence intensity and emission at maximum. (A) BSA at pH 2, purple; BSA pepsin-hydrolysate at pH 2, green; acidified, non-hydrolysed with inactive pepsin, blue; acidified, non-hydrolysed, orange; non-acidified, non-hydrolysed, red. Filled circles represent samples before hydrolysis, open circles represent samples after pepsin (BSA at pH 2) and trypsin-catalysed hydrolysis (other substrates at pH 8). (B) Expanded view of samples which emitted their maximum intensity at 336 nm. Circles represent means of duplicate measurements.

Such differences were not observed in the fluorescence spectra of the intact proteins that were previously acidified (AnH(P)) and that which was directly dissolved in pH 8 buffer (nAnH, Figure 4.3B). In previous research, the isomer formed at pH 3 was found to be thermodynamically less stable than the native protein at higher pH (Bhattacharya et al., 2011). Small-angle X-ray scattering showed that the refolding of BSA from the acid-induced expanded state into the alkaline, native state was reversible (Yeh et al., 2017). In this study, pH increase was achieved by dialysis against a neutral solution, potentially not altering too much the ionic strength. Nevertheless, it has also been suggested that an initial acidity-induced unfolding of BSA is not rapidly reversible when titrating back to alkaline pH (Steinhardt et al., 1971). This indicates that refolding might be influenced by the environment surrounding the protein. Helical structures in folded BSA are favoured by internal salt bridges (Servagent-Noinville et al., 2000). The protein in our AnH(P) sample was initially exposed not only to low pH but also to the ionic strength of the buffer. When increasing the pH to 8, we propose that BSA might have refolded less tightly because of the initial exposure to ions at pH 2, which may have blocked the formation of some salt bridges, enabling the slightly more extensive hydrolysis found for

AnH(P) compared to nAnH. In sum, we suggest that pH-induced conformational isomerisation, as a result of the substrates' pH and hydrolysis history, influences the accessibility for enzymes to the preferred peptide bonds for cleavage. The tight structure reported for BSA at alkaline pH might hinder trypsin activity and thus explain the lower hydrolysis ratio compared to the substrates that had gone through an acid-denatured state.

4.3.3 Isothermal titration calorimetry

4.3.3.1 Apparent reaction enthalpy

The ITC continuous injection assay was used to measure the heat associated with the complete conversion of substrates into products. A solution of substrate was injected into the reaction cell that had been previously loaded with the enzyme solution. Given enough time between injections, the heat rate signal returned to the initial baseline indicating that the reaction proceeded to completion.

We observed that the dilution of all substrates into pH 2 and pH 8 buffers was overall endothermic and was compensated by the ITC within approximately 100 s (dotted lines in Figure 4.4 and Figure A4.1). Meanwhile, the joint signal of dilution and reaction heat for the pepsin-catalysed hydrolysis of acidified BSA at pH 2 was overall exothermic and lasted for around 1400 s (Figure 4.4A). Trypsin catalysed hydrolysis was overall endothermic and its return to baseline happened sooner than for pepsin (Figure 4.4B and Figure A4.1). Shorter reaction times for the trypsin-catalysed reaction were likely due the lower concentrations of both protein and enzyme, compared to the pepsin assay. The total heat of the reaction was obtained by integrating the area under the curve of each injection with respect to time. To account for the dilution heat, the integrated heat from the blank experiments was subtracted from the heat of the injection of substrate into enzyme.

The reaction heat rate was used to calculate the apparent molar enthalpy of the reaction (ΔH_{app}, Equation 4.2). ΔH_{app} is the total heat produced or absorbed to convert one mol of substrate into product. To normalise with the number of susceptible peptide bonds of BSA in a given condition, the cleavage sites were considered as the substrate (Luo et al., 2018). The molar concentration of substrate used in Equation 4.2 was estimated from the hydrolysis ratio (mol free amino groups from hydrolysis/mol protein) of the first substrate addition (hydrolysis ratio$_1$, Table 4.1). Both ΔH_{app}, expressed as kJ/mol BSA and as kJ/mol cleavage sites, are given in Table 4.2.

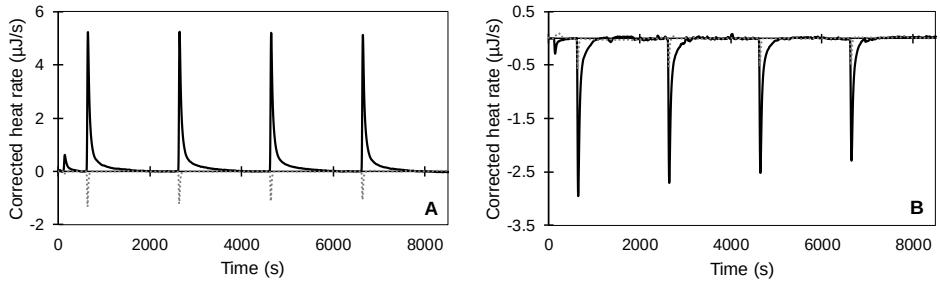

Figure 4.4 Continuous injection assay of (A) acidified BSA hydrolysed by pepsin at pH 2 and (B) BSA pepsin-hydrolysate, further hydrolysed by trypsin at pH 8. Blank injections of substrate into buffer (dotted line) and injections of substrate into enzyme solution (solid line). A small volume was injected after the initial equilibration to account for the diffusion of substrate solution from the syringe into the cell.

For the first injection, ΔH_{app} of the hydrolysis of peptide bonds in the intact protein did not differ significantly, regardless of the pH history (A catalysed by pepsin and, AnHP, AnH and nAnH catalysed by trypsin). Meanwhile, the enthalpy of the trypsin-catalysed hydrolysis of the pepsin-hydrolysate (AHP) was significantly higher than for intact BSA. This means that more heat is absorbed to hydrolyse a peptide bond within a shorter peptide chain than in a full protein.

The continuous injection assay was chosen over the more common single injection assay to gain insight into potential product inhibition (Maximova & Trylska, 2015; Maximova et al., 2018). After the first injection, the reaction medium contains enzyme, leftover substrate and products. With each subsequent injection of substrate, products continue to accumulate. Changes in the overall magnitude and shape of the curves associated to the injections would indicate an effect on the enzyme activity due to the presence of hydrolysis products. In all assays, the magnitudes and shapes of the peaks corresponding to each of the four injections appeared similar between each other, as shown by the superimposed curves from each injection (Figure A4.2). Nevertheless, a slight decrease was observed in the total area under the curve of each subsequent injection. The decrease was not significant for the ΔH_{app} associated to each injection of the pepsin-catalysed hydrolysis of peptide bonds of BSA (Table 4.2). Conversely, for the trypsin-catalysed hydrolysis of all substrates, ΔH_{app} decreased from the first to the fourth injection. This can be partly explained by the lower enzyme concentration after a new injection due to the displaced volume from the sample cell. Such a decrease in the heat signal has been associated to trypsin inhibition by products of casein hydrolysis (Maximova & Trylska, 2015). Further, product inhibition has been observed on both pepsin and trypsin-catalysed hydrolysis (Deng et al., 2018; Kitson & Knowles, 1971).

Table 4.2 Apparent molar enthalpy from the continuous injection assay of substrate into an enzyme solution. BSA at pH 2 (A) injected to a pepsin solution. BSA pepsin-hydrolysate (AHP), acidified, non-hydrolysed with inactive pepsin (AnHP), acidified, non-hydrolysed (AnH) and non-acidified, non-hydrolysed (nAnH), injected into a trypsin solution at pH 8. Standard deviation from triplicate measurements is given between brackets. Values with the same letter did not differ significantly ($p > 0.05$).

Substrate \ Injection	1		2		3		4	
	ΔHapp (kJ/mol BSA)							
A (P)	-451.9^{ab}	(17.3)	-439.3^{abc}	(7.7)	-431.4^{abc}	(11.2)	-429.6^{abc}	(1.6)
AHP (T)	500.3^{a}	(38.5)	466.7^{abc}	(20.0)	407.4^{bc}	(33.0)	364.4^{c}	(27.2)
AnHP (T)	82.9^{d}	(35.1)	90.1^{d}	(38.4)	66.1^{d}	(15.6)	45.0^{d}	(21.8)
AnH (T)	92.8^{d}	(22.5)	92.4^{d}	(1.8)	69.3^{d}	(18.0)	59.0^{d}	(25.2)
nAnH (T)	52.1^{d}	(14.1)	38.5^{d}	(17.4)	40.3^{d}	(18.4)	19.2^{d}	(5.5)
	ΔHapp (kJ/mol cleavage site)							
A (P)	-5.6^{cdefg}	(0.2)	-5.9^{cdefg}	(0.1)	-6.1^{cdefg}	(0.2)	-6.5^{cdef}	(0.0)
AHP (T)	14.5^{a}	(1.1)	11.4^{ab}	(0.5)	9.4^{bc}	(0.8)	8.3^{bc}	(0.6)
AnHP (T)	5.8^{cdef}	(2.4)	3.8^{defgh}	(1.6)	2.8^{fgh}	(0.7)	2.0^{gh}	(1.0)
AnH (T)	7.5^{bcd}	(1.8)	7.0^{cde}	(0.1)	3.2^{efgh}	(0.8)	2.7^{fgh}	(1.2)
nAnH (T)	6.4^{cdef}	(1.7)	2.5^{fgh}	(1.2)	2.1^{gh}	(1.0)	1.0^{h}	(0.3)

In contrast, we observe that the average conversion rate seems to increase with subsequent injections as shown by the hydrolysis ratio $_{1,2,3}$ (Table 4.1), challenging the notion of product inhibition. This increase in the hydrolysis ratio further reduces ΔH_{app} (kJ/mol cleavage site) after each injection, implying that by increasing the ratio of substrate to enzyme, less heat is consumed to hydrolyse one peptide bond.

4.3.3.2 Steady state enzyme kinetics

The ITC multiple injection assay was conducted to determine the kinetics of the enzyme-catalysed hydrolysis of BSA. Changes in the heat rate after each injection are related to the rate of the enzyme-catalysed hydrolysis. The progressive addition of substrate allows to estimate the reaction rate at different substrate concentrations within one assay (Figure 4.5A and Figure A4.3). The heat rate $\left(\frac{dQ}{dt}\right)$ was measured after each injection and corrected for the heat rate associated to the heat of dilution of the substrate. The reaction initial rate was estimated from the heat rate using Equation 4.3. The reaction enthalpy used for the estimation was from the first injection of the continuous injection assay as negligible product effects were observed (Table 4.2). The initial rates of the enzyme-catalysed hydrolysis of peptide bonds in BSA were calculated for each substrate-enzyme concentration combination (Figure 4.5B and Figure 4.6). The reaction rates estimated at progressive substrate concentrations were fit to the Michaelis Menten model (Equation 4.3). The turnover number and Michaelis-Menten constant were obtained (k_{cat} and K_m, Table 4.3).

A comparison was made between the initial reaction rates when protein or cleavage sites were considered as the substrate of trypsin-catalysed hydrolysis. In the former, there was a clear distinction between the hydrolysis rates for the different pH and hydrolysis history of the protein (Figure 4.6A). The hydrolysis rate as a function of BSA concentration seemingly approached a rate plateau. The slowest turnover was observed for the BSA pepsin-hydrolysate at 0.004 s^{-1}. It was followed by the previously acidified BSA, with and without inactive pepsin at 0.013 s^{-1}. Lastly, BSA directly dissolved in pH 8 buffer was hydrolysed reaching the fastest maximum reaction rate of approximately 0.017 s^{-1}. In the latter case, when the available cleavage sites were considered being the substrate of the reaction, the distinction between pH and hydrolysis history of BSA was far less evident and in some cases a plateau was not observed (Figure 4.6B). The hydrolysis of the peptide bonds in the pepsin-hydrolysate had still the slowest turnover. Nevertheless, the previously acidified intact BSA (AnHP and AHP) and the basic BSA (nAnH) swapped positions, with AnHP and AHP seemingly having the fastest turnover. The Michaelis-

Menten constant (K_m) increased accordingly (Table 4.3). The magnitude of the rates increased by a factor of up to 30 when cleavage sites were considered as the substrate.

This is not surprising as, in any case, there were more cleavage sites than moles of protein, which reduced the apparent enthalpy thus increasing the reaction rate: it can be expected that hydrolysing all the susceptible peptide bonds in a protein requires more time than a single cleavage site. Maximova and Trylska (2015) used ITC to study the trypsin-catalysed hydrolysis of casein and of a small substrate with one peptide bond that acted as a cleavage site, Nα-benzoyl-DL-arginine β-naphthylamide. They observed slower rates of hydrolysis for the protein compared to the single peptide bond. We make a similar comparison when considering BSA as a substrate as opposed to cleavage sites.

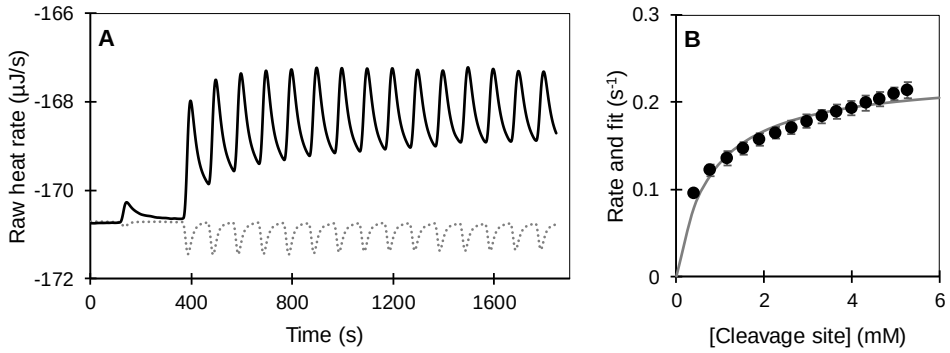

Figure 4.5 Multiple injection assay of pepsin-catalysed hydrolysis of BSA at pH 2. (A) Exemplar of calorimetric rate measurements of the blank injection of 300 µM BSA into phosphate buffer (dotted line) and reaction injection of BSA into 12.8 µM pepsin (solid line). A small volume was injected after the initial equilibration to account for the diffusion of substrate solution from the syringe into the cell. (B) Reaction rates estimated from three replicate measurements (circles), error bars represent standard deviation; line shows the fit to the Michaelis Menten model.

The differences in k_{cat} and K_m for the trypsin-catalysed hydrolysis of BSA with varying pH and hydrolysis histories might be attributed to the denatured state and resulting average size of the proteins. We speculate that the intact, previously acidified BSA (AnH(P)) was less tightly refolded when the pH was increased to 8, where the folded state prevailed again (Sadler & Tucker, 1993). Therefore, we propose that the average size of AnH(P) was slightly larger than that of the protein which was not acidified (nAnH). Naturally, the average size of the pepsin-hydrolysate (AHP) would be even smaller due to the hydrolysis. A direct relationship between polypeptide chain length and hydrolysis rate has been long proposed (Nachlas et al., 1964; Pozsgay et al., 1981). Further, according to the K_m estimates, the affinity of trypsin towards smaller structures seems to be higher, with the BSA pepsin-hydrolysate (AHP) having lower K_m compared to the intact protein (AnH(P) and nAnH). In this case, affinity might be a reflection of the poorer accessibility

of larger structures to the active site of trypsin. Previous research has related the ease of dissociation of the enzyme-product complex with the volume of the product (Shi et al., 2005). It was suggested that a product of larger volume would be more readily released due to space repulsion.

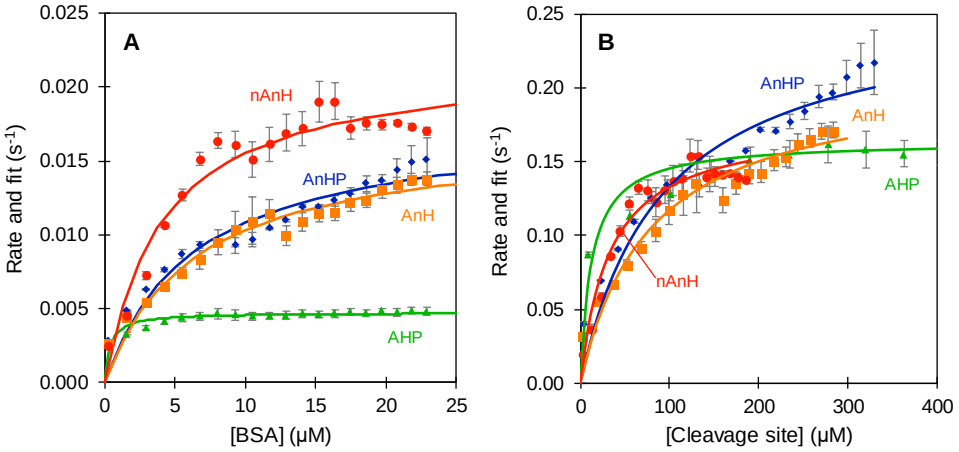

Figure 4.6 Reaction rate of trypsin-catalysed hydrolysis at pH 8 of BSA pepsin-hydrolysate (AHP, triangle), acidified, non-hydrolysed BSA with inactive pepsin (AnHP, diamond), acidified, non-hydrolysed BSA (AnH, square) and non-acidified, non-hydrolysed BSA (nAnH, circle). Error bars represent standard deviation from three replicate estimations. Fit to the Michaelis Menten model (line). Protein (A) and cleavage sites (B) considered as substrates, rates estimated from the appropriate ΔHapp (kJ/ mol BSA or kJ/ mol cleavage sites). The complete range of the BSA pepsin-hydrolysate reaction rate is shown in Figure A4.4.

As a plateau was not observed for the intact proteins, a more reliable measure of the hydrolytic efficiency towards the different substrates is the ratio of k_{cat} over K_m (Table 4.3). This ratio indicates how often a productive enzyme-substrate complex is formed; effectively, telling us how often an active site-bound sequence surrounding a susceptible peptide bond is broken. The cleavage sites of the BSA pepsin-hydrolysate (AHP) showed the best hydrolytic efficiency by trypsin. These were followed by those of BSA at pH 8 (nAnH), leading to the previously acidified BSA, with and without inactive pepsin (AnH(P)), as the least efficient substrate for trypsin-catalysed hydrolysis. As previously stated, we hypothesize that trypsin has a higher affinity towards the BSA pepsin-hydrolysate because of its smaller size and thus greater flexibility which allows easier exposure of cleavage sites, resulting in the most efficient trypsin-catalysed hydrolysis compared to the intact protein. For the intact protein, the initial exposure to low pH for AnH(P) had a significant impact in the hydrolytic efficiency compared to the protein dissolved directly in pH 8 (nAnH). Regardless of being the substrate with the lowest

hydrolytic efficiency, previously acidified BSA could potentially reach faster rates at maximum.

The hydrolysis ratio was used to convert the protein concentrations to cleavage site concentrations. This approach corrects for the enzyme's real potential to hydrolyse certain peptide bonds of a given protein with its own pH and hydrolysis history. Nevertheless, it assumes an equal hydrolysis rate on any cleavage site, when in actual fact it will be an average rate for the various cleavage sites. Within a protein, there are multiple cleavage sites with their unique exposure and surrounding amino acid sequences. It is known that pepsin and trypsin-catalysed hydrolysis, after different amino acid sequences, results in a wide range of kinetic parameters (Caprioli & Smith, 1986; Cornish-Bowden et al., 1969; Cornish-Bowden & Knowles, 1969; Hollands & Fruton, 1968; Lobo et al., 1976; Sachdev & Fruton, 1969).

Table 4.3 Kinetic parameters of the pepsin (P) and trypsin (T) catalysed hydrolysis of BSA estimated from the multiple injection assay. Standard error at the 95% confidence interval of the fitted parameters is given between brackets.

Substrate (Enzyme)	K_m ($\mu M_{cleavage\ sites}$)		k_{cat} (s^{-1})		k_{cat}/K_m (1/s mM)	r^2
A (P)	776.5	(194.2)	0.23	(0.01)	0.3	0.980
AHP (T)	12.8	(4.9)	0.16	(0.01)	12.8	0.957
AnHP (T)	92.8	(35.1)	0.26	(0.03)	2.8	0.947
AnH (T)	79.1	(24.2)	0.21	(0.02)	2.6	0.966
nAnH (T)	33.2	(11.2)	0.18	(0.02)	5.3	0.960

It has been suggested that pepsin-catalysed hydrolysis of a peptide bond within a protein is more efficient than of short, synthetic peptides (Luo et al., 2018). Our findings do not corroborate this. We believe this is because the different injection interval used in our assays. The kinetic parameters found in the current study lie between those of synthetic peptides with one cleavage site. While the specificities estimated in this study for pepsin and trypsin-catalysed hydrolysis of peptide bonds in BSA were larger than for some of the substrates reported in literature, they were still smaller than others (Table A4.1 and Table A4.2). This illustrates how the parameters estimated in this study are averages that likely disguise the influence of the amino acid sequence surrounding the to-be-cleaved peptide bond and subsite binding to the enzymes' active site. Hence, pepsin or trypsin catalysed hydrolysis of peptide bonds are not necessarily more efficient when located within a protein or within a short peptide. In our following work, we will address the role of the gastric phase on the gastro-duodenal digestion of other proteins. Future research

should assess to what extent limited trypsin-catalysed hydrolysis might influence exopeptidase action and ultimately absorption of small peptides and amino acids.

Our findings have interesting implications *in vivo*, particularly for populations for whom optimal protein utilization is essential such as athletes or aging adults. In optimal conditions, parietal and chief cells secrete adequate amounts of acid and pepsinogen to reduce the pH and catalyse the hydrolysis of proteins in the stomach. This physiological function is affected by age. A reduced output of pepsinogen has been observed in healthy aging adults (Feldman et al., 1996; Hurwitz et al., 1997). In contrast, age alone does not seem to be a determining factor of acid hyposecretion or hypochloridria. Nevertheless, hypochloridria is often observed in cases of atrophic gastritis (Kuipers & Grool, 2001), which are somewhat common among the elderly (Gao et al., 2017). In any case, gastric pH is expected to rise with increasing bolus size, requiring a longer time and higher amount of acid secretion for pH to be low enough for pepsinogen activation. Perhaps by reducing meal size and thus bolus entering the stomach, the pH can be maintained low enough for pepsinogen activation, and for protein digestion to be closer to the optimum.

4.4 Conclusion

After consuming a meal with protein, some of it might bypass the gastric environment by being emptied quickly from the stomach, some might only be present in the stomach long enough to experience the acid pH but not pepsin-catalysed hydrolysis, and lastly, some, with the longest gastric residence time, might be acidified and hydrolysed by pepsin action. We show that there are real differences in the hydrolysis because of this. The gastric phase, and the history of the protein in terms of pH and pepsin-catalysed hydrolysis, influences the extent and efficiency of trypsin activity in small intestinal conditions. Sequential pepsin and trypsin-catalysed hydrolysis of BSA results in the most efficient overall hydrolysis regarding both degree and rate of hydrolysis. Bypassing gastric hydrolysis reduced the catalytic efficiency of trypsin. Interestingly, just exposure to acid ensued the least efficient substrate while increasing the peptide bonds susceptible to tryptic hydrolysis. We propose that conformation and peptide length, as a consequence of pH and hydrolysis history of the protein, are among the main determinants of the course of trypsin-catalysed hydrolysis. Our findings indicate that eating a large meal (causing a sharp temporary increase in pH) may result in a slower or possibly incomplete digestion of proteins. Similarly, protein digestion by elderly, who generally secrete less enzyme and acid, may also be compromised for the same reason.

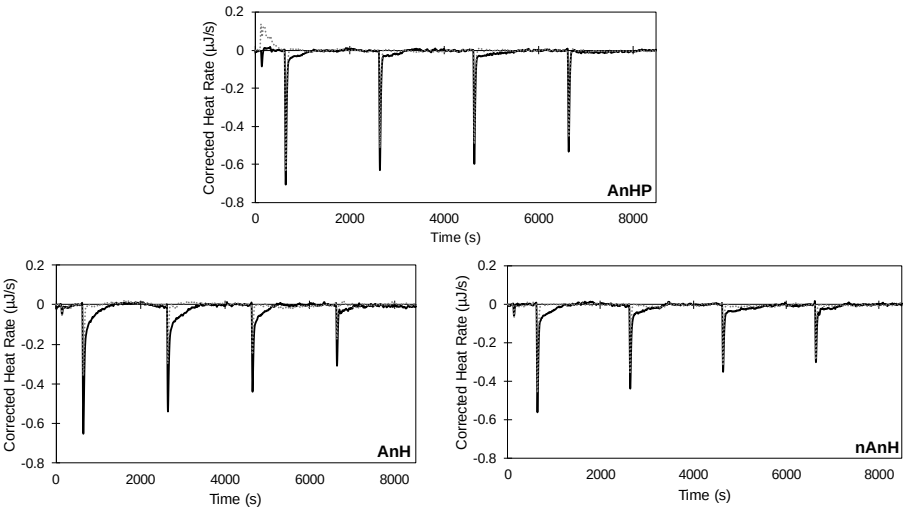

Figure A4.1 Continuous injection assay. Exemplar of calorimetric rate measurements of the blank injection of 100 µM substrate into phosphate buffer (dotted line) and reaction injection of substrate into 2.5 µM trypsin (solid line). A small volume was injected after the initial equilibration to account for the diffusion of substrate solution from the syringe into the cell. Acidified, non-hydrolysed with inactive pepsin (AnHP), acidified, non-hydrolysed (AnH) and non-acidified, non-hydrolysed (nAnH).

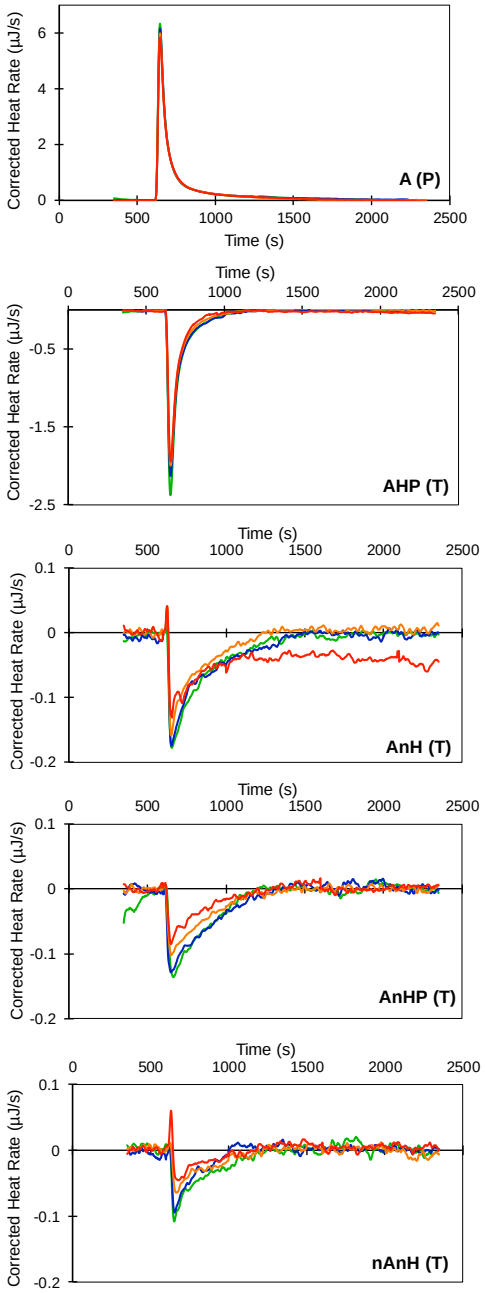

Figure A4.2 Superimposed heat rates from the continuous injection assay BSA at pH 2 (A) injected to a pepsin solution. BSA pepsin-hydrolysate (AHP), acidified, non-hydrolysed with inactive pepsin (AnHP), acidified, non-hydrolysed (AnH) and non-acidified, non-hydrolysed (nAnH), injected into a trypsin solution at pH 8. (1st injection green, 2nd blue, 3rd orange, 4th red).

Just as in the continuous injection assay, the higher amount of substrate that is injected results in a higher signal intensity of heat rate, this is the case of BSA injected into pepsin and the pepsin-hydrolysate into trypsin (Figure A4.3). Contrarily, the lower amount of cleavage sites for trypsin in the intact proteins result in a low signal intensity, in such cases the instrument drift is more noticeable than for larger signal intensities (Hansen et al., 2016). This might explain the greater error associated to the rate estimates at high substrate concentration of trypsin-catalysed hydrolysis of intact protein, especially when it has not been previously acidified (Figure 4.5).

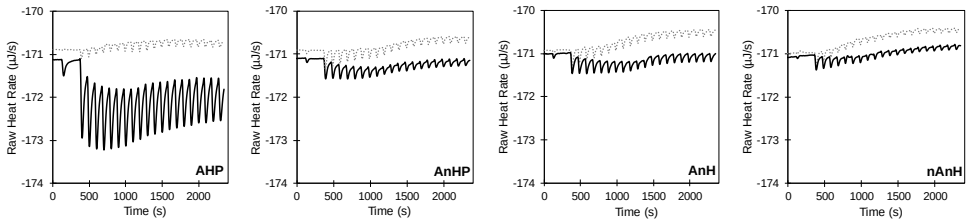

Figure A4.3 Multiple injection assay of trypsin-catalysed hydrolysis at pH 8. Exemplar of calorimetric rate measurements of the blank injection of 100 µM substrate into phosphate buffer (dotted line) and reaction injection of substrate into 2.5 µM trypsin (solid line). BSA pepsin-hydrolysate (AHP), acidified, non-hydrolysed with inactive pepsin (AnHP), acidified, non-hydrolysed (AnH) and non-acidified, non-hydrolysed (nAnH). A small volume was injected after the initial equilibration to account for the diffusion of substrate solution from the syringe into the cell.

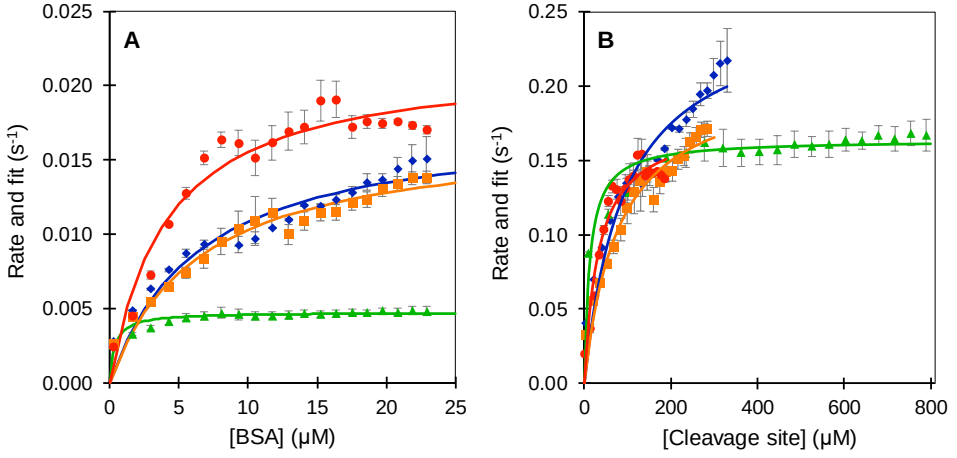

Figure A4.4 Reaction rate of trypsin-catalysed hydrolysis at pH 8 of BSA pepsin-hydrolysate (triangle), acidified, non-hydrolysed BSA with inactive pepsin (diamond), acidified, non-hydrolysed BSA (square) and non-acidified, non-hydrolysed BSA (circle). Error bars represent standard deviation from three replicate estimations. Fit to the Michaelis Menten model (line). Protein (A) and cleavage sites (B) considered as substrates, rates estimated from the appropriate ΔHapp (kJ/ mol BSA or kJ/ mol cleavage sites).

Kinetic parameters of pepsin and trypsin-catalysed hydrolysis of peptide bonds

Table A4.1 Kinetic parameters of pepsin-catalysed hydrolysis of small synthetic peptides and derivatives at pH 2 – 2.2. Selected, representative values from the references are included. Parameters are ranked according to their specificity constant (k_{cat}/K_m). Parameters estimated in this study for BSA are highlighted.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (1/s mM)	Reference
Ac-F-FNH_2	-	-	0.02	Cornish-Bowden and Knowles (1969)
Ac-F-$Y(NO_2)_2$	0.50	0.011	0.02	Cornish-Bowden et al. (1969)
Ac-F-Y-OEt	0.94	0.021	0.02	Hollands and Fruton (1968)
Ac-F-F	1.40	0.04	0.03	Cornish-Bowden et al. (1969)
Ac-$Y(NO_2)_2$-F	0.41	0.06	0.13	Cornish-Bowden et al. (1969)
Z-F-F-G-OMe3P	0.80	0.18	0.23	Sachdev and Fruton (1969)
Ac-F-F-G	1.7	0.39	0.23	Cornish-Bowden et al. (1969)
BSA A	0.78	0.23	0.30	
Z-H-F-F-OEt	0.33	0.11	0.33	Hollands and Fruton (1968)
Z-F-F-OP4P	0.71	0.49	0.69	Sachdev and Fruton (1969)
Z-F(NO_2)-F-OP4P	0.50	0.69	1.4	Sachdev and Fruton (1969)
Z-G-G-F(NO_2)-F-OP4P	1.10	8.1	7.4	Sachdev and Fruton (1969)
BSA	0.200	4.44	22.1	Luo et al. (2018)
Z-G-G-F-F-OP4P	0.80	56.5	70.6	Sachdev and Fruton (1969)

Table A4.2 Kinetic parameters of trypsin-catalysed hydrolysis of small synthetic peptides and amino acid derivatives at pH 8. Selected, representative values from the references are included. Parameters are ranked according to their specificity constant (k_{cat}/K_m). Substrates of deamidation are presented in italics. Parameters estimated in this study for BSA are highlighted.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (1/s mM)	Reference
Z-R-V-L-Nan	0.20	0.001	0.005	Pozsgay et al. (1981)
R-V-L-Nan	0.10	0.007	0.070	Pozsgay et al. (1981)
BACA	4.30	0.33	0.077	Wang and Carpenter (1968)
Ac-R-V-OMe	6.69	2.2	0.33	Lobo et al. (1976)
BLA	4.60	1.9	0.4	Wang and Carpenter (1968)
BANA	0.14	0.08	0.6	Maximova and Trylska (2015)
H-G-P-R-V-OH	1.20	1.2	1.0	Lobo et al. (1976)
BAA	2.50	2.8	1.1	Wang and Carpenter (1968)
Ac-R-G-OEt	3.00	4.2	1.4	Lobo et al. (1976)
BSA AnHP	0.08	0.21	2.6	
BSA AnH	0.09	0.26	2.9	
M-R-F-A	1.90	7.1	3.7	Caprioli and Smith (1986)
BSA nAnH	0.03	0.18	5.4	
Ac-G-R-V-OMe	20.00	3.6	0.2	Lobo et al. (1976)
Ac-G-G-R-G-OEt	2.90	19.0	6.6	Lobo et al. (1976)
Ac-V-R-G-P-R-OH	0.37	4.2	11.4	Lobo et al. (1976)
F-M-R-F(NH_2)	5.90	69.0	11.7	Caprioli and Smith (1986)
BSA AHP	0.01	0.16	12.5	
Ac-E-G-G-G-G-R-G-OEt	0.34	5.6	16.5	Lobo et al. (1976)
F-R-S-V	4.80	99.2	20.7	Caprioli and Smith (1986)
Bz-V-L-K-Nan	0.40	8.99	22.5	Pozsgay et al. (1981)
Ac-G-P-R-V-OEt	4.70	290.0	61.7	Lobo et al. (1976)
Ac-P-R-V-OMe	1.50	200.0	133.3	Lobo et al. (1976)
Bz-F-F-R-Nan	0.06	12.8	198.4	Pozsgay et al. (1981)
Ac-E-V-R-G-OMe	1.30	290.0	223.1	Lobo et al. (1976)
Bz-F-T-R-Nan	0.05	24.8	488.6	Pozsgay et al. (1981)
I-P-R-Nan	0.01	69.8	4976.7	Pozsgay et al. (1981)

Ac, Acetyl; BAA, N_α-Benzoyl-L-argininamide; BACA, N_α-benzoyl-S-2-aminoethyl-L-cysteinamide; BANA, N_α-Benzoyl-DL-arginine β-naphthylamide; BLA, N_α-benzoyl-L-lysinamide; Bz, benzoyl; Nan, p-nitroanilide; OP4P, 3-(4-pyridyl)propyl-1-oxy; P, pyridyl; Z, benzyloxycarbonyl.

CHAPTER FIVE

Gastro-small intestinal *in vitro* digestion of conventional and mildly processed pea protein ingredients

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Abstract

We report on the effect of processing, particularly heat treatment, on the digestion dynamics of pea proteins using the standardised semi-dynamic *in vitro* digestion method. Fractions with native proteins were obtained by mild aqueous fractionation of pea flour. A commercially produced pea protein isolate was chosen as a benchmark. Heating dispersions of pea flour and mild protein fractions reduced the trypsin inhibitory activity to levels similar to that of the protein isolate. Protein-rich and non-soluble protein fractions were better hydrolysed after being thermally denatured, particularly for proteins emptied at the last stages of the gastric phase. The degree of hydrolysis throughout the gastro-small intestinal digestion of these heated fractions was similar to that of the conventional isolate. Further heating of the protein isolate reduced its digestibility. Protein solubility enhances the digestibility of native proteins, while heating aggregates the proteins, which ultimately reduces the achieved extent of hydrolysis from gastro-small intestinal enzymes.

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5.1 Introduction

Plant proteins can meet consumer demands for ethical and more sustainable alternatives to animal-based products. The plant-based protein market could constitute up to 15% of the overall protein market in 2035 (Witte et al., 2021). Conventionally, plant protein-rich ingredients are extracted by a sequence of alkaline extraction and dissolution, isoelectric precipitation and dehydration, commonly spray drying (Lam et al., 2018). Proteins in commercial plant protein isolates are often denatured by the isolation process (Osen et al., 2014; Sun & Arntfield, 2010). A certain degree of denaturation is required for proteins to express their techno-functionality (Damodaran, 2005; Swanson, 1990). Furthermore, protein unfolding can facilitate the accessibility of digestive enzymes to cleavage sites (Barbé et al., 2013; Fontana et al., 1997). Conversely, it has been long proposed that excessive processing (Deshpande & Damodaran, 1989) and protein aggregation can reduce protein digestibility (Carbonaro et al., 1997; Rivera del Rio et al., 2020). Enzyme accessibility can be hindered by buried cleavage sites specific to a particular digestive enzyme within the protein structure.

Antinutritional factors in pulses, such as tannins, phytic acids and protease inhibitors, are secondary metabolites that protect the plant against biological stresses (Ryan, 1990). Protease inhibitors form stable enzyme-inhibitor complexes whose dissociation half-lives can be as long as the full digestion time of a meal, virtually sequestering enzymes. Long-term exposure to such inhibitors can induce enzyme over-production by the pancreas, leading to lesions or tumour formation (Gumbmann et al., 1989). Certainly, protease inhibitors should be inactivated prior to consumption. Efforts in plant breeding, separation techniques and structure modification by physical or chemical processes have managed to reduce or completely inactivate protease inhibitors (Avilés-Gaxiola et al., 2018; Khattab & Arntfield, 2009). For instance, trypsin inhibitors from pulses can be inactivated by heat treatment. Accordingly, the trypsin inhibitor content of conventionally produced protein isolates is low or non-detectable.

Protein digestion is a complex process which involves pH changes, mechanical breakdown of solid matrices, pH-dependent protease-catalysed hydrolysis, transit through the gastrointestinal tract and absorption. Gastric digestion has proved to be decisive in trypsin-catalysed hydrolysis of protein in small-intestinal conditions (Rivera del Rio et al., 2021). The recently proposed standardised semi-dynamic *in vitro* digestion method provides a straightforward approach to simulating the dynamic aspect of *in vivo* gastric digestion (Mulet-Cabero et al., 2020). Dynamically administered secretions allow

to reduce the pH of the food, while progressively increasing pepsin concentration, thus promoting protein hydrolysis.

It is known that native β-lactoglobulin is quite resistant to gastric digestion, while denatured β-lactoglobulin is readily digested in the stomach (Peram et al., 2013). It has been suggested that the individual units in a protein mixture, such as plant protein isolates, respond differently to denaturing agents such as heating, which in turn results in increased, reduced or sometimes unchanged overall protein digestibility (Genovese & Lajolo, 1998; Salazar-Villanea et al., 2016; C.-H. Tang et al., 2009). For instance, the i*n vitro* digestion with pepsin and pancreatin of heated, freeze-dried soybean protein isolate showed higher degrees of hydrolysis than their unheated counterpart (Ren et al., 2018; Zhang et al., 2018). Meanwhile, the work by Tian et al. (2019) illustrated the effect of heating temperature and time on the gastric digestibility of freeze-dried soybean protein isolate. The degree of hydrolysis of samples heated for 15 min at 70 or 100 °C was significantly lower than for the sample heated at 85 °C. A similar effect was observed for longer heating times at 85 °C. At any rate, unheated samples were less extensively hydrolysed than any of the heated samples.

We thus hypothesise that upon processing, denaturation and unfolding, protein will become more digestible compared to its native counterpart. Conversely, the digestibility of proteins that are already denatured in a conventional protein isolate would be reduced by further processing into a food product. We therefore here aim at studying the effect of protein denaturation on *in vitro* gastro-small intestinal digestibility of pea protein. To do so, native proteins are needed. Mild wet fractionation is not yet widely applied but can be used to extract native proteins from pulses (Geerts et al., 2017). To the best of our knowledge, the effect of heat treatment on native and denatured pea protein on their *in vitro* digestibility, using a dynamic gastric method, has not been studied previously.

5.2 Materials and methods

Dry yellow peas (*Pisum sativum L.*, Alimex, The Netherlands) were used for mild wet fractionation and industrially-produced yellow pea protein isolate (PPI, Nutralys F85M, Roquette, France) was used as a benchmark. All chemicals, unless otherwise stated, were purchased from Merck Sigma Aldrich without further purification. Milli-Q water (resistivity 18.2 MO, Merck Millipore, France) was used to prepare all samples and reagent solutions.

5.2.1 Sample preparation and characterization

Pea milling and mild wet fractionation was performed according to the method of Geerts et al. (2017) with some modifications. Briefly, grits from pin-milled peas (LV 15M, Condux-Werk, Germany) were impact-milled to obtain a fine flour (ZPS50, Hosokawa-Alpine, Germany). The temperature was monitored during impact milling between 16 and 34 °C. One part of pea flour and five parts of water were stirred for 60 min. The resulting slurry was centrifuged at 1500 g for one second at 20 °C in a Sorvall Legend XFR (Thermo Scientific, USA) centrifuge. The supernatant, henceforth referred to as the protein-rich fraction (PRF), was transferred to a new centrifuge bottle and centrifuged once more at 10000 g for 30 min at 20 °C in a Sorvall Lynx 4000 centrifuge (Thermo Scientific, USA). The resultant supernatant is here referred to as the soluble protein fraction (SPF) and the pellet as non-soluble protein fraction (NSPF).

The nitrogen content of pea flour, the resulting protein fractions and the conventional isolate was measured by Dumas analysis (Rapid N exceed, Elementar, Germany) in triplicate. A conversion factor of N x 5.52 was used (Holt & Sosulski, 1979). The protein content of pea flour and resulting fractions is presented in Figure 5.1. For the conventional pea protein isolate, the measured protein content was 74.2± 0.3% dw. Protein dispersions were prepared to match the protein content of SPF, 2.24 $g_{protein}$/100 $g_{dispersions}$, as this fraction held the most diluted protein content.

5.2.1.1 Heat treatment

90 ml of PPI dispersion, SPF, diluted pea flour slurry, PRF and NSPF in 100 ml Schott bottles were placed in a stirring dry bath (2mag Magnetic(e)motion, Germany) preheated to 120 °C. Dispersions were continuously stirred at 700 rpm. The temperature of the dispersions was monitored continuously. The dispersions were heated for 5 min after their temperature reached 90 °C.

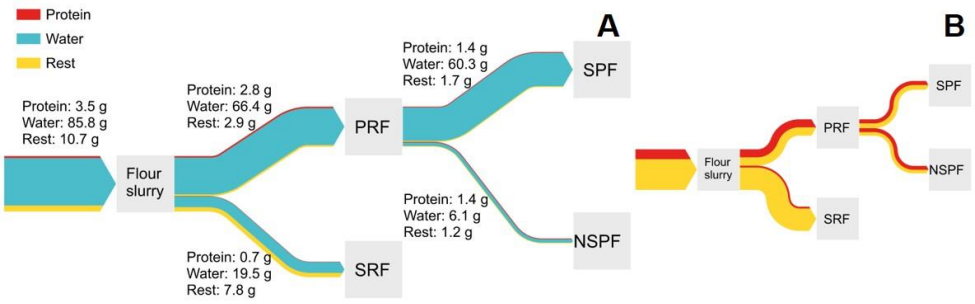

Figure 5.1 Composition of pea flour slurry, protein-rich (PRF), starch-rich (SRF), soluble protein (SPF) and non-soluble protein fraction (NSPF) on wet (A) and dry (B) bases.

5.2.1.2 Unit and subunit identification

Pea flour, mild protein fractions and conventional isolate were characterized with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with non-reducing and reducing conditions. 2x Laemmli sample buffer (Bio-Rad Laboratories Inc., USA) was mixed in equal parts with 2 $g_{protein}/l$ sample dilutions. Reducing sample buffer was prepared with 5% β-mercaptoethanol and the sample mixture was heated at 95 °C for 10 min. Sample mixtures and 10–250 kDa molecular weight standard were loaded into pre-cast mini-protean TGX gels. 10x Tris/Glycine/SDS running buffer was diluted and poured in the buffer tank. Electrophoresis was carried out at 300 V. Gels were rinsed three times with water, stained with bio safe Coomassie brilliant blue r-250 and rinsed once more, before scanning for band analysis.

5.2.1.3 Protein denaturation state

Differential scanning calorimetry was used to estimate the denaturation state of proteins in heated and unheated pea flour, mild protein fractions and conventional protein isolate. Samples were loaded and sealed onto stainless steel high volume pans. Two heating and cooling cycles, with a heating rate at 1 or 2 °C/min to 130 °C and a cooling rate at 30 °C/min to 20 °C, were conducted on a DSC 250 calorimeter (TA Instruments, USA).

5.2.1.4 Trypsin inhibitory activity

Trypsin inhibitory units were quantified based on the assay from Liu (2019), with some modifications. Briefly, trypsin inhibitors were extracted from the unheated and heated samples in an alkaline medium by slow rotation (8 rpm) of 4.12 mg protein per ml 10 mM NaOH. The pH was adjusted between 9.4 and 9.6. After 3 h of extraction, the samples were centrifuged at 3500 rpm for 10 min.

A standard substrate solution of 0.4 mg/ml Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA), pre-dissolved in dimethyl sulfoxide (0.1 $ml_{dimethyl\ sulfoxide}/ml_{standard\ substrate\ solution}$, final concentration), was prepared in 50 mM Tris·HCl buffer, pH 8.2 with 20 mM $CaCl_2$, previously warmed in a water bath at 37 °C. The enzyme solution of trypsin from bovine pancreas (15119 BAEE units/mg solid) at 20 µg/ml in a 1 mM HCl solution, with 5 mM $CaCl_2$, was stored at 4 °C until use. A 30% v/v acetic acid solution was used to stop the enzymatic reaction. For the trypsin inhibition assay blanks, standard and sample assays included the same samples and reagents with trypsin solution added before or after acidification with acetic acid. Final composition of the assay was 20% v/v water or diluted extract, 50% v/v BAPNA solution, 20% v/v trypsin solution and 10% v/v acetic acid solution. For the blanks, water or diluted sample extract, for reagent and sample blank, respectively, were mixed with BAPNA solution and preincubated at 37 °C

for 10 min. Acetic acid solution was mixed in and trypsin solution was added. For the standard and sample assays, the preincubated mixture of water or diluted extract with BAPNA was incubated at 37 °C with the trypsin solution. After 10 min, the acetic acid solution was added to inactivate trypsin. Blanks and tests were centrifuged at 10000 rpm for 15 min. Their absorbance was measured at 410 nm. Measurements were conducted in triplicate.

The inhibitor extract must be diluted appropriately to achieve trypsin inhibition (Equation 5.1) between 30 and 70%. Trypsin inhibitory units (TIU, unit/$g_{protein}$) were calculated with Equation 5.2, in which A_x, is the measured absorbance at 410 nm of the blanks, standard and extract assays, $V_{extract}$ and V_{assay} (ml) are the volumes of extract in the assay and the total volume of the assay, respectively, $[protein]_{extract}$ is the concentration of protein in the extract (g/ml), and ΔA is the difference in absorbance corresponding to one inhibitory unit per ml (0.1 ml/unit).

$$TI = 100 \cdot \frac{(A_{extract}\text{-}A_{extract\ blank})\text{-}(A_{standard}\text{-}A_{reagent\ blank})}{A_{standard} - A_{reagent\ blank}} \qquad \text{Equation 5.1}$$

$$TIU = \frac{V_{extract}}{[protein]_{extract}} \cdot \frac{V_{asssay}}{\Delta A} \cdot \left[(A_{extract}\text{-}A_{extract\ blank})\text{-}(A_{standard}\text{-}A_{reagent\ blank})\right] \qquad \text{Equation 5.2}$$

5.2.2 Semi-dynamic *in vitro* gastro-small intestinal digestion

Unheated and heated dispersions of 2.24 $g_{protein}$/100 g from pea protein, mild protein dispersions and conventional protein isolate were subjected to *in vitro* gastro-small intestinal digestion following the recently proposed semi-dynamic method from Mulet-Cabero et al. (2020). Simulated digestive fluids were prepared following the suggested salt stock solution combinations and enzyme unit concentrations.

In the oral phase, 20 ml of protein dispersion, pre-warmed at 37 °C was combined with simulated salivary fluid (SSF) at 37 °C (1:1, food dry weight : SSF). Only the SSF for pea flour digestion contained human salivary α-amylase (84 U/mg solid). After two minutes of the oral phase, the gastric phase started with 10% of the total simulated gastric fluid (SGF), including electrolyte mixture, pepsin from porcine gastric mucosa (632 haemoglobin U/mg solid), HCl and water, in a jacketed vessel connected to a water bath set at 37 °C, mixed with the oral content (1:1, oral content : total SGF). The remaining 90% of SGF (without pepsin) and pepsin were loaded in a titrator (877 Titrino plus, Metrohm, Switzerland) and a syringe pump (PHD 2000 Infusion, Harvard Apparatus, USA), respectively. The delivery rate was set following the calculations of the semi-dynamic method. The contents of the reaction vessel were stirred with a magnetic bar at

100 rpm. Five gastric emptying (GE) steps were performed by removing one fifth of the total gastric phase (oral content + total SGF), with a pipette. The duration of the gastric phase was calculated from the *in vivo* gastric half time of a 500 ml meal with an energy emptying rate of 2 kcal/min. Gastric digestion was halted by adjusting the pH to 7 with 1 M NaOH.

Digestion was continued in a static small intestinal phase in which the neutralized chyme from each of the five GE steps, was mixed with the simulated intestinal fluid (SIF) containing the appropriate electrolyte mixture, pancreatin from porcine pancreas (10.4 TAME trypsin U/mg solid) and water (1:1, acid chyme : SIF). Note that the NaOH added to the acid chyme is considered part of the SIF. The intestinal phase was performed for 2 h at 37 °C shaking at 350 rpm. Three aliquots were taken for analysis at 30, 60 and the final 120 min, digestion was halted by heating the aliquots at 95 °C for 5 min. Digestion samples were dispersed in a 2% solution of SDS to ensure optimal solubility for further analysis. Digestions were conducted in triplicate for heated and unheated, pea flour, mild protein fractions and conventional protein isolate dispersions.

5.2.2.1 Buffering capacity

Before the digestion assay can be conducted, the amount of acid needed to reduce the pH of the food to pH 2 has to be quantified. The oral phase was conducted as in the digestion assay. However, the gastric phase was modified. The total SGF was added to the reaction vessel and the pepsin solution was substituted with water. 1.5 M HCl was slowly titrated until pH 2 is reached. The required amount of acid was recorded and used to calculate the composition of SGF used in the digestion assay. The buffering capacity was calculated using these data.

5.2.2.2 Protein content

The protein content of each GE step aliquot was quantified with the Pierce™ bicinchoninic acid protein (BCA) assay kit using the microplate procedure (ThermoFisher Scientific Inc., USA). A 2 mg/ml bovine serum albumin (BSA) standard was used to prepare a standard curve. Samples were diluted to estimate concentrations within the standard curve of 20–2000 µg BSA/ml. The method followed the standard protocol, incubating the reacting samples at 37 °C for 30 min with the prepared reagent. The resulting absorbance of the colorimetric reaction was measured at 562 nm. The protein quantification was conducted in triplicate.

5.2.2.3 Degree of hydrolysis

Hydrolysis after the addition of pepsin and pancreatic enzymes was quantified by the *o*-phthaldialdehyde (OPA) colorimetric assay (Nielsen et al., 2001).The OPA reagent contained 3.81% w/v sodium tetraborate decahydrate, 0.1% w/v SDS, 0.08% w/v OPA previously dissolved in 2% v/v ethanol, and 0.088% w/v dithiothreitol. The reagent was stored protected from light after it was filtered through a 0.45 µm syringe filter. A standard curve was prepared with L-serine (Alfa Aesar, Germany) in a concentration range from 0 to 2 mM. 30 µL of sample or standard were combined with 240 µL OPA reagent in a microplate well. 3 min after the start of the reaction, the absorbance was measured at 340 nm. From the standard curve, the absorbance from unknowns was converted to [$NH_{2\,free}$] (mM). The concentration of free amino groups from hydrolysis was corrected by subtracting the contribution of the digestive enzymes.

The degree of hydrolysis (%, DH) represents the fraction of cleaved bonds from the total peptide bonds. The DH was calculated with Equation 5.3, where [protein] was obtained from the BCA assay, β accounts for the α- and ε-amino groups measured for each sample before hydrolysis; and h_{tot} is the total peptide bonds which were determined after acid hydrolysis with 6 M HCl at 95 °C for 24 h.

$$DH = 100 \cdot \frac{\left[NH_{2,free}\right]/_{[protein]} - \beta}{h_{tot}} \qquad \text{Equation 5.3}$$

5.2.2.4 Peptide size distribution

Digestion samples were analysed using an UltiMate 3000 chromatographer (ThermoFisher Scientific Inc., USA) used to perform Size Exclusion Chromatography (SEC). Samples were injected through a dual column system with TSK gel columns G3000SWXL and G2000SWXL for proteins and peptides. The eluent was an aqueous solution of 30% v/v acetonitrile and 0.1% v/v trifluoroacetic acid. UV absorbance detection at 214 nm was used to measure the resulting signals. Chromatogram integration was conducted with the Chromeleon 7.2 software (ThermoFisher Scientific Inc., USA). The data processing method was adjusted to integrate peaks between retention times corresponding to molecular weight ranges. A calibration curve was constructed with molecular weight standards, α-lactalbumin (14 kDa), aprotinin (6.51 kDa), bacitracin (1.42 kDa) and phenylalanine (147 Da).

5.2.3 Statistical analysis

SPSS Statistics software (version 25, IBM, USA) was used to perform one-way analysis of variance and post-hoc Tukey's Honestly Significant Difference test to compare significant differences between means at a confidence interval of 95% ($p \leq 0.05$). Digestion samples were compared among each gastric or small intestinal timepoint.

5.3 Results and discussion

5.3.1 Characterization of protein sources

5.3.1.1 (Sub)unit identification

SDS-PAGE was used to identify the protein units and subunits present in pea flour, PRF, SPF, NSPF and conventional PPI (Figure 5.2). Under non-reducing conditions, almost all protein units from pea flour were present in the protein fractions and the conventional protein isolate. Slightly fainter bands were observed in SPF, one just above 50 kDa and another above the 37 kDa mark. These coincide with the molecular weight of legumin A and B. Likewise, in reducing conditions, the acid chain of legumin A (at approximately 37 kDa) and the β chains of legumin A and B, as well as the α chain of legumin B (at approximately 20 kDa), were nearly absent in SPF. The absent band in NSPF, for both non-reducing and reducing conditions, at approximately 27 kDa could be assigned to lectin, which might have been separated into the soluble fraction through centrifugation. Lectins are commonly extracted by soaking pulses, suggesting that these units are rather soluble (Shi et al., 2018).

Compared to the mild protein fractions, PPI showed a somewhat faint band for legumin B and a relatively lower amount of convicilin, below the 75 kDa mark under non-reducing conditions. As expected, the smear at the top of the gel for high molecular weight units suggests a lower proportion of large proteins in the SPF compared to the other samples, particularly NSPF and PPI. It may be worth noting that recent research showed that isoelectrically precipitated PPI did not include the albumins, which generally precipitate not as readily than globulins (Kornet et al., 2021). The PRF and SPF will still contain the albumins, which are smaller and may contain more accessible dissociating groups.

5.3.1.2 Protein denaturation

The DSC thermogram of the unheated pea flour slurry showed a distinct endothermic peak at ca. 68 °C that can be associated to starch gelatinization (Figure A5.1 and Table A5.1). Somewhat overlapped, smaller peaks at ca. 77 °C and 89 °C were observed in the unheated PRF dispersion. In SPF, the peak was most predominantly at ca. 78 °C, while in flour and NSPF it was more evident at 89 °C. Our observations are in agreement

with Sim et al. (2019) who found a relatively wide peak from 73 to 88 °C attributed to the thermal denaturation of both 7S and 11S pea globulins, as well as a peak at 66 °C associated to starch gelatinization.

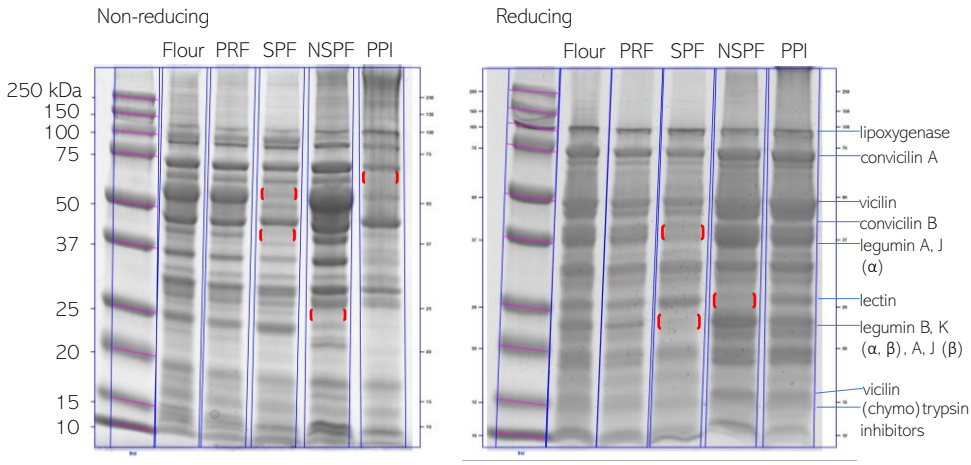

Figure 5.2 Non-reducing and reducing SDS-PAGE patterns of pea flour, protein-rich (PRF), soluble (SPF), non-soluble (NSPF) protein fractions and conventional pea protein isolate (PPI).

The enthalpy changes in the first heating ramp of the unheated dispersions of flour and mild fractions were not observed in the second heating ramp. This indicates that proteins in pea flour and mild fractions were initially in their native state. Furthermore, thermal denaturation of these proteins was irreversible as shown by the second heating ramp of the unheated samples and both heating ramps of the heated dispersions. As expected, the thermograms of both unheated and heated PPI dispersions did not show enthalpy changes or peaks associated to protein denaturation, confirming that the proteins in the commercial isolate were denatured (Shand et al., 2007).

5.3.1.3 Peptide size distribution

Size exclusion chromatography was used to estimate the molecular weight distribution of proteins and peptides in pea flour, protein fractions and protein isolate prior to their *in vitro* digestion (Figure 5.3). Fractionating PRF into SPF and NSPF leads to more small peptides (<10 kDa) into the soluble fraction and more large peptides (>10 kDa) into the non-soluble fraction.

Native proteins fractionated from pea flour were denatured by heat treatment. Heating led to protein unfolding and limited aggregation as can be observed in the peptide size distribution of the unhydrolyzed samples. In PRF and SPF, peptides with a molecular weight >10 kDa were formed from the aggregation of lower molecular weight peptides (Figure 5.3). For NSPF, less peptides >10 kDa and more peptides <4 kDa were recorded

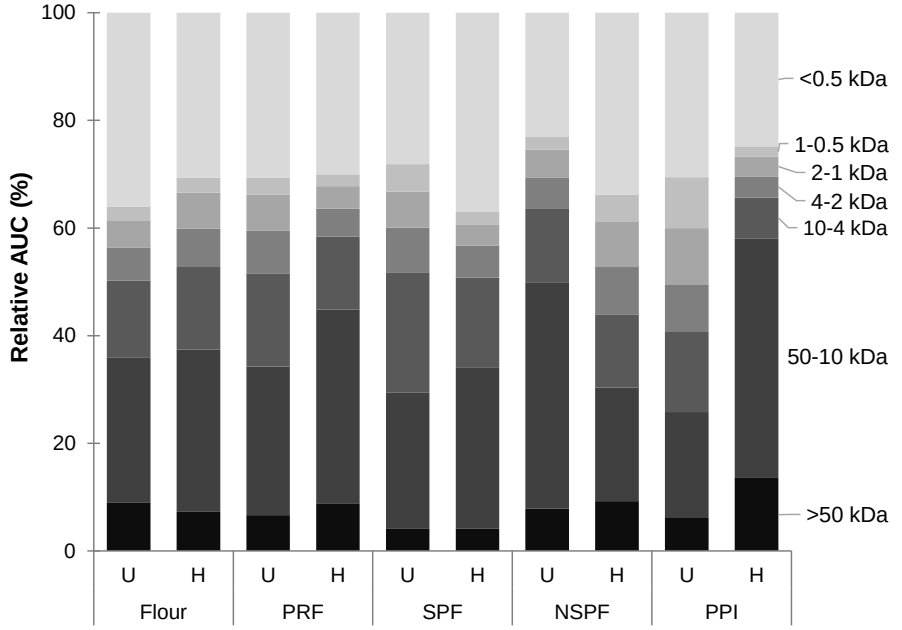

Figure 5.3 Peptide profile integrated from size exclusion chromatograms of pea flour, protein-rich (PRF), soluble (SPF), non-soluble protein (NSPF) fractions, and conventional pea protein isolate (PPI), unheated and heated (H) dispersions.

after heat treatment. Coincidently, the β-value measured for these fractions was reduced upon heating, implying less accessible NH_2 groups, i.e., aggregation, particularly for SPF for which the reduction was most significant (Table 5.1).

5.3.1.4 Trypsin inhibitory activity

The effect of processing on trypsin inhibitor contents in pea flour, mild protein fractions and conventional PPI was studied. Unheated pea flour and its PRF contained the largest amount of trypsin inhibitory units (TIU, Table 5.1). Interestingly, TIU were lower in SPF and NSPF, compared to PRF, although not significantly for SPF. Trypsin inhibitors are proteins of relatively low molecular weight. Bowman-Birk (~11 kDa) and Kunitz-type (~21 kDa) inhibitors have been identified in pea, with 7 and 2 disulphide bonds, respectively, that stabilize their structure (UniProt Consortium, 2018). It is to be expected that proteins of lower molecular weight partition into the SPF after centrifugation. Our TIU values are similar to those reported in literature for protein-rich fractions (Reinkensmeier et al., 2015).

Table 5.1 Trypsin inhibitory units (TIU), buffering capacity (BC, ΔpH from pH of food to pH 2), β-value (α and ε amino groups) and h_{tot} (total peptide bonds) of pea flour, pea protein-rich (PRF), soluble (SPF) and non-soluble protein (NSPF) fractions and pea protein isolate (PPI) in unheated and heated dispersions. Standard deviation from triplicate measurements is given between brackets. For TIU, values with the same letter did not differ significantly ($p > 0.05$).

	TIU ($U/g_{protein}$)		BC ($mmol_{H+}/\Delta pH\ g_{protein}$)		β ($meq_{NH2}/g_{protein}$)		h_{tot} ($meq_{NH2}/g_{protein}$)
	unheated	heated	unheated	heated	unheated	heated	
Flour	6.3^{a} (0.8)	0.9^{bc} (0.2)	1.04	1.15	1.1 (0.0)	1.1 (0.0)	9.1 (0.4)
PRF	6.6^{a} (0.7)	0.9^{bc} (0.2)	0.88	1.18	1.2 (0.0)	1.0 (0.0)	8.1 (0.3)
SPF	3.9^{ab} (0.2)	3.6^{abc} (0.4)	1.30	1.28	2.2 (0.0)	1.4 (0.0)	6.8 (0.2)
NSPF	2.2^{bc} (0.2)	0.7^{bc} (0.1)	0.84	1.06	1.0 (0.0)	0.8 (0.0)	9.6 (0.4)
PPI	1.4^{bc} (0.2)	0.4^{c} (0.1)	0.72	0.76	0.8 (0.0)	0.8 (0.0)	9.6 (0.1)

It has been reported that boiling inactivates or reduces trypsin inhibition in pea seeds and flour (Avilés-Gaxiola et al., 2018; Ma et al., 2011). Heating during spray drying may have the same effect, which would explain the low values for PPI. Higher TIU values have been reported for freeze-dried (Reinkensmeier et al., 2015) than for spray-dried PPI (Çabuk et al., 2018). Further heating of PPI resulted in a non-significant reduction of TIU. TIU values were also reduced upon heating of pea four, PRF and NSPF, although not significantly in the latter. No significant difference was observed between the unheated and heated SPF. We currently have no explanation for this heat resistance; it may well be that the major path towards lowering the TIU values in regular fractions is through washing and not through heating; however, this does not explain why the TIU values of the flour, PRF and NSPF were reduced strongly. Alternatively, it has been observed that the inhibitory activity of soybean flour is lost faster upon heat treatment than in the purified forms of the Bowman-Birk or Kunitz-type inhibitors; signalling some form of interaction between the inhibitors and with the matrix itself (DiPietro & Liener, 1989). Potentially, Bowman-Birk type inhibitors, the more heat-stable of the two types, were partitioned into SPF. Nevertheless, TIU in the heated flour and mild protein fractions did not present a significant difference compared to unheated PPI, indicating that a mild heat treatment is sufficient to reduce the trypsin inhibitory activity to the levels achieved by the conventional isolation process.

5.3.1.5 Buffering capacity

Proteins are the main components in food that are responsible of buffering the pH reduction upon secretion of HCl into the stomach. Considering that only 0.01 mmol H^+ is required to lower the pH of 1 ml of water to 2, the buffering capacity of pea flour, mild protein fractions and conventional protein isolate was significant (Table 5.1). The unheated flour slurry showed a relatively high buffering capacity, compared to PRF. This could be related to the presence of starch in flour taking up some of the acid and preventing the pH to drop. The buffering capacity of SPF was higher than that of NSPF, suggesting perhaps a greater exposure of dissociating groups in the former material. Similarly, heating PRF and NSPF might have induced protein unfolding and expose buffering groups, thus increasing their buffering capacity. Meanwhile, PPI showed a low buffering capacity that did not change after heat treatment.

5.3.2 Semi-dynamic *in vitro* digestion

The standardised semi-dynamic *in vitro* digestion method consists of a dynamic addition of gastric secretions to the 'bolus' from the oral phase, a stepwise emptying of gastric 'chyme' and a static small intestinal phase (Mulet-Cabero et al., 2020). In this method,

the rate of gastric emptying (GE) and, as a consequence, the duration of the gastric phase depends on the energetic content of the food. Naturally, the presence of starch in the flour slurry impacted its caloric density, and thus, the duration of the gastric phase for this dispersion was longer than for the mild fractions and PPI dispersions. For the purpose of comparison, we present the results from the digestion assays for each GE step (1 – 5) for the gastric phase and subsequent small intestinal phase (at 30, 60 and 120 min).

5.3.2.1 Unheated pea protein

The hydrolysis profiles of native pea protein in the gastric phase differed according to the matrix, i.e., pea flour, PRF, SPF and NSPF (Figure 5.4, unheated). A steady increase in DH was observed along the gastric phase, except for a slight reduction in DH at the second GE step, for flour, PRF and SPF, and at the first GE step, for NSPF. These reductions can be explained by protein clotting or precipitation around the isoelectric point of proteins in each fraction. Afterwards, flour, PRF and NSPF showed similar extents of hydrolysis, while SPF was more extensively hydrolysed through the course of the gastric phase.

The aliquots of each GE step were further digested in the small intestinal phase with pancreatic enzymes. Figure 5.4 shows the DH for each GE step after 30, 60 and 120 min in the small intestinal phase. As it is commonly observed, the extent of protein hydrolysis was significantly higher in the small intestinal phase, compared to the gastric phase for a given GE step. For each protein sample, the DH observed after 30 min of the small intestinal phase was higher for later GE steps. This suggests that a longer residence time in the gastric phase increases the DH in the small intestinal phase. Throughout the small intestinal phase (from 30 to 120 min), a slight increase in DH was observed for a given sample and GE step.

The lowest DH throughout the small intestinal phase of the native proteins was observed in PRF and NSPF, particularly for GE 3 to 5. The proteins in the flour slurry were hydrolysed to a higher extent, however not as much as SPF, which showed the highest DH for all GE steps of all native proteins studied. The higher DH observed for SPF suggests a greater exposure of cleavage sites in this fraction, perhaps due to units of smaller molecular weight being separated into the supernatant after centrifugation. This is evidenced by the high β-value and low h_{tot} measured for SPF (Table 5.1), as well as its peptide size distribution before hydrolysis which showed a smaller fraction of high molecular weight peptides (>10 kDa, Figure 5.3). Therefore, proteins in SPF, while native, do exhibit an enhanced digestibility compared to pea flour, PRF and NSPF. The smaller

proteins in SPF might be entrapped in the total PRF and therefore less accessible to protease-catalysed hydrolysis.

Conventional pea protein isolate was chosen as the benchmark of denatured protein (Shand et al., 2007). Unheated PPI was more extensively hydrolysed than native pea flour, PRF, and NSPF from GE 3 -5. Negligible hydrolysis took place in the first two GE steps due to the short residence times in the gastric phase at high pH (Figure 5.4). The DH of SPF was similar to that of unheated PPI throughout the gastric phase.

The DH of native proteins in PRF and NSPF was significantly lower than that of the denatured proteins in unheated PPI during the small intestinal phase. This would suggest that protein denaturation leads to higher protein digestibility. Nevertheless, unheated SPF was more extensively hydrolysed than PPI, highlighting the relevance of protein size and conformation above the degree of denaturation as determinants of protein digestibility.

5.3.2.2 Thermal denaturation of native proteins

The effect of heat treatment on the gastric digestion of pea flour and mild fractions was relatively small (Figure 5.4). For the most part, no significant differences between the DH of unheated and heated dispersions were observed (Table A5.2). Nevertheless, when significant differences were observed, they anticipated the effect of heat treatment of proteins in the small intestinal digestion, e.g., in the GE 4 and 5 of PRF, the heated dispersion was hydrolysed to a significantly greater extent than its unheated counterpart.

Protein hydrolysis throughout the small intestinal phase of each of the samples studied showed a distinctive behaviour as a result of heat treatment. For a direct comparison of DH from the digestion heated and unheated dispersions from each GE step, please refer to Figure A5.2. The DH was significantly smaller in the heated pea flour dispersion compared to its unheated counterpart. Here, heating does not only affect the protein but it also induces the gelatinization of starch from the flour, which might embed proteins within it and in fact form polar and non-polar starch-protein interactions, protecting it from hydrolysis (López-Barón et al., 2017). Similarly, a significant reduction in *in vitro* gastric digestibility has been previously reported for quinoa protein in the presence of gelatinized starch (Opazo-Navarrete et al., 2019).

Heat treatment of PRF resulted in a significantly higher DH by pancreatic enzymes, compared to its unheated counterpart. The DH of NSPF remained almost equal after heat treatment, with an exception for the last two GE steps after 60 min in the small intestinal phase in which the heated dispersion showed a higher DH. An opposite effect was observed for SPF, for which, heating reduced the DH achieved by pancreatic enzymes.

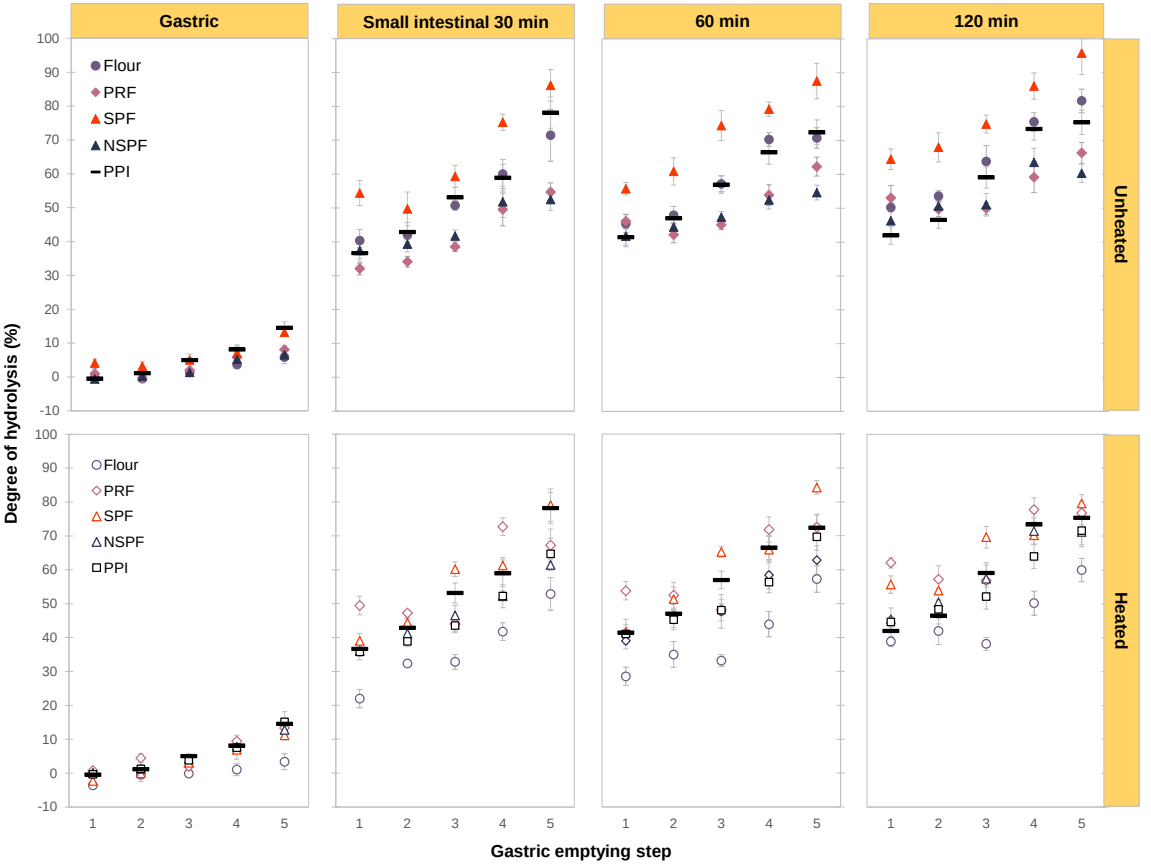

Figure 5.4 Degree of hydrolysis after five gastric emptying steps during the gastric phase and after 30, 60 and 120 min of small intestinal phase of unheated (filled symbols) and heated dispersions (open symbols) of pea flour, pea protein-rich (PRF), soluble (SPF), non-soluble protein (NSPF) fractions and pea protein isolate (PPI). For comparison, unheated PPI also shown with heated samples. Error bars represent the standard deviations of the triplicate digestion assays. For statistical significance comparisons refer to Table A5.2 and Figure A5.2.

As it has been described in the previous sections, heat treatment of SPF led to protein aggregation, as a result of protein denaturation, which might have hindered protein digestibility.

Further processing of PPI, into a heated PPI dispersion, showed no effect on the DH in the gastric phase. We previously reported no significant difference in the *in vitro* gastric digestion of conventional pea and soy protein isolates (Rivera del Rio et al., 2020), however upon close inspection, we realised that the lack of an overall effect on DH may have been caused by solubilization and aggregation balancing each other. In the unheated dispersions, the spray-dried morphology of the particles prevented them from being solubilized, however the particles were sensitive to pepsin-catalysed hydrolysis. When the dispersion was heated, the spray-dried particle released most proteins into the supernatant, while the proteins that were separated into the pellet were larger protein clusters and aggregates. The latter showed poorer digestibility compared to the proteins in the supernatant.

In the current work, heating a PPI dispersion also induced some aggregation as was observed in the peptide size distribution where more peptides >10 kDa were present in the heated dispersion than in its unheated counterpart (Figure 5.3). However, no change in β-value was observed as a result of heat treatment (Table 5.1). In the small intestinal phase, the DH of the heated PPI dispersion was, for the most part similar to that of its unheated counterpart. In a few exceptions, the DH was significantly lower for the heated dispersion.

Interestingly, the DHs of unheated SPF, and heated PRF and SPF in some timepoints, were significantly larger than both unheated and heated PPI. This might imply the presence of 'faster' proteins in the mild fractions, i.e., proteins that could be broken down more quickly and absorbed earlier into the bloodstream. As mentioned before, Kornet et al. (2021) found that that isoelectrically precipitated fractions such as PPI do not contain albumins anymore. The other fractions do, and these easily soluble albumins are smaller and may be better available for hydrolysis.

5.3.2.3 Peptide size distribution throughout digestion

Size exclusion chromatography was used to obtain the peptide size distribution of each digestion sample. Similar trends were observed in the changes within a molecular weight range throughout the complete digestion assay for all dispersions studied (Figure 5.5). During the gastric phase, proteins and peptides with a molecular weight higher than 4 kDa were hydrolysed by pepsin into 0.5 – 4 kDa peptides. The proportion of <0.5 kDa

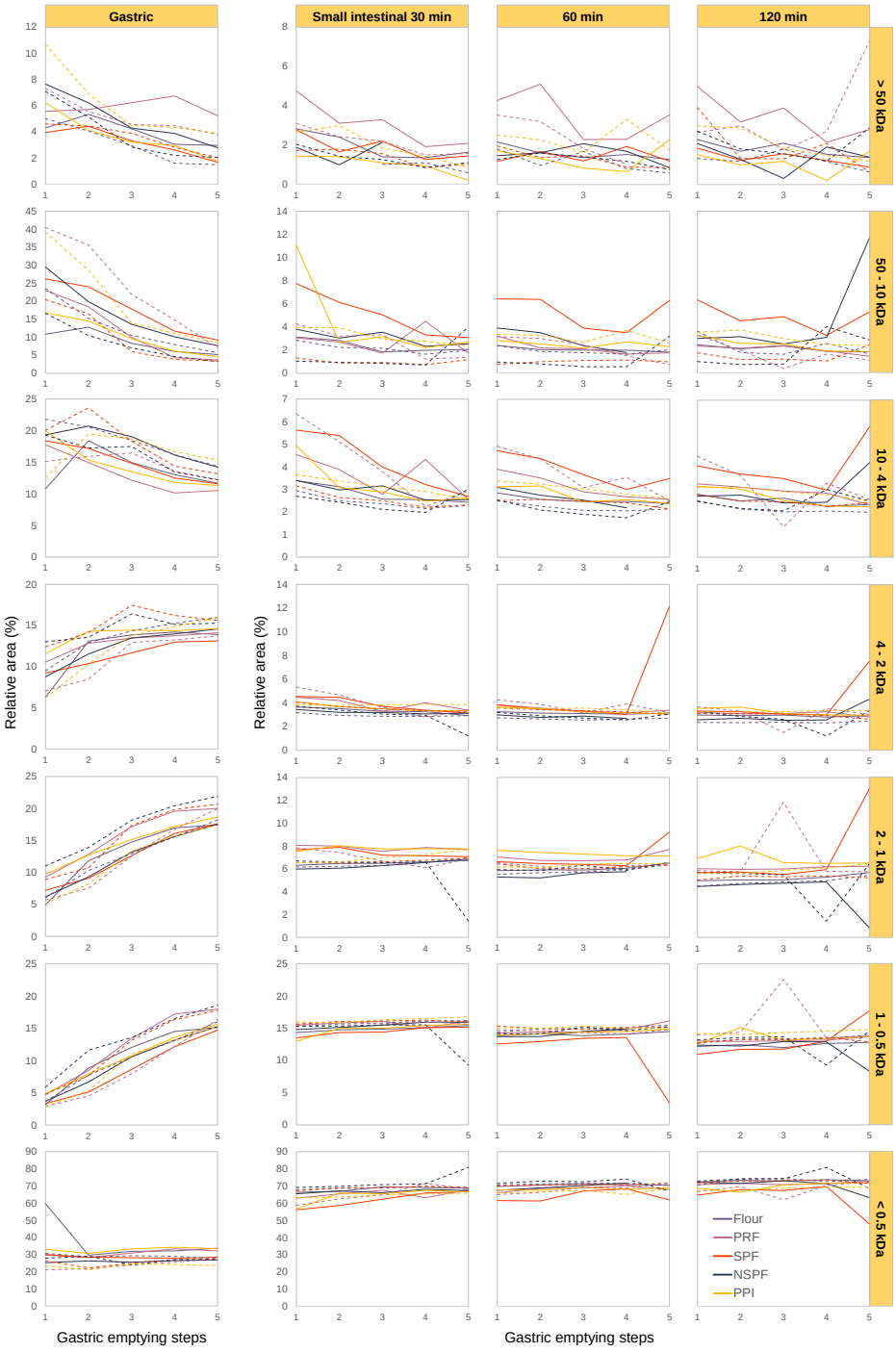

Figure 5.5 Molecular weight distribution integrated from size exclusion chromatograms after gastric and small intestinal digestion of unheated (continuous lines) and heated (dashed lines) pea flour, protein-rich (PRF), soluble (SPF), non-soluble (NSPF) protein fractions and conventional pea protein isolate (PPI) dispersions. The lines connect the experimental data points at gastric emptying steps 1 to 5. For clarity of the figures, the data points are left out.

peptides remained practically unchanged from the proportion in the samples before hydrolysis (Figure 5.3).

In the first 30 min of the small intestinal phase, for all GE steps, a sharp reduction in the proportion of 1 – 10 kDa peptides, from the gastric phase, was recorded to give rise to < 0.5 kDa peptides. The sharp increase of small peptides can be equated to the larger DH in the small intestinal phase compared to the limited DH from the gastric phase. Interestingly, most of the small peptides (< 1 kDa) were formed already after the first 30 min of the small intestinal phase of the aliquot from the first GE step. This suggests that the changes in DH observed for the different GE steps and throughout the small intestinal phase resulted from the hydrolysis of large into medium peptides without contributing significantly to the amount of smallest peptides.

5.3.3 Relevance of the gastric phase

In the standardised semi-dynamic *in vitro* digestion method, the rate of gastric emptying and thus, the duration of the gastric phase, are determined by the caloric density of the 'food' (Mulet-Cabero et al., 2020). As previously mentioned, the pea flour slurry contained the highest caloric density of all the samples studied due to the presence of starch. The duration of the gastric phase for this sample was nearly twice that of the mild fractions and conventional pea protein isolate. To evaluate a potential effect of the duration of the gastric phase on the overall DH after the gastric or small intestinal phases, the pea flour slurry was subjected to the conditions of a gastric phase without considering the contribution of starch to the caloric density of the meal. As shown in Figure A5.3, no significant difference was observed between the DH of a shorter or longer duration of the gastric phase, throughout the full digestion assay. Certainly, the total amount of acid and pepsin added to the digestion vessel remained the same; the simulated gastric fluid was only dosed at a faster rate for shorter durations of the gastric phase. Likewise, the time between GE sampling is shortened, but the intervals are proportional to the full duration of the gastric phase. Therefore, for the relatively simple, liquid 'foods' used in this study, the extent of gastric and subsequent small intestinal hydrolysis was not influenced by the duration of the gastric phase.

Nevertheless, the present study shows the effect of residence time in the *stomach* on hydrolysis by pancreatic enzymes in the small intestinal phase. Generally, we see that longer residence times in the stomach result in better digestion in the small intestine. Albeit the extent of hydrolysis in the gastric phase is relatively limited, it has a major impact on the extent of the hydrolysis achieved in the small intestinal phase. For instance, the difference in DH between the first and last GE step directly after the gastric phase of

the unheated pea flour slurry is approximately 6%, while subsequent small intestinal phase of 120 min for the same GE aliquots adds another 31%. It should be noted that the enzyme-to-substrate ratio varies among the five GE steps, both in the gastric and small intestinal phases. The first GE aliquot in the small intestinal phase has the highest concentration of protein and is met by the same amount of pancreatic enzymes as the last GE aliquot, which contains less protein. Thus, early GE steps have a lower enzyme-to-substrate ratio than later GE steps, in both the gastric and small intestinal phases.

To check for these dilution effects, we did an additional experiment where the aliquot from the first GE step of the unheated flour slurry digestion was diluted to match the protein concentration of the last GE aliquot. The diluted GE1 aliquot was subjected to the small intestinal phase as was done for the undiluted aliquots (Figure A5.4). Although slightly larger, the DH during the small intestinal phase of the diluted aliquot from the first GE step was not significantly different to that of the undiluted aliquot ($p > 0.05$, among 30, 60 or 120 min small intestinal timepoints). Therefore, the significantly larger DH from the last GE step cannot be fully attributed to the dilution effects resulting in higher enzyme-to-substrate ratios as the dynamic gastric phase proceeds to completion.

In previous work, we demonstrated that the acidification and pepsin-catalysed hydrolysis of bovine serum albumin in the gastric phase ensures better hydrolysis by trypsin in the small intestinal phase, both in terms of extent and efficiency, compared to non-acidified and non-hydrolysed protein (Rivera del Rio et al., 2021). Our findings here suggest that pepsin-catalysed hydrolysis has a preparatory effect on protein structure to be better susceptible to hydrolysis by pancreatic enzymes than an intact protein, particularly if the latter was not acidified during the gastric phase.

5.4 Conclusion

The use of a dynamic *in vitro* gastric digestion method highlights the importance of an optimal gastric digestion for an enhanced subsequent small intestinal digestion of protein. Our findings show that thermal denaturation of pea proteins improves their *in vitro* gastro-small intestinal digestibility. Nevertheless, the presence of starch in the food matrix, when gelatinized reduced the overall digestibility of protein. We found that native, soluble proteins were highly digestible. These proteins, however, aggregated as a result of a mild heat treatment which impaired the extent of hydrolysis by gastric and pancreatic enzymes. Therefore, protein size and conformation, in addition to the degree of denaturation and ultimately aggregation, are determinants for protein digestion. Pea protein isolate was chosen as a benchmark for denatured protein but also as a

commercial, widely available and used ingredient. The heated dispersions of the mild fractions were at least as digestible as both unheated and heated PPI after 120 min of the small intestinal phase. In fact, the heated PPI dispersion was less extensively hydrolysed than its unheated counterpart. Consequently, we should aim for minimal processing in the manufacture of ingredients, since the final preparation will then provide the right degree of processing to ensure good digestibility.

A5.1. Differential scanning calorimetry

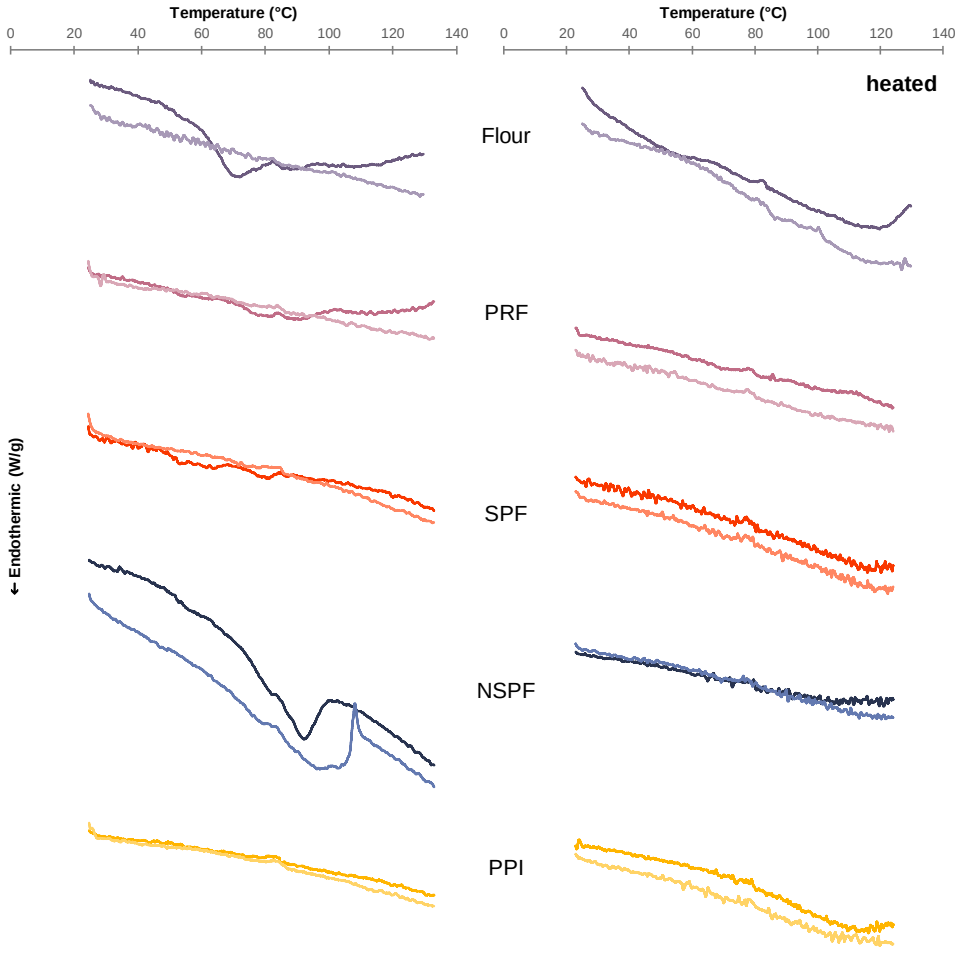

Figure A5.1 Thermograms from differential scanning calorimetry of unheated and heated flour, protein-rich (PRF), soluble (SPF) and non-soluble protein fractions (NSPF) and, pea protein isolate (PPI) dispersions. Two heating ramps at 1 °C/min with a cooling step at 30 °C/min in between (first ramp, dark-coloured and second ramp, light-coloured).

Table A5.1 Peak information from the first heating ramp (1 °C/min) from differential scanning calorimetry of unheated pea flour, starch-rich (SRF), protein-rich (PRF), soluble (SPF) and non-soluble protein (NSPF) fractions dispersions. No peaks were observed in heated dispersions of flour, mild fractions or unheated and heated pea protein isolate. Peak 1 associated to starch gelatinization, peak 2 and 3 to protein denaturation.

	Peak 1			Peak 2			Peak 3		
	ΔH (J/g)	Onset T (°C)	Peak T (°C)	ΔH (J/g)	Onset T (°C)	Peak T (°C)	ΔH (J/g)	Onset T (°C)	Peak T (°C)
Flour	0.73	62.3	69.6				0.10	83.7	88.1
SRF	1.38	59.3	67.6						
PRF				0.07	71.8	76.3	0.05	83.2	89.0
SPF				0.06	76.6	78.9			
NSPF							0.48	83.0	90.3

A5.2. Degree of hydrolysis – statistics

Table A5.2 Tukey's HSD range distribution of degree of hydrolysis of heated and unheated pea flour, protein rich (PRF), soluble (SPF), non-soluble (NSPF) protein fraction and conventional pea protein isolate (PPI), at each individual gastric or corresponding, small intestinal timepoint. Samples with the same letter did not differ significantly ($p > 0.05$, by column).

		gastric					+ 30 min small intestinal					+ 60 min					+ 120 min				
		GE1	GE2	GE3	GE4	GE5	GE1	GE2	GE3	GE4	GE5	GE1	GE2	GE3	GE4	GE5	GE1	GE2	GE3	GE4	GE5
Flour	unheated	b	c	cd	cd	de	b	b	bc	b	bc	b	bc	c	b	b	bc	bc	bc	b	b
	heated	d	c	d	d	e	d	d	g	e	e	d	d	f	d	de	e	e	g	f	e
PRF	unheated	b	bc	bcd	bc	cd	c	cd	f	d	e	b	c	e	c	cde	b	cd	f	e	de
	heated	b	ab	ab	a	ab	a	ab	cd	a	cd	a	bc	cd	b	b	a	b	cde	b	bc
SPF	unheated	a	a	a	abc	ab	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	heated	cd	bc	abc	abc	bc	b	ab	a	b	ab	bc	b	b	b	a	b	bc	ab	bcd	bc
NSPF	unheated	bc	bc	cd	bc	de	bc	bc	ef	cd	e	bc	c	de	c	e	cd	bcd	ef	de	e
	heated	b	abc	ab	ab	ab	bc	b	cde	cd	de	c	bc	de	c	cd	cd	cd	cde	bc	cd
PPI	unheated	bc	abc	a	ab	ab	bc	b	b	bc	ab	bc	bc	c	b	b	de	de	cd	b	bc
	heated	b	abc	abc	ab	a	bc	bc	def	cd	cd	bc	bc	de	c	bc	cde	cd	def	cde	cd

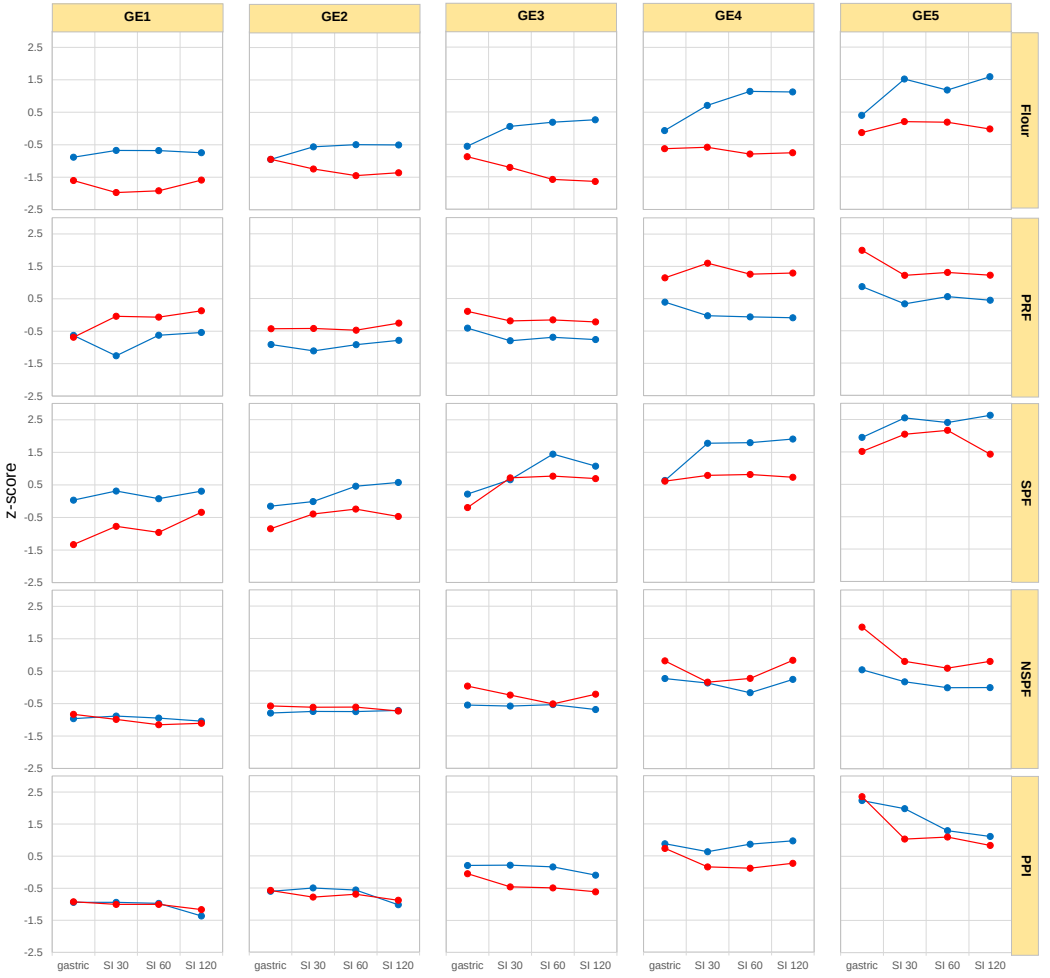

Figure A5.2 z-scores from the degree of hydrolysis after five gastric emptying steps and subsequent small intestinal phase with sampling at 30, 60 and 120 min. Scores were calculated for the gastric, small intestinal (at 30, 60 and 120 min) separately. Unheated samples (blue lines) and heated samples (red lines).

A5.3. Relevance of gastric phase

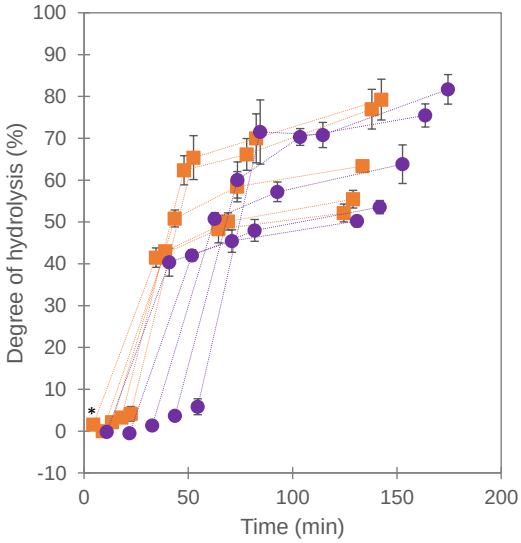

Figure A5.3 Degree of hydrolysis after five gastric emptying steps of unheated pea flour slurry. Calculated duration of the gastric phase according to the full caloric density of the 'food' (purple symbols) and duration calculated without the contribution of starch to the caloric density of the 'food' (orange symbols). Error bars represent the standard deviations of the triplicate digestion assays. t-test for independent samples showed no significant difference among individual timepoints ($p > 0.05$), difference only shown for the first gastric emptying step.

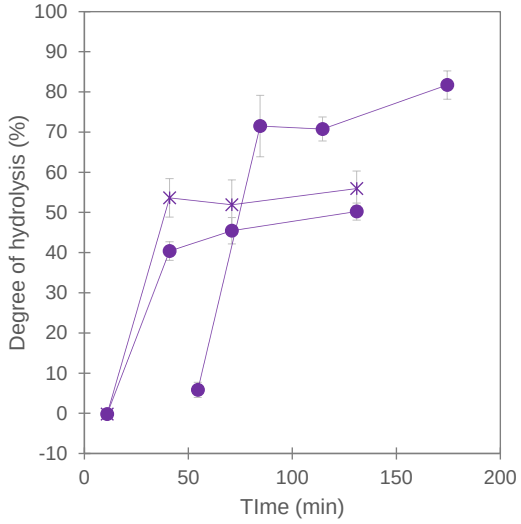

Figure A5.4 Degree of hydrolysis after the first and last gastric emptying step of unheated flour dispersion and subsequent small intestinal phase (circles). Dilution of the aliquot from the first gastric emptying step to match the protein concentration from the last gastric emptying step, and subsequent hydrolysis from small intestinal phase (star). Error bars represent the standard deviations of the triplicate digestion assays.

CHAPTER SIX

Nutritional value in sustainability assessment of protein-rich ingredients and foods: a *'farm-to-faeces'* approach

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Abstract

The environmental impact of ingredients and foods is often assessed from production of the raw materials up to consumption ('*farm-to-fork*' system boundaries). Comparing animal- to plant-based protein ingredients and foods points to plant proteins as a more sustainable alternative to animal-sourced ingredients. However, the nutritive quality of plant proteins is often deemed less than that of animal proteins, while processing also affects the nutritional value of proteins. Incorporating the protein digestibility as a functional unit into the life cycle assessment, a '*farm-to-faeces*' approach, provides a more actionable measurement to compare a set of ingredients, processes or final products. Conventionally and mildly fractionated pea protein ingredients were compared to a whey protein isolate acting as a reference of animal-sourced proteins. Processing influences both the digestibility and the overall environmental impact of the food. Processing may decrease but also enhance the digestibility. The digestibility of conventional ingredients such as a protein isolate (pea and whey) is reduced more strongly by processing than the mildly fractionated ingredients. Mild fractions always provide a more sustainable alternative to whey and pea protein isolates, in relation to both ingredient production and protein digestibility, demonstrating the usefulness of mild processing. In general, this study clearly demonstrates that processing and digestion should be included in an LCA study, to obtain a correct view of the impact of fulfilling our nutritional needs.

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6.1 Introduction

Plant proteins are more sustainable than animal proteins (Aiking, 2011; Alexander et al., 2017; Nijdam et al., 2012; Pimentel & Pimentel, 2003; Poore & Nemecek, 2018). This is mainly due to the inefficient protein conversion from feed to food. To facilitate the transition from animal to plant protein, the techno-functionalities of plant protein-rich ingredients have been capitalized to produce plant-based alternatives to animal-sourced products (Day, 2013). Nevertheless, plant proteins are considered to be less nutritious than those of animal origin (Day et al., 2022). Since one of the prime functions of these ingredients is to supply protein to the consumers' metabolic system, it is logical to consider the nutritional characteristics in a sustainability comparison. To allow for a fair evaluation, the basis for comparison, also called the 'functional unit' (FU), can be used. The FU in life cycle assessment (LCA) reflects the purpose of the analysed product or process. The nutritional characteristics can be included in the LCA by extending the FU beyond product-oriented metrics such as mass of product or protein.

Various authors proposed to include the nutritional qualities in the FU of sustainability analysis using different approaches. Weidema and Stylianou (2020) considered the extent at which a food or diet satisfies the nutritional requirements or contains dietary risk factors that should be limited. Hallström et al. (2018) reviewed twenty different studies that incorporate some dietary quality score into the environmental sustainability assessment approaches. They identified two categories of dietary quality scores; one based on the content of a certain nutrient relative to a reference intake, and the other on the degree of fulfilment of the dietary guidelines.

The digestibility and bioavailability as FU are less commonly incorporated into LCA (McAuliffe et al., 2020). Some examples worth noting include the work from Sonesson et al. (2017), in which the FUs considered were the amount (kg) of food, of protein, of digestible protein, and the amount of *protein quality index*-weighted food. The latter encompasses the context of the diet, i.e., the dietary requirements and the intake of digestible, indispensable amino acids (IAA) from a given food relative to other foods in a diet. Bread and pea soup were compared to milk, chicken fillet, minced pork and beef. As expected, the environmental impact of these animal-sourced products was higher than of those of plant origin when comparing on basis of the amount of food consumed. Since plant-based proteins were assumed to be less digestible than animal proteins, introduction of the digestible protein as measure in the FU, reduces the large disparity between the food origins. Barré et al. (2018) recommended assessing the sustainability of complete diets by including the bioavailability of protein and micronutrients. Berardy et

al. (2019) introduced the *weighted protein score* as a FU to assess the global warming potential of individual foods. This score comprises realistic consumption amounts, protein content and the digestible indispensable amino acid score (DIAAS). Finally, Moughan (2021) incorporated the daily, per capita intakes of protein (g), the digestible protein (g, from true ileal digestibility) and the utilizable protein (estimated from DIAAS calculations) for mixed diets of selected countries. Based on DIAAS calculations, lysine was consistently the first-limiting amino acid (AA) in the national diets. This study paid particular attention to the distinction between plant- and animal-sourced foods. Similar to previous studies, the initial wide gap between the land use or greenhouse gas emissions of animal- and plant-sourced foods was reduced when the amount of digestible lysine was used as a FU rather than the amount of gross protein.

One of the downsides of using the digestibility and DIAAS values reported in literature is that the effect of the processing history on the digestibility, which is specific to each ingredient, food or finished product is not considered (Li et al., 2021; Sá et al., 2020a). Generally, proteins in raw pulses are poorly digestible because of the inaccessibility of the proteins for digestive enzymes due to the cellular matrix, and because of the presence of antinutritional factors. Processing reduces the physical inaccessibility, e.g., by breaking down cell walls by milling and denaturing proteins by heating. Trypsin and chymotrypsin inhibitors are inactivated by heating, while phytic acid and tannins can be removed or reduced by soaking, germination or fermentation of whole pulses. On the other hand, extensive processing can also lead to a lower digestibility of the protein, for example by oxidation, by rendering the proteins insoluble or by incorporating them into insoluble aggregates.

Next to the digestibility, processing also affects the sustainability of the ingredients. This is because of the resources required for processing but also because part of the protein is lost during its isolation from the plant matrix, which leads to a larger environmental impact per kg of protein ingredient. Van der Goot et al. (2016) proposed the concept of tailored, functionality-driven processes that would avoid dissolution and minimize drying. Dilution reduces the amount of proteins that is lost in the typical dissolution-precipitation process for protein isolation; less extensive drying reduces the amount of energy spent for processing, in which drying is the major contributor in terms of energy use. Lie-Piang et al. (2021) used LCA to compare such mildly processed fractions to conventional, highly refined plant protein fractions and showed that reducing the degree of refining substantially decreases the environmental impact. In some cases, processing impacts even exceeded those of cultivation, which is generally the dominant life cycle stage of agri-food products (Notarnicola et al., 2015).

Fardet and Rock (2020) investigated the link between so-called 'ultra-processed foods' and sustainability. Ultra-processed foods were defined as reconstructed matrices made of isolated ingredients, that were obtained by 'cracking' of raw materials, and additives. The authors emphasised the importance of processing in food system sustainability policies, as foods from isolated ingredients generally are more energy intensive than minimally processed foods. Green et al. (2018) showed that processing contributes significantly to the environmental impact, which is in line with the work of Berners-Lee et al. (2012). While this might lead to the conclusion that food processing needs to be limited overall to achieve sustainable diets, it should not be forgotten that processing as such is essential for a viable, efficient and safe food system. Next to food safety, shelf-life and palatability are important reasons for processing, while it can also enhance the nutritional quality by applying appropriate food processes.

We thus conclude that a comprehensive LCA of protein foods should include the cultivation, the complete processing history, including the processing of the primary crop, the processing of the ingredient, and the assembly of the ingredients into foods, as well as the digestibility in the context of the food. This can be done by integrating a nutrition-based FU in a full sustainability analysis. Therefore, in this study we compare the total system, from '*farm-to-faeces*' and thus aim for the quantification of the environmental impact relative to their nutritional value, quantified in terms of protein digestibility. Appropriate FUs that capture the effect of the processing history on protein digestibility and availability are needed. These can be obtained from *in vitro* digestion assays. We illustrate this for a range of yellow pea protein-rich ingredients with various levels of refining. Whey protein isolate was adopted as reference, which has a high nutritional value but is also a relatively impactful protein ingredient of animal origin. The effect of post-fractionation processing was simulated by heating aqueous dispersions of the ingredients, after which the effect on environmental sustainability and digestibility was quantified.

6.2 Materials and methods

6.2.1 Materials

Whey protein isolate (WPI, UltraWhey90 standard, Volac International, UK) and yellow pea protein isolate (PPI, Nutralys F85M, Roquette, France) were used as reference materials. Dry yellow peas (*Pisum sativum L.*, Alimex, The Netherlands) were milled to obtain pea flour and mildly separated aqueous protein-rich (PRF), soluble (SPF) and non-soluble (NSPF) protein fractions as described by Geerts et al. (2017) and in Chapter 5 (Figure 6.1). All protein materials were dispersed or diluted into 2.24% (w/w) protein

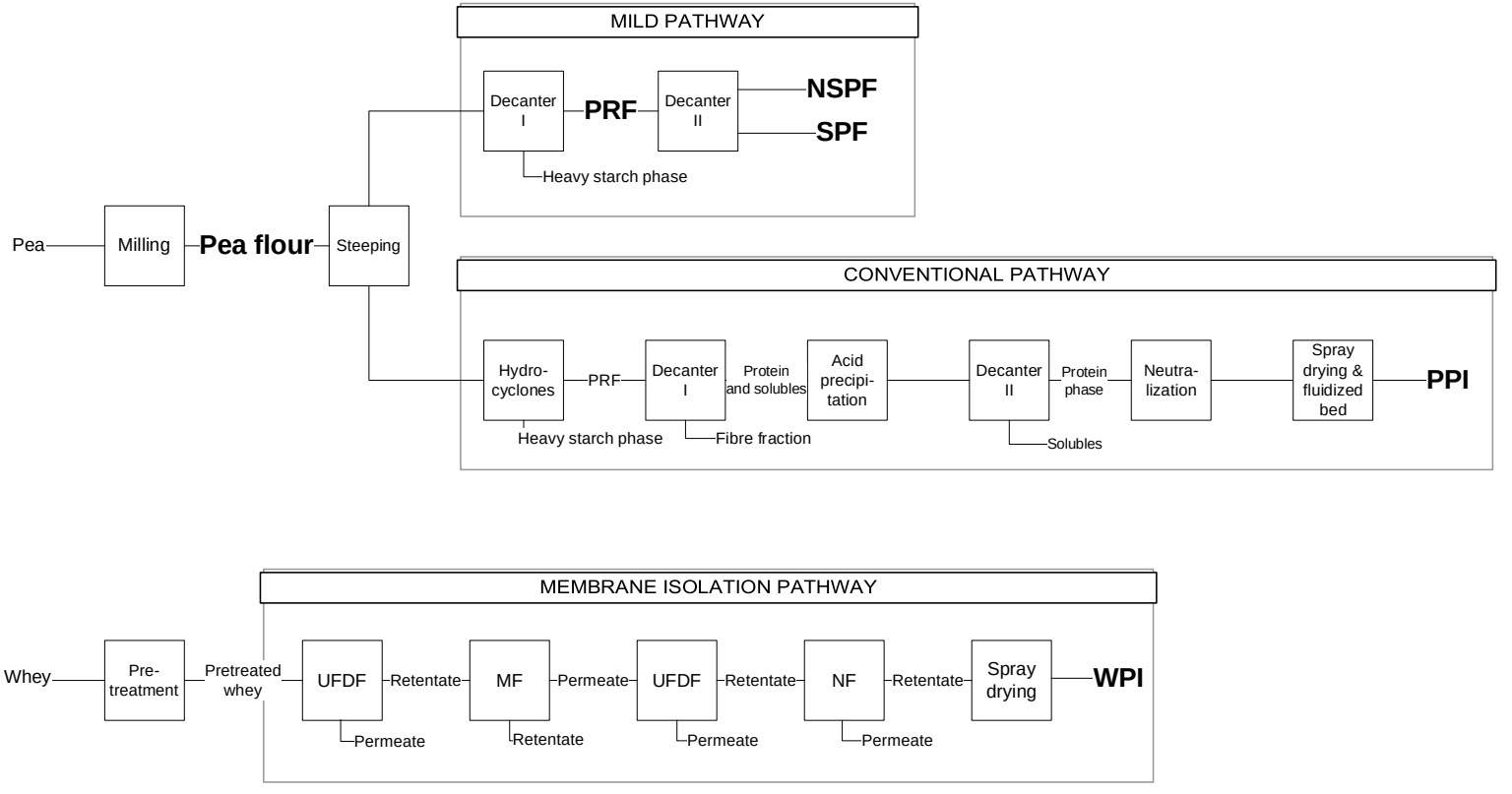

Figure 6.1 Overview of the main processing steps involved in the production of pea flour, protein-rich (PRF), soluble (SPF), non-soluble (NSPF) protein fractions, pea (PPI, Lie-Piang et al. (2021)) and whey (WPI, Tetra Pak (2015)) protein isolates.

dispersions. For the post-fractionation process, dispersions were heated at 90 °C for 5 min.

6.2.2 AA composition

The total AA profile of the protein materials (WPI, PPI, pea flour, PRF, SPF and NSPF) was analysed by Triskelion (Utrecht, The Netherlands) using liquid chromatography with UV detection after derivatization (based on AOAC 2018.06). The tryptophan content was determined by liquid chromatography with fluorescence detection (based on ISO13904).

6.2.3 *In vitro* gastro-small intestinal digestion

The standardised semi-dynamic *in vitro* digestion method (Mulet-Cabero et al., 2020) was used to digest the unheated and heated protein dispersions. Each of the five gastric emptying steps was followed by 120 min in a static small intestinal phase. To ensure the maximum possible solubilization of protein and peptides after digestion, the digestion samples were diluted with a 2% sodium dodecyl sulphate solution. The protein content in each aliquot was determined with a bicinchoninic acid kit (ThermoFisher Scientific Inc., USA). Aliquots were diluted to equal protein concentration for each digestion assay (and protein source) for further analysis. For a detailed description of the methods used, the reader is referred to Chapter 5 in this thesis. In the current chapter, we retrieve the degree of hydrolysis (DH) and molecular weight distribution data from the digestion samples after 120 min of small intestinal digestion of each of the five gastric emptying steps.

6.2.4 Life cycle assessment

For all pea ingredients, the LCA data were obtained from the work of Lie-Piang et al. (2021). For WPI, no data were available in the literature and an additional LCA was performed. The goal of the LCA was to quantify the impact of the used WPI, using the same methodology that Lie-Piang et al. (2021) used for the pea protein ingredients. The LCA system boundaries were from the cultivation up to the end of ingredient processing, in other words cradle-to-processing-gate. The impact of capital goods, distribution and consumption is excluded from the system boundaries and all WPI processing steps were assumed to take place at the same location. Allocation was done on mass (dry matter) basis. There were no streams that were considered waste and therefore allocated zero impacts, since the ultrafiltration (UF) permeate and microfiltration (MF) retentate can be valorised further by direct spray drying or subsequent purification of the lactose (in the case of the UF permeate).

6.2.4.1 Functional units

The following product-oriented FU were applied in this study: ingredient (kg), dry matter (kg), and protein (kg). Moreover, two digestibility-oriented FU were used, namely the quantity of *in vitro* hydrolysable protein (kg), which is the average DH after 120 min of small intestinal digestion of the five gastric emptying steps, determined by the *o*-phthalaldehyde method (Figure A6.3a), and the minimum digestible, limiting, IAA (kg m-IAA, Equation 6.1).

$$\text{m-IAA (\%)} = 100\ \frac{\text{digestion coefficient}_{min} \cdot \text{IAA (mg)} / \text{protein (g)}}{\text{same dietary IAA (mg)} / \text{reference protein (g)}}\text{, lowest value} \qquad \text{Equation 6.1}$$

In this equation, the digestion coefficient $_{min}$ is the minimum value of <1 kDa peptides after *in vitro* small intestinal digestion (following the five gastric emptying steps), IAA (mg/g protein) is the content of a given AA from the AA profile of the undigested protein material; the reference IAA (mg/g reference protein) was obtained from the recommended AA scoring patterns for adults (Table 6.1, FAO (2011)). The lowest value calculated, among the different IAA, is then m-IAA; the IAA to which the value belongs to is then the limiting IAA. This equation resembles the calculation of DIAAS, with the exception that individual true ileal AA digestibilities are needed for this score. The argumentation and assumptions made for this FU can be found in the appendix A6.1.

6.2.4.2 Life cycle inventory

WPI is produced from sweet whey, a co-product from cheese production. Two common production methods exist for whey protein isolation from sweet whey: ion exchange and a cascade of membrane-based filtration processes. In both cases, the fractionation methods are followed by spray drying. The WPI used in the current study was produced with a cascade of membrane fractionation processes (see Figure 6.1). After pre-treatment consisting of pasteurization and centrifugation, four membrane steps follow (Tetra Pak, 2015). First, UF in diafiltration (DF) mode to increase the fraction of whey proteins in the total solids (removing minerals, nonprotein nitrogen and lactose), followed by MF to remove fat and bacteria. Next, a second UFDF is applied to demineralize, followed nanofiltration (NF) to preconcentrate the whey protein to 35-38% dry matter. Finally, the concentrate is spray-dried into a powder. The cleaning of the membrane systems was included in the analysis, assuming a simplified average membrane cleaning process for all steps.

The foreground data were collected or deduced from literature and are listed together with the mass balances in Table 6.2. If no specific information was found, values were

Table 6.1 Indispensable amino acid (IAA) content and minimum digestibility for minimum digestible, limiting, IAA (m-IAA) of whey (WPI) and pea (PPI) protein isolate, pea flour, protein-rich (PRF), soluble (SPF) and non-soluble (NSPF) protein fractions. Limiting IAA marked in red for each ingredient. m-IAA higher than 100% marked in green.

Ingredient	Heat treatment	Minimum digestion coefficient	Histidine	Isoleucine	Leucine	Lysine	Sulphur AA	Aromatic AA	Threonine	Tryptophan	Valine
			IAA (mg/g protein)								
WPI	N	0.90	16.1	71.4	102.7	98.2	49.1	76.8	80.4	19.2	58.0
	Y	0.91	12.9	58.0	84.8	80.4	43.3	68.3	67.0	18.8	49.1
PPI	N	0.83	29.9	58.0	102.7	93.8	25.0	127.2	44.6	11.2	58.0
	Y	0.82	29.9	58.0	98.2	93.8	24.1	127.2	44.6	11.2	58.0
Pea flour	N	0.85	17.9	30.8	49.1	58.0	23.7	76.8	33.9	10.7	35.3
	Y	0.84	23.7	40.2	71.4	75.9	20.5	90.2	38.8	9.8	44.6
PRF	N	0.76	29.9	49.1	84.8	93.8	30.8	119.2	49.1	12.1	58.0
	Y	0.83	29.9	53.6	89.3	93.8	31.3	120.1	49.1	12.9	58.0
SPF	N	0.81	25.0	43.3	67.0	84.8	33.5	103.6	49.1	11.2	49.1
	Y	0.83	24.1	41.5	62.5	80.4	32.1	103.1	44.6	12.1	49.1
NSPF	N	0.81	17.0	28.6	53.6	49.1	12.9	72.3	24.6	13.4	30.8
	Y	0.84	20.1	33.5	62.5	55.8	14.1	76.3	26.8	7.1	35.7
			In vitro min digestibility for IAA (mg/g protein)								
WPI	N		14.5	64.2	92.3	88.3	44.2	69.0	72.3	17.3	52.2
	Y		11.7	52.5	76.8	72.7	39.2	61.8	60.6	17.0	44.4
PPI	N		24.9	48.3	85.4	78.0	20.8	105.8	37.1	9.3	48.3
	Y		24.4	47.3	80.0	76.4	19.6	103.7	36.4	9.1	47.3
Pea flour	N		15.1	26.0	41.5	49.1	20.0	64.9	28.7	9.1	29.8
	Y		19.8	33.7	59.8	63.6	17.2	75.5	32.5	8.2	37.4
PRF	N		22.6	37.2	64.2	70.9	23.3	90.2	37.2	9.1	43.9
	Y		24.9	44.7	74.4	78.2	26.1	100.1	40.9	10.8	48.4
SPF	N		20.3	35.2	54.4	69.0	27.2	84.2	39.9	9.1	39.9
	Y		20.0	34.5	51.9	66.8	26.7	85.7	37.1	10.0	40.8
NSPF	N		13.7	23.0	43.2	39.6	10.4	58.3	19.8	10.8	24.8
	Y		16.9	28.2	52.7	47.1	11.9	64.4	22.6	6.0	30.1
Recommended AA scoring patterns for adults (mg/g protein requirement)			16	30	61	48	23	41	25	6.6	40
		m-IAA (%)									*In vitro* min digestible reference ratio for IAA
WPI	N	90.3	0.90	2.14	1.51	1.84	1.92	1.68	2.89	2.62	1.30
	Y	73.2	0.73	1.75	1.26	1.52	1.70	1.51	2.42	2.57	1.11
PPI	N	90.4	1.55	1.61	1.40	1.62	0.90	2.58	1.48	1.41	1.21
	Y	85.4	1.52	1.58	1.31	1.59	0.85	2.53	1.46	1.38	1.18
Pea flour	N	68.1	0.94	0.87	0.68	1.02	0.87	1.58	1.15	1.37	0.75
	Y	74.8	1.24	1.12	0.98	1.32	0.75	1.84	1.30	1.25	0.93
PRF	N	101.3	1.41	1.24	1.05	1.48	1.01	2.20	1.49	1.38	1.10
	Y	113.3	1.56	1.49	1.22	1.63	1.13	2.44	1.64	1.64	1.21
SPF	N	89.2	1.27	1.17	0.89	1.44	1.18	2.05	1.60	1.37	1.00
	Y	85.2	1.25	1.15	0.85	1.39	1.16	2.09	1.48	1.52	1.02
NSPF	N	45.4	0.86	0.77	0.71	0.83	0.45	1.42	0.79	1.64	0.62
	Y	51.6	1.06	0.94	0.86	0.98	0.52	1.57	0.90	0.91	0.75

Recommended AA scoring patterns from the FAO (2011). Minimum digestion coefficient from Figure A6.3b.

estimated from representative processes. Background data, such as utilities and whey, were retrieved from Agrifootprint 5® (van Paassen et al., 2019) and are listed in Table A6.1.

Impacts for post-fractionation processing, i.e., heating the materials in aqueous dispersions at 90 °C, were also added to the inventory for the heated materials. This was done by calculating the energy needed, assuming a 75% heating efficiency and a heat capacity for each material.

6.2.4.3 Life cycle impact assessment

The life cycle impact assessment was done using SimaPro LCA software version 9.2 and the ReCiPe 2016 Midpoint (H) and Endpoint (H) v1.03 method (Huijbregts et al., 2017). SimaPro gathers the inventory substance flows and converts those into impact categories according to the characterization factors of the ReCiPe methodology. The indicators calculated with the midpoint methodology focus on single environmental issues, such as climate change (expressed in CO_2 equivalents) or land use (expressed in m_2a crop eq). Indicators calculated with the endpoint methodology aggregate the impacts on higher levels, namely the damage to human health, ecosystems and resource availability. In addition, those endpoint categories may be normalized to a single score, having the unit millipoint (mPt), representing the total environmental impact. In the current study we present environmental impact in the single score format since it aggregates all impact categories and therefore allows for easy comparison.

6.3 Results and discussion

The environmental impact of processing protein-rich ingredients was assessed using both product- and digestibility-oriented metrics, using WPI as an animal-protein reference. The results of the LCA midpoint assessment of WPI are presented in Table A6.2. The endpoint assessment results (including single score) are tabulated in Table A6.3 for all ingredients with different degrees of fractionation, and with and without the post-fractionation heat treatment. The results on endpoint damage categories or single score results, carry more uncertainty than on midpoint results. This follows naturally from applying conversion factors which add uncertainty with each aggregation step. Nevertheless, the single score allows for easy overall comparison of the environmental impacts of multiple products, demonstrating the main trends. Figure 6.2 and Figure 6.3 show the single score results (reflecting total environmental impact) for WPI and the pea ingredients, respectively. Due to the difference in scale, WPI and pea ingredients are shown in separate figures. Both figures demonstrate the effects of applying the various FUs.

Table 6.2 Life cycle inventory for processing 1000 kg of whey into WPI.

Process	Inputs		Protein content		Outputs		Protein content	
			kg/kg	kg/kg dm			kg/kg	kg/kg dm
Pre-treatment	Whey	1000 kg	0.009	0.182	Pre-treated whey	1000 kg	0.009	0.182
	Electricity[1]	15.6 MJ						
	Natural gas[1]	46.4 MJ						
UFDF-1	Pre-treated whey	1000 kg	0.009	0.182	UFDF-1 retentate[2,4]	255 kg	0.035	0.350
	Electricity[2]	36 MJ			UFDF-1 permeate	925 kg	1E-4	0.004
	Water for UFDF[3]	180 kg						
MF	UFDF-1 retentate	255 kg	0.035	0.350	MF retentate	13 kg	7E-4	0.700
	Electricity[3]	78.2 MJ			MF permeate[5,6]	242 kg	0.036	0.348
	Natural gas[3]	28.3 MJ						
UFDF-2	MF permeate	242 kg	0.036	0.348	UFDF-2 retentate	29 kg	0.292	0.974
	Electricity[2]	8.7 MJ			UFDF-2 permeate	257 kg	3E-4	0.005
	Water for UFDF[3]	44 kg						
NF	UFDF-2 retentate	29 kg	0.292	0.974	NF retentate[4]	24 kg	0.341	0.974
	Electricity[7,8,9]	0.06 MJ			NF permeate[10]	4 kg		
Spray drying	NF retentate	24 kg	0.341	0.974	WPI	9 kg	0.930	0.974
	Process steam[2]	81.3 MJ						
Membrane cleaning (ton^{-1} feed)	Electricity[3,11]	33 MJ			Waste water	420 kg		
	Natural gas[3,11]	38 MJ						
	Water[3,11]	420 kg						
	NaOH 50%[3,11]	2.7 kg						

Based on the work of [1]Bacenetti et al. (2018), [2]Schuck et al. (2015), [3]Gésan-Guiziou et al. (2019), [4]Tetra Pak (2015), [5]Saboyainsta and Maubois (2000), [6]Chen et al. (2021), [7]Wafi et al. (2019), [8]Carić et al. (2009), [9]Turan et al. (2002), [10]Suárez et al. (2006), [11]Depping et al. (2020).

6.3.1 Product-oriented metrics

The environmental impact of WPI was significantly higher than those of all pea ingredients when expressed per kg of ingredient (Figure 6.2 and Figure 6.3A). This stems largely from the impact that the raw material for WPI (whey) embodies. Most impact of the dairy production system is caused by feed and enteric fermentation. For pea production, no such feed conversions take place. In addition to the impact of the raw material, the isolation methods also add to the impact of WPI. Especially the spray drying step greatly impacts the environment, since it requires large quantities of energy that is supplied by fossil fuel. This is also the case for PPI, as shown by the higher score compared to the mild fractions (Figure 6.3A).

It is counterintuitive that the impact of the mild fractions is lower than that of pea flour, even though more processing is applied, meaning more resource use. This arises from the allocation taking place after separating the steeped flour into the protein and starch rich fractions (Figure 6.1). The impacts are allocated to the fractions based on their dry matter mass, leading to the starch-rich fraction carrying a large part of the impact. One way to overcome this effect would be to allocate impacts based on protein content instead. One should however realise that the starch fraction is also a useful and commercially valuable fraction. Next to the effect of allocation, another reason for the low impact of the mild fractions in Figure 6.3A is the high water content of the materials. The environmental impact of water is relatively low. Consequently, adding water dilutes not only the material but also the environmental impact per kg of ingredient.

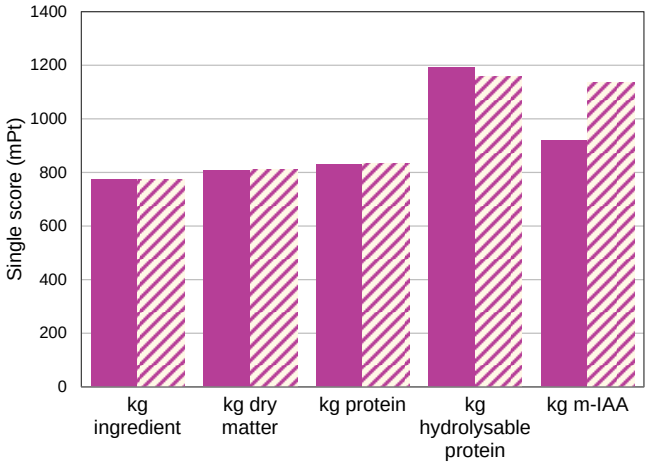

Figure 6.2 LCA single score expressed in millipoint for different functional units for the unheated (solid bars) and heated (dashed bars) dispersions of whey protein isolate.

Since the PRF, SPF and NSPF remain hydrated, in contrast to PPI and WPI, a fairer comparison is using the dry matter as FU (Figure 6.3B). The mild fractions then have roughly similar environmental impact, but the more refined PPI still has more than double the impact. Due to the lower protein purity of mild fractions, the difference between the score of PPI and that of the mild fractions (kg ingredient), was smaller when the FU considered was per kg of protein. (Figure 6.3A and C). Also the impact of pea flour increases considerably, following from its low protein content. Yet, even when the all ingredients are corrected for the dry matter and protein content, the impact of the plant-based ingredients remain all clearly lower than the impact of WPI (Figure 6.2).

The mild fractions do not carry the environmental impact related to drying. In practice, ingredients are usually dried to increase their shelf-life and reduce transport volumes. From a sustainability perspective we therefore observe a trade-off between the environmental impacts related to drying and transportation of large volumes and using chilled or frozen transport and storage (Depping et al., 2017). A first remark is that spray drying the PRF, SPF and NSPF fractions will still result in significantly lower impacts than WPI, given that spray-dried PPI is still approximately 4 times lower than WPI. However, one might also avoid this trade-off and still make use of the benefits of the mild processing which can be tailored towards a specific application (Lie-Piang et al., 2022; van der Goot et al., 2016). The ingredient fractionation could take place at the same location as the assembly of the ingredients into finished products. The original raw materials, which are generally dry and thus stable in time, can then be transported, while the need for drying is eliminated, since the wet ingredients can be used directly. This integration of ingredient fractionation and use, or process symbiosis, may reduce the environmental impact, enhance traceability and control of the ingredient history, following from societal demands for transparency and sustainability. It could also improve the functionality of proteins, since drying generally affects the functionality negatively. This obviously does depend on the possibility to locally use, handle or stabilise all produced co-fractions.

6.3.2 Digestibility-oriented metrics

Expressing the environmental impact in terms of *in vitro* digestible protein amplifies the effects observed when using protein as a FU, as not all protein is digestible. The amplification depends on the ratio of digestible versus non-digestible protein, reflected in the DH and m-IAA. Similar to the product-oriented FU, the score of the unheated and heated mild pea fractions remained lower than those of flour or PPI, when kg of hydrolysable protein was considered as the FU (Figure 6.3D). Nevertheless, the score of unheated SPF was lower compared to the other mild fractions, PRF and NSPF. This is

related to the high DH that was recorded for SPF for all small intestinal aliquots from the five gastric emptying steps (Figure A6.3a).

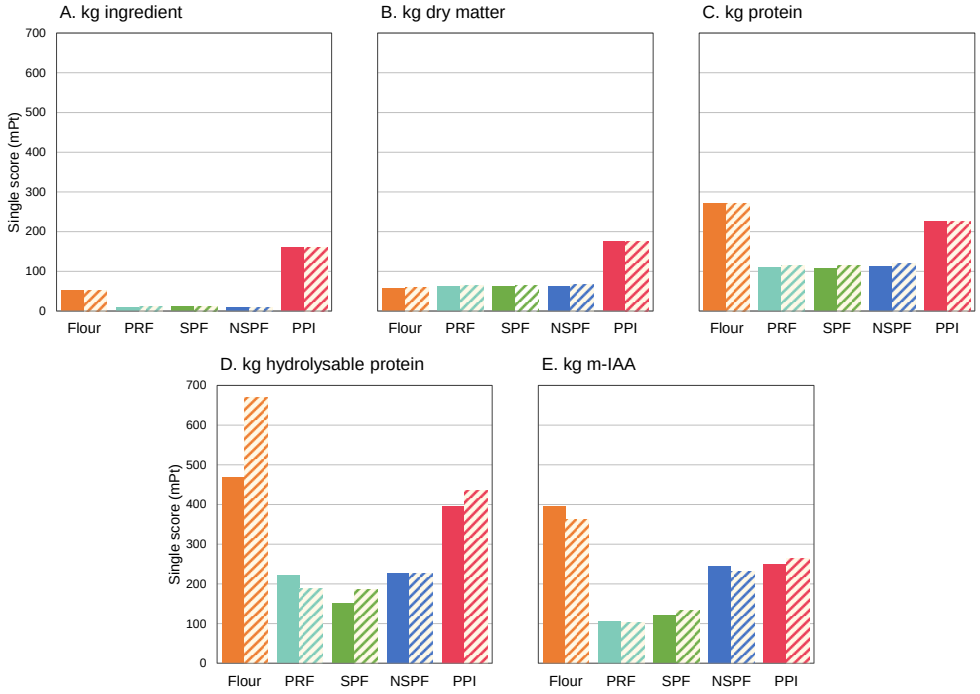

Figure 6.3 LCA single score for the unheated ingredients (solid bars) and heated dispersions (dashed bars). Scores expressed in millipoint for the functional units: per kg of (A) ingredient, (B) dry matter, (C) protein, (D) hydrolysable protein, and (E) minimum digestible, limiting, indispensable amino acid (m-IAA) in pea flour, protein-rich (PRF), soluble (SPF), non-soluble (NSPF) protein fractions and pea protein isolate (PPI).

Protein-rich ingredients are rarely consumed as is, Instead, they are further processed into finished products. In this study, heat treatment is used to represent this post-fractionation processing. The heating itself does not add a significant extra impact to the ingredients (Table A6.3). The differences between unheated and heated dispersions (solid and dashed bars in Figure 6.3D) mainly reflect the effect of heat treatment on the DH by the digestive enzymes (Figure A6.3a). The impacts of flour, SPF and PPI therefore increased as a result of the negative effect of the heat treatment on the DH, albeit in different proportions (Figure 6.3D). The WPI and PRF showed an increase in the DH and therefore the heating reduced their environmental impact (Figure 6.2 and Figure 6.3D). The DH of the NSPF fraction was barely influenced by heating and its score (per kg of hydrolysable protein) was higher than that of the other mild fractions. It is interesting to see that even a relatively small heating step can have a significant effect on the

sustainability of protein-rich materials, particularly for flour and PPI, albeit not by the heating itself but by making the protein less digestible.

Until now, we considered the DH achieved through hydrolysis by the luminal enzymes, which represents the overall degradability of proteins, but does not yet distinguish between dispensable AA and IAA. For this, we need to evaluate the availability of IAA. The FU per kg of m-IAA aims to couple the *in vitro* protein digestibility, defined as the proportion of small peptides that could be further hydrolysed by brush border peptidases and absorbed through the enterocytes, to the capacity of the food to provide the amount of IAA recommended for adults. Therefore, the amount of hydrolysable protein and the amount of m-IAA can be regarded as complementary units. The scores of PRF and SPF (per kg of m-IAA, Figure 6.3E) remained relatively low compared to the other ingredients. In contrast, the score of NSPF approached the value of PPI. The m-IAA content of the former was particularly low due to the low content of sulphuric AAs. Meanwhile, the score of PPI decreased significantly due to the high m-IAA calculated. This was also observed for unheated WPI (Figure 6.2).

The scores are similar for unheated and heated dispersions, except for WPI. The m-IAA for the heated WPI dispersion was significantly lower than for its unheated counterpart, due to a reduction in the measured histidine content (Table 6.1). It is important to observe that the scores per kg of m-IAA of the highly purified, spray-dried ingredients (WPI and PPI) increased after heat treatment. We propose that this is because of the more extensive processing involved in the fractionation process, which not only increases the impact of the material but also renders the proteins less utilizable. With mild fractions, this two-fold effect is averted, and therefore potentially more digestible and sustainable proteins can be obtained.

Although flour, PRF, SPF, NSPF and PPI were all obtained from pea, we did observe differing environmental impacts when some measure of digestion is considered as FU, before and after heating. This is an indication that assuming one true ileal digestibility or DIAAS as reported in literature for a different ingredient under study might give a rough indication of the environmental impact of the ingredient, but the effects from the processing history of an ingredient or a finished product would be dismissed. This study shows that these are important.

6.3.3 *In vitro* and *in vivo* protein digestibility in perspective

In this work, we used hydrolysis and molecular weight data from an *in vitro* digestion method, namely, the standardised semi-dynamic *in vitro* digestion method (Mulet-Cabero et al., 2020). *In vitro* methods have shown good correlation with *in vivo* data for some

pulses and protein isolates from collagen, whey and zein (Brodkorb et al., 2019; Sousa et al., 2020). This is probably not extendible to any *in vitro* method. Although the method from Mulet-Cabero et al. (2020) is based on that from Brodkorb et al. (2019), each method might require its own validation against *in vivo* measurements. The effect of processing on *in vivo* digestion is not often addressed. It is therefore worthwhile exploring the use of *in vitro* methods to quantify and, more importantly, compare the effect of the processing history of different protein sources on the extent of hydrolysis or digestibility.

The semi-dynamic nature of the used *in vitro* method allowed us to obtain richer data than with a static model. The gastric secretions were dosed into the system continuously over the course of the gastric phase, which is comparable to the *in vivo* situation. In this way, HCl and pepsin concentrations were highest towards the end of the phase. Gastric emptying was simulated by removing aliquots from the gastric phase at five timepoints. The frequency of aliquot removal was calculated to simulate a delivery of calories into the duodenum of 2 kcal/min *in vivo*. Each of the five aliquots were subjected to luminal small intestinal digestion. We thus obtained a range of DH and proportion of <1 kDa peptides from an *in vitro* gastro-small intestinal digestion.

Differences between digestion in the gastric and small intestinal phase have been observed for plant-based alternatives to animal products. Zhou et al. (2021) reported that plant-based beef was more extensively hydrolysed than beef during *in vitro* gastric digestion; but less during small intestinal digestion. These nuances could also be incorporated into a LCA depending on the purpose of the assessment or design and purpose of the food.

Besides the overall protein digestibility, the bioavailability of IAA defines the nutritional quality of proteins. Efforts towards quantifying the digestibility of individual AA *in vitro*, as well as determining an *in vitro* DIAAS have been carried out (Ariëns et al., 2021). The proposed method includes a sequence of centrifugation, concentration and ultrafiltration, followed by the determination of the IAA profile of the resulting <5 kDa fraction from the digestion samples. Our method assumes that all IAA are equally digestible and, for the most part, the calculated m-IAA (minimum digestible, limiting, indispensable AA) is roughly in accordance with the DIAAS reported in literature for WPI and pea protein concentrate (100 and 62%, and limiting IAA histidine and sulphur AA, respectively, Mathai et al. (2017)).

6.3.4 Proteins of animal and plant origin

Regardless of the FU applied, pea materials are consistently more sustainable than WPI. The differential was smaller when more specific FU were used to report the single score

of the materials. Depping et al. (2020) developed a framework to compare fractionated and non-fractionated products in which they systemised the choice of FU. When comparing products with identical functionalities they recommend defining functionalities as specific as possible. For ingredients with the purpose of supplying nutritious protein, the most specific FU would be taking digestibility into account like done in the current work using DH or m-IAA values.

The presented results considering protein ingredients confirmed the overall better sustainability of plant protein compared to animal protein, even when the nutritional quality is considered. This advantage might be maintained for structured, plant-based products. Saget et al. (2021) compared the environmental impact of plant-based and beef burger patties from different origins, using a *nutrient density unit* as a FU. This unit considers the ratio of nutrients (protein, fibre and essential fatty acids) to energy. In terms of digestibility, it considers that pea protein is 8% less digestible than animal protein. We discussed before that this probably underestimates the effect of processing on the digestibility of proteins from either source. Still, the plant-based burger showed a much lower environmental impact, particularly in regard to the *nutrient density unit*, even though more energy is necessary to produce the plant-based burgers, compared to Brazilian beef patties.

Moughan (2021) warned about the risk of underestimating both the environmental footprint and the fulfilment of dietary protein requirements of national diets, when considering production and intake of gross protein. In his approach, the gross protein intake was corrected for protein quality by calculating a DIAAS coefficient for each national diet. This approach, while suitable for estimating the environmental impact from general protein quality determinations, disregards the capability of processing to lock or unlock the digestibility and availability of proteins from animal or plant origin. Considering the processing history of foods, particularly protein-rich crops, is also important to consider as the protein transition continues.

It is clear that processing should be included in any analysis, since it significantly influences both the impact of the production, and the digestibility, both negatively and positively. Therefore, instead of just referring to the origin of a protein, it is important that chain actors also include the type of processing in their consideration. Mild processing was shown to have clear advantage over conventional processing of proteins into isolates.

6.4 Conclusion

We incorporated *in vitro* protein digestibility into the LCA of protein-rich ingredients and, potentially, of food products. In this way, the life cycle extends beyond '*farm-to-fork*' to '*farm-to-faeces*'. This approach can contribute to the discussion around real sustainable protein and thus, enable us to make better informed decisions regarding sustainable nutrition in the context of the protein transition.

The LCA, including the FUs considering *in vitro* protein digestibility, shows that the processing history of ingredients and finished products should be included, not only to assess their environmental impact, but also because of its effects on the nutritional value beyond composition, i.e., how well the nutrients can be degraded and utilized by the consumer. The latter can be positive and negative, but it clearly affects the outcome.

Mild aqueous fractionation of pea protein presents an attractive alternative to both animal-based protein (WPI) and also conventionally produced PPI as it is less environmental impactful to produce one kg of digestible protein, compared to the isolates. An interesting observation is that heating the mild ingredients had less impact on their digestibility, than it had on conventional, fully refined protein isolates. We recommend producing protein-rich ingredients using as little processing as possible, as they have overall lower environmental impact for the same nutritional effect.

Appendix

A6.1 Argumentation and assumptions for the functional unit m-IAA (minimum digestible, limiting, indispensable amino acid)

For this FU, we drew concepts from the protein digestibility-corrected AA score and the DIAAS (FAO, 2011). We assume that all AAs are equally digestible relative to each other and the protein as a whole. *Digestible protein* was defined as the fraction of <1 kDa peptides from the total solubilized protein, estimated with size exclusion chromatography.

The currently available, standardised, *in vitro* digestion methods consider gastric (pepsin) and pancreatic proteases. Thus, the action of brush border exopeptidases is not considered, as it is not readily available for incorporation in simple *in vitro* methods (Figure A6.1). Without these enzymes it is difficult to determine the proportion of ingested protein that could be hydrolysed to small enough peptides and single AAs to be available for uptake. One could assume that peptides of a certain (low) molecular weight are more likely to be hydrolysed by brush border peptidases than those of larger molecular weight. Le Roux et al. (2020) considered a molecular weight cut-off of 10 kDa to represent the potentially absorbable peptides. This molecular weight would still roughly correspond to a peptide chain of 90 AAs (considering an average molecular weight for AA of 110 Da). Rieder et al. (2021) defined '*digested protein*' as those small peptides that increased in amount at the expense of larger peptides and intact proteins throughout the *in vitro* gastro-small intestinal digestion. They identified the molecular weight cut-off point between decreasing and increasing amounts between 970 and 1100 Da.

We therefore calculated the fraction of <1 kDa peptides from the total soluble protein by integrating the area under the curve after 12.52 min in the chromatogram of the digestion samples (Figure A6.2 and Figure A6.3b), relative to the total area. It is worth noting that size exclusion chromatography data should be regarded as qualitative rather than quantitative, unless the appropriate extinction coefficients are measured for each protein material, peptide sequence or AA (Rieder et al., 2021). Thus, the value of *digestible protein* supports the other measurements, but is not sufficiently reliable on its own.

In fact, digestibility can vary among individual AA within one protein. Nevertheless, these digestibilities commonly fall within a narrow range (Guillin et al., 2021; Mathai et al., 2017). In addition, we decided against using previously reported data on individual AA digestibility as the effect of the fractionation method and processing (heating) would be lost when we assume the same digestibility for, for instance, all pea protein AA.

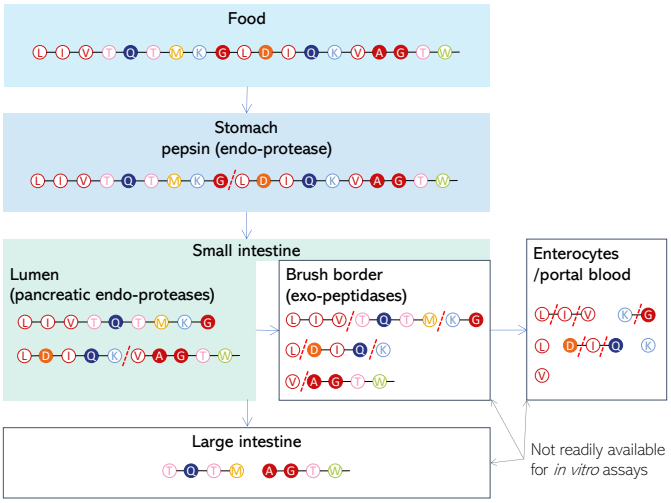

Figure A6.1 Schematised protein hydrolysis by gastric, pancreatic and brush-border enzymes throughout the gastrointestinal tract.

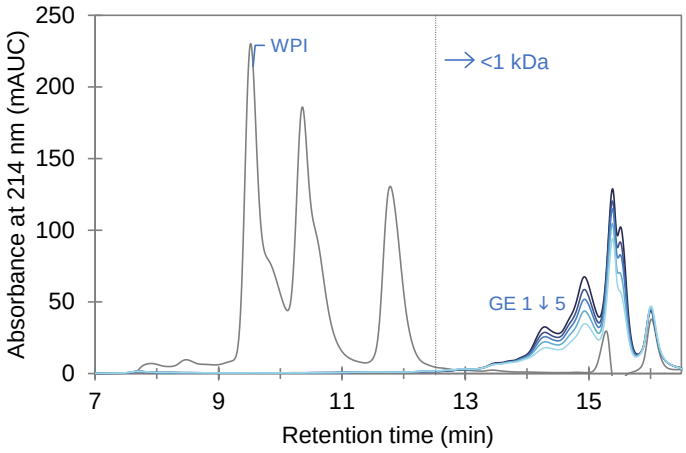

Figure A6.2. Exemplar of chromatogram for undigested whey protein isolate and the digestion products after 120 min of in vitro static small intestinal digestion from five gastric emptying (GE) steps from a dynamic gastric phase. The mark at 12.52 min retention time corresponds to the <1 kDa molecular weight cut-off.

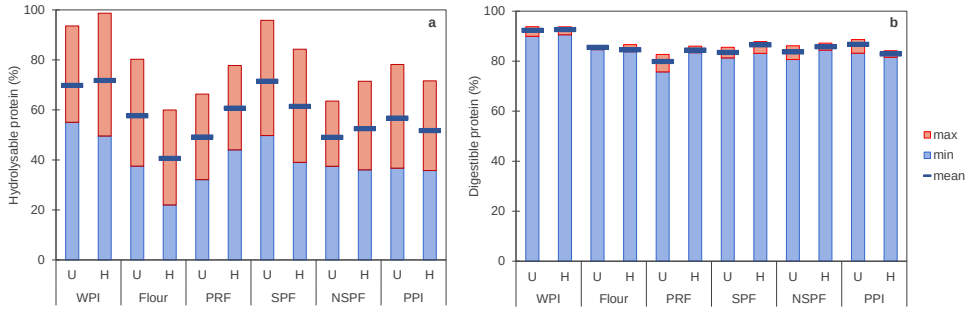

Figure A6.3. Minimum, maximum and average hydrolysable (degree of hydrolysis from OPA assay) and digestible (>1 kDa peptides estimated from size exclusion chromatography) protein of unheated (U) and heated (H) dispersions of whey (WPI) and pea (PPI) protein isolates, pea flour, protein rich fraction (PRF), soluble (SPF) and non-soluble (NSPF) protein fractions.

A6.2 Life cycle assessment

Table A6.1. Overview of the background processes used for the life cycle assessment from Agri-footprint® 5.0 (van Paassen et al., 2019).

Process	Background process AFP5
Whey	Liquid whey (Gouda 48+), at processing/NL Mass
Electricity	Electricity mix, AC, consumption mix, at consumer, < 1kV NL S System - Copied from ELCD
Natural gas	Combustion of natural gas, consumption mix, at plant/NL Mass
Water	Drinking water, water purification treatment, production mix, at plant, from surface water RER S System - Copied from ELCD
Steam	Process steam from natural gas, heat plant, consumption mix, at plant, MJ, NL S System - Copied from ELCD
NaOH 50%	Sodium hydroxide (50% NaOH) (mix), at plant/RER Mass
Wastewater	Waste water treatment, domestic waste water according to the Directive 91/271/EEC concerning urban waste water treatment, at waste water treatment plant EU-27 S System - Copied from ELCD

Table A6.2 Environmental impact results (midpoint) for 1 kg of WPI

Impact category	Unit	Value
Global warming	kg CO_2 eq	1.84E+01
Stratospheric ozone depletion	kg CFC11 eq	1.17E-04
Ionizing radiation	kBq Co-60 eq	8.02E-02
Ozone formation, Human health	kg NO_x eq	8.59E-03
Fine particulate matter formation	kg PM2.5 eq	2.93E-02
Ozone formation, Terrestrial ecosystems	kg NO_x eq	8.74E-03
Terrestrial acidification	kg SO_2 eq	2.24E-01
Freshwater eutrophication	kg P eq	5.60E-04
Marine eutrophication	kg N eq	2.95E-02
Terrestrial ecotoxicity	kg 1,4-DCB	4.34E+00
Freshwater ecotoxicity	kg 1,4-DCB	2.23E-01
Marine ecotoxicity	kg 1,4-DCB	4.96E-02
Human carcinogenic toxicity	kg 1,4-DCB	2.15E-03
Human non-carcinogenic toxicity	kg 1,4-DCB	2.04E+01
Land use	m^2a crop eq	6.58E+00
Mineral resource scarcity	kg Cu eq	2.51E-03
Fossil resource scarcity	kg oil eq	1.43E+00
Water consumption	m^3	1.56E-01

Table A6.3 LCIA endpoint results per damage category (human health, ecosystems and resources) and normalized to millipoint (mPt), for all materials (per kg). The single score is the total of all mPt from each damage category.

	Heat treatment	Human health		Ecosystems		Resources		Single score
		DALY	mPt	species.yr	mPt	USD2013	mPt	mPt
Flour	N	1.9E-06	33	3.5E-08	20	3.9E-02	0.3	52
	Y	1.9E-06	33	3.5E-08	20	4.1E-02	0.3	53
PRF	N	3.7E-07	6	6.5E-09	4	7.9E-03	0.1	10
	Y	4.0E-07	7	6.6E-09	4	1.2E-02	0.1	11
SPF	N	3.9E-07	7	6.9E-09	4	8.4E-03	0.1	11
	Y	4.2E-07	7	7.0E-09	4	1.2E-02	0.1	11
NSPF	N	3.3E-07	6	5.8E-09	3	7.1E-03	0.1	9
	Y	3.6E-07	6	5.9E-09	3	1.1E-02	0.1	9
PPI	N	6.9E-06	117	7.4E-08	41	3.3E-01	2	160
	Y	6.9E-06	117	7.4E-08	41	3.3E-01	2	160
WPI	N	4.0E-05	680	1.6E-07	90	5.0E-01	4	774
	Y	4.0E-05	681	1.6E-07	90	5.0E-01	4	774

CHAPTER SEVEN

In silico modelling of protein digestion: a case study on solid/liquid and blended meals

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Abstract

We present a dynamic, semi-mechanistic, compartmental protein digestion model to study the kinetics of protein digestion. The digestive system is described as a series of eight compartments. The digestive processes are described by a set of zero or first order differential equations. The model considers ingestion of a meal, secretion of gastric and pancreatic juices, protein hydrolysis, grinding, transit and amino acid absorption. The model was used to simulate protein digestion of a meal composed of a solid and a liquid phase or one where both phases are blended into a homogeneous phase. Luminal volumes and pH of gastric and duodenal contents were estimated for both meals. Further, gastric emptying is described as a function of the energy density of the bolus, instead of the more common mass action approach.

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7.1 Introduction

In the last decades, *in vitro* models have aided in the study of food digestion complementing *in vivo* studies (Lucas-González et al., 2018). *In silico* models have only recently gained some momentum but have the potential to aid and steer the whole field by identifying causality, and also gaps and inconsistencies in our understanding (Le Feunteun, Al-Razaz, et al., 2021; Le Feunteun et al., 2020). *In vitro* and *in silico* models are both simplified representations of the complex processes occurring during *in vivo* digestion. These models can integrate different stages of food digestion and shed more light on the underlying mechanisms.

The use of physiologically based models to describe food digestion borrows concepts from the field of pharmacokinetics, i.e., the study of the kinetics of absorption, distribution, metabolism and excretion of pharmaceutical compounds (Turfus et al., 2017). Physiological systems are described as a set of interconnected biological compartments, often anatomical parts of the organism under study. In animal nutrition works, compartmental models have been used to study digestion in pigs (Bastianelli et al., 1996; Rivest et al., 2000; Strathe et al., 2008). Halas et al. (2018) gathered a comprehensive collection of feed intake, digestion kinetics, utilization and animal growth models. Meanwhile, Le Feunteun, Al-Razaz, et al. (2021) reviewed the models and tools currently available to study food digestion in humans and provided an outlook to the potential of the developing field. Mathematical models allow the replication of the dynamic nature of digestion, not only in terms of the flow within and throughout the gastrointestinal tract, but also the physicochemical properties of the food and its environment (van der Sman et al., 2020). Provided that enough knowledge on the underlying mechanisms is available, it is also possible to incorporate feedback processes. *In silico* models can therefore aid in understanding and quantifying abstract events and processes occurring during the digestion of food. In this way, *in silico* models contribute to understanding and predicting nutrient absorption kinetics.

In this work, we developed a dynamic model of protein digestion, incorporating endogenous secretions, antral grinding, hydrolysis, gastric emptying, transit through the small intestine and ultimately, absorption. This model is based on simple mass balances and the description of physiological mechanisms. Our objective is to study the effect of the physical state of the meal on its fate in the gastrointestinal tract. We were inspired by the work of Marciani et al. (2012) in which the gastric emptying of two meals was studied; a meal composed of two phases, one solid and one liquid, or a meal where the solid and liquid components were blended into a homogeneous, single phase. In our model, gastric

emptying is described by assuming a constant energy (kcal) delivery into the duodenum compartment and an antral lag time is included to signal the start of antral grinding and gastric emptying for solid meals. To the best of our knowledge, the notion of gastric emptying as a function of the energy density of the digesta has only been explored in one recent model considering computational fluid dynamics within the stomach (Li & Jin, 2021).

7.2 Model development

The model assumes that for protein digestion, the gastro-small intestinal tract can be described with eight compartments, one for the stomach and seven for the small intestine (duodenum, jejunum 1 and 2, ileum 1, 2, 3 and 4,Figure 7.1) (Yu et al., 1996). The food bolus enters the stomach through the mouth and oesophagus. HCl and pepsin are secreted into the stomach from the parietal and chief cells, respectively. Gastric chyme is emptied from the stomach into the duodenal compartment, where $NaHCO_3$ and endoproteases such as trypsin, chymotrypsin and elastase are secreted from the pancreas. Chyme continues to flow through the jejunal and ileal compartments. Protein hydrolysis into peptides takes place in the stomach and small intestine compartments, while peptide hydrolysis into amino acids (AA) is simulated only from the jejunum 1 to the ileum 4 compartments, assuming that only brush border enzymes have exopeptidase activity and are only present in these compartments. AA are absorbed into and through the enterocytes of the jejunal and ileal compartments into the portal blood. Unabsorbed components flow out of the ileum 4 compartment into the colon, which is not part of the model anymore.

The primary dependent variables of this model are the quantities or pool sizes, Q_{ij}, of each component, *i*, in a given compartment, *j*. These are defined by ordinary differential equations with the independent variable being the time (dQ_{ij}/dt), hence the dynamic nature of the model. The rate of change in the size of each pool is described by the balance between inflows and outflows as depicted in the flow diagram.

7.2.1 General assumptions and considerations

To construct our model, we assume that the meal arrives in compartments that do not contain nutrients of prior meals. Diffusion is assumed to be instant, for instance, enzymes are in immediate contact with their substrate upon secretion. In the respect of protein or peptide hydrolysis, all peptide bonds are subject to cleavage, i.e., enzyme specificity is not considered.

No processes are modelled past the ileum 4 compartment. Proteic compounds (protein, peptide or AA pools) are considered to be undigested if they arrive in the colon. Likewise,

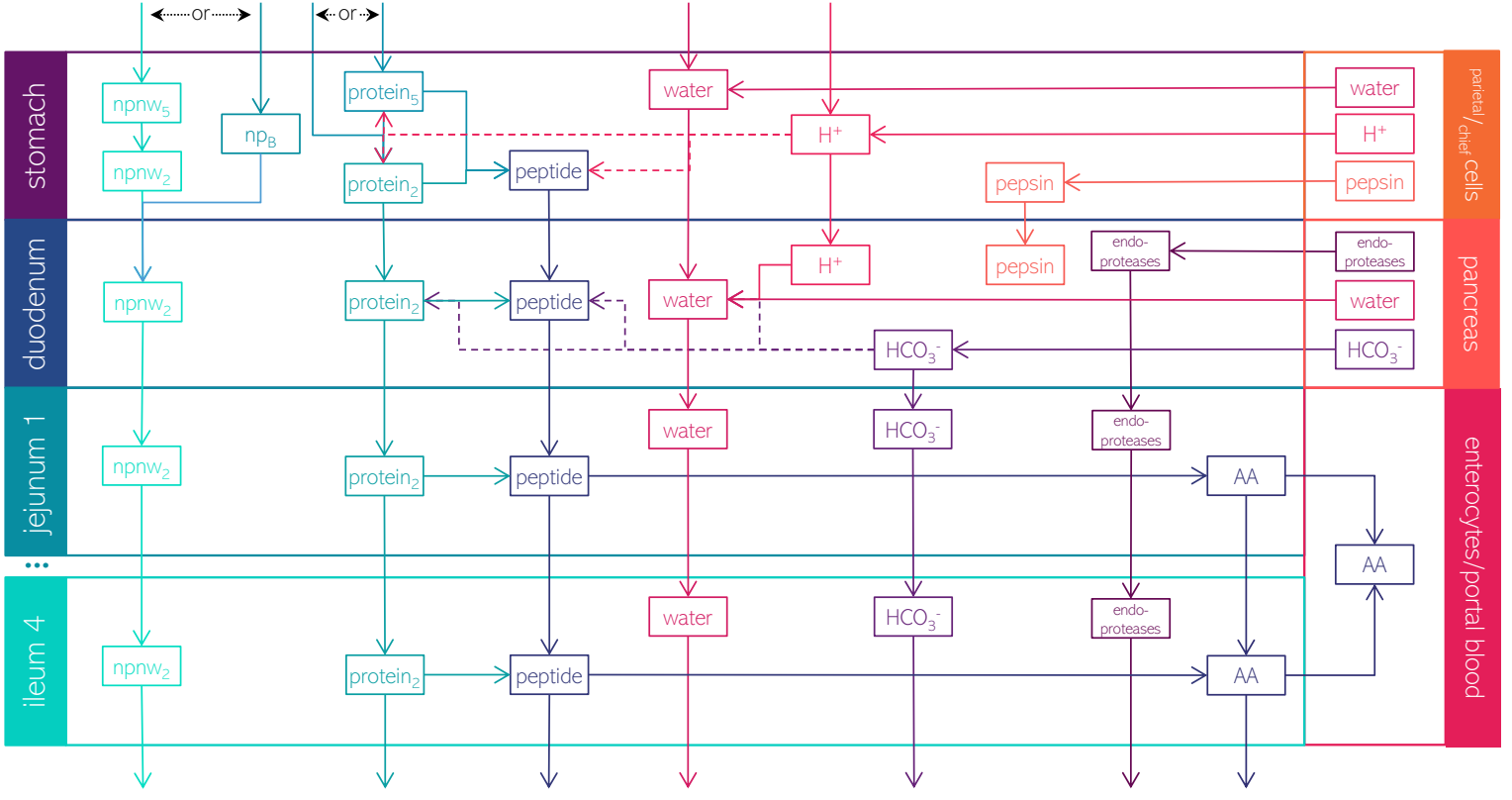


Figure 7.1 Flow diagram of the protein digestion model for solid/liquid and blended meals. Solid lines represent fluxes, dashed lines represent influential factors that do not contribute to the protein-peptide mass balance. The jejunum 2, ileum 1, 2 and 3 compartments are not shown but are located after jejunum 1, with the same pools and fluxes as this compartment. $npnw_{5,}$ non-protein/non-water 5 mm; np_B, non-protein blended; AA, amino acid.

no further metabolism is modelled after AA absorption through the enterocytes into the portal blood. Interindividual variability is not considered.

The model describes fluxes with functions that have a physiological meaning. When available, parameters were obtained from literature (Table A7.1).

7.2.2 Meal characterization

The model considers meals with two possible physical states, one where a solid phase is consumed next to liquid, e.g., water, henceforth referred to as 'solid/liquid' and one where the solid and liquid components form a single, homogeneous phase, this meal is referred to as 'blended' (Marciani et al., 2012). The composition of the solid/liquid meal is divided into three pools, protein, water, and non-protein/non-water ($npnw_5$). Meanwhile, the blended meal is described by two pools, protein and non-protein (including water, np_B) pools (Table 7.1). To further ease the description of some fluxes, the protein pool is expressed in *AA equivalent* units. This unit conversion is possible if an average molecular weight and number of AA in the protein sequence is known. In other cases, a representative protein can be chosen to estimate the weight of protein to AA equivalents considering its molecular weight and number of AA in the sequence.

Other meal characteristics required to feed into the model are the pH, the number of AA with pH buffering capacity in the acid and alkaline environment, the volumetric mass and the energy density of the full meal and its individual pools (Table 7.1).

7.2.3 Intake

A single meal, either solid/liquid or blended, is consumed at a constant rate for the duration of the meal. The solid phase (henceforth called $npnw_5$ and $protein_5$) of the solid/liquid meal is assumed to be broken down in the mouth into particles of 5 mm diameter.

7.2.4 Gastric juice secretion

The rate of secretion from the chief and parietal cells of water, pepsin and HCl, into the gastric lumen was modelled as a zero order function triggered by the arrival of bolus into the stomach (Goetze et al., 2009). When the gastric contents of food are lower than 0.5 g, either in the fasted state or after most of the chyme has been emptied into the duodenum, the rate of secretion of gastric juice is marginal.

7.2.5 Antral grinding

The 5 mm solid particles of the solid/liquid meal ($npnw_5$ and $protein_5$) that arrive in the stomach compartment are considered too large to pass through the pylorus. We

incorporated a lag time between ingestion and the onset of antral contractions and peristalsis into the model (Brown et al., 1993; Houghton et al., 1988). *In vivo*, a lag time has been experimentally observed between ingestion and emptying of solids out of the stomach, and not necessarily of the antral contractions (Camilleri et al., 1985; Siegel et al., 1988). After the lag time, antral grinding, i.e., the flux between the 5 mm pools into the 2 mm pools, starts (Figure 7.1). This size reduction is described by a zero order rate function. To signal the end of antral grinding, when most of the large particles have been ground, the flux from the 5 mm pools to the 2 mm pools is then described by a first order rate equation as a function of the remaining $npnw_5$ and $protein_5$ pools.

Table 7.1 Meal properties and composition parameters included in the model description

Meal attribute			unit
Buffering capacity			mmol/l pH
pH (homogenized meal)			
Molecular weight of protein			g/mol
Number of amino acids in sequence			aa eq
Number of glutamic and aspartic acid in protein sequence			aa eq
Number of histidine, glutamic and aspartic acid in protein sequence			aa eq
Rate of food ingestion			g/min
Duration of meal			min
	Solid/liquid meal	Blended meal	
Composition	water	-	g/g meal
	non-protein/non-water	non-protein (water included)	
	protein	protein	
Density	non-protein/non-water	non-protein (water included)	g/ml
		protein	
		food	
Energetic content	non-protein/non-water	non-protein (water included)	kcal/g
		protein	

7.2.6 Gastric emptying

Solids are retained in the stomach for a longer period of time compared to liquids. This is known as gastric sieving. Furthermore, many stimuli (mineral acids, fats and fatty acids, osmotic factors, duodenal distension) and receptors in the proximal duodenum and jejunum are involved in inhibiting gastric peristalsis and, as a result, slowing down gastric emptying (Dooley et al., 1984; Roman, 1982; Rønnestad et al., 2014). It has been observed that meals of high energy density are emptied more slowly than meals with lower energy density (Hunt & Stubbs, 1975; Keto et al., 2012). This phenomenological relation between the energy density and the gastric emptying rate probably captures the complex feedback mechanisms that in fact regulate the delivery of chyme into the small

intestine. In our model, gastric sieving is accounted for by the lag time to antral grinding and thus gastric emptying of the solid fraction of the solid/liquid meal. Gastric emptying of the non-protein and proteic ($protein_{2\,mm}$ and peptide) pools is therefore described by a constant rate of energy delivery into the duodenal compartment (2 to 2.5 kcal/min) (Hunt et al., 1985). The flux of water, H^+ and pepsin from the stomach into the duodenum compartment are described by zero order kinetics with rate constants for each of the components. When most of the gastric content has been emptied, the flux out of the stomach is described by mass action kinetics with a postprandial gastric emptying rate constant.

7.2.7 Pancreatic juice secretion

Pancreatic secretion is mediated by various hormonal pathways, triggered by the presence of acid and nutrients in the duodenal lumen, among other stimuli (Pandol, 2011; Vella, 2016). Secretion of pancreatic juice (water, HCO_3^- and endoproteases) into the duodenal compartment is described by zero order kinetics. This flux is prompted by the presence of proteic contents in the duodenum. Secretion starts when the size of the protein and peptide pools combined is larger than 0.1 aa meq, and stops when only this amount remains in the compartment. Endoprotease delivery into the jejunum and ileum compartments results from transit from the duodenum.

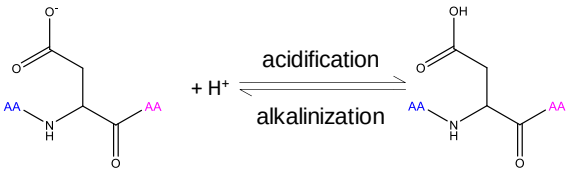
7.2.8 pH changes

The molar concentration of H^+ in a given compartment was calculated from the balance between inflows, from food and secretions, and outflows, buffering capacity, (de)protonation after hydrolysis, transit and neutralization in the duodenum. Volume of contents within compartments was also calculated dynamically considering the basal volume of gastric or pancreatic juice in a given compartment and the sum of volumes from water, proteic and non-protein pools. As the content in the compartments in this model were considered to be ideally mixed, no spatial differences in pH would be observed within the stomach compartment or small intestine compartments.

7.2.8.1 Buffering capacity

The pH in the gastrointestinal tract can be as low as 2, in the fed mid-distal stomach, and as high as 6.7 in the fed mid-distal duodenum (Abuhelwa et al., 2016; Kalantzi et al., 2006; Simonian et al., 2005). The AA with a dissociating side chain, having a pKa within this range are histidine (6.5 – 7.4), aspartic and glutamic acids (4.0 – 4.8) (Mathews et al., 2000). The buffering rate (mmol H^+/min, Equation 7.1), represents the flow out (or in) of the H^+ pool into (or out of) protein, more specifically the protonatable (or de-

protonatable) AA in the structure. However, it is a so called non-additive flux or influential factor to the protein pool, as it does not affect the protein mass balance as such.

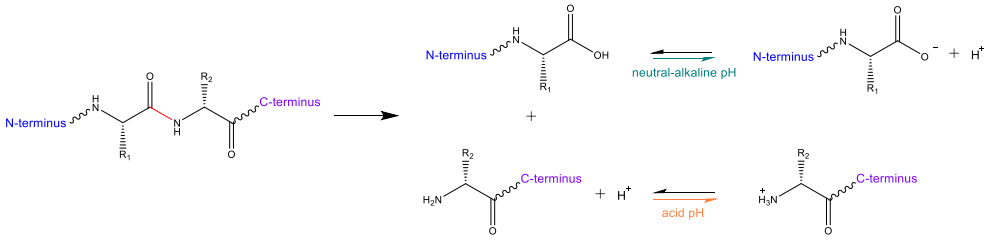


$$\text{Buffering rate} = \frac{k_f \, Q_{H^+} Q_{B^-}}{Q_w} - k_b Q_A \qquad \text{Equation 7.1}$$

In the acid-base reactions, k_f and k_b (min^{-1}) are the forward and backward rates of buffer reaction (conjugate base$^-$ + H^+ ⇆ weak acid) as described by Weinstein et al. (2013). In this expression, Q_{H+}, Q_{B-} and Q_A (mmol) are the amounts of protons, deprotonated and protonated amino acid in the protein or peptide sequence, respectively, and Q_W (mmol) is the amount of water.

7.2.8.2 (De-)protonation after hydrolysis

A proton is removed or added after a peptide bond cleavage catalysed by pepsin or pancreatic endoproteases, depending on the surrounding pH. A carboxyl and an amino group are released when a peptide bond is hydrolysed. The structure of the resulting peptides influences the pKa of the newly exposed groups. The pKa of α-COOH is between 3.5 and 4.0, while for α-NH_2 ranges between 8 and 9 (Mathews et al., 2000).



Thus, the flux of protons in or out of the recently exposed carboxyl and/or amino group (mmol H^+/min) is proposed to be equal to the rate of cleavage itself, i.e., the flux from the protein to the peptide pool (AA eq/min).

7.2.8.3 Neutralization of acid chyme

As gastric emptying starts, acidic chyme enters a more alkaline or neutral environment in the duodenum. The bicarbonate already present in the duodenum plus that which

begins to be secreted as a result of the presence of nutrients in the compartment neutralizes the acid protons entering in the acid chyme. A water molecule is produced from one molecule of sodium bicarbonate and one of hydrochloric acid. The reaction is correctly balanced with the formation of CO_2 and NaCl, however this is not considered in the model. The neutralization rate (mmol/min) in the duodenum is described by mass action kinetics considering the forward (kw, mM min^{-1}) and backward (ko, min^{-1}) rate constants of the neutralization reaction.

$$\text{Neutralization rate}_1 = kw\,[H^+]_1\,Q_{HCO3-1} - ko\,Q_{water1} \qquad \text{Equation 7.2}$$

The pH is not calculated beyond the duodenal compartment as any pH-dependency of the global activity of pancreatic proteases is not considered.

7.2.9 Intestinal transit

The compartmental transit model from Yu et al. (1996) divides the small intestine in seven compartments to better simulate the flow and axial mixing within the organ. This is presented as a suitable compromise between a complex dispersion model and a single compartment one. In this model, the duodenum represents the first half of the compartment 1, the jejunum the second half along with compartments 2 and 3; and the ileum the remaining four compartments. For simplicity, we consider the complete first compartment to represent the duodenum.

Transit through the small intestine compartments and out of the ileum 4 into the colon is described by a fractional passage rate with first order kinetics proportional to the pool size of each component in a given compartment. The fractional passage rate is the inverse of the mean retention time which is considered equal for all components of the digesta, in each compartment or the complete small intestine divided by the seven compartments.

7.2.10 Enzymatic hydrolysis

Protein hydrolysis has been simplified into the conversion from intact protein to peptides, regardless of the size or number of the latter since the proteic pools are modelled as *AA equivalents*. The protein hydrolysis rate (aa meq/min) in our model depends on a fractional conversion rate (k_E or k_N, mM min^{-1}), the pool size of protein (aa meq) and the concentration of enzyme (mM), considering the dynamicity of the volume of the contents of a given compartment (Equation 7.3).

$$\text{Hydrolysis rate}_{\text{pancreatic endoproteases}} = k_E\,[\text{endoprotease}]_j\,Q_{\text{protein}_j} \qquad \text{Equation 7.3}$$

Furthermore, the activity of digestive proteases depends on the pH (Fersht & Renard, 1974; Goldberg et al., 1969; Hess et al., 1970; Piper & Fenton, 1965). As an aspartic enzyme, pepsin behaves as a diacid and it is active to catalyse hydrolysis in its first deprotonated form. The proportion of the active form of pepsin as a function of pH was expressed by Kondjoyan et al. (2015) and originally derived by (Cornish-Bowden & Knowles, 1969) (Equation 7.4).

$$X_{pepsin_{active}} = \frac{1}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}} \qquad \text{Equation 7.4}$$

where K_{a1} and K_{a2} are the first and second dissociation constants. This results in an additional term to Equation 7.3 to consider the effect of pH on the pepsin-catalysed hydrolysis rate (aa meq/min).

$$\text{Hydrolysis rate}_{pepsin} = k_N \, X_{pepsin_{active}} \, [pepsin]_i \, Q_{protein_i} \qquad \text{Equation 7.5}$$

Residual pepsin activity might continue in the first part of the small intestine while the pH is low enough for the enzyme to be in its active form. Therefore next to hydrolysis in the gastric environment, Equation 7.3 and Equation 7.5 also express the hydrolysis that might take place in the duodenum.

7.2.11 Absorption

Peptides are assumed to be small enough to arrive at the brush border and to be the only substrates for exopeptidase-catalysed hydrolysis into single AA in the jejunal and ileal compartments (Figure 7.1). The exopeptidase-catalysed hydrolysis is described by first order kinetics, dependent on the amount of peptide present in a given compartment. As AA are produced from the peptide pools, they can diffuse or be transported through the intestinal epithelium. AA absorption is described by Michaelis Menten-type kinetics to consider the affinity and preference of AA to specific transporters (Bröer, 2008).

7.2.12 Model simulations

The model was built on the Smart (Simulation and Modelling Assistant for Research and Training) environment (Scholten et al., 1998-2020). This is a user-friendly, free package in which ordinary differential equations can be solved. Once the equations and parameters were entered, simulations were run with the fourth order Runge-Kutta fixed step length numerical integration algorithm with a time step of 6×10^{-4} min, for 420 min. Thorough instructions on the use of Smart have been detailed elsewhere (Gerrits et al., 2021).

7.3 Model output

The use of our model to simulate the digestion of protein from solid/liquid and blended meals draws inspiration from the work from Marciani et al. (2012). The participants in this study consumed a solid/liquid meal consisting of chicken, vegetables and water as beverage, or a blended meal consisting of the same components as a homogeneous liquid. The nutrient composition, meal size and duration from this study were used as input to our model. As the main source of protein in this meal is chicken meat, actin was chosen as the representative protein to convert *g protein* into *aa eq* (Murakami & Uchida, 1985; UniProt Consortium, 2018).

7.3.1 Gastric phase

7.3.1.1 Antral grinding

Particles in the food bolus from the solid/liquid meal are considered to be too large to pass through the pylorus. Therefore, a particle size reduction step is included. While it is not clear whether particles are ground during this retention time, in our model, grinding starts after an 'antrum lag' and, consequently, also gastric emptying of the ground particles. Figure 7.2 shows, first, the ingestion of 185 g of the non-protein/non-water fraction of the meal, over 15 min. Second, the 'antrum lag', during which there is virtually no change in the pool size of 5 mm particles ($Qnpnw_5$) for 30 min after ingestion. Third, the flux from $npnw_5$ to $npnw_2$ starts (Figure 7.1), as indicated by the reduction in the 5 mm pool or the increase in the cumulative pool of ground material. Small particles are emptied out of the stomach as soon as they appear. This results in a very small pool size of 2 mm particles and in an overlap of the cumulative pools of ground and emptied material.

7.3.1.2 Gastric emptying

Gastric emptying is described by a constant delivery of calories into the duodenum compartment from the nutrient pools (2 mm or blended). Meanwhile water, being a non-caloric component, was described with an independent emptying rate (explained by gastric sieving and also the so called Magenstrasse effect (Pal et al., 2007)). It should be noted that in our model, the water from the gastric juice secretion contributes to the water pool in the stomach. To show these effects, we calculated the volume of the contents in the stomach compartment (Figure 7.3). After ingestion of the solid/liquid meal, gastric emptying showed a two-phase character. Water is emptied as soon as the meal is ingested, while the emptying of solids is delayed by the 'antrum lag'. As antral grinding starts, so does the emptying of nutrients.

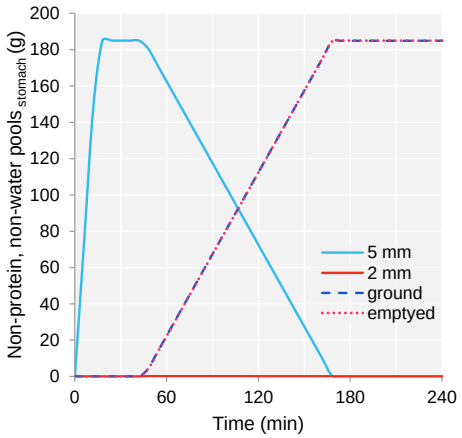


Figure 7.2 Simulated antral grinding of 5 mm particles and gastric emptying of 2 mm particles from the solid/liquid meal. Solid lines indicate absolute pools of 5 and 2 mm non-protein particles, dashed lines indicate cumulative pools of ground and emptied particles.

The blended meal was emptied at a constant rate for most of the duration of the gastric phase. This meal is emptied at a faster rate than that of the solids from the solid/liquid meal because of its lower caloric density. Regardless, the volume of gastric contents was higher from the blended meal than from the solid/liquid meal.

Including the 'antrum lag' and describing the gastric emptying of nutrients in terms of calories delivered to the duodenum, allows us to mimic the biphasic nature of gastric emptying of a solid/liquid meal and the monophasic nature of homogenized liquid meals as presented in Figure 7.3 (Marciani et al., 2012; Siegel et al., 1988). Nevertheless, in the study of Marciani et al. (2012), gastric content from both meals were emptied over a similar period of time (ca. 170 min). From our simulation, most of the contents of the stomach compartment from the blended meal are emptied nearly an hour before they are for the solid/liquid meal, suggesting that there are more phenomena that determine the duration of the gastric phase.

7.3.1.3 pH of gastric content

The contents within each compartment are assumed to be instantly mixed. Thus, a single pH throughout the stomach is estimated based on the inflows of H^+, (1) from the meal, which is rather marginal, (2) from the basal gastric juice content from the fasted state and, more significantly, (3) from the secretion of gastric juice in the fed state (Figure 7.1). The outflows are (1) the buffering capacity of the protein, (2) the protonation after protein hydrolysis and (3) gastric emptying of acid into the duodenum compartment. The volume of the gastric contents is considered to be dynamic and changing with time, which of course also affects the calculation of the H^+ concentration.

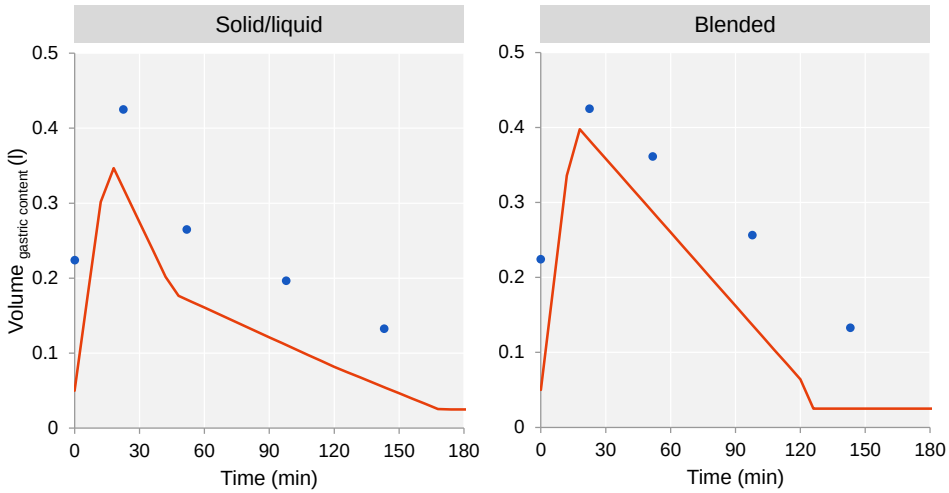


Figure 7.3 Volume of gastric content from solid/liquid and blended meals, model output in orange and data points from Marciani et al. (2012) in blue. Time zero indicates the time of meal ingestion. Note that the model was not fitted.

Gastric juice secretion is stimulated by the presence of nutrients in the stomach compartment. A basal volume of gastric juice, including acid, is present in the fasted state (Figure 7.4, time 0), even though the amount of basal gastric juice is negligible compared to the fed state secretion as can be observed in the following time points. It can be seen for the solid/liquid meal that secretion proceeds until 180 min, whereas it reaches a maximum at 120 min for the blended meal. The end of the fed secretion rate corresponds to the moment when most of the gastric contents have been emptied out. Afterwards, it returns to a postprandial or fasted secretion rate which is much lower.

The most significant outflux from the H^+ pool at the beginning of the gastric phase is the protonation of acidic AA, which accounts for the proteins' buffering capacity. This outflux increases slightly after 30 min, i.e., most of the buffering capacity is expressed as soon as the protein is ingested. It is assumed that all binding sites are exposed instantaneously after having been ingested, and readily available for protonation. By incorporating a diffusion term, the buffering capacity could be more realistically represented as a sustained flux for a longer period of time. A more sophisticated empirical model to predict the buffering capacity of protein gels and dispersions was proposed by Mennah-Govela et al. (2019). That model accounts for the exposure of acidic amino acids by considering the surface area of particles. For simplicity, this model was not used as it considers diffusion phenomena and requires a number of fitting parameters as well as an accurate characterization of the protein's amino acid profile.

The protonation flux belongs to the more interesting ones as the pepsin activity is influenced by the concentration of H^+, and protonation after pepsin-catalysed hydrolysis influences the H^+ pool. As will be discussed further, the pepsin-catalysed hydrolysis of protein progresses along the gastric phase, in part because of the decreasing pH, and hence the protonation of the resulting amino groups does as well. Less protonation was recorded for the blended meal reflecting a lower extent of protein hydrolysis.

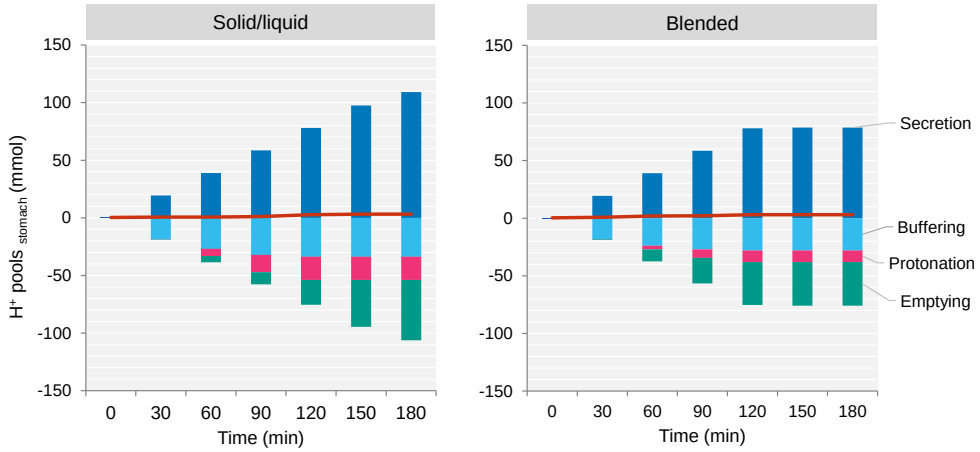


Figure 7.4 Cumulative pools from the in and out fluxes of the H^+ pool (bars) and absolute H^+ pool (line) in the stomach compartment from solid/liquid and blended meals.

Lastly, the gastric emptying of acid also influences the concentration of H^+ in the gastric contents. More acid is emptied from the stomach compartment during the digestion of the solid/liquid meal. This is again explained by the retention of the gastric contents for a longer period for this meal than for the blended meal, as the acid emptying rate constant is higher in the fed state than in the fasted state as a proxy for the overall emptying of chyme.

The pH resulting from the contribution of these fluxes to the H^+ pool as well as the dynamic volume of the gastric contents shows a different profile for the two meals (Figure 7.5). For both meals, the initial increase results from the meal ingestion and dilution of the basal gastric juice present in the compartment. Our results follow a trend qualitatively similar to that observed by Malagelada et al. (1979) as the bolus from the blended meal reaches a higher pH than that from the solid/liquid meal. The sharp decrease can be associated to both the onset of gastric secretions but also to the reduction of the gastric volume. The pH reduction is dampened by the protein's buffering capacity but more significantly by the start of the pepsin-catalysed hydrolysis. This continues until the end of the gastric phase or when most of the contents have been emptied. At this point, both the secretion and emptying slow down and the pH remains relatively constant. Unlike the

results from Malagelada et al. (1979), the pH of the solid/liquid meal is consistently higher than that from the blended meal during the acidification of the gastric chyme.

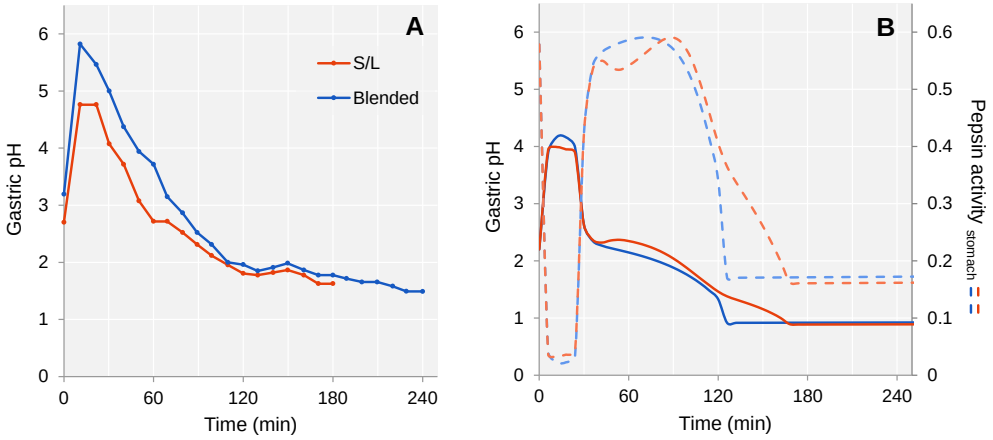


Figure 7.5 pH of gastric contents from solid/liquid and blended meals, (A) data points from Malagelada et al. (1979), (B) model output: pH (solid lines) and, fractional pepsin in its active form (dashed lines).

The concept of the gastric pH, just as that of other compartments, is rather ambiguous. It is known that pH in the stomach not only changes over time but also spatially throughout the stomach (Bornhorst et al., 2014; Simonian et al., 2005) and even within particles (van der Sman et al., 2020). Furthermore, pH will also be heterogeneous within the digesta composed of solid particles of various sizes, a liquid phase from the meal and the gastric juice. A potential approach to describe the local pHs could be to fragment the stomach compartment into several sub-compartments with their own H^+ pools and fluxes. Le Feunteun et al. (2014), for example, described the stomach as two compartments instead of one and simulated the secretion rate of the gastric juice using a gaussian step function.

- Pepsin activity

The pH influences the amount of pepsin that is in its active form. Figure 7.5 shows the time interval at which pepsin is most active, i.e., when the highest possible proportion of pepsin is in its active form. In the first 30 min, when the pH is the highest, a very small fraction of pepsin is active. As the pH drops sharply, the pepsin activity increases and is maintained roughly between pH 2.2 and 1.7. When pH decreases further until ca. 1, the pepsin activity drops accordingly.

7.3.2 Small intestinal phase

7.3.2.1 pH of duodenal contents

The influx to the H^+ pool in the duodenum compartment is the gastric emptying of acid chyme. The outfluxes are the protonation after protein hydrolysis (if pH<5) and neutralisation by the bicarbonate secreted in the pancreatic juice. As in the gastric phase, the pH profile of the duodenal contents shows the interplay of many events occurring in the gastrointestinal tract (Figure 7.6A and B). Acid chyme arrives in the duodenum compartment increasing the size of the H^+ pool and causing the pH to drop.

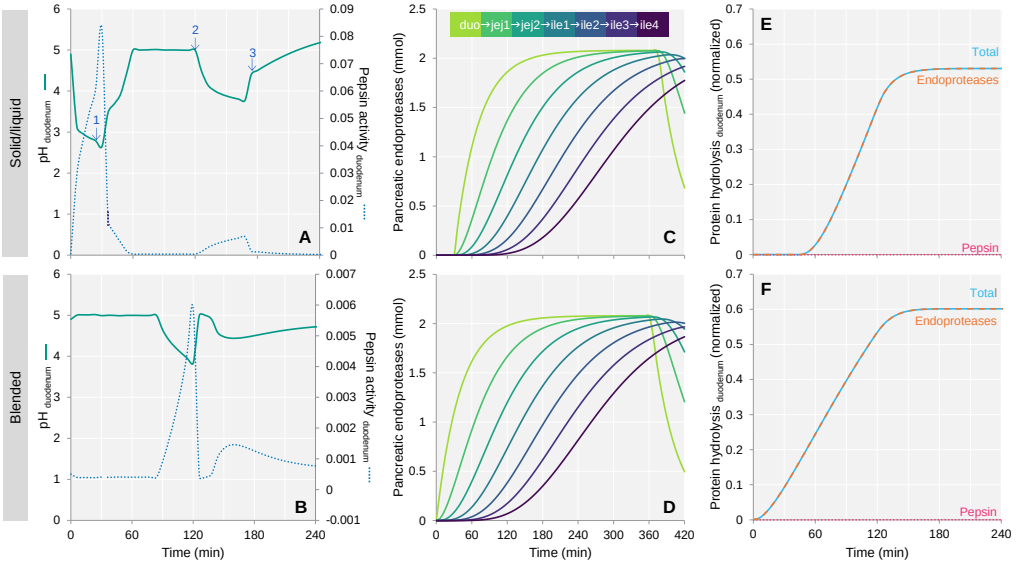


Figure 7.6 (A-B) pH and pepsin activity in the duodenum compartment, (1) start of pancreatic juice secretion, (2) maximum rate of protein hydrolysis in duodenum compartment, (3) end of gastric secretion in the fed state. (C-D) Pancreatic endoproteases in the small intestine compartments. (E-F) Protein hydrolysis in the duodenum, contribution from pepsin and endoproteases.

In our model, pancreatic secretion is stimulated by the presence of proteic (protein and/or peptide) pools in the duodenum compartment. Protein from the solid/liquid meal must be ground in the stomach before it can be emptied into the duodenum, but $protein_5$ is subject to pepsin-catalysed hydrolysis from the moment of ingestion. The resulting peptides can flow out of the stomach as they are being formed. However, only a small part of pepsin is in its active form during the first minutes of the gastric phase because of the relatively high pH. Thus, virtually no peptides are formed initially. In sum, the physical state of the meal and the pH of the gastric content cause pancreatic secretion to start only ca. 30 minutes after the meal has started. It is only then that the pH starts to return to the basal pH of the duodenum. Conversely, the pH of the duodenal contents from the blended meal

does not show a significant drop, as the protein arrives earlier in the duodenum and, as a result, the pancreatic secretion starts earlier than in the case of the solid/liquid meal.

The pH is roughly constant for 60 to 120 min for the solid/liquid meal, and until 90 min for the blended meal. Afterwards, the pH decreases. These times coincide with the rate of protein hydrolysis in the duodenum compartment starting to slow down (Figure A7.1). H^+ accumulates as there is less protonation after protein hydrolysis, and the pancreatic juice secretion is not sufficient to neutralize the incoming acid from the stomach compartment. This pH drop continues until most of the gastric contents have been emptied, and the gastric secretion and emptying slows down.

7.3.2.2 Secretion of pancreatic endoproteases

Endoproteases are secreted from the pancreas into the duodenum along with HCO_3^- and water. As described for HCO_3^- secretion, endoprotease secretion starts as proteic components arrive in the duodenum compartment. Figure 7.6C and D show the onset of pancreatic endoprotease secretion occurring earlier for the blended than the solid/liquid meal. This relates to the gallbladder contraction observations from Marciani et al. (2012), which were greater for the blended meal and thus expected to induce a higher pancreatic secretory response than the solid/liquid meal.

The pancreatic juice is modelled to only be secreted into the duodenum (Figure 7.1). The endoproteases flow from the duodenum through the jejunum and ileum compartments along with the rest of the digesta. Besides the onset of pancreatic juice secretion, no major differences emerge from the intake of the two types of meals on the size of the endoprotease pools in the small intestine compartments. The secretion of pancreatic juice stops when most of the proteic pools have flown out of the duodenum into the jejunum 1 compartment (Figure A7.2).

As previously discussed, the pepsin activity depends on the pH of the medium. Some residual pepsin-catalysed hydrolysis could be observed in the duodenum, as both pepsin and acid are emptied within the chyme from the stomach compartment. However, the fraction of active enzyme is considerably lower in the duodenum than in the stomach compartment (Figure 7.6A and B). As a result, pepsin-catalysed hydrolysis is virtually non-existent in the duodenum and nearly all of the protein hydrolysis occurs due to the action of pancreatic endoproteases (Figure 7.6E and F).

7.3.3 Transit of proteic pools

The compartmental modelling allows monitoring the concentration of a given component in a given compartment at any moment in time during digestion. Figure 7.7 shows the

transit and appearance of the intact protein, peptide and AA pools throughout the stomach, small intestine and portal blood compartments. As previously discussed, the solid protein in the solid/liquid meal is retained for a longer time in the stomach compartment than the protein in the blended meal. Interestingly, peptides resulting from pepsin-catalysed hydrolysis in the stomach compartment from the blended meal accumulate and are retained longer than those from the solid/liquid meal. As large particles are retained in the stomach during the 'antrum lag', peptides from the solid/liquid meal are the only nutrients that can be emptied out of the stomach (Figure A7.3). Meanwhile, for the blended meal, non-protein, protein and peptides are emptied at the same time. Peptides therefore accumulate in the compartment since proteins are hydrolysed and emptied at a slower rate, compared to those from the solid/liquid meal.

The earlier arrival into the duodenum of protein from the blended meal also implies earlier formation of peptides in the small intestine compartments. When protein arrives in the duodenum compartment, it initially accumulates as the rate of gastric emptying is higher than that of the protein hydrolysis in the duodenum, while the transit into the following small intestine compartment, jejunum 1 is still zero.

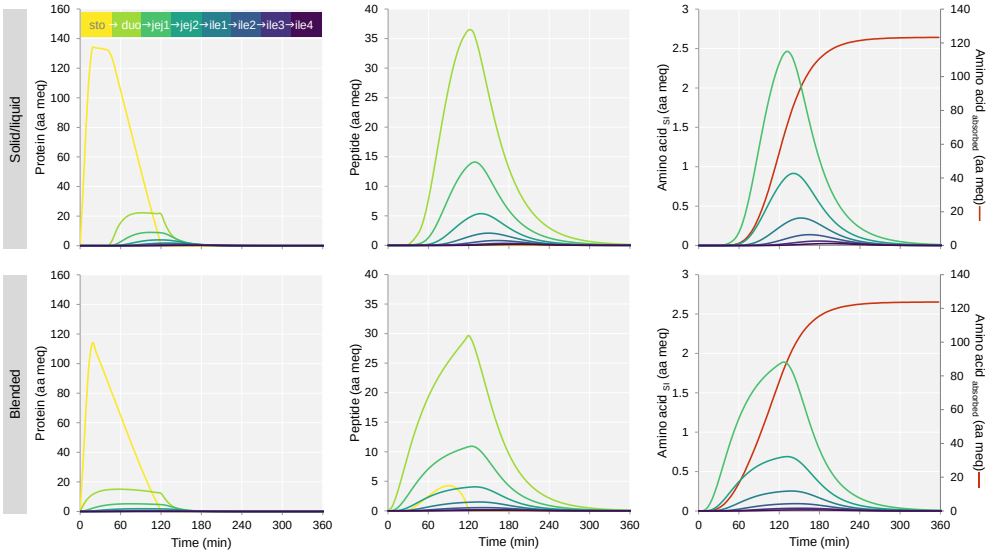


Figure 7.7 Simulated proteic (protein, peptide and amino acid) pools in the stomach, small intestine and portal blood compartments.

The exopeptidases that catalyse the hydrolysis of peptides into AA are located in the jejunum and ileum compartments. It can be seen that there is very little accumulation of AA in the jejunum and ileum compartments. This is because the AA are quickly absorbed by the enterocytes as soon as they are being formed (Figure A7.4). As expected, the

onset of the AA absorption fluxes from the jejunum 1 to ileum 4 compartments into the portal blood starts earlier for the blended than for the solid/liquid meal.

7.3.4 Protein hydrolysis

From our model, pepsin-catalysed hydrolysis is particularly significant as many events influence its extent and rate, i.e., enzyme and substrate (5 mm, 2 mm or blended) concentration in the compartment, and pH, which is in turn influenced by protonation after protein hydrolysis (Figure A7.5). We observe that protein hydrolysis does not start until about 15 min after either meal has been ingested. This is explained by the high pH, thus low proportion of pepsin in its active form, as well as the dynamic secretion of pepsin in the gastric juice.

The physical state of the meal influences protein hydrolysis and, more specifically, the extent of protein hydrolysis in a given compartment. A longer residence time in the stomach, e.g., for the solid/liquid meal where more protein is retained, results in a higher extent of hydrolysis than a shorter residence time does (Figure 7.8). For the blended meal, a small fraction of the protein is hydrolysed in the stomach compartment. Nevertheless, protein hydrolysis in the duodenum compensates for these differences. A similar extent of hydrolysis is achieved by the end of the duodenal phase for both meals, granted that the start and end of protein hydrolysis in the duodenum occur earlier for the blended meal. Most hydrolysis takes place in the duodenum, since protein hydrolysis is described by mass action kinetics from both the substrate and the enzyme, even though the initial hydrolysis in the stomach is crucial as it liquifies the digesta and makes it available for further hydrolysis.

Similarly, the fraction of peptides that is hydrolysed is highest in the jejunum 1 compartment, with respect to the other jejunum and ileum compartments. Because of the earlier arrival of protein from the blended meal into this compartment, peptide hydrolysis into AA as well as AA absorption, occurs earlier than for the solid/liquid meal. However, the extent of hydrolysis and absorption is similar with both meals.

7.3.5 AA absorption

The AA absorption curves depicted in Figure 7.7 and Figure 7.8 effectively represent the disappearance of AA from the small intestine. This can be compared to the true ileal digestibility, which has been reported to be ca. 92% for chicken meat, similar to the outcome of the model (Faber et al., 2010; Kashyap et al., 2018). The proteic content (protein, peptide and AA) that is not absorbed through the enterocytes, flows into the colon and is virtually lost for AA utilization.

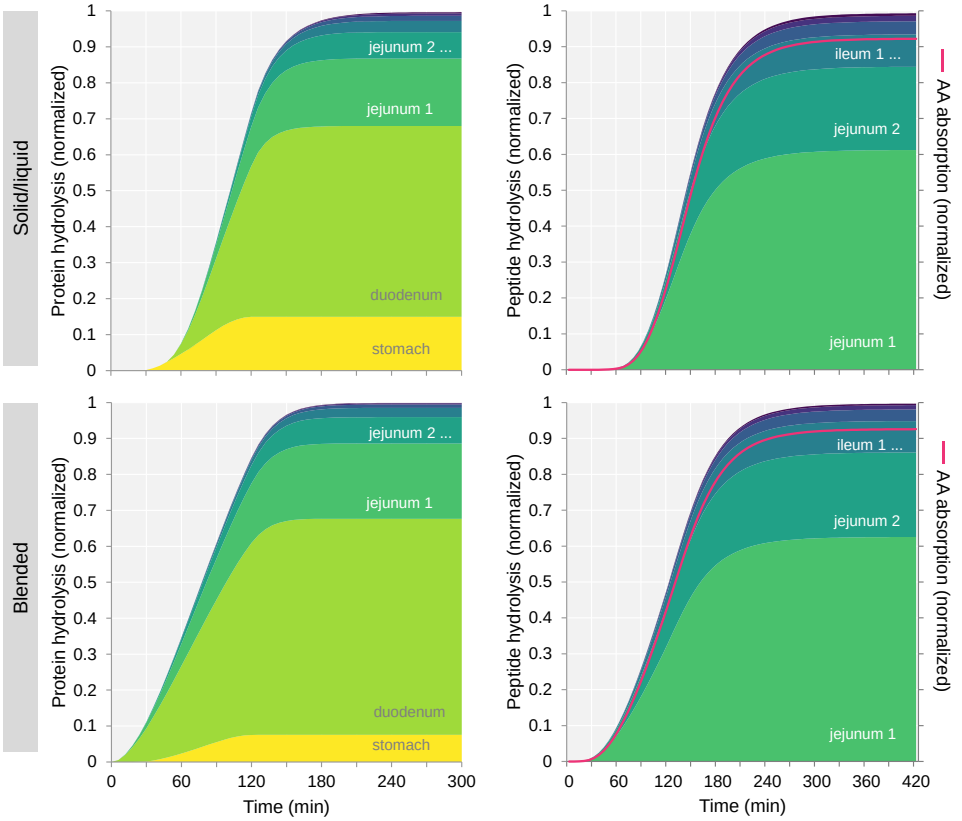


Figure 7.8 Simulated protein hydrolysis into peptides by pepsin and pancreatic endoproteases, peptide hydrolysis into amino acids by brush border exopeptidases, and cumulative pool of absorbed amino acids. Pools are normalized to the total amount of protein consumed.

Naturally, the absorbed AA do not accumulate in the portal blood. It is difficult to compare the rate of AA absorption from our model to that occurring *in vivo*. The AA metabolism has been reported to start already in the intestinal tissues during absorption, the so called first-pass metabolism (van der Schoor et al., 2002). The AA are utilized to maintain the gut mucosa, to generate intestinal energy and for oxidative purposes, before they are available for whole-body metabolism. Schop et al. (2020) argue that the quality of the net portal appearance of AA data available is insufficient to compare to simulated AA absorption kinetics.

In vitro experimental methods are advanced enough to mimic protein hydrolysis in the small intestinal lumen, however the action of brush border enzymes as well as absorption into and through enterocytes are not readily available to apply in a laboratory setting. Furthermore, predicting the effect of passage is easier when done through an *in silico* model, compared to a static or semi-dynamic *in vitro* setting. An *in silico* approach as the

one presented in this work can complement the *in vitro* observations, simulating complex processes during food digestion.

7.4 Concluding remarks

An adequate representation of the gut physiology is required to study the relevance of the physical state of meals. We chose for a dynamic, semi-mechanistic, compartmental model to describe protein digestion. The components of the digesta that were deemed most relevant for protein digestion were included. Fluxes between pools were described by simple functions with a physiological meaning.

The model uses the Smart environment for computation, which is a free and user friendly tool that is suitable as a first step into *in silico* modelling. The qualitative agreement with experiments on gastric pH and volume of the gastric contents is good considering that the model consists of independent equations and was not fitted to the data. Extensions and testing against experimental observations will improve the predicted accuracy and versatility of the model.

The model was applied to investigate the influence of the physical state of meals and was examined on transit, digestive protein hydrolysis, and amino acid absorption. The digesta from the blended meal was emptied first and faster from the stomach, compared to that of the solid/liquid meal. The model was able to predict the biphasic nature of gastric emptying from solid/liquid meals as reported from in *vivo* trials. Furthermore, we show the dynamic behaviour of the pH and its effect on the enzymatic protein hydrolysis, both in gastric as well as the duodenal environment.

Appendix

Table A7.1 Parameter values of the model simulating protein digestion

Parameter	Description	Value	Unit	Reference
Gastric juice				
Vs0	Basal gastric volume	0.025	l	Vertzoni et al. (2005)
kgj	Rate of gastric juice secretion (fed)	0.01	l/min	Versantvoort and Rompelberg (2004)
kgjfast	Rate of gastric juice secretion (fasted)	0.00001	l/min	
Wgj	Water content in gastric juice	0.999	l/l	
Hgj	H^+ content in gastric juice	65	mmol/l	
Ngj	Pepsin(ogen) content in gastric juice	0.026	mmol/l	Malagelada et al. (1976)
kn	Rate constant of pepsin-catalysed hydrolysis of protein	60	min^{-1}	
ka1	Dissociation constant for first proton pepsin	0.025	-	Kondjoyan et al. (2015)
ka2	Dissociation constant of second proton pepsin	0.003	-	
kfb	Forward rate of buffering	3000	ml/meq min	
kbb	Backward rate of buffering	1	min^{-1}	
Gastric emptying				
antrumlag	Lag time to grinding and gastric emptying of solid bolus	45	min	
kagNP	Rate of grinding of a non-protein particle from 5 to 2 mm	1.5	g/min	
kagP	Rate of grinding of a protein particle from 5 to 2 mm	1.5	aa meq/min	
Kge	Rate constant of gastric emptying of food	2.5	kcal/min	Hunt and Stubbs (1975)
kgeN	Rate constant of gastric emptying of pepsin	0.00005	mmol/min	
kgeW	Rate constant of gastric emptying of water	890	mmol/min	
kgeH	Rate constant of gastric emptying of acid	0.2	mmol/min	
kgepost	Rate constant of postprandial gastric emptying	0.5	min^{-1}	

Pancreatic juice				
V10	Basal duodenal volume	0.00614	l	
pH10	pH in mid-duodenum (fasted)	4.9	-	Ovesen et al. (1986)
kpj	Secretion rate of pancreatic juice	0.004	l/min	
Wpj	Water content of pancreatic juice	0.999	l/l	
Opj	HCO_3^- content of pancreatic juice	300	mmol/l	
kw	Forward rate of neutralization	100000	l/mmol min	
ko	Backward rate of neutralization	0.00001	min^{-1}	
Epj	Endoprotease content of pancreatic juice	13	mmol/l	
ke	Rate constant of endoprotease-catalysed hydrolysis of protein	15	l/mmol min	
Transit and absorption				
ksi	Small intestine transit constant	0.025	min^{-1}	Worsøe et al. (2011)
vx	Rate constant of exopeptidase-catalysed hydrolysis	0.06	min^{-1}	
vt	Maximum rate of aa transport	0.03	aa meq/l min	
kt	MM constant of aa transport	0.09	aa meq/l	

Table A7.2 Notation convention for components and compartments for the equations describing pools (Q_{yu}) and fluxes (F_{yuzv}) of y or z components in u or v compartments.

component		index
water		w
non-protein/non-water $_{5\ mm}$	$npnw_5$	f
non-protein/non-water $_{2\ mm}$	$npnw_2$	g
non-protein $_{blended}$		x
$protein_5$		i
$protein_2$		j
acid aa_5^+		k
acid aa_2^+		l
acid aa_b^+		m
peptide		p
aa		a
H^+		h
pepsin		n
HCO_3^-		o
endoprotease		e

compartment	index
stomach	s
parietal/chief cells	c
pancreas	q
duodenum	1
jejunum1	2
jejunum2	3
ileum1	4
ileum2	5
ileum3	6
ileum4	7
portal blood	b
colon	r

Table A7.3 Equations for state (pools) and auxiliary (fluxes) variables. Equations from the jejunum 1 compartment are exemplars for the jejunum 2, ileum 1-4 and colon compartments, indices should be changed accordingly.

Stomach					
Qns	amount of pepsin in stomach				
	in	Fncns	mmol/min	Pepsin secretion if (Qxs+Qfs+Qgs)<0.5	kgj* Ngj kgjfast* Ngj
	out	Fnsn1	mmol/min	Gastric emptying of pepsin if Qns<0.00001	kgeN Kgepost*Qns
	differential equation	dQns/dt	mmol	Fncns-Fnsn1	
			basal (initial pool size)	0.0000794	
Qhs	amount of protons in stomach				
	in	Fhdhs	mmol/min	ingestion of H+ if time>MealDuration	FoodIntake* Hfood 0
		Fhchs	mmol/min	H+ secretion if (Qxs+Qfs+Qgs)<0.5	kgj* Hgj kgjfast* Hgj
	out	Fhsks	mmol/min	Protonation of protein (5 mm) if Qhsbuffer≥aaP	(kfb*((Qhs*((aaDE/aa)*Qis))/((Qws*MwW)/1000)))-(kbb*Qks) BCfood*Qis
		Fhsls	mmol/min	Protonation of protein (2 mm) if Qhsbuffer≥aaP	(kfb*((Qhs*((aaDE/aa)*Qjs))/((Qws*MwW)/1000)))-(kbb*Qls) BCfood*Qjs
		Fhsps	mmol/min	Protonation of COO- after peptide bond cleavage	Fisps+Fjsps
		Fhsh1	mmol/min	Gastric emptying of H+ if (Qxs+Qfs+Qgs)<0.5	kgeH*Qhs Kgefast
	differential equation	dQhs/dt	mmol	Fhdhs+Fhchs-Fhsks-Fhsls-Fhsps-Fhsh1	
			basal	0.315	
Qws	amount of water in stomach				
	in	Fwdws	mmol/min	Intake of free water	FoodIntake* WmolMeal

				if time>MealDuration	0
		Fwcws	mmol/min	Water secretion	kgj* Wgj
				if (Qxs+Qfs+Qgs)<0.5	kgjfast* Wgj
	out	Fwsw1	mmol/min	Gastric emptying of water	kgeW
				if Qws<0.1	kgepost*Qws
	differential equation	dQws/dt	mmol	Fwdws+Fwcws-Fwsw1	
			basal	1387.5	
Qxs	Amount of blended non-protein in stomach				
	in	Fxdxs	g/min	Intake of non-protein (blended)	FoodIntake* NPBfood
				if time>MealDuration	0
	out	Fxsg1	g/min	Gastric emptying of non-protein (blended)	(kge*(Exs/(Exs+Egs+Ejs+Eps)))/eNPB
				if Qxs<0.1	Kgepost*Qxs
	differential equation	dQxs/dt	g	Fxdxs-Fxsg1	
Qfs	amount of non-protein food in stomach (5 mm particle)				
	in	Ffdfs	g/min	Intake of non-protein	FoodIntake* NPSfood
				if time>MealDuration	0
	out	Ffsgs	g/min	Particle reduction of non-protein	kagNP
				if time<antrumlag	0
				if Qfs<0.1	kagNP*Qfs
	differential equation	dQfs/dt	g	Ffdfs-Ffsgs	
Qgs	amount of non-protein from food (2 mm particle)				
	in	Ffsgs	g/min		
	out	Fgsg1	g/min	Gastric emptying of non-protein (solid)/non-water	(kge*(Egs/(Egs+Ejs+Eps)))/eNPS
				if Qgs<0.1	kgepost*Qgs
	differential equation	dQgs/dt	g	Ffsgs-Ffsg1	
Qis	Amount of protein (5mm) in stomach				
	in	Fidis	aa meq/min	Ingestion of protein (solid)	FoodIntake* PSeqFood

				if time>MealDuration	0
	out	Fisjs	aa meq/min	Particle reduction of protein	kagP
				if time<antrumlag	0
				if Qis<0.1	kagP*Qis
		Fisps	aa meq/min	Hydrolysis of an internal peptide bond in protein (5 mm)	kn*Nas*(Qns/(1000*Vs))*Qis
	differential equation	dQis/dt	aa meq	Fidis-Fisjs-Fisps	
Qjs	amount of protein (2 mm or blended) in stomach				
	in	Fjdjs	aa meq/min	Ingestion of protein (blended)	FoodIntake* PBeqFood
				if time>MealDuration	0
		Fisjs	aa meq/min		
	out	Fjsps	aa meq/min	Hydrolysis of an internal peptide bond in protein (2 mm)	kn*Nas*(Qns/(1000*Vs))*Qjs
		Fjsj1	aa meq/min	Gastric emptying of protein (2 mm)	(kge*(Ejs/(Exs+Egs+Ejs+Eps)))/ePeq
				if Qjs<0.1	kgepost*Qjs
	differential equation	dQjs/dt	aa meq	Fjdjs+Fisjs-Fjsps-Fjsj1	
Qps	amount of peptide in stomach				
	in	Fisps			
		Fjsps			
	out	Fpsp1	aa meq/min	Gastric emptying of peptides	(kge*(Eps/(Exs+Egs+Ejs+Eps)))/ePeq
				if Qps<0.1	Kgepost*Qps
	differential equation	dQps/dt	aa meq	Fisps+Fjsps-Fpsp1	
Duodenum					
Qn1	amount of pepsin in duodenum				
	in	Fnsn1			
	differential equation	dQn1/dt	mmol	Fnsn1	
Qh1	amount of H+ in duodenum				
	in	Fhsh1			

	out	Fh1w1	mmol/min	neutralization of H+ in duodenum	(kw*Ch1*Qo1)-(ko*Qw1)
		Fh1p1	mmol/min	Protonation after protein hydrolysis	Fj1p1
				if pH1>5	0
	differential equation	dQh1/dt	mmol	Fhsh1-Fh1w1-Fh1p1	
			basal	1.259E-5	
Qo1	amount of bicarbonate in duodenum				
	in	Foqo1	mmol/min	Bicarbonate secretion into duodenum	kpj* Opj
				if (Qj1+Qp1)<0.1	0
	out	Fo1w1	mmol/min	neutralization of HCO3- in duodenum	Fh1w1
		Fo1m1	mmol/min	Deprotonation of protein	(kfb*((Qo1*((aaDEH/aa)*Qjs))/Qw1))-(kbb*Qm1)
				if Qo1<0.1	0
		Fo1p1	mmol/min	Deprotonation after protein hydrolysis	Fj1p1
				if pH1<5	0
		Fo1o2	mmol/min	Transit of bicarbonate from duodenum to jejunum 1	ksi*Qo1
	differential equation	dQo1/dt	mmol	Foqo1-Fo1w1-Fo1m1-Fo1p1-Fo1o2	
Qe1	amount of endoproteases in duodenum				
	in	Feqe1	mmol/min	Endoprotease secretion into duodenum	kpj* Epj
				if (Qj1+Qp1)<0.1	0
	out	Fe1e2	mmol/min	Transit of endoprotease from duodenum to jejunum 1	ksi*Qe1
	differential equation	dQe1/dt	mmol	Feqe1-Fe1e2	
Qw1	amount of water in duodenum				
	in	Fwsw1			
		Fwqw1	mmol/min	Water secretion	kpj* Wpj
				if (Qj1+Qp1)<0.1	0
		Fh1w1			

	out	Fw1w2	mmol/min	Transit of water from duodenum to jejunum 1	ksi*Qw1
	differential equation	dQw1/dt	mmol	Fwsw1+Fwqw1+Fh1w1-Fw1w2	
Qg1	amount of non-protein (2 mm) in duodenum				
	in	Fxsg1 Fgsg1			
	out	Fg1g2	g/min	Transit of non-protein from duodenum to jejunum 1	ksi*Qg1
	differential equation	dQg1/dt	g	Fxsg1+Fgsg1-Fg1g2	
Qj1	amount of protein in duodenum				
	in	Fjsj1			
	out	Fj1p1	aa meq/min	Hydrolysis of protein in duodenum	(kn*((Na1*Qn1)/(1000*V1))*Qj1)+(ke*(Qe1/(1000*V1))*Qj1)
		Fj1j2	aa meq/min	Transit of protein from duodenum to jejunum 1	ksi*Qj1
	differential equation	dQj1/dt	aa meq	Fjsj1-Fj1p1-Fj1j2	
Qp1	amount of peptide in duodenum				
	in	Fpsp1 Fj1p1			
	out	Fp1p2	aa meq/min	Transit of peptides from duodenum to jejunum 1	ksi*Qp1
	differential equation	dQp1/dt	aa meq	Fpsp1+Fj1p1-Fp1p2	
Jejunum 1 to colon (compartment 2 to r)					
Qe2	amount of endoproteases in jejunum 1				
	in	Fe1e2			
	out	Fe2e3	mmol/min	Transit of endopeptidase from jejunum 1 to jejunum 2	ksi*Qe2
	differential equation	dQe2/dt	mmol	Fe1e2-Fe2e3	

Qo2	amount of bicarbonate in jejunum 1				
	in	Fo1o2			
	out	Fo2p2 *	mmol/min	Deprotonation after protein hydrolysis in jejunum 1	Fj2p2
		Fo2o3	mmol/min	Transit of bicarbonate from jejunum 1 to jejunum 2	ksi*Qo2
	differential equation	dQo2/dt	mmol	Fo1o2-Fo2p2-Fo2o3	
Qw2	amount of water in jejunum 1				
	in	Fw1w2			
	out	Fw2w3	mmol/min	Transit of water from jejunum 1 to jejunum 2	ksi*Qw2
	differential equation	dQw2/dt	mmol	Fw1w2-Fw2w3	
Qg2	amount of non-protein in jejunum 1				
	in	Fg1g2			
	out	Fg2g3	g/min	Transit of non-protein from jejunum 1 to jejunum 2	ksi*Qg2
	differential equation	dQg2/dt	g	Fg1g2-Fg2g3	
Qj2	amount of protein in jejunum 1				
	in	Fj1j2			
	out	Fj2p2 *	aa meq/min	Hydrolysis of protein in jejunum 1	(ke*(Qe2/(1000*V2))*Qj2)
		Fj2j3	aa meq/min	Transit of protein from jejunum 1 to jejunum 2	ksi*Qj2
	differential equation	dQj2/dt	aa meq	Fj1j2-Fj2p2-Fj2j3	
Qp2	amount of peptide in jejunum 1				
	in	Fp1p2			
		Fj2p2 *			

	out	Fp2a2 *	aa meq/min	Hydrolysis of peptides by exopeptidases in jejunum 1	vx*Qp2
		Fp2p3	aa meq/min	Transit of peptides from jejunum 1 to jejunum 2	ksi*Qp2
	differential equation	dQp2/dt	aa meq	Fp1p2+Fj2p2-Fp2a2-Fp2p3	
Qa2	amount of amino acids in jejunum 1				
	in	Fp2a2			
	out	Fa2ab *	aa meq/min	Absorption of aa from jejunum 1	((vt*Qa2)/(kt+(Qa2/(1000*V2))))
		Fa2a3	aa meq/min	Transit of aa from jejunum 1 to jejunum 2	ksi*Qa2
	differential equation	dQa2/dt	aa meq	Fp2a2-Fa2ab-Fa2a3	
Enterocytes/portal blood					
Qab	cumulative amount of absorbed aa				
	in	Fa2ab			
		Fa3ab			
		Fa4ab			
		Fa5ab			
		Fa6ab			
		Fa7ab			
		dQab/dt	aa meq	Fa2ab+Fa3ab+Fa4ab+ Fa5ab+Fa6ab+Fa7ab	

** indicates functions that do not occur in the colon compartment.*

Table A7.4 Additional auxiliary variables and pseudo constants

Auxiliary variables			
Nas	Proportion of active pepsin as function of pH in stomach		1/(1+(Chs/ka1)+(ka2/Chs))
Cns	Concentration of total pepsin in stomach	mmol/l	(Qns/(1000*Vs))
Vs	Gastric content volume	l	Vs0+((((Qfs+Qgs)/dNPS)+(Qxs/dNPB))/1000) +(((Qws*MwW)+(((Qis+Qjs+Qps)*MwP)/(aa*DP)))/ 1000000)
Vsw	Water gastric content volume	l	(Qws*MwW)/1000000
Chs	Molar concentration of H+ in stomach	mol/l	Qhs/(Vs*1000)
pHs	Intragastric pH		-log10(Chs)
Exs	Energy from non-protein blended in stomach	kcal	Qxs*eNPB
Egs	Energy from non-protein 2 mm in stomach	kcal	Qgs*eNPS
Ejs	Energy from protein 2 mm or blended in stomach	kcal	Qjs*ePeq
Eps	Energy from peptides in stomach	kcal	Qps*ePeq
V1	Duodenal content volume	l	V10+((Qg1/dNPS)/1000)+(((Qw1*MwW)+ (((Qj1+Qp1)*MwP)/(aa*DP)))/1000000)
Ch1	Molar concentration of H+ in duodenum	mol/l	Qh1/(V1*1000)
		if V1≤V10	pow(10, -pH10)
Co1	Molar concentration of bicarbonate in duodenum	mol/l	Qo1/(V1*1000)
pH1	pH in duodenum		-log10(Ch1)
		if Ch1<1E-10	14+log10(Co1)
Na1	Proportion of active pepsin as function of pH in duodenum		1/(1+(Ch1/ka1)+(ka1/Ch1))
Pseudo constants			
PSeqFood	Protein (solid) content in meal expressed as amino acid equivalent	aa meq/g	(PSfood* aa)*1000/ MwP
aaP	Total amount of ingested protonatable aa	aa meq	(MealDuration*(PBfood+PSfood)*FoodIntake*aaDE*1000)/MwP
PBeqFood	Protein (blended) content in meal expressed as amino acid equivalent	aa meq/g	(PBfood* aa)*1000/ MwP

WmolMeal	Water molar content in meal	mmol/g	(Wfood*1000)/ MwW
Hfood	H+ content in food	mmol/g	pow(10,-pHfood)/dfood
ePeq	Energy content of protein expressed as aa equivalent	kcal/aa meq	(eP*MwP)/(aa*1000)
V0w	Initial amount of water	l	(MealDuration*FoodIntake*Wfood)
BCfood	Buffering capacity from the meal	mmol/ pH l	BC/((MealDuration*FoodIntake)/(Dfood*1000))

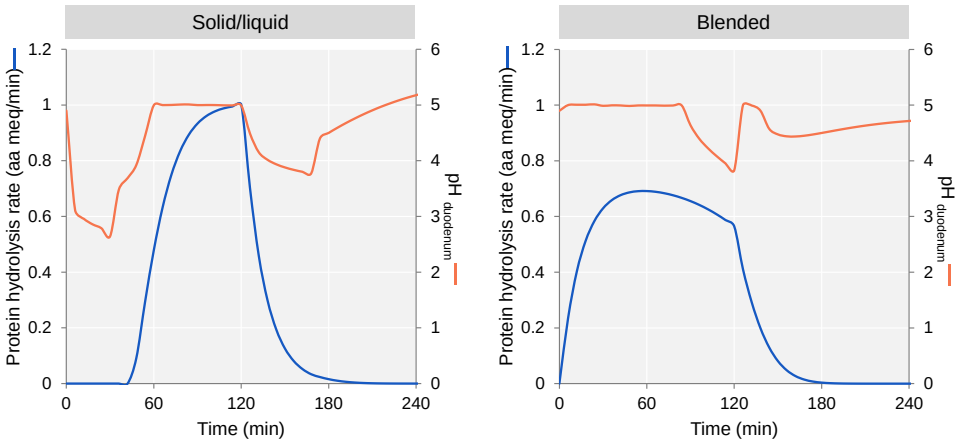


Figure A7.1 Simulated protein hydrolysis rate and pH in the duodenum compartment of solid/liquid and blended meals.

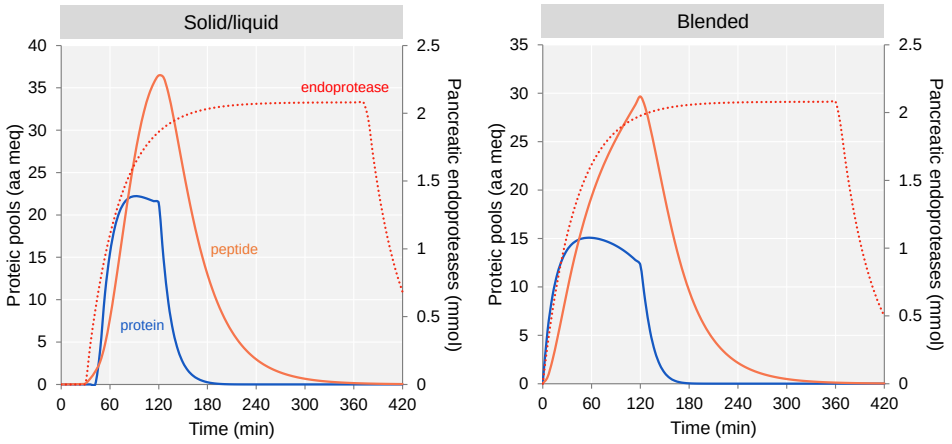


Figure A7.2 Pancreatic endoprotease secretion is stimulated by the presence of proteic pools in the duodenum compartment.

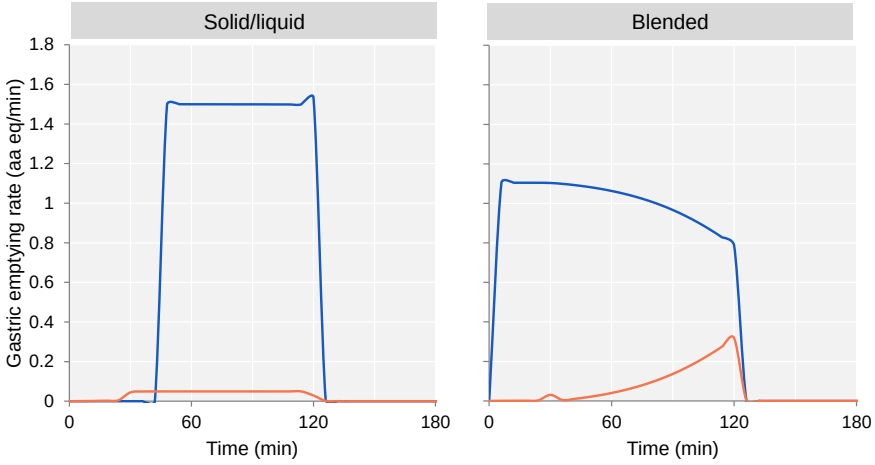


Figure A7.3 Simulated gastric emptying rate of protein (blue) and peptide (orange) pools from solid/liquid and blended meals.

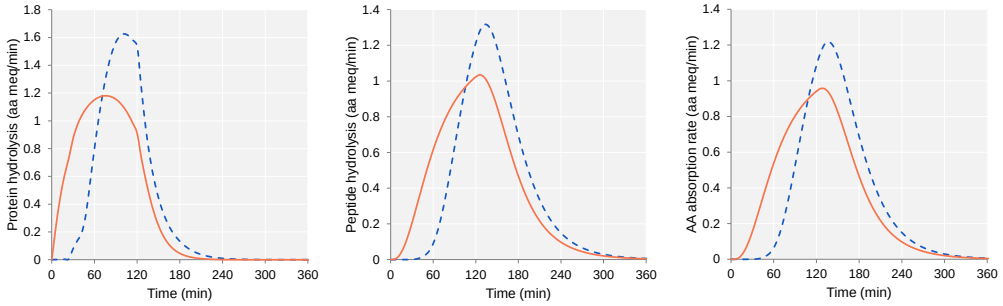


Figure A7.4 Simulated global rate of protein and peptide hydrolysis, and amino acid absorption from solid/liquid (dashed line) and blended (solid line) meals.

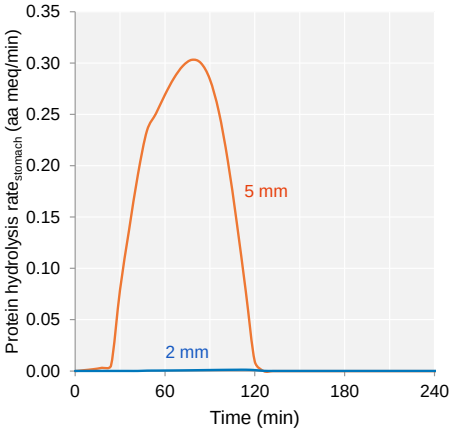


Figure A7.5 Simulated rate of protein hydrolysis in the stomach compartment from the solid/liquid meal

CHAPTER EIGHT

General discussion

The aim of this thesis was to relate the effects of processing, with emphasis on heating, on the digestibility of plant proteins, which allows us to make better informed choices when producing sustainable alternatives to animal-based products. The availability of plant protein-rich ingredients is important in the transition towards more plant-based diets. Better digestible foods can be developed by furthering our understanding of the digestion of the protein itself. Furthermore, sustainable and nutritious products can be developed if we consider their digestibility as part of their core quality requirements.

8.1 Main findings and conclusions

The effects on protein digestibility of several processes for preparation and fractionation of ingredients and their employment in foods were reviewed in Chapter 2. Some processes such as acylation or extrusion consistently enhanced the digestibility, regardless of the crop studied. Meanwhile, alkaline extraction of proteins negatively impacts the digestibility of various crops. Heat treatment is a classic example of how a certain extent of processing has positive effect, while more extensive heating reduces protein digestibility. This is why we propose a hypothetical relation between the digestibility of a protein and the extent of processing as represented in Figure 8.1.

Even though some treatments do not seem to affect the digestibility, in reality, this may be a combination of different effects that reduce and enhance the digestibility at the same time, leading to a net zero effect. Heating of soybean and pea protein isolates had no net effect on the extent of pepsin-catalysed hydrolysis (Chapter 3). However, when the full dispersion was separated into the dispersed and non-soluble fractions, we showed that heating does affect the degree of hydrolysis achieved by pepsin. After heating, proteins in the disperse phase became more digestible, while proteins in the pellet became less digestible.

The effect of a dynamic gastric phase on small-intestinal digestion was explored in Chapter 4. The hydrolytic efficiency of trypsin is affected by the pH and hydrolysis history of its substrate during the gastric phase. The substrate that was exposed to low pH and pepsin-catalysed hydrolysis, as a protein with a long residence time in the stomach would be, was more extensively and efficiently hydrolysed by trypsin than its non-acidified, non-hydrolysed counterpart.

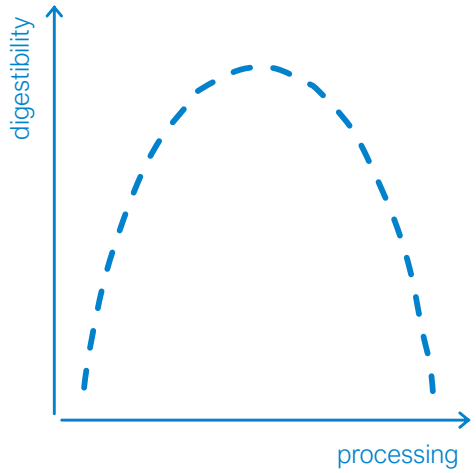


Figure 8.1 Hypothetical relation between digestibility and extent of processing.

Chapter 5 presents the semi-dynamic gastric and small intestinal digestion of pea protein. We examined the effect of thermal denaturation on the digestion of native pea protein mild fractions. The degree of hydrolysis after pepsin and pancreatin of protein rich and non-soluble protein fractions was significantly enhanced by heat-induced denaturation. Meanwhile, the soluble protein fraction was less extensively hydrolysed upon heat treatment.

The digestibility results from this chapter were used in Chapter 6 to extend the assessment of environmental impact from a *'farm-to-fork'* to a *'farm-to-faeces'* perspective; the need to consider protein digestibility to better assess the environmental impact of plant-protein rich ingredients and foods was discussed here as well. Mild fractions had notably less environmental impact than the conventionally produced pea protein isolate, with 'kg of ingredient' as the functional unit. As expected, the difference between impacts was substantially reduced when considering 'kg of protein' as functional unit, due to the lower protein purity of the mild fractions. The functional unit 'kg of hydrolysable protein' allowed the comparison of ingredients by the degradability of their proteins, which draws closer to their potential utilization by the consumer. This functional

unit becomes particularly relevant when ingredients are further processed into finished products.

Finally, a dynamic, compartmental model to study the differences in the digestive fate of solid/liquid and blended meals of identical composition was constructed (Chapter 7). The volume of the gastric contents was predicted for solid/liquid and blended meals concurring with *in vivo* experimental meals and observations. The gastric emptying rate was described as a function of the caloric density of the chyme to achieve a controlled delivery of calories into the duodenum. For solid meals, a lag time was introduced to simulate the generation of antral grinding waves.

The findings in this thesis suggest the following general conclusions:

i. Processing history affects plant protein *in vitro* digestibility

This thesis showed that protein solubility is affected by heat treatment, ultimately as a result of heat-induced changes in conformation. This in turn influences the digestibility of these proteins. However, a process will likely not have the same effect on the digestibility of proteins of any plant origin. Therefore, this research needs to be extended to a wider range of crops than could be investigated here (Chapter 2), to find a way to reliably predict the effect of processing on any plant protein.

- Protein solubility and digestion

While the solubility does influence the digestion, it is difficult to draw a straightforward relation between the two. Chapter 3 reported on the poor solubility of the unheated soybean and yellow pea protein isolate dispersions. This is not inherent insolubility but caused by spray drying. The shell-like morphology of the spray-dried particles impairs the release of protein and their suspension in the supernatant. Nevertheless, the proteins that sedimented into the pellet were easily hydrolysed by pepsin. Meanwhile, heating facilitated the release of proteins from the spray-dried particles and the suspended proteins were even more extensively hydrolysed than those separated into the pellet.

In Chapter 5, gastric and small intestinal digestion could extensively hydrolyse the soluble protein fraction from the mild aqueous fractionation of yellow pea; more so than the mildly fractionated non-soluble protein fraction and the conventionally fractionated and spray-dried pea protein isolate. Upon heating, visible, insoluble clots were formed, and the fraction was less extensively hydrolysed than its unheated counterpart. We thus propose that solubility is not a requirement for protein digestion, but that protein size and conformation are the main determinants for protein digestion.

- Conformation as a result of heat-induced denaturation and aggregation

It is generally assumed that protein unfolding allows for the exposure of cleavage sites to the digestive enzymes (Bhat et al., 2021a, 2021b; Zou et al., 2020), and that native or only partly unfolded structures cannot be extensively hydrolysed (Fontana et al., 1997). Proteins in conventionally fractionated and spray-dried protein isolates are already denatured. As previously mentioned, the degree of hydrolysis during the gastric phase was similar for unheated and heated protein isolate dispersions, under both static (Chapter 3) and dynamic (Chapter 5) conditions. Nevertheless, a significantly lower degree of hydrolysis from the small intestinal phase after dynamic gastric digestion was found for the pea protein isolate heated dispersion, compared to its unheated counterpart. Additionally, the micrographs and particle size distribution measurements from Chapter 3 illustrate the presence of heat-induced aggregates in the pellet of protein isolate dispersions. These aggregates were poorly digestible compared to their unheated counterparts and the corresponding supernatants.

Recalling the hypothesised relation between protein digestibility and extent of processing (Figure 8.1), extensive processing generally reduces the digestibility. Hence, we propose that the production of plant-protein rich ingredients aim at the lowest possible extent of processing so that further processing towards the finished product, ensures high protein digestibility.

- Other components: starch

Proteins are almost never consumed and digested in isolation. The protein from protein-rich ingredients will interact with other components in the ingredient or from other ingredients in the formulation of the finished product (Figure 8.2). While the effect of nutrient interactions on protein digestion was out of the scope of this thesis, the influence of starch is briefly touched on in Chapter 2 and 5. The presence of starch in the *food matrix*, can be influential particularly when heating. The heated flour dispersion, containing both starch and protein, was hydrolysed less extensively than its unheated counterpart. Its degree of hydrolysis was also lower than the corresponding heated protein-rich dispersion, even though heat-induced protein denaturation can have a positive effect on the extent of gastro-small intestinal hydrolysis. This lower degree of hydrolysis is attributed to the gelatinization of starch, which hinders the access of enzymes to the proteins' cleavage sites and imbibes some of the digestive fluids during the assay, leaving less for the proteins.

ii. Protein digestibility should be included in the life cycle assessment of ingredients and finished products

Plant proteins are regarded as a more sustainable alternative to animal-sourced proteins. This is generally true so long as *whole foods* are compared, e.g., cooked soybeans vs. pasteurized milk. The better sustainability becomes less obvious when proteins are extracted from crops to obtain protein-rich ingredients and subsequently processed into finished products. This requires energy and resources, while part of the protein is lost to non-ideal fractionation processes. Given the effects of processing on the nutritional quality of protein, the protein digestibility should be assessed for finished products. Once more, this directs us to the same conclusion as before, that we advise for limited processing to fractionate proteins, as better digestible finished products will also be more sustainable due to both the lower environmental impact of the process and the provision of utilizable amino acids.

iii. The gastric phase influences the efficiency of small intestinal protein digestion

It could be argued that the *processing* history of protein continues in the gastrointestinal tract. The bolus is exposed to the gastric environment(s) for varying extents of time. Bovine serum albumin, the protein studied in Chapter 4 is sensitive, just like other proteins, to changes in the environment. Fu et al. (2021) suggested that pepsin is more effective at catalysing the hydrolysis after acid-induced denaturation of native protein in the gastric environment. Similarly, native substrates were found to be poorly hydrolysed by pancreatic endoproteases. This is in line with our finding that proteins that would bypass the gastric phase by being emptied first from the stomach are the least extensively hydrolysed by trypsin. Thus, gastric and luminal small intestinal digestion act synergistically towards more extensive protein hydrolysis.

- Protein size as a determinant of digestion

In Chapter 5, the dynamic nature of gastric juice secretion and gastric emptying was simulated. It was confirmed that a longer residence time in the stomach allowed for a more extensive hydrolysis by the proteases present in pancreatin. As the pepsin-hydrolysates were better substrates for trypsin-catalysed hydrolysis, protein or peptide size, additional to conformation, are also determinants of the hydrolytic capacity of pancreatic proteases.

The products from luminal proteolysis will continue to be digested by brush border enzymes. It is uncertain whether the products with a short residence time in the *stomach*, i.e., lower degree of hydrolysis from the luminal small intestinal phase, will also be hydrolysed to a lesser extent by brush border enzymes. Alternatively, hydrolysis at the

brush border might compensate for differences in the degree of hydrolysis from luminal digestion. Further research is needed to assess the contribution of brush border enzymes to the whole of protein hydrolysis during digestion in relation to the pH and hydrolysis history of the proteins.

iv. Dynamic aspects of protein digestion can be simulated with an *in silico* model

The results from Chapters 4 and 5 highlight the relevance of the dynamic aspects of the gastric on the small intestinal phase. *In silico* modelling tools, as presented in Chapter 7, can simulate dynamic events such as gastric and pancreatic secretions, enzymatic hydrolysis and transit through the gastrointestinal tract.

8.2 Future research challenges and directions

8.2.1 Why *(continue to)* study the digestibility of plant proteins?

The health risks associated to consuming poorly digestible proteins were addressed in Chapter 1. These include dysbiosis of colonic microbiota and the production of metabolites from protein fermentation that are associated to inflammation and cytotoxicity (Amaretti et al., 2019). As the production of plant protein-rich ingredients and foods increases, so does the need to ensure an adequate digestibility, especially when less common crops or novel processes are involved.

This thesis, as well as previous works, advocates for the use of mildly fractionated ingredients, as high purity is not necessary to attain favourable functionalities (Geerts et al., 2018; Lie-Piang et al., 2021; van der Goot et al., 2016), and the mild processing that may be required, limits the modifications that might impair protein digestion. Regardless of the ingredients' purity, other components, such as carbohydrates, lipids, polyphenols or other proteins, will be present in a finished product (Figure 8.2). The nutrient interactions within a food, meal or the gastrointestinal tract should be considered to further understand and perhaps predict protein digestibility. Protein-fibre interactions have been associated with lower or slower rates of proteolysis (Boachie et al., 2021). It is known that protein carbonylation from oxidative or Maillard reactions can significantly impair protein digestion (Estévez et al., 2021). This can be prevented by the use of phenolic compounds as antioxidants; however, these compounds can also affect protein digestion, in addition to losing their antioxidant activity by interaction or complexation with proteins or proteases (Qie et al., 2021; Xu et al., 2021).

Besides composition, the food matrix and its multi-scale structures are key factors influencing protein digestion and, in fact, that of all nutrients (Aguilera, 2019; Capuano & Janssen, 2021). This thesis dealt with relatively simple systems, diluted dispersions, and

with relatively simple microstructures, such as spray-dried particles. Certainly, plant protein-rich ingredients will be used to form more complex structures, such as gels or fibrous matrices, involving greater extents of processing. As previously stated, it is crucial that the protein digestibility of such products is adequate to truly claim a health or environmental benefit.

Lastly, as plant-based food innovation continues, the effect of long-term consumption of novel foods with complex structures on muscle protein synthesis and whole body function should be investigated, according to the requirements of different age groups (Pinckaers et al., 2021).

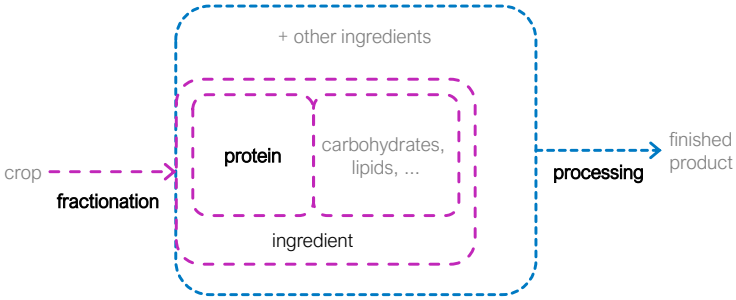


Figure 8.2. Fractionation, processing and composition of protein rich ingredients and finished products, arrows represent multi-step processes.

8.2.2 Dynamic aspects of protein digestion

Food digestion involves a delicate balance between breakdown and passage. As the digesta flow through the gastrointestinal tract, it is exposed to a cascade of changing environments that promote or delay the physical breakdown and hydrolysis by digestive enzymes. Some dynamic aspects relevant to protein digestion have been addressed in this thesis. However, other aspects remain to be studied and incorporated to the assessment of protein digestibility.

- Kinetics in lowly acidic gastric pH

Salelles et al. (2021) showed that pepsin-catalysed hydrolysis can already occur early in the gastric phase, when acidification has not occurred yet; even though it is commonly assumed that pepsin activity is pH dependent and highest at approximately pH 2. This study showed that similar extents of hydrolysis of milk casein can be achieved from pH 5 to 1, after two hours. This, however, was not observed for egg white proteins, suggesting that the activity of pepsin depends on both the pH and the type of substrate. It will be interesting to further explore this phenomenon in terms of the kinetics of hydrolysis using isothermal titration calorimetry as reported in Chapter 4. One may speculate that the peptides obtained from pepsin-catalysed hydrolysis at higher pH are not the same as

those obtained at lower pH, even when the extent of hydrolysis might be similar. If this is the case, the kinetics of trypsin-catalysed hydrolysis, or that by other pancreatic proteases, might differ between substrates from high or low pH gastric digestion.

- Small intestine: pancreatic and brush border enzymes

Similar to the study by Luo et al. (2018), in which the kinetics of pepsin-catalysed hydrolysis were determined at different pH and ionic strength, the pH-dependent trypsin-catalysed hydrolysis should be investigated using isothermal titration calorimetry. This will not only shed light on the hydrolysis occurring early in the small intestinal phase, but it will also inform the design of simple dynamic small intestinal *in vitro* digestion methods.

Another aspect yet to be addressed to obtain a more complete understanding of the sequential protein hydrolysis during gastro-small intestinal digestion is the action of brush border peptidases. It is not clear whether the location of digestion by the brush border peptidases is solely in the region adjacent to the membrane, and not in the lumen (Hooton et al., 2015). It is thus uncertain if the size of the oligopeptides resulting from hydrolysis by pancreatic enzymes is relevant for their contact with brush border peptidases. Nevertheless, the specificity of these peptidases is more diverse than that of pancreatic enzymes. This allows us to speculate whether a limited digestion by luminal proteases can be compensated by brush border peptidases.

- Small intestine: absorption

This last hydrolysis step by brush border peptidases is the gatekeeper between luminal hydrolysis and absorption into and through the enterocytes. It has been demonstrated that the rate at which dietary amino acids are absorbed matters for muscle protein accretion (Wolfe, 2002). Whey protein (considered a *fast* protein) appears earlier and faster in plasma, and thus is more effective at stimulating muscle protein synthesis than casein (*slow* protein) and even, casein hydrolysate (Pennings et al., 2011). Soybean protein isolate was found to be closer in digestive pattern to whey protein than to casein, and as such muscle protein synthesis was greater than with casein (J. E. Tang et al., 2009). This is not to imply that *slower* proteins are not relevant for muscle protein synthesis. On the contrary, their later and slower appearance in plasma can sustain protein accretion once it has been initiated by *faster* proteins.

8.2.3 Digestion models

The great complexity of studying the digestive fate of foods in the gastrointestinal tract stems from the complexity of the digestive system and that of food itself, regarding

composition and structure on multiple length scales. *In vitro* and *in silico* models aid in unravelling (part of) this complexity.

By using *in vitro* experimental models we can find associations or correlations between variables. These models are essential to obtain an understanding of the underlying processes and mechanisms, which can then be used to better understand the behaviour in more complex conditions. It is however difficult to study spatial complexities as these depend on many factors that are challenging to replicate in an *in vitro* experiment, such as, the dynamic rheological properties of the digesta, the shape and inner structure of the digestive organs, mixing and fluid dynamics.

Mechanistic and semi-mechanistic *in silico* models allow to test hypotheses of direct or indirect causations from associations observed *in vivo* or *in vitro*. It is somewhat simpler to simulate the relevant intricate processes and regulating mechanisms occurring during food digestion via *in silico* modelling. Furthermore, a wide range of parameters can be tested because of their higher throughput.

Food scientists have gathered a vast amount of knowledge on the digestive fate of foods of various origins, processing histories and multi-scale structures. We consider that it is time to shift the focus towards constructing robust, dynamic, (semi)mechanistic, compartmental models that consider these findings from *in vitro* experiments. Every new iteration will aid in reducing the simplifying assumptions that stand in the way of making better predictions and understanding the complexities. In doing so, the outcome of *in silico* models can be used to better target *in vitro* and even *in vivo* experiments which are more resource-expensive, and better understand the variations between the digestive responses among different consumers or groups of consumers.

- Gastric emptying

Future versions of the compartmental model presented in Chapter 7 should incorporate more aspects that influence gastric emptying, beyond caloric density and physical state of the meal. The composition and rheological properties of chyme are also relevant to the gastric emptying rate. For instance, it was reported that the source of the calories, i.e., from proteins, carbohydrates or fats, influences the rate of gastric emptying. Giezenaar et al. (2018) found that the rate of calorie delivery to the duodenum in the first 60 min after ingestion was 2.7 kcal/min for a drink with whey protein. An isocaloric drink with whey protein, dextrose and olive oil was emptied approximately 1.4 times faster.

The presence of dietary fibre delays and slows down the gastric emptying of protein (Bornhorst et al., 2013). This was associated to layering within the stomach of brown rice bran fragments in the antrum. Interestingly, this effect was only observed for protein and

not for starch or dry matter. Further, the viscosity of chyme has been reported to be inversely related to the rate of gastric emptying and overall transit through the gastrointestinal tract (Darwiche et al., 2003; Ménard et al., 2018).

Protein properties also influence gastric emptying. It is well known that milk proteins show different behaviour in the gastrointestinal tract; whey proteins are quickly emptied from the stomach, while casein coagulates and is emptied later and more slowly than whey proteins (Boirie et al., 1997; Huppertz & Chia, 2021).

- Other nutrients

Referencing back to the relevance of the other components of an ingredient, food or meal, we propose an extension to the model from Chapter 7 that incorporates starch digestion, particularly amylolytic activity in the stomach compartment (Figure 8.3). It is known that salivary α-amylase remains active in the stomach while the pH is still relatively high (pH 3.3 to 3.8, Fried et al. (1987)). Interestingly, amylolysis can be quite extensive in the stomach (Freitas et al., 2018). The gastric acidification slows the amylolysis but increases the rate of gastric protein hydrolysis. The longer it takes for pH to decrease, the longer the salivary α-amylase can be active in the stomach. Salivary α-amylase inactivation may happen earlier if the meals contains some component(s) with low pH. For example, Freitas and Le Feunteun (2019) found that the pH of the bolus, as well as the amylolytic activity, were rapidly reduced when consuming bread and lemon juice.

This model extension will need further studies to better describe the luminal pH. The geometric complexity of the gastrointestinal system, plus its adaptation to the food being digested are challenging aspects of digestion to model. Understanding the influence of the geometric complexity requires considerable knowledge of fluid and mixing dynamics (Harrison et al., 2018). The chyme properties also influence the behaviour of the gastrointestinal muscles and tissues and therefore also creates part of the geometric complexity. For example, intragastric pH gradient from the proximal and distal regions was reported to be narrower for chyme with softer components (Nadia et al., 2021). These aspects will require completely new features of the model but will result in much better understanding and prediction of the digestion.

8.3 Outlook : the context of foods and diets

The protein transition considers an increase in consumption of plant-based foods and a reduction of those from animal origin. This thesis indicates the importance of considering the digestibility and utilization of nutrients in plant-based foods to better assess the environmental impact of ingredients and finished products. This should however be in the context of diets as a whole (Figure 8.4).

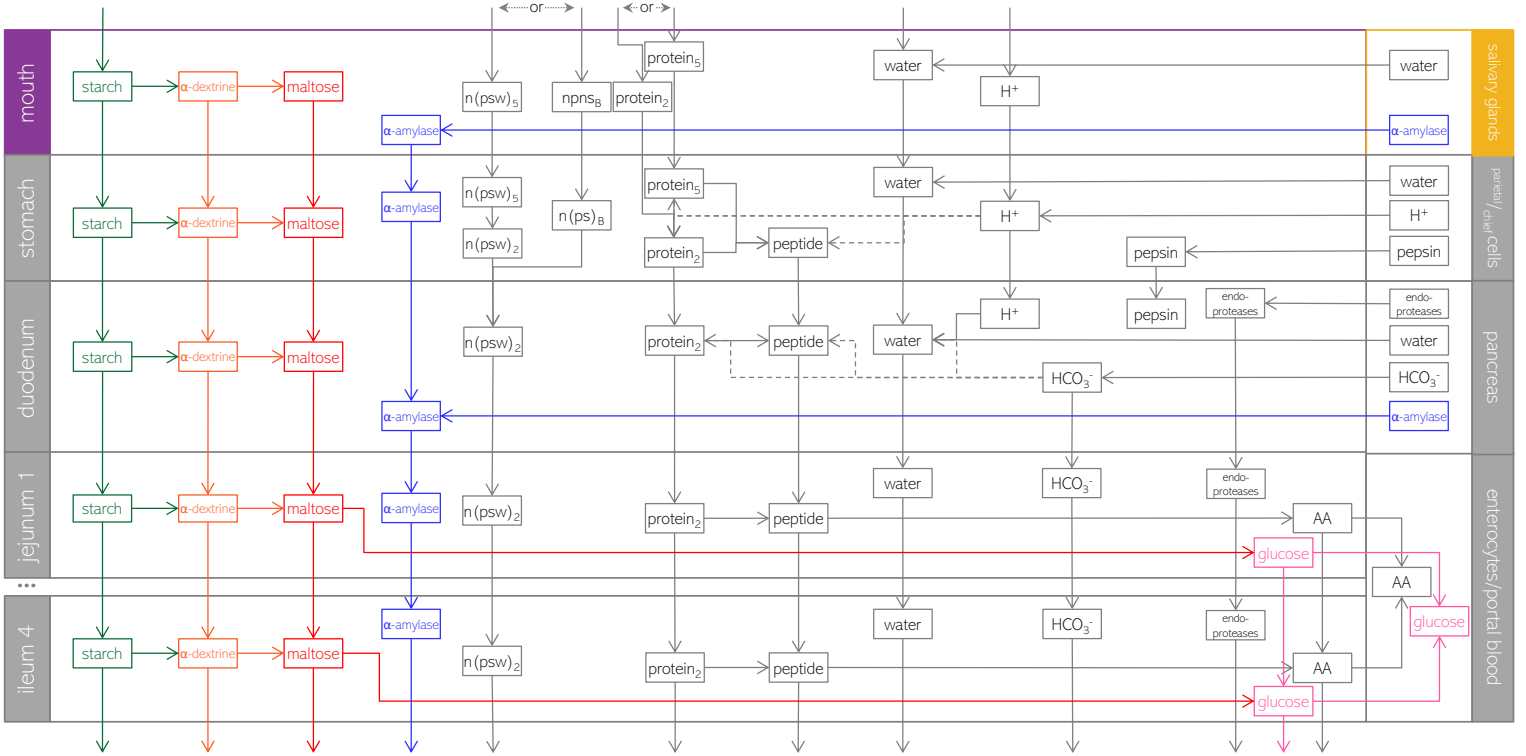


Figure 8.3. Extension of the model from Chapter 7 to consider starch digestion and residual α-amylase activity in the stomach. Solid lines represent fluxes, dashed lines represent influential factors that do not contribute to the protein-peptide mass balance. The jejunum 2, ileum 1, 2 and 3 compartments are not shown but are located after jejunum 1, with the same pools and fluxes as this compartment. $n(psw)_5$, non-(protein, starch, water) 5 mm; $n(ps)_B$, non-(protein, starch) blended; AA, amino acid.

As has been thoroughly discussed, physical, chemical and biochemical characteristics at different length scales affect the digestibility of nutrients, ingredients and foods. Through the combination of *in silico* tools, it would be possible to estimate the digestion and utilization of full meals and diets.

In terms of nutrition and sustainability, we believe that ultimately diets, as opposed to foods, should be considered. Without this, we run the risk of adopting a rather reductionist approach. It might be better to regard plant-based foods as partial rather than full replacers for foods of animal origin. Nutritionally, we should be aware of micronutrient displacement. For instance, when replacing milk with plant based alternatives, diets might become deficient in calcium and vitamin D (Itkonen et al., 2020). Van Vliet et al. (2021) found that ground meat and plant-based red meat analogues shared only 19 out of 190 metabolites, for products with similar macronutrient composition. This evidences that the foods studied are not nutritionally interchangeable.

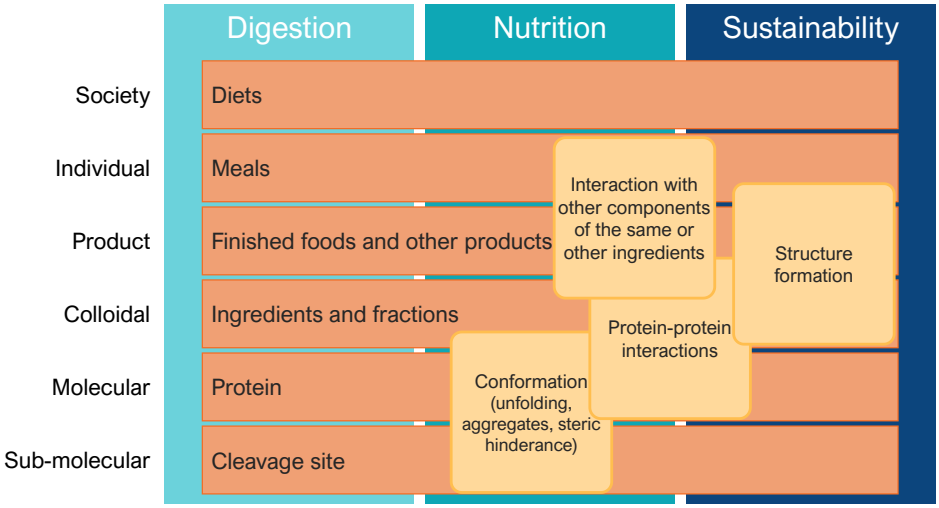


Figure 8.4. The role of protein digestion in the context of nutritious and sustainable diets.

8.4 Concluding remarks

The digestibility of plant proteins depends on their processing history. In turn, the digestibility influences the assessment of sustainability. However, digestibility is a complex and multidimensional concept. One should match the method to assess said digestibility to the exact purpose of the assessment. Dynamic methods might give richer information than those in static conditions. Dynamic models highlight the importance of the pH dependency of digestive enzymes and how this impacts their specificity and kinetics of catalysis. In the future, it will be interesting to explore the potential of the

combined effect of heat- *(from processing)* and acid- *(during digestion)* induced denaturation.

We should continue to progress towards more holistic methods to study protein digestion, considering the impact of other nutrients and the structure(s) of the food matrix. With more robust dynamic, *in silico* models, the relations between food properties and total digestion can be identified and considered. This will help us gain further insight into health implications of individual foods, meals or diets. In doing so, *in silico* models will direct, and perhaps optimize, food digestion research.

Finally, our improved understanding of the unfavourable and favourable effects of processing on the digestion of plant protein rich ingredients and foods will aid in better determining the *'farm-to-faeces'* environmental impact of these products.

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Summary

The aim of this thesis is to better relate the effects of processing, particularly heating, to the digestibility of plant proteins. Plant protein-rich ingredients are of interest to produce alternatives to animal-sourced foods. Studying protein digestion relies on the use of models that simulate some aspects of the *in vivo* digestion conditions. In this work, we explore the relevance of some dynamic aspects of the gastric phase on the overall luminal gastro-small intestinal digestion.

A narrative review of literature relating processing before, during and after fractionation, to protein digestibility of protein-rich ingredients is presented in Chapter 2. It was evidenced that some types of processing, such as dry fractionation, had mixed effects on protein digestibility, depending on the processing conditions and the crop under study. Meanwhile, other processes consistently improved or reduced digestibility, independently of the crop (e.g., acylation and alkaline treatment, respectively). Some overarching relations and knowledge gaps were identified.

Commercially available plant protein-rich ingredients are commonly produced by isoelectric precipitation and spray drying. In Chapter 3, the static *in vitro* gastric digestion of unheated and heated aqueous dispersions of commercial soybean and yellow pea protein isolates is presented. The effect of heat treatment on gastric digestion was studied in full dispersions and their insoluble and suspended phases. Spray-dried particles, although insoluble, were hydrolysed by pepsin, as evidenced by microscopy observations and high degrees of hydrolysis. Heat treatment disrupted the shell-like morphology to release the particles' content into the suspended phase. At the same time, some proteins formed heat-induced aggregates that sedimented into the pellet. Pepsin-catalysed hydrolysis was improved by heat treatment for the suspended proteins, while it was reduced for the sedimented, insoluble protein. These effects were however concealed in the extent of hydrolysis of the full dispersion that did not differ significantly as a result of heat treatment.

The relevance of the dynamic nature of the gastric phase on luminal small intestinal digestion is demonstrated in Chapter 4. Isothermal titration calorimetry was used to determine the kinetics of trypsin-catalysed hydrolysis of a model protein (bovine serum albumin). This protein was exposed to treatments that represent a full and a practically null residence time in the stomach. The protein that would be first emptied from the stomach into the small intestine, virtually bypassing gastric digestion, was only slightly hydrolysed by trypsin. However, the protein fully exposed to the gastric environment, i.e., emptied last from the stomach, which was both, more extensively and also more efficiently hydrolysed during small intestinal digestion.

This finding was confirmed in Chapter 5 where the standardised semi-dynamic *in vitro* digestion method was used to simulate the dynamicity of gastric secretions and emptying. The extent of hydrolysis by pancreatic proteases was higher for pea proteins that had a longer residence time in the gastric phase, i.e., with a lower pH and higher proportion of pepsin, than for those that were emptied sooner. The materials studied in this chapter were unheated and heated aqueous dispersions of yellow pea flour, three mild aqueous protein fractions and a commercial pea protein isolate as benchmark. The effect of thermal denaturation on digestion by pepsin and pancreatin was assessed by heating the native protein fractions. Protein denaturation, either as unfolding or aggregation, was related to the extent of processing during and after fractionation indicating that a certain extent of processing is necessary to improve protein digestion, however more extensive processing would reduce it.

In Chapter 6, data from Chapter 5 was incorporated to the life cycle assessment of pea protein-rich ingredients. A *'farm-to-faeces'* approach was proposed, over the more common *'farm-to-fork'*, to extend the assessment beyond production and include protein digestibility. In doing so, more actionable measurements can be obtained to compare ingredients or finished products from different origin or with different processing history, as it has been shown that these factors influence protein digestibility. The functional units included in this study were kg of ingredient, of dry matter, of protein, of *in vitro* hydrolysable protein and of minimum digestible, limiting, indispensable amino acid. The environmental impact of pea protein isolate, pea flour, protein-rich, soluble and non-soluble protein fractions is reported relative to that of a commercial whey protein isolate. Furthermore, heat treatment was included to represent post-fractionation processing. Mildly fractionated ingredients provided a more sustainable alternative to whey protein isolate but also to conventionally produced pea protein isolate; this, both in terms of production of ingredient and of digestible protein.

Chapter 7 presents a case study of the *in silico* digestion of protein from two meals, one with solid and liquid components and one in which both components were blended into a soup. A dynamic, compartmental, semi-mechanistic protein digestion model is proposed. Transit of digesta, secretion of digestive juices, particle breakdown, protein hydrolysis and amino acid absorption are described by zero or first order functions. Gastric emptying was described by a constant delivery of calories from the stomach into the duodenum compartment. Furthermore, a lag time to antral grinding was included for the solid phase of the solid/liquid meal. In this way, the simulated volume of gastric contents reflected relatively well the behaviour observed *in vivo* for solid/liquid and blended meals.

Finally, Chapter 8 discusses the main findings and conclusions from this thesis. We relate processing and sustainability with the digestibility of plant proteins as food ingredients. Emphasis is placed on the importance of the model choice to study, digestion with respect to its static and dynamic aspects. This chapter brings forward an outlook towards a more widespread use of *in silico* digestion models. Furthermore, the relevance of the dietary context is acknowledged.

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About the author

Andrea Rivera del Rio was raised in Mexico City. She obtained her BSc in Food Chemistry with honours from the School of Chemistry at the National Autonomous University of Mexico. Her career has focused on eating as an enjoyable, nutritious and sustainable experience. After learning Sensory Science at the University of California, Davis, she worked in the flavour and functional ingredient industry in Mexico; translating consumer insights and sensory assessment of foods into actionable recommendations for flavourists and developers.

She received her MSc degree (*cum laude*) at Wageningen University. During her MSc studies she conducted a research project at the Human Nutrition Research Centre from Newcastle University, studying product portfolios and the effect of reformulations on the dietary intake of nutrients to limit. In 2018, Andrea started her PhD in the Food Process Engineering group from Wageningen University. Her research focused on the digestion of plant protein ingredients and how it is influenced by processing during ingredient production and manufacture of final products. The results of her work are presented in this thesis.

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Publications

Rivera del Rio, A., Opazo-Navarrete, M., Cepero-Betancourt, Y., Tabilo-Munizaga, G., Boom, R. M., & Janssen, A. E. M. (2020). Heat-induced changes in microstructure of spray-dried plant protein isolates and its implications on *in vitro* gastric digestion. *LWT*, *118*, 108795.

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Rivera del Rio, A., van der Wielen, N., Gerrits, W.J.J, Boom, R. M., & Janssen, A. E. M. *In silico* modelling of protein digestion: a case study on solid/liquid and blended meals. *Accepted for publication in Food Research International.*

Draijer, N., **Rivera del Rio, A.**, Lie-Piang, A., Janssen, A. E. M. & Boom, R. M. Nutritional value in sustainability assessment of protein-rich ingredients and foods: a *'farm-to-faeces'* approach. *In preparation.*

Overview of completed training activities

Discipline specific activities	Year
Isothermal titration calorimetry (TA Instruments)	2018
Food proteins: functionality, modifications and analysis (VLAG)	2018
Intestinal microbiome of humans and animals (VLAG)	2019
33rd EFFoST conference (EFFoST)[1]	2019
Food Science Symposium (VLAG)[1]	2020
15th Plant-Based Foods & Proteins Summit (Bridge2Food, online)	2020
Transport in the digestive tract: experiments, modelling, applications to microbiology (École Normale Supérieure, online)	2020
International INFOGEST webinar series on Food Digestion (INFOGEST, online)[1]	2020-21
Virtual International Conference on Food Digestion (INFOGEST, online)[2]	2021
Healthy and sustainable diets: synergies and trade-offs (VLAG)	2021
Plant Protein Science and Technology Forum (AOCS, online)[2]	2021
Big Data Analysis in the Life Sciences (VLAG)	2021
35th EFFoST conference (EFFoST)[1]	2021
6th Food Structure, Digestion & Health conference (CSIRO & Riddet Institute, online)	2021

General courses	
VLAG PhD week (VLAG)	2018
Introduction to Systematic Review and Meta-Analysis (Johns Hopkins University, online)	2018
Introduction to R (VLAG)	2018
PhD workshop carousel (WGS)	2018

Scientific writing (Wageningen in'to languages)	2019
Project Management: Mastering Complexity (TU DelftX, online)	2021
Career Perspectives (VGS)	2021

Optionals

Preparation of research proposal	2018
PhD study tour to Canada	2018
Weekly FPE group meetings	2018-22

[1] oral presentation

[2] poster presentation

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