HEAT-INDUCED STRUCTURAL MODIFICATIONS OF PLANT PROTEINS:

IMPLICATIONS FOR PEPTIDE PATTERN AND BIOACTIVITY AFTER INFANT DIGESTION

Jiaying Tang

Propositions

1. The soluble receptor for AGEs, contrary to its name, is not a reliable tool for determining AGE levels.
   (this thesis)

2. The major bottleneck in predicting peptide bioactivity is the adequacy of the protein database.
   (this thesis)

3. Building tolerance to allergenic proteins may be a more efficient solution compared to complete avoidance.

4. Simultaneous submission to multiple journals should become a standard in academia.

5. Direct presentation of P-values is better than relying solely on arbitrary cutoffs for statistical significance.

6. Working smarter outweighs working harder.

7. The best way for motivation is self-reward after achieving a goal.

Propositions belonging to the thesis, entitled

Heat-induced structural modifications of plant proteins: implications for peptide pattern and bioactivity after infant digestion

Jiaying Tang

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Heat-induced structural modifications of plant proteins: implications for peptide pattern and bioactivity after infant digestion

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Heat-induced structural modifications of plant proteins: implications for peptide pattern and bioactivity after infant digestion

Jiaying Tang

Thesis

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CHAPTER 1

General Introduction

1.1. Plant proteins in infant formula

Driven by considerations of cow's milk allergy (CMA) and lactose intolerance, as well as sustainability, plant protein-based infant formula (IF) has emerged as a prominent alternative to dairy-based options. However, this transition introduces challenges in terms of nutritional and health aspects, including the need to achieve a proper balance of essential amino acids (EAAs), ensure adequate digestibility, mitigate allergenic risks, and limit the presence of antinutritional factors (ANFs) within plant protein sources (Hertzler, Lieblein-Boff, Weiler, & Allgeier, 2020; Martín-Cabrejas et al., 2009; Tan, Nawaz, & Buckow, 2023).

Soy protein isolate is a widely accepted dairy protein alternative for IF, authorized by the European Commission (2016). Soy protein (SP) is well tolerated by the majority of infants with CMA and has a good EAA profile, making it a viable long-term substitute for dairy proteins. However, SP is characterized by its high trypsin inhibitors (TIs) content, a type of ANF that could impede intestinal digestion (Xiao, Wood, Robertson, & Gilani, 2012). Moreover, soybean ranks among the eight common allergens, potentially triggering food allergies (Cordle, 2004). Hence, exploring novel hypoallergenic plant proteins for IF is crucial.

Various plant protein sources, including those from potatoes, quinoa, faba beans, peas, lentils, and chickpeas, have been proposed as promising dairy substitutes other than soybeans (Dakhili, Abdolalizadeh, Hosseini, Shojaee-Aliabadi, & Mirmoghtadaie, 2019; Gorissen et al., 2018; Maryniak, Sancho, Hansen, & Bøgh, 2022; Venlet, Hettinga, Schebesta, & Bernaz, 2021). Among these options, pea protein (PP) has received widespread interest, given its richness in EAAs, high digestibility, and notably low levels of ANFs and low allergenicity, appealing especially in regions where soybeans are not native crops or for infants allergic or intolerant to SP (Davidsson, Dimitriou, Walczyk, & Hurrell, 2001; Roux, Chacon, Dupont, Jeantet, Deglaire, & Nau, 2020).

In this thesis, the primary research materials selected are SP and PP. SP is chosen due to its role as the predominant plant protein source in current plant-based IF, while PP is considered a promising alternative.

1.2. Structural characteristics of soy protein and pea protein

SP and PP are predominantly constituted of globular proteins, including globulins and albumins. These globular proteins can be fractionated based on their sedimentation coefficients, resulting in four distinct groups: 2S, 7S, 11S, and 15S (Nishinari, Fang, Guo, & Phillips, 2014). In legumes, globulins are the major storage proteins, mainly distributed among the 7S, 11S, and 15S fractions. They account for more than 50% of the total protein composition (Grossmann & Weiss, 2021; Sui, Zhang, & Jiang, 2021). In contrast, the minor 2S fraction primarily contains albumins, among which are some known to act as ANFs, e.g., protease inhibitors and lectins (Shevkani, Singh, Chen, Kaur, & Yu, 2019).

SP primarily comprises two major components: β-conglycinin (7S) and glycinin (11S). β-conglycinin is composed of three subunits, namely α, α', and β, while glycinin consists of 6 subunits, each comprised of an acidic and a basic polypeptide covalently linked by a disulfide bond that can be dissociated under reducing conditions. PP, on the other hand, is mainly composed of vicilin (7S), convicilin (7-8S), and legumin (11S). Vicilin presents as a trimeric protein, with each monomer containing α, β, and γ subunits (Lam, Karaca, Tyler, & Nickerson, 2018) and notably lacks cysteine residues (Reinkensmeier, Bußler, Schlüter, Rohn, & Rawel, 2015). Convicilin is its polymorphic variant, sharing an 80% homology with vicilin. However, it can be distinguished from vicilin by its highly charged N-terminal extension region, and the presence of a cysteine residue (Reinkensmeier et al., 2015; Tzitzikas, Vincken, de Groot, Gruppen, & Visser, 2006). Similar to soy glycinin, pea legumin is a hexamer, characterized by the presence of disulfide-linked acidic and basic polypeptides.

Compared to SP, PP has a relatively higher level of lysine, while SP has higher levels of sulfur-containing AAs (Reinkensmeier et al., 2015). The different primary structures lead to different secondary structures. In general, legume proteins are abundant in β-structures (i.e., β-sheets, β-strands, and β-turns) (Shevkani et al., 2019). For SP and PP, the most abundant secondary structure is β-sheets, with 37% for SP and 38% for PP (Withana-Gamage, Wanasundara, Pietrasik, & Shand, 2011). However, this abundance in β-sheets can negatively affect their digestibility (Carbonaro, Maselli, & Nucara, 2012). In addition, as mentioned above, SP is abundant in TIs, thus the digestion may be more limited compared to PP. Nevertheless, proper heating can inactivate these TIs, and thereby increase its digestibility. For example, by heating at 75°C-100°C for 30 min, the digestibility of SP increased, coinciding with a reduced activity of TIs (Vagadia, Vanga, Singh, Gariepy, & Raghavan, 2018).

1.3. Heat-induced structural modifications

In the production of IF, three critical stages contribute to a substantial heat load to ensure the safety and quality of the final products, including pasteurization/sterilization, evaporation, and spray drying. In general, when proteins are exposed to heat, their natural rigidity can be altered, rendering them more flexible (Tian et al., 2019). During heating, various structural modifications may occur, including denaturation, aggregation, and glycation. SP and PP primarily consist of globular proteins. These globular proteins have compact three-dimensional structures that are stabilized by weak interactions such as hydrogen bonds, van der Waals forces, and hydrophobic interactions. However, when subjected to temperatures above their denaturation thresholds, these weak interactions can be disrupted, causing the proteins to lose their native conformation. This results in protein unfolding and, in the case of 11S globulins, subunit dissociation (Farjami, Babaei, Nau, Dupont, & Madadlou, 2021; Keerati-u-rai & Corredig, 2009). These structural transformations expose hydrophobic regions that are typically buried within the protein's core, hence the increased surface hydrophobicity (Wang et al., 2012; Wang, Li, Jiang, Qi, & Zhou, 2014). When these regions come into contact with each other or with other proteins, protein aggregation may occur. For globular proteins, this aggregation process typically begins with disulfide rearrangements, forming oligomers that then aggregate through non-covalent forces (Kavanagh, Clark, & Ross-Murphy, 2000; Li, Li, Hua, Qiu, Yang, & Cui, 2007). In the case of SP, research by Wang et al. (2014) showed that heat-induced denaturation led to increased surface hydrophobicity, while aggregation led to its reduction. Additionally, heating at temperatures between 70-90°C resulted in an increase in α-helix structures and a decrease in β-sheet structures in SP. Interesting, limited aggregation generated by proper heating can lead to the inactivation of TIs (Xu, Chen, Zhang, Kong, & Hua, 2012).

Glycation, also referred to as the Maillard reaction, is a non-enzymatic chemical reaction initiated by the covalent binding of a (reducing) sugar molecule to specific AA residues, typically lysine and arginine, within proteins (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016; Tamanna & Mahmood, 2015). This initial reaction generates early glycation products, such as Schiff bases and Amadori products (e.g., Nε-furoylmethyllysine), which can undergo further chemical rearrangements to create more stable and complex advanced glycation end products (AGEs). AGEs can exist in linear forms such as Nε-carboxymethyllysine and Nε-carboxyethyllysine, as well as in cross-linked forms like pentosidine. As glycation progresses to its final stage, it culminates in the production of brown nitrogenous polymers, such as melanoidins. **Figure 1.1** illustrates some representative marker molecules from early and

advanced stages of glycation. Importantly, due to the temperature dependence of glycation, the protein structural modifications by glycation and heating cannot be easily distinguished. Furthermore, the occurrence of glycation has been described as a contributing factor to protein aggregation (Feng, Berton-Carabin, Atac Mogol, Schroen, & Fogliano, 2021; Fu et al., 2021). In another study by Li et al. (2019), glycation has been linked to the increase in the surface hydrophobicity and free sulfhydryl groups of SP.

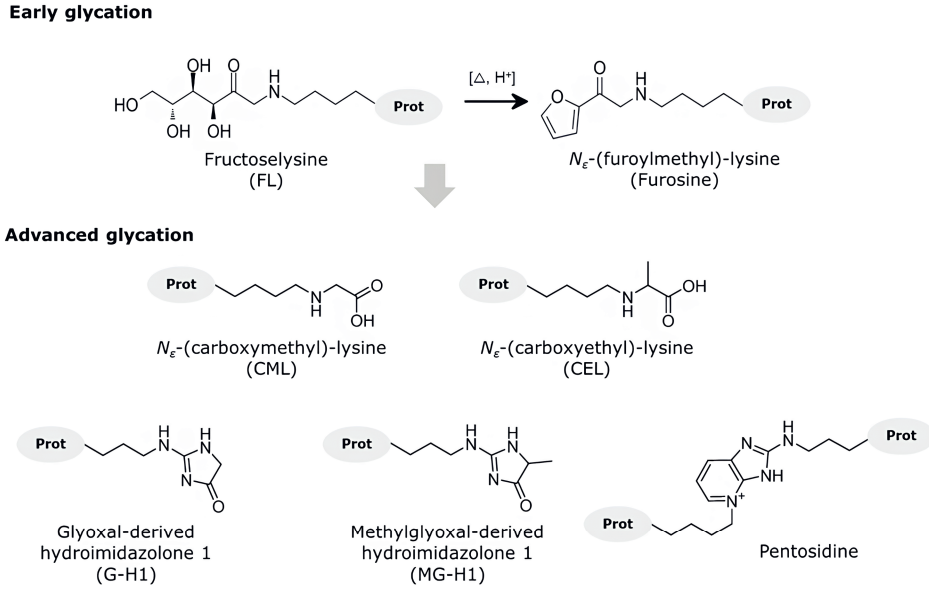

Figure 1.1. Representative marker molecules for early and advanced stages of glycation between proteins (Prot) and glucose. Modified from Brings, Fleming, Freichel, Muckenthaler, Herzig, and Nawroth (2017).

1.4. Infant digestion and formation of bioactive peptides

Infant digestion, characterized by unique enzymatic profiles and relatively higher gastric pH, primarily occurs in the intestinal phase (Bourlieu et al., 2014; Nguyen, Bhandari, Cichero, & Prakash, 2015). This distinctive digestive process, influenced by an immature digestive system and increased gut barrier permeability (Weström, Arévalo Sureda, Pierzynowska, Pierzynowski, & Pérez-Cano, 2020), leads to different digestion kinetics compared to adults and profoundly affects the release and subsequent absorption of peptides. These factors potentially contribute to variations in nutrient utilization and peptide bioactivity in infants.

Gastrointestinal digestion can result in the breakdown of dietary proteins into smaller peptide fragments, leading to the formation of bioactive peptides (BPs), whether through natural human *in vivo* digestion or simulated *in vitro* digestion (Wang, Kadyan, Ukhanov, Cheng, Nagpal, & Cui, 2022). Due to the distinct structures and AA compositions of BPs resulting from different cleavage processes, they can exhibit a wide range of physiological functions fulfilling various roles in the human body, including e.g., antioxidative, antimicrobial, antithrombotic, antidiabetic, anticancer, antihypertensive, anxiolytic, hypocholesterolemic, immunomodulatory, and opioid effects (Ashaolu, 2020; Fan, Liu, Zhang, Zhang, Liu, & Wang, 2022; Kim, Yang, & Kim, 2021). Moreover, they can also be synthesized and introduced into infant foods to enhance their functional properties. One intriguing function, especially for glycated peptides, is their binding affinity to the soluble receptor for AGEs (sRAGE). RAGE, known as a multi-ligand receptor, regulates inflammatory responses, oxidative stress, and tissue damage (Fritz, 2011; Yue, Song, Liu, Zhang, Yang, & Li, 2022). Notably, food-derived AGEs have been described as its ligands, and IF, due to its processing, contains a significantly higher amount of AGEs compared to breastmilk (Kutlu, 2016). sRAGE is the soluble form for RAGE and can also bind to AGEs. It is recognized as a decoy receptor for circulating AGEs, with higher sRAGE levels found in neonatal circulation (Fritz, 2011; Quintanilla-García et al., 2019). Therefore, evaluating sRAGE-binding affinity could provide insights into the potential immunoregulatory activity of peptides.

Furthermore, given the compositional complexity of digesta, fractionation and purification steps are required when extracting BPs (Tsou, Kao, Lu, Kao, & Chiang, 2013). Subsequently, relevant assays can be used to assess the bioactivities of these fractions, followed by the identification and synthesis of primary BPs. The functionality of these BPs can be further confirmed through either *in vivo* or *in vitro* experiments (García-Tejedor, Sánchez-Rivera, Castelló-Ruiz, Recio, Salom, & Manzanares, 2014). Additionally, some researchers begin by predicting potential BPs within the digesta and subsequently synthesize and validate their functions (Almaas et al., 2011; Asledottir et al., 2023). Furthermore, it is worth noting that BPs may exert synergistic effects contributing to overall bioactivities. As a result, many studies focus on the hydrolysates or peptide fractions as a whole rather than on individual BPs, which can make it challenging to establish a direct functional link to a specific BP (Ashaolu, 2020; Zhang, Yokoyama, & Zhang, 2012). However, this approach aligns more closely with the *in vivo* situation, where the intestinal epithelium is concurrently exposed to a multitude of peptides.

Numerous studies have investigated the bioactivities of peptides formed upon digestion of SP (Amigo-Benavent, Clemente, Caira, Stiuso, Ferranti, & del Castillo, 2014; Coscueta, Campos, Osório, Nerli, & Pintado, 2019; Staljanssens et al., 2012), and PP (Asledottir et al., 2023; Cipollone & Tironi, 2020; Jadhav, Gaonkar, & Rathi, 2021). However, it is essential to note that these investigations were conducted within the context of adult digestion and do not specifically address the unique digestion patterns of infants. Therefore, there is a significant gap in research regarding the bioactivities of these peptides in the context of infant digestion, warranting further investigation.

1.5. Immunogenicity

Immunogenicity is a broader concept than allergenicity, which encompasses a range of immune responses that a protein can evoke in an organism, including immune system activation, antibody production, and other immunological events. Although a certain protein might not cause food allergy, it may still have immunogenicity. Upon the oral intake of food proteins, they enter the gastrointestinal tract and undergo hydrolysis by digestive enzymes. Subsequently, protein fractions or peptides that manage to cross the gut barrier may have the potential to elicit immunological reactions mediated by B cells and T cells. These responses include the production of immunoglobulin G (IgG) antibodies and, in the case of allergy, IgE responses (Cano & Lopera, 2013; Sanchez-Trincado, Gomez-Perosanz, & Reche, 2017; Van Wijk, Hartgring, Koppelman, Pieters, & Knippels, 2004). The IgG response can be considered the ultimate stage in the digestive process. During this stage, IgG binds to its receptor on immune cells such as macrophages. These receptor-bound IgG antibodies function to scavenge peptides that are being endocytosed, facilitating their further destruction and degradation. In the context of allergic reactions, the immunological response involves IgE antibodies, which have a specialized role in triggering immediate hypersensitivity reactions. IgE antibodies bind to mast cells and basophils, leading to the release of histamines and other inflammatory mediators when allergens cross-link receptor-bound IgE antibodies on the surface of effector cells.

Therefore, besides evaluating the nutritional quality of SP and PP, their immunogenicity should also be taken into consideration. According to WHO/IUIS Allergen Nomenclature Sub-committee (http://www.allergen.org/, accessed October 2023), SP has eight common allergens from Gly m 1 to Gly m 8, with β-conglycinin (Gly m 5) and glycinin (Gly m 5) being able of causing severe soy allergies (Holzhauser et al., 2009; Ito et al., 2011). For PP, three allergens

have been reported, i.e., vicilin (Pis s 1), convicilin (Pis s 1), and nsLTP (Pis s 3). These proteins have potential allergenicity. Nevertheless, individual variations in immune responses and sensitivities can lead to significant differences in the likelihood of developing an allergic reaction. Moreover, specific peptides generated through digestion, whether originating from well-known allergens or other proteins not traditionally recognized as allergens, may still possess or acquire immunogenic properties. Importantly, infants with immature digestive and immune systems may face a heightened risk. Therefore, when evaluating the use of SP and PP in infant nutrition, it is essential to account for potential (residual) immunogenicity, especially in this vulnerable population.

1.6. Effect of heating on protein digestion and bioactive properties of resulting peptides

Heat processing of proteins can affect their digestibility, either positively or negatively, depending on the applied heating methods and the resulting structural modifications. Denaturation and unfolding may facilitate digestibility. This can be due to the generation of random coils resulting from the destruction of tertiary and secondary (e.g., α-helices and intramolecular β-sheets) structures, increasing accessibility of enzymes to cleavage sites within proteins (Dupont & Nau, 2019; Salazar-Villanea, Hendriks, Bruininx, Gruppen, & van der Poel, 2016). For example, autoclaving of SP can increase its digestibility due to the loss of β-sheet secondary structures (Carbonaro et al., 2012). The increase in drying temperature from 50°C to 70°C led to increased PP digestibility, also ascribed to the reduced contents of β-sheets (Gonzalez, Alvarez-Ramirez, Vernon-Carter, Reyes, & Alvarez-Poblano, 2020). Conversely, aggregation and glycation may hinder digestion by the burial of enzyme-cleavage sites. For instance, oxidation-induced cross-linking contributed to the formation of insoluble and indigestible aggregates of 11S globulins from SP (Zhang, Wang, Li, Guo, & Lv, 2022). Moreover, glycation impairs digestibility by blocking tryptic cleavage sites at lysine and arginine residues (Hemmler et al., 2019; Olsen, Ong, & Mann, 2004). Notably, in terms of TI-rich SP, heating-induced limited aggregation can also inactivate TIs to increase the overall digestibility (Vagadia et al., 2018; Xu et al., 2012).

In addition to the overall digestibility, after different heat treatments, the number and length of released peptides can vary, as well as their potential modifications, leading to distinct peptide profiles and bioactivity outcomes (Dupont et al., 2010; Kopf-Bolanz, Schwander, Gijs,

Vergères, Portmann, & Egger, 2014; Zenker, Wichers, Tomassen, Boeren, De Jong, & Hettinga, 2020; Zhang, Li, Lv, Guo, & Yang, 2023). For instance, one study showed that by heating at 90°C for 30 min, the antioxidant activity of cowpea-derived peptides was reduced (Quansah, Udenigwe, Saalia, & Yada, 2013). Besides, antioxidant peptides were found in digested egg white protein treated at 100°C for 5 min (Wang, Qiu, & Liu, 2018). Moreover, heat-induced glycation has been shown to modify specific AA residues within IgE epitopes of tropomyosin, resulting in reduced immunoreactive capacity (Bai et al., 2021).

It is important to highlight that while extensive research has been dedicated to understanding simulated protein digestion and the formation of BPs after heating, a significant research gap exists when it comes to infant digestion of plant proteins. Therefore, a more detailed investigation in this area is warranted.

1.7. Peptidomics and bioinformatics to identify bioactive peptides

To comprehensively characterize the numerous peptides generated during protein digestion, peptidomics analysis is applied. This process begins with the preparation of biological samples containing peptides, followed by their detection. Mass spectrometry (MS), particularly liquid chromatography-tandem mass spectrometry (LC-MS/MS), serves as the primary tool for high-throughput peptide identification (Giacometti & Buretić-Tomljanović, 2017; Li-Chan, 2015). MS enables the determination of both the molecular mass and structural details of peptides, allowing in principle for the identification of certain post-translational modifications, such as oxidation, phosphorylation, and glycation (Rajendran, Mason, & Udenigwe, 2016; Secher et al., 2016; Zenker et al., 2020). LC-MS/MS has been widely used in studies involving various peptide sources, such as SP, PP, faba beans, human milk, and dairy-based IF (Asledottir et al., 2023; De Angelis, Pilolli, Bavaro, & Monaci, 2017; Gan et al., 2019; Wada, Phinney, Weber, & Lönnerdal, 2017). The detected peptide data is subsequently compared to existing peptide databases or protein sequence databases to identify known peptides, while other approaches are needed for the discovery of novel peptides. In cases where identified peptides do not match any entries in existing databases, *de novo* sequencing is required for peptide identification. Additionally, peptidomics can include quantitative analysis to determine the abundance of different peptides in a sample (Panchaud, Affolter, & Kussmann, 2012).

Bioinformatics, recognized for its cost-effectiveness and high throughput, is a valuable tool that applies computational methods to analyze and interpret complex biological data (Li-Chan, 2015;

Wang, Wang, Liu, & Fu, 2020). In this thesis, it is applied to decipher the BPs derived from plant proteins during simulated infant digestion. This *in silico* study aids in predicting the potential bioactivity of many peptides simultaneously, based on their AA sequences, thereby accelerating the screening of peptides with potential for the desired biological properties (Agyei, Tsopmo, & Udenigwe, 2018). For example, BPs related to antioxidant, antimicrobial, or immunoreactive activities can be recognized (De Angelis, Bavaro, Forte, Pilolli, & Monaci, 2018; Duan, Leng, Chen, Zhang, & Li, 2023; Lynn et al., 2004; Tejano, Peralta, Yap, Panjaitan, & Chang, 2019). However, it is essential to emphasize that while bioinformatics serves as a valuable initial step, it cannot substitute experimental validation.

1.8. Research objectives and outline of this thesis

Plant proteins are promising dairy alternatives for IF, yet a comprehensive evaluation of their suitability is still needed. In this thesis, apart from the currently already used protein source of SP, also PP as a potential plant protein was chosen for investigation. Furthermore, proteins are always heat-treated to produce powdered IF. This may lead to structural modifications (including glycation and non-glycation modifications), potentially affecting the digestibility, immunogenicity, and bioactivity of the proteins and their digesta. Especially for plant proteins, an in-depth integrated analysis of the interrelationships of these aspects has yet to be performed, especially when it comes to infant digestion. To enhance our knowledge in this area, the overarching objective of this research was to investigate how heat processing of SP and PP, with a focus on structural modifications, impacts their infant digestion and peptide formation. This thesis also explored the residual immunogenicity and bioactivity of resulting peptides using bioinformatics and experimental approaches.

Chapter 2 offers a comprehensive evaluation of critical factors associated with applying plant proteins in IF, including protein quality, ANFs, allergenicity, and various technical requirements, providing insights for informed plant protein selection for IF. **Chapters 3 & 4** delved into the effect of heating on *in vitro* infant digestion. In **Chapter 3**, the structural changes of SP and PP due to wet heating and their relation to *in vitro* infant digestion were studied. In **Chapter 4**, the role of aggregation and glycation upon dry heating on *in vitro* digestion of SP and PP, in the presence or absence of glucose, was investigated. Importantly, due to the different digestion behaviors resulting from different heating conditions, the composition and physicochemical properties of the resulting peptides may differ, thus leading to different

biological activities. To further understand this, **Chapter 5** focused on understanding the differential impact of wet and dry heating on SP and PP, with an emphasis on glycation levels, peptide formation during *in vitro* infant digestion, as well as the sRAGE-binding and antimicrobial bioactivities of the resulting peptides. Furthermore, a bioinformatic tool was used to predict potential antimicrobial peptides. **Chapter 6** explored the residual immunogenicity of SP after different heating and digestion conditions. Bioinformatics was used to predict potential T-cell and linear B-cell epitopic peptides, along with the search for possible cross-reactive peptides from other legumes in the database. Finally, **Chapter 7** summarized the results of this thesis, interpreting them in the context of existing literature, and presents the main conclusions and future perspectives.

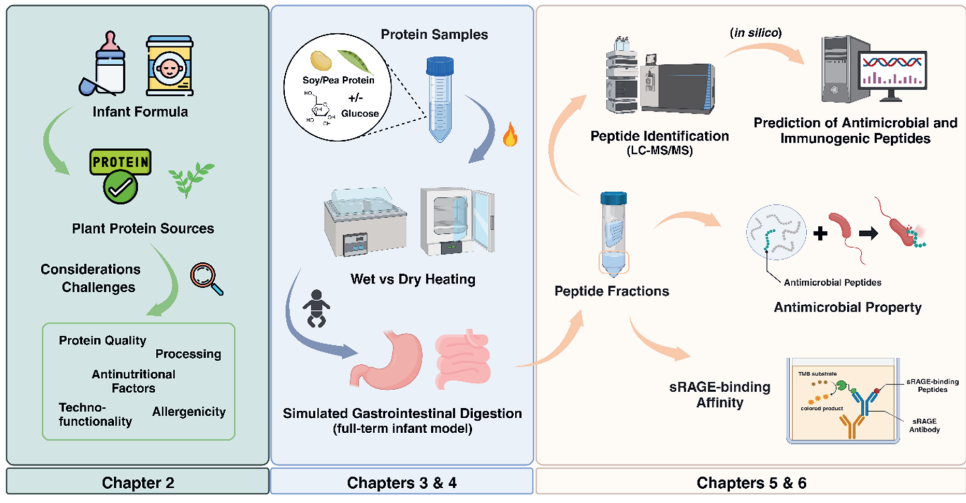

Figure 1.2. Schematic overview of this thesis.

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CHAPTER 2

Comprehensive evaluation of plant proteins as potential dairy substitutes in infant formula: A review

This chapter has been submitted as:

Tang, J., Wichers, H. J., & Hettinga, K. A. Comprehensive evaluation of plant proteins as potential dairy substitutes in infant formula: A review.

Abstract

Apart from breastmilk, infant formula is the only solution to provide adequate nutrition to ensure the growth and development of infants. While cow's milk proteins are an excellent choice for fulfilling most nutritional requirements, they can trigger allergies in some infants. As a result, plant proteins have emerged as a viable solution, as some of them are well-tolerated by infants suffering from cow's milk allergy. Furthermore, they are also vegan-friendly, environmentally sustainable, and cost-effective. Consequently, the interest in developing novel infant formula based on high-quality plant proteins as dairy substitutes is increasing. This review comprehensively evaluated the critical factors associated with applying plant proteins in infant formula, including protein quality, antinutritional factors, allergenicity, and various technical requirements such as protein content, yield, purity, extraction methods, potential contaminants, and techno-functional properties. This analysis thereby provides valuable insights for the development of innovative and functional plant-based infant formula.

2.1. Introduction

Breastmilk is widely considered as the optimal source of nutrition for infants, with the WHO recommending exclusive breastfeeding for the first six months of life. Despite this, exclusive breastfeeding rates were only 48% for infants aged 0-5 months during 2015-2021, potentially due to challenges like insufficient supply or breastfeeding difficulties (Boyd, Quigley, & Brocklehurst, 2007; UNICEF, 2022). When breastmilk is unavailable or inadequate, infant formula (IF) becomes the sole viable substitute. Currently, cow's milk-based IF dominates the market. They are formulated from milk and whey powders and supplemented with nutrients to mimic breastmilk's composition, thereby guaranteeing the fulfillment of energy and nutritional needs crucial for the growth and development of infants. Industrial production of powdered IF usually involves processing like dry storage of ingredients, wet mixing, oil blending, pasteurization, evaporation, homogenization, and spray drying (Sun, Wang, Wang, & Guo, 2018). Furthermore, as global IF demand surges and projections indicate its continued rise in the coming years, interest in exploring novel and innovative substitutes intensifies.

One such alternative being explored is plant protein-based IF, which is a promising option for infants with cow's milk allergy (CMA) (Dupont et al., 2020; Maryniak, Sancho, Hansen, & Bøgh, 2022; Venlet, Hettinga, Schebesta, & Bernaz, 2021). Furthermore, plant proteins are not only environmentally friendly, abundant, and cost-effective, but some are also nutritionally adequate. Thus, comprehending the consequences of substituting dairy proteins with plant proteins in IF, both from the nutritional and techno-functional perspectives, is necessary given the current knowledge gap.

To summarize, this review specifically focuses on plant proteins, discussing crucial aspects of quality assessment involving protein quality, antinutritional factors (ANFs), allergenicity, and other constraints such as protein content, yield, purity, extraction methods, potential contaminants, and techno-functional properties in view of their application in IF. By doing so, this review furnishes valuable insights into the industrial production of plant protein-based IF.

2.2. Protein requirements for infant growth

Proteins are an essential building block of life and are critical to support metabolism, the breakdown and uptake of which depend on factors like protein sources, food processing, food matrix, and circumstances in the digestive tract. Furthermore, the nutritional value of proteins is dependent on the types and amounts of essential amino acids (EAAs). Especially for IF, the ratio of EAAs should closely match the needs of the growing infant. In addition to the eight EAAs that adults need, infants have to absorb three extra EAAs from the diet, i.e., histidine, cysteine, and tyrosine, because their synthesis mechanisms from ribose-5-phosphate, methionine, and phenylalanine are not yet fully developed (Thompkinson & Kharb, 2007).

For infants, dietary proteins fulfill a major role in nutrition as well as in the development of e.g., the immune system. Protein digestion in infants is more limited compared to adults, due to an immature digestive system, potentially contributing to inadequate provision of EAAs and thus nutritional deficiencies. Additionally, infants are born with an immature immune system, which will gradually develop in the first years after birth. Provided the massive nutritional demands for immune development are not met, weak immunity may arise (Rijkers, Niers, Stasse-Wolthuis, & Rombouts, 2010). Dietary proteins also affect the (in)tolerance to them, as well as the potency of the digesta to initiate intestinal immunological reactions in infants. For developing infants, it is crucial to supply foods that offer an optimal potential to establish tolerance to dietary proteins (Koplin & Allen, 2014; Rijkers et al., 2010). Therefore, proteins in IF should be well-tolerated by infants, easily digestible, and mimic breastmilk's EAA composition as closely as possible to ensure proper infant development.

As mentioned, insufficient EAA intake during infancy can lead to nutritional deficiencies. Conversely, excessive protein consumption adds extra burden on organs such as the kidneys, liver, and bones, and might thereby increase the risk of metabolic imbalance and obesity later in life (Delimaris, 2013; Larqué et al., 2019). Moreover, due to physiological immaturity, especially of the kidneys, infants have much lower maximum nitrogen loads than adults, necessitating strict protein limitations. The average requirements and safe levels for infants (0-6 months) according to FAO, WHO, and UNU (2007) and the European Commission (2016), are provided in **Table 2.1**, while the recommended protein contents in IF are shown in **Table 2.2**. Due to the limited protein intake by infants, the requirements on protein composition, and especially EAAs, are thus more difficult to achieve than in adults.

Table 2.1. Protein requirements for infants (0-6 months).

Age of months	Average requirement (g protein/kg body weight per day)	Safe level
1	1.41	1.77
2	1.23	1.50
3	1.13	1.36
4	1.07	1.24
6	0.98	1.14

Table 2.2. EU-recognized infant formulas (IFs) and their recommended protein contents.

Infant formula	Minimum	Maximum	Minimum	Maximum
manufactured from	g/100 kcal		g/100 g in IFs	
Cows' milk or goats' milk proteins	1.8	2.5	1.08	1.75
Soy protein isolates	2.25	2.8	1.35	1.96
Protein hydrolysates	1.86	2.8	1.12	1.96

Table 2.3. The scoring patterns of essential amino acids (EAAs) for infants (0-6 months).

EAAs	Scoring pattern (mg/g protein)	
Reference	FAO et al. (2007)	FAO (2013)
		Refer to breastmilk
Histidine	20	21
Isoleucine	32	55
Leucine	66	96
Lysine	57	69
Methhionine + Cysteine	27	33
Phenylalanine + Tyrosine	52	94
Threonine	31	44
Tryptophan	8.5	17
Valine	43	55

2.3. Significance of plant proteins as dairy protein alternatives in infant formula

Cow's milk proteins, as the primary protein sources in IF, are notable for their rich content of EAAs, easy digestibility, and lack of ANFs (Pereira, 2014). Nonetheless, these dairy-derived products are not suitable for infants who suffer from CMA, which is a prevalent allergic condition in infancy that leads to adverse reactions, such as urticaria, vomiting, and diarrhea (Rona et al., 2007). The prevalence of CMA is relatively high in the first year after birth, due to the immaturity of the immune system of infants (Wood, 2003).

The increasing incidence of CMA has led parents of infants with this condition to seek alternative formulas (Verduci et al., 2019). The first alternative is hydrolyzed milk protein-based IF, e.g., the extensively hydrolyzed IF based on whey and/or casein proteins. This type of formula has been developed as a response to CMA in many countries. During hydrolysis, proteins are cleaved into short peptides (< 3 kDa), that no longer trigger an allergic response. Its high price may, however, discourage parents, and its bitter taste may also not be well-liked by infants.

In addition to hydrolyzed milk protein formula, plant protein-based IF is nowadays another option for infants with CMA, as plant proteins are generally well-tolerated by these infants. Plant proteins offer several benefits beyond the prevention of an allergic response. Firstly, plant-based food systems require less land, water supply, and energy than animal-based systems (Pimentel & Pimentel, 2003). Secondly, plants are easily cultivated, with for some plants their proteins being extractable and concentratable for IF production. Moreover, plant-based IF aligns well with the preferences of vegan families, and contributes to enhanced accessibility, affordability, and environmental sustainability. However, plant proteins typically fall short of the nutritional properties of dairy proteins, due to their lower digestibility and limiting EAA levels, as well as the presence of ANFs (Friedman, 1996). Consequently, only specific plant proteins, i.e., soy protein isolate and hydrolyzed rice proteins, meet the nutritional requirements of infants and are legally approved for use in IF (**Table 2.2**) . In the latter case, some EAAs need to be added for nutritional adequacy (Dupont et al., 2020). Yet, some other plant protein sources also have good AA profiles and have been proposed as potential dairy substitutes, including potato, quinoa, pea, faba bean, lentil, and chickpea (Gorissen et al., 2018; Maryniak et al., 2022; Venlet et al., 2021). Besides their nutritional properties, plant proteins differ in their structural and techno-functional properties, which pose distinctive challenges when applied in

industrial production of IF. Thus, the formula designers must carefully navigate these challenges to fulfill nutritional requirements while maintaining the desired attributes of texture, stability, and sensory properties of the final product.

2.4. Considerations and challenges

2.4.1. Protein quality

Protein quality is a composite measure influenced by both EAA levels and digestibility, reflecting a protein's capacity to fulfill the body's EAA requirements and physiological needs. Because infants have far lower recommended protein intake than adults, consuming high-quality protein is more important for them.

To evaluate protein quality, the initial step involves calculating the amino acid score (AAS), which compares the concentration of the first limiting EAA to that found in a reference pattern. An earlier report from FAO et al. (2007) provided AA scoring patterns for various age groups (toddlers, children, adolescents, and adults). Nevertheless, a more recent report from FAO (2013) introduced modifications to the 2007 report. Specifically, for infants aged 0-6 months, the recommended reference pattern is based on the EAA contents of breastmilk, aiming to predict the protein quality of infant nutrition. The two different scoring patterns of infants (0-6 months) from these two reports are presented in **Table 2.3**. This updated recommendation in the 2013 report is rooted in the understanding that the intake of breastmilk from a healthy and well-nourished mother is sufficient to meet protein requirements for this age group, although the AA intakes of infants from breastmilk are likely to be in excess of the actual demand. Furthermore, for young children aged 0.5-3 years, the reference pattern from the previous 2007 report for infants (0-6 months) is more suitable. For older children, adolescents, and adults, the recommended pattern is derived from the group of children aged 3-10 years from the 2007 report.

Subsequently, the resulting AAS values are corrected for the true fecal digestibility of the tested protein, yielding the protein digestibility-corrected amino acid score (PDCAAS), the conventional standard since 1993. Any PDCAAS value exceeding 1.0 will be truncated to 1.0 (Schaafsma, 2000). However, the use of fecal digestibility leads to the overestimation of the nutritional value of a protein, whereas the truncation to 1.0 results in higher quality proteins not being identified or highlighted. More importantly, the presence of ANFs in plant protein sources and the consequences of food processing, such as heating, are not taken into consideration,

leading to the overestimated AA bioavailability (e.g., lysine) (Leser, 2013; Marinangeli & House, 2017).

To address these concerns, the digestible indispensable amino acid score (DIAAS) emerged in 2013 as the recommended standard for protein quality assessment (FAO, 2013). DIAAS uses ileal digestibility instead of fecal digestibility, accounting for endogenous losses of AAs, thus resulting in potentially lower values compared to PDCAAS. Interestingly, despite that DIAAS values for individual ingredients may exceed 1.0 with truncation, for infants consuming sole protein-source food like IF, the DIAAS value still remains truncated at 1.0 (Marinangeli et al., 2017). However, ethical and clinical constraints, especially in the case of infants who are more vulnerable than adults, significantly impact the feasibility of the DIAAS. The preferred method involves *in vivo* infant digestion and invasive sample collection, which apparently causes issues related to the safety, well-being, and comfort of infants. Therefore, researchers rely more on *in vivo* animal models such as piglets (Bouzerzour et al., 2012) and rats (Rutherfurd, Fanning, Miller, & Moughan, 2015), simulated *in vitro* digestion methods (Maathuis, Havenaar, He, & Bellmann, 2017), and extrapolations from adult studies to infer potential DIAAS values in infants.

Given the substantial variability in the nutritional value of various plant protein sources, it is important to carefully evaluate their PDCAAS and DIAAS for informed selection in IF. Some plant proteins show promising scores in these regards. For instance, potato protein has scores of 0.87 and 0.85 for PDCASS and DIAAS, respectively, while pea protein has scores of 0.78 and 0.66 (Hertzler, Lieblein-Boff, Weiler, & Allgeier, 2020). Chickpea protein, on the other hand, scores 0.92 and 0.83, respectively (Monsonego Ornan & Reifen, 2022). Quinoa protein has an approximate PDCAAS of 0.8 (Venlet et al., 2021). It is noted that these values were calculated based on different digestion models and AA scoring patterns tailored to specific age groups. In addition, when assessing protein quality for infants aged 0-6 months, both reported scoring patterns are often simultaneously used for AAS calculation in the literature. Furthermore, the protein composition and AA profile of plant proteins can diverge depending on factors such as the selection of specific subspecies or cultivars of plants, as well as their growing conditions and processing methods. Consequently, all these factors can significantly impact the final PDCAAS and DIAAS of a given plant protein source. Thus, a detailed analysis is necessary for each specific plant protein source, and food matrix it is used in, to determine its protein quality accurately. For IF, this means that the specific plant protein sources used (including variety and growth location), as well as the protein extraction procedure used and

the IF food matrix, should be included in the protein quality assessment, instead of relying on literature values for EAA composition.

Furthermore, since the EAA profile of a plant protein usually does not fully match that of breastmilk, fortification with specific (limiting) AAs is a common strategy to enhance the nutritional quality of IF. For instance, the addition of lysine, threonine, and tryptophan ensures the aminogram of rice protein-based IF more closely to breastmilk, thus the EAA requirements of infants are met (Dupont et al., 2020). Another potential solution could be the use of a combination of various protein sources (Dimina, Rémond, Huneau, & Mariotti, 2021). However, beyond the nutritional considerations, it is important to acknowledge that in a mixed protein system, intricate interactions can take place among various protein sources. Consequently, the solubility, viscosity, and particle size may be subject to alteration, potentially affecting the overall stability and sensory attributes of the end product (Lima Nascimento et al., 2023). Therefore, assessment of protein quality of actual IF products, as mentioned above, would be important.

2.4.2. Antinutritional factors (ANFs)

What distinguishes plant proteins from animal proteins is, amongst others, also the presence of ANFs in plant protein sources. These ANFs may resist processing and undesirably remain in the final plant proteins-based products. Therefore, different processing methods can potentially yield varied outcomes in terms of the category and content of ANFs. Naturally occurring ANFs are compounds produced by plant metabolism and serve as an important protective barrier for plants against bacteria, viruses, and animals. They are widespread in the plant kingdom and are the result of long-term natural selection. ANFs can be categorized based on their chemical structure into proteins (e.g., protease inhibitors and lectins), glycosides (e.g., saponins), phenols (e.g., tannins), and others (e.g., antimetals and antivitamins).

2.4.2.1. Adverse impacts on nutritional and health aspects

Notably, consumption of plant-based IF containing ANFs can exert negative effects on nutrition and health of infants through different mechanisms. Taking specific ANFs as examples, compounds such as protease inhibitors, commonly found in soybeans, can impede protein digestion, limiting the absorption and availability of EAAs required for infant growth (Friedman & Brandon, 2001; Hathcock, 1991; Roos et al., 2013; Xiao, Wood, Robertson, & Gilani, 2012). Phytates, which are found in foods like legumes and grains, can hinder the absorption of essential minerals like calcium, iron, and zinc and also the protein availability,

potentially leading to mineral deficiencies and compromised cognitive and immune development (Carnovale, Lugaro, & Lombardi-Boccia, 1988; Masum Akond, Crawford, Berthold, Talukder, & Hossain, 2011; Reddy, 2001; Sandberg, 2002). Lectins, present in foods like beans and lentils, may affect carbohydrate availability, and may also trigger allergic reactions that could manifest as gastroenteritis or skin irritations (Barre, Damme, Simplicien, Benoist, & Rougé, 2020; Pan, Farouk, Qin, Zhao, & Bao, 2018; Sun et al., 2019). Saponins, present in e.g., beans and quinoa, can cause hemolysis by interacting with the cholesterol in the red blood cell membranes, cause indigestion through enzyme inhibition, and decrease nutrient absorption, of e.g., minerals and vitamins, by binding to small intestine cells (Popova & Mihaylova, 2019; Samtiya, Aluko, & Dhewa, 2020). Tannins, concentrated in legume brans, can not only depress digestive enzymes reducing protein digestibility, but can also interact with carbohydrates, minerals, and vitamins, thereby interfering with their absorption (Amarowicz, 2007; Joye, 2019).

Due to the low gastric acidity and high intestinal permeability of infants, ANFs are more likely to resist digestion and transfer across the intestines during early life (Weström, Arévalo Sureda, Pierzynowska, Pierzynowski, & Pérez-Cano, 2020). However, studies on the consequences of ANFs on infants are rare, and it still remains unclear to what extent ANFs would impact protein quality and digestion, and the subsequent risks of nutritional deficiencies and related health issues. Therefore, to ensure adequate nutrient intake and minimize health risks for infants, it is advisable to aim for the lowest possible level of ANFs in IF.

2.4.2.2. Inactivation and removal strategies

The residual ANFs present in plant proteins pose challenges to the utilization of plants proteins in IF. Thus, various effective inactivation and removal strategies are imperative during the extraction and processing of plant proteins to mitigate the negative effects of ANFs.

Heat treatment is one of the simplest and most efficient approaches to eliminate ANFs. One reason is that heat-sensitive ANFs are more abundant in plant foods than heat-stable ANFs (Kong, Li, & Liu, 2022). Techniques including e.g., cooking, boiling, blanching, steaming, microwaving, and roasting, are applied to denature the structure of heat-labile ANFs. Nonetheless, excessive processing may induce protein damage leading to lower protein quality, negatively affect techno-functional properties such as solubility, and destroy other important components such as heat-labile vitamins. Thus, the selection of the duration and intensity of heating is important. For instance, in the case of soybeans, subjecting them to heating at 100°C for 9 min led to the inactivation of most trypsin inhibitors (Avilés-Gaxiola, Chuck-Hernández,

& Serna Saldívar, 2018). Additionally, a 5-minute autoclaving of soybean flours removed over 90% of lectins and trypsin inhibitors, while maintaining protein solubility above 80% (Machado et al., 2008).

In addition to heating, various other strategies can reduce ANFs as well. Physical method of soaking can leach out hydrophilic ANFs including tannins, trypsin inhibitors, and saponins, but during that step, also soluble proteins might move into the discarded solution (Avilés-Gaxiola et al., 2018; Samtiya et al., 2020), which may actually be the protein fraction of interest for the production of IF. Mechanical techniques such as milling, grinding, and sieving can remove the outer layers of plants, which are rich in ANFs such as phytates, lectins, and tannins. However, the mineral content might be compromised during such processes (Gupta, Gangoliya, & Singh, 2015), although this may not be a problem for IF, as minerals would be supplemented to such products anyway. Biotechnological methods, like germination and fermentation, can activate endogenous enzymes (e.g., phytase, protease, and β-glucosidase) present in raw materials, leading to the degradation of specific ANFs and release bound nutrients (Das, Sharma, & Sarkar, 2022). Furthermore, the exogenous addition of these enzymes during processing could also yield positive outcomes. For instance, producing soy protein isolates by phytase-assisted processing can lower phytate content, resulting in increased protein content and digestibility compared to more traditional isolation methods (Wang, Chen, Hua, Kong, & Zhang, 2014). Regulation of pH has the potential to alter ANFs' structures and reduce their biological activity by chemical interactions. By the addition of 0.5% citric acid, for instance, the levels of trypsin inhibitors and lectins in soybean meal were decreased, but simultaneously resulted in a decrease of glycinin and β-conglycinin (Norozi, Rezaei, & Kazemifard, 2022). Thus, caution is necessary to prevent protein denaturation and unwanted chemical reactions, which may lower the nutritional quality that is essential for products such as IF.

Furthermore, increasing interest is found in exploring other emerging processing techniques, such as extrusion, ultrafiltration, ultrasound, high hydrostatic pressure, pulse electric field, irradiation, and genetic modification, as potential strategies to mitigate ANFs. Moreover, the combination of different approaches can have synergistic effects on ANF removal as well. However, it is noteworthy that while these methods effectively target ANFs, they may also influence the overall nutritional value and techno-functional properties of plant proteins, with some of these changes being detrimental to the quality of the final IF products. Hence, it is essential to carefully select appropriate processing for ANF removal.

2.4.3. Allergenicity

Food allergy is an adverse immune response to allergens, primarily dietary proteins, and can occur upon repeated exposure to specific foods (Boyce et al., 2011). Eight major allergic foods include milk, egg, fish, shellfish, wheat, peanut, tree nut, and soybean (Cordle, 2004), half of which originate from plant sources. This phenomenon includes three allergy types: IgE-mediated, non-IgE-mediated (e.g., cell-mediated), and a combination of both. IgE-mediated immunologic reactions generally exhibit acute symptoms such as acute urticaria; whereas non-IgE-mediated reactions (e.g., food protein-induced enterocolitis syndrome) and mixed IgE and non-IgE reactions (e.g., atopic dermatitis) manifest with delayed onset or chronic symptoms (Burks et al., 2012). Among these, IgE-mediated type I hypersensitivity reactions pose a relatively high health risk. Most type I food allergies emerge within the first two years of life (Suresh, Kim, Sicherer, & Ciaccio, 2022; Wood, 2003).

Because plant proteins can also elicit allergic responses in infants, their utilization as substitutes for dairy proteins in IF against CMA is constrained. Although soy is the primary plant protein source applied in plant-based IF, controversy persists regarding its suitability, due to its high potential for causing allergies. Besides, 10% to 14% of infants with CMA are allergic to soy proteins as well (Kattan, Cocco, & Järvinen, 2011; Klemola, Vanto, Juntunen-Backman, Kalimo, Korpela, & Varjonen, 2002; Zeiger et al., 1999). Moreover, allergenic cross-reactivity may occur between soybean and other legumes, including e.g., peanut, lentil, lupine, pea, and chickpea, which can be attributed to the presence of shared epitopes within the allergens of these legumes (L'Hocine & Boye, 2007; Verma, Kumar, Das, & Dwivedi, 2013). This emphasizes the need for cautious consideration of allergenicity when developing legume-based IF. Conversely, hydrolyzed rice protein, another plant protein source permitted in IF, appears to be a safer option for infants with CMA (Vandenplas et al., 2021). Besides rice being relatively hypoallergenic, an additional reason is the enzymatic hydrolysis that reduces the allergenicity of intact proteins (Pali-Schöll, Untersmayr, Klems, & Jensen-Jarolim, 2018). Research indicates that 92 infants with IgE-mediated CMA were well tolerating rice protein-hydrolyzed IF (Reche et al., 2010). Furthermore, other plant proteins like quinoa and potato proteins, are being considered due to their lower allergenicity and limited reported allergy cases (Maryniak et al., 2022). Nevertheless, given that quinoa proteins are not commonly consumed in western countries, the introduction of quinoa protein-based foods into the market could potentially result in an increased occurrence of sensitization among certain individuals, although no quinoa allergens are reported by WHO/IUIS Allergen Nomenclature Sub-Committee

(http://www.allergen.org/, accessed August 2023).

Allergenicity is subject to influence by both food processing and digestion. During processing, structural alterations such as denaturation, aggregation, and glycation can potentially modify epitopic properties. For example, the exposure of conformational epitopes in soy protein isolate due to heat-induced unfolding has been linked to increased IgE-binding capacity (Pi, Liu, Sun, Ban, Cheng, & Guo, 2023). Conversely, glycation-induced conformational changes and modifications in key residues within IgE epitopes of tropomyosin led to reduced allergenicity (Fu, Wang, Wang, Ni, & Wang, 2019; Han et al., 2018). Furthermore, the physicochemical changes resulting from processing can impact gastrointestinal digestion and absorbance kinetics, contributing to variations in the formation of large allergenic fragments (conformational epitopes) and linear/sequential epitopic peptides, as well as their presentation to the immune system (Rahaman, Vasiljevic, & Ramchandran, 2016; Tan, Nawaz, & Buckow, 2023). For instance, the digestibility of β-lactoglobulin was enhanced under high hydrostatic pressure, resulting in decreased allergenicity of its hydrolysate (López-Expósito, Chicón, Belloque, López-Fandiño, & Berin, 2012). Moreover, it is important to note that infants, due to their high gastric pH and gut permeability (Nguyen, Bhandari, Cichero, & Prakash, 2015; Weström et al., 2020), and immature immune system, may face a heightened risk. These factors can render allergenic fractions more resistant to gastric digestion and more likely to cross the intestinal barrier, thereby triggering allergic responses. Therefore, to prevent allergic responses to plant protein-based IF, both the source of protein as well as its processing needs to be carefully considered.

2.4.4. Other constraints

2.4.4.1. Content, yield, purity, and extraction methods of plant proteins

When selecting suitable plant protein sources as dairy substitutes in IF, it is necessary to consider protein content, as well as yield and purity of the isolated protein ingredient, in conjunction. The protein content in raw plants serves as an initial indicator. Taking the protein-rich legumes as an example, the approximately protein contents (w/w) of some legumes are: lupin (44%), soybean and faba bean (40%), pea (30%), and chickpea (25%) (Nawaz, Tan, Øiseth, & Buckow, 2022), although large variation can be expected depending on e.g., variety and environmental growing conditions. However, despite certain plants possessing a high protein content, the achievable protein yield may in some cases still be insufficient for commercial production. Protein yield signifies the fraction of proteins obtained or extracted in relation to the total protein content in the raw materials. Furthermore, residual impurities after

extraction can diminish protein purity (the net protein content within protein extracts), including unwanted impurities such as ANFs and harmless residues such as sugars.

The yield and purity can vary due to the distinct properties of proteins in diverse tissues and the specific extraction methods applied. Especially for plant sources, the extraction methods are supposed to effectively break down cell walls to free proteins. Various methods are utilized for cell lysis, including mechanical methods such as bead milling, ultrasound, and high-pressure homogenization, as well as non-mechanical methods such as the addition of chemical agents (e.g., detergents, chelating agents, acids and bases) and enzymes (e.g., cellulases, pectinases, and hemicellulases) (Stirk et al., 2020). However, the application of chemical procedures may lead to the denaturation and/or degradation of proteins, through e.g., the interactions with acidic and basic reagents. In addition, there is added concern of introducing new toxic contaminants and undesired impurities into the final protein extract (Fido, Mills, Rigby, & Shewry, 2004).

Following cell lysis, subsequent extraction methods must be applied, with two conventional ways being isoelectric precipitation and salt extraction. Research reported that, when extracting proteins from chickpea, faba bean, pea, and soybean, the isoelectric precipitation method yielded protein purities ranging from ~84% to 89%, whereas salt extraction resulted in lower purity levels of 73% to 82% (Karaca, Low, & Nickerson, 2011). Nevertheless, conventional methods have their limitations, including e.g., altered techno-functional properties, undesirable color changes, and reduced nutritional quality. To address these limitations, innovative extraction technologies have been developed, such as enzyme-assisted extraction, microwave-assisted extraction, ultrasonic-assisted extraction, and reverse micelle extraction, offering advantages like higher yield, reduced processing time, eco-friendliness, less adverse impact on techno-functional properties, and lower solvent usage (Hewage, Olatunde, Nimalaratne, Malalgoda, Aluko, & Bandara, 2022; Kumar et al., 2021). On the other hand, the extraction method of air classification can also be used. This method is relatively mild, but leads to lower purity of the resulting ingredients (Xing et al., 2020). However, many of these novel techniques are currently at the laboratory scale and face challenges in terms of scalability and cost-effectiveness for industrial adoption (Aimutis, 2022; Hewage et al., 2022). Therefore, further efforts are required to adapt and optimize these methods for large-scale industrial production of IF while controlling costs and also maintaining optimal nutritional and technical functionalities.

2.4.4.2. Plant-derived chemical contaminants

The first years of newborns are crucial since this is when their nervous, reproductive, digestive, respiratory, and immune systems undergo rapid development. However, food-borne contaminants can pose potential risks during this critical stage, with infants in the first months of life being particularly vulnerable (Hardy et al., 2017; Pereira, Almeida, Leandro, Da Costa, Conte-Junior, & Spisso, 2020). Food processing, such as protein extraction, may both lead to diminishing these contaminations, but may also unexpectedly lead to the accumulation of these harmful substances.

Heavy metals and pesticide residues are two commonly seen plant-derived chemical contaminants. Plants can absorb and accumulate heavy metals from contaminated soil, water, and air sources (Shaban, Abdou, & Hassan, 2016). Four toxic heavy metals, namely lead, arsenic, mercury, and cadmium, can have irreversible adverse effects on infant health even at small doses, leading to lowered intelligence quotient, developmental delays, and hyperactivity disorder (Bair, 2022; Choi, Chang, Hong, Shin, Park, & Oh, 2017). Thus, heavy metals must be eliminated. For instance, the high levels of arsenic and cadmium in rice must be removed before making rice-based foods (De Paiva, Morgano, & Arisseto-Bragotto, 2019), such as IF.

Pesticides are commonly used to safeguard plants from weeds, fungi, and pests like insects. However, the overuse or abuse of pesticides can lead to their incomplete removal from plants. This can result in acute toxicity in humans when consuming plant-based foods with high residual pesticide levels and chronic poisoning when exposed to low pesticide doses over time (Pereira et al., 2020; Singh, Gupta, Kumar, & Sharma, 2017). Pesticides can be categorized based on their chemical structures, into organochlorines, organophosphates, nitrogen-containing pesticides, plant-originated pesticides, and several others (Shaban et al., 2016). Four of them, organochlorines, organophosphates, carbamates, and pyrethroids, have the potential to cause developmental neurotoxicity in children, affecting cognitive, behavioral, sensory, motor, and morphological functions (Chen et al., 2014). Recent research has even identified residues of glyphosate—an organophosphates pesticide and its metabolite aminomethylphosphonic acid in commercial soy-based IF in Brazil (Rodrigues & de Souza, 2018).

Therefore, it is imperative to rigorously implement stringent quality control and safety measures to monitor the levels of chemical contaminants in the production of plant-based IF, thereby ensuring the healthy development of infants.

2.4.4.3. Techno-functional properties

The techno-functional properties of proteins, such as solubility, emulsification capacity, and viscosity, can impact their behavior throughout processing, storage, and consumption, ultimately playing a pivotal role in shaping the desired nutritional, technical, and sensory attributes of the final IF products.

In terms of solubility, proteins should readily dissolve in water to produce an IF that is uniform and easily mixable. Moreover, from a consumer perspective, protein solubility also influences the reconstitution/rehydration process of IF. Poor water solubility can lead to the formation of clumps or sediment, which, in turn, potentially gives rise to feeding challenges and infant digestive discomfort, hence reducing nutritional quality (Rodríguez Arzuaga et al., 2021). This incomplete solubilization can even increase the risk of inducing allergic reactions, as large protein fragments are more prone to being recognized as food allergens by the immune system (Ballegaard & Bøgh, 2023). Various plant protein sources exhibit different solubility characteristics. For example, commercial canola and potato protein isolates were almost fully solubilized in water, whereas e.g., faba bean protein concentrates had a comparatively lower solubility index of approximately 40-60% (Jakobson et al., 2023). This could be partly solved for plant-based IF products, but specifically utilizing the soluble fraction of the plant protein in its production.

Emulsification capacity is crucial for proteins as well, as it enables them to effectively emulsify lipids and maintain a stable and homogenous emulsion in the IF product. This property is important for ensuring that IF products have desirable sensory attributes in terms of texture and appearance, as well as that infants receive a consistent and uniform intake of essential fatty acids with each feeding. Some plant proteins, such as those sourced from soybean, pea, chickpea, and canola, possess high emulsifying properties (Sá, Laurindo, Moreno, & Carciofi, 2022).

Likewise, viscosity of protein solutions can impact the thickness of the IF, and thus have consequences for its drinkability, texture and mouthfeel (Sá et al., 2022). The IF is supposed to achieve a texture that closely resembles breastmilk, as this enhances infants' sensorial acceptance and overall feeding experience. When the formula is too thin, it may not provide the desired mouthfeel and feeling of satiety, potentially leading to overfeeding. Conversely, if it is overly thick, it is likely to be challenging for infants to consume, resulting in feeding and nutritional problems.

Moreover, different protein sources vary in their protein composition, including e.g., albumins, globulins, glutelins, and prolamins. Notably, when considering legume proteins, the globular proteins can even have different ratios of subfractions like legumin, vicilin, and convicilin. Each of these fractions possesses unique techno-functional properties and differently contributed to the overall techno-functionality of the protein source (Sim, SRV, Chiang, & Henry, 2021). In addition, glycation, a process involving the interaction of proteins with reducing sugars, may introduce variations in the techno-functional properties of plant-based proteins (Kutzli, Weiss, & Gibis, 2021). Furthermore, the utilization of different food processing technologies can also potentially cause such influence (Dhiman et al., 2023). For example, when comparing the drying methods of quinoa protein isolates, freeze drying improved emulsifying and oil-holding capacities, while spray drying resulted in higher solubility and water-holding capacity (Shen, Tang, & Li, 2021). In another study conducted by Ebert, Gibis, Terjung, and Weiss (2020), it was observed that different extraction methods yielded varying solubility levels for plant proteins. For pea protein powders, solubility ranged approximately from 9% to 50%, while wheat gluten displayed solubility levels between 8% and 29%.

Overall, the techno-functional properties of plant proteins significantly impact the behavior and quality of IF, which can be influenced by factors such as protein composition, glycation, and food processing methods. Therefore, comprehensive research in this field is necessary to better understand and optimize the use of plant proteins in IF production. This should lead to the right combination of protein source, protein extraction, and IF production processes that results in high quality IF products.

2.5. Conclusions and future perspectives

Plant-based IF offers a compelling alternative to traditional dairy-based options. However, the selection of appropriate plant protein sources for its development is accompanied by multifaceted considerations and challenges. Firstly, the chosen plant proteins need to have high protein quality to satisfy the nutritional requirements of infants. One possible solution is to use synergistic plant protein blends to complement EAA profiles. Secondly, the ANFs present in plant protein sources should be removed or inactivated by proper approaches. Protein allergenicity needs to be cautiously considered and allergenicity assessments at both molecular and cell levels are required for promising plant proteins, thereby mitigating potential risks to infant health. In addition, the protein extraction techniques should be tailored to the unique

characteristics of each plant protein and the specific requirements of their application in IF. Rigorous quality control procedures are also required to detect and eliminate plant-derived contaminants that may remain after extraction and cause potential risk to the infant's health.

The techno-functional properties of these plant proteins play a pivotal role in shaping the sensory attributes (e.g., texture, mouthfeel, and appearance) of the final IF products to ensure usability, acceptance and feeding experience for infants. Among those properties, solubility is one of the most important parameters. Given that plant proteins tend to be less soluble in water, they can be separated into distinct fractions based on their solubility to maximize source utilization. A fraction with high solubility may be more suitable to be directly used in plant-based IF. Simultaneously, the insoluble portion, while less suitable for IF, can find its purpose in the development of e.g., meat analogues, contributing to the diversification of plant-based protein products and minimizing waste in the process.

Additionally, the manufacture of plant-based IF involves a series of food processing stages, which encompass processes such as plant protein extraction and ANF removal, as well as the subsequent industrial-scale steps essential for IF production. Distinct processing stages and the associated techniques applied have the potential to impact the nutritional quality, allergenic characteristics, techno-functional properties, and sensory attributes of the final IF products in various ways. Crucially, the industrial processing techniques that have been conventionally utilized for dairy-based IF will probably require adaptation or modification to accommodate the unique characteristics of plant-based protein alternatives. The scaling up of emerging processing methods from laboratory-scale to industry-scale also demands comprehensive optimization. As such, it is essential to implement a thoughtful and tailored process for the design and production of plant-based IF.

In summary, the development and widespread adoption of plant-based IF hold significant potential for infant health, nutrition, and environmental sustainability. Future efforts should give priority to the careful selection of suitable plant proteins, with a thorough evaluation of their nutritional value, health implications (including allergy risks), and safety aspects. Furthermore, addressing the processing complexities, ensuring safety and efficacy, and gauging consumer acceptance are crucial steps. In addition, long-term clinical studies are needed to comprehensively assess the nutritional and health outcomes of infants fed with these formulas.

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CHAPTER 3

Heat-induced unfolding facilitates plant protein digestibility during *in vitro* static infant digestion

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Abstract

Soy protein is the main protein source for plant-based infant formula, whereas pea protein is considered as a potential alternative plant protein source. This study assessed the structural changes of soy and pea proteins after heating between 65°C and 100°C, and its effects on the *in vitro* digestibility in the context of infant digestion. We found that with increased heating intensity, both soy and pea proteins unfolded, manifested as the increased surface hydrophobicity, thereby potentially improving the accessibility to digestive enzymes. Their final *in vitro* digestibility increased from ~30% of non-treated samples to ~60% of 100°C-heated samples for soy protein, and from ~52% to ~65% for pea protein. Surface hydrophobicity was strongly positively correlated to the overall digestibility. Therefore, the heating temperatures that enabled protein unfolding promoted the digestibility of soy and pea proteins under infant digestion conditions.

3.1. Introduction

Infant formulas (IFs) are the only acceptable breastmilk alternative to support the growth and development of infants. Proteins in IFs supply amino acids that are important contributors to support growth and metabolism. The most common protein sources are cow's milk proteins, which contain a high level of essential amino acids (EAAs) and are able to be fully digested (Pereira, 2014). However, because of the relatively high prevalence of cow's milk allergy (CMA) amongst infants, as well as sustainability considerations to lower the economic cost and ecological footprint, plant proteins are being taken into consideration as protein sources for IFs as well. Nevertheless, it should be noticed that the presence of antinutritional factors (ANFs), such as trypsin inhibitors (TIs) in plant proteins, may restrict the amino acid bioavailability of plant proteins during digestion (Martín-Cabrejas et al., 2009). TIs are known as a group of small serine protease enzyme inhibitors that directly act on trypsin and chymotrypsin, leading to a reduction in protein digestibility (Gilani, Xiao, & Cockell, 2012; Vagadia, Vanga, & Raghavan, 2017).

Soy proteins are the most common plant-based protein source and are nutritionally adequate, as evidenced by their good amino acid balance and high EAA content (Gorissen et al., 2018). However, they may act as allergens for some infants and are high in TIs (Xiao, Wood, Robertson, & Gilani, 2012). Therefore, despite that soy protein isolate is the only approved (except for hydrolyzed rice protein) plant-based protein source for IFs (0-6 months) according to European Commission (2016), it is still needed to explore novel protein substitutes for infant nutrition. Thus, a new protein source, pea proteins, has received widespread attention, in particular in those countries/regions where soybeans are not a native crop and for infants who are allergic or intolerant to soy proteins. Pea proteins are also as rich in EAAs and highly digestible, as soy proteins, but are low in ANFs and allergenicity compared to other legumes (Gorissen et al., 2018; Roux, Menard, et al., 2020).

Heat treatment is a key factor in the production of IFs. The structure transformation/denaturation occurring in proteins due to heat processing may have a great impact on the protein's digestion and absorption. For example, heating may alter the three-dimensional structure of the protein, and thereby change its digestibility. Besides, according to previous studies, severe heat treatment can also lower the activity of TIs. For instance, TIs in soy flours can be inactivated by around 80% after hot air drying at 100°C for 2 h (Agrahar-Murugkar & Jha, 2010); and heating at 100°C for 20 min caused a decrease in TIs to 13% in

soymilk (Yuan & Chang, 2010). By such heating steps, the digestibility of the soy protein can be increased (Vagadia et al., 2017; Wallace, Bannatyne, & Khaleque, 1971).

Digestibility is one of the parameters that determines the bioavailability of amino acids, which is very important for infant nutrition, as protein intake should be low, while EAA intake should be high. Many groups studied the digestibility of plant proteins but did not relate it to heating (He, Spelbrink, Witteman, & Giuseppin, 2013; Roux, Chacon, Dupont, Jeantet, Deglaire, & Nau, 2020; Roux, Menard, et al., 2020); or they studied the effect of heating on plant protein digestibility, but these were conducted in the context of adult digestion rather than of infant digestion (Rivera del Rio, Opazo-Navarrete, Cepero-Betancourt, Tabilo-Munizaga, Boom, & Janssen, 2020; Sun, Mu, Sun, & Zhang, 2014; Zahir, Fogliano, & Capuano, 2018). It is noticed that infants have an immature digestion system, in which the gastric digestion of proteins may be limited, due to a relatively high gastric pH. This may lead to reduced digestion causing a lower bioavailability of EAAs and cause nutritional deficiency. Recently, an *in vitro* static digestion model was established for full-term newborns (one month of life), the digestion parameters (e.g., enzyme activity and pH) of which were designed considering the immaturity of the infant digestion system (Menard et al., 2018). This model has now been widely used to study infant digestion (Phosanam, Chandrapala, Huppertz, Adhikari, & Zisu, 2021; Zenker, Raupbach, Boeren, Wichers, & Hettinga, 2020).

Based on the above information, it is hypothesized that soy and pea proteins can both be affected by heating, causing changes in their protein structure, thereby having an impact on the degree of *in vitro* infant digestion. Therefore, the aim of this paper is to in-depth investigate the relations between heating, structural changes, and infant digestibility of soy and pea proteins. To achieve this aim, isolated soy and pea proteins were used to avoid interference from other ingredients in plant-based IFs. Consequences of different heating conditions for the surface hydrophobicity and protein composition were studied. Besides, an *in vitro* infant digestion model for one-month-old newborns was used to explore infant digestion. The level of digestion was monitored by the degree of hydrolysis and the disappearance of intact protein.

3.2. Materials and methods

3.2.1. Materials and chemicals

Fresh soybean (*Glycine max*; protein content: 39.3%, w/w) was obtained from Wageningen Plant Research, Lelystad, the Netherlands (courtesy ing. Ruud Timmer), and pea (*Pisum sativum* L.; protein content: 23.3%, w/w) was purchased from a local retailer (Brand name: HAK). NuPAGE LDS sample buffer (4×), NuPAGE 12% Bis-Tris Protein Gel, NuPAGE MOPS running buffer (10×), NuPAGE reducing agent (10×, dithiothreitol), and PageRuler Prestained Protein Ladder (10 to 140 kDa) were obtained from Thermo Fisher Scientific (Massachusetts, USA). Coomassie brilliant blue R-250 was obtained from Bio-Rad (California, USA). Porcine pepsin and porcine pancreatin, as well as all other chemicals, were obtained from Sigma-Aldrich (Missouri, USA).

3.2.2. Preparation of soy and pea protein

From our earlier research, we found that industrially prepared plant proteins isolates have already undergone high heat treatment and would not be sensitive to heat treatment, therefore the whole extraction is conducted at low temperature to avoid interference from heat. Soy protein (SP) and pea protein (PP) were prepared according to the methods of Karaca, Low, and Nickerson (2011) with some modifications. Briefly, fresh soybean and pea were peeled and crushed into fine flours using a 6875D Freezer/Mill cryogenic grinder (Spex SamplePrep, Rickmansworth, UK) with liquid nitrogen. Then, flours were defatted three times by the addition of petroleum ether at a ratio of 1:3 (w/v), stirring for 3 h at room temperature and discarding the supernatant. Defatted meals were left overnight in a fume hood at room temperature to allow the remaining petroleum ether to evaporate. Subsequently, isoelectric precipitation was performed to precipitate SP and PP. Defatted meals were mixed with distilled water at a ratio of 1:10 (w/v), adjusted to pH 8.0 by 2 M NaOH, and stirred for 1 h at room temperature. After centrifugation (16,000 × g, 30 min, 4°C), the supernatants were collected, and the pH was adjusted to 4.5 and 4.0 with 2 M HCl, for soy and pea, respectively. Afterward, solutions were mixed, refrigerated for 1 h, and centrifuged at 16,000 × g for 30 min at 4°C. Precipitates of SP and PP were washed with distilled water and neutralized to pH 7.0 with 2 M NaOH before freeze drying. The obtained SP and PP were stored at -20°C until use. Protein contents of powdered SP and PP were determined using DUMAS Flash EA 1112 Protein analyzer (Thermo Fisher Scientific, Massachusetts, USA) in technical duplicate, using a nitrogen-to-protein conversion factor of 5.7, according to the method from Zahir et al. (2018).

3.2.3. Heat treatment of SP and PP

Extracted SP and PP were dissolved in 10 mM PBS buffer (pH 7.4) and stirred for 2 h, followed by centrifuging (10,000 × g, 10 min, 4°C) and filtrating by a 0.45 μm filter to remove the insoluble part. Protein concentration was adjusted to 12 mg/mL to better mimic IFs, as determined by Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) with bovine serum albumin as standard (He, Hu, Woo, Xiong, & Zhao, 2021). After that, samples were heated in a water bath at 65, 75, 85, and 100°C for 30 min in duplicate, and immediately cooled down on ice to stop the heating process. Non-treated (NT) samples were used as control. All the samples were stored at 4°C until use.

3.2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein composition of SP and PP before and after heating, as well as the disappearance of intact protein at each sampling point after digestion, were analyzed by SDS-PAGE, according to Zenker, Raupbach, et al. (2020). All samples were centrifuged to remove the insoluble parts and diluted to the proper concentration before analysis. SDS-PAGE was conducted under both reducing and non-reducing conditions. Briefly, protein samples were mixed with loading buffer, which consisted of LDS sample buffer (4×) and ultrapure water, and in the case of reducing conditions also reducing agent (10×). The protein sample mixtures were heated at 70°C for 10 min in a heating block. About 2.5 μg of the undigested samples or 5 μg of the digested samples were loaded onto a 12% Bis-Tris gel, using the prestained protein ladder (10 to 140 kDa) as molecular weight marker. Gels were run with MOPS SDS running buffer at a constant 120 V for around 90 min. After that, gels were stained with Coomassie brilliant blue R-250 for approximately 1 h, followed by destaining in washing buffer (distilled water with 7.5% acetic acid and 10% ethanol) overnight.

3.2.5. Particle size distribution

Particle size distribution was determined by dynamic light scattering (DLS) using a Mastersizer 2000 with ZS Xplorer software (Malvern Instruments Ltd, UK), according to Rivera del Rio et al. (2020), with minor modifications. The NT and heated samples were diluted to 1 mg/mL with 10 mM PBS buffer (pH 7.4). After being centrifuged and filtrated using a 0.45 μm filter, 1 mL of supernatant was loaded to the cuvette to do the measurements. Refractive index was set as 1.45 for the dispersed phase and as 1.33 for the continuous phase, respectively. Results were shown as volume-weighted particle size distribution. Experiments were performed in quadruplicate.

3.2.6. Surface hydrophobicity

To determine the protein surface hydrophobicity, the 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS)-assay method was adopted according to Zenker, Teodorowicz, et al. (2020). Briefly, NT and heated SP and PP samples were diluted to 0.15 mg/mL, and then were transferred to a 96-well black polystyrene plate mixing with 0.8 mM ANS (prepared in 10 mM PBS buffer, pH 7.4) in a sample-to-ANS ratio of 1:8 (v/v). Mixtures were incubated for 10 min in the dark, and fluorescence intensity was measured in duplicate using Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland). Excitation and emission wavelengths were 390 nm and 470 nm, respectively. PBS buffer with ANS was used as blank control. The protein surface hydrophobicity was displayed as the intensity of fluorescence.

3.2.7. *In vitro* static infant gastrointestinal digestion

Infant *in vitro* gastrointestinal digestions were performed for NT and all the heated samples, according to the *in vitro* infant digestion model from Menard et al. (2018) with some modifications. Protein concentration of all the samples was 12 mg/mL. In the gastric phase (GP), pH was set to 5.3 with 1 M HCl. Samples were mixed, in a ratio of 63:37 (v/v), with simulated gastric fluid including 268 U/mL pepsin (final concentration). After incubation at 37°C for 1 h, gastric digestion was stopped by adjusting the pH to 6.6 using 1 M NaOH. In the intestinal phase (IP), digestion was performed at 37°C and pH 6.6. Gastric chyme was mixed with simulated intestinal fluid (62:38, v/v) containing 16 U/mL trypsin in pancreatin (final concentration). The ratio of samples to total simulated fluid was 39 to 61. After 1 h incubation, 5 mM Pefabloc was used to stop intestinal digestion. Samples were collected at 0 min (G0, before digestion), 10 min (G10) and 60 min (G60) of the GP, as well as 10 min (I10) and 60 min (I60) of the IP. Digestions were independently performed in duplicate. All the digested samples were stored at -20°C for further experiments.

3.2.8. Degree of protein hydrolysis (DH)

The DH after digestion was measured by determining the amount of free amino groups by *o*-phthaldialdehyde (OPA)-assay, which was modified from the method of Zenker, Raupbach, et al. (2020). GP and 3-time diluted IP samples were mixed with 1.25-fold 10% trichloroacetic acid (TCA), and then centrifuged at 10,000 × g for 30 min to remove the insoluble parts. Afterward, 10 µL TCA-containing supernatants were added to a 96-well transparent polystyrene plate and mixed with 200 µL freshly prepared OPA working reagent, prior to the incubation for 15 min in the dark at room temperature. The absorbance was measured at 340

nm using Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) with PBS buffer as blank. Standard curves were prepared using L-leucine from 0-10 mM. The DH was calculated according to the following equation:

$$DH(\%) = \frac{NH_2(final) - NH_2(initial)}{NH_2(acid) - NH_2(initial)} \times 100 \quad (3\text{-}1)$$

where NH_2(final) refers to the concentration of free amino groups by the time of sample collection, NH_2(initial) is the concentration of free amino groups in each sample before digestion (G0), and NH_2(acid) represents the concentration of the total free amino groups after acid hydrolysis (6 M HCl, 110°C, 24 h). All samples were analyzed from duplicate digestions in technical duplicate.

3.2.9. Quantification of nitrogen transfer after *in vitro* digestion

To quantify the nitrogen transfer as an indicator for protein solubilization of samples during digestion, DUMAS Flash EA 1112 Protein analyzer (Thermo Fisher Scientific, Massachusetts, USA) was utilized to measure the nitrogen content in the supernatant of the digesta (Zenker, Raupbach, et al., 2020). The digesta was centrifuged at 10,000 × g for 20 min at 4°C and 200 µL supernatant of each was transferred to a tin cup and dried at 60°C overnight before measurement. Nitrogen transfer was calculated as the percentage of the actual nitrogen concentration in the supernatant relative to the theoretical maximum nitrogen concentration, where the theoretical maximum nitrogen concentration was calculated based on complete dissolution of all protein (i.e., 12 mg protein per mL). Results were corrected for enzyme controls to compensate for the extra introduction of nitrogen. Enzyme controls contained all the elements of the GP and IP but used the water to replace the sample. All samples were analyzed from duplicate digestions in technical duplicate.

3.2.10. Statistical analysis

All experiments were carried out at least in duplicate, as specified before. Data were visualized by Origin 9.5 software and analyzed by IBM SPSS Statistics 25.0 software and GraphPad Prism 9.0 software using ANOVA for determining significant differences. Differences were considered significant when $p<0.05$. Results were shown as mean ± standard deviation (SD) of replicates.

3.3. Results and discussion

3.3.1. Preparation, quantification, and composition of SP and PP

SP and PP were extracted from soybean and pea at low temperature. Yields of SP and PP were 25.8% and 18.7%, respectively. Protein contents (purity) of the freeze-dried extracts, as quantified by DUMAS, were 87.7% for SP and 91.0% for PP, both on a dry matter basis.

Protein composition of SP and PP was determined by SDS-PAGE as shown in **Figure 3.1A**. These results were consistent with previous reports on these protein sources (L'Hocine, Boye, & Arcand, 2006; Lu, He, Zhang, & Bing, 2019). SP is composed mainly of β-conglycinin (7S) and glycinin (11S). β-conglycinin has three subunits, namely α, α' and β; while glycinin contains subunits composed of acidic polypeptides and basic polypeptides linked by disulfide bonds, labeled as glycinin A and glycinin B in **Figure 3.1A**, respectively. PP mainly contains vicilin (7S), convicilin (7-8S), and legumin (11S). Vicilin and convicilin show 80% homology. Legumin is a protein that is similar to soy glycinin, containing subunits with disulfide-linked acidic and basic polypeptides, corresponding to legumin A and legumin B in **Figure 3.1A**, respectively. Under reducing conditions, disulfide bonds of samples were broken, leading, amongst others, to the separation of the acidic and basic polypeptides of the 11S subunits; while under non-reducing conditions, the intact 11S subunits were visible.

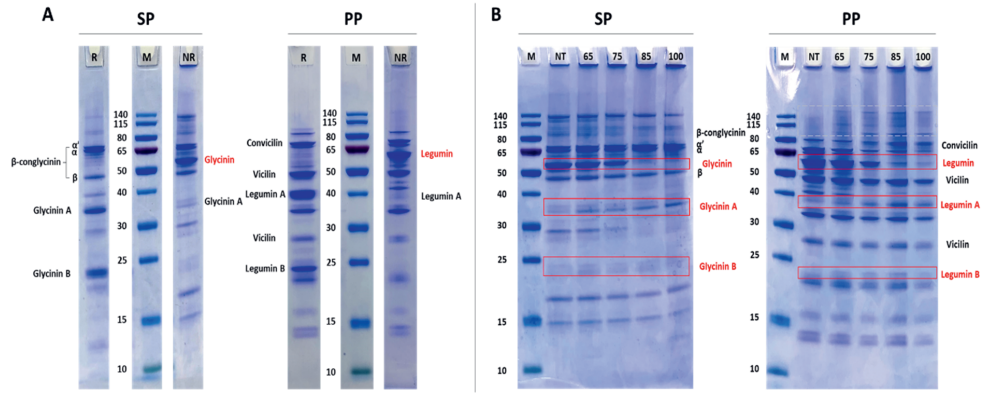

Figure 3.1. (A) SDS-PAGE of SP and PP composition. Lane R: SP/PP under reducing conditions; lane NR: SP/PP under non-reducing conditions. (B) Non-reducing SDS-PAGE of SP and PP of NT, 65, 75, 85, 100°C-heated samples. Lane M: molecular weight marker from 10-140 kDa. Solid boxes in red refer to the 11S protein subunits and the dashed box in white refers to the multimers in PP.

3.3.2. Structural changes of proteins induced by heating

3.3.2.1. SDS-PAGE

To get insights into the differences in subunit composition after heating, SDS-PAGE was conducted under both reducing and non-reducing conditions. As shown in the reducing gels in **Figure 3.S1**, the subunits were clear and remained unchanged among all the samples after the different heating conditions. In addition, there was no aggregation visible at the top of the wells. This showed that thermal treatment at 100°C or below did not destroy the protein subunits, or in other words, had little impact on the protein's primary structure.

The SDS-PAGE gel under non-reducing conditions is displayed in **Figure 3.1B**. The similarity between SP and PP was that most of the subunits remained stable, except for 11S, even after heating at 100°C. Regarding the 11S protein subunits, **Figure 3.1B** of the SP revealed that NT and 65°C-heated samples had a high intensity of the intact glycinin (11S) subunit bands. However, after heating at 75°C, the 11S subunit bands became lighter, while this band almost disappeared after heating at 85°C and 100°C. Simultaneously, aggregates, too large to migrate into the gels, were visible in the wells at the top of the gel. The detection of soluble aggregates by SDS-PAGE was also reported previously (Chen, Chen, Liang, & Xu, 2020). The soluble aggregates were most likely glycinin that aggregated at these increased temperatures. Next to that, part of the disulfide bonds of the glycinin subunits were broken, causing the appearance of the band for the acidic polypeptides of which the intensity increased with increased heating, while the basic polypeptides were never visible on the gel. PP showed a similar result compared with SP, as shown in **Figure 3.1B**. Subunits of 11S legumin became less intense on the gel when the heating intensity increased from 65°C to 75°C and most of it was no longer visible at 85°C. Similarly, aggregation, as well as increased intensity of the acidic polypeptides, were visible on the gel. But unlike SP, under mild heating at 65°C, PP formed relatively large soluble multimers with a molecular weight >100 kDa.

Aggregates visible at the top of the wells were not found in the reducing gel (**Figure 3.S1**) but only in the non-reducing gel (**Figure 3.1B**), indicating that these aggregates were formed through disulfide rearrangement. Overall, these results suggest that heating caused the partial breakage of the disulfide bonds in the 11S protein. The SDS-PAGE in combination with the DLS results described in section 3.3.2.2, shows the formation of soluble aggregates through new disulfide bonds, but without an effect on the primary protein structure.

3.3.2.2. Particle size distribution

The above SDS-PAGE results suggest the aggregation of proteins due to heating, but to better understand the particle size distribution among the samples, DLS analyses were carried out, for which the data can be seen in **Figure 3.2**. The DLS results of both SP and PP showed that thermal treatment indeed increased protein aggregation, leading to the formation of larger particles in solution.

From the DLS analysis of the SP samples (**Figure 3.2A**), both NT and 65°C-heated samples had the smallest size distribution, centered at approximately 13 nm, and their curves almost completely overlapped. This indicates that heating at 65°C hardly resulted in changes in the particle size compared with the NT sample, which was similar to the SDS-PAGE results (**Figure 3.1B**). From 75°C to 85°C, the size distribution became broader, with the size of the most abundant particles increasing from 32 nm to 38 nm, probably due to increased aggregation. After reaching 100°C, the protein aggregation was very obvious from the DLS curve, as well as the SDS-PAGE gel (**Figure 3.2A**). At this temperature, the most frequently occurring size was around 44 nm. In summary, a heating temperature of 65°C did not influence the SP particle size, while a higher heating intensity (75°C to 100°C) showed increasing protein aggregate formation.

The DLS results of PP (**Figure 3.2B**) showed that the NT sample had the smallest particle size distribution centered at around 11 nm. When the heating intensity went up to 65°C, unlike SP the most frequent particle size increased to about 15 nm. The SDS-PAGE gel (**Figure 3.2B**) showed this as well, with multimers > 100 kDa formed after heating at 65°C. At 75°C, the 11 to 15 nm-centered size fraction was at least two times less abundant than that at 65°C, and simultaneously another protein fraction that centered at around 32 nm appeared. After heating at 85°C, the smaller particles had completely disappeared, all forming a larger-sized group centered around 38 nm. At 100°C, the largest particles were formed, and the most frequently occurring size was around 44 nm. To conclude, DLS showed that PP had a similar trend of protein aggregation after heating compared with SP, with the exception that the aggregation starting from 65°C rather than 75°C, being in good agreement with the SDS-PAGE results (**Figure 3.2B**). Also, higher heating intensities caused a higher level of aggregation.

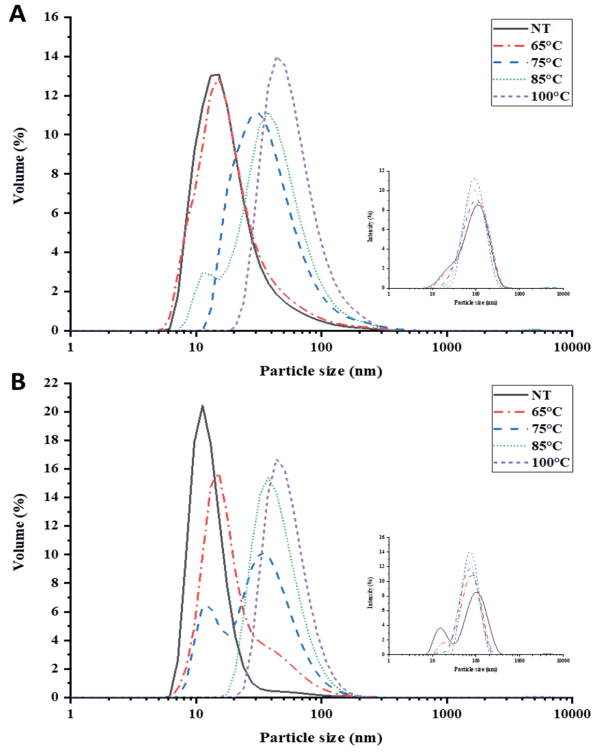

Figure 3.2. DLS analyses of (A) SP and (B) PP of NT, 65, 75, 85, 100°C-heated samples. Results were shown as volume-weighted particle size distribution.

3.3.2.3. Surface hydrophobicity

Surface hydrophobicity is related to the tertiary protein structure. When proteins are folded in water, they naturally tend to bury the hydrophobic groups inside their structure and expose the hydrophilic groups to the outside. An increase in the surface hydrophobicity thus means that the distribution of hydrophobic amino acid residues on the protein's surface is more extensive, which may be due to unfolding, as this exposes the hydrophobic inside. This agrees with the results from other studies, who have shown with different techniques that the structure of plant proteins changes extensively upon heating (Carbonaro, Maselli, & Nucara, 2012; Jiang et al., 2015; Wang et al., 2012; Wang, Li, Jiang, Qi, & Zhou, 2014).

As shown in **Figure 3.3**, as the heating temperature increased from 65°C to 100°C, the hydrophobicity of both SP and PP increased as well. Furthermore, NT samples showed the lowest hydrophobicity among all the samples, with that of NT PP being slightly higher than SP, which is consistent with previous studies (Ge, Sun, Mata, Corke, Gan, & Fang, 2021; Karaca

et al., 2011). Besides, the hydrophobicity of PP was in general still higher than that of SP after heating at different intensities. By analyzing the data in detail, for SP, the surface hydrophobicity increased by 93%, 160%, 300%, and 382% at 65, 75, 85, 100°C, respectively, compared with the NT sample. Among them, there was no significant difference ($p > 0.05$) in surface hydrophobicity between the NT and the 65°C-heated samples as well as between the 85°C and the 100°C-heated samples. For PP, compared with the NT sample, the hydrophobicity of the 65, 75, and 85°C-heated samples gradually increased by 104%, 147%, and 196%, respectively, and was significantly different ($p < 0.05$) between these temperatures. From 85°C to 100°C, the hydrophobicity only increased by ~4%, without being significant ($p>0.05$), which was similar to the results of SP.

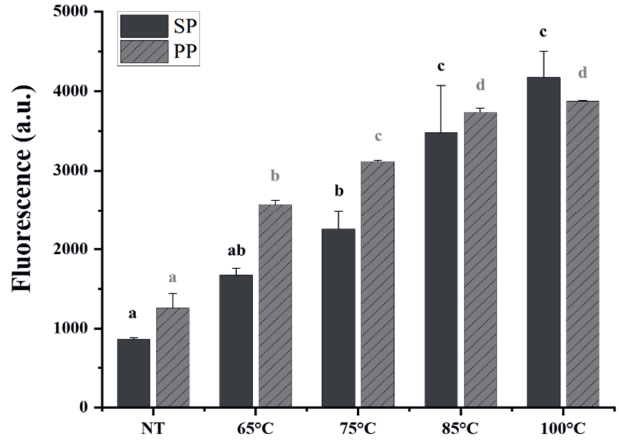

Figure 3.3. Results of ANS-assay of SP and PP of NT, 65, 75, 85, 100°C-heated samples. Error bars represent standard deviations of duplicates. Statistical differences were analyzed with ANOVA and Duncan test. Different letters above the bars represent significant differences ($p < 0.05$).

Bearing in mind that heating can cause changes in the structure of proteins, the main reason for the increase in the surface hydrophobicity was most likely the heat-induced protein unfolding and subunit dissociation, thereby causing the hydrophobic domains that were hidden inside the proteins to be exposed to the surface (Wang et al., 2012; Wang et al., 2014). In this study, despite the fact that the formation of larger soluble aggregates during heat treatment may lower the hydrophobicity, their contribution did not exceed the exposure of hydrophobic groups, as shown by the continuous increase in hydrophobicity. To summarize, in this study, heating temperatures (less than or equal to 100°C) were positively correlated with the exposed hydrophobicity for both SP and PP.

3.3.3. *In vitro* protein digestion after heating

3.3.3.1. Nitrogen transfer after digestion

When changing the pH to 5.3 for the gastric digestion, before the addition of pepsin, the SP and PP solutions turned turbid immediately, indicating that part of the proteins precipitated. Subsequently, the G0 samples (samples before digestion) were neutralized, and the supernatants were collected to perform reducing SDS-PAGE analyses (**Figure 3.S2**). As displayed, the bands became less intense with increasing heating temperature, which cannot be seen in **Figure 3.S1**, where all the bands remained the same. The sole difference between these figures was the pH adjustment, indicating that heated proteins respond differently to a decreased pH. This may be due to the changes in the electrostatic charge distribution. During heating, the changes in the protein structure, as well as the formation of soluble aggregates, possibly altered the surface charge, causing the precipitation during *in vitro* digestion.

To better monitor the nitrogen transfer from the precipitate into the digestive fluids after gastric (G60) and intestinal (I60) digestion, the nitrogen concentration of the supernatants was determined by DUMAS among all the heating conditions as well as NT samples (**Figure 3.4**). Firstly, it was obvious that from all the undigested samples (G0), the nitrogen content in the supernatants declined when heating intensity increased, being in accordance with the SDS-PAGE results (**Figure 3.S2**). The percentage of nitrogen in the supernatant decreased from ~47% to ~16% in SP and from ~53% to ~21% in PP at G0 for the different heat treatments. The relative amount of soluble nitrogen of the 85°C and 100°C-heated samples were significantly lower ($p < 0.05$) than that of the NT samples at G0, by ~22% and ~31% for SP as well as ~30% and ~32% for PI accordingly. At the end of intestinal digestion (I60), samples reached a soluble nitrogen content ranging from 66.3% (NT) to 83.3% (100°C) for SP and from 83.2% (NT) to 95.9% (100°C) for PP, without significant differences ($p>0.05$) among samples. Moreover, as digestion progressed from G0 to G60 to I60, the percentage of soluble nitrogen became higher, where higher heating conditions showed stronger increases. Especially at 85°C and 100°C, the soluble nitrogen increased by ~53% and ~68% accordingly from G0 to I60 for SP, and by ~73% and ~75% accordingly for PP. This may be due to the larger amount of the insoluble protein clot initially formed in the samples heated at those temperatures upon pH adjustment, or different digestion kinetics of the clot that may be caused by differences in its composition or structure. However, despite the upward trend in soluble nitrogen during digestion, the NT and 65°C samples showed no significant differences ($p > 0.05$) between G60 and I60, suggesting

that no more peptides went into the supernatants, although the soluble part was digested more extensively (see section 3.3.3.2).

Although heating declined the total soluble nitrogen in supernatants at G0, digestion contributed to its increase, whereas the amount of initial precipitation of the samples had little effect on the nitrogen transfer at I60.

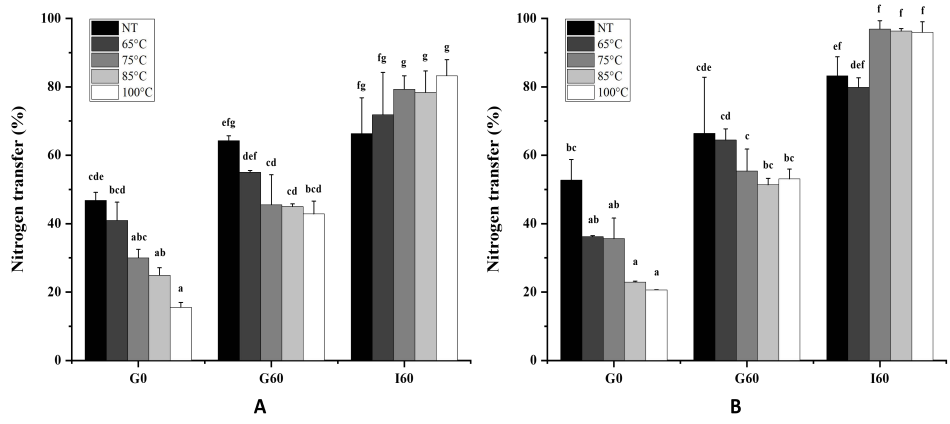

Figure 3.4. Nitrogen transfer of (A) SP and (B) PP of NT, 65, 75, 85, 100°C-heated samples, after *in vitro* infant digestion. G0: samples before digestion; G60: samples after gastric digestion for 60 min; and I60: samples after intestinal digestion for 60 min. Error bars represent standard deviations of digestion duplicates. Statistical differences were analyzed with ANOVA and Duncan test. Different letters above the bars represent significant differences among all the SP/PP samples ($p < 0.05$).

3.3.3.2. Degree of hydrolysis

The DH was monitored by measuring the free amino groups in the supernatant of the digest with the OPA-assay, as shown in **Figure 3.5**. Generally, both SP and PP samples within different digestion stages showed a similar trend that the DH increased from NT to 100°C-heated samples. For the samples heated at 85°C and 100°C, the phenomenon that heating promoted proteolysis was most obvious. Besides, SP had a relatively lower DH than PP, whether heated or not, and showed a larger increase in the DH upon heating, especially at 85°C and 100°C.

After gastric digestion for 60 min, the DH was still very low in all SP and PP samples. The highest DH was observed at G60 for 100°C-heated samples being 3.0% for SP and 3.8% for PP. In addition, no significant differences ($p > 0.05$) were found among all the samples in the GP. This low DH was due to the gastric digestion being conducted at pH 5.3 to simulate the

infant gastric environment, which is far from the optimal pH 2.0 of the used porcine pepsin (Gray et al., 2014). On top of that, the far-lower enzyme concentration used in the infant gastric digestion model further limited the DH.

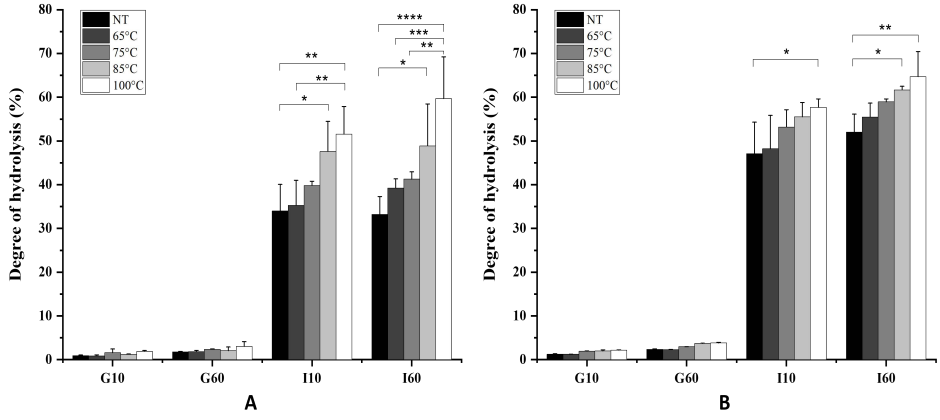

Figure 3.5. Degree of hydrolysis of (A) SP and (B) PP of NT, 65, 75, 85, 100°C-heated samples, after *in vitro* infant digestion. G10 and G60: after gastric digestion for 10 min and 60 min, respectively; and I10 and I60: after intestinal digestion for 10 min and 60 min, respectively. Error bars represent standard deviations of digestion duplicates. Statistical differences were analyzed with ANOVA and Tukey test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Nevertheless, the DH sharply increased in the intestinal digestion for both SP and PP. This can partly be explained by the pH value, as the intestinal digestion was done at pH 6.6, where porcine pancreatin has its optimal enzyme activity (optimal pH: 6-8) (Berdutina, Neklyudov, Ivankin, Karpo, & Mitaleva, 2000). For SP, after digestion in the IP for 10 min, the highest DH was still found to be 51.5% for the sample heated at 100°C, followed by 47.6% at 85°C. The DH of samples heated at both 85°C and 100°C were significantly higher ($p < 0.05$ and $p < 0.01$, respectively), by ~14% and ~18%, than that of the NT sample at I10. At the end of intestinal digestion (I60), the DH of the 100°C-heated sample showed a further increase by ~8% compared with that at I10, while the other samples did not show a further increase. Compared with the NT sample, at I60, similar to I10, the 85°C and 100°C-heated samples had significantly higher DH ($p<0.05$ and $p<0.0001$, respectively) as well, by ~16% and ~26% respectively.

Regarding PP, the DH steadily increased from NT to 100°C at the different intestinal digestion stages. After 10 min of intestinal digestion, there was a significant difference ($p < 0.05$) in the DH only between NT (47.1%) and 100°C (57.7%). At I60, the DH reached its maximum, ranging from 52.0% for the NT sample to 64.7% for the 100°C-heated sample. The samples

heated at both 85°C and 100°C had significantly higher DH ($p < 0.05$ and $p < 0.01$, respectively) than the NT sample, with a difference of ~10% and ~13%. Additionally, compared with NT SP, the DH of NT PP was ~19% higher at the end of digestion (I60). But after heating, this gap narrowed to ~13% for 85°C-heated and only ~5% for 100°C-heated samples. This indicates that the higher heating temperatures (85°C and 100°C) had more impact on the digestion of SP than PP.

3.3.3.3. Disappearance of intact protein after digestion

Disappearance of intact protein in SP and PP was analyzed through reducing SDS-PAGE, as shown in **Figure 3.6**.

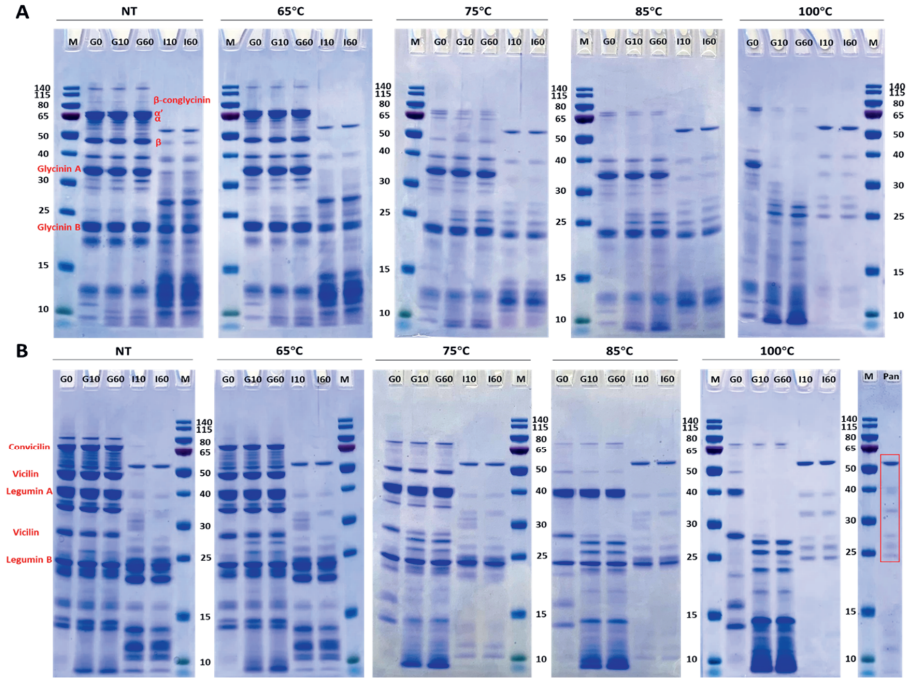

Figure 3.6. Reducing SDS-PAGE of (A) SP and (B) PP of NT, 65, 75, 85, 100°C-heated samples, after *in vitro* infant digestion. Lane G0: samples before digestion; lane G10 and G60 samples after gastric digestion for 10 min and 60 min, respectively; lane I10 and I60 samples after intestinal digestion for 10 min and 60 min, respectively; lane Pan: porcine pancreatin as control; and lane M: molecular weight marker from 10-140 kDa.

With respect to gastric digestion, many large peptides were visible. As digestion progressed, some relatively smaller peptides appeared as well. The low gastric digestibility was also confirmed by the DH results (**Figure 3.5**). Moreover, heat treatment also enhanced proteolysis, showing more small peptides, especially after heating at 85°C and 100°C (**Figure 3.6**). During intestinal digestion, the large subunits were cleaved into smaller peptides, also in the case of NT samples. Similar to gastric digestion, this cleavage into peptides became more obvious as the temperature increased. For the samples heated at 100°C, only the enzymes in pancreatin were visible on the gel, suggesting that almost all the proteins were cleaved into very small peptides that were not visible on the gel. This result was in good agreement with the DH results (**Figure 3.5**). It is worth noting that the 11S basic polypeptides had very strong resistance to pancreatin. In all heating conditions, except 100°C, even at the end of digestion (I60), clear bands of the 11S basic polypeptides could be seen on the gels. The same situation was found in another study regarding soy protein (Zhao, Qin, Sun, Zhang, & Wang, 2010), who linked this to the conformation, with the basic polypeptide chains being buried in the interior and therefore being more resistant to digestion. After heating at 100°C, its unfolding may lead to an increased accessibility of the basic polypeptides to digestive enzymes.

3.3.4. Thermal treatment facilitated the protein digestibility

Taken together, the results showed that thermal treatment altered the protein's tertiary structure. On the one hand, thiol-disulfide exchange reactions happened during heating, which enabled the breakage of part of the disulfide bonds in 11S protein, while simultaneously forming new soluble aggregates. On the other hand, hydrophobic groups were exposed more to the outside surface of proteins with increased heating and contributing to the increase in the surface hydrophobicity. Meanwhile, digestion results demonstrated that as heating intensity increased, digestibility (both degree of hydrolysis and disappearance of intact protein) increased as well.

This is because heating increased the surface hydrophobicity by unfolding, and probably through increased accessibility for the digestive enzymes, to improve the digestibility of SP and PP. To confirm this potential relation, a correlation analysis was conducted between the overall DH (at the end of intestinal digestion) and protein surface hydrophobicity, as shown in **Figure 3.S3**. Pearson correlation coefficients of SP and PP were 0.977 and 0.963, respectively ($p < 0.01$), showing that the DH was strongly positively correlated to surface hydrophobicity as heating intensity increased. Heating could lower the protein conformational stability by unfolding as displayed by the increased surface hydrophobicity, which was consistent with Wang et al. (2012). It was reported that the protein's hydrophobic interactions contribute a lot

to the protein conformational stability. When there are less hydrophobic side chains buried inside the protein, or in other words when the surface hydrophobicity is high, the protein is prone to be more conformationally unstable in solution (Loladze, Ermolenko, & Makhatadze, 2002; Pace et al., 2011; Schwehm, Kristyanne, Biggers, & Stites, 1998; Wigley et al., 1987). Furthermore, as previous research showed that changes in protein structure will influence their accessibility for digestive enzymes (Dupont & Nau, 2019; Salazar-Villanea, Hendriks, Bruininx, Gruppen, & van der Poel, 2016), we therefore hypothesize that the increase in digestibility was due to heat-induced protein unfolding, which subsequently enhanced the accessibility to enzymes. In other words, these unfolded proteins may have been more susceptible to digestive enzymes, and could therefore have been digested to a larger extent. Taken together, combining the results in **Figure 3.3** and **Figure 3.S3**, it is clear that with the heating intensity increasing, the protein became more conformationally unstable, and subsequently easier to be hydrolyzed by the enzymes, leading to a higher digestibility.

Comparing the protein sources, SP had a relatively lower DH than PP (**Figure 3.5**). There might be two main reasons for this difference. Firstly, the lower overall hydrophobicity of SP may indicate a higher protein conformational stability with a more tightly folded tertiary structure, which in turn may have led to a lower digestibility. On top of that, SP contained more TIs than PP. It was previously shown that severe heating inactivated TIs, causing a weakened TI activity and therefore a higher digestibility (Vagadia et al., 2017). Another study reported that when the heating temperature increased from 75°C to 100°C, the digestibility increased coinciding with a reduction in TIs, with the maximum digestibility reached after heating at 100°C for 30 min (Vagadia, Vanga, Singh, Gariepy, & Raghavan, 2018). Comparing that result to ours, the weakest trypsin inhibition in SP was presumably at 100°C, as well as the highest hydrophobicity, so the digestibility was the highest at this time, reaching a level close to PP. However, as digestibility already started to increase at temperatures that would not cause TI inactivation, the presence of TIs cannot be the only explanation of the increased digestibility after more intense heating. Overall, it could be elucidated that the rapidly increased digestibility of SP at 85°C and 100°C was due to a combination of their relatively higher surface hydrophobicity (**Figure 3.S4**), as well as the stronger inactivation to TIs.

3.4. Conclusion

Soy protein-based infant formula is widely marketed as dairy-based substitutes, often for the alleviation of cow's milk allergy or intolerance. Because soybeans are rich in antinutritional factors and can be allergenic, the interest in exploring pea protein-based formula is increasing. This study analyzed the heat-induced structural changes of soy and pea proteins and its relation to the level of *in vitro* infant digestion, which has not been reported before. We found that with the increased heating, both the surface hydrophobicity and *in vitro* infant digestibility increased, which were strongly positively correlated to each other. This is because the protein unfolding is related to increased hydrophobicity, which in turn may lead to the higher accessibility to digestive enzymes, thus promoting the *in vitro* infant digestion. Besides, PP had a relatively higher overall digestibility than SP, both with and without heating; but the latter was more sensitive to heat, as its digestibility increased to a larger extent as heating increased. In conclusion, all these results indicate that heating facilitated the digestion of SP and PP, mainly due to protein unfolding. This study thereby provides information that may be useful for the preparation of plant-based IFs. Also, taking into account the limitations of *in vitro* digestion, it would be interesting to confirm these findings in *in vivo* studies in future.

Supplementary information

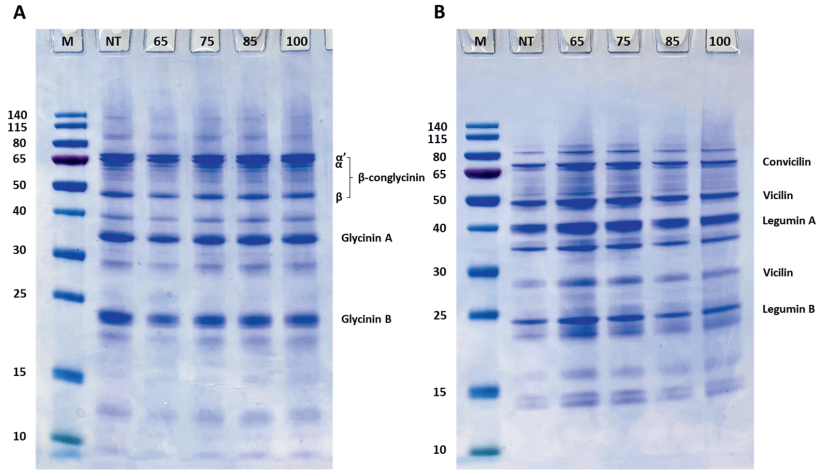

Figure 3.S1. Reducing SDS-PAGE of (A) SP and (B) PP of NT, 65, 75, 85, 100°C-heated samples. Lane M: molecular weight marker from 10-140 kDa.

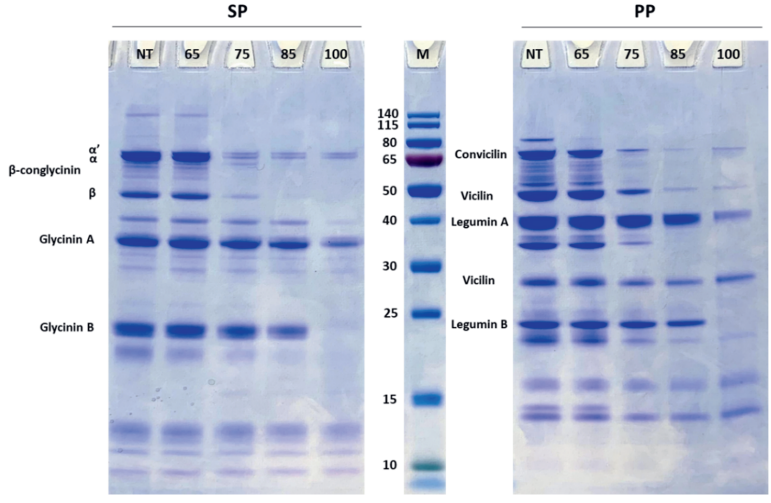

Figure 3.S2. Reducing SDS-PAGE of SP and PP of NT, 65, 75, 85, 100°C-heated samples at G0 (before digestion). Lane M: molecular weight marker from 10-140 kDa.

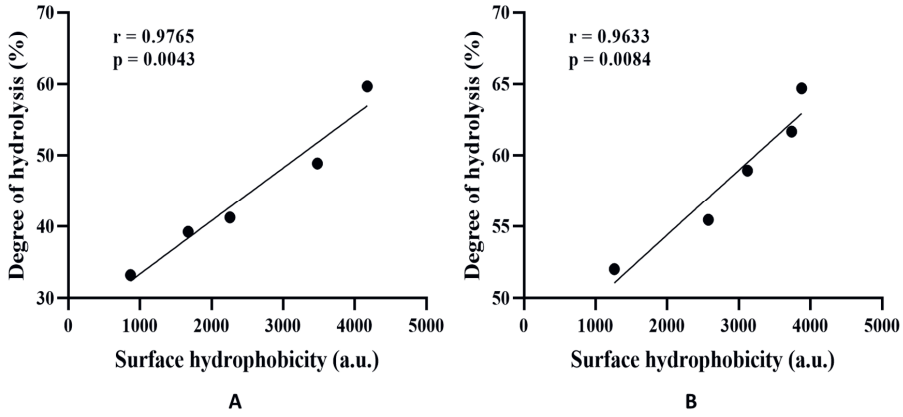

Figure 3.S3. The correlations between the overall degree of hydrolysis and surface hydrophobicity for (A) SP and (B) PP of NT, 65, 75, 85, 100°C-heated samples.

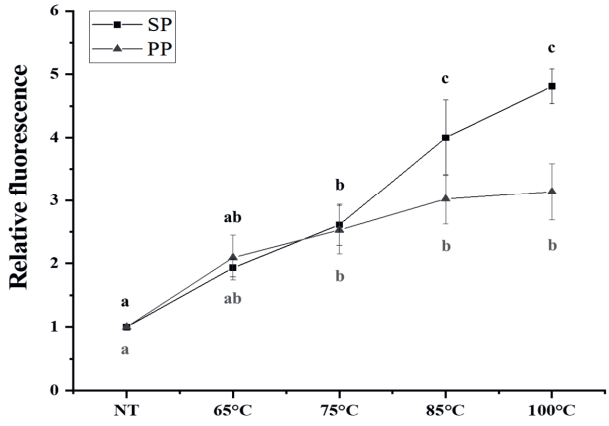

Figure 3.S4. Surface hydrophobicity of SP and PP samples relative to NT samples (=1), after thermal treatments at 65, 75, 85, 100°C for 30 min. Error bars represent standard deviations of duplicates. Statistical differences were analyzed with ANOVA and Duncan test. Different letters represent significant differences ($p < 0.05$).

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CHAPTER 4

Glycation of soy and pea proteins influences infant gastric digestibility more than intestinal digestibility

This chapter has been published as:

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Abstract

Dry heating of plant proteins occurs during production of plant-based infant formula. To study the effect of dry heating on physicochemical changes and *in vitro* infant digestion of soy and pea proteins, these proteins were mild dry heated (60°C) for 6 h and 48 h, in the presence or absence of glucose. In this study, we found that with extended dry heating, the degrees of glycation increased in the presence of glucose. After 48 h of dry heating, the degree of gastric hydrolysis decreased from 2.7% to 0.2% for soy protein and from 2.8% to 0.4% for pea protein, compared to non-treated proteins. Such resistance to gastric digestion was probably due to the extensive protein aggregation induced by glycation. However, the intestinal digestion was less affected, as the degree of hydrolysis was similar at the end of intestinal digestion, ranging from 31.0% to 35.4% for soy protein and 36.7% to 48.5% for pea protein. This may be due to the broad range of proteases in pancreatin negating the effect of glycation. By contrast, for the glucose-free samples, no glycation happened and only limited insoluble aggregates were formed during dry heating. Despite this aggregation, the variation in dry heating duration did not lead to different digestibility. Therefore, 60°C of mild dry heating will influence the gastric digestion of glucose-containing samples, but will not influence the final intestinal digestibility of all the samples with or without glucose.

4.1. Introduction

Proteins are a key factor in infant formulas (IFs) to provide sufficient essential amino acids (EAAs) to meet the needs for the growth and development of infants. In recent years, there is increasing interest to apply plant proteins, such as soy protein (SP), in IFs. SP-based IFs have been commercially available for decades. Apart from the sustainability consideration, an important objective is to alleviate cow's milk allergy (CMA). CMA occurs especially in infants because of their immature immune system. SP is the only legally accepted legume protein source in the European Union according to the European Commission (2016). However, SP can be an allergen for some infants. In addition, the trypsin inhibitors in SP, as one of the antinutritional factors, may limit the digestion and lower the bioavailability of EAAs (Martín-Cabrejas et al., 2009). Therefore, innovative legumes need to be explored as soy alternatives to develop plant-based IFs. Among all the legumes, pea protein (PP) is considered a promising alternative for SP. Compared to SP, PP still has a high PDCAAS (protein digestibility corrected amino acid score), as well as relatively lower allergenicity and fewer antinutritional factors (Gorissen et al., 2018; Roux et al., 2020; Rutherfurd, Fanning, Miller, & Moughan, 2014).

Production of powdered IFs involves thermal processing such as spray drying, pasteurization, and sterilization, during which the Maillard reaction may occur. The Maillard reaction, also known as glycation, is a spontaneous and non-enzymatic reaction between amino groups of proteins/peptides/amino acids and reducing sugars. Especially lysine residues are sensitive to glycation. Glycation can be characterized into three stages: early, advanced, and final stages; these three stages can occur simultaneously (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016). During the early stage of glycation, lysine residues covalently react with reducing sugars and initially undergo irreversible rearrangement, to form Amadori products such as furosine (Nε-2-furoylmethyllysine). It is known that lysine is one of the limiting amino acids in the diet. Therefore, the lysine damage during this stage may limit its bioavailability and thereby reduce the overall nutritional value of proteins. The advanced stage involves the further reaction of Amadori products, resulting in the formation of various advanced glycation end products (AGEs), e.g., CML (Nε-carboxymethyllysine) and CEL (Nε-carboxyethyllysine). This is followed by the formation of brown nitrogenous polymers such as melanoidins at the final stage of glycation (Tamanna & Mahmood, 2015). The mechanism of this reaction remains unclear due to the occurrence of multiple complex chemical reactions. Moreover, glycation is affected by the nature of reactants, heating temperature and time, as well as water activity and pH (Kutzli, Weiss, & Gibis, 2021). It has also been reported that glycation is more intense when reactants

are dry heated (i.e., in a powdered form), where the higher heating temperatures and longer heating durations can also increase the reaction rate (de Oliveira et al., 2016; Schong & Famelart, 2017).

During dry heating, next to glycation, also protein denaturation and aggregation normally happen. In general, the protein structural modifications by glycation and heating cannot be easily distinguished. In addition, such variation in protein structure may further influence protein digestion. Our previous study showed that the digestibility may increase when heat-induced protein unfolding partially denatures the protein, due to increased accessibility to digestive enzymes (**Chapter 3**). However, aggregation and glycation may also bury the enzyme-cleavage sites, and thereby negatively affect digestion (Corzo-Martínez, Soria, Belloque, Villamiel, & Moreno, 2010; Wada & Lönnerdal, 2014). For instance, it is known that glycation mainly occurs at the lysine residues, which are the specific cleavage sites of trypsin as well (Hemmler et al., 2019; Olsen, Ong, & Mann, 2004). Therefore, when these sites are blocked by glycation, they cannot be recognized by trypsin, and this may thus have negative consequences for intestinal digestion.

Previous research mostly focused on the glycation modification of plant proteins, for the purpose of improving their techno-functional properties (Feng, Berton-Carabin, Atac Mogol, Schroen, & Fogliano, 2021; Kutzli et al., 2021; Zhao, Huang, McClements, Liu, Wang, & Liu, 2022), whereas the effect of glycation on plant protein digestibility has been studied to a much smaller extent. Especially there is no research reported when it comes to *in vitro* infant digestion. Therefore, this study aimed at exploring the effect of glycation of SP and PP on protein structures, and their consequences for infant digestibility. To achieve this aim, we selected glucose as the reducing sugar, as glucose from corn syrup is the main reducing sugar in soy-based infant formulas. SP and PP were then dry-heated with glucose for different heating durations, to mimic different glycation levels in actual processing and storage conditions of infant formulas. Subsequently, the level of glycation and protein physicochemical changes were determined. Afterward, all the glycated proteins were subjected to a full-term infant digestion model system, and the level of digestion was measured.

4.2. Materials and methods

4.2.1. Materials and chemicals

Fresh soybean (*Glycine max*; protein content: 39.3%, w/w) was obtained from Wageningen Plant Research, Lelystad, the Netherlands (courtesy ing. Ruud Timmer), and pea (*Pisum sativum* L.; protein content: 23.3%, w/w) was purchased from a local retailer (Brand name: HAK). Protein concentration was determined through Bicinchoninic Acid (BCA) Protein Assay Kit or through DUMAS Flash EA 1112 Protein analyzer from Thermo Fisher Scientific (Massachusetts, USA). LDS sample buffer (4×), reducing agent (10×), 12% Bis-Tris gel, prestained protein ladder, and MOPS SDS running buffer were also obtained from Thermo Fisher Scientific (Massachusetts, USA). D-Glucose, porcine pepsin, and porcine pancreatin, as well as all other chemicals, were obtained from Sigma-Aldrich (Missouri, USA) unless otherwise stated.

4.2.2. Preparation of protein samples and dry heating

Soy protein (SP) and pea protein (PP) were isolated as described previously in section 3.2.2. Protein contents of powdered SP and PP were 77.0% and 79.2%, respectively, according to DUMAS, with a nitrogen-to-protein conversion factor of 5.7. SP and PP powders were dry-heated in an incubator at 60°C for 0 h (T0, i.e., non-treated control), 6 h (T6), or 48 h (T48) in the presence of glucose (SP-G and PP-G). The protein-to-glucose weight ratio was set to be 1:4, as this ratio is normal in IFs. The control groups were treated the same, but without added glucose (SP and PP). The relative humidity was controlled at 60% through a desiccator with a saturated potassium iodide solution. Heat treatment was performed in independent duplicates. All the samples were stored at -20°C until use.

4.2.3. Color measurement

The color of the dry-heated samples was measured in duplicate by using a ColorFlex CX2189 Spectrophotometer (HunterLab, Virginia, USA). The color value was expressed by three chromaticity coordinates: L^* (lightness), a^* (redness), and b^* (yellowness). Browning index (BI), as an important parameter in glycation, was calculated by equations (4-1) and (4-2) according to Consoli et al. (2018):

$$BI = \frac{x-0.31}{0.172} \times 100 \quad (4\text{-}1)$$

$$x = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)} \quad (4\text{-}2)$$

4.2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Reducing SDS-PAGE was performed to analyze protein composition and the disappearance of intact protein after digestion. Prior to analysis, all the samples were centrifuged to remove the insoluble parts. The supernatants were mixed with LDS sample buffer (4×) and reducing agent (10×), and then diluted with ultrapure water. These sample mixtures were incubated at 70°C for 10 min in a heating block. Undigested samples containing ~15 μg protein or digested samples containing ~10 μg protein were loaded onto a 12% Bis-Tris gel. A prestained protein ladder (10 to 140 kDa) was used as molecular weight marker. Gels were run under constant 120 V for 90 min with MOPS SDS running buffer. Gels were stained by Coomassie brilliant blue R-250 (Bio-Rad, California, USA) and destained by washing buffer (7.5% acetic acid, 10% ethanol).

4.2.5. Analysis of glycation markers by LC-MS/MS

Quantification of glycation markers—lysine, furosine (Nε-2-furoylmethyllysine) and CML (Nε-carboxymethyllysine) of non-treated and dry-heated samples was conducted on a high-performance liquid chromatograph coupled to a triple quadrupole mass spectrometer (LC-MS/MS, series: LCMS-8050, Shimadzu Corporation, Kyoto, Japan), according to the methods of Troise, Fiore, Roviello, Monti, and Fogliano (2015) and Zenker et al. (2019) with some modifications. Briefly, sample powders containing 2.5 mg protein (based on DUMAS results) were mixed with 4 mL 6 M HCl and heated at 110°C for 22 h in a heating block for acid hydrolysis. Nitrogen was saturated in the heating tubes to avoid interference from oxidation. After acid hydrolysis, the hydrolysates were dried under nitrogen and reconstituted in 4 mL ultrapure water, followed by filtration (0.22 μm filter) and centrifugation (10,000 × g, 10 min, 20°C) to remove insoluble parts. Subsequently, 20 μL of the filtered supernatants were mixed with 180 μL 50% (v/v) acetonitrile and 10 μL internal standard. The internal standard was 10 ppm of mixed d4-lysine, d4-furosine and d2-CML. Calibration standards were 200 μL 0-10 ppm of mixed lysine, furosine, and CML, spiked with 10 μL internal standard. For analysis, 5 μL of each sample was injected into the LC-MS/MS system and measured in duplicate. Calibration curves were linearly regressed as peak area ratio of standards/internal standard against corresponding standards' concentration. Lysine, furosine, and CML were quantified according to the calibration curves and their peak area ratio (analytes/internal standards). The final content was shown as mg/g protein for lysine and furosine, as well as mg/100 g protein for CML.

4.2.6. Particle size distribution (PSD)

PSD was determined for two fractions: the full suspension and the supernatant. The suspension was measured by laser diffraction by Mastersizer 3000 (Malvern Instruments, Worcestershire, UK); whereas the supernatant was measured by dynamic light scattering by Zetasizer Ultra (Malvern Instruments, Worcestershire, UK) (Feng et al., 2021). The refractive indexes of both systems were set to be 1.45 and 1.33 for the dispersed phase and continuous phase, respectively. Briefly, sample powders containing 0.25 g protein were dissolved in 5 mL 10 mM PBS buffer (pH 7.4) and divided into two portions. One portion of the suspensions was gradually dropped into the analyst chamber of the Mastersizer system until the obscuration rate was between 10-20%, after which the PSD was measured in triplicate. Another portion was centrifuged (10,000 × g, 10 min, 20°C) and filtrated with a 0.45 μm filter. The supernatants were diluted to 1 mg/mL according to DUMAS results. About 1 mL of the sample solutions were transferred into the Zetasizer system and measured in triplicate. All the results were shown as volume-weighted PSD.

4.2.7. Surface hydrophobicity

The 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANSA) fluorescence assay was performed to measure the surface hydrophobicity of soluble proteins, as previously described in section 3.2.6. Briefly, samples were dissolved in 10 mM PBS buffer (pH 7.4) and centrifuged. All the supernatants were diluted to 0.15 mg/mL. Subsequently, 25 μL of the supernatants were mixed with 200 μL 0.8 mM ANSA reagent (prepared in 10 mM PBS buffer, pH 7.4) in a 96-well black polystyrene plate. After incubation in the dark for 10 min, the fluorescence intensity was measured in duplicate at 390 nm (excitation) and 470 nm (emission) by Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland). PBS buffer with ANSA was set as blank. Results were displayed as fluorescence intensity corrected for protein concentration (based on BCA results) and blank.

4.2.8. *In vitro* static infant gastrointestinal digestion

Infant *in vitro* digestion was performed with a full-term infant digestion model according to Ménard et al. (2018). All the sample powders were dispersed in 10 mM PBS buffer (pH 7.4), and their protein concentrations were adjusted to 12 mg/mL to mimic infant formula. For the gastric phase (GP), digestion was conducted for 60 min at 37°C with 268 U/mL pepsin at pH 5.3 and stopped by increasing the pH to 6.6. For the intestinal phase (IP), digestion was conducted for 60 min at 37°C with 16 U/mL trypsin in pancreatin at pH 6.6 and stopped by

adding 5 mM Pefabloc. Sampling points were G0 (0 min, before digestion), G10 and G60 (10 min and 60 min of the GP), as well as I10 and I60 (10 min and 60 min of the IP). Digestions were independently performed in duplicate. All the samples were stored at -20°C for later use.

4.2.9. Degree of hydrolysis

Degree of hydrolysis (DH) after digestion was determined by o-phthaldialdehyde (OPA) assay according to Mulet-Cabero, Rigby, Brodkorb, and Mackie (2017) with some modifications. Briefly, GP and 3-fold diluted IP samples were mixed with 10% cooled trichloroacetic acid (TCA, w/v) in a volume ratio of 1:8 and then centrifuged at 10,000 × g for 30 min. Afterward, 10 μL of all the TCA-containing supernatants were added to a 96-well transparent polystyrene plate with 200 μL OPA working reagent. The plate was incubated for 15 min in the dark, and the absorbance was measured in duplicate at 340 nm by Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland). The calibration curve was made by using 0-10 mM L-leucine. The DH was calculated by the following equation (4-3):

$$DH(\%) = \frac{NH_2(final) - NH_2(initial)}{NH_2(acid) - NH_2(initial)} \times 100 \quad (4\text{-}3)$$

where NH_2(final) refers to the concentration of free amino groups At different sampling points, NH_2(initial) refers to the concentration of free amino groups before digestion (G0), and NH_2(acid) refers to the concentration of the total free amino groups after acid hydrolysis (6 M HCl, 110°C, 24 h).

4.2.10. Quantification of nitrogen transfer after *in vitro* digestion

Nitrogen transfer represented the total soluble nitrogen transfer from the insoluble sample material to the digestive fluids during digestion. The soluble nitrogen contents of samples at G0, G60, and I60 were measured by DUMAS, according to Zenker, Raupbach, Boeren, Wichers, and Hettinga (2020). The percentage of nitrogen transfer was calculated as the soluble nitrogen content of the digesta relative to the theoretical maximum nitrogen content (i.e., assuming 12 mg/mL of proteins were completely dissolved). The nitrogen contents in enzyme controls were subtracted to compensate for the extra introduction of nitrogen. Enzyme controls were the same as other digestion samples, but the protein samples were replaced by ultrapure water. All samples were analyzed from duplicate digestions in technical duplicate.

4.2.11. Statistical analysis

All the data were visualized by GraphPad Prism 9.0 software and analyzed through IBM SPSS 25.0 software by one-way ANOVA with Duncan post-hoc test. Differences were considered significant when $p < 0.05$.

4.3. Results and discussion

4.3.1. Level of glycation

4.3.1.1. Quantification of glycation markers by LC-MS/MS

Lysine, furosine, and CML are regarded as glycation markers, reflecting the different glycation levels. Lysine residues react with reducing sugars during dry heating, forming furosine at the early stage and CML at the advanced stage of glycation.

Regarding the glucose-free systems (SP and PP), no obvious glycation happened upon dry heating in the absence of reducing sugar (**Figure 4.1**), as expected. Almost no significant differences ($p > 0.05$) were found in the contents of lysine, furosine and CML among the different heating durations (T0, T6, and T48).

For the glucose-containing systems (SP-G and PP-G), as shown in **Figure 4.1A**, the lysine contents were 100.0 mg/g protein and 114.4 mg/g protein at T0 for samples of SP-G and PP-G, respectively. After dry heating for 6 h (T6) to 48 h (T48), the lysine content of both SP-G and PP-G decreased, by approximately 25% at T6 and 57% at T48 for SP-G, as well as by 23% and 57% for PP-G, compared to T0, with these decreases being significant ($p < 0.05$). These results suggest that glycation was aggravated in SP-G and PP-G systems after dry heating from 6 h to 48 h. Furosine contents can be seen in **Figure 4.1B**. The starting furosine contents were 1.4 mg/g protein in SP-G and SP samples and 1.3 mg/g protein in PP-G and PP samples. At T6, for SP-G and PP-G the furosine level was about 16 times higher than at T0, reaching approximately 22 mg/g protein; from T6 to T48, the furosine contents further increased by ~58% in SP-G and by ~60% in PP-G. This sharp increase in furosine in the first 6 h was due to the active Amadori rearrangement happening at the early stage of glycation. As displayed in **Figure 4.1C**, for the T0 samples, the CML contents were 44.7 mg/100 g protein in SP-G and 30.5 mg/100 g protein in PP-G. For SP-G, the contents of CML at T6 and T48 were ~2 and 3 fold higher than at T0, respectively. For PP-G, the contents of CML at T6 and T48 were ~3 and 7 times higher than at T0, respectively. This suggests that glycation for PP-G was more advanced than for SP-G. To

the best of our knowledge, there have not been other studies simultaneously comparing the CML contents of SP-G and PP-G.

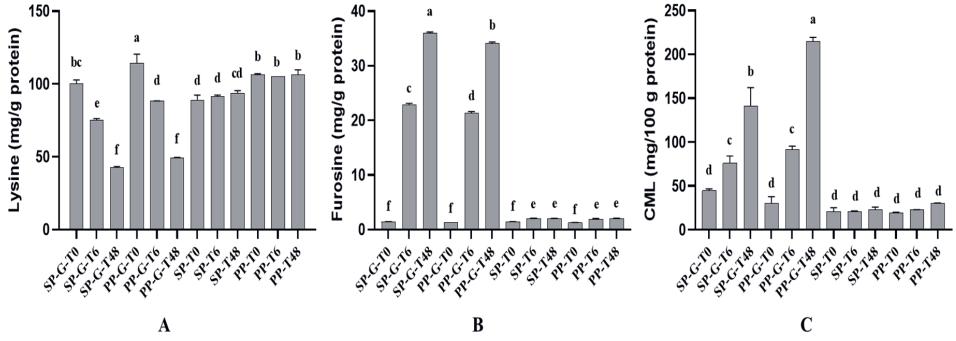

Figure 4.1. Contents of glycation markers (A) lysine, (B) furosine, and (C) CML of SP-G, PP-G, SP and PP upon different heating durations, as measured by LC-MS/MS. Error bars represent standard deviations of duplicates. Statistical differences were analyzed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$).

4.3.1.2. Color

The browning index (BI) was used to monitor color development and obtain information on final stage glycation levels. In **Table 4.1**, for the glucose-containing samples, the increase in BI was approximately 41% in SP-G and 23% in PP-G from T0 to T6, whereas at T48, the BI was ~6 and ~5 times higher than at T0 for SP-G and PP-G, respectively. Thus, dry heating of SP-G and PP-G for 48 h, as expected, showed a much larger extent of the final stage of glycation than dry heating for 6 h. In addition, for glucose-free samples, the increase in BI was slower. However, it was noticed that from T6 to T48, BI increased by around 42% in SP while only by around 12% in PP. The color development of SP was thus more obvious than of PP at T48, although no significant formation of CML was found (**Figure 4.1C**). Therefore, the BI increase in SP was not attributed to glycation but may be due to the browning of flavonoids during dry heating (Muliterno, Rodrigues, de Lima, Ida, & Kurozawa, 2017). First, soybean has a higher total flavonoid content than pea seed (Xu, Yuan, & Chang, 2007). Second, during acid extraction of proteins, the hydrophobic association between proteins and flavonoids led to their co-precipitation (Charlton et al., 2002; Li et al., 2014). Taken together, we speculate that SP will have a relatively higher amount of flavonoids than PP, which may result in non-glycation browning after 48 h of dry heating.

Table 4.1. Color values (L^* = lightness, a^* = redness, and b^* = yellowness) and browning index (BI) of powdered SP-G, PP-G, SP and PP upon different heating durations. Statistical differences were analyzed with ANOVA and Duncan post-hoc test. Different letters within different index columns represent significant differences ($p < 0.05$).

	T0				T6				T48			
	L^*	a^*	b^*	**BI**	L^*	a^*	b^*	**BI**	L^*	a^*	b^*	**BI**
SP-G	89.54 ± 0.01^{a}	-0.65 ± 0.01^{h}	6.17 ± 0.10^{l}	6.36 ± 0.11^{j}	88.81 ± 0.01^{b}	-0.77 ± 0.01^{i}	8.42 ± 0.11^{j}	8.97 ± 0.13^{i}	76.93 ± 0.09^{f}	3.58 ± 0.09^{b}	24.69 ± 0.14^{a}	40.82 ± 0.27^{a}
PP-G	89.04 ± 0.16^{b}	-0.49 ± 0.01^{g}	7.87 ± 0.02^{k}	8.52 ± 0.02^{i}	88.12 ± 0.02^{c}	-0.52 ± 0.02^{g}	9.44 ± 0.01^{i}	10.50 ± 0.02^{h}	74.04 ± 0.33^{g}	4.05 ± 0.05^{a}	23.38 ± 0.03^{b}	40.73 ± 0.32^{a}
SP	85.13 ± 0.16^{d}	-0.50 ± 0.01^{g}	11.11 ± 0.07^{h}	13.06 ± 0.05^{g}	85.55 ± 0.16^{d}	-0.37 ± 0.01^{f}	12.20 ± 0.04^{g}	14.52 ± 0.10^{f}	85.26 ± 0.01^{d}	0.34 ± 0.01^{d}	16.19 ± 0.04^{c}	20.62 ± 0.07^{b}
PP	83.57 ± 0.18^{e}	-0.07 ± 0.02^{e}	13.40 ± 0.11^{f}	16.80 ± 0.12^{e}	84.08 ± 0.26^{e}	-0.09 ± 0.01^{e}	14.09 ± 0.23^{e}	17.62 ± 0.25^{d}	83.74 ± 0.14^{e}	0.64 ± 0.04^{c}	15.13 ± 0.23^{d}	19.80 ± 0.33^{c}

4.3.2. Physicochemical changes

4.3.2.1. Protein composition

The reducing SDS-PAGE results represent the subunit composition of soluble soy and pea proteins with/without glucose after dry heating (**Figure 4.2**). Native SP and PP were mainly composed of globular proteins, namely β-conglycinin (7S) and glycinin (11S) for SP, and vicilin (7S), convicilin (7-8S), and legumin (11S) for PP, which was in line with our previous study in section 3.3.1.

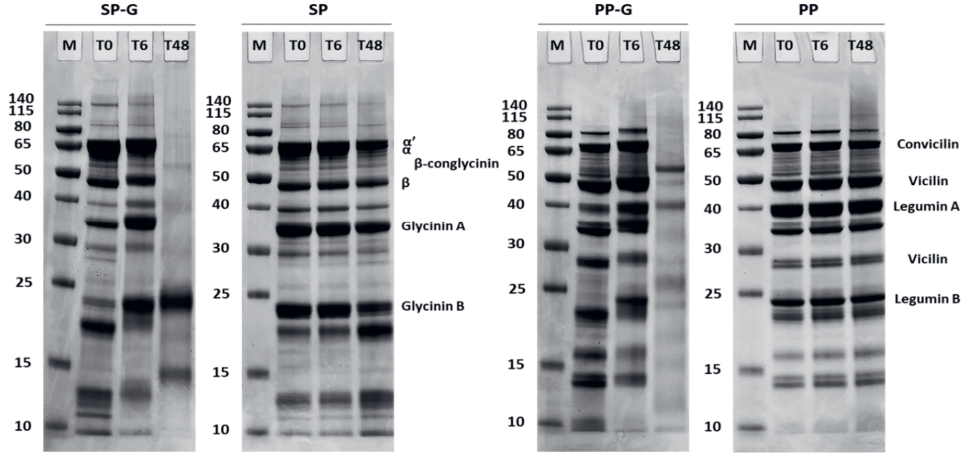

Figure 4.2. Protein composition of soluble SP-G, SP, PP-G and PP upon different heating durations. Lane M: molecular weight marker from 10-140 kDa.

For glucose-free SP and PP, the subunit composition remained almost unchanged when dry heated from 6 h to 48 h, compared to samples at T0. For glucose-containing SP-G and PP-G, glycation became more intense when dry heating lasted 48 h, as represented by the subunits shifting up and some of them becoming lighter or even disappearing, which is consistent with a previous study by Fu et al. (2021). The shifting up of subunits was due to glucose being attached to proteins by covalent reactions during glycation, hence the increase in molecular weights. Moreover, the decrease and disappearance of the subunits were very clear in the samples with glucose at T48. The reason is that, at the advanced and final stages of glycation, cross-linking and aggregation occurred among glycated proteins, leading to larger aggregates being formed (Wang, Yuan, Zhou, & Gu, 2021). Those aggregates would mostly be left in the precipitate after centrifugation of the samples, thus not being visible on the gels.

4.3.2.2. Particle size distribution

The PSD of both suspension and supernatant after dry heating is shown in **Figure 4.3**. For the suspensions in general (**Figure 4.3 A&B**), the particle sizes of proteins heated with glucose were larger than those without glucose. Moreover, as the heating time increased, the small-sized fractions decreased, while the large-sized fractions increased simultaneously. The particle sizes were mostly in the range of 10-100 μm. Only the samples of SP-G-T48 and PP-G-T48 had extremely large particles, the sizes of which were mostly centered at around 310 μm and 220 μm, respectively. These results indicate that dry heating did enhance protein aggregation, but glycation that happened in the glucose-containing samples contributed much more to the overall level of aggregation.

For the supernatants containing only the soluble proteins (**Figure 4.3 C&D**), the PSD curves almost overlapped. The most frequently occurring sizes were around 12 nm for SP-G and SP, as well as around 10 nm for PP-G and PP, as these numbers were the sizes of the monodispersed protein molecules. However, strong aggregation was observed in the samples of SP-G-T48 and PP-G-T48, similar to the suspensions. This is because larger soluble aggregates formed, which was further confirmed by the surface hydrophobicity data at T48 (see section 4.3.2.3).

Regarding the mechanisms of aggregation, for the glucose-free systems, the formation of large particles may be due to heat-induced aggregation. According to Kavanagh, Clark, and Ross-Murphy (2000) and Li, Li, Hua, Qiu, Yang, and Cui (2007), globular proteins first form oligomers by heat-induced disulfide rearrangement and subsequently form aggregates by non-covalent forces (e.g., hydrogen bonds and hydrophobic interactions). For the glucose-containing systems, it is estimated that proteins and glucose were first covalently conjugated

during glycation, and then the conjugates were further aggregated mainly by hydrophobic-hydrophobic interactions (Wang et al., 2021). For SP-G-T48 and PP-G-T48, the reason why soluble aggregates formed was likely to be that the net charges on the surface and repulsive forces kept them soluble (Spotti, Loyeau, Marangón, Noir, Rubiolo, & Carrara, 2019).

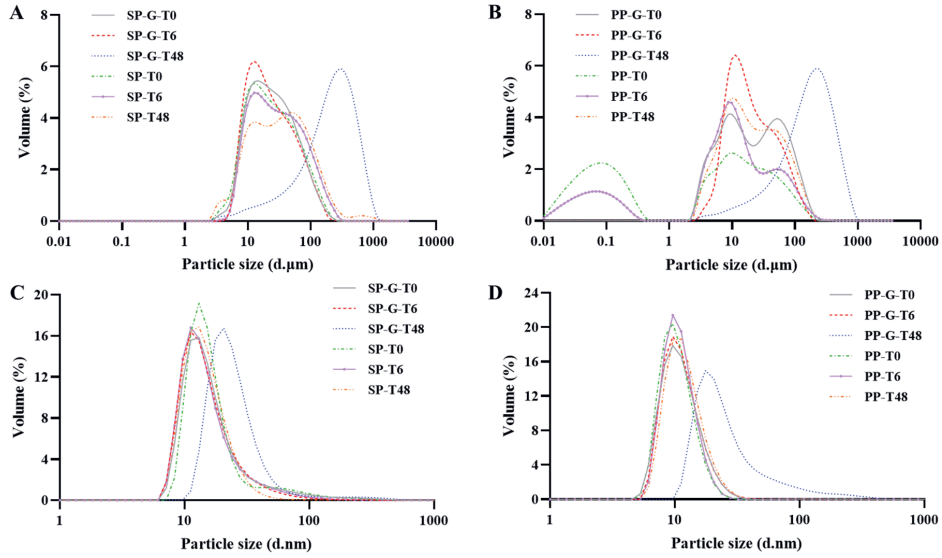

Figure 4.3. Particle size distribution for suspension (A) & (B) and supernatant (C) & (D) of SP-G, PP-G, SP and PP upon different heating durations, respectively. Results were shown as volume-weighted particle size distribution.

4.3.2.3. Surface hydrophobicity

Surface hydrophobicity is an indicator for the distribution of hydrophobic amino acid residues on the protein surface. The surface hydrophobicity of soluble proteins was shown in **Figure 4.4**. Generally, glucose-containing proteins had lower surface hydrophobicity than glucose-free proteins, due to glucose being covalently bound with lysine residues and forming hydrophilic glycation products on the protein surface after dry heating. In SP-G and PP-G, the surface hydrophobicity at T48 was about 13 and 6 times lower than that at T0, respectively, which could be attributed to the highest level of glycation at T48. Furthermore, the surface hydrophobicity in SP and PP remained stable after heating, except that SP-T48 showed a significant decline by approximately 43% in the surface hydrophobicity ($p < 0.05$), compared to SP-T0. One possible reason may be that the long-time dry heating (T48) led to the hydrophobic binding between SP and flavonoids, thereby decreasing the fluorescence intensity, as previously shown by Yuksel,

Avci, and Erdem (2010). Thus, this decline is unlikely to represent that the tertiary structure of SP was affected.

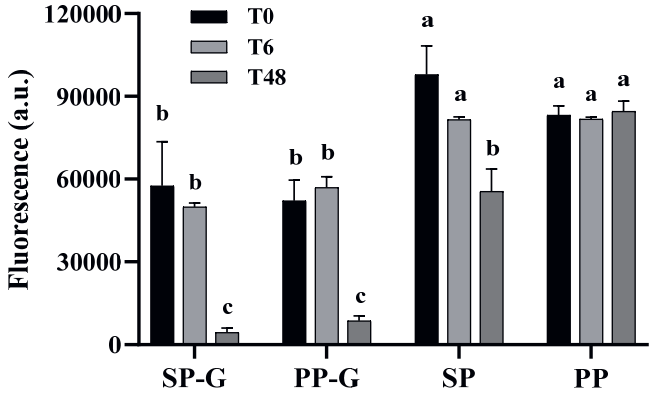

Figure 4.4. Surface hydrophobicity of soluble SP-G, PP-G, SP and PP upon different heating durations. Error bars represent standard deviations of duplicates. Statistical differences were analyzed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$).

4.3.3. *In vitro* protein digestion of dry-heated samples

4.3.3.1. Degree of hydrolysis

The degree of hydrolysis (DH) after digestion were shown in **Figure 4.5**. Generally, the phenomenon that glycation limited the DH was obvious in gastric digestion, and the overall DH of glucose-containing samples after intestinal digestion was relatively lower compared to glucose-free samples. Moreover, SP had a relatively lower DH than PP, whether in the presence or absence of glucose.

In the GP, the DH of all the samples ranged from 0.2% to 2.8%. The reason for the low gastric DH was the relatively high pH of 5.3, and the low pepsin activity of 268 U/mL, reflecting infant digestion conditions. Both SP-G and PP-G showed a similar declining trend in the DH with extended dry heating, with significant differences between T6 and T48 ($p < 0.05$). As digestion progressed from G10 to G60, the DH increased significantly ($p < 0.05$) for the samples at T0 and T6, whereas that at T48 remained unchanged ($p > 0.05$). At the end of gastric digestion (G60), the DH was only 0.2% and 0.4% for SP-G-T48 and PP-G-T48, respectively. These results indicate that highly glycated proteins have a strong resistance to gastric digestion. This was due to the glycation-induced aggregation, which will be further explained later in section 4.3.4. For SP and PP, no significant differences in the DH were found after different heating

durations ($p > 0.05$), whereas the differences from G10 to G60 were significant ($p < 0.05$). Therefore, different heating intensities would not influence the gastric digestibility of these proteins in the absence of reducing sugars, being in line with previously published research (Rivera del Rio, Opazo-Navarrete, Cepero-Betancourt, Tabilo-Munizaga, Boom, & Janssen, 2020).

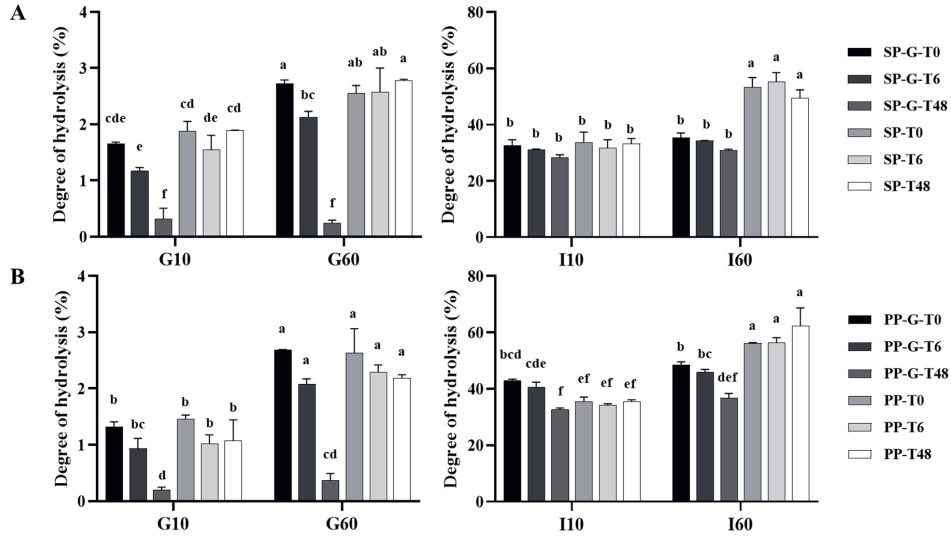

Figure 4.5. Degree of hydrolysis of (A) SP-G and SP as well as (B) PP-G and PP upon different heating durations, after *in vitro* infant digestion. G10 and G60: samples after gastric digestion for 10 min and 60 min, respectively; and I10 and I60: samples after intestinal digestion for 10 min and 60 min, respectively. Error bars represent standard deviations of duplicates. Statistical differences were analyzed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$).

The DH in the IP was much higher than in the GP, as the intestinal digestion was conducted at pH 6.6, near the optimal pH of the enzymes in pancreatin. The values of the DH among all the samples in the IP were between 28.2% and 62.3%. From T0 to T48, the DH of both SP-G and PP-G tended to decrease. However, this trend was not significant in SP-G ($p > 0.05$), but significant only from T6 to T48 in PP-G ($p < 0.05$). This indicates that PP-G was more sensitive to dry heating than SP-G, which was also confirmed by its relatively higher CML levels (**Figure 4.1C**), indicating that especially the advanced stage of glycation reduces the digestibility of proteins. Regarding SP and PP, the results in the IP were all the same ($p > 0.05$) after different heating durations. This suggests that the effect of different dry heating times of full suspensions (without reducing sugars) on intestinal digestion was negligible, similar to gastric digestion. In addition, from I10 to I60, SP and PP increased about 20% in the DH ($p < 0.05$). But interestingly,

for SP-G and PP-G, this increasing trend was not significant ($p > 0.05$), with their DH increasing no more than ~6%. Therefore, at the end of intestinal digestion, samples with glucose had a relatively lower overall DH than those without glucose, independent of heating time.

Comparing the protein sources, the overall DH (I60) of PP was higher than that of SP. Likewise, the overall DH of PP-G was higher than that of SP-G. This is probably because SP contains more trypsin inhibitors than PP, and 60°C of mild dry heating as applied here will not have fully inactivated these trypsin inhibitors, leading to the lower DH in SP and SP-G (Gilani, Cockell, & Sepehr, 2019; Stewart, Raghavan, Orsat, & Golden, 2003).

4.3.3.2. Nitrogen transfer

Nitrogen transfer during digestion was determined by monitoring the nitrogen solubilization in the digestive fluids (**Figure 4.6**).

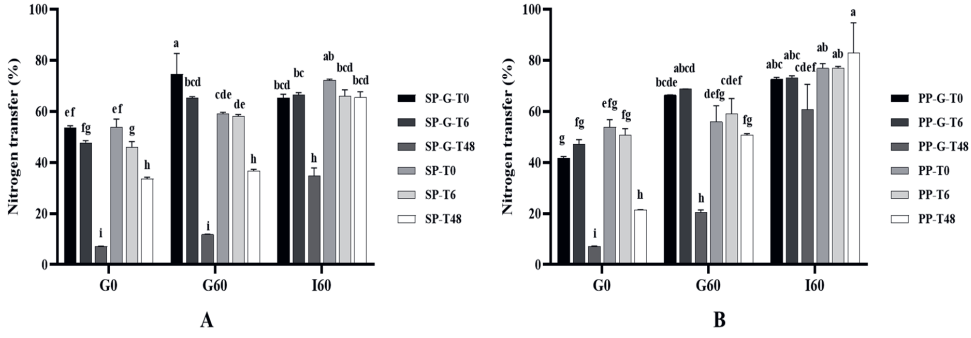

Figure 4.6. Nitrogen transfer of (A) SP-G and SP as well as (B) PP-G and PP upon different heating durations, after *in vitro* infant digestion. G0: samples before digestion; G60: samples after gastric digestion for 60 min; and I60: samples after intestinal digestion for 60 min. Error bars represent standard deviations of digestion duplicates. Statistical differences were analyzed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$).

Before digestion (G0), the percentage of soluble nitrogen in the supernatants was representative of the protein solubility to some extent. For all the samples, the solubility was around 50% for T0; but decreased to only 7.2% (SP-G and PP-G), 33.5% (SP), and 21.4% (PP) for T48. This decrease in solubility reflects the formation of aggregates, as confirmed by the PSD results (**Figure 4.3**). For those T48 samples, the initial content of soluble nitrogen (at G0) was the lowest among all samples. During digestion, the increase in the nitrogen transfer of SP-G and SP was not significant between G0 and G60 ($p > 0.05$), but significant between G60 and I60 ($p < 0.05$). In comparison, for PP-G and PP, the increasing trend in nitrogen transfer was significant ($p < 0.05$) between all different digestion stages (G0, G60, and I60).

In addition, at the end of digestion (I60), all the samples reached their highest level of soluble nitrogen, being similar among all samples, except for SP-G-T48 and PP-G-T48 with relatively lower levels. However, despite so, these similar levels of nitrogen transfer did not translate to similar DH levels at I60. As discussed earlier, SP and PP had a higher overall DH (I60) than SP-G and PP-G. Therefore, the absence of a correlation between DH and nitrogen solubilization indicates that not all the solubilized proteins during digestion are actually hydrolyzed.

4.3.3.3. Disappearance of intact protein

Disappearance of intact protein was monitored by reducing SDS-PAGE, as shown in **Figure 4.7**.

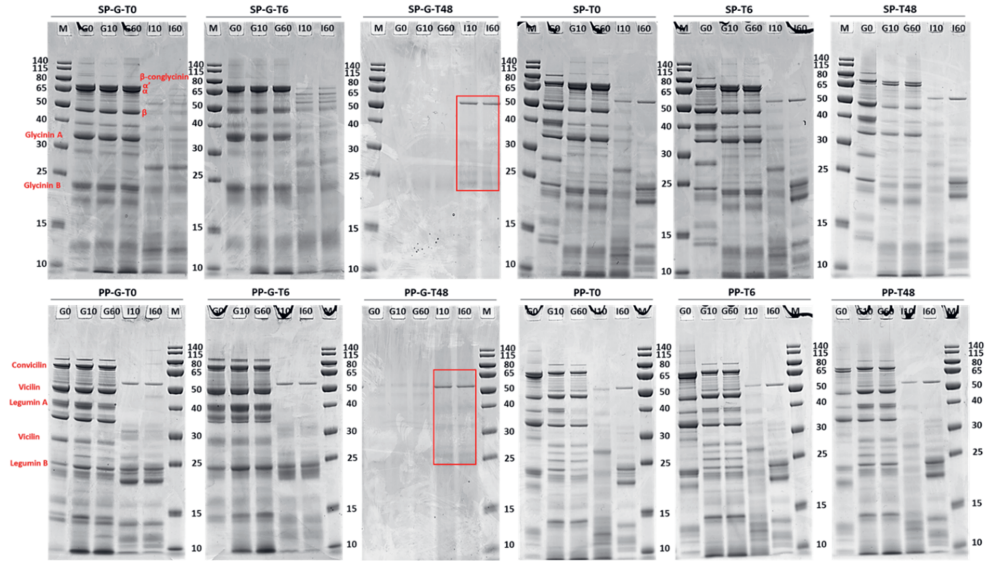

Figure 4.7. Disappearance of intact protein of SP-G, PP-G, SP and PP upon different heating durations, after *in vitro* infant digestion. Lane G0: samples before digestion; lane G10 and G60: samples after gastric digestion for 10 min and 60 min, respectively; lane I10 and I60: samples after intestinal digestion for 10 min and 60 min, respectively; and lane M: molecular weight marker from 10-140 kDa. Solid boxes in red refer to the digestive enzymes from pancreatin.

During gastric digestion, proteins were slightly digested, and large peptides around 10 kDa appeared on the gels. The low gastric disappearance of intact protein agrees with the low DH in the GP (**Figure 4.5**). After 10 min in the IP, large peptides were cleaved into much smaller peptides. From I10 to I60, it is obvious in SP and PP samples that this cleavage into peptides further increased. However, for SP-G and PP-G, the peptides were not digested further, agreeing with the DH results that no significant increase in DH was found between I10 and I60 (**Figure 4.5**).

Furthermore, the bands of the highly glycated proteins (T48) were faint, and almost only the digestive enzymes were visible on the gels. On the one hand, both SP-G and PP-G had the lowest starting solubility at T48, and they were more likely to be digested in the form of insoluble aggregates. During digestion, the formed peptides of low molecular weights went into the digestive fluids. On the other hand, samples were centrifuged to remove the insoluble parts, and only the supernatants were used for SDS-PAGE. Taken together, although the DH and the soluble nitrogen increased, the bands were still not visible on the gels, due to the absence of intact proteins in the supernatants.

4.3.4. Effect of dry heating and glycation on protein digestibility

To summarize, for glucose-containing SP-G and PP-G, longer dry heating contributed to a higher level of glycation, which was manifested by the decrease in lysine and the increase in furosine and CML (**Figure 4.1**), as well as the enhanced browning (**Table 4.1**). The variation in the subunit composition of soluble proteins also confirmed this (**Figure 4.2**). Furthermore, aggregation happened for all the glycated samples (**Figure 4.3**); glycation lowered the surface hydrophobicity of soluble proteins, because of the formation of hydrophilic glycation products on the protein surface (**Figure 4.4**). Among all the samples, the highly-glycated samples at T48 had the largest aggregates as well as the lowest surface hydrophobicity.

However, for glucose-free SP and PP, no glycation occurred during dry heating (**Figure 4.1**). Thus, the heating itself and not the glycation was the dominant driving force of the physicochemical changes. To be specific, the subunit composition and the surface hydrophobicity of soluble proteins remained unchanged after dry heating (**Figures 4.2 & 4.4**). In addition, no soluble aggregates were found in the supernatants, but aggregation in the protein suspensions was seen (**Figure 4.3**). It was noticed that dry heating contributed to a weaker aggregation when compared to heat-induced glycation, as confirmed by the PSD results and the reduced solubility (**Figures 4.3 & 4.6**).

In terms of the overall digestibility, i.e., degree of hydrolysis and the disappearance of intact protein (**Figures 4.5 & 4.7**), for glucose-free samples, no significant difference was found among different dry heating durations. Despite the increased aggregation during dry heating, these aggregates were mostly formed via non-covalent forces and therefore were easy to break down during digestion. Their accessibility to enzymes was thus not hindered. To conclude, the gastro-intestinal digestion was not affected for glucose-free samples.

As for SP-G and PP-G, the resistance to gastric digestion was apparent, especially in the samples at T48. It is known that pepsin preferentially cleaves at hydrophobic amino acid residues (Hall & Moraru, 2022). With enhanced glycation, the surface hydrophobicity of glucose-containing samples was decreased, while aggregation happened simultaneously (**Figures 4.3 & 4.4**). It is possible that when dry heating is extended, the hydrophobic areas of proteins are buried inside, leading to reduced accessibility to pepsin. Furthermore, in terms of intestinal digestion, glycation nearly had nearly no impact on it. Only the DH of PP-G-T6 and PP-G-T48 significantly differed (**Figure 4.5**). This can be explained by the use of pancreatin in this study. Although the tryptic cleavage sites might be blocked by glycation, other proteases and peptidases present in pancreatin, such as chymotrypsin, can still contribute to the protein digestion and compensate for the limited tryptic hydrolysis, being in line with the conclusion from Zenker et al. (2020). However, as discussed before, PP-G was more sensitive to dry heating, at T48, the glycation was more intense than SP-G (**Figure 4.1**). Due to that, the tryptic digestion of PP-G was more inhibited, and such inhibition was not fully offset by other pancreatic enzymes, hence the significant decrease in the DH between T6 and T48. Therefore, it seems that the final intestinal digestion of glucose-containing samples was not affected. However, the different gastric digestion behavior of the glycated proteins may generate differences in peptides in the intestinal phase, and may therefore alter the form in which proteins are presented to the immune system, leading to different immunological responses.

4.4. Conclusion

In the presence of glucose, dry heating at 60°C led to the glycation and aggregation of soy and pea proteins. These structural changes had a negative effect on gastric digestion, whereas the intestinal digestion was influenced to a much smaller extent. Moreover, for those proteins that were heated without glucose, longer dry heating only led to limited aggregation, but had no consequences for both gastric and intestinal digestion. Furthermore, future work should study the peptidomics of the intestinal digesta, to see the overall picture of peptides, as well as the immunoreactive peptides that may affect the immunological response. In addition, using *in vivo* studies to further confirm our findings might be interesting.

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CHAPTER 5

sRAGE-binding and antimicrobial bioactivities of soy and pea protein after heating and *in vitro* infant digestion

This chapter has been submitted as:

Tang, J., Teodorowicz, M., Boeren, S., Wichers, H. J., & Hettinga, K. A. sRAGE-binding and antimicrobial bioactivities of soy and pea protein after heating and *in vitro* infant digestion.

Abstract

This research assessed the impact of different modes of heating on soy protein (SP) and pea protein (PP), focusing on glycation levels, peptide formation during *in vitro* infant digestion, and the sRAGE-binding and antimicrobial activities of the resulting peptides. Dry heating led to increased glycation and glycated peptide production, particularly with higher glycation in PP than SP. Moreover, PP exhibited an overall stronger sRAGE-binding capacity than SP, regardless of heating and digestion conditions. Regarding antimicrobial activity, both SP and PP-derived peptides displayed reduced effectiveness against *Enterobacter cloacae* after dry heating. Additionally, *Staphylococcus epidermidis* was differently inhibited, where PP-derived peptides showed inherent inhibition. The primary determinant of sRAGE-binding and antimicrobial potential in digestion-derived peptides was the protein source. Subsequent bioinformatics analysis predicted 519 and 133 potential antimicrobial peptides in SP and PP, respectively. This study emphasizes the importance of protein source for infant formula to ensure infant health.

5.1. Introduction

Soy protein (SP) constitutes the primary protein source within plant-based infant formula (IF), while a promising alternative, pea protein (PP), is also under consideration (Gorissen et al., 2018). Once infants consume IF, the dietary proteins within these formulas will be digested, leading to the release of peptides and free amino acids (AAs), to support infant growth and development. An important category is that of the bioactive peptides, which, apart from the primary nutritional role, possess a diverse range of potential bioactivities related to health, such as antioxidant, anti-inflammatory, and antimicrobial effects (Daliri, Oh, & Lee, 2017; Li, Dhordain, Hearn, Martin, & Bennett, 2023). During IF production, external factors such as wet and dry heating conditions, as well as the presence of reducing sugars, can induce changes in protein structure, such as denaturation, aggregation, and glycation (i.e., the Maillard reaction). These structural modifications can subsequently affect the process of infant digestion and thereby have consequences for the release of bioactive peptides.

Infant digestion is characterized by its distinct enzymatic profile and relatively higher gastric pH than that of adults (Bourlieu et al., 2014; Nguyen, Bhandari, Cichero, & Prakash, 2015), leading to the digestive process predominantly occurring in the intestinal phase. After gastrointestinal digestion, the released peptides may enter the blood via the intestinal epithelial barrier to exert their potential physiological activity (Amigo & Hernández-Ledesma, 2020; Xue et al., 2021). This process is influenced by the immature digestive system in infants, which tends to restrict protein hydrolysis, thereby resulting in a greater production of peptides rather than free AAs. Additionally, the underdeveloped infant gut barrier exhibits increased permeability, allowing for the absorption of larger molecules, e.g., peptides (Weström, Arévalo Sureda, Pierzynowska, Pierzynowski, & Pérez-Cano, 2020). Consequently, this combination of factors contributes to the release and absorption of a greater number of bioactive peptides in the infant, which can serve as functional molecules (Li et al., 2023).

The assessment of the binding capacities of the peptides to the receptor for advanced glycation end products (RAGE) is particularly important for infants, as their developing immune and metabolic systems are sensitive to changes in cellular responses. RAGE is a multi-ligand receptor that regulates inflammatory responses, oxidative stress, and tissue damage, making it a key player in various physiological processes (Fritz, 2011; Yue, Song, Liu, Zhang, Yang, & Li, 2022). sRAGE is its soluble isoform that binds to the same ligands. Various ligands include advanced glycation end products (AGEs) formed by glycation, S100 protein, high-mobility group protein 1, and amyloid β protein, etc. Quintanilla-García et al. (2019) reported that higher

sRAGE levels in neonatal circulation of neonates compared to maternal circulation, suggesting placental origin of the receptor and its possible role as a decoy protein for circulating AGEs. Therefore, assessing sRAGE binding potential could offer valuable insights into the pro-inflammatory activity of food proteins and peptides.

In addition to their sRAGE-binding properties, the antimicrobial capacity of the SP and PP-based peptides formed during digestion is also interesting to study. Antimicrobial peptides (AMPs) serve as a natural defense mechanism against potential threats. Infants are vulnerable to microbial infections. When released during digestion, these peptides act as a first line of defense within the infant's gastrointestinal tract (Benkerroum, 2010), endogenously preventing the colonization and proliferation of harmful microorganisms to maintain a healthy gut microbiota. Apart from that, those digestion-resistant AMPs, sourced from harmless and cost-effective origins, can be synthesized and introduced into infant foods to enhance their functional properties. Furthermore, glycated AMPs are also an emerging research field offering potential advantages in enhancing stability, targeting, pathogen specificity, microbicidal potency, and reducing microbial resistance (Bednarska, Wren, & Willcocks, 2017; Bellavita, Braccia, Galdiero, & Falanga, 2023).

In this study, we aimed at exploring the effect of different heating conditions on the sRAGE-binding and antimicrobial capacities of peptides derived from SP and PP in the context of infant digestion. Therefore, soy and pea proteins were wet or dry-heated and then *in vitro* digested. The released peptides were identified by LC-MS/MS, and their sRAGE-binding and antimicrobial capacities were determined.

5.2. Materials and methods

5.2.1. Materials and chemicals

Fresh soybean (*Glycine max*; protein content: 39.3%, w/w) was obtained from Wageningen Plant Research, Lelystad, The Netherlands (courtesy ing. Ruud Timmer). Fresh pea (*Pisum sativum*; protein content: 23.3%, w/w) was purchased from a local retailer (Brand name: HAK). Protein contents were determined by DUMAS Flash EA 1112 Protein analyzer (Thermo Fisher Scientific, Waltham, Massachusetts), using a nitrogen-to-protein conversion factor of 5.7. Recombinant human sRAGE was purchased from R&D systems (Minneapolis, Minnesota). Goat anti-human IgG-HRP antibody was purchased from SouthernBiotech (Birmingham, Alabama). Enhanced K-Blue® TMB Substrate was purchased from NEOGEN (Lansing, Michigan). Ovalbumin (OVA) was purchased from InvivoGen (San Diego, California). β-Amyloid peptide 1-42 (AMB 1-42) was purchased from HelloBio (Bristol, UK). All the chemicals were obtained from Sigma-Aldrich (Saint Louis, Missouri) unless otherwise stated.

5.2.2. Sample preparation and heat treatment

Soy protein (SP; protein content: 77.9%, w/w) and pea protein (PP; protein content: 78.8%, w/w) were extracted and quantified, as described in section 3.2.2. Heating treatments were performed in independent duplicates, involving wet heating (W) at 85°C for 30 min and dry heating (D) at 60°C for 48 h, in the presence of a 4-fold excess of glucose (w/w). Non-treated (NT) samples were used as controls.

5.2.3. Quantification of glycation markers

Lysine, furosine (Nε-2-furoylmethyllysine), CML (Nε-carboxymethyllysine), and CEL (Nε-carboxyethyllysine) of all samples were quantified by LC-MS/MS according to our previously established method in section 4.2.5. The final results were displayed as mg/g protein for lysine and furosine, and mg/100 g protein for CML and CEL.

5.2.4. Simulated in *vitro* infant digestion

Simulated *in vitro* infant digestion was conducted according to Ménard et al. (2018) with minor modifications. Briefly, SP and PP were dissolved in 10 mM PBS (pH 7.4) to mimic 1.2% (w/v) protein in IF. A total of 60 min gastric digestion and 60 min intestinal digestion were performed. Sampling was done at 10 min and 60 min of intestinal digestion (I10 and I60). The digestion process was stopped by immediately transferring samples into liquid nitrogen. Digests were

filtered through 10 kDa cut-off filters to collect peptide fractions, which were kept at -20°C until usage.

5.2.5. Peptidomics analysis

Peptide sequences and their glycation-related modifications were identified according to Dingess et al. (2017) and Zenker, Wichers, Tomassen, Boeren, De Jong, and Hettinga (2020) with modifications. Specifically, peptide samples were purified through a solid phase extraction C18 column and were then analyzed through LC-MS/MS by MaxQuant 2.2.0.0 with an "unspecific" digestion mode. Soy and pea protein databases were downloaded from UniProt (https://www.uniprot.org, accessed June 2022) for Andromeda searches with peptide lengths ranging from 8 to 25 AAs to minimize false identifications. Glycation-related modifications at lysine residues included hexose modification (+162 Da), CML modification (+58 Da), and CEL modification (+72 Da); as well as modifications at arginine residues included methylglyoxal hydroimidazolone (MG-H) modification (+54 Da) and glyoxal hydroimidazolone (G-H) modification (+40 Da). Only peptides detected in both duplicate digestions were considered valid. Peptides with a score ≥ 80 were included in the overall profiles and classified into non-glycated and glycated peptides. In addition, for the prediction of antimicrobial peptides, only the peptides with a score ≥ 100 were considered.

5.2.6. Inhibition sRAGE ELISA

Inhibition ELISA-based sRAGE binding assay was conducted following the method described by Teodorowicz et al. (2021) with some modifications. A transparent high-binding ELISA plate was coated with soy protein (G90) glycated under specific conditions (wet heated with glucose, 90 min, at 100°C). This plate was incubated overnight at 4°C, followed by washing with 0.05% Tween-20 in 10 mM PBS. Washing was carried out after each step of the ELISA process. Afterward, the plate was blocked with 3% BSA in 10 mM PBS for 1 h at room temperature. Simultaneously, sample solutions and sRAGE receptor were mixed in a 1:1 volume ratio in the dilution buffer (1.5% BSA and 0.025% Tween-20 in 10 mM PBS), resulting in final protein concentrations of 50 and 25 μg/mL, with the final sRAGE concentration reaching 1 μg/mL. The mixture of the sample/sRAGE solutions was then incubated on a NuncTM polystyrene plate for 1 h at 37°C, followed by transfer to the ELISA plate for an additional 1-hour incubation at 37°C. After the addition of goat anti-human IgG-HRP antibody, the plate was incubated for 30 min at room temperature. Color development was achieved by TMB solution and was stopped by adding 2% HCl. Absorbance (Abs) was measured in duplicate at 450 nm with a reference wavelength of 620 nm, by a Filter Max F5 multi-mode microplate reader (Molecular Devices,

San Jose, California). Results were manifested by the percentage of inhibition, with high inhibition (%) indicating high receptor binding affinity. Inhibition (%) was calculated by the following equation (5-1):

$$Inhibition\ (\%) = \frac{Abs_{sRAGE} - Abs_{sample}}{Abs_{sRAGE}} \quad (5\text{-}1)$$

where Abs_{sRAGE} represented the absorbance for sRAGE without samples, and Abs_{sample} represented the absorbance for the mixture of the sample/sRAGE solutions. All the absorbance measurements were corrected for the dilution buffer used as a blank control.

5.2.7. Surface hydrophobicity

Surface hydrophobicity of digestion-derived peptides was determined by 8-anilino-1-naphthalenesulfonic acid ammonium salt fluorescence assay (see section 3.2.6), with a minor modification that the samples were diluted to 0.5 mg/mL in 10 mM PBS.

5.2.8. Culture of bacterial strains

Gram-negative *Enterobacter cloacae* (DSM 30054) and gram-positive *Staphylococcus epidermidis* (DSM 20044) were purchased from DSMZ (Göttingen, Germany) for use in the antimicrobial assay according to Xiong, Boeren, Vervoort, and Hettinga (2021). These two strains were cultured overnight in specific growth media and temperatures: nutrient broth medium at 30°C for *Enterobacter cloacae*, and tryptic soy broth medium at 37°C for *Staphylococcus epidermidis*. After culturing, the optical density (OD) of the bacterial solution was measured at a wavelength of 600 nm. Subsequently, the bacteria solutions were diluted with PFZ (peptone physiological salt solution; Tritium Microbiology, Eindhoven, The Netherlands) and plated onto their respective agar medium plates, and then cultured for 48 h. Only plates with 20-300 colonies were used for counting. The colony numbers obtained were correlated with their corresponding OD values to draw calibration curves. These curves were then used to calculate the bacterial concentrations needed for subsequent inoculation. All the experimental procedures were conducted under sterile conditions.

5.2.9. Antimicrobial assay

Antimicrobial activity was measured based on the methods of Xiong et al. (2021) and Tidona et al. (2011) with some modifications. Briefly, peptide samples were diluted with 10 mM PBS to achieve a final nitrogen concentration of 0.2 mg/mL (based on DUMAS results), followed by sterile filtration using 0.2 μm filters. Subsequently, 135 μL of the diluted samples were transferred into a transparent 96-well plate. Following this, 15 μL of each bacterium solution

was inoculated into the samples, resulting in a final bacterial concentration of approximately 10^5 CFU/mL. Positive controls involved replacing the sample solutions with broth medium, while the negative controls replaced the sample solutions with distilled water.

Peptide samples and bacteria were co-incubated at their respective optimal temperature for specific durations: 15 h for *Enterobacter cloacae* and 12 h for *Staphylococcus epidermidis*. The selection of these incubation times was informed based on our preliminary study, with the selected time point representing the end of exponential growth (Data not shown). After incubation, the OD_{600} values were measured by SpectraMax ABS Plus microplate reader from Molecular Devices (San Jose, California). The final results were displayed as the bacterial growth rate, with a lower growth rate indicating higher antimicrobial activity. The experiment was conducted in duplicate under sterile conditions. Equation (5-2) was used to calculate the growth rate as follows:

$$Growth\ rate = \frac{OD(t) - OD(t_0)}{OD(t_0)} \quad (5\text{-}2)$$

where OD(t) referred to the OD values measured after incubation, and $OD(t_0)$ referred to the OD values measured before incubation.

5.2.10. Prediction of antimicrobial peptides

The peptides identified by LC-MS/MS were submitted to the $CAMP_{R4}$ webserver (http://www.camp.bicnirrh.res.in/predict/, accessed July 2023) to predict their potential as antimicrobial peptides with a random forest algorithm (Gawde et al., 2022). Prediction mode was set as “natural”, and a threshold of 0.5 was selected.

5.2.11. Statistical analysis

GraphPad Prism 9.0 was used for data visualization and unpaired t-test. IBM SPSS 25.0 was used for analysis by ANOVA with Duncan post-hoc test. Statistical significance was determined at $p < 0.05$.

5.3. Results and discussion

5.3.1. Level of glycation

Lysine, furosine, CML, and CEL are important glycation markers to evaluate the level and stage of glycation after heating of SP and PP. This is due to the covalent reaction between lysine residues and reducing sugars, leading to the formation of furosine at the early stage and CML and CEL at the advanced stage of glycation. The contents of glycation markers are shown in **Figure 5.1**.

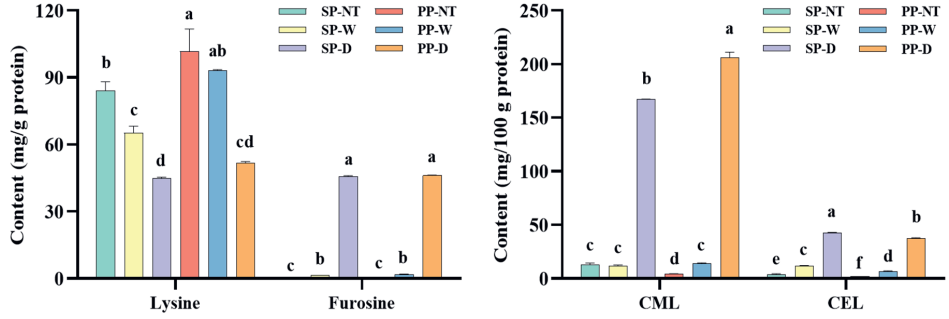

Figure 5.1. Contents of glycation markers—lysine, furosine, CML, and CEL of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP). Error bars represent standard deviations of duplicates. Statistical differences were analysed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$).

Regarding lysine, the initial lysine content of SP before heating was 84.2 mg/g protein. After wet heating, it decreased by ~22%; while after dry heating, it decreased by ~47%. For PP, its initial lysine content was the higher at 101.7 mg/g protein, which decreased by ~8% and 49% after wet and dry heating, respectively. Interestingly, despite PP having a higher initial lysine content than SP, after dry heating, their lysine levels became comparable. In terms of furosine contents, only a slight increase was observed in the wet-heated (W) SP and PP samples compared to the non-treated (NT) samples, by 1.4 and 1.9 mg/g protein for SP and PP, respectively. However, after dry heating, the furosine contents of SP and PP sharply increased by ~46 mg/g protein. As for CML, compared to the NT samples, after wet heating, no increase was found in the CML contents for SP and only a slight 2-fold increase was observed for PP. Moreover, in the dry-heated SP and PP samples, the contents of CML were ~12 and 48-fold higher than those of the NT samples, respectively. Likewise, the CEL contents show similar trends compared to CML. Only slight increases were found after wet heating, of around 2-fold. After dry heating, their contents were approximately 9 and 16 times higher than the NT samples,

respectively. All these results, as expected, indicate that dry heating contributed more to glycation than wet heating, and glycation of PP was more extensive than SP.

5.3.2. Identification of glycated peptides in the digests

Peptides released after 10 min (I10) and 60 min (I60) of intestinal digestion were analyzed by LC-MS/MS. The subsequent data analysis separated the glycated from non-glycated peptides. Results are presented in **Figure 5.2**, showing the relative intensity and number of glycated peptides to the total peptides detected. Intensity refers to the cumulative abundance of peptides, while number represents the diversity of peptides.

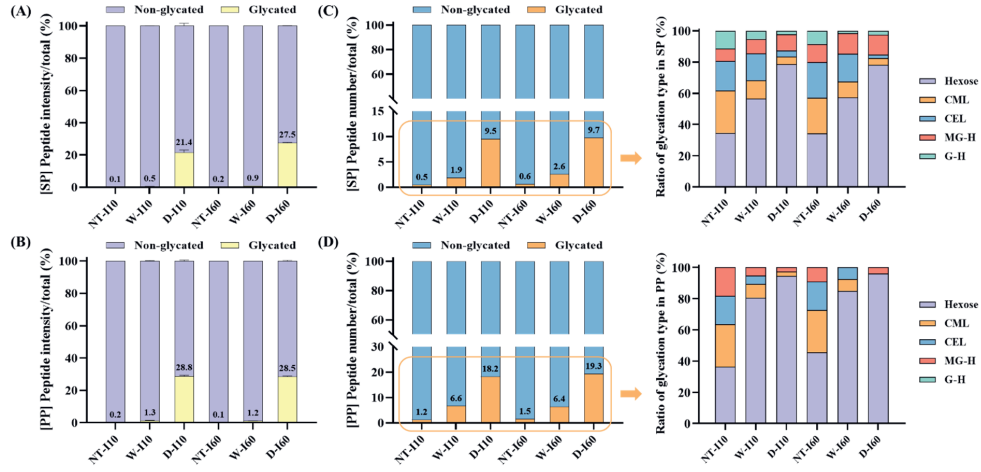

Figure 5.2. Relative intensity and number of glycated peptides to the total peptides in each sample, as well as the ratio of glycation types within the glycated peptides. Peptides were fractionated from non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), after *in vitro* infant digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively.

In general, a trend of increases in both the relative intensity and number of glycated peptides was found after wet and especially dry heating. However, as digestion progressed from I10 to I60, no clear further changes were found. Specifically, by the end of intestinal digestion (I60), the wet-heated (W) SP and PP exhibited only minor increases compared to the NT samples. In the dry-heated (D) samples, the relative intensities of SP and PP increased to ~28% and 29%, respectively; for the relative numbers, the percentages were ~10% and 20%, respectively. These results indicate that wet, and particularly, dry heating contributed to the formation of glycated peptides. In addition, PP is more sensitive to dry heating, as manifested by the higher levels of relative counts than that of SP. These findings are in agreement with the results of the level of glycation in section 5.3.1 **(Figure 5.1**).

Further analysis of the modification types within the glycated peptides is displayed in **Figure 5.2 C&D**. A total of five different types of glycation-related modifications were measured, including modifications at lysine (K) residues (Hexose, CML, and CEL modifications), as well as modifications at arginine (R) residues (MG-H and G-H modifications). Specifically, the K modifications were the dominant modification types among all the differently-treated samples, accounting for more than 80% of the glycated peptides for both SP and PP. Notably, hexose-modified peptides were the most abundant modifications. Specifically for the D samples, which were glycated to the largest extent, around 80% of glycated peptides were hexose-based in SP, while for PP, nearly 95% of them were hexose-based. As for the R modifications, both MG-H and G-H-modified peptides were found in SP. However, in the PP-derived glycated peptides, only MG-H modification was observed. Overall, lysine-based glycation was the main type of glycation for both SP and PP of peptides formed during digestion.

5.3.3. sRAGE-binding affinity

The sRAGE-binding affinity (% inhibition) of the SP and PP samples before and after digestion upon wet and dry heating was measured by sRAGE inhibition ELISA, with a protein concentration of 25 μg/mL (**Figure 5.3**). Before digestion, both SP and PP samples exhibited significantly high binding affinity (> 50% inhibition) to sRAGE, regardless of whether they were heated or not. The NT sample for SP exhibited the highest inhibition, whereas for PP, the W sample did so. Furthermore, both the SP-D and PP-D samples had the lowest inhibition, at ~50% and 53%, respectively. Notably, under the same heating conditions, PP exhibited higher inhibition compared to SP. This phenomenon was also found in the samples with a higher protein concentration of 50 μg/mL (**Figure 5.S1**).

After digestion at I10 and I60, the binding affinity of the peptide fractions was sharply reduced to less than 18% (**Figure 5.3**). This decline suggests that digestion can lower the binding capacity of proteins to sRAGE, which in turn may contribute to reducing the risk of inflammatory processes linked to conditions such as diabetes, immune disorders, and chronic inflammation. Generally, the inhibitions for all the SP samples ranged from no inhibition (-3%) to about 4%, whereas for PP, the inhibitions were between ~6% and 18%. Thus, similar to the undigested samples, digestion-derived PP samples had significantly higher inhibition than SP, at both protein concentrations of 25 μg/mL ($p = 0.001$) and 50 μg/mL ($p = 0.017$) (**Figures 5.3 & 5.S1**). To be specific, for SP, no significant differences in inhibition were found among the samples from different heating and digestion conditions. While for PP, significances were observed between different heating conditions, with the NT samples exhibiting the highest

inhibitions. After dry heating, the inhibitions dropped by ~7% at I10 and ~9% at I60. Moreover, the inhibition percentages between I10 and I60 were not significantly different. This might be because most of the sRAGE-binding peptides were formed during the first 10 min of intestinal digestion.

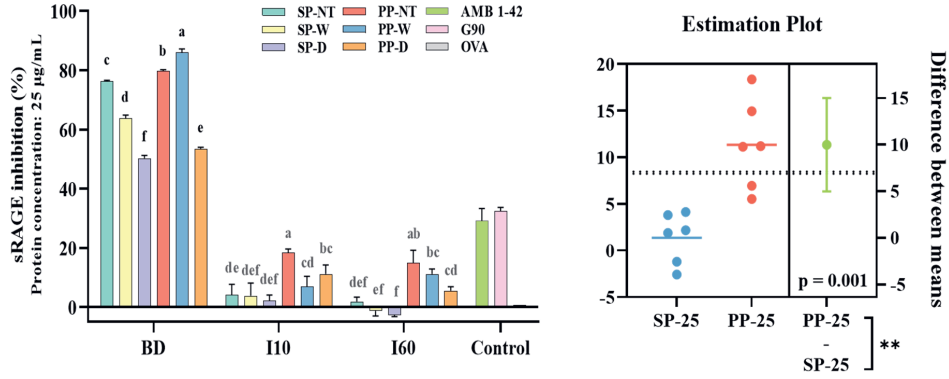

Figure 5.3. sRAGE inhibition ELISA of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), before and after *in vitro* infant digestion, using a protein concentration of 25 µg/mL. BD refers to the samples before digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively. β-Amyloid peptide 1-42 (AMB 1-42) and soy protein glycated with glucose for 90 min at 100°C (G90) were used as positive controls, while ovalbumin (OVA) was used as a negative control. Error bars represent standard deviations of duplicates. Statistical differences of samples before and after digestion were analysed with ANOVA and Duncan post-hoc test. Different letters in black (for proteins before digestion) and in grey (for peptides) above the bars represent significant differences within the group ($p < 0.05$). Statistical differences between peptide groups of SP and PP were analysed with unpaired t-test: *$p < 0.05$ and **$p < 0.01$.

Regarding mechanisms, various factors could contribute to the sRAGE binding, including surface hydrophobicity, aggregation, glycation-induced AGE formation, and β-sheet structures (Fritz, 2011; Liu, Teodorowicz, Wichers, van Boekel, & Hettinga, 2016; Sugihara et al., 2012; H. E. Zenker et al., 2020). All these factors involve RAGE recognizing the negative charge and/or hydrophobic domains on its ligands (Bongarzone, Savickas, Luzi, & Gee, 2017; Deane, 2012). For SP and PP, both contain more than 30% β-sheet structures (Shevkani, Singh, Chen, Kaur, & Yu, 2019). In addition, research reported that the surface charge of SP and PP were comparable, but the surface hydrophobicity of PP was considerably higher than SP (Karaca, Low, & Nickerson, 2011). Thus, in this study, before digestion, the general higher sRAGE binding capacity of PP than SP was more due to the higher level of surface hydrophobicity of PP. Furthermore, the reduced inhibition observed in dry-heated proteins could be attributed to a) reduced surface hydrophobicity by the formation of hydrophilic AGEs on the outer surface of SP and PP, and b) the increased covalent aggregation by glycation leading to the burial of

sRAGE recognizing sites. The related results were shown in **Chapter 4**. In other words, despite that AGEs are the main ligands for RAGE, their contribution to binding is limited, potentially even hindering it. On the contrary, the high surface hydrophobicity facilitated the interactions of SP and PP with RAGE. In terms of digestion-derived peptides, surface hydrophobicity no longer appeared to be a significant factor. As shown in **Figure 5.S2**, the surface hydrophobicity of all the peptide samples did not show significant differences among them. Nevertheless, the overall inhibition of SP was lower than that of PP. Thus, it was probably the inherent protein characteristics that governed the binding affinity of peptides to sRAGE after digestion. Variations in protein structure and the AA sequences contributed to the different digestive selectivity, resulting in diverse peptide compositions between SP and PP, and consequently, distinct sRAGE-binding affinities. This phenomenon was primarily linked to the primary sequence composition of the peptides and was no longer influenced by the advanced protein structure.

In summary, the undigested samples exhibited significantly higher sRAGE-binding affinities, compared to the digested peptides. Moreover, PP demonstrated a higher overall inhibition than SP, both before and after digestion. In addition, dry heating-induced glycation did not increase the binding capacity to sRAGE, but rather diminished it to some extent. Notably, after digestion, sRAGE binding to the samples seemed more closely related to the inherent protein characteristics rather than variations in heating conditions and digestion times. Ultimately, the differences in sRAGE-binding capacities of peptides were primarily attributed to the source of proteins.

5.3.4. Antimicrobial property

5.3.4.1. Antimicrobial activity of peptides

Antimicrobial activity of SP and PP-derived peptides was assessed based on the bacterial growth rate, where a low growth rate indicates a high capacity to inhibit the growth of microorganisms. **Figure 5.4** shows the growth rates of *Enterobacter cloacae* and *Staphylococcus epidermidis* in each sample.

In relation to *Enterobacter cloacae* (**Figure 5.4A**), wet heating had minimal effects on bacterial growth; dry-heated protein samples did not inhibit bacterial growth but rather facilitated it, resulting in a growth rate surpassing even the positive control. The growth rate of *Enterobacter cloacae* in the presence of the dry-heated SP increased significantly by ~32% and 28% at I10 and I60, respectively, compared to the NT samples; for PP, it increased by ~54% and 59%,

respectively. Reasons might be, firstly, members of the Enterobacteriaceae family possess the inherent capability to ferment sugars, and they also previously exhibited an enhanced growth response when stimulated by glycated proteins compared to non-glycated ones (Dominika, Arjan, Karyn, & Henryk, 2011; Singh, Sharma, & Nara, 2015). Secondly, the process of digestion enzymatically transformed intact proteins into peptides, enabling them to be more easily absorbed, transported, and utilized by microorganisms. Consequently, *Enterobacter cloacae* could effectively use glycated peptides. Furthermore, under the same heating conditions, no notable differences in growth rate were observed between I10 and I60, due to the high intestinal digestibility within the initial 10 min leading to the formation of the majority of peptides at I10 with only minor changes between I10 and I60.

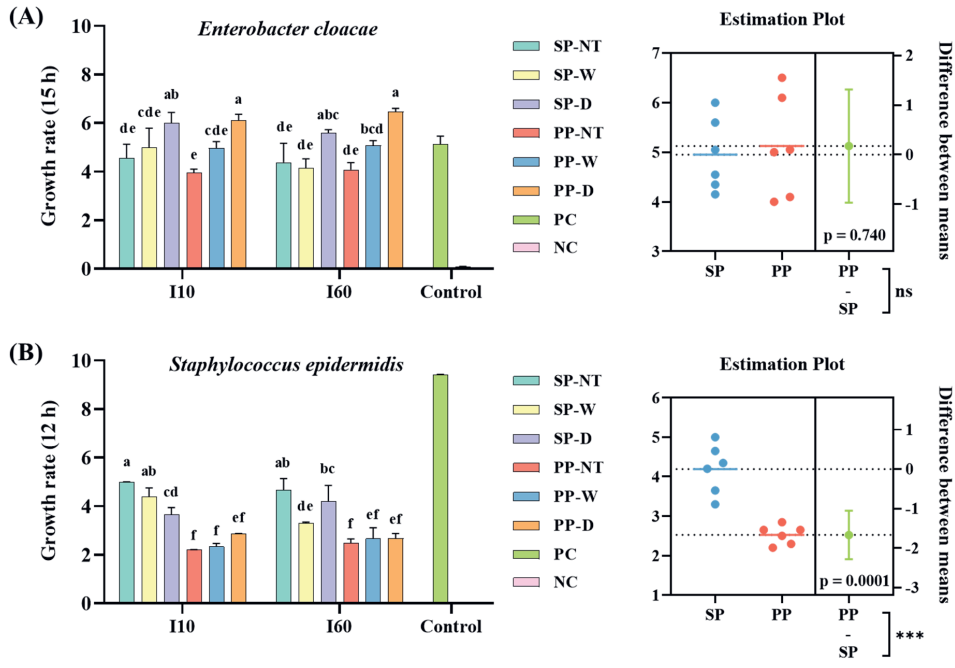

Figure 5.4. Growth rates of (A) *Enterobacter cloacae* and (B) *Staphylococcus epidermidis* of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), after *in vitro* infant digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively. PC refers to the positive controls replacing the sample solutions with broth medium. NC refers to the negative controls replacing the sample solutions with distilled water. Error bars represent standard deviations of duplicates. Statistical differences were analysed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$). Statistical differences between peptide groups of SP and PP were analysed with unpaired t-test: ns $p > 0.05$ and ***$p < 0.001$.

For *Staphylococcus epidermidis* (**Figure 5.4B**), the SP and PP samples behaved differently regarding their growth rate. For SP, the NT samples were associated with the highest growth rate, and heating decreased bacterial growth to some extent. After dry heating, the growth rate at I10 declined by ~37%, whereas after wet heating, it decreased by ~41% at I60. For PP, the growth rates were all at a comparable level, irrespective of the extent to which the PP samples were heated and digested. Furthermore, similar to *Enterobacter cloacae*, from I10 to I60, the growth rates of *Staphylococcus epidermidis* were not significantly different. More importantly, the overall growth rate of *Staphylococcus epidermidis* in PP was significantly lower than in SP ($p = 0.0001$). The divergent primary AA compositions could be an inherent factor driving the microbial behavior, similar to sRAGE-binding.

To conclude, *Enterobacter cloacae* displayed no specific preference for SP- and PP-based peptide samples, and dry heating did not inhibit its growth but instead facilitated it. For *Staphylococcus epidermidis*, heating lowered its growth rate in the SP digests, with PP samples significantly inhibiting bacterial growth, independent of heating, when compared to SP. In addition, the extension of digestion time did not influence the bacterial growth of both SP and PP. These results highlight the differential responses of bacterial strains to peptide samples derived from digestion of different protein sources and heating conditions, suggesting potential implications for their antimicrobial activities.

5.3.4.2. Prediction of antimicrobial peptides

To identify the antimicrobial peptides (AMPs) in the digests for samples with different heating and digestion conditions, the online prediction tool $CAMP_{R4}$ was used. As a result, 519 peptides were found to be AMPs in SP, with 9 of them being glycated. For PP, 133 distinct AMPs were predicted, including 6 that were glycated. Moreover, at the end of intestinal digestion (I60), the SP samples had 361 AMPs for NT, 208 for W, and 120 for D, while the PP samples had 76 AMPs for NT, 70 for W, and 34 for D. These endogenously formed AMPs during digestion can support immunity and defense against microbial infections, and hold the potential to be valuable supplements in infant foods. For a complete list of all identified AMPs, please refer to **Tables 5.S1 & 5.S2**. The AMP probability score reflects the likelihood of a peptide having antimicrobial capabilities. For SP, a total of 103 peptides had a score over 0.6. Among them, peptide "KIGGIGTVPVGRVETGVLKPGM" (A0A0R0FER5, aa 243-264) achieved the highest score of 0.91. However, in the case of PP, only 13 peptides had a score above 0.6, with the peptide "VVIIPAGHPVA" (Q9M3X6, aa 513-523) having the highest score of 0.68. Furthermore, as shown in **Figure 5.5**, the D samples exhibited the highest abundance of

glycated AMPs: approximately 5.6% at I10 and 3.3% at I60 for SP, and respectively 10% and 11.8% for PP. The W samples showed a relatively lower abundance, while the NT samples contained the least. This trend was similar to the percentage of glycated peptides to the total peptide numbers (**Figure 5.2**).

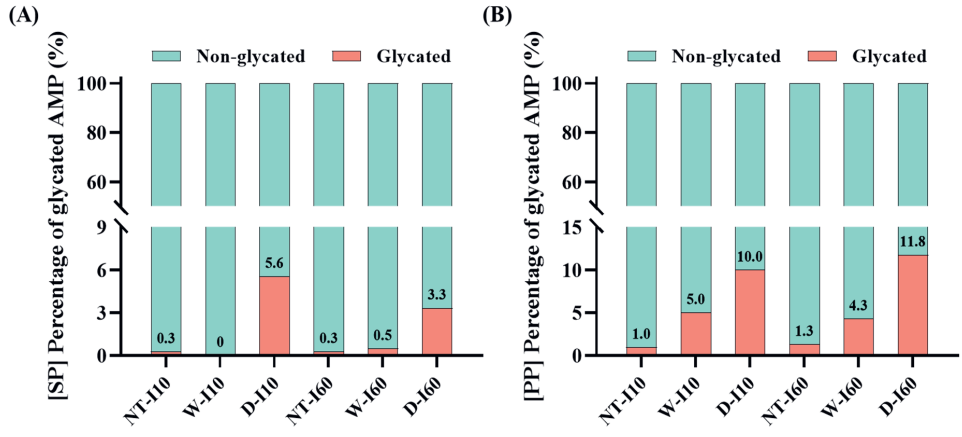

Figure 5.5. Count ratio of predicted non-glycated and glycated antimicrobial peptides (AMPs) in the digests of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP). I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively.

Given the above-mentioned potential benefits of glycated AMPs, we conducted a focused analysis of 9 glycated AMPs derived from SP and 6 from PP. Their detailed information and intensity changes are illustrated in **Table 5.1**. The highest probability score of 0.67 was found to be peptide "TVGIGGPVGTGKT" in SP. On the other hand, in PP, peptides "IVTVTEGKGDFE", "IVTVTEGKGDFEL", and "LNNNQNLKPL" all shared an equal highest score of 0.53. The underlined AAs refer to the glycation sites. Notably, the hexose (K) modification was the dominant modification type amongst all the glycated AMPs. Besides, the SP peptide "NFNNPTRSKNP" had two modifications: G-H (R) modification and hexose (K) modification. An intriguing discovery was the identification of CML-modified peptides "NKLQGKIA" (SP) and "GDAIKLPAGTI" (PP), which were not generated from the D samples but were already present in the NT samples.

Table 5.1. List of potential glycated antimicrobial peptides (AMP) of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), after in vitro infant digestion. Glycation sites at lysine (K) or arginine (R) residues were highlighted in bold and underlined. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively.

#	Glycated AMP of SP	AMP Probability	Glycation Type (Site)	Protein Group	Protein ID	Position	Length	Peptide Intensity					
								NT-I10	W-I10	D-I10	NT-I60	W-I60	D-I60
1	IAQG**K**GALGV	0.62	Hexose (K)	Glycinin G4	P02858	94-103	10	0	0	2.93×10^{8}	0	0	0
2	**K**GNVGGLIGTGL	0.54	Hexose (K)	Lipoxygenase	I1M596	41-52	12	0	0	3.06×10^{7}	0	0	1.67×10^{7}
3	NFNNPT**R**S**K**NP	0.5	Hexose (K); G-H (R)	Eukaryotic translation initiation factor 3 subunit D	I1MEK0	56-66	11	0	0	0	0	0	2.06×10^{8}
4	N**K**LQGKIA	0.55	CML (K)	Uncharacterized protein	A0A0R0I4F6	38-45	8	2.57×10^{7}	0	0	3.24×10^{7}	0	0
5	NVDNVFEDPDGL**K**	0.53	Hexose (K)	Beta-amylase	Q42795	25-37	13	0	0	0	0	1.75×10^{6}	1.41×10^{8}
6	TVGIGGPVGTG**K**T	0.67	Hexose (K)	Ni-binding urease accessory protein UreG	Q9XGS2	85-97	13	0	0	4.15×10^{7}	0	0	2.77×10^{7}
7	VETGVL**K**PGMVV	0.54	Hexose (K)	Elongation factor 1-alpha	A0A0R0FER5	255-266	12	0	0	4.44×10^{7}	0	0	0
8	VETGVV**K**PGMVV	0.55	Hexose (K)	Elongation factor 1-alpha	A0A0R0JTV5	255-266	12	0	0	5.65×10^{7}	0	0	0
9	VGSVEVG**K**LAD	0.5	Hexose (K)	Urease	I1K3K3	693-703	11	0	0	1.27×10^{8}	0	0	0

#	Glycated AMP of PP	AMP Probability	Glycation Type (Site)	Protein Group	Protein ID	Position	Length	Peptide Intensity					
								NT-I10	W-I10	D-I10	NT-I60	W-I60	D-I60
1	FNTDYEEIE**K**	0.5	Hexose (K)	Vicilin	P13918	186-195	10	0	1.06×10^{7}	8.99×10^{7}	0	4.10×10^{6}	1.45×10^{7}
2	GDAI**K**LPAGTI	0.52	Hexose (K)	Vicilin 47k	Q43626	118-128	11	0	2.13×10^{6}	1.98×10^{7}	0	9.87×10^{4}	6.07×10^{5}
3	GDAI**K**LPAGTI	0.52	CML (K)	Vicilin 47k	Q43626	118-128	11	3.66×10^{6}	1.22×10^{7}	0	1.45×10^{6}	0	0
4	IVTVTEG**K**GDFE	0.53	Hexose (K)	Vicilin 47k	D3VNE0	309-320	12	0	2.20×10^{7}	5.26×10^{8}	0	4.50×10^{6}	2.82×10^{7}
5	IVTVTEG**K**GDFEL	0.53	Hexose (K)	Vicilin 47k	D3VNE0	309-321	13	0	1.07×10^{6}	1.71×10^{7}	0	0	0
6	LNNNQNL**K**PL	0.53	Hexose (K)	Seed linoleate 9S-lipoxygenase-3	P09918	460-469	10	0	0	1.69×10^{8}	0	0	8.49×10^{7}

When comparing the various intensities of glycated AMPs after heating and digestion, only the two above-mentioned CML-modified peptides were present in the NT samples at both I10 and I60. Furthermore, as digestion progressed from I10 to I60, a decreasing trend in intensity was observed for nearly all the glycated AMPs, with some even becoming undetectable at I60. This was due to the further degradation of the AMPs during digestion. In contrast, only two SP-derived peptides showed detectable intensities at I60, whereas they were non-detectable at I10. Specifically, the intensity of peptide "NFNNPTRSKNP" was 2.0×10^8 at D-I60. As for peptide "NVDNVFEDPDGLK", its intensity was 1.8×10^6 at W-I60 and 1.4×10^8 at D-I60. The increase in intensity at I60 might be attributed to the further degradation of larger peptides or the intact protein, leading to the formation of peptides possessing antimicrobial properties.

5.4. Conclusion

The heating of both SP and PP samples, particularly through dry heating, led to increased glycation levels and subsequent formation of glycated peptides during digestion. PP exhibited a higher degree of glycation compared to SP after heating. Moreover, PP consistently demonstrated higher sRAGE-binding affinity than SP, regardless of heating and digestion phase. This difference between SP and PP was expected to be related to their inherent physicochemical characteristics. Regarding antibacterial properties, dry heating resulted in reduced antimicrobial activity against *Enterobacter cloacae*, in both SP and PP. However, the response of SP and PP-derived peptides differed for *Staphylococcus epidermidis*. Specifically, heating increased the antimicrobial activity of SP, while PP exhibited intrinsic inhibitory effects on its growth, independent of heating. Therefore, the protein source is the primary factor shaping the sRAGE-binding and antimicrobial potentials of digestion-derived peptides. This emphasizes the significance of selecting suitable protein sources in IF, as they could play an important role in promoting health and combatting against infections during infancy.

Subsequent bioinformatics analysis predicted 519 potential SP-based AMPs, 9 of which were glycated. Similarly, for PP, a total of 133 AMPs were predicted, with 6 that were glycated. AMPs have gained recognition as a promising avenue for developing therapeutic agents to combat microbial infections. Given this context, an intriguing subsequent step could involve synthesizing the predicted AMPs to evaluate both their antimicrobial capacity and their resilience against digestion. Moreover, a deeper exploration into the analysis of glycated AMPs could enhance the comprehensiveness of our investigation.

Supplementary information

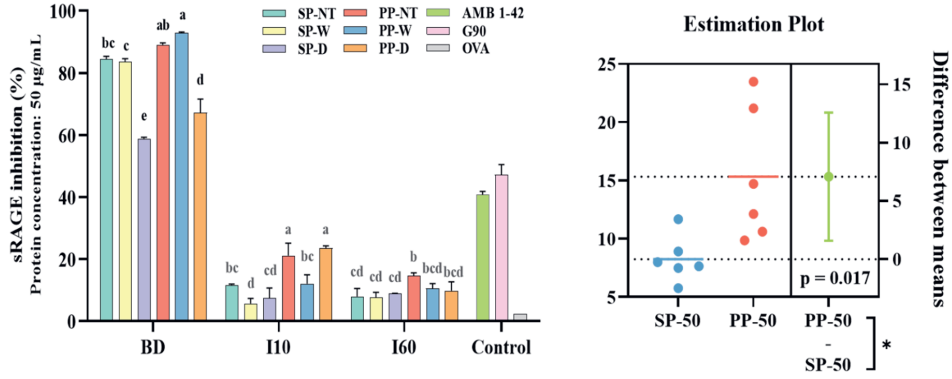

Figure 5.S1. sRAGE inhibition ELISA of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), before and after in vitro infant digestion, using a protein concentration of 50 µg/mL. BD refers to the samples before digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively. β-Amyloid peptide 1-42 (AMB 1-42) and soy protein glycated with glucose for 90 min at 100°C (G90) were used as positive controls, while ovalbumin (OVA) was used as a negative control. Error bars represent standard deviations of duplicates. Statistical differences of samples before and after digestion were analysed with ANOVA and Duncan post-hoc test. Different letters in black (for proteins before digestion) and in grey (for peptides) above the bars represent significant differences within the group ($p < 0.05$). Statistical differences between peptide groups of SP and PP were analysed with unpaired t-test: *$p < 0.05$ and **$p < 0.01$.

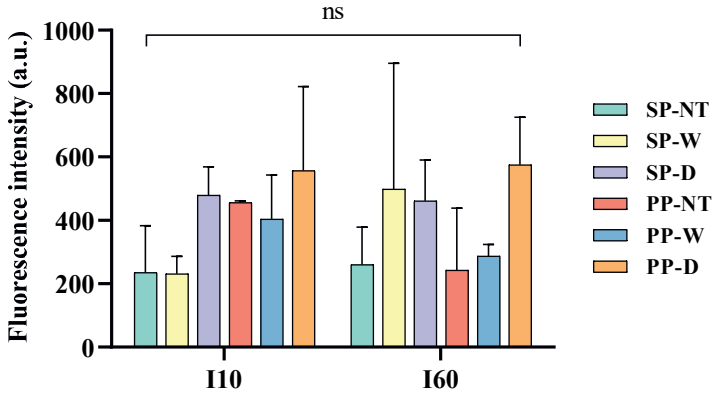

Figure 5.S2. Surface hydrophobicity of peptides in the digests of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), after *in vitro* infant digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively. Error bars represent standard deviations of duplicates. Statistical differences of samples were analysed with ANOVA and Duncan post-hoc test: ns as no significance ($p > 0.05$).

Tables 5.S1 & 5.S2. List of all predicted antimicrobial peptides (AMPs) of non-treated (NT), wet-heated (W), and dry-heated (D) soy protein (SP) and pea protein (PP), after *in vitro* infant digestion. I10 and I60 represent the samples after 10 min and 60 min of intestinal digestion, respectively. Please scan this QR code to access these supplementary tables, or visit: https://figshare.com/s/3c77b25ad3b7b8acca74.

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CHAPTER 6

Residual immunogenicity of soy-derived peptides released after *in vitro* infant digestion upon different heating conditions

This chapter has been submitted as:

Tang, J., Boeren, S., Wichers, H. J., & Hettinga, K. A. Differential effects of heating modes on the immunogenic potential of soy-derived peptides released after *in vitro* infant digestion.

Abstract

During production of soy-based infant formula, soy protein undergoes heating processes. This study investigated the differential impact of heating on the residual immunogenicity of peptides in soy protein digests. Wet or dry heating were applied, followed by *in vitro* gastrointestinal infant digestion. The released peptides were analyzed by LC-MS/MS, and bioinformatics tools were utilized to predict and identify potential linear B-cell and T-cell epitopes, as well as to explore cross-reactivity with other legumes. The peptide intensities of potential epitopes were subsequently compared between heating methods. We confirmed the previously observed enhancing effect of wet heating on infant digestion and inhibitory effect of dry heating. A total of 29 potential T-cell epitopes and 27 potential B-cell epitopes were identified. Cross-reactivity between soy and other legumes, including peanut, pea, chickpea, lentil, kidney bean, and lupine, was also detected. Overall, heating and digestion time could modulate the potential to trigger peptide-induced immune responses.

6.1. Introduction

Soy protein-based infant formula (IF) is commercially used as a substitute for dairy-based IF, as it is suitable for infants with cow's milk allergy (CMA), lactose intolerance, or those from vegan families. However, approximately 10-14% of infants with CMA also exhibited allergic reactions to soy protein (Kattan, Cocco, & Järvinen, 2011; Klemola, Vanto, Juntunen-Backman, Kalimo, Korpela, & Varjonen, 2002; Zeiger et al., 1999). This is because people with one food allergy may be more prone to developing additional allergies due to a heightened immune system response (Hill & Spergel, 2018). Furthermore, according to WHO/IUIS Allergen Nomenclature Sub-committee (http://www.allergen.org/), eight common soy allergens involve hydrophobic protein (Gly m 1), defensin (Gly m 2), profilin (Gly m 3), pathogenesis-related protein (Gly m 4), β-conglycinin (Gly m 5; vicilin, 7S globulin), glycinin (Gly m 6; legumin, 11S globulin), seed biotinylated protein (Gly m 7), and 2S albumin (Gly m 8). Amongst them, β-conglycinin and glycinin have been extensively studied due to their association with severe soy allergies (Holzhauser et al., 2009; Ito et al., 2011). Furthermore, individuals with a soy allergy may exhibit sensitivity to other legumes. This phenomenon, referred to as allergic cross-reactivity, occurs when a secondary allergen is capable of cross-reacting with a primary allergen, leading to the recognition of immunoglobulin E (IgE) antibodies or the activation of cellular responses, such as T cell response. In this case, the immune system identifies both allergens as either identical or similar due to the presence of shared epitopes (Cox, Eigenmann, & Sicherer, 2021; Kamath, Bublin, Kitamura, Matsui, Ito, & Lopata, 2023).

Heat treatments, such as pasteurization, sterilization, and spray drying are essential in the production of IF to ensure microbiological safety and maintain high product quality during shelf life. However, denaturation and aggregation may occur during these steps, as well as glycation in the presence of reducing sugars, resulting in potential alteration of epitopic properties, hence differences in immunogenicity of these proteins. For instance, heating can increase allergenicity by inducing protein unfolding and aggregation exposing new epitopes (Li, Yang, Zou, Shu, Han, & Yang, 2022; Pi, Liu, Sun, Ban, Cheng, & Guo, 2023). On the other hand, heating can also decrease allergenicity through glycation, which involves the formation of soluble aggregates and/or covalent modification on lysine and arginine residues within epitopes, resulting in a reduced capacity for IgE binding (Bai et al., 2021; Han et al., 2018).

Furthermore, during digestion, proteins undergo enzymatic breakdown into smaller peptides within the gastrointestinal tract. These resulting peptides may have different immunogenicity compared to the intact proteins due to changes in epitope structure (Li, Zhu, Zhou, Peng, &

Guo, 2013). Nevertheless, certain peptides derived from digestion, whether from common allergens or other proteins not known to be an allergen, can still retain, or even develop, immunogenic properties and have the potential to elicit immune responses mediated by B cells and T cells through distinct mechanisms (Cano & Lopera, 2013; Sanchez-Trincado, Gomez-Perosanz, & Reche, 2017). B-cell immune responses involve the recognition of specific peptides by B-cell receptors, leading to the production of antibodies, including IgG, and in the case of an eventual allergic reaction, also IgE (Van Wijk, Hartgring, Koppelman, Pieters, & Knippels, 2004). Once bound to effector cells such as mast cells or basophils, these antibodies can bind to the peptides and trigger allergic reactions upon subsequent exposure. T-cell immune responses, particularly T-helper (Th) cell responses, involve the recognition of exogenous digestion-derived peptides by major histocompatibility complex class II (MHC-II) molecules on the surface of antigen-presenting cells. This recognition activates the differentiation of CD_4^+ T-cells into Th2 cells and initiates the release of cytokines, particularly interleukin-4 (IL-4), which further stimulates B-cell activation and IgE antibody production, as well as up-regulation of MHC-II expression. These immune responses contribute to the development of allergic reactions and inflammation in individuals with certain protein allergies, playing a key role in the immunogenic response after digestion.

To our knowledge, only a few studies regarded residual immunogenicity, including e.g., milk proteins (Nutten et al., 2020; Picariello et al., 2010), rice prolamins (Moreno, Muñoz-Suano, López-Casado, Torres, Sousa, & Cebolla, 2016), peanut (Di Stasio et al., 2017), and soy protein (De Angelis, Pilolli, Bavaro, & Monaci, 2017; Li et al., 2013). However, none of these studies were conducted in the context of infant digestion. Infants possess an immature digestive and immune system, which can make them more susceptible to certain components, such as soy protein, present in IF. The lowered proteolytic capacity of infants keeps proteins and peptides more intact, and thus the potential residual immunogenicity may differ from that of adults. Additionally, the impact of different heating processes on residual immunogenicity has been relatively understudied, warranting further investigation. Therefore, understanding these factors is crucial for evaluating the risk of immune reactions in infants consuming soy-based IF. This can provide insights into the persistence or elimination of immunogenic peptides during infant digestion and aid in the development of safer IF for susceptible individuals.

Therefore, this study aimed at investigating the influence of different heating processes on the residual immunogenicity of soy peptides after *in vitro* infant gastrointestinal digestion. To achieve this aim, soy protein was heated in wet and dry conditions and then subjected to one-

month *in vitro* infant digestion, followed by peptidomics analysis based on LC-MS/MS. After that, online bioinformatics tools that are time-saving, cost-effective, sensitive, and high-throughput (Wang, Wang, Liu, & Fu, 2020), were used to predict and identify the potential T-cell and linear B-cell epitopic peptides in the digests. The intensities of the epitopic peptides upon different heating and digestion conditions were analyzed compared to the non-treated condition. Finally, all the putative epitopes were further searched in the UniprotKB/Swiss-Prot database to find possible cross-reactive peptides from other legumes.

6.2. Materials and methods

6.2.1. Materials and chemicals

Fresh soybean (*Glycine max*; protein content: 39.3%, w/w) was obtained from Wageningen Plant Research, Lelystad, The Netherlands (courtesy ing. Ruud Timmer). All the chemicals were obtained from Sigma-Aldrich (Missouri, USA) unless otherwise stated.

6.2.2. Preparation of soy protein samples

Soy protein (protein content: 77.9%, w/w) was extracted and identified according to section 3.2.2. After the addition of glucose in a weight ratio of 1:4 to mimic the ratio in IF, sample mixtures were wet-heated (W) at 85°C for 30 min, or dry-heated (D) at 60°C for 48 h, with non-treated (NT) samples as control. Heat treatments were carried out in independent duplicates.

6.2.3. *In vitro* static infant gastrointestinal digestion

Simulated *in vitro* infant digestion was performed in duplicate based on the protocol of Ménard et al. (2018). The protein concentration was adjusted to 1.2% (w/v) with 10 mM PBS buffer (pH 7.4) to mimic IF. Subsequently, 60 min of gastric digestion was conducted for all the samples, after which the gastric chyme was submitted to intestinal digestion. Samples were taken out after 10 min and 60 min in the intestinal phase (I10 and I60) and were immediately transferred into liquid nitrogen to stop the digestion process. Digests were stored at -20°C until use.

6.2.4. Degree of hydrolysis

The degree of hydrolysis was measured according to section 3.2.8. All samples that underwent duplicate digestions were analyzed in technical duplicate.

6.2.5. Peptidomics analysis

To identify the peptides after *in vitro* digestion of wet and dry heated soy protein, as well as those in the NT samples, sample digests were pre-treated as described by Dingess et al. (2017) with some modifications. Briefly, spin filters with a 10 kDa cut-off membrane (Sigma-Aldrich, Missouri, USA) were used to fractionate the peptide in the digests, removing intact protein and digestive enzymes. Subsequently, the peptide fractions underwent a purification process through a homemade solid phase extraction (SPE) C18 column, after which they were concentrated to offset any dilution that may have occurred during the SPE stage. The purified peptides were loaded onto a Thermo nLC 1000 system (Thermo, Waltham, MA, USA) coupled to an LTQ orbitrap XL (Thermo Fisher Scientific, Breda, The Netherlands). LC-MS/MS was run by MaxQuant 1.6.3.4 with "unspecific" digestion mode. The instrument parameters were set according to Zenker, Wichers, Tomassen, Boeren, De Jong, and Hettinga (2020).

A soy database (UP000008827) containing 74,863 protein sequences downloaded from Uniprot (https://www.uniprot.org, accessed June 2022) was used to analyze in MaxQuant, together with another database for common contaminants, including e.g., bovine trypsin (P00760), porcine trypsin (P00761), and human Keratin K2C1 (P04264). Peptides were identified with unspecific enzyme cleavage and with lengths from 8 to 25 AAs. To conduct the analysis, peptides that were present in both duplicate digestions were considered valid. Peptides with a score ≥ 80 were included in the overall profiles, while only those peptides with a score ≥ 100 were considered for epitopic analysis.

6.2.6. T-cell epitope prediction

T-cell epitopes in the digests were predicted by using the online IEDB MHC-II Binding Prediction tool (http://tools.iedb.org/mhcii/, accessed April 2023), which specifically targets the exogenous antigens, e.g., digestion-derived peptides. MHC-II binding epitopes were reported to range from 15 to 24 AAs in length (Knol, de Jong, Ulfman, & Tiemessen, 2019). Peptides falling within these length range, as identified by LC-MS/MS, were submitted to the prediction software. The default "IEDB recommended 2.22" method was chosen, and 27 HLA class II alleles were used for the prediction. Two key factors were used to filter the results: percentile ranking value and inhibitory concentration 50 (IC50) value. Both values represented the binding affinity between peptides and alleles, with lower values indicating higher binding affinity. Therefore, to screen the potential T-cell epitopes, the maximum percentile ranking value was set to 10, and the maximum IC50 score was set to 250 nM, according to Zenker et al. (2020) and Zhou, He, Zhang, Wang, Sun, and Liu (2022) with some modifications.

Subsequently, these potential epitopes were applied to an online IL4pred tool (https://webs.iiitd.edu.in/raghava/il4pred/predict.php, accessed April 2023) to predict the ability to induce IL-4 secretion by Th2 cells (Dhanda, Gupta, Vir, & Raghava, 2013). Prediction mode was set as hybrid (SVM + motif), and an SVM threshold of 0.2 was selected.

6.2.7. Linear B-cell epitope identification

A soy B-cell epitope database was downloaded (https://www.iedb.org/, accessed March 2023) from the IEDB database (Vita et al., 2018). The search settings were: "linear peptide", with "human" specified as the host, and "*Glycine max*" as the organism. The search yielded a total of 550 epitopes. Notably, B-cell epitopes are known to exist in both linear and conformational forms. However, due to the data available, which consisted of peptide sequences formed during infant digestion, we focused on linear epitopes specifically. The peptide sequences in the digests were compared against this database, and only those peptides with a length longer than or equal to that of soy B-cell epitopes were reported. Moreover, a further filter was applied to select peptides with a matching rate ≥ 60%. The matching rate refers to the ratio of the length of B-cell epitopes to that of the peptides in the digests. In general, a higher matching rate indicates a higher likelihood that the peptides may serve as B-cell epitopes and could be recognized by the receptors.

6.2.8. Alignment of potential epitopic peptides with other legume proteins

Both potential epitopic peptides of T-cell and B-cell were applied to the online BLASTp tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins, accessed April 2023) to search for similar peptide sequences in other legume species. The search was conducted in the UniprotKB/Swiss-Prot database. The maximum number of aligned sequences was set to 50, and the expect threshold was set to be 0.05. The results were filtered to include only sequences containing at least 6 consecutive and no more than 3 different AAs, as well as to exclude any peptides with a percent identity score lower than 80. In most situations, the cross-reactivity requires a percent identity score over 70 (Aalberse, 2000; Bublin & Breiteneder, 2020), but to increase reliability, in our study, this number was set to 80. Percent identity is a quantitative result, as defined by the percentage of the number of identical AAs in matching peptides from other legumes to the length of the aligned sequence in the soy epitopic peptides.

6.2.9. Statistical analysis

All the data were visualized by GraphPad Prism 9.0 and analyzed based on R 4.2.1 and RStudio 2022.07.0. Statistical analyses were performed by using IBM SPSS 25.0, using one-way ANOVA with Duncan post-hoc test for statistical differences, with $p < 0.05$ being considered significant.

6.3. Results and discussion

6.3.1. Effect of different heating on infant digestion

The degree of hydrolysis (DH) after *in vitro* digestion is shown in **Figure 6.1**.

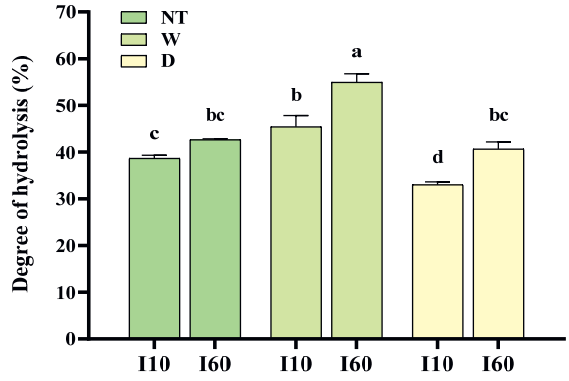


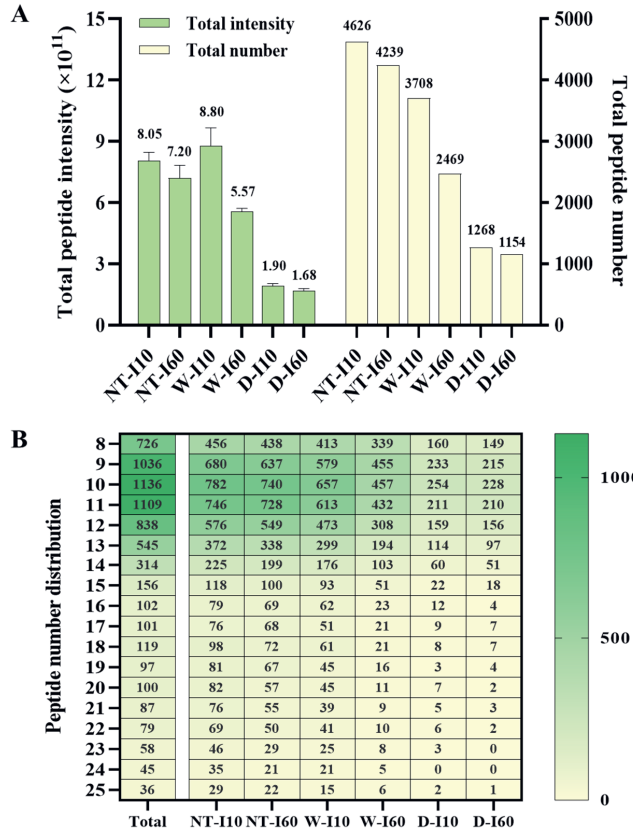
Figure 6.1. Degree of hydrolysis of soy protein upon wet (W) or dry (D) heating, after *in vitro* infant digestion, with non-treated (NT) samples as control. I10 and I60 represent 10 min and 60 min of intestinal digestion, respectively. Statistical differences were analyzed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$).

At 10 min of intestinal digestion (I10), the DH of the non-treated (NT), wet-heated (W), and dry-heated (D) samples were 38.7%, 45.4%, and 33.0%, respectively. As digestion progressed to 60 min (I60), the DH values of the NT, W, and D samples were increased to 42.7%, 55.0%, and 40.7%, respectively. These findings demonstrate an increase in the DH during prolonged digestion, as well as highlighting the enhancing effect of wet heating on infant digestion and the inhibitory effect of dry heating. Nonetheless, no significant difference was found in the DH between the NT and D samples at I60. These results reinforce the conclusions drawn from our previous research in **Chapters 3 & 4**. Specifically, wet heating promoted protein unfolding and exposed enzyme-cleavage sites to enhance digestion. In contrast, despite that glycation-induced aggregation upon dry heating led to the burial of these sites, the presence of multiple enzymes

in pancreatin negated the detrimental effects of glycation, as evidenced by the similar DH between the NT and D samples observed at I60.

6.3.2. Peptide identification in the digests

Peptide release after 10 min and 60 min of intestinal digestion upon different heating conditions was analyzed by LC-MS/MS. A total of 8,546 peptides were detected, although only 6,684 peptides with a score over 80 were retained for further analysis. Details of peptide intensity and number were demonstrated in **Figure 6.2**. Due to the limitation of the analysis method, which only targets peptides with 8 to 25 amino acids (AAs), longer or shorter peptides were not detected. Therefore, the results do not directly reflect the degree of digestion.



	Total	NT-I10	NT-I60	W-I10	W-I60	D-I10	D-I60
8	726	456	438	413	339	160	149
9	1036	680	637	579	455	233	215
10	1136	782	740	657	457	254	228
11	1109	746	728	613	432	211	210
12	838	576	549	473	308	159	156
13	545	372	338	299	194	114	97
14	314	225	199	176	103	60	51
15	156	118	100	93	51	22	18
16	102	79	69	62	23	12	4
17	101	76	68	51	21	9	7
18	119	98	72	61	21	8	7
19	97	81	67	45	16	3	4
20	100	82	57	45	11	7	2
21	87	76	55	39	9	5	3
22	79	69	50	41	10	6	2
23	58	46	29	25	8	3	0
24	45	35	21	21	5	0	0
25	36	29	22	15	6	2	1

Figure 6.2. (A) Total intensity and number of peptides. (B) Peptide number distribution with length from 8 to 25 amino acids. Total refers to all the diverse peptides found in all the samples. Peptides were fractionated from the digests upon wet (W) or dry (D) heating, with non-treated (NT) samples as control. I10 and I60 represent 10 min and 60 min of intestinal digestion, respectively.

Figure 6.2A illustrated the total intensity and number of peptides upon different heating and digestion conditions. Total intensity represents the overall abundance or signal strength of peptides in a given heating and digestion condition, with the higher total intensity signifying a higher cumulative abundance of peptides; total peptide number represents the diversity of peptides detected, and each identified peptide contributes to the count, regardless of its abundance. In general, both the total intensity and number declined from NT to W to D, as well as from I10 to I60. Specifically, the total intensity (**Figure 6.2A**, left), showed that the D sample had the lowest intensity among all the samples, being 4.2 and 4.3 times lower than that of the NT sample at I10 and I60, respectively. In addition, with prolonged digestion, the NT and D samples experienced a reduction of 10% and 12% in the total intensity, respectively, whereas the W sample exhibited the highest decrease of 37%. Regarding the total peptide number (**Figure 6.2A**, right), the NT sample exhibited the highest peptide count, followed by the W sample, while the D sample had the lowest amount. Their reductions from I10 to I60 were 8%, 33%, and 9% for the NT, W, and D samples, respectively, showing the most substantial decrease in the W sample, similar to the intensity.

It is hypothesized that the lowest overall intensity and number of peptides observed in the D sample may be attributed to the potential presence of cross-linked compounds (e.g., pentosidine) formed on the protein surface through glycation. These compounds may still exist in the peptide fragments even after digestion; thereby these peptides were not detected by the LC-MS/MS system. Moreover, the decrease in the total peptide intensity and count with extended digestion might attribute to improved digestion (especially in the case of the W sample), resulting in the production of shorter peptides with lengths less than 8 AAs, which could not be identified by the LC-MS/MS method used.

Peptides might be potential antigens to trigger immune responses mediated by B cells and T cells. Peptide length is important as it affects the accessibility and recognition of B-cell epitopes and the binding and activation of T-cell receptors. B-cell epitopes are mainly 5-22 AAs long (Singh, Ansari, & Raghava, 2013), whereas MHC-II binding T-cell epitopes are usually ranging from 15 to 24 AAs (Knol et al., 2019). **Figure 6.2B** illustrates the peptide number distribution in the length range of 8-25 AAs, providing information on the peptide diversity that may be responsible for the residual immunogenicity after digestion. It was observed that approximately 80% of the total peptides were 8-13 AAs long. This percentage varied between 78% and 91% among different heating and digestion conditions. In addition, around 98% of the peptides were in the length range of B-cell epitopes (in our study, 8-22 AAs); while only 14% matched the

length criteria for T-cell epitopes (15-24 AAs). This indicates that the immune response mediated by B cells may be more readily activated by digestion-derived peptides, than a response mediated by T cells. Nevertheless, apart from peptide length, peptide sequence is crucial for the recognition and activation of immune cells as well, as the specific AA sequence of the peptide can determine its binding affinity to the T-cell receptors or B-cell receptors. Hence, all peptides that meet the length criteria for epitopes were further studied using a sequence analysis, which will be described in sections 6.3.3 and 6.3.4.

6.3.3. T-cell epitopes prediction results

As shown in **Table 6.1**, a total of 29 peptides in the digests were predicted to be potential MHC-II binding T-cell epitopes. Among these peptides, a substantial portion originated from specific proteins, with lipoxygenase accounting for 7 peptides, followed by alcohol dehydrogenase, β-conglycinin, and glycinin, each contributing 4 peptides. Conversely, Bet v I/Major latex protein domain-containing protein, oleosin, and other proteins yielded only 1 peptide each. Amongst all, glycinin (Gly m 6), β-conglycinin (Gly m 5), and Bet v I/Major latex protein domain-containing protein (Gly m 4) are considered the main soy allergens. Therefore, despite that common soy allergens were extensively hydrolysed after intestinal digestion, intact linear epitopic sequences were still present in the digests, which may potentially elicit immune reactions. Interestingly, lipoxygenase, alcohol dehydrogenase, and other proteins, which are not seen as common soy allergens, exhibited residual immunogenicity after digestion as well. This intriguing finding may be attributed to the broader concept of immunogenicity beyond just allergenicity. While certain food proteins may not cause allergic responses, they might still trigger immune system activation, antibody production, or other immunological events (Leviatan, Vogl, Klompus, Kalka, Weinberger, & Segal, 2022; Van Wijk et al., 2004).

Table 6.1. List of potential T-cell epitopes recognized in the digests, along with HLA class II alleles and other relevant information.

#	Potential T-cell Epitope	Protein Group	Protein ID	Position	Length	HLA class II alleles	Method	IL4 Pred	IL4 Inducer
1	DLEGKTVGTVGAGRIGK	Formate dehydrogenase, mitochondrial	I1N5S0	199-215	17	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	1.416	Yes
2	IDTNSFQNQLDQMPR	Glycinin G3	P11828	163-177	15	HLA-DRB1*04:05	Consensus (smm/nn/sturniolo)	1.277	Yes
3	IKQVHQLNANPDVHG	Methenyltetrahydrofolate cyclohydrolase	C6TMV9	79-93	15	HLA-DRB1*13:02	Consensus (smm/nn/sturniolo)	1.266	Yes
4	KEIFRTDGEQALKFPPPK	Lipoxygenase	B3TDK6	332-349	18	HLA-DRB3*01:01	Consensus (comb.lib./smm/nn)	1.245	Yes
5	KEIFRTDGEQALKFPPPKVIQ	Lipoxygenase	B3TDK6	332-352	21	HLA-DRB3*01:01	Consensus (comb.lib./smm/nn)	1.225	Yes
6	GHEASGIVESVGKGV	Alcohol dehydrogenase 1	A0A0R4J4U4	68-82	15	HLA-DQA1*05:01/HLA-DQB1*03:01/HLA-DRB1*13:02	Consensus (comb.lib./smm/nn), Consensus (smm/nn/sturniolo)	1.037	Yes
7	PRIFGHEAGGIVESVGEGV	Alcohol dehydrogenase	I1KAJ4	66-84	19	HLA-DQA1*05:01/HLA-DQB1*03:01/HLA-DRB1*13:02	Consensus (comb.lib./smm/nn), Consensus (smm/nn/sturniolo)	0.967	Yes
8	NSIPAFRKGSIPGVTSDHM	Uncharacterized protein	C6T0B5	46-64	19	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	0.308	Yes
9	MRKNVLDFNSVADLT	Lipoxygenase	I1M596	26-40	15	HLA-DRB1*13:02	Consensus (smm/nn/sturniolo)	0.305	Yes
10	SKHFLAQSFNTNEDTAE	Cupin type-1 domain-containing protein	A0A0R0GMV1	233-249	17	HLA-DPA1*01:03/HLA-DPB1*04:01	NetMHCIIpan	0.300	Yes
11	SKHFLAQSFNTNEDIAE	Glycinin G4	P02858	234-250	17	HLA-DPA1*01:03/HLA-DPB1*04:01	NetMHCIIpan	0.288	Yes
12	SVDINEGALLLPHFNSKA	Beta-conglycinin beta subunit 2	F7J077	276-293	18	HLA-DRB1*13:02	Consensus (smm/nn/sturniolo)	0.253	Yes
13	SSVDINEGALLLPHFNSKA	Beta-conglycinin beta subunit 2	F7J077	275-293	19	HLA-DRB1*13:02	Consensus (smm/nn/sturniolo)	0.238	Yes
14	SVENVEGNGGPGTIK	Bet v I/Major latex protein domain-containing protein	C6T588	40-54	15	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	0.172	No
15	GHEAGGIVESVGEGV	Alcohol dehydrogenase	I1KAJ4	70-84	15	HLA-DQA1*05:01/HLA-DQB1*03:01/HLA-DRB1*13:02	Consensus (comb.lib./smm/nn), Consensus (smm/nn/sturniolo)	0.018	No

Table 6.1. (continued) List of potential T-cell epitopes recognized in the digests, along with HLA class II alleles and other relevant information.

#	Potential T-cell Epitope	Protein Group	Protein ID	Position	Length	HLA class II alleles	Method	IL4 Pred	IL4 Inducer
16	GHEASGIVESVGEGV	Alcohol dehydrogenase 1	I1MA91	60-74	15	HLA-DQA1*05:01/HLA-DQB1*03:01/HLA-DRB1*13:02	Consensus (comb.lib./smm/nn), Consensus (smm/nn/sturniolo)	-0.001	No
17	YREEELKSLRGNGTGE	Lipoxygenase	A0A0R0H569	161-176	16	HLA-DRB1*01:01	Consensus (comb.lib./smm/nn)	-0.019	No
18	VEYREEELKSLRGNGTGE	Lipoxygenase	A0A0R0H569	159-176	18	HLA-DRB1*01:01	Consensus (comb.lib./smm/nn)	-0.058	No
19	NSLLNALPEEVIQHT	Glycinin G1	P04776	449-463	15	HLA-DRB1*09:01	Consensus (comb.lib./smm/nn)	-0.068	No
20	KTNDTPMIGTLAGANS	Glycinin G1	P04776	435-450	16	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	-0.092	No
21	VEYREEELKSLRGNGTGERKEYD	Lipoxygenase	A0A0R0H569	159-181	23	HLA-DRB1*01:01	Consensus (comb.lib./smm/nn)	-0.118	No
22	YREEELKSLRGNGTGERKEYD	Lipoxygenase	A0A0R0H569	161-181	21	HLA-DRB1*01:01	Consensus (comb.lib./smm/nn)	-0.190	No
23	AFPGSAQAVEKLLKNQ	Beta-conglycinin alpha subunit 2	P0DO15	558-573	16	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	-0.237	No
24	LADELINAAKGSSNS	Ribosomal protein S7 domain-containing protein	I1L2H4	173-187	15	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	-0.278	No
25	SDVLTDAKEKLPADKA	SMP domain-containing protein	I1L849	201-216	16	HLA-DRB1*03:01	Consensus (smm/nn/sturniolo)	-0.325	No
26	LSIVDMNEGALLLPH	Beta-conglycinin alpha subunit 2	P0DO15	440-454	15	HLA-DQA1*01:02/HLA-DQB1*06:02/HLA-DRB1*13:02/HLA-DRB3*02:02	Consensus (comb.lib./smm/nn), Consensus (smm/nn/sturniolo), NetMHCIIpan	-0.791	No
27	THRYEAGVVPPGARFEPPR	Oleosin	I1N747	16-34	19	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	-0.903	No
28	NGGVVLGADSRTSTGV	Proteasome subunit beta	I1K964	23-38	16	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	-1.115	No
29	KIAQGVVGAVKDKGSV	Inosine-5'-monophosphate dehydrogenase	I1MJD7	426-441	16	HLA-DQA1*05:01/HLA-DQB1*03:01	Consensus (comb.lib./smm/nn)	-1.151	No

Moreover, regarding the length of the potential T-cell epitopes, 10 peptides consisted of 15 AAs, while 16 peptides were 16-19 AAs long, with these specific lengths thus accounting for almost 90% of the total 29 peptides. Thus, despite that the length criterium for MHC-II binding T-cell epitopes was 15-24 AAs, peptides in the soy digests with 15-19 AAs were more often predicted to be T-cell epitopes. This might be due to the low number of longer peptides present in the digests. Furthermore, the peptide with sequence "LSIVDMNEGALLLPH" (P0DO15, AA 440-454) from β-conglycinin can bind to 4 different HLA II alleles, indicating that a larger population may have the potential to recognize and interact with this particular peptide.

Afterward, the IL4 pred tool was used for further analysis, and only the peptides with prediction scores over 0.2 were seen as potential IL-4 inducers. A higher prediction score indicates a greater predicted ability to stimulate Th2 cells to secrete IL-4. Result show that 13 out of 29 peptides were predicted to be IL-4 inducers. Amongst these peptides, the peptide "DLEGKTVGTVGAGRIGK" (I1N5S0, AA 199-215) had the highest score, followed by "IDTNSFQNQLDQMPR" (P11828, AA 163-177), "IKQVHQLNANPDVHG" (C6TMV9, AA 79-93), and "KEIFRTDGEQALKFPPPK" (B3TDK6, AA 332-349). Moreover, peptides "GHEASGIVESVGKGV" (A0A0R4J4U4, AA 68-82) and "PRIFGHEAGGIVESVGEGV" (I1KAJ4, AA 66-84) could bind to 3 different HLA II alleles, suggesting that they could be recognized by a larger population.

Overall, 29 potential T-cell epitopes were found in the digests, with 13 of them recognized as IL-4 inducers. Generally, the epitopic peptides were more likely to have 15-19 AAs. In addition, aside from the 9 peptides derived from the main soy allergens, an additional 20 peptides from minor or non-allergic soy proteins demonstrated residual immunogenicity as well.

6.3.4. B-cell epitopes identification results

By aligning the peptide sequences to the soy linear B-cell epitopes database from IEDB, 116 peptides were found, with 27 peptides with a matching rate over 60% being filtered as potential B-cell epitopes (**Table 6.2**). Among these, 10 peptides were derived from glycinin (Gly m 6), 10 peptides from 2S albumin (Gly m 8), and 1 peptide from β-conglycinin (Gly m 5), which are known as common soy allergens. Furthermore, 3 peptides from lectin and 3 peptides from basic 7S globulin were also recognized as B-cell epitopes, indicating that some minor soy allergens still had residual immunogenicity after *in vitro* digestion, agreeing with the results of potential T-cell epitopes.

Table 6.2. Comparison of peptides identified in the digests and B-cell epitopes reported in the IEDB database, with a matching rate over 60% being included in the list. The peptide sequence in bold matches 100% with the B-cell epitopes reported.

#	Peptide	Peptide length	Matching B-cell Epitope	Matching rate (%)	Epitope ID	Protein Group	Protein ID	Epitope Position
1	**FSFREQPQ**	8	FSFREQPQ	100	913228	Glycinin G3	P11828	20-27
2	**NQSEELEEK**	9	NQSEELEEK	100	913848	2S Albumin	P19594	120-128
3	ENQSEELEEK	10	NQSEELEEK	90.0	913848	2S Albumin	P19594	120-128
4	NQSEELEEKQ	10	NQSEELEEK	90.0	913848	2S Albumin	P19594	120-128
5	IYIQQGSGI	9	YIQQGSGI	88.9	914294	Glycinin G3	P11828	92-99
6	SFASNLPH	8	FASNLPH	87.5	913189	Lectin	P05046	260-266
7	MENQSEELEEK	11	NQSEELEEK	81.8	913848	2S Albumin	P19594	120-128
8	GGLSVISPK	9	GLSVISP	77.8	913280	Glycinin G4	P02858	268-274
9	SFASNLPHA	9	FASNLPH	77.8	913189	Lectin	P05046	260-266
10	IMENQSEELEEK	12	NQSEELEEK	75.0	913848	2S Albumin	P19594	120-128
11	LGTRQLEE	8	GTRQLE	75.0	913313	Basic 7S globulin 2	Q8RVH5	392-397
12	QQFHNQDI	8	QQFHNQ	75.0	913988	Basic 7S globulin 2	Q8RVH5	237-242
13	FSFREQPQQNE	11	FSFREQPQ	72.7	913228	Glycinin G3	P11828	20-27
14	LFGEEEEQRQ	10	LFGEEEE	70.0	913609	Beta-conglycinin beta subunit 1	P25974	196-202
15	EGGLSVISPK	10	GLSVISP	70.0	913280	Glycinin G4	P02858	268-274
16	SFASNLPHAS	10	FASNLPH	70.0	913189	Lectin	P05046	260-266
17	KIMENQSEELEEK	13	NQSEELEEK	69.2	913848	2S Albumin	P19594	120-128
18	MENQSEELEEKQK	13	NQSEELEEK	69.2	913848	2S Albumin	P19594	120-128
19	IMENQSEELEEKQK	14	NQSEELEEK	64.3	913848	2S Albumin	P19594	120-128
20	MENQSEELEEKQKK	14	NQSEELEEK	64.3	913848	2S Albumin	P19594	120-128
21	SFQNQLDQMPR	11	FQNQLDQ	63.6	913224	Glycinin G3	P11828	168-174
22	VEGGLSVISPK	11	GLSVISP	63.6	913280	Glycinin G4	P02858	268-274
23	YQGNSGPLVNP	11	NSGPLVN	63.6	913858	Glycinin G5	P04347	510-516
24	APEFLEHA	8	FLEHA	62.5	913209	Glycinin G3	P11828	228-232
25	NALEPDHR	8	LEPDH	62.5	913606	Glycinin G4	P02858	40-44
26	KIMENQSEELEEKQK	15	NQSEELEEK	60.0	913848	2S Albumin	P19594	120-128
27	VTLGTRQLEE	10	GTRQLE	60.0	913313	Basic 7S globulin 2	Q8RVH5	392-397

In terms of the length distribution of linear B-cell epitopes, 21 out of 27 peptides had 8-11 AAs, suggesting the shorter peptides were relatively easier to be recognized by B cells, while longer peptides may contain multiple epitopes or have complex structures that hinder binding to B-cell receptors. Furthermore, it is noteworthy that only 2 peptides demonstrated a perfect matching rate of 100%, showing that these peptides have already been identified as B-cell epitopes before. One peptide is "FSFREQPQ" from glycinin (P11828, AA 20-27) with the epitope ID 913228, and another peptide is "NQSEELEEK" from albumin (P19594, AA 120-128) that matches the epitope ID: 913848.

To sum up, 27 out of 116 peptides were seen as potential linear B-cell epitopes, with 2 of them already documented in the database. Out of these, 21 peptides were derived from common soy allergens, while 6 peptides originated from less allergic soy proteins. The majority of these potential epitopes had lengths ranging from 8 to 11 AAs.

6.3.5. Evaluation of potential cross-reactivity compared to other legumes

To identify cross-reactivity between soy proteins and other legume proteins, the potential epitopic peptides were aligned to the UniprotKB/Swiss-Prot database via a BLASTp search. By IEDB bioinformatics tools, we have already predicted potential T-cell epitopic peptides in the digests, as well as identified B-cell epitope-containing peptides from the database (sections 6.3.3 and 6.3.4). But to increase the reliability, we only selected 13 potential T-cell epitopic peptides that were IL-4 inducers and 2 linear B-cell epitopes that had a 100% match.

The results in **Table 6.3** show that several T-cell epitopic peptides shared identical or similar sequences with other legumes, while no B-cell epitopic peptides did so. In total, 28 matching peptides from other legume proteins were identified to align to 7 potential T-cell epitopic peptides, with the highest occurrences observed in *Lupinus angustifolius* (11 peptides, narrow-leaved blue lupine), followed by *Pisum sativum* (8, pea), *Lens culinaris* (2, lentil), *Phaseolus vulgaris* (2, kidney bean), *Lupinus albus* (2, white lupine), *Arachis hypogaea* (2, peanut), and *Cicer arietinum* (1, chickpea). Interestingly, if an epitope is from one specific protein, its matching peptides were always derived from a protein with the same or similar sequence and function. For instance, soy-derived peptides from lipoxygenase were aligned to that of *Pisum sativum*, *Phaseolus vulgaris*, and *Lens culinaris*, while peptides from soy β-conglycinin matched to conglutin from *Lupinus* family and matched to Ara h 1 from *Arachis hypogaea*. In addition, of all the identified peptides, the sequence "PRIFGHEAGGIVESVGEGV" from alcohol dehydrogenase was found to be identical in both soy and pea. Thus, this peptide is more likely to cause cross-reactivity.

Table 6.3. Comparison of potential epitopes in the digests and proteins belonging to other legume species. All the matching peptides are obtained through BLASTp search in the UniprotKB/Swiss-Prot database, with at least 6 consecutive and no more than 3 different amino acids compared to epitopic peptides. The peptide sequence in bold matches 100% with the epitopic peptide. Different amino acids are highlighted in red.

Epitopic Peptide	Protein Group	Matching Peptide	Identity	Protein Group	Protein ID	Position	Organism	General Name
IDTNSFQNQLDQMPR	Glycinin G3	IDTSSFQNQLDQMPR	93.3	Legumin	Q9SMJ4	165-179	*Cicer arietinum*	Chickpea
KEIFRTDGEQALKFPPPK	Lipoxygenase	KEIFRTDGENTLKYPPPK	83.3	Seed linoleate 9S-lipoxygenase-3	P09918	336-353	*Pisum sativum*	Garden pea
		KEIFRTDGEQVLKFTPP	88.2	Seed linoleate 9S-lipoxygenase-2	P14856	340-356	*Pisum sativum*	Garden pea
		KEILRTDGEQVLKFPPP	88.2	Linoleate 9S-lipoxygenase 1	P27480	337-353	*Phaseolus vulgaris*	Kidney bean
		ELFRSDGEAALKFPPPK	82.4	Linoleate 9S-lipoxygenase	P38414	341-357	*Lens culinaris*	Lentil
KEIFRTDGEQALKFPPPKVIQ	Lipoxygenase	KEIFRTDGENTLKYPPPKVIQ	85.7	Seed linoleate 9S-lipoxygenase-3	P09918	336-356	*Pisum sativum*	Garden pea
		KEIFRTDGEQVLKFTPPHVI	85.0	Seed linoleate 9S-lipoxygenase-2	P14856	340-359	*Pisum sativum*	Garden pea
		KEILRTDGEQVLKFPPPHVI	85.0	Linoleate 9S-lipoxygenase 1	P27480	337-356	*Phaseolus vulgaris*	Kidney bean
		ELFRSDGEAALKFPPPKVIQ	85.0	Linoleate 9S-lipoxygenase	P38414	341-360	*Lens culinaris*	Lentil
GHEASGIVESVGKGV	Alcohol dehydrogenase 1	GHEAGGIVESVGEGV	86.7	Alcohol dehydrogenase 1	P12886	69-83	*Pisum sativum*	Garden pea
		GHEAAGIVESVGEGV	86.7	Alcohol dehydrogenase class-3	P80572	67-81	*Pisum sativum*	Garden pea
PRIFGHEAGGIVESVGEGV	Alcohol dehydrogenase	**PRIFGHEAGGIVESVGEGV**	100	Alcohol dehydrogenase 1	P12886	65-83	*Pisum sativum*	Garden pea
		ILGHEAAGIVESVGEGV	88.2	Alcohol dehydrogenase class-3	P80572	65-81	*Pisum sativum*	Garden pea

Table 6.3. (continued) Comparison of potential epitopes in the digests and proteins belonging to other legume species. All the matching peptides are obtained through BLASTp search in the UniprotKB/Swiss-Prot database, with at least 6 consecutive and no more than 3 different amino acids compared to epitopic peptides. The peptide sequence in bold matches 100% with the epitopic peptide. Different amino acids are highlighted in red.

Epitopic Peptide	Protein Group	Matching Peptide	Identity	Protein Group	Protein ID	Position	Organism	General Name
SSVDINEGALLLPHFNSKA	Beta-conglycinin beta subunit 2	INEGALLLPHYNSKA	93.3	Conglutin beta 2	Q6EBC1	371-385	*Lupinus albus*	White lupine
		EINEGALLLPHYNSKA	87.5	Conglutin beta 1	Q53HY0	370-385	*Lupinus albus*	White lupine
		EINEGALLLPHYNSKA	87.5	Conglutin beta 1	F5B8V9	441-456	*Lupinus angustifolius*	Narrow-leaved blue lupine
		EINEGALLLPHYNSKA	87.5	Conglutin beta 7	F5B8W5	439-454	*Lupinus angustifolius*	Narrow-leaved blue lupine
		EISEGALLLPHYNSKA	81.3	Conglutin beta 5	F5B8W3	472-487	*Lupinus angustifolius*	Narrow-leaved blue lupine
		IEINEGALLLPHYNSKA	82.4	Conglutin beta 6	F5B8W4	431-447	*Lupinus angustifolius*	Narrow-leaved blue lupine
		IEINEGALLLPHYNSKA	82.4	Conglutin beta 4	F5B8W2	428-444	*Lupinus angustifolius*	Narrow-leaved blue lupine
		IEINEGALLLPHYNSKA	82.4	Conglutin beta 3	F5B8W1	418-434	*Lupinus angustifolius*	Narrow-leaved blue lupine
		VEIKEGALMLPHFNSKA	82.4	Allergen Ara h 1	P43238	436-452	*Arachis hypogaea*	Peanut
		VEIKEGALMLPHFNSKA	82.4	Allergen Ara h 1	P43237	428-444	*Arachis hypogaea*	Peanut
SVDINEGALLLPHFNSKA	Beta-conglycinin beta subunit 2	EINEGALLLPHYNSKA	87.5	Conglutin beta 1	F5B8V9	441-456	*Lupinus angustifolius*	Narrow-leaved blue lupine
		EINEGALLLPHYNSKA	87.5	Conglutin beta 7	F5B8W5	439-454	*Lupinus angustifolius*	Narrow-leaved blue lupine
		IEINEGALLLPHYNSKA	82.4	Conglutin beta 6	F5B8W4	431-447	*Lupinus angustifolius*	Narrow-leaved blue lupine
		IEINEGALLLPHYNSKA	82.4	Conglutin beta 4	F5B8W2	428-444	*Lupinus angustifolius*	Narrow-leaved blue lupine
		IEINEGALLLPHYNSKA	82.4	Conglutin beta 3	F5B8W1	418-434	*Lupinus angustifolius*	Narrow-leaved blue lupine

Many studies have demonstrated that cross-reactivity can occur between soy and other legumes (or legume-based food additives), such as peanut, pea, chickpea, lima bean, green bean, white bean, lentil, guar gum, carob bean, tragacanth, and liquorice (L'Hocine & Boye, 2007; Verma, Kumar, Das, & Dwivedi, 2013). According to our results, apart from peanut, pea, chickpea, and lentil that were already mentioned above, potential epitopic peptides from kidney bean and lupine family may cause cross-reactions as well. However, these results are based on prediction *in silico*, thus further confirmation is required, e.g., through serum testing or other similar experiments. Despite so, these results indicate that consumption of other legumes may result in the generation of cross-reactive peptides upon digestion, and in this way cause an allergic reaction, even when the individual has only been sensitized to soy allergens.

6.3.6. Residual immunogenicity after digestion upon different heating

To evaluate the residual immunogenicity after *in vitro* infant digestion under different heating conditions, we specifically selected 15 potential epitopic peptides as described in section 6.3.5. The alterations in the intensity of these epitopic peptides are illustrated in **Figure 6.3**.

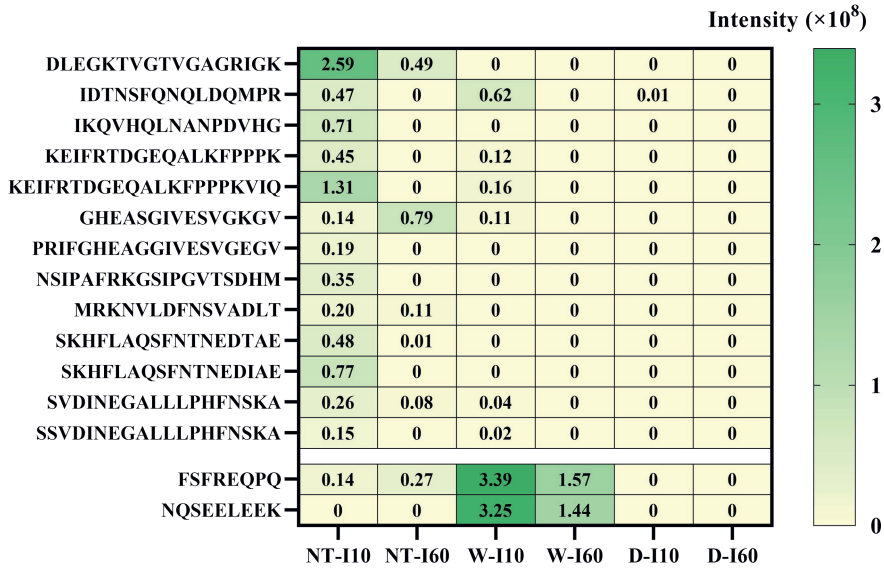


Figure 6.3. Intensity of epitopic peptides in the digests upon wet (W) or dry (D) heating, with non-treated (NT) samples as control. I10 and I60 represent 10 min and 60 min of intestinal digestion, respectively. Peptide sequences are displayed on the left side of their respective rows. The first 13 peptide sequences refer to the IL-4 inducing T-cell epitopes, while the last 2 sequences refer to the 100% matching B-cell epitopes.

Please note that the intensity of a peptide reflects its cumulative abundance, which can be influenced by factors such as peptide length and its specific sequence. Therefore, it is important

to only compare the intensities of the same peptide across different experimental conditions, but not make direct comparisons of intensity between different peptides as the factors affecting their abundance may vary.

For potential T-cell epitopes, in general, wet, but especially dry heating decreased their intensities, based on which digestion would lead to a further decrease, indicating a reduction in the likelihood of triggering immunogenic responses mediated by T cells. To be specific, without heating, all 13 peptides had detectable intensities at I10. After wet heating, 12 peptides experienced a decrease in intensity, with 7 of them becoming non-detectable. Similarly, after dry heating, the intensity of 12 out of 13 peptides became non-detectable. Notably, the peptide "IDTNSFQNQLDQMPR" had contrasting trends between wet and dry heating. Its intensity significantly rose by 32% after wet heating but decreased by around 40-fold after dry heating, compared to the non-treated condition. In addition, the peptide "DLEGKTVGTVGAGRIGK" demonstrated the most significant decline, with its intensity reducing from 2.6×10^8 at I10 to non-detectable at I60 after both wet and dry heating. As digestion progressed from I10 to I60, the intensity of 12 peptides in the NT sample showed a decrease, with 8 of them eventually becoming non-detectable. However, 1 peptide, "GHEASGIVESVGKGV", showed an opposite trend, with an increase of 4.9-fold in the intensity from I10 to I60. In the case of the heated samples at I10, the intensities of most peptides were already decreased due to the heating process, except for the peptide "IDTNSFQNQLDQMPR" mentioned earlier. Only 6 peptides in the wet heated sample and 1 peptide in the dry heated sample had a detectable intensity at I10. However, after prolonged digestion, all of these peptides became non-detectable at I60, regardless of whether heating had initially increased or decreased their intensity.

In terms of linear B-cell epitopes, after wet heating, both peptides "FSFREQPQ" and "NQSEELEEK" displayed an increasing trend in intensity. The intensities of both peptides increased by 3.3×10^8 at I10, starting from 1.4×10^7 and 0 (i.e., non-detectable), respectively. From I10 to I60, despite the initial increase in the intensity caused by wet heating, digestion subsequently decreased their intensities by 1.2- and 1.3-fold, respectively. In contrast, these peptides were non-detectable after dry heating at either I10 or I60. Therefore, unlike T-cell epitopes, B-cell epitopes exhibited an increase in intensity due to wet heating, and these epitopes were not completely eliminated during the following digestion. In other words, the residual immunogenicity associated with B-cell epitopes eventually increased after wet heating, compared to the non-treated condition.

Regarding the mechanisms causing these differences, combining the conclusions drawn from our earlier research (**Chapters 3 & 4**), it is most likely that wet heating contributed to a looser tertiary structure resulting in increased accessibility to digestive enzymes, thus leading to two opposite results: the breakdown of the epitopic peptides, or the breakdown of longer peptide fragments into shorter epitopic peptides. For dry heating, the glycation-induced aggregation caused the formation of soluble aggregates and complicated cross-linked peptides after digestion, leading to the inability to detect single epitopic peptides. While for extended digestion, similar to wet heating, epitopic peptides were further digested and no longer existed in the digests, or conversely, longer peptides were hydrolysed to certain peptides that possessed epitopic properties.

6.4. Conclusion

This study investigated the soy protein-derived peptides released during *in vitro* infant digestion after wet and dry heating in comparison to a non-treated sample. The peptides showed a general decline in total intensity and number from non-treated to wet-heated to dry-heated samples, as well as from 10 min to 60 min of intestinal digestion. The subsequent bioinformatics analysis predicted 29 potential T-cell epitopes among all peptides, 13 of which were predicted to have the capacity to induce IL-4 secretion. Moreover, 27 possible linear B-cell epitopes were found, with 2 of them perfectly matching previously reported B-cell epitopes. Furthermore, the potential cross-reactivity of putative epitopic peptides between soy proteins and other legumes including peanut, pea, chickpea, lentil, kidney bean, and lupine was explored. Finally, we found that the intensity of most T-cell epitopic peptides declined after either mode of heating, while that of B-cell epitopes increased after wet heating but decreased after dry heating. After digestion, the intensities of these epitopic peptides eventually declined. However, the predicted epitopic peptides present at the initial stage of intestinal digestion (I10) may still trigger an immune response, despite their subsequent breakdown during later digestion. These findings suggest that certain linear peptides released during digestion could potentially trigger immune responses, and heat treatment and digestion time could modulate this likelihood. However, these results were only obtained by bioinformatics tools, thus still requiring further validation through experiments, such as synthesizing these putative epitopes to conduct antibody binding assays and to evaluate their ability to activate T cells.

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CHAPTER 7

General Discussion

7.1. Background

Cow's milk allergy represents a significant issue associated with dairy consumption, particularly in infants. In response to these challenges, plant protein-based infant formula (IF) has been developed to support infant nutrition and health. While plant proteins offer notable advantages in terms of sustainability and affordability, they often exhibit lower protein quality and higher levels of antinutritional factors (ANFs). Efforts have been made by researchers and formula designers to identify suitable plant protein sources for IF, including options like soy protein (SP) and hydrolyzed rice protein, which have received legal approval (European Commission, 2016). Furthermore, alternative plant proteins such as pea protein (PP), quinoa protein, and potato protein are being studied for their potential use. Nevertheless, the process of selecting appropriate plant proteins for IF remains a challenging task. Therefore, **Chapter 2** thoroughly evaluated the key considerations and challenges related to their application in IF, including aspects like nutritional value, potential health implications (including allergy risks), and safety. Additionally, this chapter underscored the significant role of food processing in IF production, as different processing stages and techniques can influence the nutritional quality, allergenicity, techno-functionality, and sensory characteristics of the final IF products in various ways.

Considering that heat processing is crucial for IF production, this thesis focused on the effect of heating on plant proteins. Both wet and dry heating were studied, as both may be used in practice. SP and PP were selected as research subjects, as SP is the primary plant protein source used for IF nowadays, and PP is a promising alternative. **Chapter 3** focused on the effect of wet heating on physicochemical properties of SP and PP, and associated it with *in vitro* infant digestion. Results show that wet heating of SP and PP facilitated protein unfolding, as manifested by the increased surface hydrophobicity, thus leading to the exposure of enzyme-cleavage sites and hence was related to increased digestibility. **Chapter 4** targeted dry heating, finding that in the presence of glucose, glycation occurred, although to different extents in SP and PP. In addition, glycation-induced aggregation primarily hindered gastric digestion, and due to the covalent binding of glucose to lysine residues in proteins covering trypsin-cleavage sites, the intestinal digestibility of SP and PP was differentially influenced.

Furthermore, these different digestion behaviors may result in different compositions and functional properties of the resulting peptides, which play a role in various physiological processes. **Chapter 5** evaluated the formation of peptides released from SP and PP under wet and dry heating after *in vitro* infant gastrointestinal digestion. Especially after dry heating, many

glycated peptides were identified. Moreover, this chapter described the antimicrobial activity of peptides and their binding affinity to the soluble receptor for advanced glycation end products (sRAGE). This investigation was due to that food-derived advanced glycation end products (AGEs) have been described as potentially involved in regulating immunogenicity (Uribarri et al., 2015; Zenker et al., 2019), which may occur through RAGE. Besides, antimicrobial peptides (AMPs) are natural defenders against potential threats. It was found that the differences in sRAGE-binding and antimicrobial potentials between SP and PP-derived peptides were primarily related to the inherent characteristics of the protein sources, with heating and digestion conditions playing a comparatively lesser role. Additionally, AMPs were predicted by a bioinformatics tool, where glycated AMPs were found as well.

Chapter 6 delved deeper into the examination of the residual immunogenic potential of the peptides released during *in vitro* infant digestion, based on various bioinformatics tools. This analysis focused solely on SP-derived peptides due to the availability of well-established online protein and peptide databases for SP. This chapter predicted the presence of 29 T-cell epitopes and 27 linear B-cell epitopes in the SP digests. In addition, potential cross-reactive epitopes from other legumes were found, including peanut, pea, chickpea, lentil, kidney bean, and lupine. The results also emphasized the influence of heat treatment and digestion time on the generation of these epitopic peptides, which could potentially impact immune responses.

Combining all these results, the interrelationship between heating, protein modifications, digestion, and peptide bioactivity (including immunogenicity) can be linked. Therefore, this thesis provides valuable information for the development of better plant-based formulas for infants with specific dietary needs.

7.2. Structural modifications of SP and PP upon wet and dry heating

The application of heating induces a range of structural modifications in proteins, including denaturation, aggregation, and, in the presence of reducing sugars, glycation. In general, when proteins are exposed to temperatures exceeding their denaturation temperatures (typically above 65°C for globular proteins), their tertiary and secondary structures experience partial denaturation, leading to protein unfolding and the exposure of hydrogen bonds, hydrophobic groups, disulfide bonds, and sulfhydryl groups (Wang, Yuan, Zhou, & Gu, 2021). As proteins denature through heating, they can interact with other proteins via intermolecular sulfhydryl-disulfide interchange reactions and hydrophobic interactions, resulting in aggregate formation

(Spotti, Loyeau, Marangón, Noir, Rubiolo, & Carrara, 2019). When proteins are heated in the presence of reducing sugars, glycation occurs, involving the covalent binding of proteins to carbohydrates. This glycation process further contributes to protein aggregation and cross-linking (Aronson, 2003; Chevalier, Chobert, Mollé, & Haertlé, 2001; van Lieshout, Lambers, Bragt, & Hettinga, 2020). It is noteworthy that certain aggregates can remain soluble, a phenomenon attributed to the net surface charge and repulsive forces (Wang et al., 2021).

SP and PP are mainly composed of 7S and 11S globulins. Notably, 11S globulins are characterized by the presence of disulfide-linked acidic and basic polypeptides in their subunits, and can be dissociated under reducing conditions (Lu, He, Zhang, & Bing, 2019; Sui, Zhang, & Jiang, 2021). Our SDS-PAGE results (**Figure 3.1A**) confirmed these findings, indicating that disulfide rearrangement is an important mechanism underlying SP and PP aggregation.

The impact of wet and dry heating of SP and PP on their structures was studied in the absence of glucose (**Chapters 3 & 4**). Wet heating was carried out within the temperature range of 65-100°C for 30 min, while dry heating was conducted at 60°C for 6 h and 48 h. These heating conditions were selected to acquire different levels of structural modifications. The results show that no differences were found in the subunit compositions of SP and PP between wet and dry heating (**Figures 3.S1 & 4.2**). This suggests that different heating intensities did not cause significant damage to the protein subunits. However, upon wet heating, particularly at temperatures exceeding 75°C for SP and 65°C for PP, the disulfide rearrangement in 11S globulins was more pronounced, leading to the formation of soluble aggregates (**Figures 3.1B & 3.2**). For SP and PP that were dry-heated at 60°C for 6 h and 48 h, only limited insoluble aggregates were detected (**Figures 4.3 & 4.6-G0**), which primarily formed through non-covalent forces. Furthermore, wet heating resulted in increased surface hydrophobicity for SP (starting from 75°C) and PP (starting from 65°C), indicating protein unfolding at these temperatures (**Figure 3.3**). By contrast, after dry heating (60°C), the tertiary structures remained unchanged (**Figure 4.4**). To summarize, mild dry heating at 60°C had nearly no effect on protein structures of both SP and PP, regardless of the duration. Conversely, in the case of wet heating, increased temperatures (above 65°C) led to concurrent protein unfolding and the formation of soluble aggregates.

As glycation may occur during IF production, SP and PP were also subjected to different heating conditions in the presence of the reducing sugar glucose in a weight ratio of 1:4, which reflects the common composition in plant-based IF (**Chapter 4**). Glucose was selected because glucose from corn syrup and/or maltodextrin is the main carbohydrate used in soy-based IF.

Upon wet heating at 85°C, both SP and PP exhibited low levels of glycation (**Figure 5.1**). By contrast, dry-heated samples showed considerably higher glycation levels (**Figures 4.1 & 5.1** and **Table 4.1**). Therefore, the structural analysis was only conducted in the samples dry heated with glucose. It was noticed that as the dry heating duration increased from 6 h to 48 h, glucose induced a greater covalent binding to proteins, resulting in increased molecular weights of protein subunits (**Figure 4.2**) and an increased formation of insoluble aggregates (**Figures 4.3 & 4.6-G0**), both induced by glycation. Moreover, the surface hydrophobicity of both proteins dropped significantly after 48 h of dry heating, which may be related to the formation of more hydrophilic AGEs on the protein surface (**Figure 4.4**). Therefore, in the presence of glucose, long-time (48 h) mild dry heating at 60°C led to glycation and aggregation of SP and PP, potentially reflecting the conditions of IF after long-term storage.

7.3. Effect of structural modifications on protein digestion

The structural modifications in proteins resulting from heating, as discussed in section 7.2, can influence digestion kinetics (van Lieshout et al., 2020). Denaturation and unfolding, in particular, have a positive impact on digestibility by disrupting β-sheet structures and exposing intramolecular enzyme-cleavage sites (Carbonaro, Maselli, & Nucara, 2012; Dupont & Nau, 2019; Gonzalez, Alvarez-Ramirez, Vernon-Carter, Reyes, & Alvarez-Poblano, 2020). Typically, this process coincides with an increase in surface hydrophobicity, since hydrophobic groups migrate to the protein surface (Wang et al., 2012; Wang, Li, Jiang, Qi, & Zhou, 2014). Consequently, assessing the correlation between digestibility and surface hydrophobicity serves as a valuable approach to validate the role of unfolding in the digestion process. Our results confirmed the positive role of unfolding by wet heating (**Chapter 3**). As heating intensity increased from 65 to 100°C, the surface hydrophobicity and digestibility of both SP and PP increased as well (**Figures 3.3 & 3.5 & 3.6**). A deeper analysis of Pearson correlation coefficients for SP and PP revealed a strong positive correlation between these two factors (**Figure 3.S3**), indicating that it was primarily the unfolding induced by wet heating that was related to the increased digestibility of SP and PP.

Interestingly, aggregation can have a positive correlation with digestion, which was particularly evident in the case of wet-heated SP (**Chapter 3**). SP is characterized by its abundance of trypsin inhibitors (TIs), which can affect its intestinal digestibility (Gilani, Xiao, & Cockell, 2012). Research has shown that with increased heating temperature (from 75°C to 100°C), TIs

were more effectively inactivated, leading to enhanced digestibility (Vagadia, Vanga, Singh, Gariepy, & Raghavan, 2018). This is due to the incorporation of TIs into protein aggregates, which results from sulfhydryl-disulfide interchange reactions, ultimately leading to the inactivation of TIs (Xu, Chen, Zhang, Kong, & Hua, 2012). Thus, for SP, limited aggregation facilitated digestion, due to TI inactivation.

However, in most cases, aggregation tends to impede the digestive process by burying enzyme-cleavage sites (Zhang, Wang, Li, Guo, & Lv, 2022). **Chapter 4** indicated that glycation-induced aggregation was the primary factor hindering gastric digestion. The highly glycated SP and PP samples demonstrated the most substantial aggregation (**Figures 4.3 & 4.6-G0**) and the lowest gastric digestibility (**Figures 4.5 & 4.7)**. Moreover, glycation can impede intestinal digestion by modifying lysine and arginine residues within proteins, as these are also the tryptic digestion sites (Deng, Wierenga, Schols, Sforza, & Gruppen, 2017; Hemmler et al., 2019; Olsen, Ong, & Mann, 2004; Zenker, van Lieshout, van Gool, Bragt, & Hettinga, 2020). Notably, this study found that the intestinal digestibility of dry-heated PP was more inhibited than SP, due to more trypsin-cleavage sites being glycated (**Figures 4.1 & 4.5**).

In summary, protein unfolding facilitated the digestibility of both SP and PP, while glycation decreased it. Limited aggregation, particularly in SP, contributed to improved digestion due to TI inactivation.

7.4. Peptide patterns of SP and PP upon wet and dry heating

Digestion kinetics can be influenced by heat-induced structural changes, leading to potential differences in the peptide profiles of SP and PP. To explore this further, we selected two distinct heating conditions: wet heating at 85°C for 30 min and dry heating at 60°C for 48 h, both in the presence of a 1:4 ratio of protein to glucose (w/w). This was to ensure consistent composition and induce substantial structural modifications. Subsequently, peptidomics analysis was conducted on the peptides formed during *in vitro* infant gastrointestinal digestion, based on LC-MS/MS and MaxQuant.

Figure 7.1 illustrates the enhancing effect of wet heating on infant digestion and the inhibitory effect of dry heating (primarily due to glycation), as previously demonstrated with other analytical approaches in **Chapters 3 & 4**. In addition, it was observed that SP generally exhibited lower overall digestibility compared to PP. Subsequently, we performed an analysis of peptides with lengths ranging from 5 to 40 amino acids (AAs), resulting in the identification

of 10,352 distinct peptides in the intestinal digests of SP and 1,399 in the case of PP. To ensure data accuracy, these peptides were further filtered to only include those with a MaxQuant score exceeding 80, resulting in final counts of 7,022 for SP and 1,299 for PP. Importantly, it is worth noting that a significantly greater number of peptides were identified in SP compared to PP. This discrepancy can be attributed to the substantial difference in the sizes of their respective protein databases obtained from Uniprot (https://www.uniprot.org, accessed June 2022). Specifically, the soy database comprises 74,863 protein sequences, while the pea database only includes 2,508 protein sequences. This is because SP is a widely studied plant protein, and researchers have accumulated a substantial amount of its data. In contrast, PP, although gaining popularity, has a smaller database because it has not been studied as extensively. As only peptides originating proteins present in a database can be identified, there is a clear correlation between database size and number of identifications.

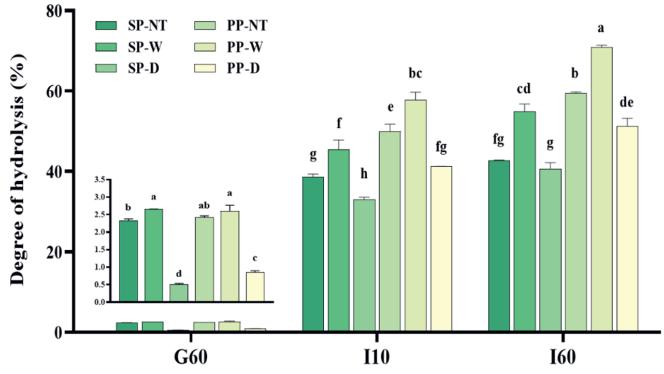

Figure 7.1. Degree of hydrolysis of SP and PP after wet (W) or dry (D) heating, after *in vitro* infant digestion, with non-treated (NT) samples as control. G60 represents 60 min of gastric digestion; I10 and I60 represent 10 min and 60 min of intestinal digestion, respectively. Statistical differences were analyzed with ANOVA and Duncan post-hoc test. Different letters above the bars represent significant differences ($p < 0.05$).

As shown in **Figure 7.2A**, in the case of SP and PP-based peptides (5-40 AAs), their identifiability decreased after wet heating and reached the lowest levels after dry heating, compared to non-treated conditions. There are multiple reasons for this decreased identifiability. For wet heating, protein unfolding leads to the exposure of more enzyme-cleavage sites, facilitating the further cleavage of larger peptides into smaller ones, and even single AA that cannot be identified. For dry heating, peptides modified by cross-linked AGEs (e.g., pentosidine) were not identified by the LC-MS/MS system due to limitations in the data analysis. Therefore, although the overall digestibility of dry-heated SP and PP was not low (**Figure 7.1**), the number of identified peptides was limited. Furthermore, both dry-heated SP and PP contained shorter

peptides (**Figure 7.2B**). Apart from the analytical limitation mentioned above, another factor contributing to the reduced detection of longer peptides might be that highly glycated proteins tend to be digested in the form of insoluble aggregates (as discussed in section 4.3.3) and the formed peptides may be preferentially further digested, instead of digestion of insoluble aggregated proteins. Comparing the peptide sizes of dry-heated SP and PP, the longest peptides from SP (25 AAs) were longer than those from PP (18 AAs). Two reasons may underlie this difference: 1) the smaller pea than soy database may have led to less peptide identifications, and 2) the higher digestibility of PP than SP led to greater formation of non-detectable short peptides (< 5 AAs) and single AAs.

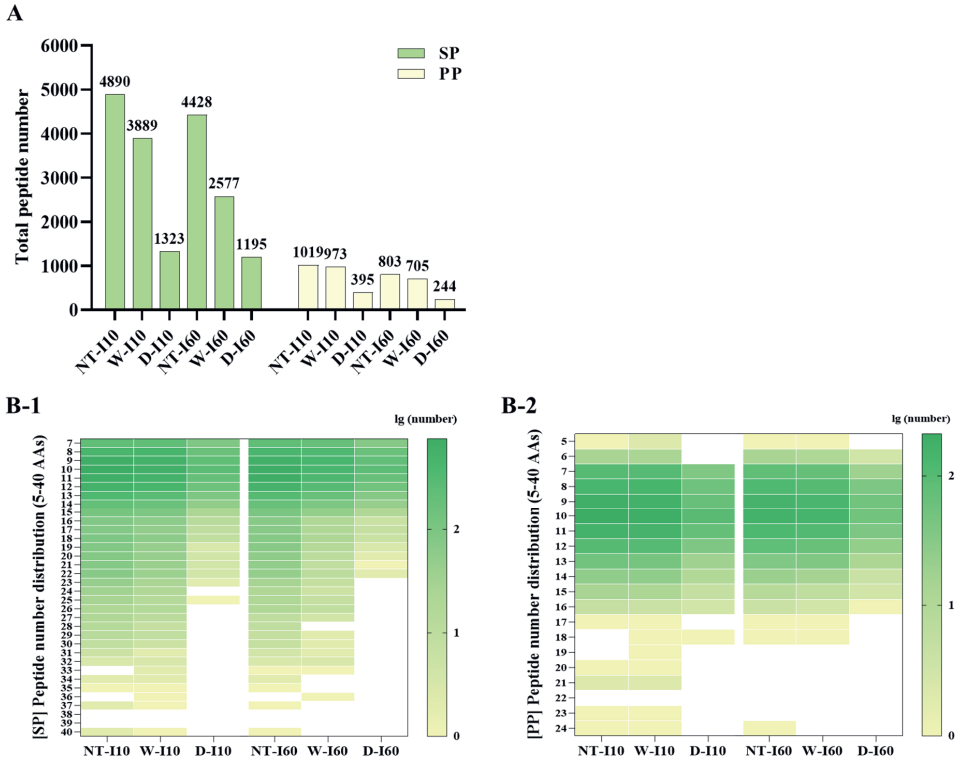

Figure 7.2. (A) Total peptide numbers in SP and PP digests with lengths from 5 to 40 amino acids (AAs). (B) Peptide number distribution of (1) SP and (2) PP. Peptides were fractionated from the digests after wet (W) or dry (D) heating, with non-treated (NT) samples as control. I10 and I60 represent 10 min and 60 min of intestinal digestion, respectively.

Quantifying glycated peptides in the digests offers a reliable method to estimate the level of glycation in SP and PP. To limit false identifications, the analysis focused on peptides with lengths of 8-25 AAs. Subsequently, after filtering (MaxQuant score ≥ 80), 6,684 out of 8,546 SP-based peptides and 1,119 out of 1,162 PP-based peptides were identified. The total number of glycated and non-glycated peptides was counted in the samples after different heat treatments and digestion durations (**Figure 7.3**). As SP and PP differed pronouncedly in their peptide numbers, the results were expressed as the percentage of glycated peptides relative to the total peptides in each sample. As shown in **Figure 5.2**, wet, and particularly dry heating contributed to the formation of glycated peptides. In addition, the higher digestibility of PP contributed to a relatively higher abundance of glycated peptides identified in PP compared to SP.

In summary, the dry-heated samples exhibited the lowest total peptide counts, yet they also contained the highest levels of glycated peptides. Additionally, considering the limited size of the pea database, it would be interesting to apply *de novo* sequencing for peptide identification in future studies, as this method does not rely on reference sequences in a protein database.

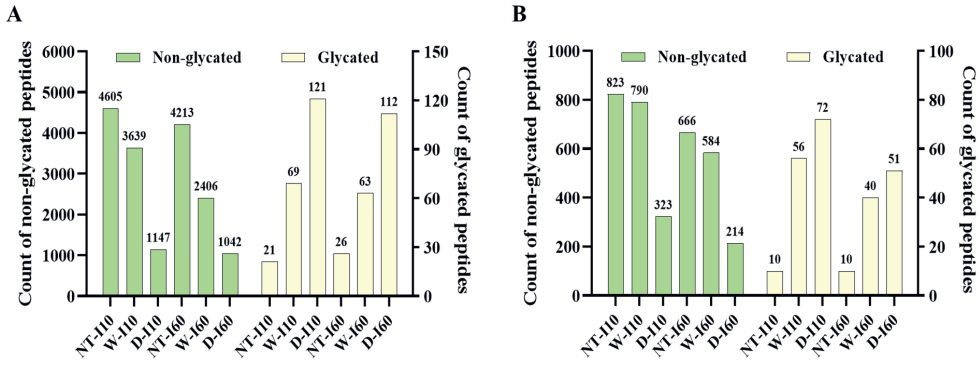

Figure 7.3. Count of glycated and non-glycated peptides in (A) SP and (B) PP. Peptides were fractionated from the digests after wet (W) or dry (D) heating, with non-treated (NT) samples as control. I10 and I60 represent 10 min and 60 min of intestinal digestion, respectively.

7.5. Potential bioactivity of resulting peptides

Bioactive peptides (BPs) are important as they can have significant physiological and health-related effects, such as antimicrobial and immunoregulatory activity. Investigating and characterizing BPs using both experimental and bioinformatics approaches can contribute to the development of functional IF designed to support infant health and well-being. During gastrointestinal digestion, BPs are released from their parent proteins and can exert their

bioactivity locally in the intestinal tract or after they have been transported to other target organs (Toldrá & Mora, 2021). Many studies have explored peptidic bioactivities after simulated *in vitro* digestion (Chen & Li, 2012; Giromini, Fekete, Givens, Baldi, & Lovegrove, 2017; Giromini et al., 2021; Torres-Fuentes, Contreras, Recio, Alaiz, & Vioque, 2015) or simulated *in silico* digestion (Barati et al., 2020; Li, Dhordain, Hearn, Martin, & Bennett, 2023). Nevertheless, the information about BPs is still lacking in the context of infant digestion of plant proteins.

Furthermore, glycated peptides formed during digestion of heat-treated foods might also possess the bioactive potential and participate in physiological processes and thus are also of interest to study. For example, AGE-modified peptides could interact with AGE receptors on antigen-presenting cells to regulate the inflammatory response (Teodorowicz, Van Neerven, & Savelkoul, 2017; Xue et al., 2011). Additionally, glycated AMPs offer promising advantages, such as improved peptide stability and pathogen specificity (Bednarska, Wren, & Willcocks, 2017; Bellavita, Braccia, Galdiero, & Falanga, 2023).

Chapters 5 & 6 aimed at assessing the antimicrobial and immunomodulatory properties of peptides released during *in vitro* infant digestion of SP and PP, based on experimental and bioinformatic methods, which will be further discussed in sections 7.5.1-7.5.3. Given the distinct peptide profiles arising from various heating and digestion conditions, the bioactivity of resulting peptides can be expected to differ. Furthermore, it is important to note that the differences in database sizes of SP and PP can also significantly influence the number of identified peptides, thereby affecting the bioinformatic prediction of BPs.

7.5.1. Antimicrobial properties

AMPs serve as a first line of defense within the gastrointestinal tract of infants against potential microbial infections (Benkerroum, 2010; Salas, Badillo-Corona, Ramírez-Sotelo, & Oliver-Salvador, 2015). Some BPs have the ability to maintain a healthy balance of gut microbiota. In this study, Gram-negative *Enterobacter cloacae* (DSM 30054) and Gram-positive *Staphylococcus epidermidis* (DSM 20044) were selected to determine the antimicrobial properties of the peptide fractions of SP and PP. These bacterial strains are found in the human gastrointestinal tract (Ferry et al., 2020; Garcia-Gutierrez et al., 2020) and are considered opportunistic pathogens, especially posing increased infection risks for infants with underdeveloped immune systems (Fusco et al., 2018). Our study revealed the promoting effect of glycation on the growth of *Enterobacter cloacae* in both SP and PP-derived peptides, without a distinct preference for either protein source (**Figure 5.4A**). Moreover, the growth rate of

Staphylococcus epidermidis was reduced in SP after heating, while PP possessed an inherently stronger microbial inhibition activity compared to SP, independent of heating (**Figure 5.4B**). Therefore, these findings underscored the varying reactions of bacterial strains to peptide samples originating from the digestion of distinct protein sources after heating at different conditions. However, focusing solely on these two bacterial strains is insufficient. Other pathogens, such as *Cronobacter* sp. and *Salmonella enterica*, are the primary microorganisms linked to the contamination of powdered IF (Kent, Fitzgerald, Hill, Stanton, & Ross, 2015). Therefore, exploring these specific pathogens in future research would be of great interest to see whether SP or PP digests can inhibit their growth.

Subsequently, an online bioinformatics tool CAMP$_{R4}$ (http://www.camp.bicnirrh.res.in/predict/, accessed July 2023) was used to predict potential AMPs in SP and PP digests. This prediction was based on peptides with lengths between 8-25 AAs and a peptide score over 100 for more reliable identifications. Both glycated and non-glycated AMPs were found, with 9 out of 519 AMPs being glycated in SP and 6 out of 133 being glycated in PP. Notably, due to differences in the number of peptides available for prediction, more AMPs were predicted in SP than in PP. Furthermore, **Figure 7.4** demonstrates that a reduction of the distinct AMPs was found from non-heated to wet-heated to dry-heated SP and PP. This was attributed to fewer total identified peptides after heating, as also discussed in section 7.4. However, the relative number of glycated peptides was still the highest in dry-heated SP and PP (**Figure 5.5**).

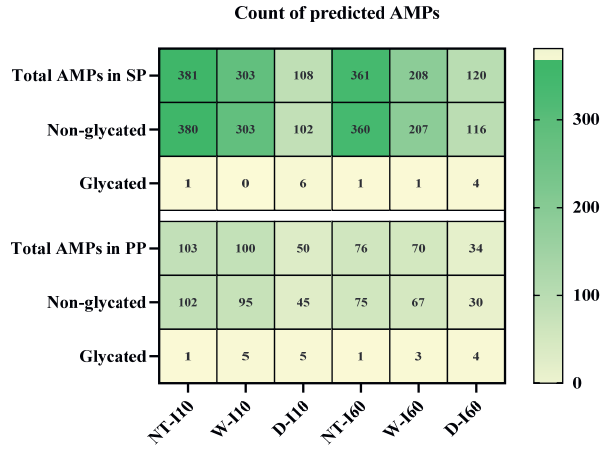

Figure 7.4. Count of glycated and non-glycated AMPs in SP and PP. Peptides were fractionated from the digests after wet (W) or dry (D) heating, with non-treated (NT) samples as control. I10 and I60 represent 10 min and 60 min of intestinal digestion, respectively.

Interestingly, when comparing SP and PP, more SP-derived AMPs had high scores according to CAMP, indicating a higher antimicrobial potential of SP digests (**Tables 5.S1 & 5.S2**). However, experimental results revealed different behavior of microorganisms in different digested samples. Especially PP-derived peptide fractions showed an inherent inhibitory effect on *Staphylococcus epidermidis*. Thus, we cannot directly link *in vitro* and *in silico* results. This may be due to the fact that the *in vitro* antimicrobial experiments only targeted specific bacteria and applied the whole peptide fractions, while *in silico* bioinformatics studies focused on the antimicrobial potential of individual peptides, including anti-virus, anti-fungal, and anti-bacterial properties. The whole range of AMPs present in actual digesta samples may have synergistic or antagonistic effects within the complex food matrix, which may differently contribute to the overall microbial inhibition. Studying more bacteria, as also mentioned before, as well as other types of microorganisms, may give a more complete overview of the actual antimicrobial activity of digests, which can then be compared to *in silico* predictions.

7.5.2. Immunomodulatory potency

Peptides can have the potential to act as immunomodulators (Gokhale & Satyanarayanajois, 2014). In this study, our primary focus was on analyzing the binding affinity of digestion-derived peptides to sRAGE, as determined by inhibition ELISA. Especially glycated peptides are possible ligands for RAGE. RAGE is a cell surface receptor known for its role in regulating inflammation and immune responses. In addition, sRAGE, its soluble form, is recognized as a decoy receptor for circulating AGEs, with higher sRAGE levels found in neonatal circulation (Fritz, 2011; Quintanilla-García et al., 2019), suggesting its possible immunomodulatory potency, particularly in infants. Results on sRAGE binding are shown in **Figure 5.3**. The sRAGE-binding affinity was notably higher in PP peptides, although heating had a diminishing effect on this affinity. In contrast, SP peptides exhibited an overall lower binding capacity to sRAGE, regardless of the heating process. It was noticed that RAGE primarily recognizes negative charge and/or hydrophobic domains on its ligands (Bongarzone, Savickas, Luzi, & Gee, 2017; Deane, 2012). However, most conformational structures were destroyed after digestion. Thus, the unique peptide profiles, arising from the digestive process of the two protein sources, may have contributed to the differential sRAGE-binding affinities observed between SP and PP. Given that aggregation can also promote sRAGE-binding affinity (Liu, Teodorowicz, Wichers, van Boekel, & Hettinga, 2016), there is a possibility that peptides themselves may induce self-aggregation, leading to sRAGE binding (Bellesia & Shea, 2007). To validate this hypothesis, future research can explore the physiochemical properties of the

peptide fractions beyond just their bioactivity, in order to gain a more comprehensive understanding of the underlying mechanisms.

Furthermore, the immunogenic potential of individual peptides was predicted, with only those peptides with a MaxQuant score over 100 being included. Due to the non-availability of the database for pea B-cell epitopes and only the peptide "VRIVNSEGNKVFDDK" (O24294, AA 470-484) from PP being predicted as a potential T-cell epitope, immunogenic analyses were only further conducted on SP-derived peptide samples. **Tables 6.1 & 6.2** demonstrated all the possible peptides in SP that may exhibit immunogenic properties, predicting 13 potential T-cell epitopes as IL-4 inducers and recognizing 2 B-cell epitopic peptides that were reported previously. Interestingly, none of these peptides were glycated. Moreover, different heat treatments and digestion durations can modulate their potential to trigger immune responses (**Figure 6.3**). In addition, cross-reactivity was found between SP and other legume proteins, including those sourced from peanuts, peas, chickpeas, lentils, kidney beans, and lupine family (**Table 6.3**). This suggests that individuals with sensitivities or allergies to SP may also react to identical or similar epitopes in other legume proteins, potentially triggering immune responses.

7.5.3. Prospects for future research integrating experimental and bioinformatic findings

Building upon the bioinformatic findings that predicted potential AMPs and immunogenic peptides, an intriguing next step would involve the synthesis of these peptides for further validation of their bioactive properties at both the molecular and cellular levels. Furthermore, although both *in vitro* and *in silico* studies on peptide fractions were carried out, the results obtained from these approaches did not exhibit strong associations. This discrepancy may be attributed to the fact that, in our study, bioinformatics was primarily focused on a macroscopic perspective, predicting individual peptides with antimicrobial and immunomodulatory potentials. In contrast, experimental investigations delved into the more detailed functions of entire peptide fractions in digesta, assessing their antibacterial activity against specific strains and their sRAGE-binding capacity.

Therefore, to establish a more cohesive link between experimental and bioinformatic studies, a hybrid approach can be explored. Future research could begin with the gradual fractionation of peptides with different molecular weights. Then, related assays can be applied to analyze the bioactivity of these peptide fractions, along with peptide identification through LC-MS/MS. Simultaneously, using bioinformatic tools, the most potent BPs within these fractions can be predicted, after which these can be synthesized for validation of their functions. These BPs can

naturally be generated after digestion and exert their bioactivity in the human body, or they can be synthesized and added to IFs as functional supplements for infants with specific needs.

7.6. Transition from simplified protein models to simulated plant-based infant formula

This thesis has primarily focused on the study of simplified model systems of SP and PP in the presence or absence of the reducing sugar glucose, with particular attention to their digestive behavior after different heating processes, as well as the bioactivity of the resulting peptides in the digesta. While these model systems provide valuable insights into the behavior of individual proteins, it is essential to acknowledge that IF is a complex mixture of various components. In addition to proteins and carbohydrates, IF contains lipids, vitamins, minerals, and other bioactive compounds. The interactions between these components during IF production can impact the overall properties and nutritional value of the final product. Therefore, further studies at a complete IF level would be beneficial for better understanding. Furthermore, conducting a comparative analysis between dairy-based and plant-based IFs would be of significant interest, as it could offer deeper insights into the differential effects when compared to traditional milk-based IF.

In addition, the selection of plant protein sources is also important for future studies. As discussed in **Chapter 2**, infants, due to their physiological immaturity, necessitate high-quality proteins to facilitate growth and development. Moreover, they require the intake of three additional essential AAs (EAAs): histidine, cysteine, and tyrosine from the diet, compared to adults. However, plant protein sources usually do not provide a complete profile of EAAs that matches breastmilk. Hence, the consideration of alternative plant protein sources, beyond SP and PP, such as quinoa protein and faba bean protein (Roux et al., 2020; Venlet, Hettinga, Schebesta, & Bernaz, 2021), as well as the utilization of plant protein blends to achieve a more balanced EAA composition (Dimina, Rémond, Huneau, & Mariotti, 2021), can offer nutritional benefits. Exploring a variety of plant protein sources for IF broadens the range of alternative formulas available to consumers.

Another consideration is the utilization of different infant digestion models. In this study, a full-term static *in vitro* infant digestion model was applied (Ménard et al., 2018), which is a simple and controllable model. However, it cannot fully replicate the dynamic and complex conditions within the infant gastrointestinal tract, including peristaltic movements, pH variations,

contribution of brush border-located proteases, and enzyme secretion. Furthermore, it only simulates one-month-old infants, and thus cannot reflect the digestion kinetics of infants at different ages (Miltenburg, Bastiaan-Net, Hoppenbrouwers, Wichers, & Hettinga, 2024). Moreover, *in vivo* models such as mice and piglets may increase understanding of the function of plant proteins in IF, as they provide insights into the physiological responses and health implications that cannot be fully captured *in vitro*. Taken together, future research can expand by incorporating static, dynamic, and age-specific infant digestion models, either *in vitro* or *in vivo*.

To summarize, these further explorations can inform the design of better plant protein-based IF that aligns with the nutritional needs and digestive capacities of infants at various stages of development.

7.7. Overall conclusions

This thesis first thoroughly evaluated the key considerations and challenges related to the application of plant proteins in IF, including aspects like nutritional value, health implications (including allergy risks), safety, and processing complexities. Subsequently, SP and PP were selected as the primary research materials, as SP is the predominant plant protein source in current plant-based IF and PP is a promising alternative protein source. The relationship between heating, structural modifications, *in vitro* infant gastrointestinal digestion, peptide formation, and peptide bioactivity was explored. The main findings and conclusions of the research presented in this thesis are:

(1) After wet heating, protein unfolding increased the overall digestibility of SP and PP. Moreover, the inactivation of TIs by limited aggregation also contributed to increased overall digestibility of SP after heating.

(2) Dry heating (60°C) without glucose only led to limited aggregation and did not affect the overall digestion of SP and PP.

(3) Dry heating (60°C) with glucose led to glycation-induced aggregation and cross-linking, thereby decreasing gastric digestion of both SP and PP. In addition, glycation of trypsin-cleavage sites within proteins hindered intestinal digestion of SP and particularly PP.

(4) Distinct peptide patterns were identified in SP and PP among different heating and digestion conditions. More glycated peptides were found in dry-heated samples with glucose.

(5) SP and PP-based peptide fractions differed in sRAGE-binding and antimicrobial capacities, which were more associated with protein sources than heat treatment.

(6) Upon different heating and digestion conditions, both glycated and non-glycated AMPs were predicted in the peptide fractions, with 9 glycated out of 519 for SP and 6 glycated out of 133 for PP.

(7) In SP peptide fractions, 29 T-cell epitopes and 27 B-cell epitopes were predicted, with none being glycated. Cross-reactivity was predicted between SP and other legume proteins. Furthermore, heating and digestion time may modulate peptide-induced immune responses.

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Summary

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The growing interest in replacing dairy proteins with plant proteins in infant formula (IF) is driven by concerns about cow's milk allergy, lactose intolerance, and sustainability. Currently, only soy protein (SP) and hydrolyzed rice protein are legally approved for IF in the European Union, although some other plant proteins like pea protein (PP), quinoa protein, and faba bean protein, are also promising sources. However, a comprehensive assessment of the suitability of these plant proteins remains incomplete. Furthermore, the heat processing involved in IF production can induce protein modifications, including denaturation, aggregation, and glycation, which may impact infant digestion and immune responses. To address these issues, the research described in this thesis initially explored the challenges and considerations associated with the use of plant proteins in IF. Subsequently, it focused on SP and PP as research materials to investigate the differential effects of wet and dry heating on their structural modifications and linked these modifications to digestion kinetics, peptide patterns, and peptide bioactivity.

Chapter 2 offers a comprehensive evaluation of critical factors related to applying plant proteins in IF, including protein quality, ANFs, allergenicity, and various requirements such as protein content, yield, purity, extraction methods, potential contaminants, and techno-functional properties. This information covers aspects of nutritional value, health implications (including allergy risks), and safety. This chapter also highlighted the complexities of processing and its important role in IF production. Thereby, it offers valuable insights to guide the informed selection of plant proteins for use in IF.

Chapters 3 & 4 investigated the influence of heating on the structural changes of SP and PP, as well as their digestion within an *in vitro* static full-term infant digestion model. **Chapter 3** specifically focused on wet heating at temperatures ranging from 65°C to 100°C for 30 min. Interestingly, as the heating temperature exceeded 75°C for SP and 65°C for PP, more soluble aggregates were formed due to disulfide rearrangement, and the proteins underwent increased unfolding, as indicated by greater surface hydrophobicity. The results revealed that higher heating temperatures led to improved overall digestibility for both SP and PP, primarily because unfolding exposed more enzyme-cleavage sites. An intriguing finding was that inactivating trypsin inhibitors through limited aggregation enhanced the digestibility of SP.

Chapter 4 focused on mild dry heating at 60°C for 6 h and 48 h, in the presence or absence of glucose. The addition of glucose was done to explore the effects of glycation. As expected, no

significant glycation occurred in the glucose-free SP and PP. These samples displayed only minor aggregation, which did not affect the overall digestion of SP and PP. Furthermore, the highest glycation levels were observed in SP and PP samples dry-heated with glucose for 48 h, which coincided with the highest level of aggregation and lowest gastric digestibility. This glycation-induced aggregation buried enzyme-cleavage sites, primarily impeding gastric digestion. By contrast, the impact on intestinal digestibility was less pronounced. This was because despite glycation modifying trypsin-cleavage sites, the presence of multiple pancreatic enzymes compensated for the limited tryptic hydrolysis. Notably, PP, being more heavily glycated, experienced more significant inhibition of its intestinal digestion compared to SP.

To further explore the distinct digestion behaviors of SP and PP, and their potential impact on peptide patterns and bioactivity, SP and PP were wet heated at 85°C for 30 min and dry heated at 60°C for 48 h, both at a 1:4 ratio of protein to glucose (w/w). This was to ensure consistent composition and induce substantial structural modifications. **Chapter 5** focused on the composition of peptides released from SP and PP after wet and dry heating, with analysis being conducted using LC-MS/MS and MaxQuant. The results revealed that far more peptides were identified in SP than in PP and that heating and digestion reduced the peptide numbers, with factors of analytical limitations, glycation levels, and digestion behaviors contributing to these outcomes. Moreover, a greater presence of glycated peptides was detected in glycated SP and PP samples. This chapter subsequently delved into assessing the antimicrobial activity of the peptide fractions and their binding affinity to the soluble receptor for advanced glycation end products (sRAGE). Notably, glycation was found to promote the growth of *Enterobacter cloacae*, whereas PP digests displayed a stronger inhibition against *Staphylococcus epidermidis* compared to SP. Furthermore, SP digests exhibited inherently low sRAGE-binding affinity. These results highlighted the important role of protein sources, as opposed to the specific heat treatment. Subsequent bioinformatics analysis predicted the presence of both glycated and non-glycated antimicrobial peptides (AMPs). In SP, 9 out of 519 AMPs were glycated, while in PP, 6 out of 133 AMPs were glycated.

Following **Chapter 5**, **Chapter 6** further explored the residual immunogenicity of SP-derived peptides, based on various bioinformatic tools. This investigation was exclusively conducted on SP, due to the availability of its well-established online protein and peptide databases. As a result, 29 potential T-cell epitopic peptides were predicted, with 13 of them having the capacity to induce the secretion of interleukin-4. Additionally, 27 potential B-cell epitopes were identified, including 2 epitopes that perfectly matched previously reported B-cell epitopes. The

investigation also evaluated the potential cross-reactivity of these putative epitopic peptides between SP and other legumes, including peanut, pea, chickpea, lentil, kidney bean, and lupin. Overall, the study showed that heating and digestion time could modulate the peptides' potential to trigger immune responses.

In conclusion, this thesis provides guidelines for the informed selection of plant protein sources for use in IF. In addition, it revealed that heat-induced modifications, including denaturation, aggregation, and glycation, played a significant role in shaping the diverse digestion behaviors and overall digestibility of both SP and PP. Consequently, these modifications affected the patterns and bioactivity of the resulting peptides. These insights hold implications for the development of IF, where the heat processing and the selection of plant protein sources can significantly affect the nutritional and functional aspects of the final product.

Abbreviations

Abbreviations

AA	Amino acids
AAS	Amino acid score
Abs_{sample}	Absorbance for the mixture of the sample/sRAGE solutions
Abs_{sRAGE}	Absorbance for sRAGE without samples
AGEs	Advanced glycation end products
AMB 1-42	β-Amyloid peptide 1-42
AMPs	Antimicrobial peptides
ANFs	Antinutritional factors
ANS/ANSA	8-anilino-1-naphthalenesulfonic acid ammonium salt
BCA	Bicinchoninic acid
BI	Browning index
BPs	Bioactive peptides
CEL	N_{ε}-carboxyethyllysine
CMA	Cow's milk allergy
CML	N_{ε}-carboxymethyllysine
D	Dry heating/dry-heated
DH	Degree of hydrolysis
DIAAS	Digestible indispensable amino acid score
DLS	Dynamic light scattering
EAAs	Essential amino acids
Furosine	N_{ε}-2-furoylmethyllysine
G	Glucose
G0	Before digestion
G10	10 min after gastric digestion
G60	60 min after gastric digestion
G90	Soy protein glycated with glucose for 90 min at 100°C
G-H	Glyoxal hydroimidazolone
GP	Gastric phase
I10	10 min after intestinal digestion

I60	60 min after intestinal digestion
IC50	Inhibitory concentration 50
IF	Infant formula
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-4	Interleukin-4
IP	Intestinal phase
K	Lysine
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MG-H	Methylglyoxal hydroimidazolone
MHC-II	Major histocompatibility complex class II
MS	Mass spectrometry
NH_2(acid)	Concentration of the total free amino groups after acid hydrolysis
NH_2(final)	Concentration of free amino groups by the time of sample collection
NH_2(initial)	Concentration of free amino groups in each sample before digestion
NT	Non-treated
$OD_{(t)}$	Optical density values measured after incubation
$OD_{(t0)}$	Optical density values measured before incubation
OPA	o-phthaldialdehyde
OVA	Ovalbumin
PDCAAS	Protein digestibility-corrected amino acid score
PP	Pea protein
PSD	Particle size distribution
R	Arginine
RAGE	Receptor for advanced glycation end products
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	Soy protein
SPE	Solid phase extraction
sRAGE	Soluble receptor for advanced glycation end products

T6	Dry heating at 60°C for 6 h
T48	Dry heating at 60°C for 48 h
TCA	Trichloroacetic acid
Th	T-helper
TIs	Trypsin inhibitors
W	Wet heating/wet-heated

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们总能找到共同的话题。**叶衔佩**和**段睿**，从本科到博士，我们已经认识了 10 年了。惊觉总是我一直在远行，我们总是聚少离多，但是却从不生疏。让我们一起变老，到了 80 岁也要一起打麻将、蹦迪！睿宝，愿你永远自由、热烈且真诚。佩宝，请记住，我永远是你的最佳听众。“愿岁并谢，与长友兮”。

感谢我的**家人 (to my families)**。感谢**爸爸妈妈**对我无条件的爱与支持。你们用无私的爱陪伴我成长，让我成为了今天的我。无论我面临什么挑战，你们始终鼓励我勇敢追求自己的梦想，并对我充满信心。谢谢你们给我足够的空间，让我自由地去探索、去尝试、去犯错误，然后从中学习和成长。你们的理解和包容，让我更加坚定地走自己的路，追求自己的目标。正是因为有你们的支持，我才能勇敢地面对一切。谢谢**舅舅舅妈**，让我在异国他乡也有了家人的陪伴。记得我刚来荷兰时，你们来看望我，为手忙脚乱的我准备各种生活必需品，为我做了第一顿热乎乎的饭菜。谢谢你们！感谢**表姐媛媛**的每一通视频，每一句问候，感谢所有的无话不谈和牵挂。感谢**所有家人**的陪伴和对我所有决定的支持和鼓励。我会继续努力，让你们为我骄傲。

最后一段话我想送给**自己 (to myself)**:

> 愿你行远自迩，登高自卑，笃行不怠，未来可期。
>
> 愿你长歌有和，独行有灯，光而不耀，静水流深。
>
> 愿你松花酿酒，春水煮茶，青松皓鹤，绵绵度岁。

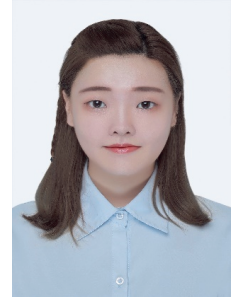
Jiaying Tang 唐佳颖

January 16th, 2024

Wageningen

About the author

Author Biography



Jiaying Tang was born on 29th July 1995, in Chengdu, China. She obtained her bachelor's degree in Food Science and Engineering from Sichuan Agricultural University in 2017 and master's degree in Nutrition and Food Safety from China Agricultural University in 2019. Her master thesis focused on the effect of thermal treatment on the structural stability of plant ferritin under the supervision of Prof. dr. Guanghua Zhao. During her master, she showed a strong interest in protein biochemistry and alternative proteins.

Sponsored by the China Scholarship Council, in October 2019, Jiaying moved to the Netherlands to pursue a doctoral degree in the Food Quality & Design Group at Wageningen University & Research. She worked under the supervision of Prof. dr. Kasper Hettinga and Prof. dr. Harry Wichers. During the PhD project, she studied the impact of heat-induced structural modifications on infant digestion of plant proteins, as well as their resulting peptide pattern and bioactivity. The results of this research are presented in this thesis.

Jiaying can be reached via email: tangjiaying0729@outlook.com.

List of publications

This thesis:

1. **Tang, J.**, Wichers, H. J., & Hettinga, K. A. (2023). Glycation of soy and pea proteins influences infant gastric digestibility more than intestinal digestibility. ***Food Hydrocolloids***, 136, 108251. https://doi.org/10.1016/j.foodhyd.2022.108251.
2. **Tang, J.**, Wichers, H. J., & Hettinga, K. A. (2022). Heat-induced unfolding facilitates plant protein digestibility during *in vitro* static infant digestion. ***Food Chemistry***, 375, 131878. https://doi.org/10.1016/j.foodchem.2021.131878.
3. **Tang, J.**, Wichers, H. J., & Hettinga, K. A. Comprehensive evaluation of plant proteins as potential dairy substitutes in infant formula: A review. (*Submitted to journal for publication*)
4. **Tang, J.**, Teodorowicz, M., Boeren, S., Wichers, H. J., & Hettinga, K. A. sRAGE-binding and antimicrobial bioactivities of soy and pea protein after heating and *in vitro* infant digestion. (*Submitted to journal for publication*)
5. **Tang, J.**, Boeren, S., Wichers, H. J., & Hettinga, K. A. Differential effects of heating modes on the immunogenic potential of soy-derived peptides released after *in vitro* infant digestion. (*Submitted to journal for publication*)

Others:

1. **Tang, J.**, Yu, Y., Chen, H., & Zhao, G. (2019). Thermal Treatment Greatly Improves Storage Stability and Monodispersity of Pea Seed Ferritin. ***Journal of Food Science***, 84:1188-1193. https://doi.org/10.1111/1750-3841.14581.
2. Mei, Y., **Tang, J.**, He, H., & Ao, X. (2017). Determination of protease activity of dominant microorganism from moldy bean. ***Food and Fermentation Industries***, 43(9):130-136. (in Chinese). https://doi.org/10.13995/j.cnki.11-1802/ts.014512.

Overview of completed training activities

Discipline specific activities			
Courses			
Food Proteins	University of Copenhagen	Virtual	2020
Dairy Protein Biochemistry	VLAG*	Wageningen, NL	2022
Conferences			
IMGC VIRTUAL Symposium	University of California, Davis	Virtual	2020
NIZO Plant Protein Functionality Conference [a]	Elsevier	Virtual	2020
Dairy Science and Technology Symposium	Aarhus University	Virtual	2021
7th International Conference on Food Digestion [a]	INFOGEST	Cork, Ireland	2022
6th Young AGErs Symposium [b]	Abertay University	Dundee, UK	2022
IMGC HYBRID Symposium	University of California, Davis	Virtual	2022
General courses			
VLAG PhD week	VLAG	Baarlo, NL	2020
Research Data Management	WGS*	Wageningen, NL	2020
Searching and Organising Literature	WGS	Wageningen, NL	2020
The Essentials of Scientific Writing & Presenting	WGS	Wageningen, NL	2020
Reviewing a Scientific Manuscript	WGS	Wageningen, NL	2020
Scientific Publishing	WGS	Wageningen, NL	2020
Competence Assessment	WGS	Wageningen, NL	2021

Bridging Across Cultural Differences	WGS	Wageningen, NL	2021
Scientific Writing	WGS	Wageningen, NL	2021
Other activities			
Preparation of research proposal	FQD*	Wageningen, NL	2019
PhD study tour to Spain	FQD	Spain	2022
Weekly group meetings	FQD	Wageningen, NL	2019 -2024
Teaching obligations			
Supervision of BSc students	FQD	Wageningen, NL	2021
Case Studies Product Quality	FQD-24306	Wageningen, NL	2021
Quality Systems Operations	FQD-20804	Wageningen, NL	2021 -2023

[a] Poster presentation; [b] Oral presentation
* VLAG: Graduate School for Biobased, Biomolecular, Chemical, Food, and Nutrition Sciences
* WGS: Wageningen Graduate School
* FQD: Food Quality & Design Group

Colophon

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