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

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ABSTRACT

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

Industrial applications

Consumer awareness of reducing the use animal-based products has been stimulated worldwide as a result of environmental concerns, vegetarianism trends and consumer acceptance of plant-based products (Pojić, Mišan, & Tiwari, 2018). Replacing animal-centric products with plant-based products is a challenge because of functional and nutritional differences. One example of the nutritional differences is related to calcium, which is mostly provided via animal products such as dairy foods in many diets. The high level of sodium in foods in modern diets is a concern (Kloss, Meyer, Graeve, & Vetter, 2015). Sodium is added for structure and taste during the development of soy products, but it is also introduced via the use of acids and bases during the fractionation process of soy protein ingredients. Therefore, achieving high-calcium and low-sodium levels in most plant-based protein products is a challenge. Using soybean as the starting material, we proposed the use of $Ca(OH)_2$ instead of NaOH during an alternative mild fractionation process. The composition and functionalities of the ingredients were then evaluated. Based on these results, soy foods can be developed with enriched calcium content and reduced sodium content using alternative mild fractionation. The proposed methodology would be potentially applicable to other plant-based resources such as pea, chickpea, and lupin.

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, Dekkers, & van der Goot, 2019). Among those functionalities, protein solubility, water-holding capacity (WHC) and viscoelastic behaviour are considered the most important (Alves & Tavares, 2019; Batista, Portugal, Sousa, Crespo, & Raymundo, 2005; Ce et al., 2006; Zare & 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 and sodium 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 calcium, therefore plant-based alternatives must also

Abbreviations: CCR, closed cavity rheometer; FFSF, full-fat soy flour; MF-SPF, mildly fractionated soy protein fraction; NSI, nitrogen solubility index; SEM, scanning electron microscopy; SF-SPF, simply fractionated soy protein fraction; SPF, soy protein fraction; WHC, water-holding capacity

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provide calcium if they are to replace animal-based products in diets. This is especially important for those who need calcium supplementation, such as children, pregnant women and elderly people (Golden et al., 2014; Hofmeyr, Lawrie, Atallah, & Duley, 2010). By contrast, in the western diet, sodium 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, Qing Zhu, Xu Hong, & Huai Sun, 2014). Thus, creating meat analogue products that do not increase the sodium intake in the diet above the recommended level is important. Excessive intake of sodium increases the risk of many diseases, including hypertension and coronary heart disease (Mitchell, 2019). Traditionally, sodium is added to enhance the flavour and texture of a final product, as well as to extend the shelf life due to its ability to lower water activity (Ruusunen & Puolanne, 2005). However, sodium can also originate from the use of NaOH during the fractionation of ingredients (J. Jiang, Chen, & Xiong, 2009). For that reason, the sodium 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 sodium before processing (Verma & 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 sodium (https://fdc.nal.usda.gov/fdc-app.html#/food-details/174276/nutrients). The high sodium content of SPI results from the precipitation and neutralization steps during fractionation. Therefore, achieving suitable calcium and sodium levels is a challenge when developing next-generation plant-based products. Effective methods are needed to address enrichment of the calcium content and reduction of the sodium content in the plant-based food industry.

The attractive price of $Ca(OH)_2$ ($116/t) compared with NaOH ($450/t) and the reduced safety issues make it an interesting option (D. Jiang et al., 2017). Moreover, calcium 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, Perera, & Valiyaveettil, 2006). Therefore, replacing NaOH with $Ca(OH)_2$ during the protein fractionation process could produce plant protein ingredients that are high in calcium and low in sodium. Currently, the strategies used in the formulation of reduced-sodium and enriched-calcium products are mainly focused on the processing steps; for example, reducing the amount of sodium chloride in the recipe or adding calcium fortifier into products such as soy milk (Dötsch et al., 2009; Verma & Banerjee, 2012). To date, there is hardly any information on how the fractionation process can play a role in producing high-calcium, low-sodium 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, De Lamballerie, & Speroni, 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, van Riemsdijk, Van Der Goot, & Boom, 2007; Wang, Tian, Boom, & van der Goot, 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, Mejia, Craig, Jaceldo-Siegl, & Sabaté, 2019). Traditionally, soy protein ingredients are produced through wet 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 & 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, Boom, & Van Der Goot, 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 behaviour of the soy protein fractions (SPFs) obtained were evaluated. The aim of this study was to create soy protein ingredients with high-calcium and low-sodium content, as well as specific functionalities for developing soy-based products.

2. Materials and methods

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.

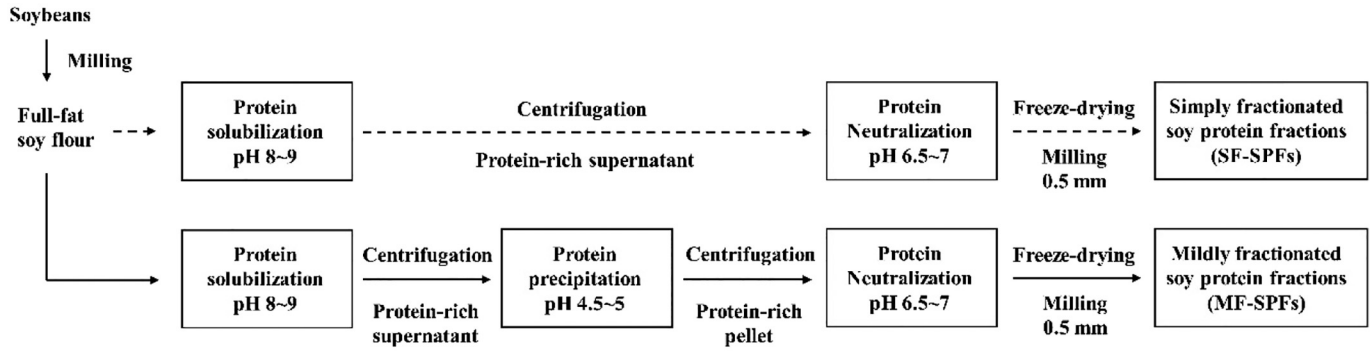
2.2. Methods

2.2.1. Preparation of full-fat soy flour

A pin mill LV 15M (Condux-Werk, Wolfgang bei Hanau, Germany) was used to pre-mill soybeans into soy grits. Then, the soy grits were further milled into full-fat soy flour (FFSF) using a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) with a classifier wheel speed of 2500 rpm, air flow of 0.8 m^3/h and impact mill speed of 8000 rpm. The feed rate of the impact mill was set around 3–5 rpm. Large batch of FFSF was prepared and stored in a cooling room (4 °C) until further analysis and processing. The FFSF contained 38.9% ± 4.2% of protein and 20.6% ± 0.6% of oil on a dry basis.

2.2.2. Preparation of soy protein fractions

The soy protein fractions 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. 1. The mild fractionation process was based on previous research (Geerts, Dekkers, van der Padt, & van der Goot, 2018; Peng, Kersten, Kyriakopoulou, & van der Goot, 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 performed 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 10,000 rpm 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/



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. 1. The mild and simplified fractionation processes and abbreviations for the SPFs.

$Ca(OH)_2$ 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.

2.2.3. Chemical composition

The protein content of SPFs was measured using a nitrogen analyser (Dumas method; FlashEA 1112 Series, Thermo Scientific, the Netherlands) with a conversion factor of 5.7. The oil content was measured using the Buchi extraction system (Buchi Labortechnik AG, Flawil, Switzerland) with petroleum ether as the extraction solvent. The calcium and sodium 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 analysed in triplicate.

2.2.4. Powder morphology

The morphology of all the SPFs was determined by scanning electron microscopy (SEM, Phenom Pure G2, Phenom-world BV, Eindhoven, the Netherlands). The SPF was evenly placed on an aluminium sample holder using double-sided adhesive conductive carbon tape, and the microstructure was observed with an accelerating voltage at 5 kV.

2.2.5. Nitrogen solubility index and water-holding capacity

A 2% (w/w) protein dispersion of each SPF was prepared with water in a centrifuge tube, rotated overnight and centrifuged (10,000 rpm, 30 min, 25 °C). The supernatant was discarded and the wet pellet was weighed. Subsequently, the wet pellet was oven-dried and weighed again. The nitrogen content in the dry pellet was measured using Dumas analysis. The WHC was calculated as the ratio of the wet pellet weight to the dried pellet weight. The nitrogen solubility index (NSI) was calculated as the ratio of soluble nitrogen to the total initial nitrogen content present in the SPFs (Geerts et al., 2018). All measurements were performed in triplicate for each produced sample.

2.2.6. Thermal properties

The thermal properties of SPFs were assessed by differential scanning calorimetry (Diamond DSC, PerkinElmer). A 20% (w/w) protein dispersion of each SPF was prepared and placed in high-volume aluminium 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 aluminium pan was used as a reference. Measurements were analysed with Start Pyris Software for the denaturation temperature and enthalpy of transition. All measurements were performed at least three times for each sample.

2.2.7. Rheological behaviour

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}.

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

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

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

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 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.

2.3. 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.

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. 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 analyse some functional properties, such as gelling properties.

3.1. Chemical composition

The compositions of all the SPFs are summarized in Table 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, Kyriakopoulou, Suarez-Garcia, van den Berg, & van der Goot, 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, Campbell, Almeida, Glatz, & Johnson, 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, Hamoen, Boom, & van der Goot, 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, van der Goot, & Nikiforidis, 2019; Romero-Guzmán, Jung, Kyriakopoulou, Boom, & Nikiforidis, 2020).

Ca and Na contents differed significantly among the different fractions produced (Table 2). It was found that using $Ca(OH)_2$ in the solubilization step during simplified fractionation (SF-Ca) improved the calcium content (5.45 mg/g SPF) and reduced the sodium content (0.23 mg/g SPF) compared with the corresponding process using NaOH (SF-Na). However, the differences in the calcium 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 calcium content was observed, showing that the precipitation and neutralization steps had a significant impact on the chances of calcium remaining in the protein-rich fraction. Acidic conditions reduced the amount of calcium 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 calcium ions bound. Based on this information, it can be hypothesized that the calcium ions added in the solubilization step (pH 8–9) were mostly bound and remained in the supernatant after the first centrifugation. The calcium content was further enriched, however, when $Ca(OH)_2$ was used in the neutralization step. In parallel a decrease in the sodium content was observed for samples where NaOH was used in the solubilization step. For example, the calcium 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 sodium 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 calcium and sodium 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 calcium in the fraction was mainly enriched when applied in the neutralization step. Therefore, a high-calcium low-sodium 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

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

Soy protein fraction: group	Soy protein fraction: name	Protein (%)	Oil (%)	Na content (mg/g SPF)	Ca content (mg/g SPF)	Ca content (mg/g protein)
SF-SPFs	SF-Na	57.22 ± 0.51^{a}	1.21 ± 0.50^{a}	5.10 ± 0.44^{a}	1.46 ± 0.11^{a}	2.70 ± 0.18^{a}
	SF-Ca	56.85 ± 0.42^{a}	1.04 ± 0.06^{a}	0.23 ± 0.12^{b}	5.45 ± 0.34^{b}	10.10 ± 0.63^{b}
MF-SPFs	MF-Na-Na	73.63 ± 2.62^{b}	1.13 ± 0.56^{a}	12.00 ± 0.93^{c}	0.44 ± 0.04^{c}	0.63 ± 0.05^{c}
	MF-Ca-Na	81.13 ± 1.73^{c}	1.34 ± 0.65^{a}	13.48 ± 0.37^{d}	1.22 ± 0.21^{a}	1.58 ± 0.29^{d}
	MF-Na-Ca	74.42 ± 0.81^{b}	3.00 ± 0.96^{b}	0.61 ± 0.03^{e}	12.27 ± 0.63^{d}	16.98 ± 1.07^{e}
	MF-Ca-Ca	79.60 ± 1.18^{d}	1.50 ± 0.55^{a}	0.10 ± 0.07^{b}	14.35 ± 1.26^{e}	18.68 ± 1.51^{f}

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

SF-SPFs. Therefore, the results indicated that calcium 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 calcium ions could further decrease soy protein solubility under acidic conditions (Manassero, David-Briand, Vaudagna, Anton, & Speroni, 2018; Ono, Katho, & Mothizuki, 1993). A possible explanation might be that the binding of calcium ions to carboxyl groups of soy proteins brings about the association of proteins and accelerates the formation of aggregation from hydrated proteins (Mohamed, May, & Morris, 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 calcium content of the fractions and lowered the sodium content.

3.2. Morphology

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. 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).

3.3. Nitrogen solubility index and water-holding capacity

Protein solubility is an important functionality of soy protein ingredients for general applications (Thrane, Paulsen, Orcutt, & Krieger, 2016). The WHC, which is defined as the ability of a sample to hold water (Peters, Vergeldt, Boom, & van der Goot, 2017), is also important. The NSI and WHC were evaluated for all SPFs, and the results are presented in Fig. 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 calcium 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. 3A). This variation can be correlated to the calcium content in the fractions (Table 2). Fractions with a calcium content less than 3 mg/g protein (SF-Na, MF-Na-Na and MF-Ca-Na) showed relatively high NSI. SF-Ca with a calcium content of 10.1 mg/g protein showed a reduction in the NSI by a third, whereas the SPFs with the highest calcium 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 calcium up to 5 mg/g protein did not significantly affect the solubility of SPI, but when more calcium 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^{2+} and decreased sharply between 6 mM and 8 mM (Ono et al., 1993). A possible explanation for these findings is that the presence of calcium ions promoted the formation of aggregates. These soy protein aggregates were still soluble up to certain concentrations, but at a higher calcium level, the aggregates became insoluble due to their large size (Añón et al., 2012; Yuan et al., 2002). Therefore, controlling the amount of calcium ions added during the fractionation process could lead to SPFs with specific solubility, designed according to the requirements of specific food applications.

Apart from the protein solubility, the $Ca(OH)_2$ added during the fractionation procedure also seemed to affect the WHC of the SPFs (Fig. 3B). However, the relationship between addition of calcium and the WHC of the fractions was not clear. Calcium-enriched SF-Ca showed higher WHC than SF-Na. By contrast, calcium-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.

3.4. Thermal properties

The values for the enthalpy and denaturation temperature (T_d) of SF-SPFs and MF-SPFs are presented in Table 3. All the SPFs exhibited two thermal transitions, which correspond to the reported denaturation temperature of β-conglycinin (7S) and glycinin (11S) (Ce 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.

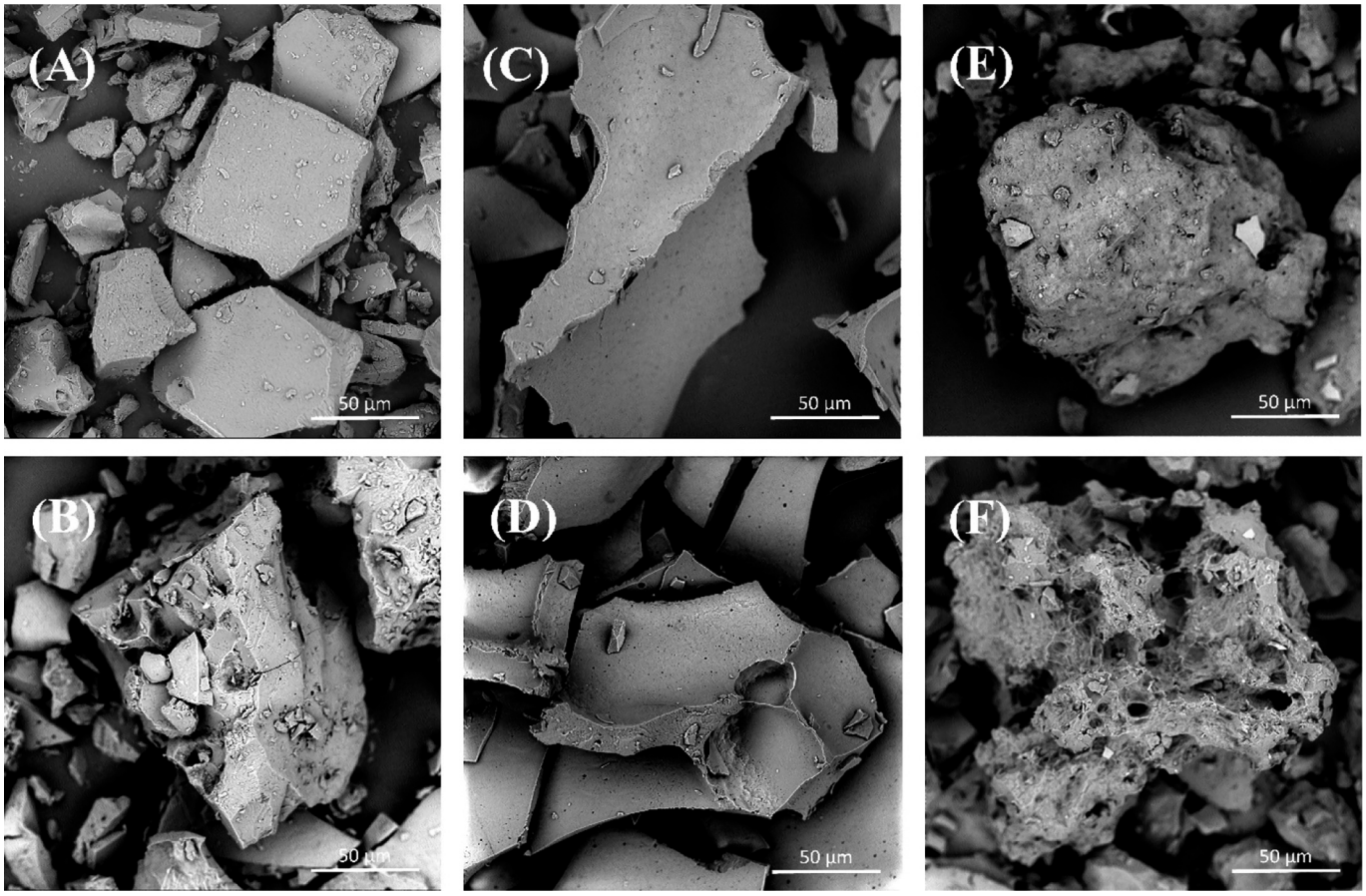


Fig. 2. SEM images of (A) SF-Na, (B) SF-Ca, (C) MF-Na-Na, (D) MF-Ca-Na, (E) MF-Na-Ca and (E) MF-Ca-Ca.

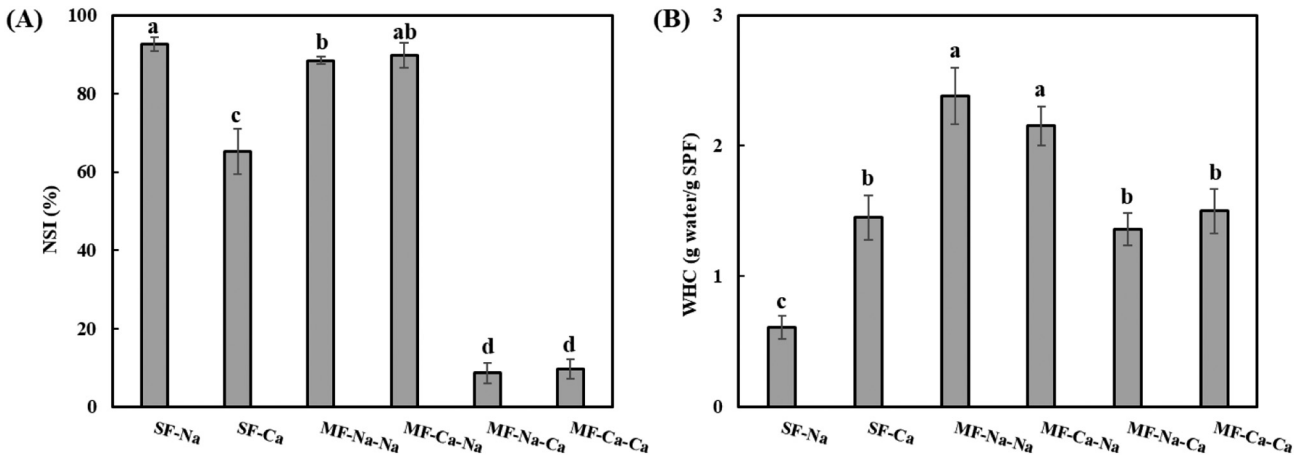


Fig. 3. (A) Nitrogen solubility index and (B) water-holding capacity all the SPF samples.

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 calcium 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 calcium concentration, whereas the T_d of 7S increased significantly when the calcium 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 calcium 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).

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

3.5. Rheological properties

The rheological properties of SPFs at different protein concentrations are presented in Figs. 4 and 5. All SPF dispersions showed

Table 3
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^{a}	1.21 ± 0.39^{a}	97.44 ± 1.88^{a}	5.76 ± 0.63^{a}
SF-Ca	75.94 ± 2.16^{a}	1.27 ± 0.34^{a}	99.42 ± 1.39^{b}	5.93 ± 1.04^{a}
MF-Na-Na	76.25 ± 1.32^{a}	1.85 ± 0.51^{ab}	94.16 ± 1.76^{c}	7.26 ± 0.57^{b}
MF-Ca-Na	77.29 ± 1.19^{a}	2.03 ± 0.64^{b}	95.62 ± 1.76^{c}	8.22 ± 0.20^{b}
MF-Na-Ca	80.68 ± 2.20^{b}	3.67 ± 0.52^{c}	100.94 ± 0.64^{b}	5.69 ± 3.86^{b}
MF-Ca-Ca	83.30 ± 1.59^{c}	3.26 ± 0.59^{bc}	103.82 ± 1.52^{d}	9.95 ± 2.62^{b}

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

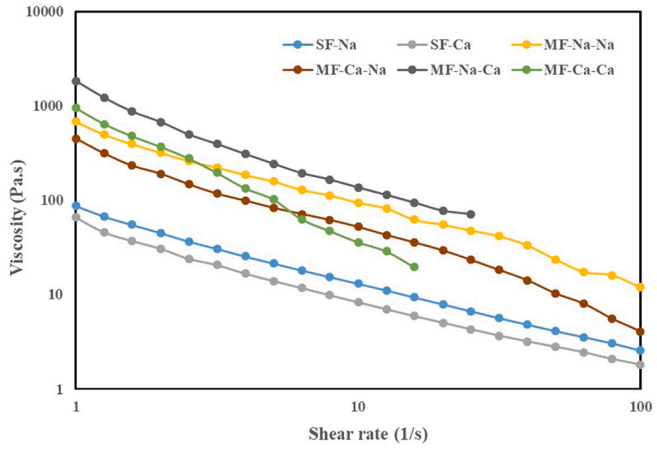


Fig. 4. Viscosity of the all the SPFs (30% protein concentration).

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, calcium-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.

The rheological properties of denser protein blends (34% protein concentration) were analysed 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. 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 calcium remaining in the SPFs increased the G^* value. This finding can be associated with the higher thermal stability of calcium-enriched SPFs (Section 3.4). The presence of calcium ions may promote the formation of aggregates, which are stabilized by hydrophobic interactions and/or calcium bridges between polypeptides during the temperature sweep (Speroni et al., 2010). As a result, the G^* value of calcium-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.

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-calcium and low-sodium 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

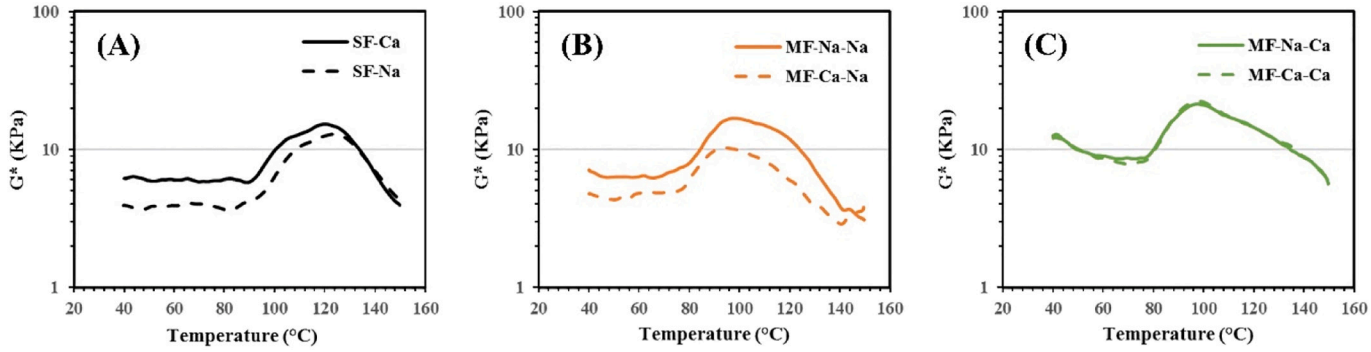


Fig. 5. Complex modulus (G^*) measured (34% protein concentration) as a function of the temperature of (A) SF-Na and SF-Ca, (B) MF-Na-Na and MF-Ca-Na and (C) MF-Na-Ca and MF-Ca-Ca.

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.

CRediT authorship contribution statement

Yu Peng: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Visualization, Validation, Writing - Original Draft

Desak Putu Ariska Pradnya Dewi: Investigation, Data Curation, Visualization

Konstantina Kyriakopoulou: Conceptualization, Validation, Writing - Review & Editing, Supervision

Atze Jan van der Goot: Conceptualization, Writing - Review & Editing, Supervision

Declaration of competing interest

None.

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