Soy fractionation pathways
for food applications

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Propositions

1. Novel soy-based ingredients should be valued on more characteristics than the protein content only.

   (this thesis)

2. The washing process is an underestimated route for protein enrichment.

   (this thesis)

3. An thorough analysis of current consumer preferences helps to predict the developments in food and nutritional science.

4. Limited availability of sample stimulates thinking about a smart experimental set-up.

5. Novelty-seeking keeps researchers experimenting.

6. To reduce current and future meat consumption, it is a good strategy to expose the young generation to traditional soy-based food products.

7. A pandemic gives a reasonable excuse for introverted people to minimize social contacts.

Propositions belonging to the thesis entitled

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Contents

Chapter 1

General introduction

1.1 Soybeans in food and feed

Soybeans were originally cultivated and consumed in the North-Eastern part of China a few thousand years ago (Hymowitz, 1970). During the course of soybean domestication, various forms of soy-foods were gradually created and promoted by the Chinese, such as soymilk, tofu, soy paste, and soy sauce. Around 1,000 years ago, along with the cultivation of soybean, the ways of preparing soy-foods had gradually spread to the nearby countries and regions. More types of products were developed to suit local taste, such as yuba, natto and tempeh (Liu, 2004). In general, these Oriental soy-foods are also known as traditional soy-foods, and they are produced by various methods like fermentation, coagulation, and film formation. A common feature of the traditional soy-foods is that those are directly made from the whole soybeans, which means the soy ingredient fractionation was not included in the processing chain of traditional products.

Traditional soy-foods, though, have less appeal to the Western consumers because of their unfamiliar taste and texture (Liu, 1997). It explains why the industrial development patterns of soybean usage in the Western countries were completely different from the traditional patterns encountered in East Asia. At the beginning of the 20th century, with the help of technological improvements, soybean was primarily recognized for its high oil content (Hymowitz, 2008; Thrane et al., 2017). Industrial soybean milling flourished in Western countries such as England, Germany, and the U.S. At that time, milling companies separated the soybeans via mechanical pressing or solvent extraction into an oil fraction and a meal fraction (Prodöhl, 2013). This marked the start of soybeans fractionation as part of modern food production chains. The oil fraction was firstly used to produce non-food products like soaps, cosmetics, candles, paints, etc. The use of solvents such as benzene or gasoline allowed a high-yield oil extraction, but the obtained oil fraction was not safe for human consumptions at that time. Major advances in oil refining gradually increased the acceptability of soybean oil as food (Hymowitz and Newell, 1981). Then, in the 1930s, the demand for low-cost and highly functional edible oils increased quickly due to the increased consumption of processed foods like margarine (Durkee, 1936). The growth of the soybean oil industry was further supported by the continuous multiplication of potential uses such as mayonnaise, salad oils and dressings (Schaub et al., 1988). In the 1970, soybean oil has dominated the world market of edible oils with a share exceeding 52% (Mcansh, 1973; Mehr, 1972), and till now, it is a leading edible oil at the global level (El-Hamidi and Zaher, 2018).

The soy oil production resulted in large quantities of a by-product, being a de-fat soy meal (DFSM). DFSM was initially applied as a nitrogen fertilizer for food production in China and Japan till the early 1930s, when chemically fertilizers took over the market (Park and Slipher, 1939). Around that time, soybean meal containing protein, vitamins, and minerals was recognized as a valuable ingredient, and served in feed for the livestock industries (Hayward et al., 1935, 1937; Wightman et al., 1944). During the 1950s, great advances in the science of animal nutrition were made, and the advantages of soybean meal over most other oilseed meals were established with respect to digestible energy and unique composition of amino acids (Patrick, 1951). The majority of the soybean meal production was then served as animal feed throughout the world (Stein et al., 2008), and only a share of 2-3% of the production is directly consumed by humans in the forms of food products ever since (Goldsmith, 2008).

The nutritional values of meal fraction for human consumption were noticed as well. This led to the development of new industrial processes to fractionate the edible soybean meal further into soy protein ingredients, such as soy protein isolate (SPI) and soy protein concentrate (SPC). By creating these advanced ingredients, the economics of the soy refinery drastically improved (Senti, 1972). Nowadays, soy protein ingredients are used in various food applications and are the mostly used protein sources for novel products like meat analogues. Consumption of soy-based products might have several effects on health. The consumption of soy products in the diet may help to reduce the cholesterol level (Shu et al., 2009; Zhuo et al., 2004) and improve metabolism and bone mineral density (Cederroth and Nef, 2009; Wei et al., 2012). The health claim for soy protein, approved by the Food and Drug Administration (FDA), has promoted a strong consumer interest in soy foods and impacted significantly on the use of soy protein ingredients in food applications (Food and Drug Administration, 1999). This explains why numerous soy-enriched foods have been developed by incorporating soy protein ingredients into mainstream foods like bread, pasta, and cereals to boost protein content and increase the consumption of soy.

Today, soybean is a versatile and demanded crop with a global production of 336.6 million metric tons in the 2019/2020 market year (USDA, 2021). Although traditional soy-foods have been consumed throughout East Asia for more than two thousand years, in Western countries they are not well incorporated in the diet. Nevertheless, also in Western countries soy has grown in importance significantly. Soy-based products have become an economical and high-quality plant protein source for a broad range of food applications. Unfortunately, the processes to make modern soy-based ingredients and then soy-based products is much more extensive and, in many cases, less efficient compared to making of the traditional products described at the start of this section.

1.2 Overview of processes to make soy protein ingredients and products

Soybean is rich in protein and oil. It also contains soluble and insoluble carbohydrates (Thrane et al., 2017). Soy protein-rich ingredients are available in various forms, including soy flour, SPC, and SPI. Soy flours are the least refined fractions, and therefore, have the lowest protein content among all the soy protein ingredients. They are obtained by milling soybean directly (full-fat soy flour) or milling the DFSM after oil extraction (de-fat soy flour). As the by-product of soy oil production, part of DFSM forms the basis for more refined ingredients such as SPC and SPI, while more than 95% of DFSM is used in animal feed applications though (Singh et al., 2008).
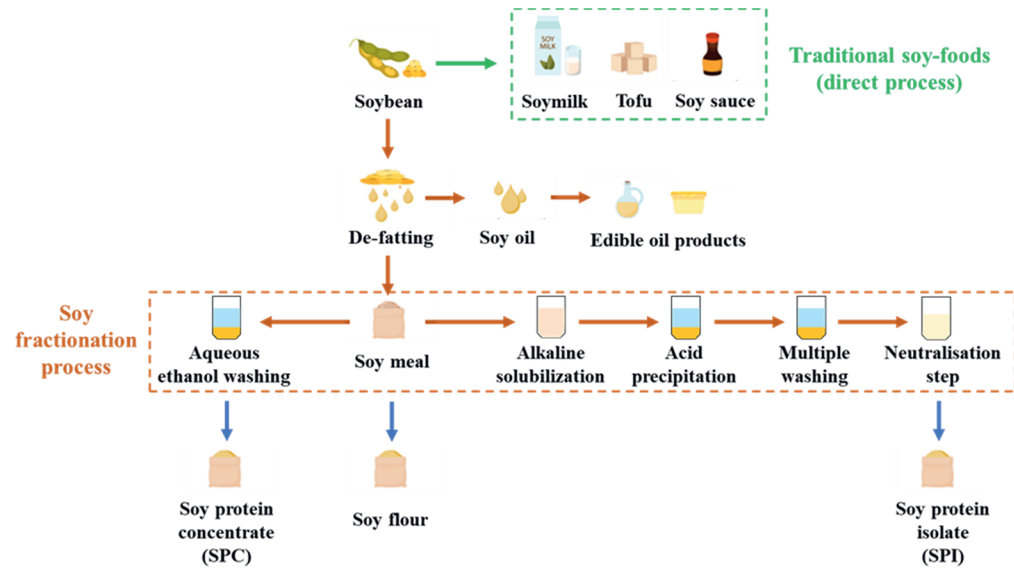

Fig. 1-1 Soy fractionations and applications.

Two fractionation pathways are commonly used in the industry for SPC and SPI (Fig. 1-1). SPC is made by washing DFSM with hot water, acidic solution or aqueous ethanol, with the latter being the most commonly used industrially (Alibhai et al., 2006). During the process, the protein is mainly kept in the solid phase with the insoluble carbohydrates, while the soluble carbohydrates are removed with the solvent. The process for making SPI normally starts with an alkaline solubilization step, in which the protein in the DFSM is extracted in the liquid phase, and the insoluble carbohydrates are separated with the solid phase (Deak and Johnson, 2007). In this step, high protein solubility is desired to achieve maximal protein extraction, and thus, to increase the yield. The soluble carbohydrates are further removed

when the protein is precipitated. This process makes SPI to be the most refined soy protein ingredient with the highest protein purity (Table 1-1).

Table 1-1 Typical composition of various soy protein-rich ingredients.

	Full-fat soy flour	De-fat soy flour	SPC	SPI
Protein, % (N× 5.7)	37.0	55.9	61.7	83.3
Oil, %	21.8	0.5	0.0	0.0
Carbohydrate, %	34.5	37.2	33.0	13.3
Ash, %	6.7	6.9	5.3	3.4

Soy protein is broadly applied in all types of products as a functional ingredient (Table 1-2) or a cheap substitute. For example, de-fat soy flour can be used as an alternative to non-fat milk powder or other costly dairy components in some bakery products such as cookies and cakes (Cotton, 1974). With improved color and flavor, SPC is commonly used in processed meat, fish, and poultry products (Thrane et al., 2017). SPI that possesses high viscosity and solubility is applied in soups, sauces, and beverages, while SPI that possesses forming and whipping characteristics can work effectively in replacing the egg white (Egbert, 2004). Texturized soy proteins, which are characterized by having structural integrity and chewy texture, can be transformed into fibers, shreds, chunks, granules, or other forms. These are used as cost-effective ingredient to meat products to reduce ingredient cost (Heywood et al., 2002). The starting material for making texturized soy protein can be de-fat soy flour, SPC, SPI, or blends of several proteinaceous products (Riaz, 2001).

For all these food products described above, the required level of soy protein ingredients is low to achieve desired effects or make health claims. The reason for not using high levels of soy is the negative attributes such as off-flavors, odor problems, or undesired textual contributions when introduced to the finished products (Waggle et al., 1981). Accordingly, the current soy fractionation processes are thus optimized to achieve high protein purity, or to obtain specific functionalities such as high solubility, high water absorption or high viscosity to maximize the functions and minimize the negative impacts. However, the enhanced interest of next generation of soy-foods such as meat analogues brings new requirements towards the protein purity and functionality of soy protein ingredients.

Table 1-2 Functionality performed by soy protein ingredients in actual food systems (Kinsella, 1979).

Functionality	Food system	Soy protein ingredients used
Solubility	Beverages	Soy flour, SPC, SPI
Water absorption and binding	Meats, sausages, breads, cakes	Soy flour, SPC
Viscosity (thickening)	Soups, gravies	Soy flour, SPC, SPI
Gelation	Meats, curds, cheese	SPC, SPI
Cohesion-adhesion	Meats, sausages, baked goods, pasta products breads, cakes	Soy flour, SPC, SPI
Fat absorption and emulsification	Meats, sausages, soup, cakes	Soy flour, SPC, SPI
Flavor-binding	Simulated meats, bakery	SPC, SPI
Foaming	Whipped toppings, chiffon desserts, cakes	SPI
Color control	Bread	Soy flour

1.3 Trends towards new products and the challenges

The market for plant-based foods has been stimulated worldwide as a result of environmental and human health concerns among consumers (Pojić et al., 2018; Tilman and Clark, 2014). The current generation of plant-based dairy, egg, and meat products are increasingly competitive with animal products (Cameron and Neill, 2019). According to U.S. retail sales, all categories of plant-based foods showed growth across the board, with the highest rate in plant-based meat products, which is up to 23% in 2019. Among all the plant resources, soy protein is by far the largest segment in the plant-based proteins industry, and it accounts for nearly 60% of the global market (Expert Market Research, 2021), followed by wheat, pea, and several niche types, such as chickpea, rapeseed, and lupin (Bashi et al., 2019).

Soy protein forms the basis for the majority of meat analogue products in the market. Soy-based products like tofu, tempeh and yuba can be counted as traditional alternatives for meat (Shurtleff and Aoyagi, 2014). However, these foods are not developed to resemble meat in appearance, structure, and taste, and therefore, they are not well-accepted to substitute meat broadly, especially in Western countries. The new generation of meat analogue products target to mimic real meat with respect to their sensory and textural properties, as well as the nutritional values. In those products, soy protein ingredients, like SPI and SPC are used at substantial levels in the formulations, being one of the bulk ingredients. These bulk ingredients are expected to offer the needed protein level, and to dominate the texture formation for the products.

As can be derived from the information above, the existing soy ingredients in the forms of soy flour, SPC and SPI were never specifically designed for the target application with regards to composition and functionality. From the composition side, both oil and insoluble carbohydrate may contribute to the fibrous structure formation for meat analogue applications (Grabowska et al., 2016). Besides, high protein purity, which was previously desired for application in many products, may not be absolutely needed. Though SPI-only has been reported to form fibrous materials using extrusion (Wittek et al., 2021), it is generally combined with other components, such as wheat starch (Lin et al., 2002; Macdonald et al., 2009), wheat gluten (Grabowska et al., 2014; Krintiras et al., 2016, 2015) or pectin (Dekkers et al., 2016) to obtain a fibrous-structured product.

As for the functionality, the optimum condition of soy protein for novel products may differ from traditional standards, so additional processing steps were incorporated. For example, soy protein with high solubility is not preferred for meat analogue application, and an extra toasting step was needed to alter the functionality in the desired direction (Geerts et al., 2018). The intensive processing steps and harsh processing conditions applied in the current soy fractionation processes led to some inefficiencies to the product processing chain. Moreover, the additional steps further enlarged the adverse effects, sometimes even conflicting with consumers' initial drives for dietary transitions (van der Goot et al., 2016). An additional drawback of the use of SPI in food applications is the high Na content in the ingredient, which is a result of the fractionation process (Ruusunen and Puolanne, 2005).

Overall, novel fractionation routes should be developed to deliver ideal ingredients for next generation soy-foods including but not limited to meat analogue products. To face the challenges, one should consider that the functional requirements of the ingredients for novel products can be quite different than traditional definitions. This might lead to a need for a wider range of soy protein ingredients with different forms of compositions and nutritional aspects. Further, novel ingredients should be made using mild and simplified fractionation process to reduce the environmental impacts and to better align with modern consumer demands.

1.4 Aim and outline of the thesis

To meet the demand of next generation of soy-foods with soy protein ingredients as a bulk ingredient, it is necessary to rethink the fractionation process of soy. This thesis describes the exploration of novel routes to fractionate soy into functional ingredients. Our approach is to simplify the current fractionation process to different extents with parameter modifications, and to explore the potential of washing processes. The overall aim of this thesis is to understand how functionality of the soy protein ingredients and the resulting composition are affected by the fractionation process applied, and to quantify the relative importance of those factors.

Chapter 2 describes the consequences of simplifying the current aqueous fractionation by omitting the organic solvent de-fatting step and multiple washing steps. With a centrifugation step, the majority of the soy oil is removed. The final neutralization step is replaced by a pH adjustment step to achieve different final pH of the protein-rich dispersions. The relationship between processing pH and the resulting functionalities of soy protein-rich fraction (SPF) is discussed.

Based on the developed simplified fractionation process, **Chapter 3** explores the effect of an additional moisture heating treatment prior to drying on the functionality of soy protein. After the protein neutralization step, a moisture heating step is performed to the protein-rich dispersion for 30 min before freeze-drying. The heating temperature is set in a relatively mild range between 60 and 100°C, while a reference sample is kept at room temperature during the full fractionation process. The functionalities of the obtained SPF are compared, and the relationship between processing temperature and SPF functionality is discussed.

Chapter 4 describes the effect of further simplification of the fractionation process by omitting the acid precipitation step in addition to the step omitted in **Chapter 2** (de-fatting and washing steps). Besides, the effect of the use of Ca(OH)$_2$ during the fractionation is explored. Current aqueous fractionation applies NaOH as the basic solution to adjust the pH of the dispersion, resulting in extra Na introduced in the final soy ingredients. Ca(OH)$_2$ could be an alternative to NaOH in the protein solubilization and neutralization steps to lower the addition of Na, and potentially enhance the Ca content in the SPF.

Chapter 5 continues to explore the use of Ca(OH)$_2$ in the neutralization step to gain more in-depth information. Instead of replacing NaOH completely by Ca(OH)$_2$, here, mixtures of NaOH and Ca(OH)$_2$ in different ratios with a fixed pH are used to neutralize the protein-rich dispersions. The effect of Ca enrichment on the functionalities of soy protein is discussed.

The aqueous fractionation process focuses on solubilizing the soy protein first to achieve high protein purity. **Chapter 6** describes the potential of using aqueous ethanol washing process to produce soy protein ingredients. Cold-pressed de-fat soy meal (DFSM) is used as the starting material. This material is washed using several mixtures with different ethanol ratios.

Chapter 7 summarizes all the results reported in this thesis, and places those in a broader perspective. The results of a pilot scale production of new soy protein ingredients are discussed, and the potential applications for the side streams of the suggested fractionation processes are explored. The chapter ends with a future outlook about the potentials and challenges of developing new soy-based ingredients for modern food applications.

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Chapter 2

Functional properties of mildly fractionated soy protein as influenced by the processing pH

Abstract

In this study, an alternative mild fractionation process for the extraction of soy protein is investigated; aqueous fractionation, in which oil extraction and intensive washing steps are omitted. Moreover, a pH adjustment is proposed instead of the conventional neutralization step. The mildly fractionated soy protein fractions (SPFs) showed higher protein and oil content compared to commercial soy protein isolate. The process retained the proteins' native state. SPFs adjusted at pH 4.5 and 5.5 (close to pI) formed a powdery texture, resulting in larger size particles after dispersion in water. Despite their low nitrogen solubility index, water holding capacity and viscosity, when mixed with flour these SPFs presented the highest G* values. A flaky texture and reversed properties were observed with SPF adjusted at pH away from the pI. The range of properties achieved exhibits new routes in creating soy protein ingredients with desired functionality, avoiding over-processing due to post-treatment modifications.

2.1 Introduction

Nowadays, plant-based protein demand is increasing rapidly around the world because of the awareness among the consumers of sustainable diets and food production (Reipurth et al., 2019; Rizzo and Baroni, 2018). To meet the market's demands, the industry is in search of protein-rich crops and more sustainable protein extraction methods. The primary use of several crops is reconsidered, and with the development of new technologies, their potential use is also broadened. An important source in this transition is soybean, which currently already presents a production of 344 million metric tons in the 2017/2018 market year. The reason for soybean production is to produce oil for food and bioenergy, and the meal is mostly destined for animal feed (Stutte et al., 2018). Approximately 2% of soybean protein is nowadays consumed directly by humans (Nishinari et al., 2017), but its potential to produce protein-rich food in a palatable form is gaining attention (Kumar et al., 2017; Post, 2012). Hence, soybean protein ingredients, which were co-products from oil extraction before, are becoming gradually one of the main products of soybean processing for human consumption.

Novel products like soy-based protein-rich beverages and meat analogues have one thing in common, the use of soy protein products, isolates (SPI) or concentrates (SPC), as ingredients instead of whole soybeans or soybean flour, which are used for more traditional products like soy milk and tofu. It means that the fractionation process is indispensable in the processing chain of such modern foods. However, the conventional approach of producing SPI and SPC often involves oil extraction steps (using organic solvents) and/or several washing steps, which are energy and resource-intensive (Berghout et al., 2014). The large requirements of water and solvents reduce the sustainability potential of novel soy-based foods, sometimes even leading to the opposite effect than the initial purpose (J. A.M. Berghout et al., 2015). Moreover, except for the adverse effects of intensive fractionation, the functionalities of SPI and SPC are not targeting these novel products. Soy protein ingredients are mostly used as industrial materials like adhesives or as food additives (Nishinari et al., 2017; Vnučec et al., 2017). In case of food applications, functionality is focused on their use in general applications for maximizing stability of liquid products and high-fat food systems (Rizzo and Baroni, 2018), which could be achieved best with high protein solubility. However, for novel applications like soy burgers or soy sausages, soy ingredients are expected to form a gel or a fibrous structure. This means different

functionalities like gelling, water holding capacity and viscoelastic properties are requested rather than only solubility. Overall, a fractionation process with minimal environmental impact is needed, while aiming in parallel for specific functional properties.

To meet the requests from the new market, mild fractionation process with pH adjustment step is explored in this study. Mild fractionation process is based on conventional aqueous fractionation but with some steps modified. Soy oil is removed by centrifugation instead of organic solvent extraction, and intensive washing steps are omitted. The whole soybean is taken as the starting material, and water is used as an extracting medium. Although the oil is separated by simple centrifugation, such process performed in oilseeds was found to recover equally high oil yields as with organic solvent extraction (Campbell et al., 2011; Rosenthal et al., 1996). The oil extracted in the form of oil-bodies also can be further used as ingredients for emulsion stabilization (Karefyllakis et al., 2019b, 2019a). Furthermore, pH adjustment is applied instead of neutralization step during fractionation. It has been reported that the functional properties of soy protein can vary dramatically at different pH-values (Idris et al., 2003; Voutsinas et al., 1983). But most studies have investigated the effect of pH on the functional properties of plant protein by adding an extra pH adjustment step to dried isolates or concentrates (Benelhadj et al., 2016; Jiang et al., 2018, 2010; Kim et al., 2016), which introduces more salt in the system as well. Limited experiments were designed to achieve specific functional properties of plant protein by adjusting pH value directly during fractionation process.

In this study, soy protein fractions (SPFs) were obtained by mild fractionation and standardized on different final pH-values. The fractions were tested on their NSI, WHC, microstructure, denaturation and structural behaviors to evaluate their potential for use in multiple novel soy-food applications. The objective of this research was to gain insight on how the processing pH impacts the functional behavior of mildly fractionated soy protein. We believe this will unlock new routes in creating protein-rich ingredients with desired functionality for specific applications, avoiding over-processing due to post-treatment modifications.

2.2 Methods and methods

2.2.1 Materials

Dry, full soybean was obtained from FRANK Food Products (the Netherlands) and was harvested in Canada in October/November of 2016. The HCl and NaOH used for pH adjustments during fractionation were purchased from Sigma Aldrich (Germany). The water used was purified in a Milli-Q Lab Water System (Milli-Q IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany).

2.2.2 Soy flour preparation

Firstly, soybeans were pre-milled by using a pin mill LV 15M (Condux-Werk, Wolfgang bei Hanau, Germany) into grits. Then, the soy grits were further milled by a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) into full-fat soy flour (FFSF). The impact mill was set according to the parameters described by Pelgrom (Pelgrom et al., 2015) with slight changes: feed rate was around 5 rpm, impact mill speed was 8000 rpm, airflow at 80 m³/h and a classifier wheel speed of 2500 rpm. FFSF was stored at 4°C for further use.

2.2.3 Mild fractionation process and pH adjustments

The mild fractionation process used in this study was based on previous research (Geerts et al., 2018) with additional modification for a pH adjustment step. Five different soy protein fractions (SPFs) were produced using a final step with pH varying from 3.5-7.5. An overview of all processing conditions can be found in Fig. 2-1. FFSF was firstly mixed with water (20% w/w). The pH of the mixture was adjusted between 8 and 9 with 1 M NaOH solution. Then, the suspension was stirred for 1 h to solubilize the proteins and, subsequently, centrifuged (18,670 *g*, 30 min, 25°C). After centrifugation, the supernatant was poured through a cheese-cloth to separate the semi-solid cream layer from the soluble proteins. The cream layer and pellet were discarded. The pH of the protein-rich supernatant was adjusted between 4.5 and 5 by adding 1 M HCl. The suspension was stirred for 1 h and subsequently centrifuged again (18,670 *g*, 30 min, 20°C). The protein-rich pellet was collected for further pH adjustments.

Each protein-rich pellet was mixed with water and stirred for at least one hour. The pH of the suspension was adjusted to various levels (3.5, 4.5, 5.5, 6.5 and 7.5) with 1 M HCl or 1 M NaOH, and was checked for every half an hour. Once the pH value became constant,

the suspensions were stirred overnight and subsequently freeze-dried (Christ, Germany). The freeze-dried SPFs were milled (Rotormill Pulverisette 14, Fritsch Germany) into powder using a sieve ring with a perforation size of 0.5 mm and a rotation speed of 6000 rpm. The obtained SPFs were stored in the refrigerator for further analysis. All the SPFs were prepared in triplicate.
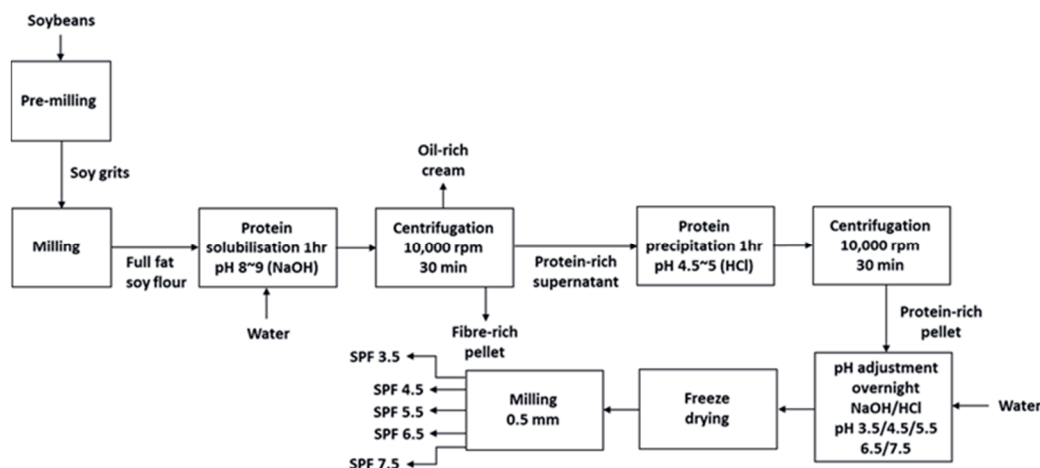

Fig. 2-1 Mild fractionation process and pH adjustment for all the SPF samples.

2.2.4 Composition analysis and yield

The protein content was determined by using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, the Netherlands) with a nitrogen-to-protein conversion factor of 5.7. The oil content was determined by using petroleum ether as an extraction solvent with a Buchi extraction system B-811LSV (Buchi Labortechnik AG, Flawil, Switzerland). The ash content was determined by AACC official method (AACC-International, 1999). The carbohydrate content was calculated as the difference between the dry matter content and the other components that were measured before. The protein yield was calculated according to Eq. (2.1). All the measurements were performed in triplicate.

$$Protein\ yield\ (\%) = \frac{Total\ protein\ in\ dried\ SPF}{Total\ protein\ in\ starting\ FFSF} \times 100\%\ Eq.(2.1)$$

2.2.5 Nitrogen solubility index (NSI) and water holding capacity (WHC)

The WHC-values of SPFs were determined using the method described by Geerts (Geerts et al. 2018) with slight modifications. For each SPF, a 2% (w/v) dispersion was placed in a centrifuge tube and shaken overnight. Then, the dispersion was centrifuged

(18,670 g, 30 min, 25°C) to separate the supernatant and pellet. The tubes were drained on tissue paper and the pellets were weighed. Subsequently, the pellets were oven-dried and weighed again. The nitrogen contents in the oven-dried pellets were measured by using Dumas analysis. The NSI was calculated by the ratio of soluble nitrogen over the total initial nitrogen content present in the SPFs. The WHC was calculated according to Eq. (2.2). All the measurements were performed in triplicates.

$$WHC\left(\frac{g\ water}{g\ dry\ SPF}\right) = \frac{M_{wet\ pellet} - M_{dry\ pellet}}{M_{dry\ SPF}}\ Eq.(2.2)$$

2.2.6 Microscopic analysis

Scanning electron microscope (SEM, Phenom Pure G2, Phenom-world BV, Eindhoven, The Netherlands) was performed for viewing the microstructures of the SPF powders with different processing pH. Each powder sample was evenly fixed on an aluminum sample holder by carbo tabs. In addition, 1% (w/v) protein dispersions were evaluated as well. For these measurements, an upright microscope Axioscope (Carl Zeiss Microscopy, LLC, USA) was used for the visualization. The protein dispersions were prepared by mixing different SPF powders with water for around 1 h under magnetic stirring. The images were captured by the connected video camera and acquisition software.

2.2.7 Particle size analysis

The particle size distribution of SPFs produced under different processing pH was measured with a laser light scattering instrument (Mastersizer 3000, Malvern Instruments Ltd, United Kingdom) and a wet module (Hydro SM, Malvern Instruments Ltd, United Kingdom). For these measurements, 1% (w/v) protein dispersions were prepared with Milli-Q water. A refractive index of 1.45 was used for the dispersion phase and 1.33 for the water continuous phase. All the measurements were performed in triplicates.

2.2.8 Denaturation behaviors

Differential scanning calorimetry (Diamond DSC, PerkinElmer, USA) was used to measure the denaturation temperature and the enthalpy of the transition of the SPF samples. Firstly the DSC was calibrated with indium, and an empty stainless pan was used as a reference. The SPF powders were mixed with water (20 g sample/100 g total). The samples were scanned from 20°C to 150°C with a heating rate of 5°C/min. Measurements were

analyzed with Start Pyris Software for denaturation temperature and enthalpy of transition. All the measurements were performed in triplicates.

2.2.9 Rheological behaviors

Two types of rheometers were used to analyze the rheological properties of SPF samples: stress-controlled rheometer (Anton Paar GmbH MCR502, Austria) and closed cavity rheometer (CCR, RPA elite, TA instrument, USA).

In the stress-controlled rheometer, a plate-plate geometry (PP-25/P2) was used to obtain the rheological properties of SPF dispersions. Each SPF powder was suspended in water (12 g sample/100 g total) and was stirred for 1 h before transferring to the rheometer. Then, the SPF dispersion was equilibrated for 5 min, and a shear rate sweep was performed at 25°C in steady-state with an increasing shear rate. The range of the shear rate was set from 1 to 100 s^{-1} (Berghout et al., 2014).

Based on previous research, a mixture of SPF with FFSF in a ratio 70/30 was prepared, and the SPF-FFSF mixture was added to the NaCl solution (1 wt.% NaCl in the total blend) to obtain a 44% dry matter (Dekkers et al., 2018; Geerts et al., 2018). The rheological properties of soy protein fraction-full fat soy flour (SPF-FFSF) blends were determined with a closed cavity rheometer (CCR). After the blends were hydrated in vacuum bags for 30 mins, approximately 5 g sample was placed between two plastic films and transferred to a CCR. An oscillation time sweep was performed with frequency 10 Hz, strain 80% for 15 mins under 140°C. In order to prevent water evaporation during measurement, a down pressure of 4.5 bar was used to close the CCR.

2.2.10 Statistical analysis

Data were collected for each sample from triplicate experiments or from more experiments in specified cases. IBM SPSS Statistics Version 23.0 was used to analyze the variance, and Duncan's test was performed to determine the statistical significance between samples at an α level of 0.05. All the results are displayed as mean values ± standard deviations.

2.3 Results and discussion

In this study, mildly fractionated SPFs were obtained with different processing pH values. The fractions were examined on their chemical composition, microstructure and functional properties. The connection between mild fractionation, processing pH value and functional properties of SPFs was analyzed, and the potential application of the produced SPFs was further discussed based on process parameters and properties.

2.3.1 Chemical composition

The chemical composition of all the SPFs was identical since the pH adjustment step was performed as the last step before drying. The results showed that mild fractionation process achieved SPFs with protein content around 85.3%, while the commercial SPI was 83.3% (Table 2-1). This was unexpected since the intensive washing steps during conventional fractionation were skipped in this study. The yield of mild fractionation process reached up to 55.8%, which was also higher than that reported yields of isolates produced from soy flour (30-40%) by the conventional processes (Alibhai et al., 2006; De Moura et al., 2011).

In terms of oil content, the start material, FFSF, contained around 21.8% of oil. During the mild fractionation process, after alkaline treatment, the centrifugation step was meant to separate protein-rich supernatant from the fiber-rich pellet, however the oil was separated as well because of the low density. Therefore, no extra defatting step was needed. 2.3% oil remained in SPFs while no residual oil was left in commercial SPI. Nevertheless, compared with organic solvent defatting step, mild fractionation can lead to soy protein ingredients with potentially clean labels and less environmental impact, which is of great interest in the industry currently.

Table 2-1 Composition of a number of different SPFs, commercial SPI, and full-fat soy flour (mean value ± standard deviation (n=3), dry basis).

	Protein (N × 5.7)	Oil	Ash	Carbohydrate	Protein yield (%)
Full-fat soy flour	37.0 ± 1.1	21.8 ± 0.4	6.7 ± 0.7	34.5 ± 2.2	
Commercial SPI	83.3 ± 0.7	0.0 ± 0.0	3.4 ± 0.0	13.3 ± 0.7	
SPFs	85.3 ± 6.2	2.3 ± 0.0	2.1 ± 0.1	10.3 ± 6.3	55.8 ± 6

2.3.2 Nitrogen solubility index (NSI) and water holding capacity (WHC)

Protein solubility and water holding capacity (WHC) are fundamental functional properties of soy protein ingredients and can be influenced by many processing parameters during fractionation, such as pH, salt content and drying methods (Kinsella, 1976). Among those, the processing pH can influence the protein's net charge and conformation, which could lead to the exposure or burial of the water binding sites of the proteins, thus to different properties regarding NSI and WHC.

In this study, dispersions of SPFs (2%, w/v) were used for the NSI and WHC measurements. The pH of the SPF dispersions was not adjusted further since it was quite close to the initial processing pH (results not shown). The plots of NSI and WHC values against the processing pH resulted in characteristic U-shaped curves (Fig. 2-2). The lowest values of NSI occurred at processing pH of 4.5 and 5.5, which is around the isoelectric pH (pI) of soy protein according to literature (Arrese et al., 1991). The NSI of SPF increased dramatically when adjusting the processing pH away from pI in both sides, for example, in the case of pH 6.5 or higher, or pH below 3.5. Specifically, SPF 7.5 showed the highest NSI (mean value 91.6%), which was even higher than the commercial SPI (mean value 62.2%) measured in this study.
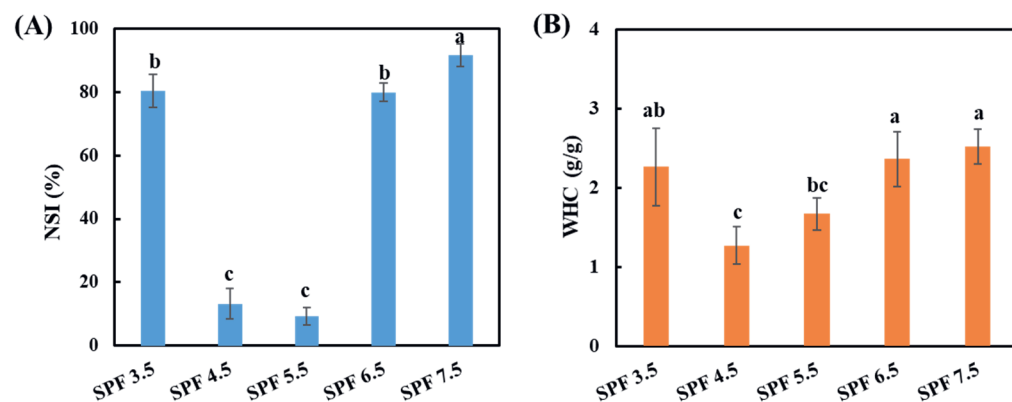

Fig. 2-2 *(A) nitrogen solubility index (NSI) and (B) water holding capacity (WHC) for SPFs with different processing pH. . *The values in the figure are compared and different top letters indicate a significant difference (P < 0.05).*

The WHC of the SPF 4.5 was the lowest as well, and the WHC increased when more basic or more acidic pH was used. This may be because the net charge on the soy protein is around zero when the processing pH is around the isoelectric region. At that point, the

protein-protein interactions are maximal, and less water binding sites are exposed (Idris et al., 2003). Subsequently, with an increase in the net charge of protein, the polarity of protein increases, resulting in an increase in the amount of bound water. Thus, compared with other SPFs, SPF 7.5 exhibited the maximum value of the WHC (mean value 2.52 g/g SPF), while higher WHC (mean value 9.17 g/g SPI) was detected from the commercial SPI.

To further investigate the effect of the fractionation process in combination with the pH adjustment to the NSI and WHC, commercial SPI was dissolved and its pH was adjusted prior to drying similarly to the SPFs. In this case, the reducing trends of both NSI and WHC were observed instead of typical U-shaped curves when the pH of SPI adjusted from 7.5 to 3.5 (App. 2-1). This argues that an extra pH adjustment step for commercial SPI could produce ingredients of varying specifications in terms of NSI and WHC, but different than preforming the pH adjustment during fractionation process. Moreover, this additional dissolution and drying steps also reduce the sustainability of the overall soy protein process and produced more salt in the ingredient, which is undesirable for future product development.

2.3.3 Microstructure and particle size distribution (PSD)

At the end of the mild fractionation process, all the SPFs were freeze-dried and milled into powder. The processing pH strongly influenced the microstructure of SPFs, as revealed by microscopy and particle size distribution analysis. Visible inspection of the powders revealed that SPF 4.5 and SPF 5.5 form finer, sand-like powders, while SPF 3.5, SPF 6.5 and SPF 7.5 resulted in more glassy-like flakes. The structural differences between SPFs became more obvious under SEM (Fig. 2-3). Glass-like pieces were observed in the SEM images of SPF 3.5, SPF 6.5 and SPF 7.5 while more blocky and spherical pieces were seen in SPF 4.5 and 5.5. These differences can be explained by protein net charge and freeze-drying process. When the protein net charge was low (processing pH around pI), most of the soy proteins were present in highly aggregated and even precipitate state and, thus, large particles were formed in the system and spherical structures were observed. In contrast, at pH far away from pI, most of the proteins would be completely solubilized in the suspensions, as a result, a continuous structure was formed and glass-like structures were observed after milling. The pH adjustment step resulted in these microstructural differences, as evidenced also from the SEM image of SPF 5.5 (Fig. 2-3C), because both spherical and glass-like shapes were found, indicating that transformation happened when the processing pH increased from

4.5 (Fig. 2-3B) to 6.5 (Fig. 2-3D). The glassy textures were also found in other freeze-dried plant protein materials such as mild-fractionated lupine protein isolate with neutralization step (Berghout et al., 2015).
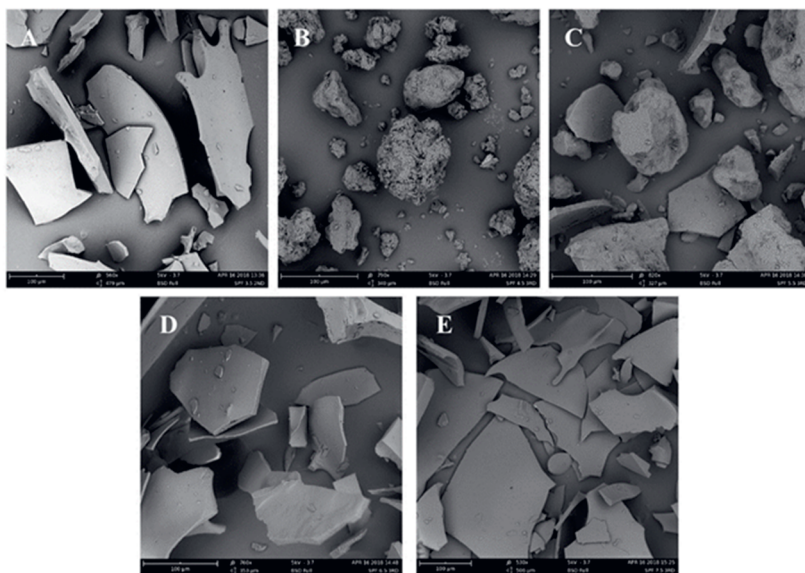

Fig. 2-3 *SEM images of freeze-dried (A) SPF 3.5, (B) SPF 4.5, (C) SPF 5.5, (D) SPF 6.5 and (E) SPF 7.5.*

The differences between SPFs due to pH adjustment were also observed after adding water. The microstructure and particle size distribution of 1% (w/v) SPF dispersions were shown in Fig. 2-4 and Fig. 2-5, respectively. The dispersions of SPF 4.5 and SPF 5.5 had larger particles under light microscopy, while no distinctive particles were detected in the images of SPF 3.5, SPF 6.5 and SPF 7.5. These differences can be further validated by the results of particle size distribution. SPF 3.5, SPF 6.5 and SPF 7.5 exhibited remarkably smaller particle size compared to SPF 4.5 and SPF 5.5, and also smaller than all the commercial SPI with adjusted pH (App. 2-2). Moreover, as for commercial SPI with adjusted pH, we saw that the particle size of all the SPI dispersions did not change despite the pH difference and the lower solubility exhibited. Previous research reported that the free surface of the protein increases by reducing the size of particles, which further affects the protein functionalities such as solubility, turbidity, heat stability and gelation (Jambrak et al., 2014; Song et al., 2013). Therefore, pH adjustment during mild fractionation process has a significant influence on the particle size and morphology, leading to more variations in the properties.
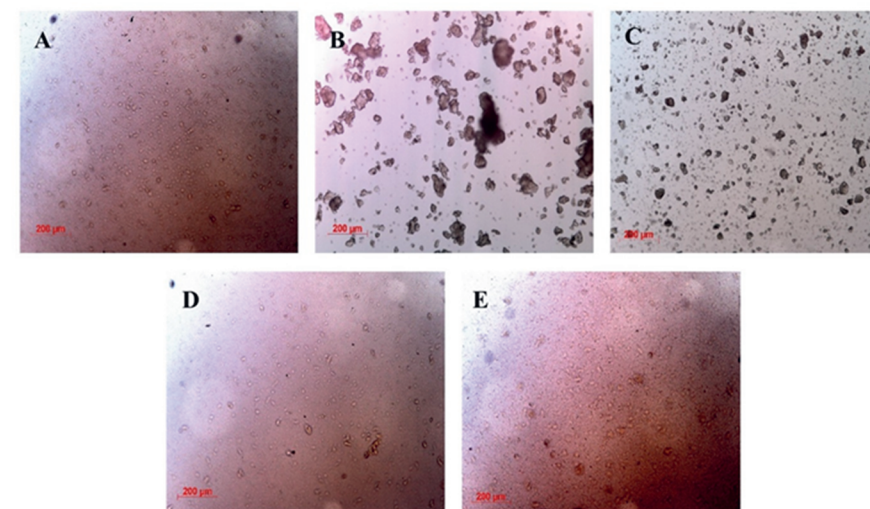

Fig. 2-4 *Light Microscopy images of (A) SPF 3.5, (B) SPF 4.5, (C) SPF 5.5, (D) SPF 6.5 and (E) SPF 7.5 dispersions. The scale bar correspond to 200 μm.*
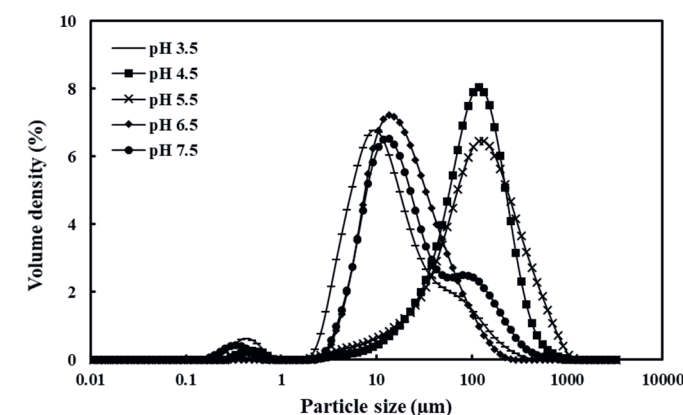

Fig. 2-5 *Particle size distributions of SPFs with different processing pH.*

2.3.4 Denaturation behaviors

The denaturation temperature (T_d) of SPFs and enthalpy of transition were summarized in Table 2-2. Two separate peaks were identified in the DSC measurements for all the SPFs, which corresponded to the denaturation behaviors of β-conglycinin (7S) and globulins glycinin (11S), respectively (Mujoo et al., 2003). On the contrary, no peak was detected from commercial SPI. This means mildly fractionated SPFs still kept their native state while commercial SPI was fully denatured after processing.

Despite the native state, different processing pH led to some differences in the denaturation behaviors of SPFs. SPF 3.5 had the lowest T_d with both 7S (mean value 60.02°C) and 11S proteins (mean value 81.75°C), which indicated that SPF 3.5 was the least thermal stable protein fraction as compared with other SPFs. For 7 S protein, T_d of SPF increased with the increase of processing pH. Hence, the highest T_d occurred with SPF 7.5 (mean value 75.60°C). This might be explained by the lower molecule weight and higher flexibility in the structure of 7S protein compared with 11S protein; the structure of 7S protein is easier to be influenced by the net charge distribution. Previous research also found that increasing the pH from 3.8 to 7.6 caused the denaturation temperature of β-conglycinin shift to higher values (Renkema et al., 2000). However, as for 11S protein, the highest T_d showed up with SPF 5.5 (mean value 95.22°C) while the processing pH was closer to pI. This was also in line with the result reported previously that soy glycinin was found to be most stable against denaturation at a pH of 5, because hydrophobic interactions that favor the folded state of 11S protein are weakened due to a low net charge, protecting the protein against denaturation (Hermansson, 1986, 1978). Moreover, the denaturation behaviors of SPFs after pH adjustment step are considered to be reversible, on the contrary to the adjustment of pH in commercial SPI (results not shown). However, any additional pH adjustment step introduces more salt in the system, which influences the ionic strength and thus the structure of soy protein (Jiang et al., 2015).

Overall, the mild fractionation process did not result in full denaturation of soy protein, but pH adjustment step during process led to different denaturation behaviors for SPFs, which could further link to the changes of protein functionalities.

Table 2-2 The denaturation temperature and enthalpy of transition of soy protein fractions processed at different pH, and commercial SPI (ND=not detected).

	7S T_d (°C)	Enthalpy (J/g)	11S T_d (°C)	Enthalpy (J/g)
SPF 3.5	60.02 ± 0.27 [c]	1.35 ± 0.64 [a]	81.75 ± 0.31 [e]	2.64 ± 0.29 [c]
SPF 4.5	68.73 ± 0.96 [b]	0.41 ± 0.06 [b]	92.96 ± 0.49 [c]	4.51 ± 1.47 [b]
SPF 5.5	68.77 ± 0.52 [b]	1.11 ± 0.23 [a]	95.22 ± 0.29 [a]	6.51 ± 0.64 [a]
SPF 6.5	73.11 ± 3.77 [a]	1.41 ± 0.23 [a]	93.60 ± 0.44 [b]	5.39 ± 0.94 [ab]
SPF 7.5	75.60 ± 0.07 [a]	1.29 ± 0.29 [a]	91.88 ± 0.25 [d]	6.92 ± 0.76 [a]
Commercial SPI	ND	ND	ND	ND

The values in the table are compared in columns and different letters indicate a significant difference (P < 0.05).

2.3.5 Rheological behaviors

It is known that structure formation in food materials is influenced by the ingredient properties and processing conditions (van der Goot et al., 2008). For better soy-based product development, it is important to understand the food structure formed. In this study, the rheological behaviors of SPFs with different concentrations were determined by a stress-controlled rheometer and a closed cavity rheometer (CCR), which can provide information on the structuring potential of SPFs for different food applications.
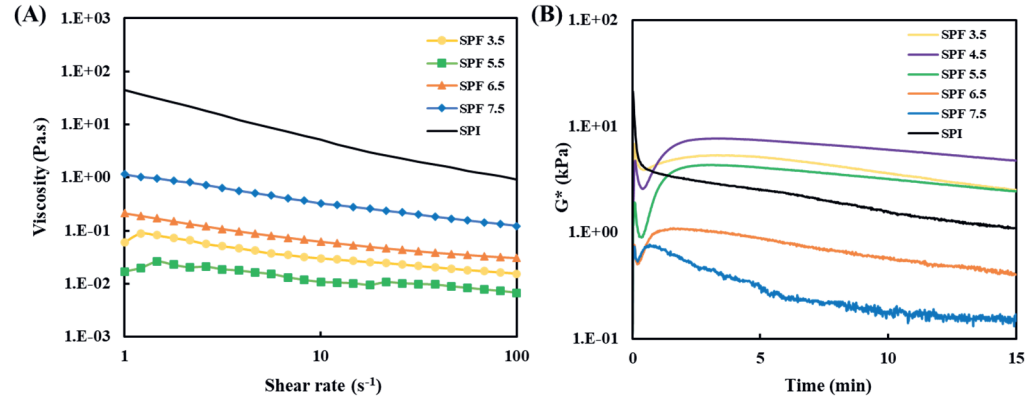
Fig. 2-6 (A) viscosity measured as a function of shear rate and (B) complex modulus (G*) measured as a function of time at 140°C of SPFs processed at different pH.

Soy beverages such as soymilk smoothie and soy yogurt are novel soy-based products that developed as the alternatives to dairy drinks. For this liquid-like applications, the rheological properties of 12 wt.% SPF dispersions were analyzed in this study by a stress-controlled rheometer with shear rate sweep (Fig. 2-6A). Most SPFs were possible to be measured expect for SPF 4.5 due to its low solubility and limitation in its hydration process. Beyond that, all the examined SPF dispersions showed similarities in behavior especially with respect to typical shear thinning behavior; upon increasing shear rate from 1 to 100 s^{-1} the viscosity values of SPF dispersions decreased. Fig. 2-6A shows that, among the SPFs, SPF 7.5 had the highest viscosity, followed by SPF 6.5, SPF 3.5 and SPF 5.5 during the shear sweep measurements. However, when compared to commercial SPI dispersions, the latter had a much higher viscosity and a stronger shear thinning behavior than all the SPF dispersions at similar protein concentrations. This means more SPF can be added into

products than SPI to achieve certain level of viscosity, bringing more potentials for SPF to develop high-protein soy drinks.

Soy-based meat analogue products are made by soy protein ingredients with an aim to mimic specific types of meat, including protein content, taste and structure as well. The creation of a fibrous structure from mildly fractionated SPFs, that could be used as a basis for meat analogue products has been reported earlier (Geerts et al., 2018). In that study, a specific blend recipe (SPF/FFSF, 70/30) was considered a successful recipe, since it exhibited certain rheological properties and lead to desired fibrous structure (Geerts et al., 2018). Therefore, similar rheological experiments were implemented to evaluate the properties of SPF blends with different processing pH. A high frequency and high strain treatment were performed under 140°C and the complex modulus (G*) was used to describe the entire rheological behavior of the SPF/FFSF blends (Fig. 2-6B). Similar trends were detected in the G* curve of all the SPF/FFSF blends, an apparent valley showed up in the first 2~3 min followed by a retarded steady-state creep. However, different processing pH also caused some variations in the curve, including the valleys and the G* values. For the valleys, it is hypothesized that the denaturation behavior of SPF/FFSF blends might be responsible for the appearances since no valley was detected in the commercial SPI. This hypothesis was also particularly evident by the results of DSC (Table 2-2). SPF 3.5 had the lowest T_d for both 7 S and 11S protein, while it also showed the smallest valley in the G* curve. For the G* values, SPF 4.5/FFSF blend and SPF 5.5/FFSF had higher G* while the SPF 7.5/FFSF blend had the lowest one, which indicated that SPFs showed more potentials for structure formation when the processing pH was close to pI.

To sum up, pH adjustment during the mild fractionation process can lead to a range of soy protein products with different rheological properties, which can be used in tailor-made applications according to the requirements.

2.4 Conclusion

The demand of novel soy-protein products is increasing, and also the need for sustainable soy-based ingredients. Therefore, the application of a mild fractionation process and pH adjustment was explored in this study. Mildly fractionated SPFs showed higher protein and oil content as compared to commercial SPI. Proteins were still in their native state after processing. The processing pH altered the functionality of the SPFs and two clusters could be distinguished; one close and one away from the pI. SPF 4.5 and SPF 5.5, processed around isoelectric point, formed a powdery texture with lower NSI, WHC and viscosity. However, in combination with FFSF, they presented higher G* value as compared to other SPFs under high temperature, which brought more potentials for structure formation. SPFs processed away from pI in both sides, presented increased NSI, WHC, and viscosity, but decreased particle size and G* value. A glass-like microstructure was also observed. Recognizing the difference this pH adjustment step can have on the functionality of the SPFs, it is recommended to adjust the mild fractionation based on the requirement on the functional properties of multiple soy-based products. Forthcoming studies could up-scale the quantities of SPFs and work with specific applications such as structure formation for meat analogue.

Appendix
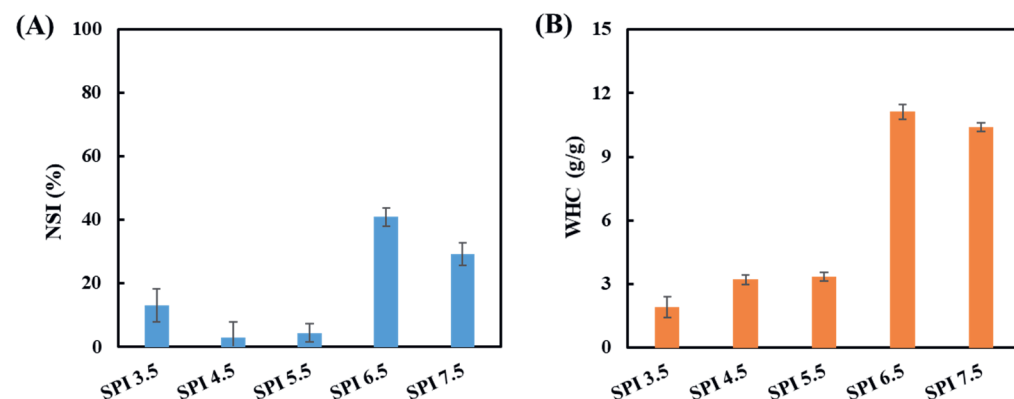
App. 2-1 *(A) nitrogen solubility index (NSI) and (B) water holding capacity (WHC) for commercial SPI with adjusted pH.*
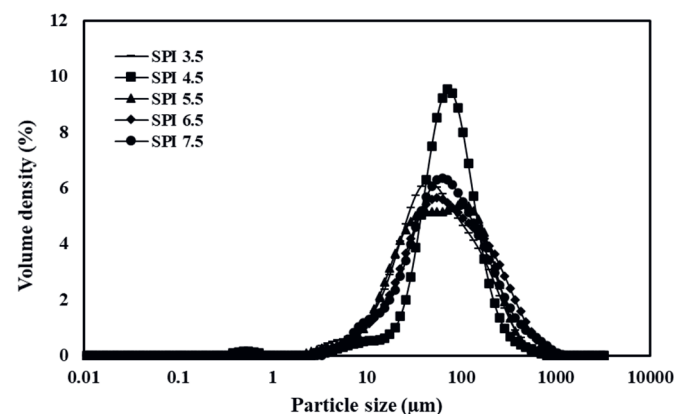
App. 2-2 *Particle size distributions of commercial SPI with adjusted pH.*

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Chapter 3

Isochoric moisture heating as a tool to control the functionality of soy protein

Abstract

In this study, we investigated the effect of isochoric moisture heating on the functionalities of mildly fractionated soy protein. Soy protein-rich fractions (SPFs) were produced through a mild fractionation process, in which the defatting and intensive washing steps were omitted. After a neutralization step, the protein-rich dispersions were heated up to various temperatures ranging from 60-100°C and subsequently freeze-dried. It was found that the denaturation level, the water holding capacity and the viscosity of SPFs increased as the processing temperature rose, whereas the solubility of SPFs decreased. Additionally, among the different conditions, an intermediate heating temperature of 70°C resulted in SPFs having a gel network with the highest elasticity. Overall, isochoric moisture heating coupled to simplified fractionation can be used to precisely control the functionality of soy protein ingredients with mild heating conditions. This work provides insights on the production of ingredients with desired functionality for novel soy-based applications like soy meat analogues.

3.1 Introduction

Currently, diets containing more plant protein are stimulated due to reasons of environmental concerns, vegetarianism trends and increased consumer acceptance of plant-based products (Pojić et al., 2018). These changed diet habits lead to a higher demand for plant-based ingredients with the proper functionality to make new products. To meet the demands, multiple novel technologies have been explored to improve the yield, functional and nutritional properties of plant-protein ingredients (Liu, Gasmalla, Li, & Yang, 2016). Conventional aqueous fractionation based on alkaline solubilization and isoelectric precipitation is the industrialized and widely applied process for soy protein production because the process provides highly purified protein isolate at a low price (Alibhai et al., 2006; Nazareth et al., 2009). There is a growing interest to develop simplified/mild aqueous fractionation to avoid the use of organic solvents (especially during the oil extraction step) and intensive washing steps (Berghout, Boom, & van der Goot, 2014; Campbell et al., 2011; Peng, Dewi, Kyriakopoulou, & van der Goot, 2020). Therefore, simplified fractionation can be rated as applicable, effective and potentially as a more sustainable alternative enabling solvent-free label status (Geerts, van Veghel, Zisopoulos, van der Padt, & van der Goot, 2018; Nazir, Asghar, & Aslam Maan, 2016).

Soy-based ingredients are getting a lot of attention, because of their potential to be used in many novel products, such as meat analogues (Kyriakopoulou et al., 2021). Traditionally, high solubility of soy-based ingredients is considered as the most important functionality for food and industrial applications (Wang and Johnson, 2001). However, new generations of soy-based products require different ingredients functionalities. For example, the water holding capacity (WHC) of soy protein gels is a significant parameter for soy-based yogurt manufacturing, because it is related to syneresis and water may be lost due to passive diffusion after extensive storage time (Cruz et al., 2009; Kovalenko and Briggs, 2002). For soy cheese analogues, high water retention properties coupled with increased viscoelasticity improve the quality attributes of final products (Jeewanthi and Paik, 2018). In the case of meat analogue products, soy protein ingredients are desired to have intermediate solubility, superior absorption of water and oil, and to form a meat-like fibrous structure (Geerts et al., 2018; Jones, 2016).

Heating is a commonly used method to alter the functionalities of protein, and it can be introduced with different treatments, such as boiling, autoclaving, ultra-heating and toasting (Hickisch et al., 2016; Obatolu et al., 2007; Zhang et al., 2020). Heating can be applied in different stages during the protein fractionation process as well. For example, the starting materials (soybean or full-fat soy flour) can be toasted before the protein extraction, the dispersions can be heated during extraction, or the obtained fractions can be processed after extraction (Alavi et al., 2021; Das et al., 2006; Deak and Johnson, 2007). However, heating before or during the protein extraction stage has an impact on the purity, yield and functionality of the obtained protein fractions. Deak & Johnson (2007) heated the soy protein during the solubilization step and found that soy protein isolates extracted at 80°C had a lower yield and purity as compared to room temperature extraction. Therefore, to modify the functionalities of protein, but minimize the influences on the purity and yield, it is suggested to perform the heating step after the protein extraction stages, before or after the final drying process.

Our previous study observed that mildly fractionated SPF presents high solubility and low WHC (Peng et al., 2020b). Generally, those properties are highly desired for functionalities like foaming and emulsification but are less desired for meat analogue-like applications (Moure et al., 2006). WHC and gelling properties are more important. Unfortunately, the WHC-value of SPF is lower than commercial SPI and SPC reported in the literature (Geerts et al., 2018), which can be attributed to differences in drying methods. Freeze drying was performed for the production of SPF while spray drying was applied industrially. Spray drying probably leads to higher WHC due to the denaturation caused by the heat applied to the soy ingredients , and limited impacts can be observed for protein isolates on the functional behaviors before and after freeze-drying (Berghout et al., 2015). To modify the functionalities of protein-rich fraction, toasting is a heating process performed in a dry state after the drying step. Toasting treatment requires a high temperature (up to 150°C) to change the functional properties of the protein in the desired direction. However, this severe heating could induce additional protein changes, which are less favorable, such as color formation (Bühler, Dekkers, Bruins, & van der Goot, 2020; Geerts et al., 2018). In this study, moisture heating treatment was explored with milder temperature (60-100°C) after the protein neutralization step and before the freeze-drying. The heating treatment is relatively easy to apply in the industry, while no extra step was required after the drying and the heating.

The novelty of our approach lies in the introduction of a mild heating step in a simplified fractionation process. It will be investigated how, the functionality of the ingredients can be tuned through physical means, as an alternative for chemical modification or the use of additional ingredients. The objective of this research is therefore to better understand how moisture heating influences the functional behavior such as protein solubility, viscosity and gelling properties of simply fractionated SPF. It is expected that the results of this study can aid in the development of soy protein ingredients processed under mild conditions, thereby expanding the current market of novel soy-based products for sustainable diets.

3.2 Methods and methods

3.2.1 Materials

Dry, full soybean was obtained from FRANK Food Products (the Netherlands) and was harvested in Canada in October/November of 2016. The HCl and NaOH used for pH adjustments during fractionation were purchased from Sigma Aldrich (Germany). The water used was purified in a Milli-Q Lab Water System (Milli-Q IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany).

3.2.2 Soy flour preparation

Firstly, soybeans were pre-milled by using a pin mill LV 15M (Condux-Werk, Wolfgang bei Hanau, Germany) into grits. Then, the soy grits were further milled by a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) into full-fat soy flour (FFSF). The impact mill was set according to the parameters described by Pelgrom (Pelgrom et al., 2015) with slight changes: feed rate was around 5 rpm, impact mill speed was 8000 rpm, airflow at 80 m³/h and a classifier wheel speed of 2500 rpm. Full-fat soy flour was stored at 4°C for further use.

3.2.3 Mild fractionation process and isochoric moisture heating

The mild fractionation process performed in this study is presented in Fig. 3-1. In more detail, FFSF was used as the starting material that was mixed with water in a ratio of 1:5 (w/w). The dispersions were adjusted to pH 8.5-9 by adding 1 M NaOH solution. The dispersion was stirred for 1 h and centrifuged at 18,670 *g* for 30 min. After centrifugation, the semi-solid oil-rich cream layer, the protein-rich supernatant and the fiber-rich pellet can be easily separated by a cheese-cloth. The protein-rich supernatant was further purified by isoelectric precipitation at pH 4.5-5 with 1 M HCl, stirred for 1 h and then centrifuged (18,670 *g*, 30 min). The protein-rich pellet was then mixed with water and neutralized at pH 6.5~7. Once the pH value became constant, the dispersion was transferred to a closed flask to avoid water evaporation, and subsequently heated on a heating plate to a set temperature (60, 70, 80, 90 and 100°C), where it remained for 30 min. A reference sample was prepared without any moisture heating. The abbreviations of the obtained soy protein fractions were referred in this study as SPF WH (without heating), SPF 60, SPF 70, SPF 80, SPF 90 and SPF 100. All the soy protein dispersions were cooled to room temperature before the flask was opened.

After that, the soy protein dispersions were freeze-dried (Freeze dryer, Epsilon 2-6D, Martin Christ, Germany) and milled (Rotormill Pulverisette 14, Fritsch, Germany) into powder by using a sieve ring with a perforation size of 0.5 mm and a rotation speed of 11,202 *g*. The obtained SPFs were stored in a cooling room (4°C) till further analysis. Each procedure was performed in duplicate, and all the mass balances were recorded for the yield calculations.
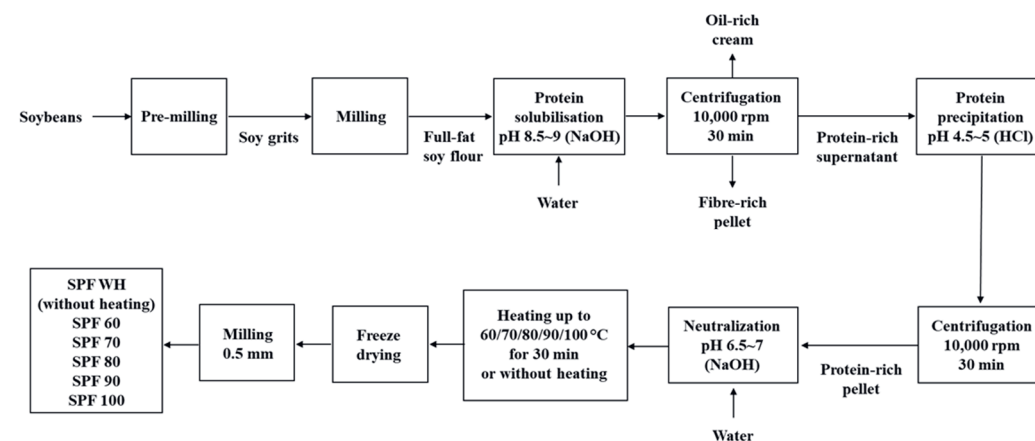

Fig. 3-1 Mild fractionation process and isochoric moisture heating for all the SPF samples.

3.2.4 Composition and yield analysis

The protein content of the FFSF and SPFs was determined by using a Nitrogen Analyzer (Flash EA 1112 series Dumas, Thermo Scientific, The Netherlands). A nitrogen-to-protein conversion factor of 5.7 was used (Krul, 2019). The oil content was measured through the Soxhlet extraction procedure (B-811 Buchi Extractor, Buchi, Switzerland). In short, the petroleum ether was heated up and evaporated, after which the vapor was condensed and dropped into the chamber with the sample inside. Subsequently, the oil in the sample was extracted, collected and measured. All measurements were performed in triplicate. The dry matter yield was calculated using Eq.(3.1), while the protein yield was calculated according to Eq. (3.2):

$$Dry\ matter\ yield\ (\%) = \frac{Weight\ of\ dried\ SPF}{Weight\ of\ starting\ FFSF} \times 100\% \quad Eq.(3.1)$$

$$Protein\ yield\ (\%) = \frac{Total\ protein\ in\ dried\ SPF}{Total\ protein\ in\ starting\ FFSF} \times 100\% \quad Eq.(3.2)$$

3.2.5 Color analysis

The color was measured using a Chroma Meter (CR-400, Konica Minolta, USA) in terms of *L** (lightness), *a** (redness and greenness) and *b** (yellowness and blueness). The instrument was calibrated with a standard black and white tile followed by measurement of samples. A glass cell containing the SPF was placed above the light source and *L**, *a** and *b** values were recorded. Color measurements were taken in triplicates.

3.2.6 Denaturation behaviors

Differential scanning calorimetry (Diamond DSC, PerkinElmer, USA) was used to measure the denaturation temperature and the enthalpy of the transition of the SPF samples. Firstly the DSC was calibrated with indium, and an empty stainless pan was used as a reference. The SPF powders were mixed with water (20 g sample/100 g total). The samples were scanned from 20°C to 150°C with a heating rate of 5°C/min. Measurements were analyzed with Start Pyris Software for denaturation temperature and enthalpy of transition. All the measurements were performed in triplicates.

3.2.7 Microscopic analysis

Scanning electron microscope (SEM, Phenom Pure G2, Phenom-world BV, Eindhoven, The Netherlands) was performed for viewing the microstructures of the SPF powders. Each SPF was evenly fixed with double-sided adhesive conductive carbon tabs on an aluminum sample holder. The accelerating voltage was 5 kV.

3.2.8 Particle size analysis

A 1% (w/v) dispersion of each SPF sample was prepared with Milli-Q water and divided into two parts: one part was stirred under room temperature for 1 h, and one part was heated at 95°C for 30 min. The particle size distribution was determined by a static laser light scattering (Malvern Master Sizer 3000, Malvern Instruments, UK) with a wet module. To determine the size of small particles (0.5nm - 5μm) in the dispersions, another 1% (w/v) dispersion of each SPF sample was well mixed with Milli-Q water and centrifuged at 2,000 *g* for 10 min. After centrifugation, the supernatant was collected and divided into two parts: one part was measured directly by a Zetasizer (Zetasizer Ultra, Malvern Instruments, UK), while the other part was heated at 95°C for 30 min, and subsequently cooled down and

measured by the Zetasizer. A refractive index of 1.45 was used for the dispersion phase and 1.33 for the water continuous phase. All the measurements were performed in triplicate.

3.2.9 Nitrogen solubility index (NSI)

The nitrogen solubility index (NSI) is routinely used to evaluate protein solubility (Wang and Johnson, 2001). A 2% (w/w) dispersion of each SPF sample was placed in a centrifuge tube and moderately shaken overnight. Subsequently, the sample was centrifuged at 18,670 *g* for 30 min to separate the supernatant and pellet. The pellet was oven-dried at 105°C and weighed. The nitrogen contents in the oven-dried pellets were measured by using Dumas analysis. The NSI was calculated by the ratio of soluble nitrogen over the total initial nitrogen content present in the SPFs. All the measurements were performed in triplicate.

3.2.10 Water holding capacity (WHC) and oil absorption capacity (OAC)

The water holding capacity (WHC) of SPFs was measured using the method described by Peters (Peters et al., 2017) with slight modification. A 2% (w/v) SPF dispersion was prepared with Milli-Q water and shaken overnight. Then, the dispersion was centrifuged at 18,670 *g* for 30 min to separate the supernatant and the wet pellet. The wet pellet was collected and weighed. Subsequently, the wet pellet was oven-dried overnight at 105°C and weighed again to determine the mass of the dry pellet. The WHC was calculated according to Eq. (3.3), and the WHC of the pellet (WHC$_P$) was determined according to Eq. (3.4).

$$WHC\left(\frac{g\ water}{g\ dry\ SPF}\right) = \frac{M_{wet\ pellet} - M_{dry\ pellet}}{M_{dry\ SPF}} \quad Eq.(3.3)$$

$$WHC_P\left(\frac{g\ water}{g\ dry\ pellet}\right) = \frac{M_{wet\ pellet} - M_{dry\ pellet}}{M_{dry\ pellet}} \quad Eq.(3.4)$$

The oil absorption capacity (OAC) of SPFs was measured according to a previously described method (Lin et al., 1974) with minor modifications. Around 0.5 g of the SPF and 10.0 ml of sunflower oil were added to a centrifuge tube, and mixed for 3 min with a vortex mixer to disperse the sample into the oil. After a holding period of 30 min, the tube was centrifuged for 25 min at 3050 *g*. The separated oil was then removed with a pipette, and the tube was inverted for 25 min to drain the oil before reweighing. The OAC was expressed according to Eq. (3.5). All the measurements were performed in triplicate.

$$OAC\left(\frac{g\ oil}{g\ dry\ SPF}\right) = \frac{M_{pellet} - M_{dry\ SPF}}{M_{dry\ SPF}} \quad Eq.(3.5)$$

3.2.11 Viscosity analysis

The viscosity of the SPFs at different concentrations (5-30%, dry basis) was analyzed by a rheometer (Anton Paar Physica MCR301, Graz, Austria) with a concentric cylinder (CC-17) or a cone-plate geometry (CP-20-2). The samples were equilibrated for 5 min. Viscosity was determined at 25°C using a shear rate range from 1 to 100 s^{-1}. The viscosity at a shear rate of 28 s^{-1} was used to quantify the effect of SPF concentration. The lowest shear rate reported corresponded with the shear rate at which the torque fell within the minimum torque of the rheometer to measure accurately. All the measurements were performed in triplicate.

3.2.12 Gelling behavior analysis

Oscillatory rheological measurements of SPF samples were carried out using a rheometer (Anton Paar Physica MCR301, Graz, Austria) with a plate-plate geometry (PP-50). SPF with 15 wt.% concentration was selected due to their consistency and requirements for gel formation. Samples were prepared and kept for 30 min before transferring to the rheometer.

Strain sweep experiments (0.01-100%) were performed at a constant frequency of 1 Hz to determine the limit of the linear viscoelastic region (LVR) of each SPF. Temperature and frequency sweeps were carried out afterward within the determined range. A thin layer of high-temperature-resistant silicone oil was covered on top of the samples to prevent water evaporation during heating. After an initial equilibration of samples at 40°C for 3 min, ramp heating occurred at 2°C/min to an endpoint of 95°C at a strain of 1% and frequency of 1 Hz. The samples were then immediately cooled back to 25°C at a rate of 5°C/min and equilibrated for 5 min. Subsequently, a frequency sweep was carried out from 0.01 to 10 Hz at a strain of 1%. All the rheological measurements were carried out in triplicate.

3.2.13 Statistical analysis

Data were collected from triplicate experiments or in specified cases from more experiments. IBM SPSS Statistics Version 23.0 was used to analyze the variance, and Duncan's test was performed to determine the statistical significance between samples at an α level of 0.05. All the results were displayed by mean values ± standard deviations.

3.3 Results

3.3.1 Composition, yield and color analysis

In this study, the protein and oil content of all the SPFs were quite similar since the isochoric moisture heating step was carried out after all the separation steps before freeze-drying (Table 3-1). The mild fractionation process resulted in protein-rich fractions with approximately 75% protein and 2% oil (dry basis). The product yield was an average of 27% while the protein yield reached up to 56%. These results were in line with the yields reported by our previous research (Peng et al., 2020a). All the SPFs obtained in this study presented lower protein content and higher oil content than commercial SPI, as commercial SPI contains around 83.3% of protein and 0% oil. This is due to the simplification of the fractionation processes compared to the conventional wet procedure. However, the protein yield of SPFs (55.7 ± 2.8%) was higher than that reported yields of lab-produced SPI (30-50%) (Jung et al., 2006; L'hocine et al., 2006).

SPFs processed at different heating temperatures showed changes in their color values (Table 3-1). Visually, all the SPFs were yellowish, but with increased heating temperature from 60 to 100°C, the color parameter $L*$ of SPF decreased from 79.0 to 72.3. No regular pattern was observed in the color values of $a*$ and $b*$ when changing temperature.

Table 3-1 *Protein, oil content and color analysis of FFSF, commercial SPI and all the SPFs in dry basis.*

Sample names	Protein, % (N × 5.7)	Oil, %	$L*$	$a*$	$b*$
FFSF	36.8 ± 1.3 [e]	21.9 ± 0.1 [a]	-	-	-
Commercial SPI	83.3 ± 0.7 [a]	0.0 ± 0.0 [d]	-	-	-
SPF WH	75.0 ± 0.4 [c]	2.6 ± 0.7 [b]	80.2 ± 0.2 [a]	1.5 ± 0.0 [d]	20.4 ± 0.2 [bc]
SPF 60	77.8 ± 0.7 [b]	2.4 ± 0.6 [b]	79.0 ± 0.4 [b]	2.3 ± 0.1 [b]	20.6 ± 0.3 [abc]
SPF 70	75.0 ± 1.4 [c]	1.5 ± 0.2 [c]	76.5 ± 0.2 [c]	2.5 ± 0.0 [a]	21.1 ± 0.1 [a]
SPF 80	73.2 ± 0.4 [d]	2.0 ± 0.3 [bc]	78.5 ± 0.2 [b]	1.9 ± 0.1 [c]	19.5 ± 0.5 [d]
SPF 90	75.9 ± 1.1 [c]	2.6 ± 0.4 [b]	75.4 ± 0.2 [d]	2.3 ± 0.1 [b]	20.7 ± 0.4 [ab]
SPF 100	75.1 ± 1.0 [c]	2.4 ± 0.1 [b]	72.3 ± 0.8 [e]	1.8 ± 0.2 [c]	20.1 ± 0.3 [cd]

The values in the table are compared in columns and different letters indicate a significant difference (P < 0.05).

3.3.2 Denaturation behaviors

DSC analysis was performed to assess the effect of moisture heating on SPF denaturation levels (Table 3-2). SPF WH and SPF 60 exhibited two heat absorption peaks, though SPF WH had a higher enthalpy value of 7S than SPF 60. The results obtained in this study were in line with the previously reported data on the peak denaturation temperatures of 7S (68.0 to 79.4°C) and 11S (88.0 to 96.4°C) (Damodaran, 1988; Guo et al., 2012). Only one peak was detected in SPF 70 and SPF 80, suggesting that the 7S was fully denatured when the moisture heating temperature was 70°C or higher. Also, here, the enthalpy of denaturation of the remaining peak became lower when the sample was treated at a higher temperature. No peaks were identified anymore when measuring SPF 90 and SPF 100, meaning that all protein was fully denatured. Thus, SPFs were gradually denatured with the increase of the moisture heating temperature.

Table 3-2 *The denaturation temperature and enthalpy of transition of all the SPFs (ND =not detected).*

Sample names	7S T_d (°C)	Enthalpy (J/ g)	11S T_d (°C)	Enthalpy (J/ g)
SPF WH	71.25 ± 0.99 [b]	1.31 ± 0.55 [a]	94.52 ± 0.81 [b]	3.22 ± 0.71 [ab]
SPF 60	72.82 ± 0.75 [a]	0.65 ± 0.17 [b]	93.57 ± 0.06 [c]	3.54 ± 0.79 [ab]
SPF 70	ND	ND	95.05 ± 0.83 [b]	4.15 ± 1.00 [a]
SPF 80	ND	ND	96.07 ± 0.25 [a]	2.87 ± 0.25 [c]
SPF 90	ND	ND	ND	ND
SPF 100	ND	ND	ND	ND

*The values in the table are compared in columns and different letters indicate a significant difference (P < 0.05).

3.3.3 Microstructure and particle size distribution (PSD)

All the SPF powders were obtained by freeze-drying followed by milling, using a sieve ring with a perforation size of 0.5 mm. The application of the same procedure to all samples is probably the reason that similar powder particle sizes were obtained. SEM images revealed further that moisture heating treatment played a minor role in the powder morphology of SPF, as can be seen from Fig. 3-2. Irregular and compact flakes, surrounded by small fragments were observed for all the SPFs. These are typical particle structures obtained after freeze-drying for proteins (Dehnad et al., 2016; Zhao et al., 2013). In contrast,

commercially available SPI and soymilk powder are mainly processed with spray drying and present individual particles with spherical shapes (Porras-Saavedra et al., 2015).
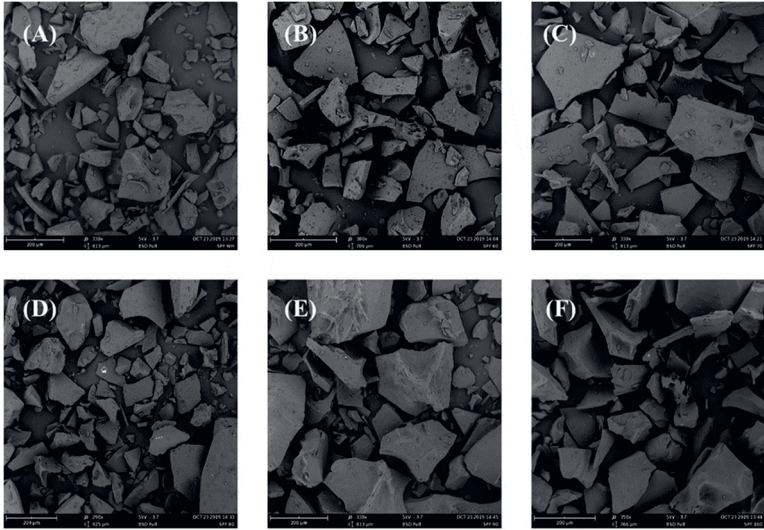

Fig. 3-2 *SEM images of freeze dried (A) SPF WH, (B) SPF 60, (C) SPF 70, (D) SPF 80, (E) SPF 90 and (F) SPF 100. The scale bar is 200 µm.*
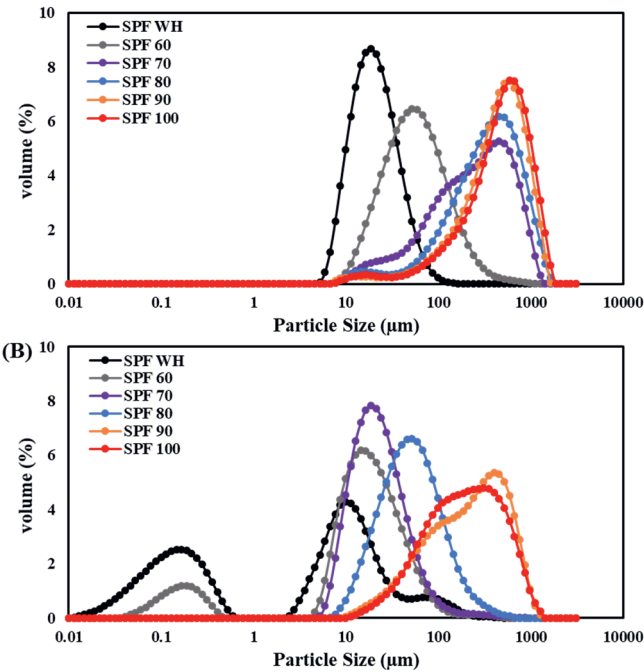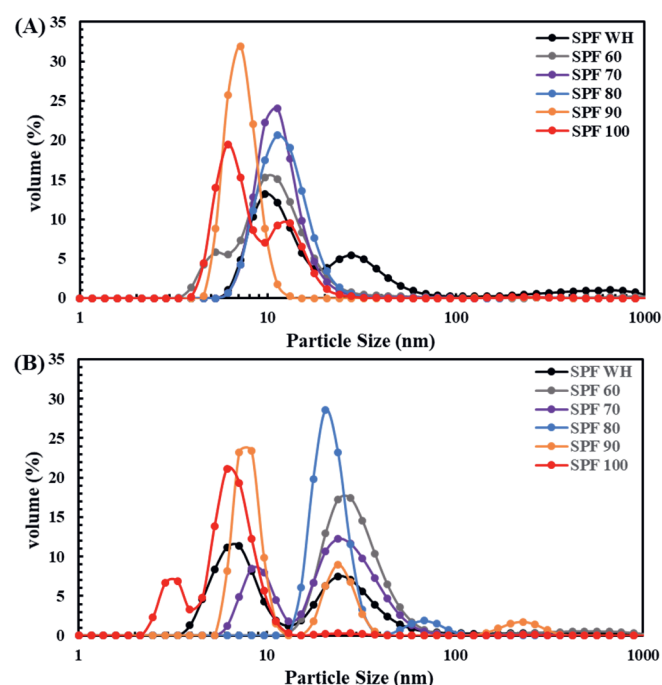

Fig. 3-3 *Particle size distribution (0.01-10,000 µm) of all the SPFs (A) mixed and stirred after 1 h and (B) heated under 95°C for 30 min.*

Differences between SPFs became clear when dispersing the powders in water for 1 h. The dispersion of SPF 100 exhibited the largest particle size while the SPF WH the smallest. The particle size of SPF dispersion increased with an increase in the moisture heating temperature applied during fractionation (Fig. 3-3A). After additional heating of the SPF dispersions to 95 °C for 30 min (Fig. 3-3B), the particles became smaller for all the SPF dispersions. However, this heating step did not alter the size sequence between samples. The dispersion of SPF 100 still presented the largest particle size among all the SPF dispersions. In addition, both SPF WH and SPF 60 showed a bimodal distribution in Fig. 3B. SPF WH presented a small fraction of particles ranging from 0.01 to 0.6 μm and a large volume percentage with particles from 2.4 to 454 μm, while for SPF 60, the ranges for two peaks were 0.04 to 0.46 μm and 4.58 to 516 μm respectively.

After centrifugation, as observed from Fig. 3-4, the particles left in the supernatant of SPF WH showed the largest size, while these remained in the supernatant of SPF 100 had the smallest size. In this particle size range, the particles of all the SPF supernatants grew upon heating but the change of size in SPF 90 and SPF 100 were less noticeable (Fig. 3-4B).

Fig. 3-4 *Particle size distribution (1-1,000 nm) of all the SPFs after the centrifugation at 2000 g for 10 min (A) mixed and stirred after 1 h and (B) heated under 95°C for 30 min.*

3.3.4 Nitrogen solubility index (NSI), water holding capacity (WHC) and oil absorption capacity (OAC)

The nitrogen solubility index (NSI) is routinely used to evaluate protein solubility (Wang and Johnson, 2001). SPF WH presented the highest NSI-value among all the SPFs, which was around 83%, as shown in Fig. 3-5A. The NSI of SPF was reduced by the application of moisture heating during fractionation. The greatest reduction occurred when the heating temperature increased from 60 to 70°C; where the NSI of SPF decreased from 75 to 38%. A small change in NSI occurred when the heating temperature increased from 90 to 100°C.
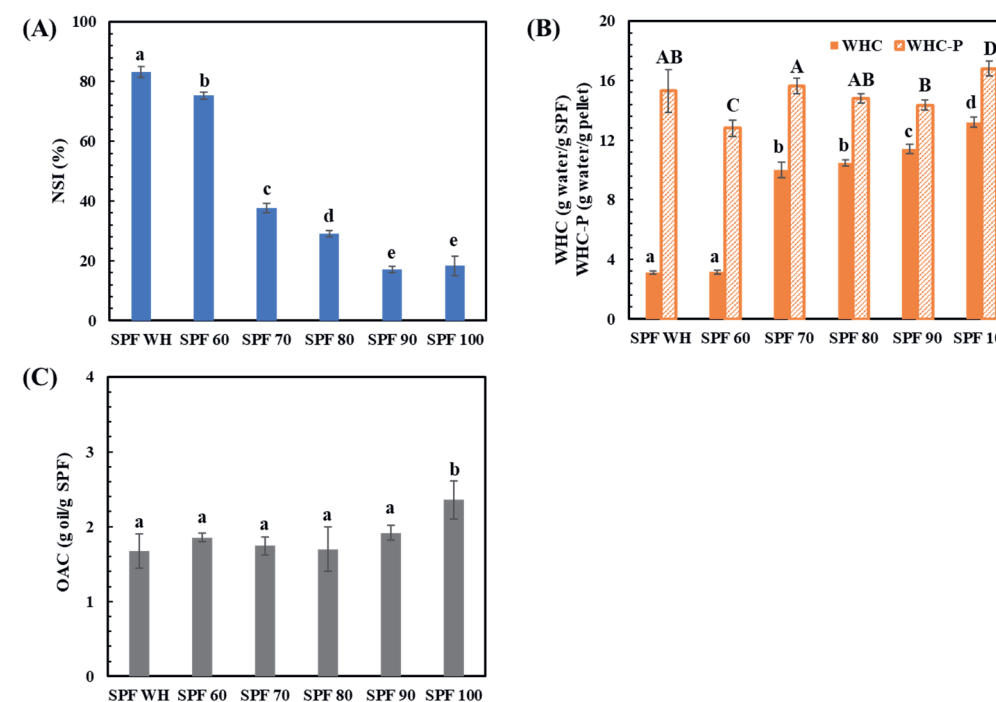
Fig. 3-5 *(A) Nitrogen solubility index (NSI), (B) water holding capacity (WHC) and (C) oil absorption capacity (OAC) of all the SPFs. *The values in the figures with different top letters indicate a significant difference (P < 0.05).*

Water holding capacity (WHC) is defined as the ability of a protein sample to hold water while the water holding capacity of the pellet (WHC$_P$) is defined as the ability of the insoluble part in a protein sample to hold water (Peters et al., 2017). Fig. 3-5B shows that the moisture heating step had limited impacts on the WHC of SPF when the heating temperature was 60°C or below. The WHC of SPF increased dramatically from 3.1 to 10.0 g water/g SPF when the heating temperature changed from 60 to 70°C, and reached the highest value of

13.2 water/g SPF when the heating temperature approached 100°C. However, the difference of WHC$_P$ values became smaller between SPFs and ranged from 12.8 to 16.8 g water/g pellet. Regarding the OAC, the difference between SPFs was not distinct when the moisture heating temperature was 90°C or below (Fig. 3-5C), and the highest OAC value of 2.4 g oil/g SPF was observed for the SPF 100.

3.3.5 Viscosity

All the SPFs presented the typical shear thinning behavior when dispersed in water with concentrations ranging from 5 to 30 wt.%. The increase of the shear rate from 1 to 100 s^{-1} led to a decrease of the viscosity values. Fig. 3-6A showed an example of SPF dispersions with 10 wt.% of concentration. The greatest reduction in viscosity took place for all the SPFs when the shear rate was lower than 10 s^{-1}, and the change was less steep with a higher shear rate. To quantify the effect of SPF concentration on the viscosity changes of all the SPFs, a shear rate of 28 s^{-1} was used and the viscosity values for other SPF samples were determined (Fig. 3-6B).

For all the SPF samples, the viscosity became larger with the increase of SPF concentrations in the range of 5 to 30 wt.%. A tendency to the plateau was observed for SPF samples as the moisture heating temperature was increased, especially when the moisture heating temperature was 80°C and above. Additionally, with a fixed concentration, the SPF dispersions became more viscous when the moisture heating temperature was increased, which can be beneficial for food applications that require a certain viscosity without further heating.
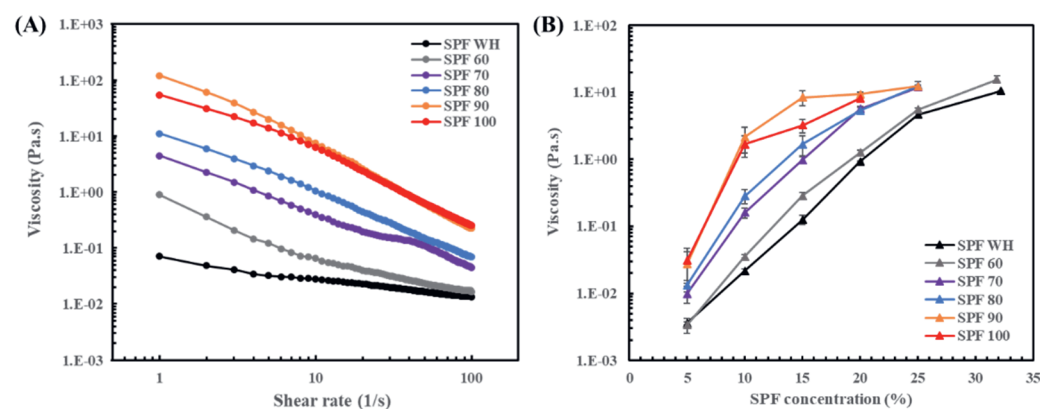

Fig. 3-6 *(A) Viscosity measured as a function of shear rate with the 10 wt.% SPF concentration, and (B) viscosity as a function of the SPF concentration at a shear rate of 28 s^{-1}.*

3.3.6 Gelling behaviors

All SPF dispersions had a constant storage modulus (G') up to a certain strain, after which the modules decreased (App. 3-1). The strain point at which G' began to drop by 5% from its maximum value was determined as a critical strain limit (Rueb and Zukoski, 1997), indicating a transition from linear to non-linear behavior. The results showed that the critical strain limit of SPF dispersions was between 1.7 to 10.3%, and it increased with the increasing temperature. SPF 100 presented the highest value, which indicated that the dispersion of SPF 100 contained a more stable and less easily fractured network than other SPF dispersions (Li et al., 2013). Overall, the strain was selected as 1% for our following temperature and frequency sweep measurements.
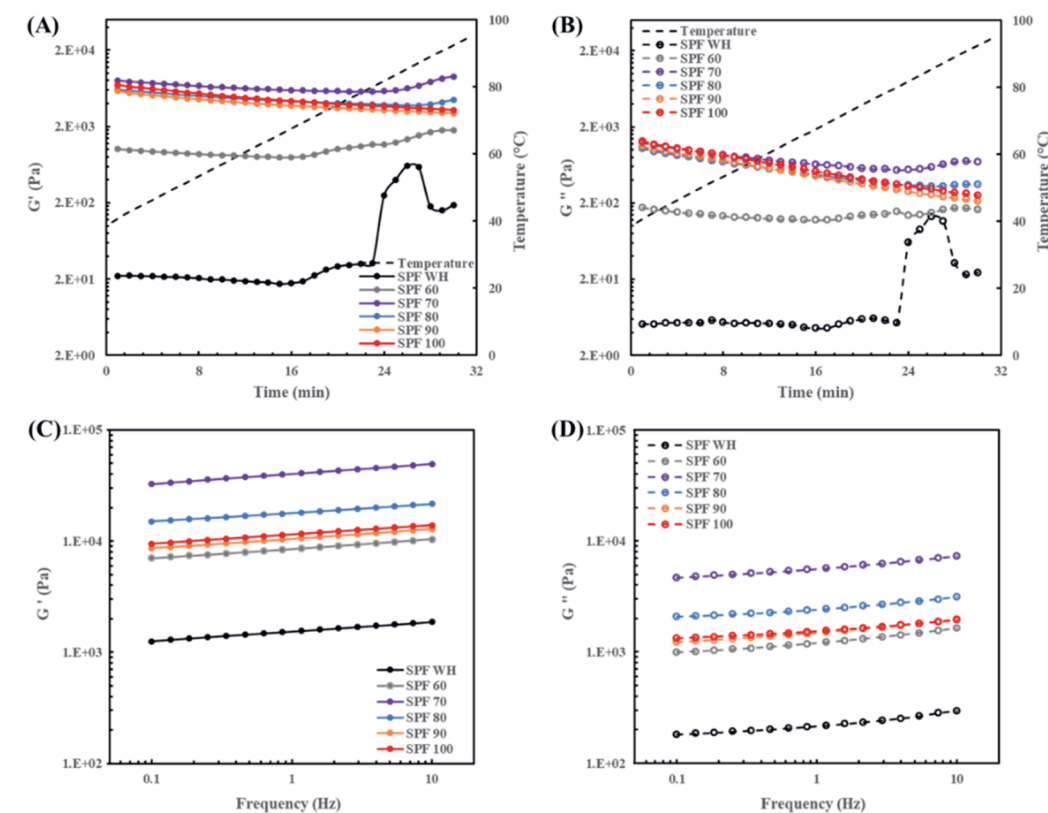

Fig. 3-7 *(A) G' and (B) G'' values during the temperature sweep; (C) G' and (D) G'' values during the frequency sweep after heating for all the SPF dispersions (15 wt.%) at a strain of 1%.*

The results of G' and G" from temperature sweeps are presented in Fig. 3-7A and 7B, respectively. During the temperature sweep from 40 to 95°C, the G' values of all the SPF dispersions were consistently higher than G", indicating that 15 wt. % was sufficient to induce solid-like behavior in all SPF-dispersions before heating already. After heating, SPF WH showed the lowest G' and G" values while SPF 70 presented the highest one. Additionally, with the increase of temperature, the G' and G" values of SPF 90 and SPF 100 decreased. The G' values of SPF WH and SPF 60, though, increased when the temperature exceeded 70°C, while for SPF 70 and SPF 80, the uptrend started around 85°C.

After the temperature sweep, the SPF samples were immediately cooled back to 25°C and the frequency sweep was performed. Fig. 3-7C and 3-7D shows that the G' and G" of all the SPFs increased after cooling and were relatively independent of the frequency applied. Also after cooling, SPF WH had the lowest G' and G" values among all the SPFs, respectively, while SPF 70 gave gels with the highest values for G' and G". Table 3-3 showed the G' and tan δ value of all the gelled soy protein samples obtained at a frequency of 1 Hz after temperature sweep. The gel formed by SPF 70 showed the highest G'-value, while the tan δ value of all the samples were comparable and below 0.15, indicating that all the SPF gels exhibited solid-like behaviors.

Table 3-3 *Average elastic moduli (G') and loss factors (tan δ) of the gelled soy protein fractions obtained at a frequency of 1 Hz after temperature sweep.*

Sample names	G' (Pa)	tan δ
SPF WH	1296 ± 368 [e]	0.14 ± 0.00 [ab]
SPF 60	8406 ± 280 [d]	0.13 ± 0.01 [bc]
SPF 70	37813 ± 4193 [a]	0.14 ± 0.00 [abc]
SPF 80	17942 ± 247 [b]	0.13 ± 0.00 [c]
SPF 90	14265 ± 5218 [c]	0.14 ± 0.00 [a]
SPF 100	11778 ± 177 [cd]	0.13 ± 0.00 [bc]

*The values in the table are compared in columns and different letters indicate a significant difference (P < 0.05).

3.4 Discussion

In this study, a mild fractionation process was used to obtain SPFs. Without additional processing, an SPF was obtained with high purity, high solubility and low WHC. For modern applications though, like meat analogues, other functionalities are required, such as high WHC and the ability to form strong gels. An isochoric moisture heating treatment was performed following the extraction stage and before the final freeze drying to drive functionality in the desired direction. We studied isochoric moisture heating as this step can be easily applied in an existing industrial fractionation process, since no additional process equipment is required. Besides, soy ingredients with a broad range of functionalities can be created without using severe heating temperatures, like in the case of toasting, chemicals or other additives.

The results showed that higher moisture heating temperature contributed to higher denaturation levels of SPFs. The hydrophobic groups located in the interior of the molecule could be exposed for interaction with water, and therefore, causing the reduction of NSI for SPFs (Arrese, Wagner, Añón, & Sorgentini, 1991). The SPF formed new aggregated structures in the dispersion. This aligns with the outcomes of the WHC-measurements and the viscosity measurements. WHC_P of all the SPFs remained comparable, which hinted that the insoluble protein aggregates newly formed as a result of protein denaturation had similar properties. This means that the higher WHC observed can be attributed to a higher amount of insoluble aggregates rather than altered water-binding properties of that insoluble phase.

The viscosity of all the SPFs increased with the increase of SPF concentration, but all samples showed a tendency to a plateau value for the viscosity. This plateau-value can be related to the phase transition of the dispersion. Once the concentration of SPF approached the point, the dispersion with colloidal protein aggregates might undergo a liquid-solid transition to the soft glass phase state (Xu et al., 2018). Above this concentration, the flowability of SPF particles wares measured rather than the viscosity of the SPF dispersions. This result can be correlated to the particle size distribution under room temperature, while larger aggregates are more efficient in obstructing the flow than the ones that are heated at lower temperatures or without heating and formed smaller aggregates, thus, the plateaus differ. Besides the concentration, the increased viscosity caused by higher moisture heating temperature might correspond with the NSI and WHC_P of SPF. The SPFs processed with

higher temperature mainly consisted of large protein aggregates that obstruct the flow, thereby increasing the viscosity. In addition, the WHC$_P$ results showed that the insoluble fractions in SPFs could take up water from the aqueous phase. This lowered the water in the aqueous phase and increased the hydrodynamic volume in the system, and thus the effective protein concentration (Purwanti et al., 2012; Rickert et al., 2004).

For the gelling behaviors, the G' values were higher than G" of all the SPF dispersions within the linear viscoelastic region (LVR), showing that a kind of network was formed for all the SPF samples with 15 wt.% concentration already at room temperature. The results from the temperature sweep were correlated to the denaturation level of the SPFs and the onset T_d of 7S and 11S. In thermal gelation, the protein unfolds, exposes hydrophobic patches that produce a gel network, while the further association of the proteins follows (Hermansson, 1986). After the onset of thermal gelation, more denatured proteins become incorporated into the initial network leading to a further build-up of structure, and therefore, an increase in G' can be observed on further heating (Renkema and Van Vliet, 2002). SPF WH and SPF 60 were still in their native state; when the heating temperature was above the onset T_d of 7S, the structure of 7S proteins was able to change thereby contributing to the gel formation. Similarly, when the temperature was approaching the T_d of 11S, the G' of SPF 70 and SPF 80 started to increase. The decreased G'-values found for SPF 90 and SPF 100 might be caused by the fact that no additional protein interactions could be formed due to the fact that the proteins were fully denatured by the moisture heating with high temperature already. Commercial SPI and SPC also showed a similar decreasing trend in G' value during temperature sweep measurement as SPF 90 and SPF 100, and they were fully denatured as well before the measurements (Ahmed et al., 2006; Li et al., 2007).

The G' and G" of all the SPFs increased after cooling. The gel formed by SPF 70 showed the highest elastic behavior among all the SPFs, followed by SPF 80, which could be considered as the result of partial denaturation. The gel network is formed by cross-linked soy proteins and their aggregates, while the participation of 7S and 11S subunits in the gel network is different (Wu et al., 2018). In both SPF 70 and SPF 80, the 7S protein was denatured while 11S remained native before the measurements. This means the dissociated subunits of 7S probably have unfolded, aggregated and networked evenly with themselves. Subsequently, the temperature sweep till 95°C caused the denaturation of 11S, allowing the dissociated subunits of 11S to associate with the networks of 7S to form a more ordered and

strong gel structure (Liu, Chang, Li, & Tatsumi, 2004; Peng, Ren, & Guo, 2016). Unlike SPF 70 and SPF 80, both 7S and 11S denatured simultaneously during the process for SPF 90 and SPF 100, lacking the ability of 11S proteins to interact with the network of 7S proteins formed during the moisture heating treatment (Tang, 2007). This hypothesis can be partially proven by the results of particle size distribution. The changes in the particle size of SPF 90 and SPF 100 were relatively small as compared to other SPFs while additional heating was applied. Besides, the results presented in this paper are aligned with previous studies, which have demonstrated that SPI with pre-denaturation degree (PDD) of 22.28% and 86.11% had higher G' than the native SPI (PDD 0%) and the completely pre-denatured SPI (PDD 100%) during gel formation process, and the formed gel was denser with more uniform gel networks (Li et al., 2020). Moreover, practically, researchers have reported that separate denaturation of 7S and 11S by two-step heating significantly increased the tofu's apparent Young's modulus and breaking strength during tofu production (Liu & Chang, 2007; Liu et al., 2004; Lu, Lu, Yin, Cheng, & Li, 2010). Overall, soy proteins with selective denaturation levels could form a network with high gel elasticity, while complete denaturation was not absolutely required. This concept can be used optimally when developing fractionation processes that use mild conditions that allow controlled protein denaturation, which can be used to create a much broader range of functional properties using SPF.

3.5 Conclusion

Modern and new food products require soy ingredients with different functionalities than originally aimed for. Mildly fractionated SPF without heating treatment (SPF WH) presented properties that are very suitable for applications like emulsification and foaming, but less optimal for modern applications such as meat analogue, while isochoric moisture heating step with mild conditions investigated in this study can be used to precisely control properties of soy ingredients without impacting the composition, yield and powder morphology. With increasing temperature, proteins in SPFs became more denatured, with complete denaturation at the highest temperature applied. In addition, WHC and viscosity of SPFs gradually increased correlated with the lower NSI. Besides, SPF 100 exhibited the highest OAC value, while the use of partial denaturation led to gels with the highest elastic behaviors. Overall, mild heating conditions allow the possibility to tune properties of simply fractionated SPFs through the moisture heating step, and therefore, can be potentially desired for specific novel applications.

Appendix
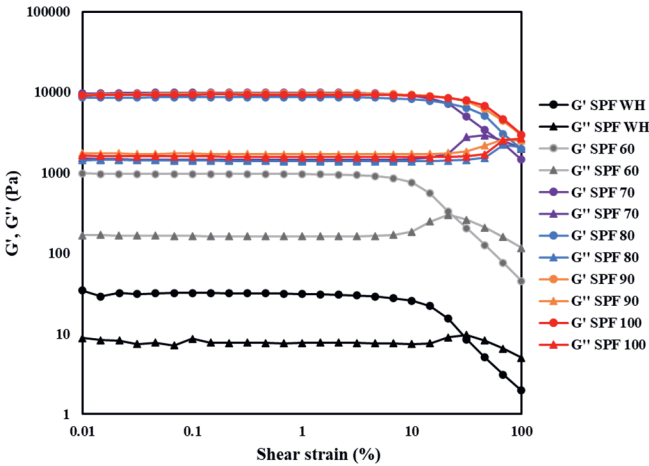
App. 3-1*. Critical strain limits of 15 wt.% SPF dispersions at a constant frequency of 1 Hz.*

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Chapter 4

Effect of calcium hydroxide and fractionation process on the functional properties of soy protein

Abstract

The demand for plant-based ingredients is continuously increasing, but achieving optimal calcium (Ca) and sodium (Na) supplies in plant-based food is a challenge. To this end, alternative fractionation processes were explored for the production of soy protein-rich fractions (SPFs), in which use of $Ca(OH)_2$ instead of NaOH has been exemplified to produce high-Ca, low-Na protein-rich fractions with adjustable functionalities. The use of $Ca(OH)_2$ could lead to SPFs with a protein purity of 81% (with a conversion factor of 5.7). Further, it could lead to increased Ca content in the SPF. $Ca(OH)_2$ treatment decreased the solubility of the fractions from 89.7% to 8.6%, and enhanced their thermal stability and viscoelastic behavior. The outcomes of this study could expand the applications of soy protein with suitable Ca and Na levels.

4.1 Introduction

The market for plant-based foods, such as meat analogues, has been stimulated worldwide as a result of the trend among consumers to reduce their consumption of animal protein (Jones, 2016). Therefore, developing ingredients optimally suitable (structurally and nutritionally) for these novel products is necessary. To date, many plant proteins such as soy, pea, and lupin have been assessed on key functionalities for developing meat analogues and meat extenders (Kyriakopoulou et al., 2019). Among those functionalities, protein solubility, water-holding capacity (WHC) and viscoelastic behavior are considered the most important (Alves and Tavares, 2019; Batista et al., 2005; L'hocine et al., 2006; Zare and Pletch, 2010). In addition, in terms of nutrition, many studies have focused on evaluating the composition of essential amino acids and the digestibility of plant proteins (Opazo-Navarrete, Altenburg, Boom, & Janssen, 2018; Ruiz et al., 2016; Zhang et al., 2016). However, nutritive values, such as the calcium (Ca) and sodium (Na) content, which are also important when developing plant-based alternatives to animal products, are not widely examined.

Calcium accounts for 1-2% of human body weight, and dietary calcium plays an important role in bone metabolism and bone health (Cashman, 2002). Animal products such as meat and dairy foods are the main source of Ca, therefore plant-based alternatives must also provide Ca if they are to replace animal-based products in diets. This is especially important for those who need Ca supplementation, such as children, pregnant women and elderly people (Golden et al., 2014; Hofmeyr et al., 2010). By contrast, in the western diet, Na intake often exceeds the World Health Organization's recommendation of <2000 mg (5 g of salt) as optimal for adults (Data, 2014; Kloss et al., 2015; Mei Kong et al., 2014). Thus, creating meat analogue products that do not increase the Na intake in the diet above the recommended level is important. Excessive intake of Na increases the risk of many diseases, including hypertension and coronary heart disease (Mitchell, 2019). Traditionally, Na is added to enhance the flavor and texture of a final product, as well as to extend the shelf life due to its ability to lower water activity (Ruusunen and Puolanne, 2005). However, Na can also originate from the use of NaOH during the fractionation of ingredients (Jiang et al., 2009). For that reason, the Na content in current meat analogue products is equal to or even higher than in meat. For example, in the case of 100 g of unmarinated meat or meat analogue product, the meat contains less than 100 mg of Na before processing (Verma and Banerjee, 2012); a corresponding meat analogue consisting of around 30 g of soy protein isolate (SPI)

(to achieve similar protein content) contains around 300 mg of Na (https://fdc.nal.usda.gov/fdc-app.html#/food-details/174276/nutrients). The high Na content of SPI results from the precipitation and neutralization steps during fractionation. Therefore, achieving suitable Ca and Na levels is a challenge when developing next-generation plant-based products. Effective methods are needed to address enrichment of the Ca content and reduction of the Na content in the plant-based food industry.

The attractive price of $Ca(OH)_2$ ($116/tonne) compared with NaOH ($450/tonne) and the reduced safety issues make it an interesting option (Jiang et al., 2017). Moreover, Ca salts are used in traditional soy-based food products; for example, calcium sulphate is used as a coagulant during tofu processing due to its precipitant properties (Prabhakaran et al., 2006). Therefore, replacing NaOH with $Ca(OH)_2$ during the protein fractionation process could produce plant protein ingredients that are high in Ca and low in Na. Currently, the strategies used in the formulation of reduced-Na and enriched-Ca products are mainly focused on the processing steps; for example, reducing the amount of sodium chloride in the recipe or adding Ca fortifier into products such as soy milk (Dötsch et al., 2009; Verma and Banerjee, 2012). To date, there is hardly any information on how the fractionation process can play a role in producing high-Ca, low-Na plant protein ingredients. Technically, NaOH is used during the fractionation of plant protein to adjust the pH of the system, especially in the protein solubilization and neutralization steps. $Ca(OH)_2$ can also be used to adjust the pH. Several challenges with the use of $Ca(OH)_2$ have been reported; for example, the low solubility complicates its use in the fractionation process, and calcium ions decrease the solubility of the plant protein and would further affect functionalities within food systems (Añón et al., 2012).

Previous studies have revealed that protein ingredients should have sufficient WHC and swelling capacity to form a meat-like structure (Grabowska et al., 2016). High solubility is not required. In the case of a dairy protein (casein), it has been shown that the use of different specific hydroxides can play a role on structure formation. Calcium caseinate, which is treated with $Ca(OH)_2$, allows the formation of a highly anisotropic structure (meat-like) in a shear cell device at 50°C. In contrast, sodium caseinate only forms an isotropic structure under similar processing conditions (Manski et al., 2007; Wang et al., 2019). This is indicative of the effect that the different hydroxides have on the properties of proteins.

Therefore, the use of $Ca(OH)_2$ instead of NaOH during plant protein fractionation could have potential for new ingredients and food product development.

In this study, soybean was selected as the starting material due to its predominance in the plant-based food market (Kyriakopoulou et al., 2019), as well as its high protein content (Fresán et al., 2019). Traditionally, soy protein ingredients are produced through aqueous fractionation. Oil extraction steps (using organic solvents) are followed by alkaline solubilization, and acid precipitation steps are performed to achieve SPI with high purity (Deak and Johnson, 2007). However, in this study, a mild fractionation process was selected in which the oil extraction steps (using organic solvents) and subsequent washing steps were omitted (Berghout et al., 2014). The fractionation was further simplified by omitting the protein precipitation step. In the proposed ingredient production lines, $Ca(OH)_2$ and NaOH were used during fractionation. The composition and functional properties, including solubility, WHC and structural behavior of the soy protein fractions (SPFs) obtained were evaluated. The aim of this study was to create soy protein ingredients with high-Ca and low-Na content, as well as specific functionalities for developing soy-based products.

4.2 Materials and methods

4.2.1 Materials

Dry, full soybeans were purchased from FRANK Food Products (the Netherlands). NaOH, $Ca(OH)_2$ and HCl were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO). Ultrapure water was purified with a Milli-Q Lab Water System (Milli-Q IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany) and was used throughout unless stated otherwise.

4.2.2 Soy flour preparation

Firstly, soybeans were pre-milled by using a pin mill LV 15M (Condux-Werk, Wolfgang bei Hanau, Germany) into grits. Then, the soy grits were further milled by a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) into full-fat soy flour (FFSF). The impact mill was set according to the parameters described by Pelgrom (Pelgrom et al., 2015) with slight changes: feed rate was around 5 rpm, impact mill speed was 8000 rpm, airflow at 80 m³/h and a classifier wheel speed of 2500 rpm. Full-fat soy flour was stored at 4°C for further use.

4.2.3 Mild and simplified fractionation process

The soy protein fractions (SPFs) used in this study were obtained by two procedures: mild fractionation and simplified fractionation. An overview of the processing conditions and abbreviations can be found in Fig. 4-1. The mild fractionation process was based on previous research (Geerts et al., 2018; Peng et al., 2020). However, due to the solubility limitation of $Ca(OH)_2$, the maximum concentration that can be prepared was around 0.023 M based on our preliminary experiments. More pre-experiments (results not shown) were preformed to adapt the solid: liquid ratio and pH of the fractionation processing steps in order to be comparable with the methods using NaOH. Here, FFSF was mixed with 0.03 M NaOH or 0.015 M $Ca(OH)_2$ (1:5 w/w) to achieve a dispersion with a pH between 8 and 9, and stirred at room temperature for 1 h to solubilize the protein (protein solubilization step). Subsequently, the dispersion was centrifuged at 18,670 g for 30 min (25°C). After centrifugation, the supernatant was poured through a cheesecloth to separate the semi-solid cream layer from the soluble protein, and the insoluble pellet was discarded. The pH of the protein-rich supernatant was adjusted to between 4.5 and 5 by adding 1 M HCl. The added

amount of HCl was weighed. The dispersion was stirred for 1 h and subsequently centrifuged as above (protein precipitation step). The protein-rich pellet was neutralized to pH 6.5–7 with 0.03 M NaOH or 0.015 M $Ca(OH)_2$ (protein neutralization step) and freeze-dried (Freeze Dryer, Martin Christ, Osterode, Germany). The added amount of NaOH/$Ca(OH)_2$ was also weighed. Four different mildly fractionated soy protein fractions (MF-SPFs) were obtained as a result of the different combinations of NaOH and $Ca(OH)_2$ used in the solubilization and neutralization steps.

The simplified fractionation process was based on the mild fractionation process but the protein precipitation step was omitted. After the protein solubilization and centrifugation, the insoluble pellet and semi-solid cream layer were removed. The protein-rich supernatant was directly neutralized to pH 6.5-7 by adding 1 M HCl and subsequently freeze-dried. Two different simply fractionated soy protein fractions (SF-SPFs) were obtained according to whether NaOH or $Ca(OH)_2$ was used in the protein solubilization step.

Before any analysis, all the freeze-dried SPFs were milled into powder using a Rotormill (Pulverisette 14, Fritsch, Idar-Oberstein, Germany) with 0.5-mm sieve ring perforation size and a rotation speed of 6000 rpm. The powders obtained were stored in a cooling room (4°C) until further analysis. All the samples were prepared in triplicate.
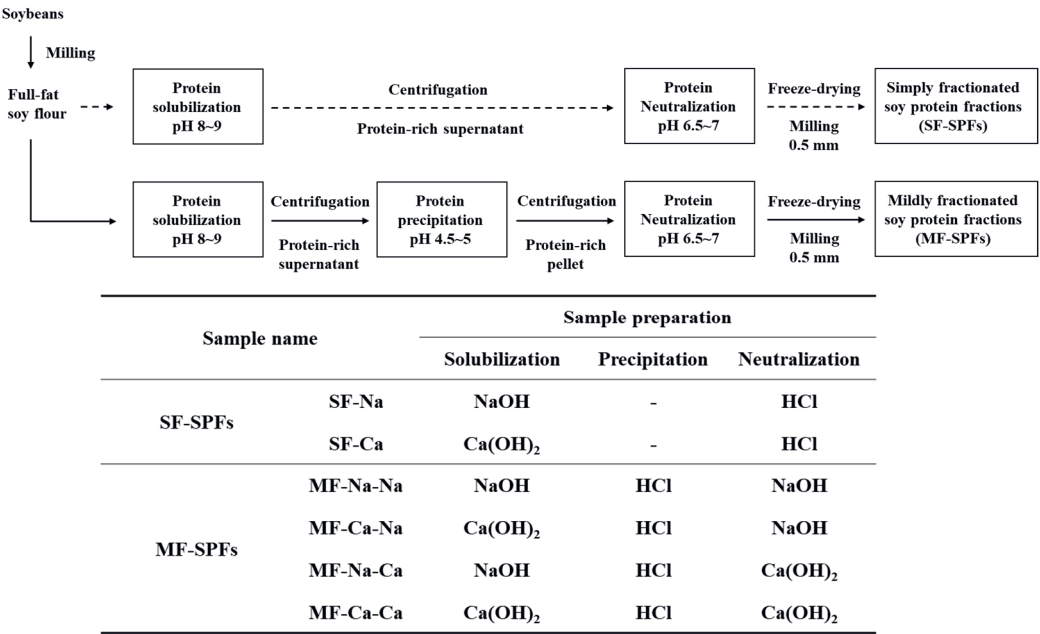

Sample name		Sample preparation		
		Solubilization	Precipitation	Neutralization
SF-SPFs	SF-Na	NaOH	-	HCl
	SF-Ca	$Ca(OH)_2$	-	HCl
MF-SPFs	MF-Na-Na	NaOH	HCl	NaOH
	MF-Ca-Na	$Ca(OH)_2$	HCl	NaOH
	MF-Na-Ca	NaOH	HCl	$Ca(OH)_2$
	MF-Ca-Ca	$Ca(OH)_2$	HCl	$Ca(OH)_2$

Fig. 4-1 *The mild and simplified fractionation processes and abbreviations for the SPFs.*

4.2.4 Composition analysis and yield

The protein content was determined by using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, the Netherlands) with a nitrogen-to-protein conversion factor of 5.7. The oil content was determined by using petroleum ether as an extraction solvent with a Buchi extraction system B-811LSV (Buchi Labortechnik AG, Flawil, Switzerland). The Ca and Na content was determined by a standard procedure using inductively coupled plasma optical emission spectrometry (AVIO 500, PerkinElmer, Waltham, MA). A multi-element standard (Merck P/N 1.11355) was used, and the results are expressed as the total concentration of Ca and Na ions compared with the standard sample. Each produced sample was analyzed in triplicate. The protein yield was calculated according to Eq. (4.1):

$$Protein\ yield\ (\%) = \frac{Total\ protein\ in\ dried\ SPF}{Total\ protein\ in\ starting\ FFSF} \times 100\% \quad Eq.\,(4.1)$$

4.2.5 Microscopic analysis

Scanning electron microscope (SEM, Phenom Pure G2, Phenom-world BV, Eindhoven, The Netherlands) was performed for viewing the microstructures of the SPF powders. The SPF was evenly placed on an aluminum sample holder using double-sided adhesive conductive carbon tape, and the microstructure was observed with an accelerating voltage at 5 kV.

4.2.6 Nitrogen solubility index (NSI) and water holding capacity (WHC)

The WHC-values of SPFs were determined using the method described by Geerts (Geerts et al. 2018) with slight modifications. For each SPF, a 2% (w/v) dispersion was placed in a centrifuge tube and shaken overnight. Then, the dispersion was centrifuged (18,670 g, 30 min, 25°C) to separate the supernatant and pellet. The tubes were drained on tissue paper and the pellets were weighed. Subsequently, the pellets were oven-dried and weighed again. The nitrogen contents in the oven-dried pellets were measured by using Dumas analysis. The NSI was calculated by the ratio of soluble nitrogen over the total initial nitrogen content present in the SPFs. The WHC was calculated according to Eq. (4.2). All the measurements were performed in triplicates.

$$WHC\left(\frac{g\ water}{g\ dry\ SPF}\right) = \frac{M_{wet\ pellet} - M_{dry\ pellet}}{M_{dry\ SPF}} \quad Eq.\,(4.2)$$

4.2.7 Denaturation behaviors

Differential scanning calorimetry (Diamond DSC, PerkinElmer, USA) was used to measure the denaturation temperature and the enthalpy of the transition of the SPF samples. A 20% (w/w) protein dispersion of each SPF was prepared and placed in high-volume aluminum pans, which were then sealed. The differential scanning calorimeter was calibrated with indium, and the sample was scanned at 5°C/min from 20°C to 150°C. An empty aluminum pan was used as a reference. Measurements were analyzed with Start Pyris Software for the denaturation temperature and enthalpy of transition. All the measurements were performed in triplicates.

4.2.8 Rheological behaviors

The rheological properties of the SPFs were determined using two types of rheometers with different sensitivity and temperature range. In order to ensure that the sample quantities were sufficient for measuring these properties, three batches produced under each fractionation process were mixed into a pooled sample.

The Anton Paar rheometer (MCR502, Anton Paar GmbH, Graz, Austria) with a plate-plate geometry (PP-25/P2) was used to obtain the viscosity of the SPF dispersions. Each SPF was mixed with water to achieve 30% total protein content in the mixture and hydrated for 30 min. Due to the differences in protein content between the SPFs, the dry matter content of the mixture was varied. Then, the SPF dispersions were equilibrated for 5 min, and a shear rate sweep was performed at 25°C in steady state with an increasing shear rate. The range of the shear rate was set from 1 to 100 s^{-1}.

A closed cavity rheometer (CCR; RPA elite, TA Instruments, New Castle, DE) was used to determine the complex modulus (G*) of the SPF samples as a function of temperature. Each SPF was mixed with water to achieve 34% total protein content in the mixture (Table 4-1) and hydrated for 30 min before the measurement. Approximately 5 g of mixture was then placed between two plastic films and transferred to a CCR. A temperature sweep was performed from 40°C to 150°C at a heating rate of 5°C/min. All analyses were performed at a frequency of 1 Hz and a strain of 1%, which resulted in measurements in the linear

viscoelastic region. That regime was determined before the measurements. To prevent water evaporation, CCR was closed using a down pressure of 4.5 bar before the measurement.

Table 4-1 Sample preparation for CCR measurements to obtain a total weight of 5 g and 34% protein concentration.

	SPF (g, wet basis)	Solid content of SPF (%)	Water (g)	Solid content of the mixture (%)
SF-Na	3.19 ± 0.23 [a]	93.18 ± 0.57 [c]	1.81 ± 0.23 [b]	59.6 ± 0.04 [b]
SF-Ca	3.18 ± 0.58 [a]	94.35 ± 0.50 [b]	1.82 ± 0.58 [b]	60.0 ± 0.11 [a]
MF-Na-Na	2.52 ± 0.14 [b]	94.38 ± 0.51 [b]	2.48 ± 0.14 [a]	47.6 ± 0.03 [c]
MF-Ca-Na	2.16 ± 0.09 [b]	94.77 ± 0.31 [ab]	2.84 ± 0.09 [a]	41.0 ± 0.02 [f]
MF-Na-Ca	2.38 ± 0.17 [b]	94.99 ± 0.69 [ab]	2.62 ± 0.17 [a]	45.2 ± 0.03 [d]
MF-Ca-Ca	2.21 ± 0.05 [b]	95.60 ± 0.15 [a]	2.79 ± 0.05 [a]	42.2 ± 0.01 [e]

*The values in the table are compared in columns and different letters indicate a significant difference (P < 0.05).

4.2.9 Statistical analysis

Data were collected for each sample from triplicate experiments or from more experiments in specified cases. IBM SPSS Statistics Version 23.0 was used to analyse the variance, and Duncan's test was performed to determine the statistical significance between samples at an α level of 0.05. All the results are displayed as mean values ± standard deviations.

4.3 Results and discussion

In this study, two classes of SPFs (MF-SPFs and SF-SPFs) were obtained through the mild and simplified fractionation processes, respectively. As shown in Fig. 4-1, the simplified and mild fractionation processes have the same solubilization step; an additional precipitation step takes place for the mild fractionation. $Ca(OH)_2$ and NaOH were used in the protein solubilization and/or neutralization step. The variations in the processing steps led to differences in the chemical composition, particle morphology and functional properties of the SPFs. However, as will be shown in this section, the solubility of certain soy fractions was low. That limited the options to analyze some functional properties, such as gelling properties.

4.3.1 Chemical composition and yield

The compositions of all the SPFs are summarized in Table 4-2. Focusing on the protein, mild fractionation resulted in a mean protein content ranging from 73.6% to 81.1%, and the simplified fractionation led to a mean protein content of 56.9% to 57.2%. For the mild fractionation process, the additional precipitation step allowed the removal of soluble carbohydrate from the fraction; therefore, the protein content was increased compared with the simplified fractions. However, not only impurities are removed with any purification step. It is difficult to avoid a certain loss of protein (Tamayo Tenorio et al., 2018). In this study, the mild fractionation resulted in lower protein yield (mean value ranging from 45.9% to 52.4%) compared with simplified fractionation (mean value ranging from 55.6% to 58.9%). Both fractionation processes led to higher yields than reported for isolates produced from soy flour (30%-40%) by conventional wet processes (Moura et al., 2011).

Moreover, more chemicals and intense processes can have an impact on the functionality of the proteins. For certain food applications such as meat analogues, it was reported that multicomponent systems provided the desired functionality for the formation of meat-like fibrous structures (Dekkers et al., 2018). Specifically, soy protein concentrate containing both proteins and carbohydrates formed better structure than soy protein isolate, which had high protein purity (Grabowska et al., 2016). Therefore, in this case, purity is not an absolute requirement, and it can even be a drawback for novel food development (van der Goot et al., 2016).

In terms of oil content, residual oil was detected in both SPF groups. Mean values ranged from 1.04% to 3.00%, which is significantly lower than the 20.6% of oil in the starting material (in this case FFSF). These results indicate that even though the aqueous extraction processes strive to extract protein, most of the oil can be efficiently removed by a simple centrifugation step. This observation is in line with previous research showing that only the centrifugation step could recover most of the oil (Campbell et al., 2011). An additional advantage of the suggested process is that oil extracted through the centrifugation step in the form of oil bodies can be used to make novel food emulsions (Karefyllakis et al., 2019; Romero-Guzmán et al., 2020).

Table 4-2 Chemical composition of all the SPF samples in dry basis.

SPF name	Protein (%)	Oil (%)	Na content (mg/g SPF)	Ca content (mg/g SPF)	Ca content (mg/g protein)
SF-Na	57.2 ± 0.5 [d]	1.2 ± 0.5 [b]	5.1 ± 0.4 [c]	1.5 ± 0.1 [d]	2.7 ± 0.2 [d]
SF-Ca	56.9 ± 0.4 [d]	1.0 ± 0.1 [b]	0.2 ± 0.1 [e]	5.5 ± 0.3 [c]	10.1 ± 0.6 [c]
MF-Na-Na	73.6 ± 2.6 [c]	1.1 ± 0.6 [b]	12.0 ± 0.9 [b]	0.4 ± 0.0 [e]	0.6 ± 0.1 [f]
MF-Ca-Na	81.1 ± 1.7 [a]	1.3 ± 0.7 [b]	13.5 ± 0.4 [a]	1.2 ± 0.2 [d]	1.6 ± 0.3 [e]
MF-Na-Ca	74.4 ± 0.8 [c]	3.0 ± 1.0 [a]	0.6 ± 0.0 [d]	12.3 ± 0.6 [b]	17.0 ± 1.1 [b]
MF-Ca-Ca	79.6 ± 1.2 [b]	1.5 ± 0.6 [b]	0.1 ± 0.1 [e]	14.4 ± 1.3 [a]	18.7 ± 1.6 [a]

*The values in the table are compared in columns and different letters indicate a significant difference (P <
0.05).

Ca and Na contents differed significantly among the different fractions produced (Table 4-2). It was found that using Ca(OH)₂ in the solubilization step during simplified fractionation (SF-Ca) improved the Ca content (5.45 mg/g SPF) and reduced the Na content (0.23 mg/g SPF) compared with the corresponding process using NaOH (SF-Na). However, the differences in the Ca content were less noticeable when a precipitation step and a neutralization step using NaOH were applied. Comparing MF-Ca-Na with the corresponding simplified fraction SF-Ca, a significantly lower Ca content was observed, showing that the precipitation and neutralization steps had a significant impact on the chances of Ca remaining in the protein-rich fraction. Acidic conditions reduced the amount of Ca ions bound to soy protein. This observation corresponds to an earlier study (Kroll, 1984), in which it was found that a small decrease in pH below 7 resulted in a large reduction in the amount of Ca ions bound. Based on this information, it can be hypothesized that the Ca ions added in the solubilization step (pH 8-9) were mostly bound and remained in the supernatant after the first

centrifugation. The Ca content was further enriched, however, when Ca(OH)₂ was used in the neutralization step. In parallel a decrease in the Na content was observed for samples where NaOH was used in the solubilization step. For example, the Ca content of MF-Na-Ca was 12.27 mg/g SPF, which is around 28 times higher than that of MF-Na-Na, and the Na content of MF-Na-Ca (0.61 mg/g SPF) was about 5% of the level found in MF-Na-Na (12 mg/g SPF). In addition, the Ca and Na content of MF-Ca-Ca was closer to MF-Na-Ca than MF-Ca-Na, which could also prove the hypothesis described above that the Ca in the fraction was mainly enriched when applied in the neutralization step. Therefore, a high-Ca low-Na SPF can be prepared best by replacing NaOH with Ca(OH)₂ in the neutralization step.

The use of Ca(OH)₂ in the solubilization step during mild fractionation also enhanced the protein content in the MF-SPFs. The protein content in MF-Ca-Na and MF-Ca-Ca was significantly higher than that in MF-Na-Na. However, the protein enhancement was not observed for SF-SPFs. Therefore, the results indicated that Ca ions might promote additional aggregation of soy proteins during precipitation step at pH 4.5-5 instead of solubilization step, thus achieving a higher protein content in the final MF-SPFs. This hypothesis is supported by previous studies, in which it is reported that the addition of a low concentration of Ca ions could further decrease soy protein solubility under acidic conditions (Manassero et al., 2018; Ono et al., 1993). A possible explanation might be that the binding of Ca ions to carboxyl groups of soy proteins brings about the association of proteins and accelerates the formation of aggregation from hydrated proteins (Mohamed et al., 1988).

Overall, mild fractionation leads to higher protein content and lower yield of fractions compared with simplified fractionation. The use of Ca(OH)₂ in the solubilization step enhanced the protein content of mildly fractionated fractions, and its use in the neutralization step increased the Ca content of the fractions and lowered the Na content.

4.3.2 Microstructure

The powder particle morphology of SPFs was dependent on the fractionation processes and calcium addition in the different steps, as can be seen from the SEM images presented in Fig. 4-2.

For the SF-SPFs, both SF-Na and SF-Ca showed irregular and compact flakes typical of products that are freeze-dried. However, SF-Na had flakes mainly with smooth surfaces, whereas SF-Ca tended to have flakes with rugged surfaces surrounded by many

broken fragments. For the MF-SPFs, both MF-Na-Na and MF-Ca-Na showed irregular and flaky plates with a smooth surface. MF-Na-Ca and MF-Ca-Ca had a coarse and porous structure with small fragments on the surface, and MF-Ca-Ca exhibited more and larger pores on the surface of the flakes.

These morphology variations between SPFs are likely to be related to the calcium content in the SPFs. Fractions with significantly higher calcium content (SF-Ca, MF-Na-Ca, and MF-Ca-Ca) formed honeycomb-like structures with rough surfaces and more broken fragments. These observations are in agreement with Kao, Su, and Lee (2003), who found that during tofu processing, the network of soy protein with 0.2% $CaSO_4$ revealed a rough and discontinuous structure with many fragments and large pores on the surface. In addition, previous research also reported that a calcium-induced structure could have an impact on the functional properties of soy protein, such as solubility and gelation (Chen & Ono, 2014; Zhang, Liang, Tian, Chen, & Subirade, 2012).
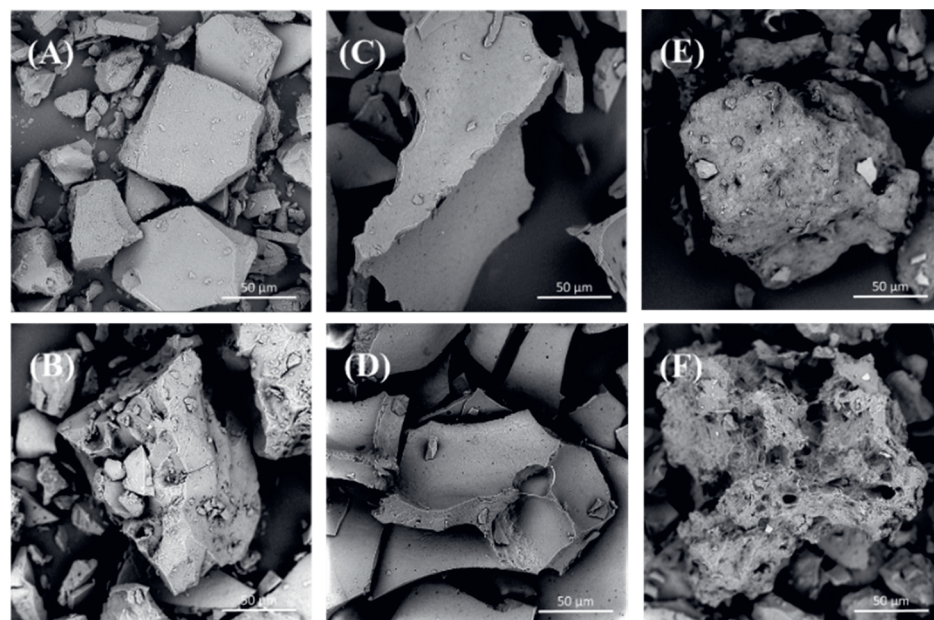
Fig. 4-2 *SEM images of (A) SF-Na, (B) SF-Ca, (C) MF-Na-Na, (D) MF-Ca-Na, (E) MF-Na-Ca and (F) MF-Ca-Ca.*

4.3.3 Nitrogen solubility index (NSI) and water holding capacity (WHC)

Protein solubility is an important functionality of soy protein ingredients for general applications (Thrane et al., 2017). The WHC, which is defined as the ability of a sample to hold water (Peters et al., 2017), is also important. The NSI and WHC were evaluated for all SPFs, and the results are presented in Fig. 4-3. Clear differences can be observed between the samples regarding these properties, indicating that adjustments in the fractionation procedure, and specifically the use of $Ca(OH)_2$, can lead to protein-rich ingredients with different functionalities.

For the simplified fractionation, SF-Na exhibited a high NSI around 90%. However, the NSI decreased to 65% after replacing NaOH with $Ca(OH)_2$ in the solubilization step (SF-Ca). In the case of MF-SPFs, a similarly high NSI was observed for MF-Na-Na (mean value 88.48%), followed by MF-Ca-Na (mean value 86.59%). These results indicated that the use of $Ca(OH)_2$ in the solubilization step did not significantly affect the NSI of MF-SPFs, which also suggested that the influences of Ca on the NSI of soy protein were reversible. However, if the replacement happened in the neutralization step, the NSI of MF-SPFs decreased greatly, as can be seen from the NSI of MF-Na-Ca and MF-Ca-Ca (Fig. 4-3A). This variation can be correlated to the Ca content in the fractions (Table 4-2). Fractions with a Ca content less than 3 mg/g protein (SF-Na, MF-Na-Na and MF-Ca-Na) showed relatively high NSI. SF-Ca with a Ca content of 10.1 mg/g protein showed a reduction in the NSI by a third, whereas the SPFs with the highest Ca content (MF-Na-Ca and MF-Ca-Ca) showed the lowest NSI values. These results are in line with previous research showing that the presence of Ca up to 5 mg/g protein did not significantly affect the solubility of SPI, but when more Ca ions were added, the solubility decreased accordingly (Scilingo & Añón, 2004). It was also reported that the solubility of raw soy milk (unheated) decreased gradually above 2 mM Ca ions and decreased sharply between 6 mM and 8 mM (Ono et al., 1993). A possible explanation for these findings is that the presence of Ca ions promoted the formation of aggregates. These soy protein aggregates were still soluble up to certain concentrations, but at a higher Ca level, the aggregates became insoluble due to their large size (Añón et al., 2012; Yuan et al., 2002). Therefore, controlling the amount of Ca ions added during the fractionation process could lead to SPFs with specific solubility, designed according to the requirements of specific food applications.
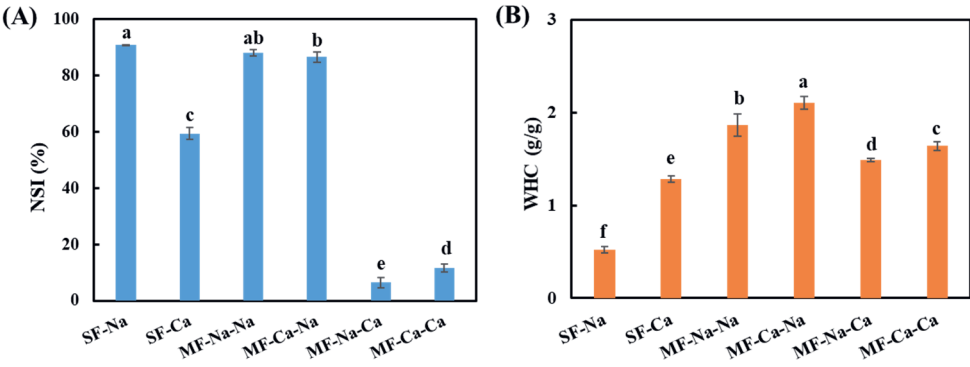
Fig. 4-3 (A) Nitrogen solubility index and (B) water-holding capacity all the SPF samples. *The values in the figures are compared and different letters indicate a significant difference (P < 0.05).*

Apart from the protein solubility, the Ca(OH)$_2$ added during the fractionation procedure also seemed to affect the WHC of the SPFs (Fig. 4-3B). However, the relationship between addition of calcium and the WHC of the fractions was not clear. Ca-enriched SF-Ca showed higher WHC than SF-Na. By contrast, Ca-enriched MF-Na-Ca and MF-Ca-Ca exhibited lower WHC than MF-Ca-Na and MF-Na-Na. However, as MF-Ca-Na and MF-Na-Na also exhibited the highest NSI, the results indicated that the insoluble components of these two fractions had the strongest ability to hold water compared with other MF-SPFs. In the literature, commercial SPI was reported to have a WHC between 5 and 10 g water/g of SPI (Geerts et al., 2018; Kaushik et al., 2016), higher than all the SPFs in this study. Part of the difference could be related to the different drying methods applied. Freeze drying was applied in this study, whereas commercial SPI is normally spray dried. This might lead to differences in the degree of denaturation of the proteins, and thereby affect the WHC of soy protein.

4.3.4 Denaturation behaviors

The values for the enthalpy and denaturation temperature (T$_d$) of SF-SPFs and MF-SPFs are presented in Table 4-3. All the SPFs exhibited two thermal transitions, which correspond to the reported denaturation temperature of β-conglycinin (7S) and glycinin (11S) (L'hocine et al., 2006). Previous research revealed that no peak was detected from commercial SPI (Peng et al., 2020), implying full denaturation. The results indicated that all the SPFs were still partly native after the fractionation process regardless of the fractionation procedure used.

Within the SF-SPFs, the use of Ca(OH)$_2$ in the solubilization step did not affect the T$_d$ of 7S and the enthalpy values but increased the T$_d$ of 11S. The use of Ca(OH)$_2$ in both the solubilization and neutralization steps led to an increase in the T$_d$ of both 7S and 11S. In the case of 11S, the increase in T$_d$ occurred at low Ca concentrations. Similar observations were also reported by Speroni, Jung, and De Lamballerie (2010), who found that in the case of SPIs, the T$_d$ of 11S increased at low Ca concentration, whereas the T$_d$ of 7S increased significantly when the Ca concentration was higher than 25 mM. Scilingo and Añón (2004) also reported that the glycinin structure was stabilized more than that of β-conglycinin by the presence of Ca ions. It was likely that soy proteins aggregated and formed compact structures in the presence of calcium ions, even when proteins were not completely unfolded. This increased the stabilization of the protein structure and, possibly explained the increased T$_d$ of soybean proteins (Ryan et al., 2008; Scilingo & Añón, 1996).

Overall, it was observed that both simply and mildly fractionated protein fractions remained in their native state, however the Ca-enriched fractions showed higher thermal stability.

Table 4-2 *The denaturation temperature and enthalpy of the transition of the all the SPF samples*

Soy protein fraction name	7S T_d (°C)	Enthalpy (J/g)	11S T_d (°C)	Enthalpy (J/g)
SF-Na	76.44 ± 1.82 [c]	1.21 ± 0.39 [c]	97.44 ± 1.88 [c]	5.76 ± 0.63 [b]
SF-Ca	75.94 ± 2.16 [c]	1.27 ± 0.34 [c]	99.42 ± 1.39 [b]	5.93 ± 1.04 [b]
MF-Na-Na	76.25 ± 1.32 [c]	1.85 ± 0.51 [bc]	94.16 ± 1.76 [d]	7.26 ± 0.57 [a]
MF-Ca-Na	77.29 ± 1.19 [c]	2.03 ± 0.64 [b]	95.62 ± 1.76 [d]	8.22 ± 0.20 [a]
MF-Na-Ca	80.68 ± 2.20 [b]	3.67 ± 0.52 [a]	100.94 ± 0.64 [b]	5.69 ± 3.86 [a]
MF-Ca-Ca	83.30 ± 1.59 [a]	3.26 ± 0.59 [ab]	103.82 ± 1.52 [a]	9.95 ± 2.62 [a]

The values in the table are compared in columns and different letters indicate a significant difference (P < 0.05).

4.3.5 Rheological properties

The viscosity of SPFs is presented in Fig. 4-4. All SPF dispersions showed similarities in behaviour, especially with respect to typical shear thinning behaviour. The viscosity of SF-SPFs (both Na and Ca) was lower than that of the MF-SPFs at the same protein concentration. Thus, SF-SPFs need a higher protein concentration to achieve the same viscosity value as MF-SPFs, which shows the potential use of SF-SPFs to develop high-

protein products. Within the SF-SPFs, SF-Ca displayed a slightly lower viscosity than SF-Na. This indicates that, during the simplified fractionation, the use of $Ca(OH)_2$ resulted in a lower viscosity of SF-SPFs. By contrast, Ca-enriched MF-Na-Ca and MF-Ca-Ca exhibited slightly higher viscosity than the other two fractions when the shear rate was relatively low (below 5 s^{-1}). These results suggested that the increase in viscosity was not directly related to calcium enrichment for the SPFs, and it might be affected by the hydration properties. However, at a high shear rate (100 s^{-1}), protein sedimentation was observed for MF-Na-Ca and MF-Ca-Ca during the measurements (data points not shown). The sedimentation might be caused by their low solubility and limitation in the hydration process.
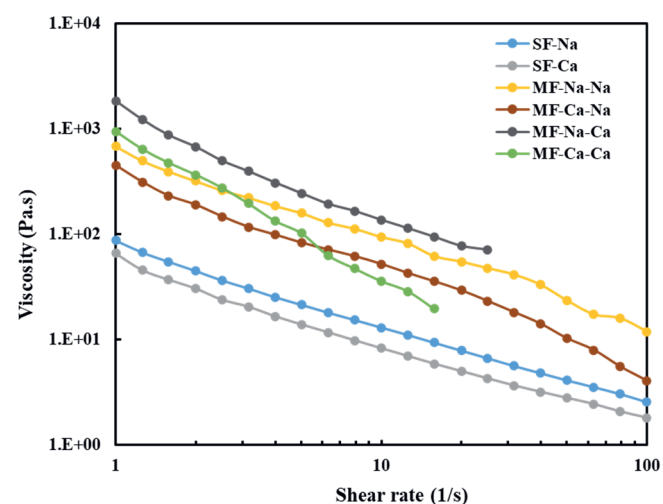

Fig.4-4 Viscosity of the all the SPFs (30 wt.% protein concentration).

The rheological properties of denser protein blends (34 wt.% protein concentration) were analyzed using a CCR. The higher concentrations were measured because those dispersions could be used as the starting material for the formation of novel soy-based products such as meat analogues and vegetarian burgers (Schreuders et al., 2019). Rheology can be used to understand the structure formation for these novel applications (Dekkers et al., 2018). The change in complex modulus G* with respect to the temperature can be seen in Fig. 4-5. All the SPFs exhibited three distinct stages: a decrease or a constant stage in G* at low temperature (stage 1); followed by a sharp increase (stage 2) and ending with a decrease at high temperature (stage 3). For all the SF-SPFs, stage 2 started at around 85°C and stage 3 at about 120°C. For all the MF-SPFs, stage 2 started at around 75°C and stage 3 at around 95°C. A temperature difference for the stage change within the group was not

observed. However, beyond the stage changes, SF-Ca displayed higher G* values than SF-Na, and MF-Ca-Ca and MF-Na-Ca presented higher G* values than MF-Na-Na and MF-Ca-Na. In general, the data suggested that Ca remaining in the SPFs increased the G* value. This finding can be associated with the higher thermal stability of Ca-enriched SPFs (Table 4-3). The presence of Ca ions may promote the formation of aggregates, which are stabilized by hydrophobic interactions and/or Ca bridges between polypeptides during the temperature sweep (Speroni et al., 2010). As a result, the G* value of Ca-enriched SPFs increased.

In summary, SF-SPFs showed lower viscosity and different temperatures for stage changes compared with MF-SPFs. In addition, calcium-enriched fractions showed higher G* during temperature sweep measurements.
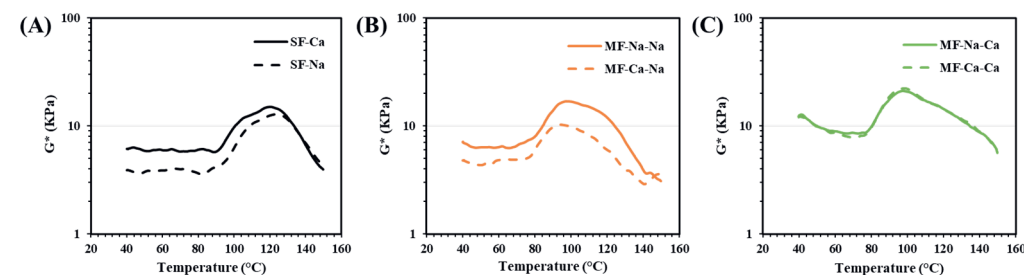

Fig. 4-5 Complex modulus (G) measured (34% protein concentration) as a function of the temperature of (A) SF-Na and SF-Ca, (B) MF-Na-Na and MF-Ca-Na and (C) MF-Na-Ca and MF-Ca-Ca.*

4.4 Conclusion

Novel plant-based products require ingredients with the right functional properties and nutritional value. In this study, we exemplify an alternative fractionation processes omitting organic solvents and intensive washing steps to obtain plant-based protein-rich ingredients using soy flour as starting material. $Ca(OH)_2$ instead of NaOH was used in different processing steps and was found to be a feasible approach to produce high-Ca and low-Na soy protein ingredients with varied functional properties. The SPFs produced had a protein content varying between 56.2% and 81.1% and were all still in their native state. The use of $Ca(OH)_2$ in the solubilization step increased protein recovery, whereas replacing NaOH with $Ca(OH)_2$ in the neutralization step recovered high-calcium low-sodium fractions. However, calcium enrichment in the fractions resulted in a decrease in NSI, and an increase in thermal stability and the G* value was observed. Overall, soy protein ingredients processed with these alternative fractionation procedures and $Ca(OH)_2$ could offer widespread functionality for soy-based food applications. The proposed methodology is potentially applicable to other plant materials, such as pea, chickpea, and lupin.

In summary, SF-SPFs showed lower viscosity and different temperatures for stage changes compared with MF-SPFs. In addition, Ca-enriched fractions showed higher G* during temperature sweep measurements.

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Chapter 5

Effect of calcium enrichment on the composition, conformation, and functionalities of soy protein

Abstract

Plant-based diets with sufficient calcium (Ca) supplements are needed to protect the body from Ca deficiencies. The Ca enrichment of protein ingredients during fractionation can provide a new route to increase the Ca content in plant-based products. We, therefore, investigated if the partial replacement of NaOH by $Ca(OH)_2$ in the fractionation process affects the functional properties of the protein-rich fractions. The protein and oil content of the obtained soy protein-rich fractions (SPFs) were not affected by the use of $Ca(OH)_2$. Moreover, when the Ca content in the SPF was lower than a certain threshold value (6.5 mg Ca/g protein), the functional properties and conformation of SPF did not change significantly. Higher Ca content in the SPF, however, led to protein-rich fractions with larger particle size, higher thermal stability, lower NSI and WHC_P values, and weaker gel networks. Thus, any addition of Ca higher than a certain threshold changes the properties of the proteins significantly, which can alter the applicability of the fractions in plant-based food products. Nonetheless, this study showed that with partial replacement of NaOH by $Ca(OH)_2$, the enrichment of SPF with Ca is possible without strongly influencing the SPF properties.

5.1 Introduction

Plant-based diets aim to minimize animal foods, such as meat, dairy, and eggs (Lynch et al., 2018; Tuso et al., 2013). They have become more widespread among the population in the promotion of physical and environmental health (Rizzo and Baroni, 2018). But despite several health benefits, such as the reduced risk of heart disease, diabetes, and constipation (Fraser, 2009; Key et al., 1999), it is also necessary for plant-based diets to be properly balanced and to provide all essential nutrients. In contrast to animal-based foods such as dairy products which are naturally rich in calcium (Ca), common plant sources including most vegetables, fruits, and cereal grains are lack in Ca (Keller et al., 2002). Additionally, protein-rich sources like dried beans have substantially lower Ca bioavailability because of the presence of phytate, which can inhibit Ca absorption (Weaver, Heaney, Proulx, Hinders, & Packard, 1993). Early studies showed that vegans have a considerably lower average Ca intake than recommended levels compared to non-vegans (Davey et al., 2003; Larsson and Johansson, 2002). This low Ca intake increases the risk of bone fracture and osteoporosis because Ca is essential for bone health (Appleby et al., 2007; Wójcik et al., 2020). Thus, it is advisable for individuals who choose a plant-based diet and limit dairy products to include Ca-enriched foods or supplements.

Among plant-based diets, soy and soy foods have attracted industrial interest due to their high nutrient content and versatile properties (Hoffman and Falvo, 2004). Soybeans are rich in phytate, but the Ca-enriched soy-foods are, exceptionally, good sources of bioavailable Ca for humans. Previous researches have been proven that Ca-fortified soy milk and Ca-set tofu present equivalent Ca absorption to cow's milk at similar Ca loads (Weaver et al., 2002; Zhao et al., 2005). Ca enrichment can be done in different steps throughout the process chain of soy foods. For example, $CaSO_4$ is used as a coagulant for tofu production (Rekha and Vijayalakshmi, 2013), while Ca fortifiers are often added after the soy milk preparation (Li et al., 2011; F Yazici et al., 1997). $CaCl_2$ has been used for the Ca enrichment of commercially available soy ingredients such as soy protein isolate (SPI) (Manassero et al., 2015; Piccini et al., 2019), which is a popular ingredient for novel soy-foods such as meat and dairy analogues (Baldassarre et al., 2020; Bhatia and Greer, 2008).

To obtain soy protein-rich ingredients from soybeans or de-fatted soy meals, an aqueous fractionation with alkaline solubilization and isoelectric precipitation steps is

required to achieve high protein purity. NaOH is commonly used for the pH adjustments during the fractionation, which introduces a certain amount of Na to the ingredients (Deak and Johnson, 2007). In the human diet, extra Na intake can raise blood pressure, which increases the risk of cardiovascular disease (Strazzullo et al., 2009). It might also enhance urinary Ca losses, leading to the opposite effect of our initial purpose (Itoh and Suyama, 1996; Matkovic et al., 1995; Mitchell, 2019). Therefore, we see the need for a new route to enrich the Ca and reduce the Na content broadly for novel soy-foods via the ingredient production route.

Several attempts have been made to enrich the Ca content in SPI. For example, Manassero et al. re-dispersed the SPI powder into water, and added the $CaCl_2$ in the dispersions afterward (Manassero et al., 2015); Piccini et al. fractionated the SPI first and added $CaCl_2$ before or after denaturing treatments (Piccini et al., 2019). These types of Ca enrichment occurred after the protein separation stage, so the post-processing steps are needed, and the NaOH has already been introduced for the pH adjustments during the fractionation process. In our previous work, we used a simplified aqueous fractionation, in which the de-fatting and washing steps were omitted. The $Ca(OH)_2$ was explored as an alternative to NaOH in the alkaline solubilization and neutralization step. This strategy was found beneficial in providing higher Ca content and lowering Na content in soy ingredients. It was concluded that replacing NaOH with $Ca(OH)_2$ in the solubilization step increased protein recovery, while the replacement in the neutralization step achieved high-Ca low-Na fractions (Peng et al., 2020a). However, a complete replacement of NaOH with $Ca(OH)_2$ greatly varied the functionalities of soy protein such as protein solubility, which could bring challenges for further applying the ingredients in soy-based products.

We hypothesize that there is a critical Ca concentration upon which the soy protein functionalities are affected. Thus, the present study investigates a step-wise increase of $Ca(OH)_2$ in the neutralization step by mixing NaOH and $Ca(OH)_2$ in different ratios in a simplified fractionation process. The obtained soy protein-rich fractions (SPFs) are examined with respect to composition, protein conformation (FT-IR), charge density (zeta-potential), morphology (SEM), aggregate size, nativity (DSC), and relevant functionalities such as protein solubility, water holding capacity, and gelling properties. The results also provide more insights into how Ca ions bind to soy. With those experiments, we aim to create soy

protein ingredients with enriched Ca content, as well as the desired range of functionalities for developing novel soy-based products.

5.2 Materials and methods

5.2.1 Materials

Dry, full soybeans were purchased from FRANK Food Products (the Netherlands). NaOH, Ca(OH)$_2$ and HCl were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO). Ultrapure water was purified with a Milli-Q Lab Water System (Milli-Q IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany) and was used throughout unless stated otherwise.

5.2.2 Soy flour preparation

Firstly, soybeans were pre-milled by using a pin mill LV 15M (Condux-Werk, Wolfgang bei Hanau, Germany) into grits. Then, the soy grits were further milled by a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) into full-fat soy flour (FFSF). The impact mill was set according to the parameters described by Pelgrom (Pelgrom et al., 2015) with slight changes: feed rate was around 5 rpm, impact mill speed was 8000 rpm, airflow at 80 m^3/h and a classifier wheel speed of 2500 rpm. Full-fat soy flour was stored at 4°C for further use. The FFSF contained 38.9% ± 4.2% of protein and 20.6% ± 0.6% of oil on a dry basis.

5.2.3 Mild fractionation process and Ca enrichment

Soy protein-rich fractions (SPFs) were fractionated based on the process developed earlier (Peng et al., 2020a). An overview of the processing steps and parameters can be found in Fig.5-1. Briefly, FFSF was mixed with 30 mM NaOH to achieve a dispersion with a pH between 8 and 9 (alkaline solubilization step), which was subsequently centrifuged at 20,000 g for 30 min (25°C). After centrifugation, the protein-rich supernatant was collected, and the pH of the supernatant was adjusted between 4.5 and 5 by adding 1 M HCl (acid precipitation step). The dispersion was stirred for 1 h and centrifuged as above. The protein-rich pellet was neutralized to pH 6.5-7 with a NaOH and Ca(OH)$_2$ mixture (protein neutralization step). All the mixtures were prepared in advance with a fixed OH- concentration of 30 mM, which was achieved through applying combinations of NaOH and Ca(OH)$_2$ solutions (Table 5-1). The pH of the mixtures was between 12.4 to 12.5. The NaOH and Ca(OH)$_2$ concentrations were selected based on the solubility limitation of Ca(OH)$_2$ and preliminary experiments.

After the protein neutralization step, the protein-rich dispersions were freeze-dried (Freeze Dryer, Martin Christ, Osterode, Germany). The dried materials were milled into powders and stored in a cooling room (4°C) until further analysis. All samples were prepared in duplicate.
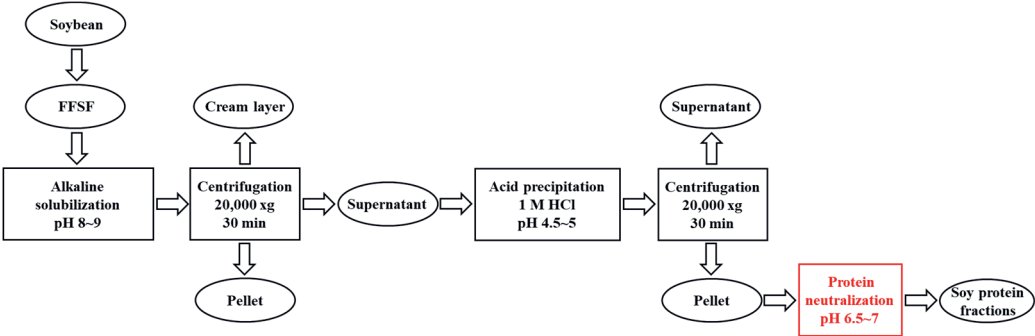

Fig. 5-1 *The mild fractionation processes and Ca enrichment for the SPFs.*

Table 5-1 *Concentrations of NaOH and Ca(OH)$_2$ for preparing the alkaline mixtures used at the protein neutralisation step to investigate a step-wise increase of the Ca content in the different SPFs..*

Sample name	NaOH concentration [mM]	Ca(OH)$_2$ concentration [mM]	Total OH$^-$ concentration [mM]	pH
SPF 1	30	0	30	12.48
SPF 2	25	2.5	30	12.44
SPF 3	20	5	30	12.43
SPF 4	15	7.5	30	12.44
SPF 5	10	10	30	12.43
SPF 6	5	12.5	30	12.41
SPF 7	0	15	30	12.41

5.2.4 Composition analysis

The protein content was determined by using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, the Netherlands) with a nitrogen-to-protein conversion factor of 5.7. The oil content was determined by using petroleum ether as an extraction solvent with a Buchi extraction system B-811LSV (Buchi Labortechnik AG, Flawil, Switzerland). The Ca and Na content was determined by a standard procedure using inductively coupled plasma optical emission spectrometry (AVIO 500, PerkinElmer, Waltham, MA). A multi-element standard (Merck P/N 1.11355) was used, and the results are

expressed as the total concentration of calcium and sodium ions compared with the standard sample. Each produced sample was analyzed in triplicate.

5.2.5 Microscopic analysis

Scanning electron microscope (SEM, Phenom Pure G2, Phenom-world BV, Eindhoven, The Netherlands) was performed for viewing the microstructures of the SPF powders. The SPF was evenly placed on an aluminum sample holder using double-sided adhesive conductive carbon tape, and the microstructure was observed with an accelerating voltage at 5 kV.

5.2.6 Particle size analysis

The particle size distribution (PSD) of the SPFs was measured with a laser light scattering instrument (Mastersizer 3000, Malvern Instruments Ltd, United Kingdom) and a wet module (Hydro SM, Malvern Instruments Ltd, United Kingdom). For these measurements, 1% (w/v) SPF dispersions were prepared with Milli-Q water. A refractive index of 1.45 was used for the dispersion phase and 1.33 for the water continuous phase.

5.2.7 FT-IR analysis

The effect of Ca or Na on the conformation of the SPFs was analysed using a Confocheck™ Invenio-S Fourier Transform Infrared (FT-IR) spectrometer equipped with a temperature-controlled Bio ATR2 unit and a MCT detector (Bruker Optics, CITY, Netherlands) optimized for protein analytics in solutions as previously described by Kayser et al. (Kayser et al., 2020). 1% (w/v) SPF dispersions (neutral pH) were prepared, and the measurements were conducted at a temperature of 25 ˚C against the respective solvent mixtures without protein as background and averaged over 120 scans at a resolution of 0.7 cm. The second derivative of the amide I band (1590-1700 cm^{-1}) was calculated using nine smoothing points.

5.2.8 Zeta potential analysis

The charge density of the SPFs at different pHs was determined with a dynamic light scattering apparatus (Zetasizer Nano ZS, Malvern Instruments Ltd, United Kingdom). A disposable cuvette (Folded Capillary Zeta Cell DTS1070, Malvern Instruments Ltd, United Kingdom) was filled with a 1% (w/w) SPF dispersion. Titration was performed (MPT-2 autotitrator, Malvern Instruments Ltd, United Kingdom) to adjust the pH of the dispersions

from 3 to 9 in steps of 0.5 either with a HCl (0.25 M) or a NaOH (0.25 M). The refractive indices used were 1.43 for the dispersed phase and 1.33 for the continuous phase.

5.2.9 Nitrogen solubility index (NSI)

The nitrogen solubility index (NSI) is routinely used to evaluate protein solubility (Wang and Johnson, 2001). A 2% (w/w) dispersion of each SPF sample was placed in a centrifuge tube and moderately shaken overnight. Subsequently, the sample was centrifuged at 18,670 g for 30 min to separate the supernatant and pellet. The pellet was oven-dried at 105°C and weighed. The nitrogen contents in the oven-dried pellets were measured by using Dumas analysis. The NSI was calculated by the ratio of soluble nitrogen over the total initial nitrogen content present in the SPFs. All the measurements were performed in triplicate.

5.2.10 Denaturation behaviors

Differential scanning calorimetry (Diamond DSC, PerkinElmer, USA) was used to measure the denaturation temperature and the enthalpy of the transition of the SPF samples. A 20% (w/w) protein dispersion of each SPF was prepared and placed in high-volume aluminum pans, which were then sealed. The differential scanning calorimeter was calibrated with indium, and the sample was scanned at 5°C/min from 20°C to 150°C. An empty aluminum pan was used as a reference. Measurements were analyzed with Start Pyris Software for the denaturation temperature and enthalpy of transition. All the measurements were performed in triplicates.

5.2.11 Water holding capacity (WHC)

The water holding capacity (WHC) of SPFs was measured using the method described by Peters (Peters et al., 2017) with slight modification. A 2% (w/v) SPF dispersion was prepared with Milli-Q water and shaken overnight. Then, the dispersion was centrifuged at 18,670 g for 30 min to separate the supernatant and the wet pellet. The wet pellet was collected and weighed. Subsequently, the wet pellet was oven-dried overnight at 105°C and weighed again to determine the mass of the dry pellet. The WHC was calculated according to Eq. (5.1), and the WHC of the pellet (WHC$_P$) was determined according to Eq. (5.2).

$$WHC \left(\frac{g\ water}{g\ dry\ SPF}\right) = \frac{M_{wet\ pellet} - M_{dry\ pellet}}{M_{dry\ SPF}}\ Eq.\,(5.1)$$

$$WHC_P \left(\frac{g\ water}{g\ dry\ pellet}\right) = \frac{M_{wet\ pellet} - M_{dry\ pellet}}{M_{dry\ pellet}} \quad Eq.\ (5.2)$$

5.2.12 Viscosity analysis

SPF dispersions standardized on 9 wt.% protein (approximately 12 wt.% SPF) were prepared and stirred for around 1 h. A stress-controlled rheometer (Anton Paar Physica MCR 301, Graz, Austria) combined with a sand-blasted CC-17 concentric cylinder geometry was used to determine the viscosity. The SPF dispersion was equilibrated for 5 min, and a shear rate sweep was performed at 25°C from 0.1 to 100 s^{-1}.

5.2.13 Gelling behaviors

A stress-controlled rheometer (Anton Paar Physica MCR 301, Graz, Austria) combined with a sand-blasted CC-17 concentric cylinder geometry was used. SPF dispersions standardized on 9 wt.% protein concentration (approximately 12 wt.% SPF) were prepared and stirred for 1 h before the measurements. A thin layer of high-temperature-resistant silicone oil was covered on top of the samples to prevent water evaporation during heating. A temperature, frequency, and strain sweep were performed sequentially. The temperature sweep was done by increasing the temperature from 20 to 95°C at a rate of 3°C /min, followed by 10 mins at 95°C before cooling back to 20°C at a rate of 3°C /min. Subsequently, the samples were exposed to a frequency sweep from 0.01 to 10 Hz (at a strain of 1%), and a strain sweep from 0.1 to 1000% (at a frequency of 1 Hz). The storage (G') and loss modulus (G") dependency on temperature, frequency, and strain were recorded.

5.2.14 Statistical analysis

Data were collected from triplicate experiments or in specified cases from more experiments. IBM SPSS Statistics Version 23.0 was used to analyze the variance, and Duncan's test was performed to determine the statistical significance between samples at an α level of 0.05. All the results were displayed by mean values ± standard deviations.

5.3 Results

In this study, SPFs were obtained through a simplified fractionation process and neutralized by NaOH and Ca(OH)$_2$ in different ratios to identify critical ratios that affect the protein functionality. The variations in the neutralization step led to differences in the composition, particle morphology, charge density, and functional properties of the SPFs.

5.3.1 Chemical composition

The protein content of all SPFs was similar and around 75% on a dry basis (Table 5-2). The oil content varied from 1.21% to 2.53%, which was much lower than the oil content in the start material FFSF. These results also agreed with the values reported by our previous study (Peng et al., 2020a). In terms of Na and Ca content, the differences between SPFs were significant. SPF 1, for which only NaOH was used in the neutralization step, had the highest amount of Na (15.9 g/g protein) and the lowest amount of Ca (0.6 g/g protein). As expected, we observed a higher Ca content and lower Na content when using more Ca(OH)2 to neutralize the SPF dispersions. The Ca content of SPF 7 (16.6 mg/g SPF) was around 29 times higher than that of SPF 1, while the Na content of SPF 7 (0.8 mg/g SPF) was only 5% of the level found in SPF 1. In addition, the exact Ca and Na contents measured for all SPF samples were quite close to the amount of Ca and Na added in the neutralization step during the fractionation.

Table 5-2 Chemical composition of all the SPF samples in dry basis

Sample name	Protein (%)	Oil (%)	Na content (mg/g protein)	Ca content (mg/g protein)
SPF 1	75.06 ± 2.17 [a]	1.52 ± 0.52 [bc]	15.91 ± 1.29 [a]	0.57 ± 0.06 [g]
SPF 2	74.53 ± 1.20 [a]	1.21 ± 0.06 [c]	11.02 ± 0.75 [b]	1.90 ± 0.12 [f]
SPF 3	74.70 ± 0.65 [a]	1.76 ± 0.26 [abc]	9.80 ± 0.05 [c]	4.28 ± 0.02 [e]
SPF 4	75.27 ± 0.75 [a]	2.07 ± 0.28 [abc]	7.47 ± 0.47 [d]	6.47 ± 0.40 [d]
SPF 5	74.34 ± 1.85 [a]	2.53 ± 0.90 [a]	5.67 ± 0.24 [e]	9.84 ± 0.41 [c]
SPF 6	75.55 ± 0.93 [a]	2.04 ± 0.70 [abc]	2.84 ± 0.09 [f]	12.62 ± 0.29 [b]
SPF 7	75.73 ± 0.28 [a]	2.18 ± 0.67 [ab]	0.81 ± 0.02 [g]	16.55 ± 0.66 [a]

The values in the table are compared in columns and different letters indicate a significant difference (P < 0.05).

5.3.2 Microstructure

SPFs prepared with different Ca(OH)$_2$ and NaOH ratios appeared to have some differences in their microstructure (Fig. 5-2). SPF 1, neutralized by NaOH during the fractionation process, showed a lamellar glass-like structure with a smooth clean surface (red arrow). Although no major differences were observed between SPF 1 and SPF 2, while increasing the Ca content (SPF 4 and SPF 5), a rougher flake surface was observed (blue circle) next to the glassy structure. At the highest Ca content of SPF 6 and SPF 7, the lamellar structures largely disappeared and a pumice stone structure with a rough sponge-like surface emerged.
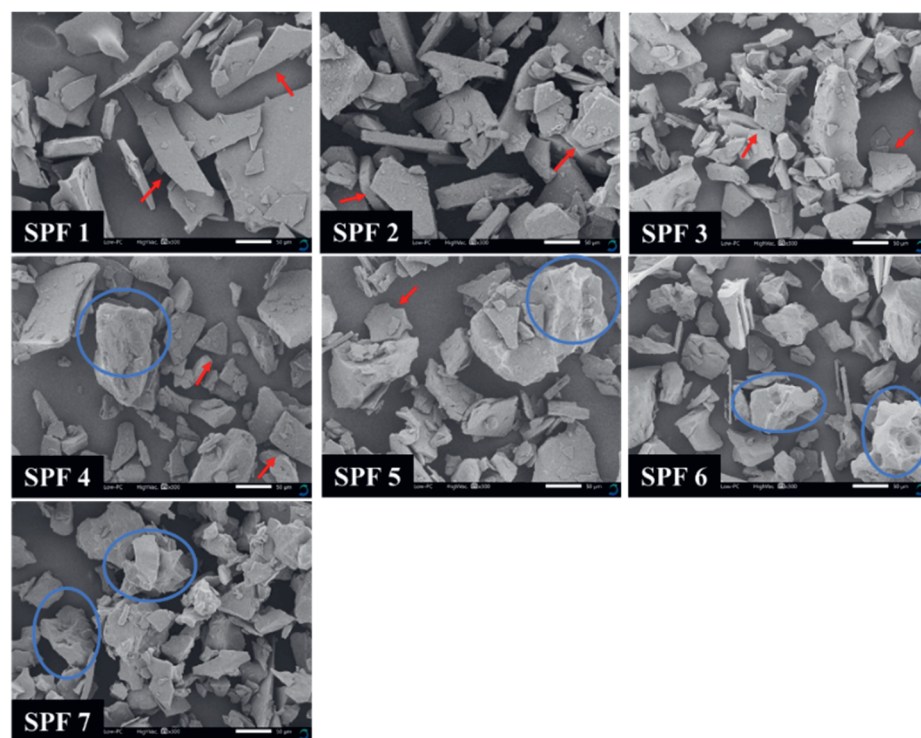

Fig. 5-2 *Representative SEM images of the SPFs. The scale bar is 50 μm. The red arrows indicate glass-like structures with smooth surfaces, the blue circles emphasize rough surface structures.*

5.3.3 Particle size distribution (PSD)

The PSD curves of 1% SPF dispersions in water (Fig. 5-3) showed a gradual shift to the right with the increase of the Ca content in the SPFs from SPF 1 to SPF 7. The distributions of SPF 1 and SPF 2 were quite similar with a single peak around 48 μm and 55 μm, respectively. For SPF 3 and SPF 4, the PSD became wider towards the right side, while SPF 4 had two peak values around 38 μm and 92 μm. The rest of the curves were single-peaked, while for SPF 6 and SPF 7, a relatively narrow distribution of larger size was observed.
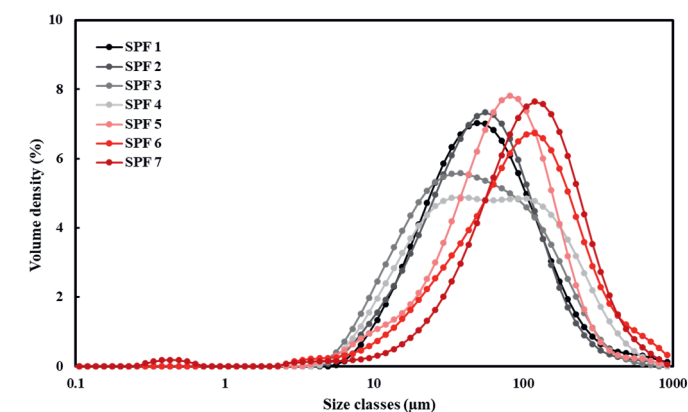

Fig. 5-3 *Particle size distribution of 1% (w/v) SPF dispersions.*

5.3.4 Secondary Structure

Fig. 5-4 shows the mean second derivative FTIR spectra in the amide I region of the SPFs. The bands corresponding to intramolecular β-sheets were obtained in the frequency region of 1635-1640 cm^{-1} and 1670-1690 cm^{-1}, while a band for α-helix appeared in the frequency around 1650-1660 cm^{-1}. The random coil structure had a band close to 1645 cm^{-1} (Cobb et al., 2020; Kong and Yu, 2007). Early studies suggested that 7S soy protein consists mainly of α-helix and antiparallel β-sheet structures (Nagano et al., 1994), while the major secondary structures of 11S soy protein are β-sheet, followed by β-turn, α-helix, and random coil (Long et al., 2015). In contrast, Zhao et al. described similar conformation for 7S and 11 S, with approximately 46.7% β-sheet structures in aqueous solution (Zhao et al., 2008). Here, the strong band at ~1635 cm^{-1} confirms a predominant β-sheet conformation of all fractions.

With the variations of Ca and Na content, some minor structural changes were observed between SPFs. The bands that corresponded to the α-helix signal at 1656 cm^{-1} were

continuously decreasing with increasing the Ca content. There is a minor increase of the 1619 cm^{-1} waveband, which is indicative for intermolecular β-sheets and thus for protein aggregation for SFP 5, 6, and 7. All other structural elements were randomly changing with increasing Ca content, and thus within the natural deviation or repeated measurements.
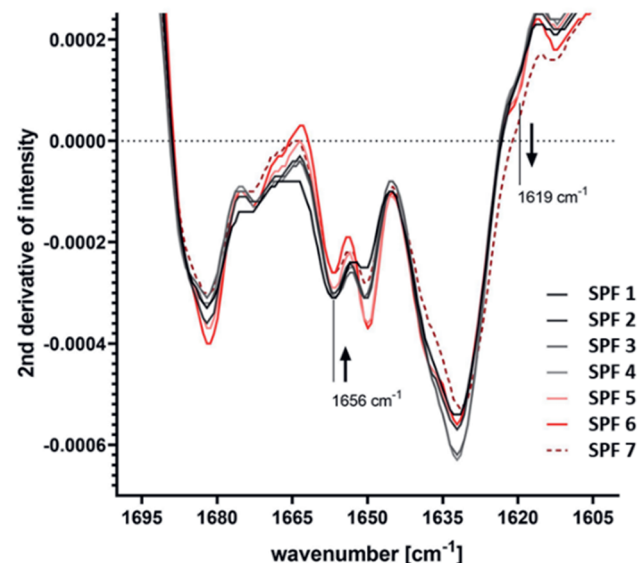

Fig. 5-4 *Deconvoluted second derivative FT-IR spectra of SPF samples (mean of three individual measurements). The arrows indicate structural elements where the intensity increases or decreases continuously with increasing Ca content.*

5.3.5 Net charge

The zeta potential is related to the net charge residing on the surface or near-surface of a suspended particle (Song et al., 2013), and is affected by many factors such as pH, ionic strength, and temperature (Malhotra and Coupland, 2004). The measured zeta potential of the SPF dispersions changed from positive to negative values when increasing pH from 3 to 9 (Fig. 5-5). All samples had a neutral charge around pH 5, suggesting a similar isoelectric point (pI) and thus no influence by the exact calcium and sodium content. When the pH was further increased from 5 to 9, the zeta potential of all the SPFs became negative. In this pH range, the curves began to diverge and the differences between SPFs became visible. SPF 1 acquired the most negative charges as compared to other SPFs, while SPF 7 acquired the least amount.
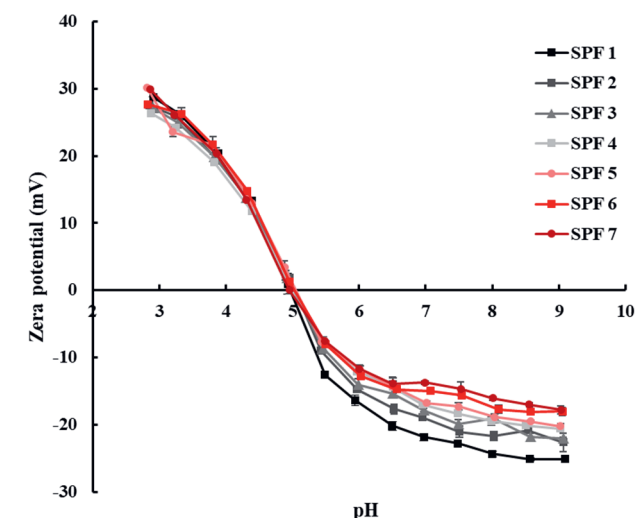

Fig. 5-5 *Zeta potential of all the SPF dispersions as related to the pH*

5.3.6 Nitrogen solubility index (NSI)

The degree of protein solubility is usually represented by the nitrogen solubility index (NSI). The NSI of all the SPFs was measured at pH 3 and pH 7. When the pH was 7, the NSI decreased with higher Ca content in the SPF. SPF 1 showed a NSI of around 88.8% while the NSI-value for SPF 7 was only 10.3%. The greatest reduction in NSI happened between SPF 4 and SPF 5 where the NSI decreased from 56.9% to 26.9%. At pH 3, a higher Ca content had a similar effect on the NSI, but the NSI variation was smaller, with values ranging between 57.1% and 86.0%. The NSI measured at pH 3 was generally higher than the NSI obtained at pH 7, and the difference becoming larger with the increase of Ca content in the SPF.
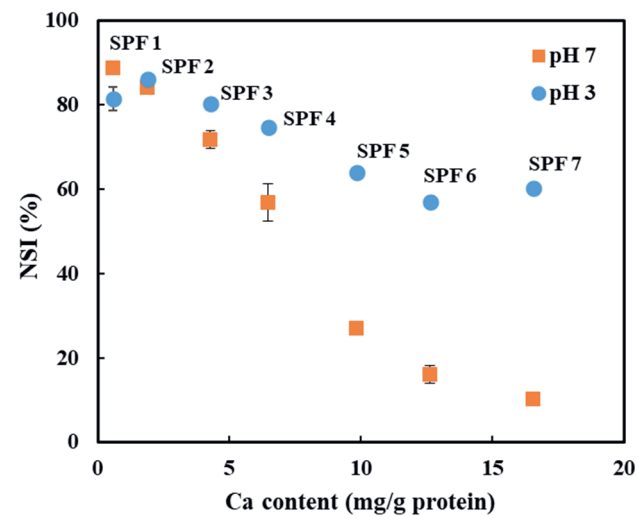
Fig. 5-6 *Nitrogen solubility index (NSI) of all the SPFs as related to the calcium content.*

5.3.7 Denaturation behaviors

Two typical endothermic peaks were observed in all the SPFs, corresponding to the denaturation of β-conglycinin (7S) and glycinin (11S) (Table 5-3). It was observed that the T_d of 7S and 11S was influenced by the Ca levels in the SPFs. Up to 6.47 mg/g protein, the increase of Ca content hardly altered the T_d of 7S, while an increase from 75.50 to 81.97°C was found once the Ca content was higher than that value. In contrast, the T_d of 11S increased already upon an increase of Ca content from 0.57 to 1.90 mg/g protein, which means that Ca influenced the T_d of 11S more than the T_d of 7S. These results are in agreement with previously reported results, which showed that no changes in T_d of 7S were found when lower Ca content was added in SPI dispersions, while higher T_d of 11S was found with the presence of Ca (Scilingo & Añón, 1996). The enthalpies corresponding to the denaturation of 7S and 11S proteins are shown in Table 4-3. The increase in Ca content in SPFs was accompanied by an increase in the enthalpy of 11S, while this effect was not significant for 7S. Overall, higher Ca content increased the thermal stability of SPF.

Table 5-3 *The denaturation temperature and enthalpy of the transition of the SPFs.*

Sample name	Ca content (mg/g protein)	7S T_d (°C)	Enthalpy (J/g)	11S T_d (°C)	Enthalpy (J/g)
SPF 1	0.57 ± 0.06 [g]	75.22 ± 0.32 [d]	1.64 ± 0.20 [a]	92.76 ± 0.30 [e]	2.45 ± 0.18 [bc]
SPF 2	1.90 ± 0.12 [f]	75.55 ± 0.56 [d]	1.39 ± 0.20 [abc]	94.20 ± 1.09 [d]	2.27 ± 0.07 [c]
SPF 3	4.28 ± 0.02 [e]	74.71 ± 0.70 [d]	1.31 ± 0.16 [bc]	94.94 ± 1.37 [d]	2.16 ± 0.19 [c]
SPF 4	6.47 ± 0.40 [d]	75.50 ± 0.41 [d]	1.22 ± 0.11 [bc]	96.53 ± 0.32 [c]	2.68 ± 0.35 [bc]
SPF 5	9.84 ± 0.41 [c]	77.35 ± 0.44 [c]	1.16 ± 0.14 [c]	98.54 ± 0.54 [b]	2.84 ± 0.19 [bc]
SPF 6	12.62 ± 0.29 [b]	79.56 ± 0.28 [b]	1.47 ± 0.24 [ab]	100.25 ± 0.25 [a]	3.06 ± 0.23 [b]
SPF 7	16.55 ± 0.66 [a]	81.97 ± 0.99 [a]	1.46 ± 0.22 [abc]	101.13 ± 0.71 [a]	3.88 ± 1.04 [a]

*The values in the table are compared in columns and different letters indicate a significant difference ($P < 0.05$).

5.3.8 Water holding capacity (WHC)

WHC is generally defined as the amount of water bound per gram of dry matter, here SPF on a dry basis. Fig. 5-7 shows that the WHC of all the SPFs is in the range of 1.30 to 3.90 g water/g SPF, and the peak value occurs at SPF 4 which contains around 6.47 mg Ca/g protein. In general, the WHC of all the SPFs was lower than the reported WHC of commercial SPI, but the peak value is comparable with the WHC of commercial SPC (Geerts et al., 2018). The lower values could be due to differences in drying. Spray drying, as applied industrially probably leads to an increased WHC, due to the heat applied to the ingredients.

The WHC can be also defined based on the pellet weight. The WHC_P represents the amount of water bound per gram of insoluble pellet and thus accounts for the effect of solubility on WHC (Peters et al., 2017). As the soluble part of the protein does not contribute to the WHC_P, the protein ingredients with lower NSI could potentially have more insoluble components which can hold water. However, a sharp decline of WHC_P with the increase of Ca content in the SPFs was observed, which was similar to the trend of NSI. In other words, SPF 7 showed the lowest protein solubility under pH 7 as compared to other SPFs, while it also had the lowest value of WHC_P, which means the majority of the protein in the SPF 7 is not soluble and has the weakest ability to interact with water.
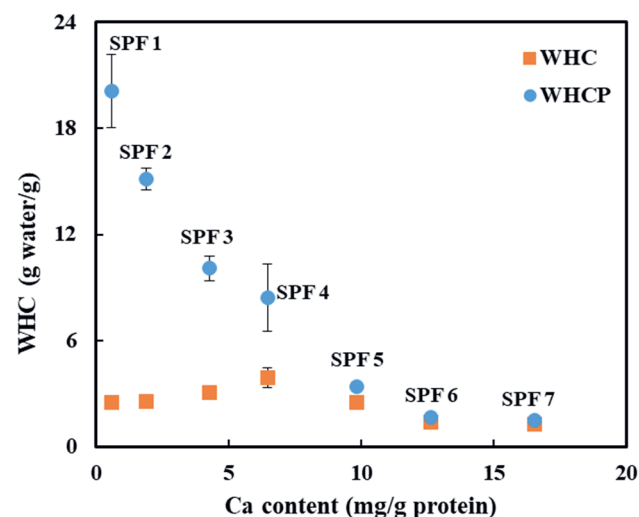
Fig. 5-7 *Water holding capacity (WHC) and water holding capacity of the pellet (WHC$_P$) of all the SPFs as related to the Ca content.*

5.3.9 Viscosity

All the SPF samples were prepared with 9 wt.% protein concentration (around 12 wt.% dry matter), and the flow curves are presented in Fig. 5-8. SPF 2 and SPF 3 presented the highest viscosity with a similar trend: a slight increase at very low shear rates suggesting shear-thickening, followed by a shear thinning behavior. The viscosities of SPF 1 and SPF 4 were similar, while the shear thinning behavior was also observed once the shear rate was higher than 1 s^{-1}. As for the SPF 5, SPF 6, and SPF 7, data points with torque values at or below the manufacturer reported sensitivity was omitted, and the viscosity measured at high shear rates was in the range of 0.001 to 0.01 Pa.s, almost approaching the reported dynamic viscosity of water (0.89 mPa.s, 25°C) (https://wiki.anton-paar.com/nl-en/water/).
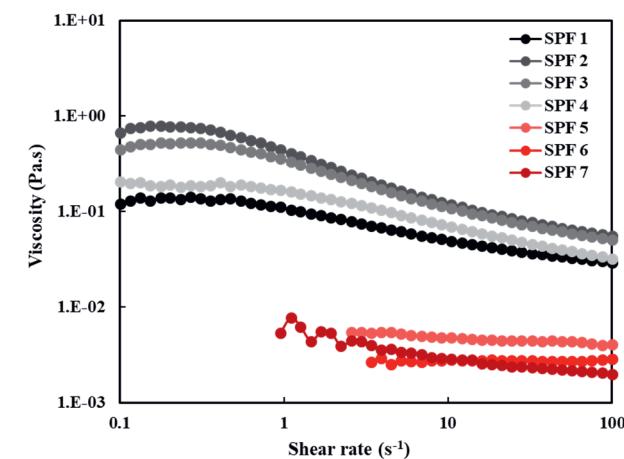

Fig. 5-8 *Viscosity curves of the SPFs (9 wt.% protein concentration) as related to the shear rate.*

5.3.10 Gelation behaviors

The gelation behaviors of all the SPFs upon and after heating treatment were measured and are shown in Fig. 5-9. The left panels present the results of the temperature sweeps, in which two types of curves were observed with an increase in temperature from 20 to 95°C. For SPF 1, SPF 2, SPF 3, and SPF 4, a decrease of G' and G" occurred first, followed by a steep increase. However, for SPF 5, SPF 6, and SPF 7, the curve began with an increase of G' and G", followed by a slight decrease and a sharp increase afterward.

From SPF 1 to SPF 5, the G' was initially lower than G", so the gelation temperature (T_{gel}) for these SPFs can be detected as the crossover temperature where G' exceeds G" (Table 5-3). The T_{gel} of SPF 1 was around 64.54°C, while the peak T_{gel} value was observed at SPF 4, which was approximately 94.93°C. By contrast, the G' of SPF 6 and SPF 7 was constantly higher than G", indicating a solid-like structure was formed before heating already. Therefore, T_{gel} was not reported in the table. Similarly to the trend of viscosity, the initial values of G' and G" for SPF 1, SPF 2, SPF 3, and SPF 4 were higher than SPF 5, SPF 6, and SPF 7. Besides, the gel firmness for all the SPFs increases upon cooling.

The G' and G" related to the frequency were examined through a frequency sweep in a range of 0.1-10 Hz (middle panels). For all the SPFs, the G' and G" remained generally steady over this frequency range. After frequency sweep, the length of the linear viscoelastic (LVE) regime was determined by a strain sweep in the range of 0.1-1000% with a constant frequency of 1 Hz (right panels). It was found that with the increase of the Ca content in the

SPF, the LVE regime of the corresponded gel became shorter. In other words, SPF 1 has the longest LVE regime, whereas SPF 7 has the smallest maximum linear strain. These results indicate that the gel network structure of SPF 7 can be easily disrupted as compared to other SPFs, while SPF 1 gave the strongest gel.
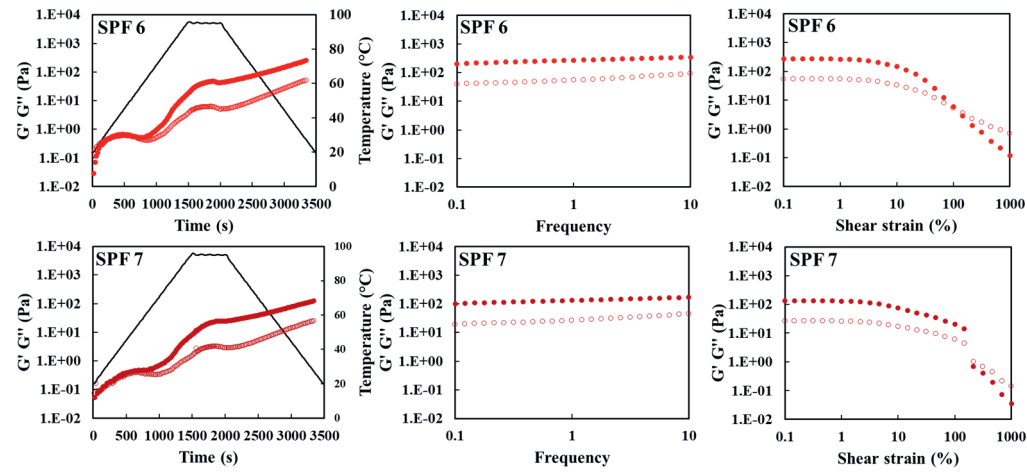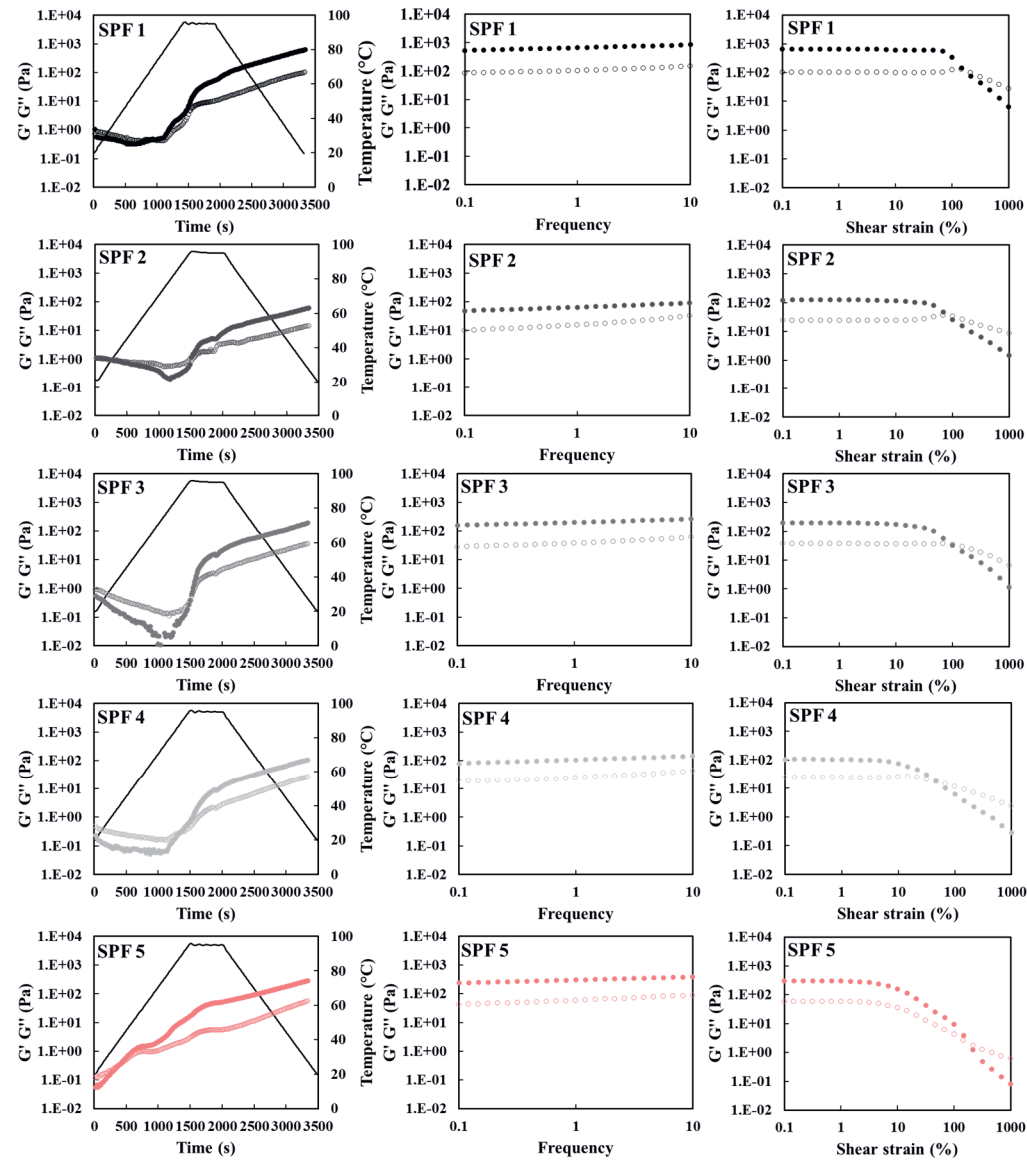
Fig. 5-9 *Temperature (1st column), frequency (2nd column), and strain sweep (3rd column) sequentially applied on all the SPFs (9 wt.% protein concentration). G': closed symbols, G'': open symbols.*

Table 5-4 *The gelation temperature of the SPFs.*

Sample name	Ca content (mg/g protein)	T_{gel} (°C)
SPF 1	0.57 ± 0.06 [g]	64.54 ± 2.59 [d]
SPF 2	1.90 ± 0.12 [f]	91.87 ± 0.52 [b]
SPF 3	4.28 ± 0.02 [e]	94.93 ± 0.54 [a]
SPF 4	6.47 ± 0.40 [d]	80.33 ± 5.10 [c]
SPF 5	9.84 ± 0.41 [c]	40.22 ± 1.58 [e]
SPF 6	12.62 ± 0.29 [b]	\
SPF 7	16.55 ± 0.66 [a]	\

*The values in the table are compared in columns and different letters indicate a significant difference (P < 0.05).

5.4 Discussion

In this study, SPFs were obtained through a simplified fractionation process and neutralized by different amounts of NaOH and $Ca(OH)_2$ during the process. Since the neutralization step was performed after the protein extraction stage, the protein and oil content of all the SPFs were quite similar (Table 5-2). The Ca and Na content in the SPFs varied based on the ratio of $Ca(OH)_2$ and NaOH used during the protein neutralization step (Table 5-1), which provides the possibility to control the Ca and Na content precisely in the ingredients via the fractionation process. From SPF 1 to SPF 7, the Ca content in the SPF gradually increased from 0.57 to 16.55 mg/g protein, while the Na content decreased from 15.91 to 0.81 mg/g protein. It was observed that the Ca content was more profound than Na in affecting the morphology, charge density, and functionalities of the SPFs. Hence, the following discussions mainly focused on the influences lead by the Ca content.

The powder morphology of the SPF changed from lamellar glass-like structure to pumice stone structure when higher Ca content presented in the SPF (Fig. 5-2). These findings were in line with the previously reported observations, in which freeze-dried SPI had a sheet-like flaky structure (Hu et al., 2013), while Ca-fortified SPI showed well defined honeycomb-like network with a certain regularity (Lee and Rha, 1978). When the Ca concentration increased, the structure of soy protein became more porous and compact, and the surface was not as smooth as that obtained with lower Ca concentration (Kao et al., 2003).

The overall decreasing amount of helix elements for SPF with high Ca content observed in the FT-IR (Fig. 5-4) reveal some minor structural rearrangements of the proteins between Na and Ca additions. Previous studies reported that an increasing concentration of Ca ions likewise decreased the α-helix band of bovine serum albumin due to the direct binding of the metal ions to the protein (Alhazmi, 2019). Besides that, we also observed an increase in the 1619 cm^{-1} region at higher Ca levels (9.8 mg/g Ca, SFP 5, and above) in the present study, which hints at protein-protein interactions probably mediated by cross-linking with the bivalent metal ion (Alhazmi, 2019). Stronger protein-protein interactions are also consistent with the increasing particle size of SPF 5 to SPF 7 observed with static light scattering (Fig. 5-3). The 7S and 11S soy proteins each may have a different affinity for Ca binding and precipitation. Previous research found that the 11S fraction of soybean protein can be preferentially precipitated with Ca ions prior to 7S (Mitsuda et al., 1965), and the binding of Ca ions is highly dependent on the pH (Appurao, A. G., 1975). It was evidenced

that there are two types of binding sites for Ca ions on the protein molecules: the imidazole group, and the side-chain carboxyl groups of the aspartic and glutamic acid residues. 11S exhibits a higher affinity with a larger number of binding sites for the Ca ions, while 7S is a smaller protein and has a higher charge density (Kroll, 1984; Sakakibara and Noguchi, 1977). Besides, the precipitations of 7S and 11S begin after a threshold concentration of Ca ions is reached, while the number of Ca ions required to precipitate 7S was much larger than the amount needed for 11S (Yuan et al., 2002).

It is likely that also affects the tested functional properties of the SPFs. The pI measured for all the SPFs was around pH 5 (Fig. 5-5), which was in line with previously reported data showing a pI of soy protein to be between 4.5 and 5 (Jaramillo et al., 2011; Preece et al., 2017; Thrane et al., 2017). The decrease in electrostatic repulsions at the pI is a property that was used in this study to extract soy protein by isoelectric precipitation. When the pH was below the pI, the soy proteins were positively charged and likely repulse Ca or Na ions. As a result, the zeta potential variation of all the SPFs was similar and not influenced by the Ca and Na contents. By contrast, when the pH was above the pI, all the SPFs were negatively charged but varied between -18 mV (high Ca content) and -25 mV (low Ca content). Both Ca and Na ions could shield the electric charges of soy protein by physical attraction (Yuan et al., 2002), but Ca ions are divalent cations that lead to stronger attractive interactions. Thus, the SPFs with higher Ca content would gain fewer negative charges at a given pH higher than pI. This hypothesis is in agreement with earlier studies where pH values between pI to pH 7 increased soy protein-bound Ca ions, while at pH 7 and above, little change in bound Ca ions were seen (Kroll, 1984). Similarly, in the present study, the absolute value of zeta potential increased sharply from pH 5 to pH 7 for all the SPFs and approached a plateau in the range of pH 7 to 9.

Generally, all the SPFs exhibited higher NSI values at pH 3 than pH 7 except for SPF 1, which indicated that the effect of Ca content on the NSI of soy protein was highly dependent on the pH and the Ca-binding was reversible to a certain extent (Fig. 5-6). Therefore, the protein solubility changes caused by pH shift should be taken into consideration for further product development. Besides, SPFs with higher Ca content presented larger particle size and lower NSI at pH 7. These might be attributed to both hydrophobic and electrostatic interactions between soy protein molecules, leading to compact aggregates. The latter is further enhanced by the lower net charge that weakens electrostatic

repulsion between proteins and lowers the solubility of SPFs. The greatest changes in particle size and NSI happened between SPF 4 and SPF 5, in which the Ca content increased from 6.47 to 9.84 mg/g protein. This might be caused by the precipitation of 7S. It was hypothesized that the Ca content in SPF 5 was sufficient to precipitate 7S, leading to the greatest changes in protein structure (FT-IR), particle size, and NSI. The differences in the precipitation of 7S and 11S of soy protein allow the production of 7S-rich fraction and 11S-fraction by utilizing calcium salts as fractionating agents with optimal concentration (Deak et al., 2007, 2006; Teng et al., 2009).

In addition, the change of Ca(OH)$_2$ and NaOH ratio modified the thermal behavior of 7S and 11S differently. A stabilization of 7S and 11S (increase in T_d) in SPF was induced by the increase of the Ca content, but a lower Ca content is required to change the T_d of 11S than that of 7S. The T_d of 7S started to increase significantly when the Ca content changed from 6.47 to 9.84 mg/g protein (SPF 4 to SPF 5), while the T_d of 11S started to change when the Ca content was above 0.57 mg/g protein. Similar results were reported in the literature, where a Ca level above 5 mg/g in SPI was found to protect the soy proteins and partially prevent their denaturation (Scilingo & Añón, 1996). The addition of Ca ions in SPI dispersions produced higher T_d of both 7S and 11S, and increased the enthalpy of denaturation, mainly the 11S fraction (Scilingo & Añón, 2004). These findings can be linked to the protein aggregation discussed above, and the cross-linking effects probably increase the thermal stability of the SPFs.

The greatest change of viscosity and gelation behaviors of all the SPFs was also observed between SPF 4 and SPF 5 (Fig. 5-8). The viscosities of SPF 5, SPF 6, and SPF 7 measured at high shear rates were similar and much lower than SPF 4. The viscosity of high-Ca SPFs was close to the viscosity of water, which hinted that the larger aggregates induced by high-Ca content became denser and insoluble, and therefore, rarely interacted with water and contributed to the viscosity afterward. This hypothesis can be confirmed by the results of NSI and WHC$_P$. With the presence of higher Ca content, the decrease of NSI and WHC$_P$ values indicated that the insoluble aggregations had limited ability to hold water, which means the volume of the aqueous phase was not highly influenced. As a result, the viscosity of SPF 5, SPF 6, and SPF 7 was much lower than the other SPFs.

The gelation ability of soy proteins is important for the textural properties of food systems. Soybean proteins can form heat-induced and cold-set gels (Zheng et al., 2019).

Generally, G" is greater than G' when a gel network structure is not formed, while when G' is greater than G", this suggests that a gel network structure has formed. For SPF 1, SPF 2, SPF 3, and SPF 4, the G' and G" declined as the temperature rose during the initial stage of heating (Fig. 5-9). As heating continued, a rapid increase of G' and G" was observed, which originates mainly from protein denaturation and network formation. Similar gelation curves were also observed from SPI upon the heating (Li et al., 2020). The gelation temperature (T_{gel}) was around 65.54°C for SPF 1, and increased to around 94.93°C for SPF 3. This trend was in line with the uptrend of T_d for the SPFs (Table 5-3). However, the T_{gel} of SPF 4 and SPF 5 was dropped to 80.33°C and 40.22°C, respectively. This result can be linked to the PSD of the SPF dispersions (Fig. 5-4). The PSD of SPF 1, SPF 2, and SPF 3 was similar and single-peaked, while for SPF 4 and SPF 5, the PSD became wider towards the right side, which probably indicates rearrangements of Ca bridges and the formation of larger aggregations. An earlier study reported that the soybean protein aggregation promoted by Ca allows the gelation of proteins that were less denatured (Speroni et al., 2010). For SPF 6 and SPF 7, the G' was higher than G" even without heating. This means the gels were formed under room temperature, suggesting the formation of Ca-induced cold-set gels. Previous research reported that a cold-set gel of soy protein can be formed depending on the protein concentrations and CaCl$_2$ level (Maltais et al., 2005). In the presence of a certain amount of Ca, the additional cross-linking could promote the formation of salt bridges between protein aggregates, allowing them to form a space-filling network at an early stage of protein unfolding (Lu et al., 2010).

Even though SPFs with relatively high Ca content (SPF 4 to SPF 7) can form gels at lower or even at room temperature, the LVE regime of the corresponding gels was shorter with the increase of the Ca content, which means that the gel broke at smaller deformation. The results of PSD, NSI, and WHC$_P$ (Fig. 4, 6, and 7) suggested that the larger Ca-induced aggregates were quite dense and inert, which could lead to the reduction of gel strength. Additionally, the lower net charge of SPFs with higher Ca content may shield electrostatic interactions at pH 7 between protein aggregates (Fig. 5-5), resulting in a weaker gel network. Other researchers also reported that the addition of Ca softened the gel strength of soy milk yogurt (Yazici et al., 1997).

5.5 Conclusion

In this study, a simplified fractionation process was employed using different alkali solutions with ranging NaOH and $Ca(OH)_2$ contents in the neutralization step. SPFs were produced with around 75%wt protein and increased Ca and reduced Na content at different levels. Ca enrichment gradually promoted the formation of larger particles, increased the thermal stability, reduced the NSI, WHC_P values, and changed the gelation properties of the SPFs. These effects were highly dependent on the Ca content in the SPFs. Although the morphology and functionalities of SPFs varied greatly at a high Ca level, at a lower Ca enrichment (< 6.5 mg/g protein) they were affected to a limited extent. This indicated that a critical Ca and Na level can be achieved for simply fractionated soy protein without causing profound changes in its conformation and functional properties. Thus, Ca enrichment during the fractionation process can provide the possibility to increase the Ca content and reduce Na content of soy protein ingredients without compromising their use for novel soy-based foods.

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Chapter 6

Characteristics of soy protein

prepared by aqueous ethanol washing

process

Abstract

Currently, the predominant process for the production of soy protein concentrate (SPC) is aqueous ethanol washing of hexane-extracted soy meal, through which the oil and soluble carbohydrates are mostly removed. However, the use of hexane is less desired, explaining the increased interest in cold-pressing for oil removal. Therefore, cold-pressed soy meal was used as the starting material in this study, and a range of water-ethanol ratios was used for the washing process to produce soy protein-rich fractions (SPFs). The effect of this so-called aqueous ethanol washing processes on the composition and the functionalities of SPFs was investigated. Washing enriched the protein content for the SPFs, regardless of the solvent used. However, all results considered, we conclude that washing with only water or solvents with high ethanolic ratio can be more advantageous. Washing with high ethanolic ratio (60% and above) gave the highest yield in protein, as well as SPFs with the highest protein solubility and water holding capacity. Water-washed SPF (0% ethanol) showed the highest viscosity, and it formed gels with the highest gel strength and hardness among all the SPFs at similar protein concentration. The variations in the functionality among the SPFs were attributed in changes of protein, though effects of non-protein constitutes such as sugar and oil might also played a role. Overall, the aqueous ethanol washing combined with cold-pressed soy meal created SPFs comparable to SPC in terms of composition, but with a range of functionalities that are relevant for the development of novel soy-food applications.

6.1 Introduction

Soybean has been recognized as a powerhouse of nutrients, rich in protein and oil, as well as carbohydrates (Liu, 2004). After fractionation, various protein-rich ingredients can be obtained from it, which have gained great popularity due to their high nutritional value and versatile functional properties (Hoffman and Falvo, 2004). Three forms of soy protein-rich ingredients are commercially available according to the protein content ($N \times 6.25$), namely: soy flour (50-65%), soy protein concentrate (SPC, 65-90%), and soy protein isolate (SPI, > 90%) (FAO, 1989). Among them, SPC was the last one to be developed; however, it nowadays provides broader application range than soy flour, and is cheaper than SPI (Kinsella et al., 1985). While the production of SPI uses alkaline conditions to maximally solubilize protein, SPC is made under conditions where the majority of the soy proteins become insoluble allowing the non-protein constituents to be washed away (Preece et al., 2017). Different washing processes developed for the production of SPC include: water washing of heat-denatured soy meal, acid washing at the isoelectric pH, and aqueous ethanol washing (Chajuss, 2004). Aqueous ethanol washing gradually became the predominant process because high yields could be attained, and the obtained SPC is relatively bland in taste with less beany flavors (Chajuss, 2001). Moreover, the process was found to eliminate the allergenicity of soy proteins (Hancock et al., 1990).

Aqueous ethanol washing is based on the ability of aqueous ethanol solutions to extract soluble fractions such as free sugars and other low-molecular-weight constitutes of de-fat soy meal (DFSM) without solubilizing the soy protein. DFSM, a by-product of the soy-oil production, is created usually after hexane extraction of soybeans. The use of hexane leads to extraction yields of crude oil greater than 95% (Johnson and Lusas, 1983). However, hexane is highly explosive and nowadays considered as toxic. The concerns about its safety and emission restrictions have stimulated interest in alternative defatting techniques (L'hocine et al., 2006). Among those, cold pressing is regarded as a rapid and environmental-friendly technique without using the toxic solvents (Koubaa et al., 2016). Besides, the relatively low operation temperature can preserve the protein nativity (Fetzer et al., 2018). Nevertheless, cold pressing is not so efficient in oil extraction as the hexane extraction.

The research to date focusses on using aqueous ethanol washing process to produce SPC with low content of residual oil and sugars, while hexane-extracted soy meal is

commonly used as the starting materials. To the best of our knowledge, limited information is available on the use of aqueous ethanol washing in combination with cold-pressed soy meal, especially the effect of water-ethanol ratios on the overall composition and thus the functionality of soy protein ingredients. Therefore, in this study we used cold-pressed DFSM as the starting materials instead of hexane-extracted DFSM. Besides, the potential to use a water-only washing process is not sufficiently described as a way to create soy protein ingredients under clean label . We hypothesize that the protein, oil and carbohydrate content of the produced fractions, as well as the final functionality can be tuned by choosing specific water-ethanol ratios. To prove this, we used aqueous ethanol washing with different ethanolic ratios (0-100%) to produce soy protein-rich fractions (SPFs) with varying composition. The composition, yield and functional properties such as solubility, water holding capacity, thermal stability of the SPFs were investigated. Besides, the rheological properties of the SPFs were evaluated with multiple protein concentrations (6, 9 and 15 wt.%). Since the developed process leaves some oil in the SPFs, we also measured the primary and secondary oil oxidation products to evaluate the oil-containing soy protein ingredients. The objective of this research was to examine the current process for SPC and adjust it for producing hexane-free or even completely organic solvent-free soy protein ingredients with a range of compositions and functionalities.

6.2 Materials and methods

6.2.1 Materials

De-fat soy meal (DFSM) obtained after mechanical cold pressing was provided by Avril group (France). No organic solvent was used for de-fatting treatment according to the supplier. The DFSM contained 43.1% ± 0.6% of protein and 10.3% ± 0.9% of oil on a dry basis. Commercial SPC was obtained from Solae (Europe S.A.).

For aqueous ethanol washing and composition analysis, 96% ethanol and petroleum ether were purchased from EMD Millipore Corporation (Germany). For phenol content analysis, Folin-Ciocalteau (FC) reagent was obtained from MP Biomedicals (France). Sodium carbonate (anhydrous ≥ 99.5% ACS) was obtained from VWR International (Germany).

For oil oxidation analysis, iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), cumene hydroperoxide solution (80%), n-hexane, sodium chloride (NaCl), para-anisidine were purchased from Sigma-Aldrich (U.S.A); hydrochloric acid (37%), acetic acid (glacial), barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$), ammonium thiocyanate (NH_4SCN), 2-propanol, 1-butanol were obtained from Merck Millipore (Germany); methanol was purchased from Actu-All Chemicals B.V. (The Netherlands).

The ultrapure water used in this study was purified with a Milli-Q Lab Water System (Milli-Q IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany).

6.2.2 Aqueous ethanol washing process

A scheme for the aqueous ethanol washing process can be found in Fig. 6-1. Aqueous ethanol solutions were prepared in advance by mixing water with 96% ethanol in a Schott glass bottle with the right volume. The ethanol to the total solvent volume ratio is referred in this study as ethanolic ratios of 0%, 20%, 40%, 60%, 80% and 100%.

DFSM was mixed with aqueous ethanol solution in a solid: liquid ratio of 1:10 (w/v) and stirred at room temperature (25°C) for 30 min. Then, the dispersion was centrifuged (20,000 g, 30 min, 25°C) to separate the supernatant and pellet. The supernatant was collected as the extract, and the pellet was transferred to a fume hood overnight to evaporate the ethanol. Subsequently, the pellet was freeze-dried (Freeze Dryer, Martin Christ, Osterode, Germany) and milled into powder, which is reported in this study as dried soy protein-rich

fraction (SPF). All the extracts and SPFs processed with varied water-ethanolic ratios were prepared in triplicate and stored at 4°C for further analysis.
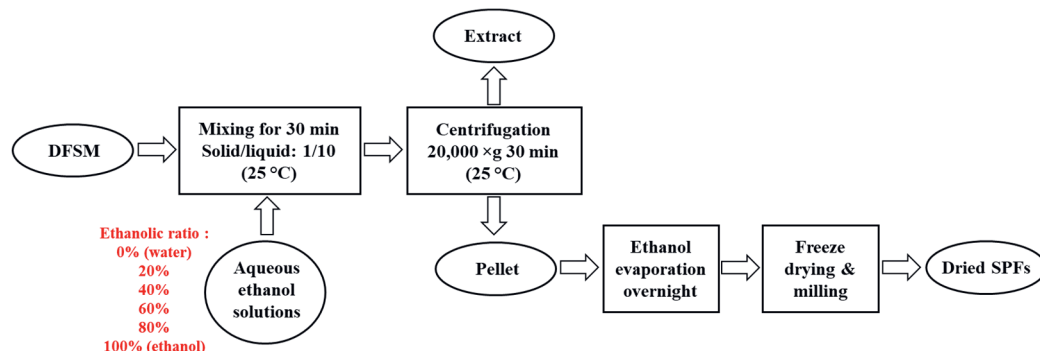

Fig. 6-1 The aqueous ethanol washing process for the SPFs.

6.2.3 Microscopic analysis

Scanning electron microscope (SEM, Phenom Pure G2, Phenom-world BV, Eindhoven, The Netherlands) was performed for viewing the microstructures of the SPF powders. The SPF was evenly placed on an aluminum sample holder using double-sided adhesive conductive carbon tape, and the microstructure was observed with an accelerating voltage at 5 kV.

6.2.4 Composition analysis and yield

The protein content of the FFSF and SPFs was determined by using a Nitrogen Analyzer (Flash EA 1112 series Dumas, Thermo Scientific, The Netherlands). A nitrogen-to-protein conversion factor of 5.7 was used (Krul, 2019). The oil content was determined by using petroleum ether as an extraction solvent with a Buchi extraction system B-811LSV (Buchi Labortechnik AG, Flawil, Switzerland). The sugar content was determined with the K-RAFGL assay from Megazyme (Megazyme International Ireland, Wicklow, Ireland), in which the method was adapted from McCleary et al. (McCleary et al., 2006). The total free sugars were the sum total amount of glucose, sucrose, and galactosyl-sucrose oligosaccharides (GOS). All measurements were performed in triplicate.

The dry matter yield was calculated using Eq. (6.1), while the protein yield was calculated according to Eq. (6.2):

$$Dry\ matter\ yield\ (\%) = \frac{Weight\ of\ dried\ SPF}{Weight\ of\ starting\ DFSM} \times 100\%\ Eq.\,(6.1)$$

$$Protein\ yield\ (\%) = \frac{Total\ protein\ in\ dried\ SPF}{Total\ protein\ in\ starting\ DFSM} \times 100\%\ Eq.\,(6.2)$$

6.2.5 Soluble protein molecule profile with SDS-PAGE

The protein molecular profile of all the SPFs and commercial SPC was characterized by SDS-PAGE. The soy protein samples were prepared with water to reach a concentration of 2 mg protein per ml in a falcon tube and were centrifuged (20,000 g, 30 min, 25°C). Afterwards, the insoluble parts were removed, and the supernatant of each fraction was used for the SDS-PAGE under reducing condition. Around 20 μl of each protein supernatant was mixed well with 1 μl β-mercaptoethanol and 20 μl Bio-Rad sample buffer (62.5 mM Tris-HCl, 25% v/v glycerol, 4% w/v SDS and 0.01% w/v bromophenol blue) and heated at 95°C for 10 min. After heating, the sample was centrifuged at 10,000 g for 1 min. Mini-protein gel was used while 5 μl of SDS-PAGE marker and 15 μl of the sample was loaded onto the gel wheels. The electrophoresis was performed at 200 V in a Mini-Protean II electrophoresis cell (Bio-rad, Veenendaal, Netherlands) with the diluted Bio-Rad running buffer (0.025 M Tris, 0.19 M glycine and 0.1% SDS). After approximately 40 min, the gels obtained after electrophoresis were stained using Bio-safe Coomassie Blue for around 1 h, and washed with water multiple times before transferring to gel scanner (Biorad-GS900, Veenendaal, Netherlands).

6.2.6 Oil oxidation: primary and secondary products

The oil oxidation level in the DFSM and SPFs was analyzed by measuring the primary (hydroperoxides concentration) and secondary (aldehydes) reaction products.

6.2.6.1 Hydroperoxide concentration

The hydroperoxide concentration (i.e., primary oxidation product) was measured according to the method described by Shanta et al. (Shantha and Decker, 1994) and Feng et al. (Feng et al., 2021) with slight modifications. Around 0.3 g of DFSM or SPFs was mixed with 1.5 ml n-hexane/2-propanol (3:1, v/v), vortexed 3 ×10 s with 20 s intervals and centrifuged at 10,000 g for 2 min. Subsequently, 0.2 ml of the supernatant was mixed well with 2.8 ml of the methanol/1-butanol (2:1, v/v) and 3.94 M ammonium thiocyanate/ferrous iron solution (1:1, v/v; 30 μl) for the reaction. After 20 min, the absorbance was measured at 510 nm with a DU 720 UV–visible spectrophotometer (Beckman Coulter, Woerden, the

Netherlands). The hydroperoxide concentration was calculated using a cumene hydroperoxide standard curve.

6.2.6.2 Para-anisidine value (pAV)

Total aldehydes (i.e., secondary oxidation product) were indicated by the para-anisidine value (pAV), using the AOCS Official Method (AOCS, 2017). Around 1 g of DFSM or SPCs was mixed with 1 ml saturated sodium chloride solution and 5 ml n-hexane/2-propanol (3:1, v/v), vortexed 3 ×10 s with 20 s intervals and centrifuged at 2000 g for 8 min. The absorbance of 1 ml of the supernatant (Ab) was measured at 350 nm using hexane as a blank. Subsequently, 1 ml of supernatant or hexane was mixed with 0.2 ml of 2.5 g/l para-anisidine in acetic acid solution. After 10 min, the absorbance (As) was measured at 350 nm, using hexane with para-anisidine solution as a blank. The pAV was calculated using Eq.(6.3), and the m is the mass of oil per ml hexane (g/ml):

$$pAV = \frac{1.2 \times As - Ab}{m} \quad Eq.(6.3)$$

6.2.7 Total phenolic content (TPC) in the extract

The total phenol content (TPC) in the extract was determined by using the Folin-Ciocalteu (FC) method described by Singleton, Orthofer, & Lamuela-Raventós (Singleton et al., 1999) with slight modification. Around 100 µl of sample taken from each extract was added to 7.9 ml of water and mixed well. Subsequently, 500 µl of FC reagent and 1.5 ml of 20% (w/v) sodium carbonate were added, mixed thoroughly with a vortex, and heated at 40°C for 30 min. The absorbance was measured at 750 nm with a spectrophotometer (DR3900 Laboratory VIS Spectrophotometer, Hach, USA). Different aqueous ethanol ratios were used as the blank. The TPC was calculated based on a calibration curve plotted with gallic acid (0.025 to 4 mg/ml) as a standard. The final results were expressed as mg of gallic acid equivalents (GAE) in per ml of extract (mg GAE/ml extract).

6.2.8 Particle size analysis

The particle size distribution (PSD) of the SPFs was measured with a laser light scattering instrument (Mastersizer 3000, Malvern Instruments Ltd, United Kingdom) and a wet module (Hydro SM, Malvern Instruments Ltd, United Kingdom). For these measurements, 1% (w/v) SPF dispersions were prepared with Milli-Q water. A refractive index of 1.45 was used for the dispersion phase and 1.33 for the water continuous phase.

6.2.9 Denaturation behaviors

Differential scanning calorimetry (Diamond DSC, PerkinElmer, USA) was used to measure the denaturation temperature and the enthalpy of the transition of the SPF samples. A 20% (w/w) protein dispersion of each SPF was prepared and placed in high-volume aluminum pans, which were then sealed. The differential scanning calorimeter was calibrated with indium, and the sample was scanned at 5°C/min from 20 to 150°C. An empty aluminum pan was used as a reference. Measurements were analyzed with Start Pyris Software for the denaturation temperature and enthalpy of transition. All the measurements were performed in triplicates.

6.2.10 Nitrogen solubility index (NSI)

The nitrogen solubility index (NSI) is routinely used to evaluate protein solubility (Wang and Johnson, 2001). A 2% (w/w) dispersion of each SPF sample was placed in a centrifuge tube and moderately shaken overnight. Subsequently, the sample was centrifuged at 18,670 g for 30 min to separate the supernatant and pellet. The pellet was oven-dried at 105°C and weighed. The nitrogen contents in the oven-dried pellets were measured by using Dumas analysis. The NSI was calculated by the ratio of soluble nitrogen over the total initial nitrogen content present in the SPFs. All the measurements were performed in triplicate.

6.2.11 Water holding capacity (WHC) and oil absorption capacity (OAC)

The water holding capacity (WHC) of SPFs was measured using the method described by Peters (Peters et al., 2017) with slight modification. A 2% (w/v) SPF dispersion was prepared with Milli-Q water and shaken overnight. Then, the dispersion was centrifuged at 18,670 g for 30 min to separate the supernatant and the wet pellet. The wet pellet was collected and weighed. Subsequently, the wet pellet was oven-dried overnight at 105°C and weighed again to determine the mass of the dry pellet. The WHC was calculated according to Eq. (6.4), and the WHC of the pellet (WHC$_P$) was determined according to Eq. (6.5).

$$WHC \left(\frac{g\ water}{g\ dry\ SPF}\right) = \frac{M_{wet\ pellet} - M_{dry\ pellet}}{M_{dry\ SPF}} \quad Eq.(6.4)$$

$$WHC_P \left(\frac{g\ water}{g\ dry\ pellet}\right) = \frac{M_{wet\ pellet} - M_{dry\ pellet}}{M_{dry\ pellet}} \quad Eq.(6.5)$$

The oil absorption capacity (OAC) of SPFs was measured according to a previously described method (Lin et al., 1974) with minor modifications. Around 0.5 g of the SPF and 10.0 ml of sunflower oil were added to a centrifuge tube, and mixed for 3 min with a vortex mixer to disperse the sample into the oil. After a holding period of 30 min, the tube was centrifuged for 25 min at 3050 *g*. The separated oil was then removed with a pipette, and the tube was inverted for 25 min to drain the oil before reweighing. The OAC was expressed according to Eq. (6.6). All the measurements were performed in triplicate.

$$OAC \left(\frac{g\ oil}{g\ dry\ SPF}\right) = \frac{M_{pellet} - M_{dry\ SPF}}{M_{dry\ SPF}}\ Eq.\ (6.6)$$

6.2.12 Viscosity analysis

SPC dispersions were prepared and stirred for around 1 h before the viscosity measurement. The protein concentration for all the dispersions was standardized on 6 wt.% according to different protein content in the SPFs (Table 6-1). A stress-controlled rheometer (Anton Paar Physica MCR 301, Graz, Austria) combined with a sand-blasted CC-17 concentric cylinder geometry was used to determine the viscosity. The SPF dispersion was equilibrated for 5 min, and a shear rate sweep was performed at 25°C from 0.1 to 100 s^{-1}, and back to 0.1 s^{-1} again. 100 data points were collected using a constant interval of 20 s between points during the measurements.

6.2.13 Gelling behaviors

A stress-controlled rheometer (Anton Paar Physica MCR 301, Graz, Austria) combined with a sand-blasted CC-17 concentric cylinder geometry was used. SPF dispersions standardized on 9 wt.% protein concentration were prepared and stirred for 1 h before the measurements (Table 6-1). A thin layer of high-temperature-resistant silicone oil was covered on top of the samples to prevent water evaporation during heating. A temperature, frequency, and strain sweep were performed sequentially. The temperature sweep was done by increasing the temperature from 20 to 95°C at a rate of 3°C /min, followed by 10 mins at 95°C before cooling back to 20°C at a rate of 3°C /min. Subsequently, the samples were exposed to a frequency sweep from 0.01 to 10 Hz (at a strain of 1%), and a strain sweep from 0.1 to 1000% (at a frequency of 1 Hz). The storage (G') and loss modulus (G") dependency on temperature, frequency, and strain were recorded.

6.2.14 Textural analysis of soy protein gels

To make the soy protein gels, 15 wt.% protein concentration was selected based on preliminary experiments to make sure all the SPFs can form a firm gel (Table 6-1). SPF was mixed with water and hydrated for 30 min before transferring to stainless steel gelation vessel (internal height 5 mm, radius 12.5 mm). The vessel was hermetically sealed and heated in a water bath of 95°C for 30 min. After heating, the vessel was cooled by running water for 15 min and subsequently the gel was removed.

Texture profile analysis (TPA) was carried out on gels using a Texture Analyzer (Instron, Norwood, U.S.A) under room temperature. Double compression mode was exerted by a cylindrical probe with a flat section (diameter 75 mm) at a displacement speed of 1 mm/s. The compression distance was 3 mm (60%) while the relaxing time between two compressions was 30 s. The tests generated a plot of force vs. time, from which the hardness, springiness, cohesiveness and chewiness were calculated.

Table 6-1 Sample preparation to obtain the SPF samples with 6, 9, and 15 wt.% protein concentration.

| Ethanolic ratio	Protein content of SPF, db.%	Solid content of the SPF samples, %		
		6 wt.% protein concentration	9.wt% protein concentration	15.wt% protein concentration
0%	46.48 ± 0.29	12.91 ± 0.08	19.36 ± 0.12	32.27 ± 0.20
20%	56.43 ± 0.47	10.63 ± 0.09	15.95 ± 0.13	26.58 ± 0.22
40%	59.49 ± 1.11	10.09 ± 0.19	15.13 ± 0.28	25.22 ± 0.47
60%	57.36 ± 0.60	10.46 ± 0.11	15.69 ± 0.16	26.15 ± 0.27
80%	52.10 ± 0.92	11.52 ± 0.20	17.27 ± 0.31	28.79 ± 0.51
100%	50.70 ± 0.21	11.83 ± 0.05	17.75 ± 0.07	29.58 ± 0.12
Commercial SPC	61.68 ± 0.00	9.73 ± 0.00	14.59 ± 0.00	24.32 ± 0.00

6.2.15 Statistical analysis

Data were collected from triplicate experiments or in specified cases from more experiments. IBM SPSS Statistics Version 23.0 was used to analyze the variance, and Duncan's test was performed to determine the statistical significance between samples at an α level of 0.05. All the results were displayed by mean values ± standard deviations.

6.3 Results and discussion

### 6.3.1 Protein content, yield, composition and microstructure

The powder morphologies of the DFSM and all the SPFs were visualized with SEM (Fig. 6-2). Before the washing process, cold-pressed DFSM presented a globular and compact structure, with some small fragments on the surface. After washing with water (0% ethanol), the surface of SPF was irregularly wrinkled with spongy porous structure. This feature was possibly attributed to the predominant insoluble carbohydrates such as cellulose and hemicellulose that remained after washing (He et al., 2017). Thus, this can be taken as a sign for both protein loss and removal of other soluble components. When the ethanolic ratio was 20% and 40%, some attached spherical shapes were observed on the cratered surface of SPFs. These may be the protein bodies released from cellular matrixes (Jia et al., 2021). The use of a high ethanolic ratio (80% and 100%) resulted in a dense structure with no obvious open pores on the surface.
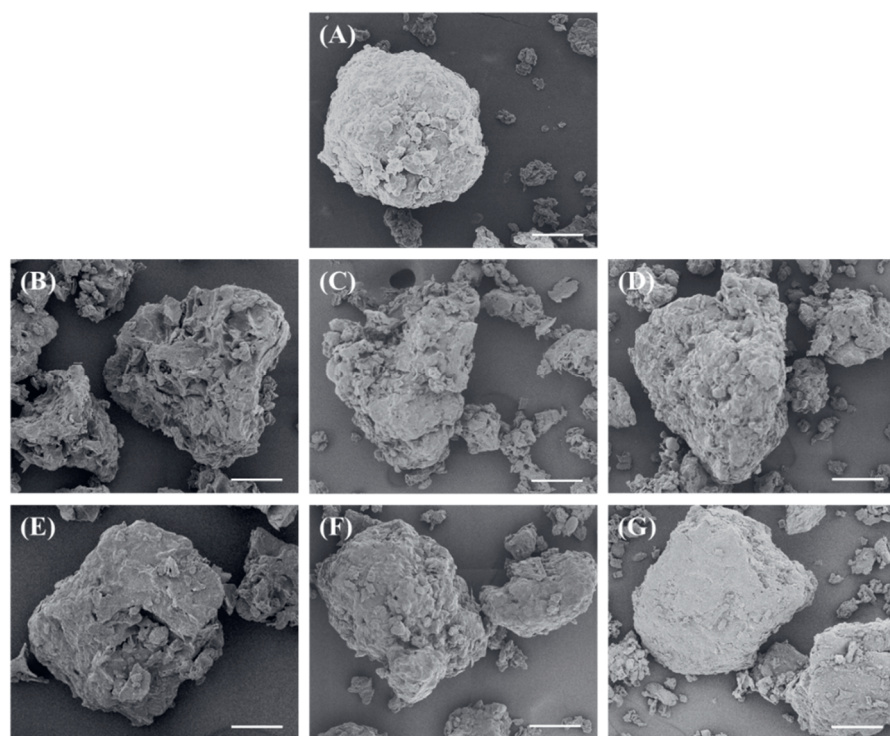
Fig. 6-2 *SEM images for the (A) DFSM and (B–G) aqueous ethanol washed SPFs with ethanolic ratio of 0%, 20%, 40%, 60%, 80% and 100%. The scale bar is 50 μm.*

The obtained SPFs presented higher protein content (between 46.48 and 59.49%) than the start material DFSM (43.14%), confirming that the aqueous ethanol washing processes can enrich the protein in the ingredients (Fig. 6-3A). With the increase of ethanolic ratio from 0 to 100%, the protein content of SPF increased until it reached a maximum with 59% protein at 40% ethanol and then decreased again. This high protein concentration approaches the measured protein content of commercial SPC (61.68%). By contrast, a protein enrichment of only 3.4% (from 43.1% to 46.5%) was achieved by washing with only water (0% ethanol). Previous research applying the aqueous ethanol washing process to de-fat sunflower kernels reported a similar change in protein content with the increase of ethanolic ratio (0-100%) (Jia et al., 2021). The uptrend of protein content may result from the removal of oil and sugars, while the downtrend might due to the low polarity of ethanol, which influences the protein extractability in the solvent (Boulet et al., 2001). Washing with water also induced the SPF with the lowest dry matter yield and protein yield, which may indicate the highest loss of both protein and non-protein constitutes. The highest yield was found once 80% ethanol was applied, being around 81% for the total mass and 96% for the protein. By interpreting these results after taking into consideration of the SEM-pictures, it seems that an open structure could be a result of protein loss. This means that the remaining insoluble carbohydrates become better visible (Fig. 6-2).

The protein compositions of all the obtained SPFs and commercial SPC were analyzed by SDS-PAGE under reducing conditions (Fig. 6-3B). Similar bands were observed in all the samples, which were identified as 7S α, α' and β subunits; 11S A3 subunit, acidic and basic proteins according to the mass (kDa) (Iwabuchi and Yamauchi, 1987). 7S and 11S are the two main types of storage protein in soybean (Badley et al., 1975), and similar protein profiles were found also in other soy products, such as soy flour (Liu and Kuo, 2016), soymilk (Hsiao et al., 2015; Hsieh et al., 2012), SPC and SPI (Duque-Estrada et al., 2019). Besides, washing with high ethanol level achieved SPFs with more low-molecular-weight subunits and less high-molecular weight proteins. This means that the average molecular weight of the protein present in the SPF decreases when washing with higher ethanolic ratios.
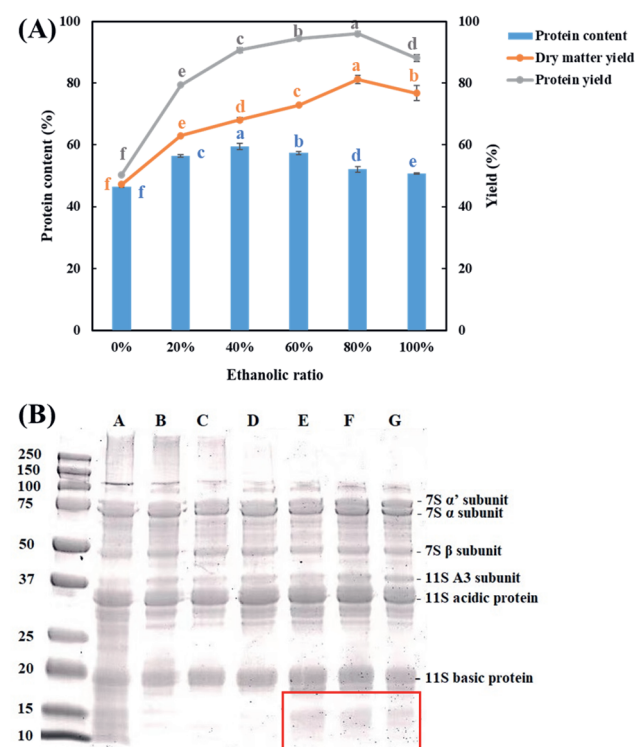
*Fig. 6-3 (A) Protein content, protein yield and dry matter yield of all the SPFs. *The values in the figure are compared and different top letters indicate a significant difference (P < 0.05). (B) SDS-PAGE electrophoresis of soluble proteins in soy samples. Lane A: commercial SPC; Lane B-G: SPFs obtained by washing process with the ethanolic ratio of 0%, 20%, 40%, 60%, 80% and 100%.*

6.3.2 Oil content and oil oxidation

The DFSM used in this study was obtained after cold pressing, without using hexane solvents. It contained around 10.3% of residual oil before washing, which was significantly higher than the DFSM obtained after intensive hexane extraction (~0.15%) (Nieh and Snyder, 1991). Aqueous ethanol washing extracted part of the oil from the DFSM, and the oil content of SPF became lower with the increase of ethanolic ratio (Fig. 6-4A). The lowest oil content in SPF was around 2.13% with an ethanolic ratio of 100%, confirming that the ethanol can work as an efficient oil-extracting solvent (Gandhi et al., 2003). However, less than 1% of oil was removed by water (0% ethanol) and approximately 9.41% of oil was left in the SPF. The use of other ethanol-water ratios removed roughly half of the oil.

The oxidation level of the oil that remained in the SPF, should remain low, because too high oxidation can produce rancidity and potential toxicity, leading to off-flavors (Schaich et al., 2012), and thus limiting applicability. Moreover, oxidized oil may result in

co-oxidation of protein, and thus negatively affecting the protein functionality (Meissner et al., 2020). Fig. 6-4C and 4D presents the primary and secondary oil oxidation products, respectively. The hydroperoxide concentration in the starting material DFSM was around 0.39 meq/kg of oil, lower than the concentration of hydroperoxide in hexane-extracted soy oil (0.60 meq/kg) (De Moura Bell et al., 2013). The pAV value of oil in the DFSM, reflecting the content of secondary oil oxidation products such as aldehyde content, was higher than the value of oil in the fresh full-fat soy flour (1.13) (Duque-Estrada et al., 2020), as well as solvent-extracted soy oil (1.88) (De Moura Bell et al., 2013). In general, combination of the results of PV and pAV indicated that aqueous ethanol washing process increased the oil oxidation in the SPFs. Among all the ethanolic ratios, the SPF prepared with 20% ethanol showed the highest amount of primary and secondary oil oxidation products. It also exhibited obvious oil oxidized off-odor, while the oil oxidation level in all the other SPFs is low and acceptable.

There are several reasons for the high oxidation values found after washing with 20% ethanol: one cause could be the low remaining phenol content in that SPF, which reduces the anti-oxidant capacity of the total sample (Bellaloui, 2012). Many phenolic compounds and plant phenolic extracts have been demonstrated to retard oil oxidation in different foods (Maqsood et al., 2014). This is, though, not a full explanation because the use of only water as solvent removed even more phenolic compounds (Fig. 6-4B), while the oxidation level was much lower in this SPF. There are, however, other possible explanations. It has been reported that the activity of lipoxygenase (LOX) is highly correlated to the oil oxidation level, resulting in the generation of odor compounds in soybean and soy-foods (Wolf, 1975). An early study investigated the effects of ethanolic soaking on the LOX activity in soybean, and reported that ethanol ratio 30-50% was more effective for inhibiting lipoxygenase than 10-30% (Borhan and Snyder, 1979). Herein, the SPF washed with 20% ethanol may have higher LOX activity than other SPFs, leading to the highest oil oxidation level. Another explanation might be that 20% ethanol washed mostly non-oxidized oil (intact oil bodies), so that only the oxidized oil remains in the SPF. Further analyses should be conducted to better understand the underlying reason.
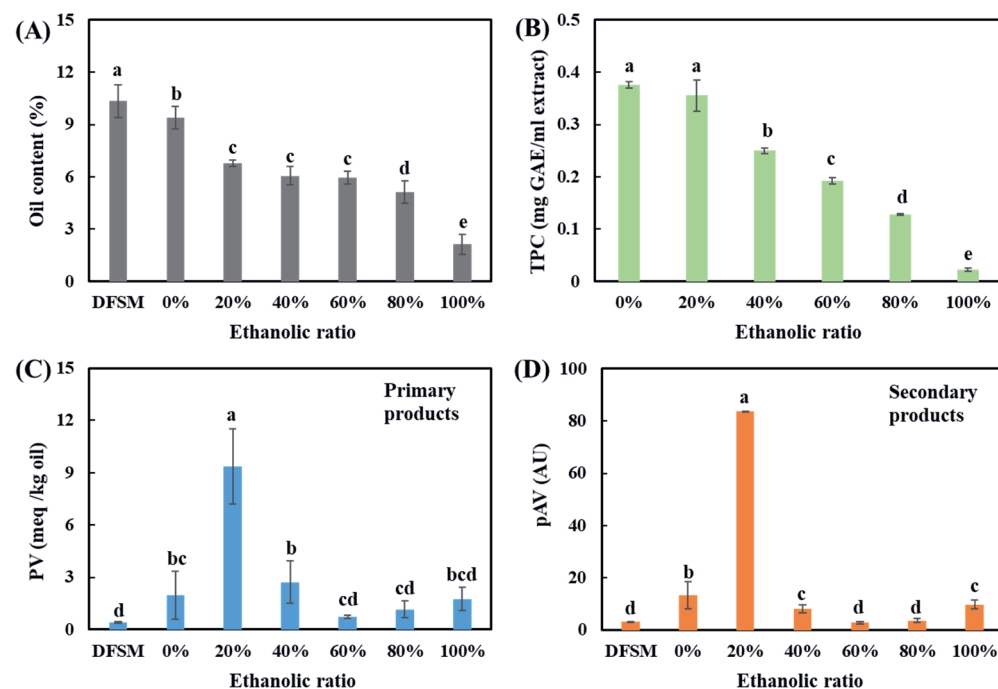
Fig. 6-4 *(A) Oil content of DFSM and all the SPFs; (B) total phenolic content (TPC) of all the extracts; (C) hydroperoxide concentration (PV) and (D) p-anisidine value (pAV) of soybean oil contained in DFSM and SPFs. *The values in the figure are compared and different top letters indicate a significant difference (P < 0.05).*

6.3.3 Total free sugars

Before the washing process, DFSM contained around 8 % of total free sugars (Fig.6-5). With the increase of ethanolic ratio from 0 to 100%, the free sugar content of SPF became lower firstly, and then increased as the ethanolic ration increased above 60%. Although water as solvent for the removal of free sugars seems promising, the lowest value was found when 40% ethanol was used for washing. The trend was in line with previous studies in which it was reported that 50% ethanol can achieve best removal of free sugars from toasted soybean meal than 80% ethanol (Johansen et al., 1996). The sugar content remained above 5% in SPF once the ethanolic ratio in the solvent was 80% and higher. The SPF processed with 100% ethanolic ratio showed the highest free sugar content of 10.57%, even higher than the value of DFSM, which could be caused by the simultaneous removal of oil and part of the protein at this high ethanol content. During the production of commercial SPC, the free sugars are mostly removed and end up in the soy molasses as a by-product (Choct et al., 2010).

In general, the amount of sucrose in the DFSM and SPFs was greater than the total amount of GOS, while the content of glucose was low. The impacts of ethanolic ratio on the sucrose and GOS content were similar and in line with the change of total free sugars. This means the lowest sucrose and GOS contents were also found when 40% ethanol was applied for washing, while above 40%, the sucrose and GOS remained in the SPF mostly. Washing with a higher ethanolic ratio generally led to an increased dry matter yield (Fig. 6-3A), which may suggest that more free sugars were kept in the SPF. A similar trend was also reported in a previous study that described a complete extraction of GOS from green pea when washed with 50% ethanol. A further increase in ethanol concentration to 80% reduced the extraction efficiency of GOS at room temperature (Ekvall et al., 2007).
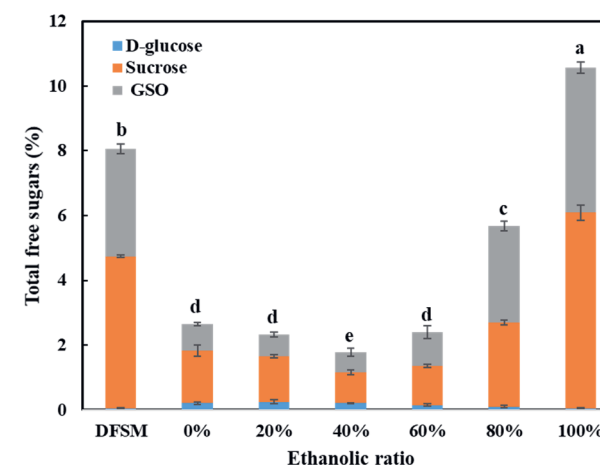

Fig. 6-5 *Total free sugars (D-glucose, sucrose and GSO) in the DFSM and all the SPFs. *The values in the figure are compared and different top letters indicate a significant difference (P < 0.05).*

6.3.4 Protein denaturation in SPFs

The thermal transition characteristics (denaturation temperature T_d and protein denaturation enthalpy) of all the SPFs are presented in Table 6-2. Two denaturation peaks were detected in all the samples, which can be related to the denaturation changes of 7S and 11S protein (Hashizume et al., 1979). The T_d of 7S in the various SPFs was between 74.6 and 76.1°C, but the differences were not significant. However, the enthalpy of denaturation was altered by the use of different washing solvents. SPFs washed with water and 20% ethanol however exhibited the highest enthalpy values, meaning least denaturation was occurred during washing process. The enthalpy values revealed that washing with 40% and 60% ethanol induced SPFs with relatively higher degree of denaturation in 7S. No significant

differences in the degree of denaturation were observed in the case of 11S. The variation in the T_d of 11S proteins in SPFs was larger than 7S. The T_d of 11S ranged from 92.4 to 96.7°C, while the SPFs prepared with 40% and 60% ethanol presented the lowest T_d values. Previous studies reported that ethanol treatment leads to the denaturation of soy protein (Griebenow and Klibanov, 1996). Also in the present study, the lowest degree of protein denaturation was observed when low ethanolic ratio (0 and 20%) was applied for washing. However, at high ethanol ratios the denaturation is not extreme. Early studies found that the presence of sucrose stabilizes the structure of proteins, for example whey protein and bovine serum albumin (Devi et al., 2009; Kulmyrzaev et al., 2000). This is also the reason why sugars are often added to protein solutions as lyoprotectants. Considering that for the SPFs washed with high ethanolic ratio (80 and 100%), the sucrose mostly remained after washing (Fig. 6-5). This may prevent the soy protein from the ethanol-induced denaturation. As a result, 40% and 60% ethanol-washed fractions presented the highest level of protein denaturation among all the SPFs. No denaturation peak was detected from the commercial SPC. This complete denaturation might be caused by the intensive aqueous ethanol washing steps, or heat treatments during the process, including spray drying for the final dry powder production.

*Table 6-2 The denaturation temperature and enthalpy of the transition of the SPFs.*

Ethanolic ratio	7S T_d (°C)	Enthalpy (J/g)	11S T_d (°C)	Enthalpy (J/g)
0%	75.72 ± 0.44 [a]	1.73 ± 0.36 [a]	94.30 ± 0.21 [d]	3.31 ± 0.31 [ab]
20%	75.20 ± 0.19 [a]	1.85 ± 0.12 [a]	95.05 ± 0.04 [c]	3.59 ± 0.64 [ab]
40%	75.94 ± 1.45 [a]	0.70 ± 0.04 [c]	92.44 ± 0.19 [e]	3.98 ± 0.74 [a]
60%	74.64 ± 1.56 [a]	0.75 ± 0.13 [c]	92.74 ± 0.49 [e]	3.38 ± 0.27 [ab]
80%	76.11 ± 0.33 [a]	1.33 ± 0.07 [b]	95.76 ± 0.09 [b]	3.48 ± 0.14 [ab]
100%	76.08 ± 0.14 [a]	1.19 ± 0.14 [b]	96.73 ± 0.27 [a]	3.14 ± 0.31 [b]

* The values in the table are compared in columns and different top letters indicate a significant difference (P < 0.05).

6.3.5 Particle size distribution (PSD)

Fig. 6-6 shows that the washing process using solvents with higher ethanolic ratio led to a wider PSD range of SPF dispersions, while higher ethanolic ratio resulted to a gradual shift in major PSD peak towards larger sizes. This indicated the occurrence of ethanol-induced aggregation in these samples. When the ethanolic ratio was 0%, the major PSD peak appeared with particle size of approximately 90 μm; the size of the major peak was similar

and around 160 μm when the ethanolic ratio was 20% and 40%. The major peak size became around 600 μm when the ethanolic ratio was 60% and above. These results are in line with those of previous studies. It was reported that the ethanol treatment of soy β-conglycinin with increasing ethanolic ratio from 0-80% (v/v) led to a gradual shift in major PSD peaks towards greater sizes, while the greatest change occurred when the ethanolic ratio was above 40% (Peng et al., 2020). Besides, chicken egg albumin underwent certain structural changes in water-ethanol solution, and the degree of aggregation was linearly depending upon the ethanol concentration (Kant Yadav et al., 2012). We therefore think that ethanol-induced aggregation may be taking place between soy proteins and/or other constitutes. For example, ethanol can induce changes in the conformations of globular proteins as a result of disruption of noncovalent interactions (Romero et al., 2007; Uversky et al., 1997). Addition of ethanol is generally considered to weaken noncovalent bonds in protein, including hydrogen and ionic bonds and hydrophobic interactions. This may result in destabilization of protein and increased formation of aggregations (Singh et al., 2010). The aggregation may be dominated by the non-protein constitutes as well, such as cellulose and semi-cellulose, because cellular matrixes have been observed from the SEM images (Fig. 6-2). The hydrophobicity of these matrixes may be increased when washing with higher ethanolic ratio, and therefore, they formed larger aggregates. In addition, the milling process after freeze drying may also have an effect. Washing with high ethanolic ratio resulted in the inclusion of more sugars in the SPFs, the higher sugar content may lead to more ductile powder that is more difficult to mill, and therefore, the size of particle remains large after dispersing in the water.
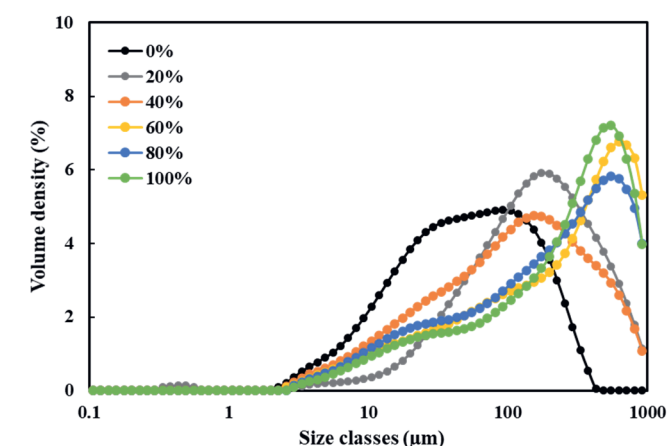
Fig. 6-6 The particle size distribution of 1% (w/v) SPF dispersions determined by Mastersizer.

6.3.6 Nitrogen solubility index (NSI)

The protein solubility was determined as nitrogen solubility index (NSI) and presented in Fig. 6-7. When the ethanolic ratio was 60% or lower, the variations in NSI were small between the SPFs. Once the ethanolic ratio increased from 60 to 80%, the solubility increased significantly. This outcome was consistent with other research which found that the protein solubility of ethanol-treated lentil protein isolate (LPI) was below 10% when 35-55% ethanol was applied, and increased to around 30% when the ethanolic ratio increased from 55% to 75% (Chang et al., 2019). Similarly, a higher NSI-value for de-fat soy meal was found when 100% ethanol was used for oil extraction as compared with 88% ethanol (Sawada et al., 2014).

There are several possible explanations for this result. It was found that 40% and 60% ethanol-washed SPF presented the highest degree of protein denaturation among all the SPFs (Table 6-2), which might result in more exposure of hydrophobic groups, and therefore, relatively lower NSI. The NSI of commercial SPC was around 20.41%, which was lower than the values of all the SPFs obtained in this study. This also correlates well with the observed complete denaturation of the commercial SPC. Another possible explanation for this is that at high ethanolic ratio (80 and 100%), a higher proportion of water-soluble components (including proteins) remained in the solid phase, leading to higher NSI values. The result from SDS-PAGE (Fig. 6-3B) also showed the bands of protein with low molecular weight. These proteins are small and may contribute to the increase of overall protein solubility. Although the size of aggregates was more pronounced when the ethanolic ratio was 60% and above (Fig. 6-6), these aggregates are likely caused by the other constitutes in the SPFs rather than soy protein.
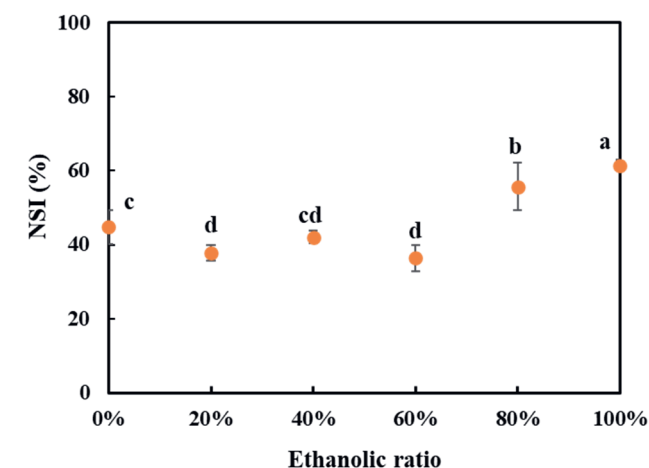

Fig. 6-7 *The nitrogen solubility index (NSI) of all the SPFs. *The values in the figure are compared and different top letters indicate a significant difference (P < 0.05).*

6.3.7 Water holding and oil absorption capacity

The water holding capacity (WHC) and oil absorption capacity (OAC) are important functional properties for developing meat-related and meat analogue products (Cornet et al., 2021; Kinsella, 1976). The WHC is defined here as the amount of water bound per gram of SPF on a dry basis, while the WHC_P represents the amount of water bound per gram of insoluble pellet (Peters et al., 2017). It was found that the WHC_P increased significantly when the ethanolic ratio increased from 0% to 20%, and from 40% to 60%, which is consistent with the change of PSD (Fig. 6-6). These insoluble aggregates can hold a certain amount of water as well. Furthermore, when the ethanolic ratio was 60% and above, the WHC of SPF was negatively correlated to the ethanolic ratio. It seems that the decrease of WHC may result from the increase of NSI (Fig. 6-7), but the WHC_P and PSD were not influenced. This might confirm that the insoluble aggregates are dominated by the non-protein constitutes such as cellulose and semi-cellulose, and therefore, not depended on the change of protein solubility. The WHC and WHC_P value of commercial SPC was 7.2 g water/g and 9.8 g water/g, respectively. These were higher than the values of all the SPFs in this study. It was reported that an additional heating treatment during the fractionation process increases the WHC and WHC_P of soy protein, suggesting that the WHC of SPF can be further increased by incorporating a heating step in the washing process (Peng et al., 2021).

The OAC value of SPF was negatively affected by an increase of ethanolic ratio during washing process, with SPF washed with water presenting the highest value of 1.3 g oil/g. A similar finding was also previously reported for the OAC of pea flour, which gradually decease from 0.96 to 0.76 g oil/g when the alcohol concentration increased from 0 to 80% during alcohol washing process (Wang et al., 2020). In addition, this trend was consistent with the trend of oil left in the SPFs, namely the lower amount of oil left in the SPF, the lower OAC value was observed. In case high oil-absorption is desired, water-only washing seems a good option.
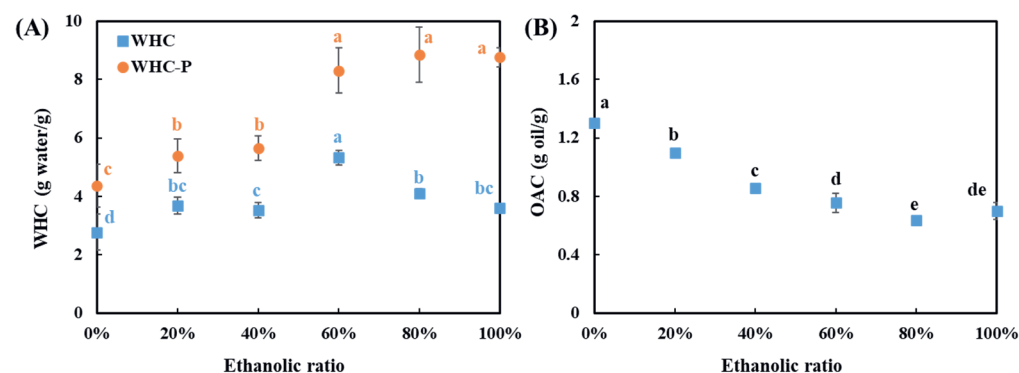

Fig. 6-8 (A) *The WHC, WHC_P and (B) the OAC of all the SPFs. *The values in the figure are compared and different top letters indicate a significant difference (P < 0.05).*

6.3.8 Viscosity analysis

For this analysis, the protein concentration for all the SPF dispersions was standardized on 6 wt.% according to different protein content in the SPFs (Table 6-1). With 6 wt.% protein concentration, all the SPF samples were still fluid based on visual observation. When measured with rheometer, a decrease in viscosity was observed with increasing shear rate, indicating that all the SPF dispersions exhibited a similar shear-thinning behavior under room temperature (Fig. 6-9). The observed shear-thinning behavior of all the SPFs was not influenced by the variation of particle size (Fig. 6-6), which may imply that the shear-thinning was caused by flow alignment more than shear-induced breakage of the smaller aggregates. Except for the SPF processed with water, the dispersions of all the other SPFs and commercial SPC exhibited similar viscosity values, despite the variation in the non-protein constitutes in the fraction. This result suggests that the oil and carbohydrate contents have minor impact on the viscosity of these mixtures. Previous study compared the viscosity of

pea protein fractions with different protein purity, and reported that the viscosity was closely related to the protein of the fractions, while the soluble and insoluble carbohydrate had limited impact indeed (Kornet et al., 2020). Although a higher soy oil volume fraction in the range of 5% to 35% led to a higher viscosity of heated SPI emulsions (Nicole et al., 2016), it is not expected that the oil content has a significant influence on the viscosity of non-heated mixtures. Therefore, the highest viscosity value of water-washed SPF may be mainly attributed to the change of soy protein. Most likely, this is a result of the fact that the average molecular weight of the proteins in water-washed SPF is higher, as revealed by SDS-PAGE.
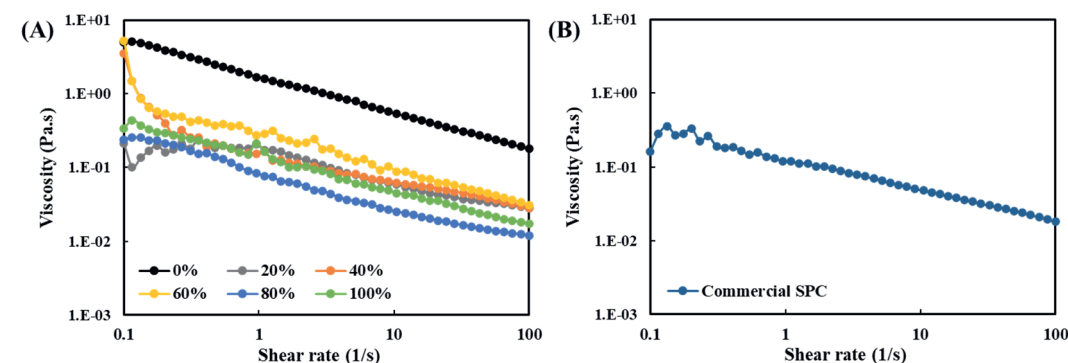

Fig. 6-9 *Viscosity as a function of shear rate at 25°C of (A) aqueous ethanol washed SPFs and (B) commercial SPC dispersions (standardized 6 wt.% protein content).*

6.3.9 Gelation behaviors

The gelation behaviors of the SPFs upon and after thermal treatment was studied with a temperature, frequency and strain sweep, and are shown in Fig. 6-10. The protein concentration for all the samples was standardized on 9 wt.% according to different protein content in the SPFs (Table 6-1). For all the SPF samples, the storage modules (G′) was constantly higher than loss modules (G″) even without heating. This indicated that a dispersion with 9 wt.% protein concentration is able to form solid-like structures at room temperature. During the temperature sweep, similar G′ and G″ changes were observed for all the SPF samples. An abrupt increase of G′ and G″ was found upon heating the sample in the temperature range from 70 to 95°C, which may be caused by the gel formation. During the constant temperature regime at 95°C, G′ increased somewhat, while G″ was about stable. After that, a slight decrease followed for both. During cooling, both G′ and G″ increased strongly for all the SPFs, indicating that the gel structures were further improved by the formation of additional bonds (Zhao et al., 2020). Commercial SPC showed similar gelation

behaviors under the same measurements. When considering the values of G′ and G″ obtained from different SPFs, the SPF processed with 0% ethanol had the highest values before heating, which was similar to the finding of viscosity. However, for the SPFs processed with 80% and 100% ethanol, the G′ and G″ values increased more pronounced than other SPFs upon heating and cooling. The final G′ and G″ values of these two SPF samples were comparable to the SPF processed with 0% ethanol after the temperature sweep.

During the frequency sweep, the G′ and G″ of all the SPFs were relatively constant over the frequency range of 0.1 to 10 Hz. The highest values were found from SPFs processed with 0, 80 and 100% ethanol, which were in the range of 1,000 to 10,000 Pa, approximately 10 times higher compared with SPFs processed with 40 and 60% ethanol. The high value of water-washed SPF may be caused by the formation of lipid-protein complex after heating, since washing with water hardly remove any oil from the DFSM. For the high ethanol-washed (80% and 100%) SPFs, the presence of low-molecular-weight proteins may work actively and contribute to the high G′ and G″ values. The 2S albumins are a group of storage proteins in soybean with molecular weights of approximately 18 kDa. It contains thiol groups that could lead to disulphide bridge formation at heating stage (Sok et al., 2006).

The length of the linear viscoelastic (LVE) regime of all the SPFs was studied by a strain sweep at constant frequency afterward. For all SPF gels, a linear response was observed wherein G′ and G″ were independent of strain. When the strain was further increased, G′ and G″ decreased sharply, suggesting breakage of bonds in the gel network and transition from linear to non-strain behavior (Ould Eleya et al., 2004). The length of the LVE regimes of SPF gels (80% and 100% ethanol washed) was rather similar, and longer than other SPFs, which can be related to a higher gel strength (Hesarinejad et al., 2014).
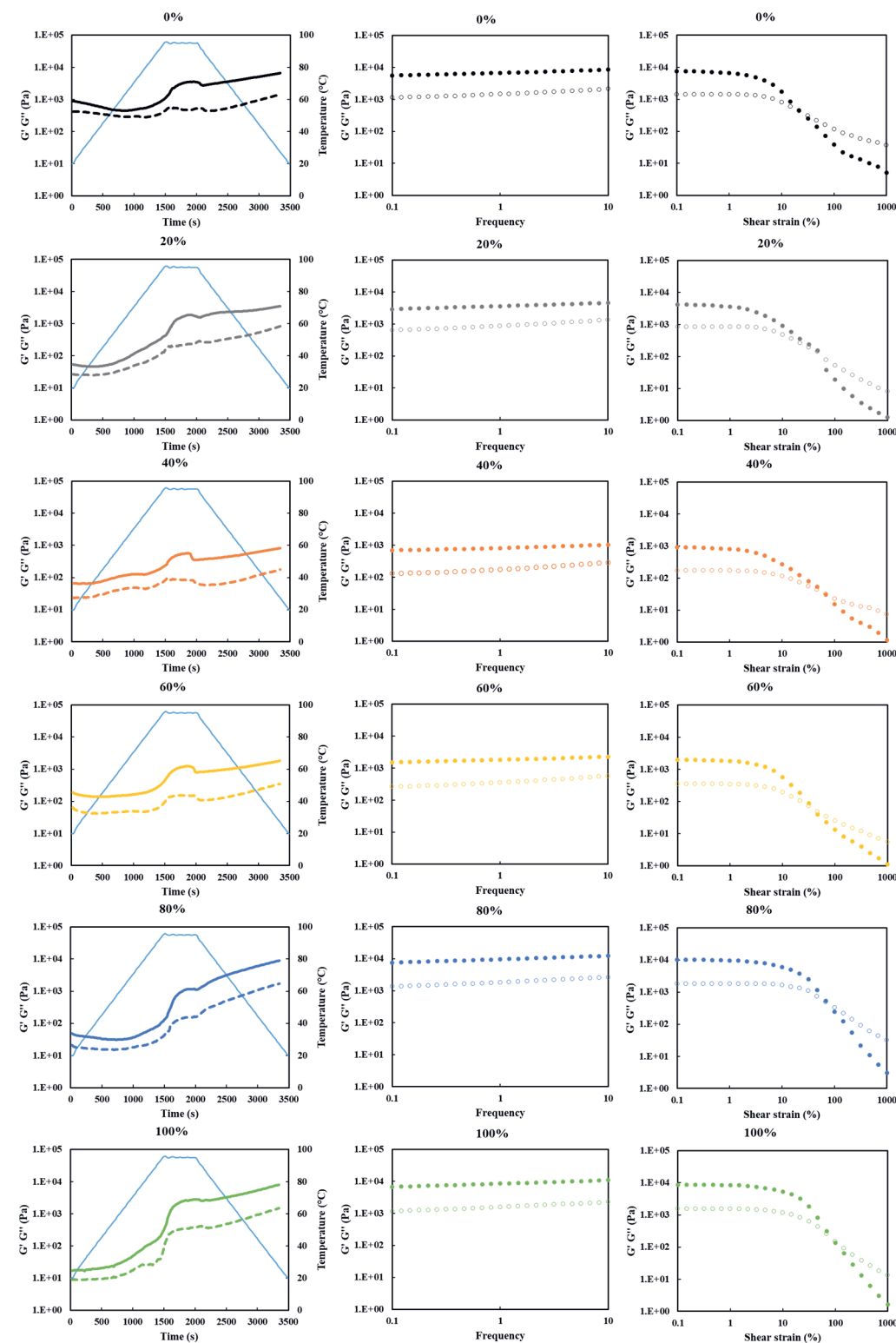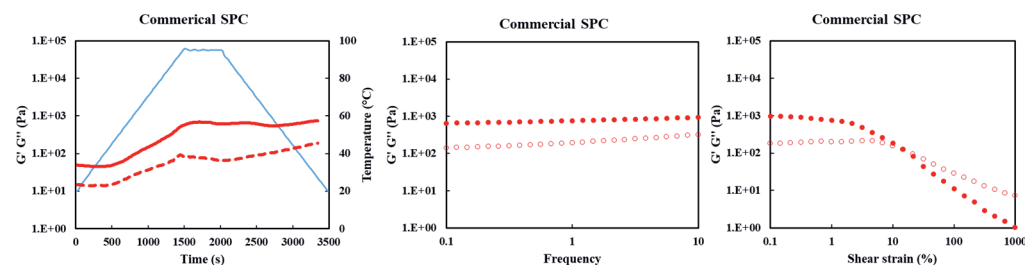
Fig. 6-10 *Temperature (1ˢᵗ column), frequency (2ⁿᵈ column), and strain sweep (3ʳᵈ column) sequentially applied on all the SPFs and commercial SPC (standardized 9 wt.% protein content). G': closed symbols, G": open symbols. Temperature: solid blue line.*

6.3.10 Textural analysis of soy gels

The ethanolic ratio during the washing process affected the soy gel textures, as shown in Fig. 6-11. Firm gels can be formed by all the SPFs after heating with a standardized 15 wt.% protein concentration. The textural properties of the gels formed by all the SPFs were comparable to the gel formed when using commercial SPC, with the exception of water-washed SPF. That gel showed the highest hardness, chewiness and cohesiveness among all the SPFs. The remarkable high values of hardness, chewiness and cohesiveness are probably related to the high molecular weight of the protein present. Also, the high residual oil in the SPFs might play a role. It is known that various lipid substances have impacts on the gelation of protein. Previous research reported that soybean oil addition (1-5%) enhanced the hardness and cohesiveness of heat-induced SPI gels under both 20% and 60% deformation (Miura and Yamauchi, 1984). The increasing hardness with increasing oil content in the whole soybean protein gel was also described by Yamamo (Yamano et al., 1981).

In the previous section, the SPF gels (80% and 100% ethanol washed) presented the highest G′ values as well as the longest length of the LVE regimes among all the gels after heating, which can be related to a higher gel strength. In contrast to earlier findings, however, no evidence can be found here that the gel characteristics of 80% and 100% washed SPF gels were more prominent than others. A possible explanation might be that the higher gel strength was within the small deformation range, and correlated to the displacement of protein aggregates or other components, while the gel characteristics were determined within the large deformation range, referring to the disruption of a gel network (Kornet et al., 2021).
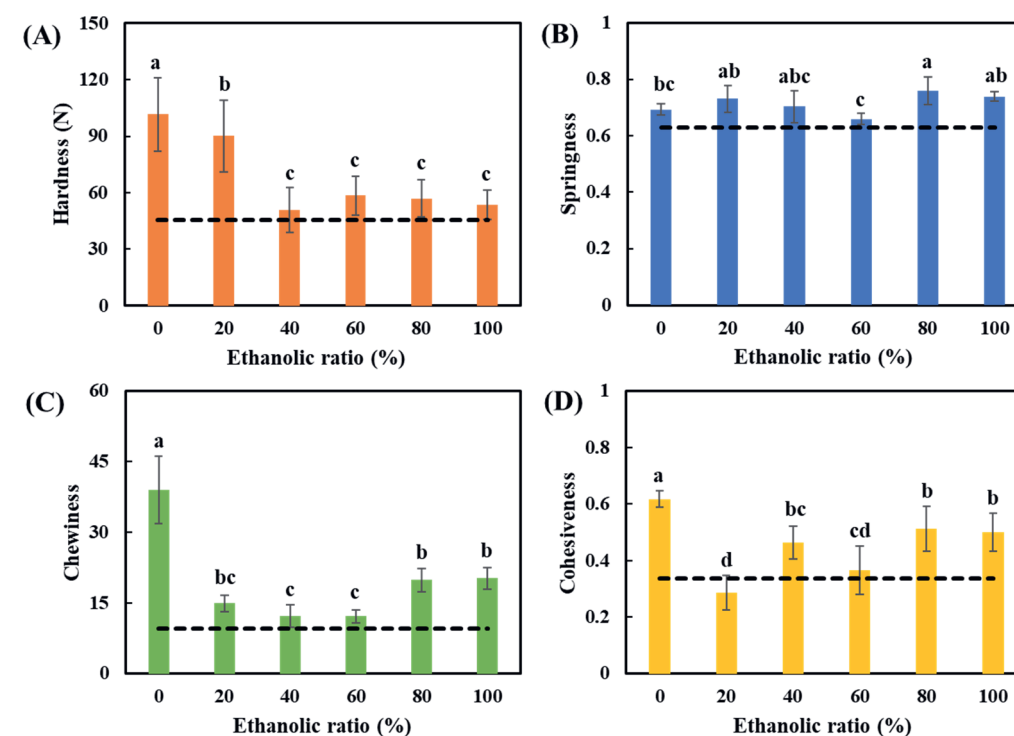
Fig. 6-11 *Textural analysis of all the soy gels (standardized 15 wt.% protein content). The black square dot line represents the reference value measured from commercial SPC gels. *The values in the figure are compared and different top letters indicate a significant difference ($P < 0.05$).*

6.4 Conclusion

This research examined the effect of aqueous ethanol washing on cold-pressed DFSM. Different water-ethanol ratios were used to investigate the effect of ethanol on the overall composition, yield and functionality of SPFs. Based on the results, the aqueous ethanol washing process enhanced the protein content in SPF, while all the obtained SPFs after the washing process still contained oil. Water-washed (0% ethanol) SPF however presented increased oil content with low oil oxidation, and the best OAC, while the water washing treatment seemed to obtain a relatively native ingredient. With regards to the rheological properties, water-washed SPF dispersions had the highest viscosity, and it formed the gel with the highest gel strength and hardness. An additional advantage of using water-only after cold pressing is that it is a completely solvent-free process. Among all the water-ethanol ratios, washing with high ethanol level (60% and above) led to SPFs with low oil content and high yield. These SPFs also presented a low degree of denaturation, a better protein solubility and a higher water holding capacity.

Overall, it can be concluded that the water-ethanol ratio is an interesting parameter to control the protein composition, yield and functional properties of the SPF. Most interesting functional properties were obtained when using either water-only solvent or solvent with high ethanol ratios. When using moderate ethanol concentrations, less favorable properties were found, including high oil oxidation levels. Nevertheless, soy protein ingredients can be produced with comparable composition as commercial SPC, but with a range of functionaries that are relevant for the development of novel soy-food applications.

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Chapter 7

General discussion

7.1 Introduction

Soybeans are used a lot in both traditional foods and new food applications. For the latter, soybeans are first fractionated into ingredients, which are then assembled into final products. The current fractionation pathways target producing soy protein ingredients with high purity or specific functionality to suit a broad range of food applications. In those applications, soy protein ingredients are added to a product for an intended effect at low use level, without causing any negative contributes to taste, flavor, and texture (Reipurth et al., 2019; Skarra, 2004). With the development of the new generation of soy-based foods, soy protein has turned towards becoming a bulk ingredient, which drives the use levels far above those needed as additives. The best form to deliver functionality may differ greatly in those new products. Therefore, it is needed to reconsider the fractionation pathways for making soy protein ingredients, which was indeed the main reason for this thesis. The aim of the thesis was to investigate how the functionality of soy protein ingredients and their resulting composition are determined by the fractionation process applied. These insights allow development of route to produce functional soy protein ingredients for novel food applications such as meat analogues, with respect to composition (the inclusion of oil and carbohydrate), as well as nutrients (Ca enrichment and Na reduction), and technical properties.

7.2 Main findings

In **Chapter 2 and 3**, the mostly described aqueous fractionation procedure was simplified by removing the organic solvent de-fatting step and the final washing steps. Full-fat soy flour (FFSF) was taken as the starting material. Under alkaline condition, soybean oil was removed efficiently as a cream layer by centrifugation. The functional properties of the obtained soy protein-rich fractions (SPFs) were greatly depended on the final pH change or a mildly heating treatment prior to drying, while the composition of the SPFs was not influenced. In **Chapter 2**, the change of processing pH in the range of 3.5 to 7.5 made the biggest changes on protein solubility, but all the SPFs were still in their native state. In **Chapter 3**, moisture heating treatment was applied with a temperature between 60 and 100°C, which resulted in SPFs with different degrees of 7S and 11S protein denaturation. Generally, higher protein denaturation levels resulted in SPFs with higher water holding

capacity (WHC) and viscosity, but the partially denatured soy protein contributed to a gel network with the highest gel elasticity.

In **Chapter 4**, next to the established aqueous fractionation process (introduced in **Chapter 2**), a further simplified fractionation was evaluated, where the acid precipitation step was omitted. The protein content of the SPF was reduced after this simplification but was still comparable to the purity of commercial SPC. While NaOH is normally used to increase pH, here the use of $Ca(OH)_2$ was investigated as an alternative to shift the pH. The purpose of using $Ca(OH)_2$ was for Ca-enrichment and Na-reduction in the SPFs. It was found that the protein content of the SPFs increased when the $Ca(OH)_2$ was applied in the alkaline solubilization step, and the Ca content of SPF only became higher by applying the $Ca(OH)_2$ in the protein neutralization step. That lowered the Na content as well. However, complete replacement of NaOH by $Ca(OH)_2$ in the neutralization step altered the functionality of SPF drastically as well, especially the protein solubility, in such a way that the SPF became less applicable as an ingredient in meat analogue applications. That is why in **Chapter 5**, combinations of $Ca(OH)_2$ and NaOH in different ratios were further explored in the final neutralization step. No separation step was performed after the neutralization step, so the Ca and Na content in the SPF was precisely controlled by adjusting the ratios. Higher Ca content led to SPFs with larger particle size, higher thermal stability, lower protein solubility, and weaker gel networks. However, a critical Ca concentration was observed, below which the functional properties and conformation of SPF did not change significantly. Therefore, it was concluded that $Ca(OH)_2$ can be used to a certain level without strongly impacting the applicability of SPF.

Chapter 6 describes the findings on the potential use of aqueous ethanol washing process for the fractionation of cold-pressed de-fat soy meal (DFSM) to create soy protein fractions that can compete with commercial SPC. The use of different washing conditions led to SPFs with different compositions and functionality. Overviewing all results, we conclude that either pure water should be used as washing solvent or solvents with high ethanolic ratio. High ethanolic ratio (60% and above) as solvents gave the highest yield in protein, as well as the highest protein solubility and water holding capacity. Among all the SPFs, when adjusted at similar protein concentration, water-washed SPF (0% ethanol) showed the highest viscosity, and it formed the gel with the highest gel strength and hardness.

The variations in the functionality were attributed in changes of protein, though effects of non-protein constitutes such as sugar and oil might also have played a role.

Overall, the research described in this thesis shows that that the fractionation process for soy can be adjusted to control the composition and functionality of soy protein fractions. This gives the opportunity to create a wider range of ingredients to be selected for development of novel soy-based products.

7.3 Fractionation towards targeted food applications

Currently, the market price of animal feed ingredients is positively correlated with their protein content, because protein is considered as the valuable component for the applications (Teekens et al., 2016). When a protein source is applied for food application, the prices can significantly increase compared with its use in animal feeds (Mulder et al., 2012). For soy protein ingredients, it is also reported that a higher protein content leads to a higher market price (Voudouris et al., 2017). Soybean's high protein content and well-balanced amino acid composition make it an important source of plant protein, with great potential to replace animal proteins in our daily diet (Day, 2013). However, protein ingredients with high purity are not absolutely required for food applications. Instead, less refined ingredients with proper functional behaviors can be beneficial for many food applications (van der Goot et al., 2016). Hence, the assessment of soy protein should be oriented to both composition and functionality.

Aqueous fractionation process and aqueous ethanol washing process are applied to produce commercial SPI and SPC, respectively. During the aqueous fractionation, the oil, insoluble carbohydrate, and soluble carbohydrate in the soybean are removed step-wise by de-fatting, alkaline solubilization, and acid precipitation step. This eventually leads to a fraction with high protein purity. If such a high purity is not necessary, the aqueous fractionation can be simplified by omitting certain processing steps such as oil extraction, allowing part of non-protein components to remain in the ingredients. On the contrary, for the aqueous ethanol washing process, the soy protein remains insoluble while all the water/ethanol-soluble components removed. Insoluble carbohydrates, however, will remain in the ingredient, meaning that the protein-enriching ability of the washing process is limited. However, the properties of a concentrate mainly consisting of proteins and insoluble carbohydrates have shown its importance in meat analogues applications (Kyriakopoulou et al., 2019).

For novel food applications, the focus on the actual protein content has become less important, which means that the selection of the soy fractionation pathway should be based on other considerations. For the development of meat analogues, the insoluble carbohydrate may be beneficial for the structure formation even, which would suggest the use of the washing process. But for the development of some soy-foods such as soy beverages or dairy

analogues, possibly ingredients containing soluble components only are preferred, meaning that the simplified aqueous fractionation is more applicable than the washing process. An additional feature of the aqueous fractionation process is that it allows the inclusion of Ca in the soy ingredients (**Chapter 4 and 5**).
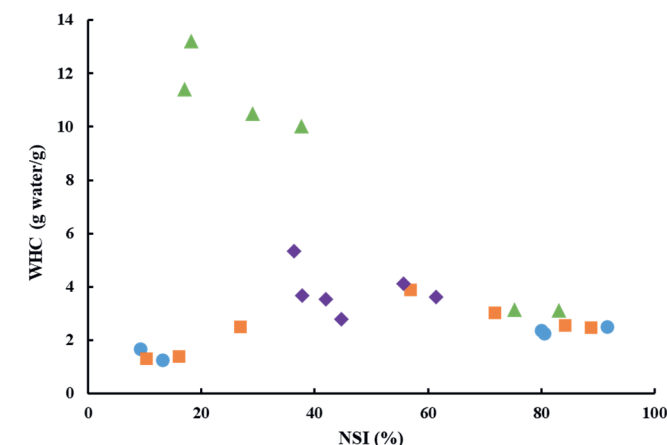

Fig. 7-1 The NSI and WHC of the soy protein fractions produced with pH adjustment (blue cycle), moisture heating treatment (green triangle), Ca enrichment (orange square), and aqueous ethanol washing process (purple rhombus).

A shown in this thesis, it is possible to create SPF with high similarity in composition but differences in functionality. This finding creates the opportunity for a reversed engineering approach. If the desired functionalities are known from an intended application, the fractionation process of soy protein can be tailor-designed to avoid potential post-processing. Nitrogen solubility index (NSI) and water holding capacity (WHC) are used as examples to show the effects from different processing parameters (Fig. 7-1). By changing the final pH during the simplified aqueous fractionation, the NSI of the fraction ended up in two extreme directions, either very low (below 20%) or very high (above 80%) (**Chapter 2**). By contrast, moisture heating treatment (**Chapter 3**) and Ca enrichment (**Chapter 5**) can lead to a stepwise reduction of NSI, which provided the possibility to control the protein solubility in a relatively accurate range by using different heating temperature or varied amount of $Ca(OH)_2$ during the process. Moreover, moisture heating treatment (above 60°C) increased the WHC of protein, which is similar to the change of WHC induced by the dry heating of protein (Bühler et al., 2020). So, the heating treatment can be performed during or after the fractionation if a high WHC value is desired for the applications. The fractions obtained by aqueous ethanol washing presented intermediate NSI and WHC values (**Chapter**

6). It might be difficult for these fractions to have high solubility due to the principle of the washing process, but the peak WHC-value of the washed fraction was higher than all the unheated samples discussed above. So, if the intermediate values of NSI and WHC are vital for the products, the washing process can be recommended rather than aqueous fractionation.

In addition to functionality, the fractionation processes should also be evaluated on their performance with respect to protein yield. We, therefore, plotted yield and purity for all processes tested in a so-called yield-purity curve (Tamayo Tenorio et al., 2018). What we noticed is that at a given purity, the yield is higher in the case of washing processes (Fig. 7-2). For example, simplified aqueous fractionation (**Chapter 4**) and aqueous ethanol washing (**Chapter 6**) can both achieve a fraction with around 56% protein content. The protein yield of washing process, though, was around 85%, which is clearly higher than the yield of simplified aqueous fractionation, which was approximately 57%. This suggests that washing process leads to lower protein losses, which makes this process more efficient on raw material use.
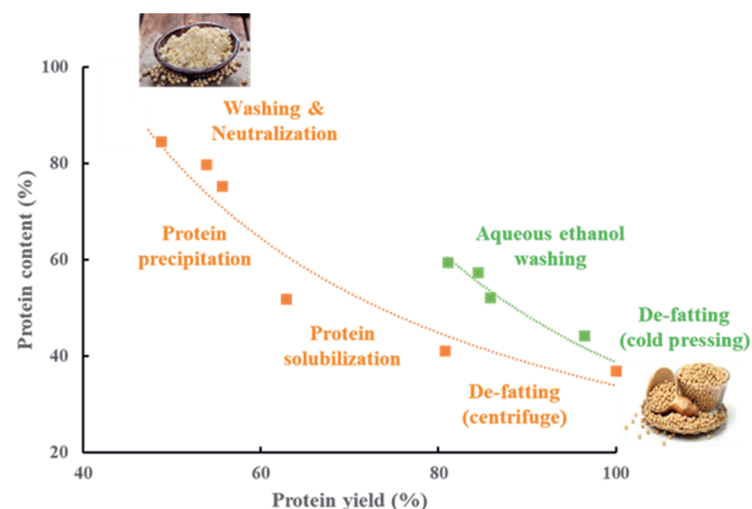
Fig. 7-2 *Schematic representation of protein content as a function of protein yield upon aqueous fractionation (orange) and aqueous ethanol washing (green) of soy protein.*

Overall, novel soy-based food products will require novel soy ingredients with specific composition and functionality. We showed that with proper design of the fractionation process, the condition of soy protein ingredients can be tailor-made, after which the post-processing can be potentially eliminated. In addition, the resource efficiency may be improved for example by the use of washing instead of extraction in the case of the production of concentrates.

7.4 Pilot-scale production: potential and challenges

The thesis describes a broad range of fractionation pathways to make SPF at a lab scale. We tested a few recipes at pilot-scale studies to explore the scalability of the process. Results from **Chapter 2** were taken as the basis for a simplified aqueous fractionation at pilot scale. It was found that not all lab-scale processing steps could be applied in the exact same manner, meaning that some processing steps were adjusted for the feasibility and applicability of pilot-scale production (Fig. 7-3). The differences are outlined below.

The pilot trials were based on the use of de-fat soy meal (DFSM) as the starting material instead of full-fat soy flour (FFSF). DFSM was produced via a cold-pressing technique to remove the oil from the soybeans. The cold-pressed DFSM contained 10.3% of residual oil, while the oil content of FFSF was around 21.8%. Centrifugation at the pilot scale was done at lower *g*-forces compared to the lab process because of limitations to apply those extremely high *g*-forces on pilot scale. Lastly, one batch of protein-rich dispersion was spray-dried, instead of freeze-dried, leading to high similarly to the process of commercial SPI. The other batch was adjusted to a pH of 5.5 and freeze-dried. This final pH-value was chosen based on promising outcomes in **Chapter 2**. Both batches of soy protein fractions (SPFs) had a similar chemical composition, since the neutralization/pH adjustment was performed after the protein separation stages prior to drying. The protein content was 77% (N × 5.7), comparable to the protein content of lab-made SPF (~75%), suggesting that the parameter adjustments between lab-scale and pilot-scale production did not impact the protein extraction efficiency. Since the final pH and the applied drying method of these two SPFs were different, they exhibited differences in color, morphology, and structural behaviors. Freeze-dried SPF adjusted at pH 5.5 had a yellowish color, with blocky and porous microstructures, while spray-dried SPF at pH 7 presented darker color and individual particles with spherical shape (Fig. 7-3).
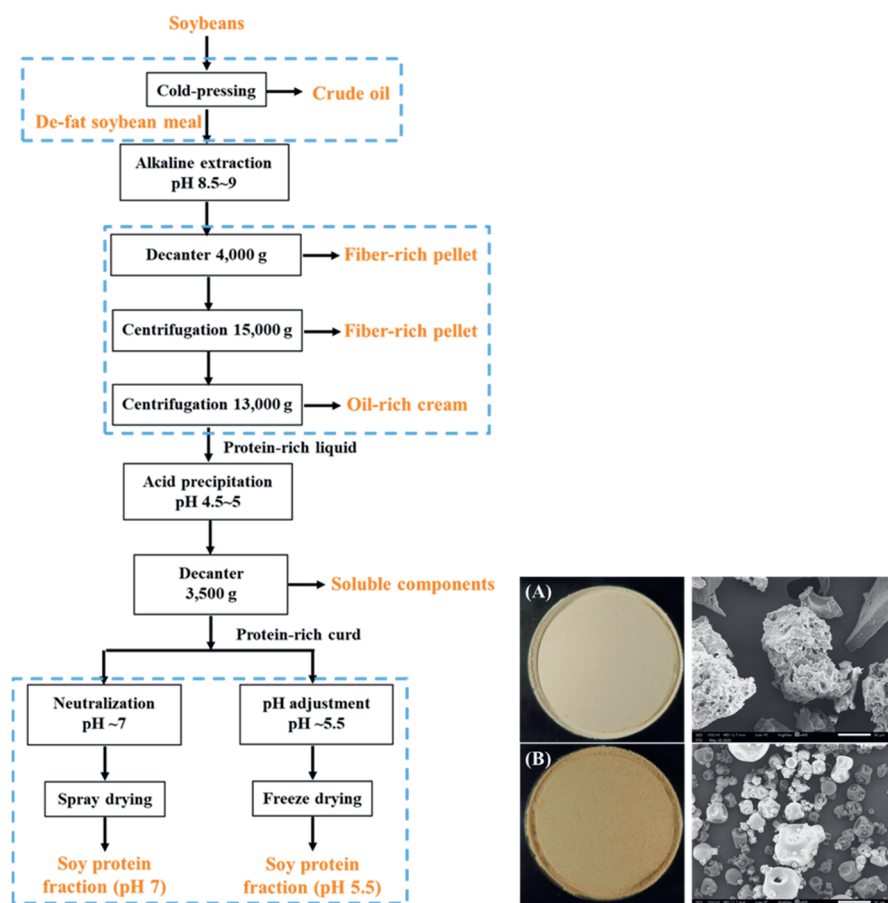
Fig. 7-3 Left: mild aqueous fractionation at a pilot-scale production. Right: visual appearance and powder morphology (SEM) of (A) freeze-dried SPF 5.5 and (B) spray-dried SPF 7.

The SPFs obtained from the pilot-scale production had a higher oil content than the SPFs produced at lab scale. Three times of centrifugation on the pilot scale with lower *g*-forces hardly removed any oil from the cold-pressed DFSM, leading to a residual oil content in the obtained SPF of 8.9%. The pilot-scale SPFs were also processed in the high-temperature shear cell (HTSC) to investigate their structural potentials for meat analogue applications. Based on the results discussed in **Chapter 2**, low-solubility fractions with a final pH close to pI were thought to have most promising rheological behaviors for meat analogue applications, which was the main reason to produce freeze-dried SPF 5.5 on a pilot scale. Unfortunately, a fibrous structure was not formed by SPF 5.5 after processing at 140°C in HTSC. Instead, fragile and homogeneous structures were exhibited (Fig. 7-4A). The spray-dried SPF 7 was obtained using similar pH and drying method of commercial SPI. It formed

rubbery and isotropic structures after processing (Fig. 7-4B), similarly to the structure that formed by commercial SPI (Dekkers et al., 2016). This suggested that the high oil content in the spray-dried SPF 7 did not markedly influence its structuring potential. To better mimic the composition of SPF 7, oil was added to SPI. After shear cell processing, the blend of SPI and oil showed clear oil droplets on the surface of the sample (Fig. 7-4C), and on the surface of the shear cell equipment after taking out the sample. This oil deposition was not found in the samples formed by spray-dried SPF 7. Hence, the residual oil in the ingredient can be better retained in the final product after shearing than that oil added as separate ingredient to the formulations. Based on previous results, we can expect that the addition of gluten or pectin will give fibrous materials with SPF 7.
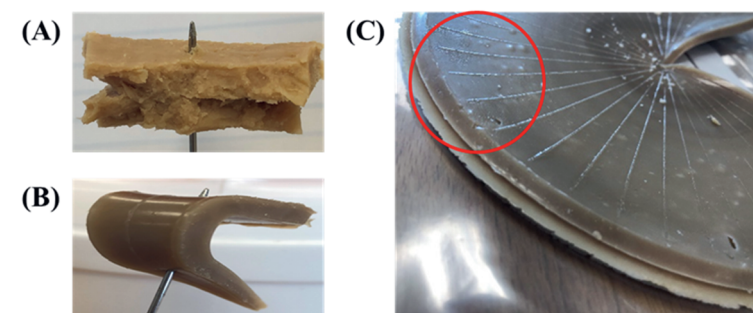

Fig. 7-4 Macrostructures after shear-induced structuring of samples consisting of (A) SPF 5.5, (B) SPF 7, and (C) commercial SPI with additional oil.

It should be noted that some oil left in the soy protein ingredient could be beneficial for functions such as juiciness, tenderness, and overall palatability of the products (Kyriakopoulou et al., 2021). A possible downside of the oil-containing ingredients is that oil oxidation might impact their applicability in food formulations. Previous research reported that oil oxidation hardly occurs in FFSF over 250 days of storage (Duque-Estrada et al., 2020). The results from **Chapter 6** also showed that the cold-pressed DFSM had a relatively low oil oxidation level. Unfortunately, when inspecting the SPFs obtained from pilot-scale production, we noticed a strong off-flavor from both SPF 5.5 and SPF 7. Additional analysis revealed that the oil was highly oxidized in both fractions. Since odor was not found from the SPF produced at the lab scale, we suspect that the oil oxidation might be related to the cold-pressing treatment with DFSM. The pressing force probably breaks the structure of the oil bodies and increases the oil's susceptibility to oxidation especially during the fractionation process. It seems therefore that a choice has to be made between keeping

the oil in their native environment, being the oil bodies and thus avoiding strong mechanical forces, or removing oil more effectively, probably via the use of organic solvents.

Overall, the lab-scale simplified fractionation could be scaled up by adjusting some parameters, without requiring extra facilities. The pilot-scale production produced SPFs with high oil content and different functionalities. The use of oil-containing ingredients showed an advantage in eliminating oil leakage during product development, while the risk of oil oxidation can be carefully considered and further investigated in future work.

7.5 Routes to introduce new soy-based ingredients

The global soybean meal consumption was around 253 million metric tons in the 2020/2021 market year (USDA, 2021), most of which has been used in the animal feed industry, and only a share of 2-3% was directly processed by the food industry (De Maria et al., 2020). For leading manufacturers in oilseed processing such as Cargill, the estimated annual soybean consumption was around 1.45 million metric tons in 2017 (Voora et al., 2020). Large production runs of a single ingredient might be preferred to have the lowest cost for the ingredient manufacturers (Skarra, 2004). However, a single ingredient may not always have the optimal option for all applications. The development of novel soy-foods, such as meat analogues, requests soy ingredients with customized composition and functionalities. Compared with a large production center that ships ingredients worldwide, small-scale manufacturers that address the ingredient production locally may present opportunities to adapt new fractionation processes, and therefore, provide a much broader range of soy ingredients to serve the needs of innovative products. Micro-fractionation plants enable not only localization in manufacturing, but allow nimbler and more flexible innovations potentially at higher efficiency. Besides, localized manufacturers stay in closer proximity to customer tastes and preferences, which may be beneficial for launching products that suit the particular markets (Paloviita, 2010).

In addition, in the case of conventional soybean processes, revenues are based on both oil and meal. Initially, soybean oil was the main product while soybean meal is the by-product from the oil production (Durkee, 1936). The soybean oil refinery chain has been well-developed and is highly optimized with the crude oil produced by solvent-extraction or mechanical pressing. However, it can be expected that the use of organic solvent like hexane is or will become undesired from an environmental and consumer point of view for oil extraction. This means that its use will possibly meet restrictions in future. During the simplified aqueous fractionation, centrifugation after alkaline solubilization was found to be a solvent-free alternative method to efficiently separate oil from FFSF. However, it delivers the oil as an emulsified cream, which differs from the solvent-extracted and mechanical-pressed crude oil. When considering future profits to drive the supplier to introduce new soy-based ingredients, the potential use of soybean oil as an emulsified cream needs to be recognized, and the existing market channels need to be extended. It has been reported that this emulsified cream is stable to oil oxidation over time, which makes its applications possible in food products (De Moura Bell et al., 2013). The cream separated by centrifugation during aqueous fractionation is not pure oil yet, so more processing steps are needed afterward to increase the oil purity. Depending on the applied oil recovery method, two forms of products are generally obtained: a liquid o/w natural emulsion or a semi-solid cream (Nikiforidis et al., 2014). With regard to liquid emulsions, the potential food applications could be the development of plant-based milk beverages and other oil-rich products such as salad dressings (Gallier et al., 2012). Besides, the semi-solid cream can be used to develop plant-based emulsion products such as mayonnaise (Romero-Guzmán et al., 2020), or incorporated into a protein-based matrix such as meat analogue products, because ideally, meat analogue products can be considered as a protein gel matrix in which oil is embedded (Kim et al., 2001).

Overall, the fractionation process proposed in this thesis might not lead to standardized ingredients, but ingredients that can be produced without organic solvent and have interesting applications. To introduce those specific ingredients, smaller-scale production facilities seem more appropriate.

7.6 Development of next generation of soy-foods

Traditional soy-foods have been consumed throughout East Asia for more than two thousand years. The processing chains of traditional soy-foods are relatively short because almost all products are made directly from whole soybeans. The new generation of soy-foods is manufactured by using soy protein ingredients mostly, which extends the processing chain with a soy fractionation process. Current soy protein ingredients have only developed over the last 60 years, and the time for the consumption of novel soy-foods for Western diets is

even shorter. Therefore, the best forms to develop and deliver soy-foods with good quality are not yet systematically built.

Product developers have been forced to use the current forms of soy protein ingredients, which were not designed for developing those novel foods. The research described in this thesis outlines how the current soy fractionation pathways can be adjusted to improve the link between fractionation and food applications. Though the analysis and discussions on the composition and functionality of the obtained ingredients can provide the information to product developers, it is also important to investigate the actual behavior of soy protein ingredients in full food formulations. Therefore, feedback can be collected from product developers when a material can or cannot perform proper functions in food products. This can give hints to the ingredient suppliers and the researchers on how to make proper ingredients (Fig. 7-5).

Overall, the development of next-generation of soy-foods is still challenging because it involves the contributions from food researchers, ingredient suppliers, and product producing companies over an extended food supply chain. To transfer the current scientific outcomes towards the soy protein supplying industry and soy-foods manufacturers, the communication should enhance the odds that soy-foods with good quality could deliver.
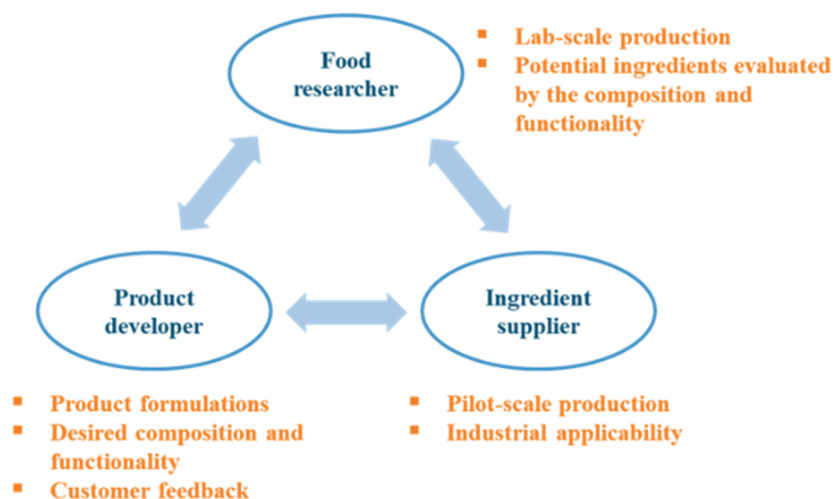

Fig. 7-5 *Cooperation between food researcher, ingredient supplier, and product developer.*

7.7 Concluding remarks

Traditionally, protein-rich ingredients were designed for high purity and certain functionalities, such as solubility, when used as additives (Kinsella, 1976). Working as a bulk ingredient for developing novel products like meat analogue, other functionalities, such as intermediate or low protein solubility may favor the applicability of soy protein. Unfortunately, lower solubility highly restrains the chemical and functional analyses of protein such as amino acid compositions, surface hydrophobicity, and protein oxidation, which severely hinders understanding the protein functionality. Only the soluble part of the protein is often analyzed, or specific steps such as hydrolysis are applied to solubilize the protein and obtain a sufficient quantity for the measurements (Trimpin and Brizzard, 2009). As a result, the relevance of the gained information may be limited. We have investigated several processing parameters to control the functionality of soy protein, of which the outcomes could be more constructive for adjusting the fractionation of other oilseeds or legumes if more fundamental analyses can be performed with protein. Therefore, the use of more innovative methods and techniques to characterize the insoluble proteins in their original state after fractionation will help to expand the current outcomes to the fractionation of more plant resources.

For less refined fractions, the functional contribution of non-protein components such as oil, sugar and fiber cannot be neglected. A route to understand the contribution of each component in SPF is to take highly purified soy protein ingredients such as SPI as the base, and to systematically add each individual component such as oil, sucrose, and cellulose to SPI in different quantities. A critical concentration may be found for each component, above which the functionalities of soy fraction can be significantly influenced. A model system can be built by incorporating all the components in SPI to mimic the composition of less refined fraction, and then the effects might be recognized. However, it should be noted that the configured fraction may behave differently than the fractionated fraction though their composition is similar. Therefore, more advanced methodologies can be explored to build better understandings of the functional changes of less purified ingredients.

With the parameter modifications during the fractionation process, we obtained a wide range of soy protein fractions. With the application of the meat analogue application in mind, some fractions showed more promising functionalities than others. Nevertheless,

fractions less suitable for meat analogue applications may have some interesting properties such as high foaming capacity or good emulsifying properties for developing other types of soy-derived products, for example, soy ice-cream, soy cheese analogue, or soy yogurt. Therefore, the exploration of other functionalities of the obtained soy ingredients is also very interesting.

To conclude, we explored the potential of using simplified fractionation and washing process to make novel soy ingredients. The production of less refined ingredients usually involves fewer processing steps, which may reduce energy consumption and increase resource use efficiency. The residual oil in the ingredients can possibly decrease the use level of refined oil in meat analogue formulations. However, the sustainability of the proposed fractionation pathways needs to be further quantified to be able to select the more sustainable process for novel soy-foods like meat analogue. A further step is to extend this research to other oilseeds or legumes.

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Summary

The market for plant-based alternatives for meat products is growing for about a decade now (Ismail et al., 2020). Initially, traditional soy-foods were consumed for thousands of years, and remained popular in Eastern diets nowadays also as alternative to meat. More recent though, the versatile properties, health benefits and relatively low price of soy has made it a source for the production of protein ingredients, which in their turn have stimulated the innovation of soy-based products, especially meat analogues, for broad markets (Thrane et al., 2017).

Novel soy-foods are mainly developed using soy protein ingredients. However, the current forms of commercially available soy protein ingredients are not optimized for these products. Specifically, current fractionation processes of soy protein focus on achieving high protein purity, or specific protein functionality to fit a broad range of applications, for many of which, soy is used as functional ingredient at low addition levels. Unfortunately, the process of making soy protein ingredients involved resource- and energy-intensive processing steps. Therefore, it is important to investigate more effective fractionation pathways to produce functional soy protein ingredients aimed at use as bulk ingredient in novel food applications such as meat analogues.

The current aqueous fractionation process of soy protein isolate includes sequential oil extraction, alkaline solubilization, acid precipitation and intensive washing steps to remove oil, insoluble carbohydrate and soluble carbohydrate in the soybean, respectively. This process leads to ingredients with a protein content over 85%. The oil extraction and intensive washing steps require the use of organic solvents (such as hexane) and large amounts of water respectively, which are undesired from an environmental point of view for soy fractionation. Therefore, in **Chapter 2** we explored the simplification of the current fractionation process by omitting these steps. The majority of oil was successfully removed from full-fat soy flour (FFSF) by centrifugation, and the protein content of soy protein-rich fractions (SPFs) was enriched to around 75% (N $\times$ 5.7). Further, we performed a pH adjustment step as an alternative to the neutralization step to produce the SPFs with a range of solubilities because high protein solubility may not be essential for developing novel soy-foods, such as meat analogues. We concluded that the change of processing pH in the range of 3.5 to 7.5 markedly altered the functional behaviors of obtained SPFs without influencing their compositions, especially solubility. The nitrogen solubility index (NSI) of freeze-dried

SPF was at a high level (above 80%) when the processing pH was away from the isoelectric point (pI) of soy protein, and at a low level (below 20%) when approaching the pI.

The SPFs obtained in **Chapter 2** were still in their native state. Therefore, we continued with the investigation of the effect of heating treatment on the denaturation level and functional properties of SPFs in **Chapter 3**. After the neutralization step, a moisture heating step was applied to the protein-rich dispersion with a relatively mild temperature between 60 and 100°C. The protein and oil content of all the SPFs were identical because the additional heating step was incorporated in the process after the protein extraction stage. The degrees of protein denaturation of the SPFs, turned out to be dependent on the heating temperature, and the differences in protein denaturation explained the differences in functional behaviors. Interestingly, partially denatured soy protein contributed to a gel network resulting in a gel highest elasticity after a second heating step.

In **Chapter 4**, next to the simplified aqueous fractionation developed in **Chapter 2**, we simplified the process further by removing the acid precipitation step. The protein content of SPF was reduced after additional simplification but still comparable to the purity of commercial SPC. During the aqueous fractionation, NaOH is regularly used as the basic solution for pH shifts in the alkaline solubilization and neutralization step. In this way, extra Na content is introduced in the soy ingredient. With the use of $Ca(OH)_2$ as an alternative, the pH shifts provided the chance for Na reduction, as well as Ca enrichment. We found that the use of $Ca(OH)_2$ in the alkaline solubilization step enhanced the protein content of SPF. The Ca content of the SPF was almost completely determined through the use of $Ca(OH)_2$ in the neutralization step. That lowered the Na content as well. However, complete replacement of NaOH by $Ca(OH)_2$ altered the functionality of SPF drastically, especially the reduction of protein solubility, in such a way that the SPF became less suitable for application as ingredient in meat analogue applications.

Therefore, in **Chapter 5** we continued the research on the use of $Ca(OH)_2$ in the neutralization step to gain more in-depth understanding on how the Ca enrichment affects the soy protein. Instead of completely replacing NaOH by $Ca(OH)_2$, combinations of NaOH and $Ca(OH)_2$ in different ratios were applied to neutralize the protein-rich dispersions, and the effects of Ca enrichment on the conformation and functionalities of soy protein were discussed. No separation step was performed after the neutralization step, so the Ca and Na content in the SPF could be precisely controlled by adjusting the ratios. The functional changes of SPF were impacted mainly by the Ca content. However, we observed a critical Ca concentration (6.5 mg Ca/g protein), below which the structural and functional properties of soy protein did not change significantly. Therefore, the Ca content in the SPF can be enriched to a certain level without strongly impacting the applicability of SPF.

In **Chapter 6** we focused on using aqueous ethanol washing process to produce SPF that can compete with SPC. Cold-pressed de-fat soy meal (DFSM) was used as the starting material. Different water-ethanol ratios were investigated for the washing process, and we conclude that either pure water should be used as washing solvent or solvents with high ethanolic ratio. High ethanolic ratio (60% and above) as solvents gave the highest yield in protein, as well as the highest protein solubility and water holding capacity. Among all the SPFs, when adjusted at similar protein concentration, water-washed SPF (0% ethanol) showed the highest viscosity, and it formed the gel with the highest gel strength and hardness. The variations in the functionality were attributed in changes of protein, though effects of non-protein constitutes such as sugar and oil might also have played a role. The fractions obtained after washing has comparable composition as commercial SPC, but with a range of functional properties that can be relevant to the development of novel soy-foods.

Chapter 7 summarizes all the results reported in this thesis and provides an overview on how the fractionation process can be modified or selected to produce new soy protein ingredients for innovative applications such as meat analogues. Besides, the results of the pilot scale productions of SPF are discussed, and the potential applications for the side streams of the suggested fractionation processes are explored. The chapter ends with a future outlook about the potentials and challenges of developing simplified soy fractionation processes.

Appendix

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While I am writing this acknowledgements, my PhD journey is approaching to an end. Looking back on the last four years, I realize that doing a PhD is not only an adventure for me in academia, but also a period during which I gradually learn to know myself, to be respectful, to think independently, to behave bravely, and to appreciate all the kindness and supports I have ever received.

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About the author

Yu Peng was born on May 26, 1992 in Changsha, China.

In 2010, she started her study of Food Science and Technology at China Agricultural University, and got her BSc degree there in 2014. In the same year, Yu continued her master program at the National Engineering Research Center for Fruit and Vegetable Processing at China Agricultural University, under the supervision of Prof. Yuanying Ni. In the second year of her MSc study, she got a scholarship from China Scholarship Council (CSC) to visit Purdue University in the U.S.A, where she completed her master thesis *Effects of twin-screw high shear processing technology on functional and physicochemical properties of corn* under the supervision of Prof. Qin Xu and Prof. Srinivas Janaswamy.

In 2017, Yu received a grant from CSC again to pursue a doctorate abroad. In September, she started working as a PhD candidate at the Laboratory of Food Process Engineering at Wageningen University & Research, the Netherlands. This thesis entitled *Soy fractionation pathways for food applications* is the result of her PhD project.

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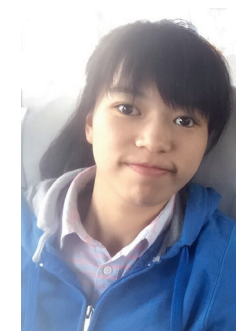
Publications

This thesis

Peng, Y., Kersten, N., Kyriakopoulou, K., & van der Goot, A. J. (2020). Functional properties of mildly fractionated soy protein as influenced by the processing pH. *Journal of Food Engineering*, 275, 109875. https://doi.org/10.1016/j.jfoodeng.2019.109875

Peng, Y., Dewi, D. P. A. P., Kyriakopoulou, K., & van der Goot, A. J. (2020). Effect of calcium hydroxide and fractionation process on the functional properties of soy protein concentrate. *Innovative Food Science & Emerging Technologies*, 102501. https://doi.org/10.1016/j.ifset.2020.102501

Peng, Y., Kyriakopoulou, K., Rahmani, A., Venema, P., & van der Goot, A. J. (2021). Isochoric moisture heating as a tool to control the functionality of soy protein. *LWT*, 150, 111979. https://doi.org/10.1016/j.lwt.2021.111979

Peng, Y., Kyriakopoulou, K., Keppler, J., Venema, P., & van der Goot, A. J. Effect of calcium enrichment on the composition, conformation, and functional. *Submitted.*

Peng, Y., Kyriakopoulou, K., Ndiaye, M., Bianeis, M., Keppler, J., & van der Goot, A. J. Characteristics of soy protein prepared by aqueous ethanol washing process. *Submitted.*

Other publication

Peng, Y., Yao, T., Xu, Q., & Janaswamy, S. (2021). Preparation and characterization of corn flours with variable starch digestion. *Food Chemistry*, 366, 130609. https://doi.org/10.1016/j.foodchem.2021.130609

Overview of completed training activities

Discipline specific activities

Courses

Healthy Food Design (VLAG, NL)	2018
Masterclass "Dairy Protein Biochemistry" (VLAG, NL)	2018
Food Proteins: Functionality, Modifications and Analysis (VLAG, NL)	2018
Microscopy and Spectroscopy in Food and Plant Sciences (VLAG & EPS, NL)	2019
17th European School on Rheology (KU Leuven, BE)	2019

Conferences

Science and Technology for Meat Analogues (Wageningen, NL)	2018
2nd Innovations in Food Science & Technology (Amsterdam, NL) [a]	2019
33rd EFFoST International Conference 2019 (Rotterdam, NL) [b]	2019
NIZO Plant Protein Functionality Conference (Online) [b]	2020
Science and Technology for Meat Analogues (Online) [a]	2021

[a] Oral presentation; [b] Poster presentation

General courses

VLAG PhD week (VLAG, NL)	2017
The Essentials of Scientific Writing and Presenting (WGS, NL)	2018
PhD Workshop Carousel (WGS, NL)	2018
Scientific Writing (WGS, NL)	2019
Supervising BSc & MSc Thesis Students (WGS, NL)	2019
Research Data Management (WGS, NL)	2019
Searching and Organising Literature (WGS, NL)	2019
Scientific Publishing (WGS, NL)	2019
Scientific Artwork, Data Visualisation and Infographics with Adobe Illustrator (WUR Library, NL)	2020
Career Perspectives (WGS, NL)	2021

Other activities

Preparation of Research Proposal	2017
PhD Study Tour to Canada	2018

FPE Weekly Group Meetings	2017-2021
FPE Group Days	2017-2021
Food Structure Journal Club	2018-2021