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

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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 during the neutralization step of 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 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.

1. Introduction

Plant-based diets aim to minimize animal foods, such as meat, dairy, and eggs (Lynch, Johnston, & Wharton, 2016; Tuso, Ismail, Hs, & Bartolotto, 2013). They have become more widespread among the population in the promotion of physical and environmental health (Bizzo & Baroni, 2019). Therefore, it is important that plant-based diets are balanced, and that they can provide all essential nutrients. Animal-based foods such as dairy products are naturally rich in calcium (Ca), while their Ca bioavailability is also high. This is because Ca is chelated in casein micelles enabling high solubility in food and intestinal lumen (Gauschner, 2005). However, common plant sources including most vegetables, fruits, and cereal grains are lacking in Ca (Keller, Lanou, & Barnard, 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). This poses a hurdle specifically for vegans to reach the recommended dietary Ca intake of 1000 mg per day (Institute of Medicine (US), 2011). Early studies showed that vegans have a considerably lower average Ca intake than recommended levels compared to vegetarians and omnivores (Davey et al., 2003; Larsson & Johansson, 2002). This low Ca intake increases the risk of bone fracture and osteoporosis because Ca is essential for bone health (Appleby, Roddam, Allen, & Key, 2007; Wojcik 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 & Falvo, 2004). Soybeans are rich in phytate, but the Ca-enriched soy-foods such as Ca-fortified soy milk (1000 mg Ca/L) and Ca-set tofu (117 mg Ca/100g) have been proven to be good sources of bioavailable Ca for humans (Weaver et al., 2002; Y. Zhao, Martin, & Weaver, 2005). Different soluble and less-soluble calcium salts can be used for Ca enrichment of soy protein, such as calcium chloride, calcium carbonate, calcium phosphate, calcium citrate, and calcium gluconate. The use of certain salts however may lead to changes in the physico-chemical properties of protein as well as the Ca bioavailability (Acosta et al., 2020). Moreover, Ca enrichment can be done in different steps

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throughout the process chain of soy foods. For example, CaSO₄ is used as a coagulant for tofu production (Rekha & Vijayalakshmi, 2013), while Ca fortifiers are often added after the soy milk preparation (Z.-G. Li, Yue, Liu, & Zhang, 2011; Yazici, Alvarez, Mangino, & Hansen, 1997). CaCl₂ has been used for the Ca enrichment of commercially available soy ingredients such as soy protein isolate (SPI) (Manassero, Vaudagna, Anón, & Speroni, 2015; Piccinii, Scilingo, & Speroni, 2019), which is a popular ingredient for novel soy-foods such as meat and dairy analogues (Bal-dassarre et al., 2020; Bhatia & 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 can be used 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 (Nicolas A. Deak & Johnson, 2007). In the human diet, extra Na intake can raise blood pressure, which increases the risk of cardiovascular disease (Strazzullo, D’Elia, Kandala, & Cappuccio, 2009). It might also enhance urinary Ca losses, leading to the opposite effect of our initial purpose (Itoh & 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.

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 CaCl₂ in the dispersions afterward (Manassero et al., 2015; Piccinii et al. fractionated the SPI first and added CaCl₂ before or after denaturing treatments (Piccinii 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, Dewi, Kyriakopoulou, & van der Goot, 2020, p. 102501). However, both Na and Ca ions can screen electrostatic interactions between charged protein molecules, whereas divalent salt ions like Ca ions also have the ability to cross-link negatively charged carboxylic acid groups on neighboring protein molecules (Hongsprabhas & Barbut, 1997). Thus, the added effect from the formation of salt bridges altered the functionalities of proteins. Our previous study reported that a complete replacement of NaOH with Ca(OH)₂ greatly varied the 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 (FTIR), 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.

2. Materials and methods

2.1. Materials

Dry, full soybeans (with hulls) were obtained from FRANK Food Products (Twello, The Netherlands) and stored in a cooling room (4 °C). Analytical grade NaOH, Ca(OH)₂, and HCl were purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.A). Ultrapure water from a Milli-Q Lab Water System (Milli-Q IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany) was used throughout the study unless stated otherwise.

2.2. Methods

2.2.1. Full-fat soy flour (FFSF) preparation

Full-fat soy flour was prepared according to the methods developed earlier (Peng, Kersten, Kyriakopoulou, & van der Goot, 2020, p. 102501). In short, full soybeans (with hulls) were pre-milled to soy grits, and the soy grits were further milled into FFSF. Around 5 kg of FFSF were produced and stored in a cooling room (4 °C) for 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. Soy protein-rich fractions preparation

Soy protein-rich fractions (SPFs) were fractionated based on the process developed earlier (Peng, Dewi, et al., 2020, p. 102501). An overview of the processing steps and parameters can be found in Fig. 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 1). The pH of the mixtures was between 12.4 and 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.

2.2.3. Composition analysis

The protein content of FFSF and SPFs was determined by using Dumas analysis (Rapid N exceed, N/Protein Analyzer, Elementar, Germany) with a conversion factor of 5.7 (Krul, 2019). The oil content was measured using the Buchi extraction system (Buchi Labortechnik AG, Flawil, Switzerland) with petroleum ether as the extraction solvent. The Ca and Na contents in the SPFs were 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.

2.2.4. Powder morphology analysis

The morphology of all the SPF powders was determined by scanning electron microscopy (SEM) (JCM-7000 NeoScope™ Benchtop SEM, JEOL Ltd., Japan). The protein powder was placed evenly on an aluminium sample holder using double-sided adhesive conductive carbon tape, and the powder morphology was observed with an accelerating voltage at 5 kV.

2.2.5. 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
SPF dispersions (neutral pH) were prepared, and the measurements were conducted at a temperature of 25 °C. Protein analytics in solutions as previously described by Kayser et al. (Kayser, Arnold, Steffen-Heins, Schwarz, 2020) were carried out in two steps: heating from 20 °C to 150 °C with a gradient of 5 °C/min, and subsequent cooling step from 150 to 20 °C with a gradient of 10 °C/min. An empty high-volume aluminium pan was used as a reference. The denaturation temperature (T_d) and enthalpy of transition were determined from the DSC graph by Start Pyris Software (PerkinElmer, USA), and the denaturation temperature was defined as the temperature at which a maximum occurred in the endothermic peaks.

2.2.9. Denaturation behaviour analysis

The denaturation behaviour of the SPFs was determined by differential scanning calorimetry (Diamond DSC, PerkinElmer, USA). A 20% (w/w) protein dispersion of each powder was prepared in a high-volume aluminium pan, which was then well sealed. The DSC analysis was carried out in two steps: heating from 20 °C to 150 °C with a gradient of 5 °C/min, and subsequent cooling step from 150 to 20 °C with a gradient of 10 °C/min. An empty high-volume aluminium pan was used as a reference. The denaturation temperature (T_d) and enthalpy of transition were determined from the DSC graph by Start Pyris Software (PerkinElmer, USA), and the denaturation temperature was defined as the temperature at which a maximum occurred in the endothermic peaks.

2.2.10. Water holding capacity

The water holding capacity (WHC) of the SPFs was determined by using the method described by Peters (Peters, Vergeldt, Boom, & van der Goot, 2017) with slight modification. SPF sample (M_f) was placed in a centrifuge tube with water to make a 2% (w/v) SPF dispersion and rotated overnight. Then, the dispersion was centrifuged at 20,000 × g for 30 min (25 °C). The supernatant was discarded and the wet pellet was collected and weighed (M_d). Subsequently, the wet pellet was oven-dried overnight at 105 °C and weighed again to determine the mass of the dry pellet (M_d). The WHC was calculated according to Equation (1) and the WHC of the pellet (WHC_p) was calculated according to Equation (2).

\[
WHC = \frac{g \text{ water}}{g \text{ dry SPF powder}} = \frac{M_d - M_f}{M_f} \quad (1)
\]

\[
WHC_p = \frac{g \text{ water}}{g \text{ dried pellet}} = \frac{M_d - M_f}{M_f} \quad (2)
\]

2.2.11. 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\(^{-1}\).

2.2.12. Gelation behaviour analysis

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 min at 95 °C before cooling back to 20 °C at a rate of 3 °C/min. The storage (G') and loss modulus (G″) were recorded as a function of time. The point where the storage and loss modulus crossed was taken as the gel point, while the temperature at that point was defined as the gelation temperature (Tgel) (Spotti, Tarhan, Schafter, Corvalan, & Campanella, 2017). 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 frequency, and strain were recorded.

2.2.13. Statistical analysis

The different fractionation schemes (with the varied neutralization conditions) were performed in duplicate, and if not stated otherwise, all data were collected for each sample from at least triplicate measurements. SPSS Statistics Version 23.0 (IBM, USA) 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

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.

3.1. Composition

The protein content of all SPFs was similar and around 75% on a dry basis (Table 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, Dewi, et al., 2020, p. 102501). In terms of Na and Ca content, the differences in the neutralization step led to differences in the composition, particle morphology, charge density, and functional properties of the SPFs.

3.2. Powder morphology

SPFs prepared with different Ca(OH)₂ and NaOH ratios appeared to have some differences in their microstructure (Fig. 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.

3.3. Particle size distribution (PSD)

The PSD curves of 1% SPF dispersions in water (Fig. 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-peak, while for SPF 6 and SPF 7, a relatively narrow distribution of larger size was observed.

3.4. Secondary structure

Fig. 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, Zai-Rose, Correia, & Janorkar, 2020; Kong & Yu, 2007). Early studies suggested that 7S soy protein consists mainly of α-helix and antiparallel β-sheet structures (Nagano, Mori, & Nishimura, 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 75 and 11 S, with approximately 46.7% β-sheet structures in aqueous solution (X. Zhao, Chen, Xue, & Lee, 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 SPF 5, 6, and 7. All other structural elements were randomly changing with increasing Ca content, and thus within the natural deviation or repeated measurements.

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, Zhou, Fu, Chen, & Wu, 2013), and is affected by many factors such as pH, ionic strength, and temperature (Malhotra & 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). 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

Table 2

| Sample name | Protein (%) | Oil (%) | Na content (mg/g protein) | Ca content (mg/g protein) |
|-------------|-------------|---------|--------------------------|--------------------------|
| SPF 1       | 75.06 ± 2.17 | 1.52 ± 0.52 | 15.91 ± 1.29 | 0.57 ± 0.06 |
| SPF 2       | 74.53 ± 1.21 | 1.21 ± 0.20 | 11.02 ± 0.75 | 1.90 ± 0.12 |
| SPF 3       | 74.70 ± 1.76 | 1.76 ± 0.26 | 9.80 ± 0.05 | 4.28 ± 0.02 |
| SPF 4       | 75.27 ± 0.65 | 2.07 ± 0.26 | 7.47 ± 0.47 | 6.47 ± 0.40 |
| SPF 5       | 74.34 ± 0.75 | 2.53 ± 0.28 | 5.67 ± 0.24 | 9.84 ± 0.41 |
| SPF 6       | 75.55 ± 1.85 | 2.04 ± 0.90 | 2.84 ± 0.09 | 12.62 ± 0.29 |
| SPF 7       | 75.73 ± 0.93 | 2.18 ± 0.70 | 0.81 ± 0.02 | 16.55 ± 0.66 |

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

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.

3.7. Denaturation behaviours (DSC)

Two typical endothermic peaks were observed in all the SPFs, corresponding to the denaturation of $\beta$-conglycinin (7S) and glycinin (11S) (Table 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 $^\circ$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 & Anón, 1996). The enthalpies corresponding to the denaturation of 7S and 11S proteins are shown in Table 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.
3.8. Water holding capacity (WHC)

The ability to hold water is an important functionality for protein-rich ingredients, while various definitions and terms of WHC can be found in the literatures (Kneifel, Paquin, Abert, & Richard, 1991; Zayas, 1997). WHC is defined as the amount of water bound per gram of dry

Fig. 4. Deconvoluted second derivative FTIR 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.

Fig. 5. Zeta potential of all the SPF dispersions as related to the pH.
The denaturation temperature, enthalpy of the transition and gelation temperature of the SPFs.

Table 3

| Sample name | Ca content (mg/g protein) | 7S Td (°C) | Enthalpy (J/g) | 11S Td (°C) | Enthalpy (J/g) | Tgel (°C) |
|-------------|---------------------------|------------|----------------|-------------|----------------|----------|
| SPF 1       | 0.57 ± 0.06               | 75.22      | 1.64 ± 0.20    | 92.76       | 2.45 ± 0.18    | 64.54    |
| SPF 2       | 1.90 ± 0.12               | 75.55      | 1.39 ± 0.20    | 94.20       | 2.27 ± 0.07    | 91.87    |
| SPF 3       | 4.28 ± 0.02               | 74.71      | 1.31 ± 0.16    | 94.94       | 2.16 ± 0.19    | 94.93    |
| SPF 4       | 6.47 ± 0.40               | 75.50      | 1.22 ± 0.11    | 96.53       | 2.68 ± 0.35    | 80.33    |
| SPF 5       | 9.84 ± 0.41               | 77.35      | 1.16 ± 0.14    | 98.54       | 2.84 ± 0.19    | 40.22    |
| SPF 6       | 12.62 ± 0.29              | 79.55      | 1.47 ± 0.24    | 100.25      | 3.06 ± 0.23    | 1.58     |
| SPF 7       | 16.55 ± 0.66              | 81.97      | 1.46 ± 0.22    | 101.13      | 3.88 ± 1.04    | \        |

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

**Fig. 6.** Nitrogen solubility index (NSI) of all the SPFs as related to the calcium content.

Previous research suggested that the water bound by the insoluble pellet is not necessarily a measure of water bound by the protein particles (Peters, Luyten, Alting, Boom, & Van der Goot, 2015), therefore, the WHC of 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, which means the majority of the protein in the SPF 7 is not soluble and has the weakest ability to interact with water.

### 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. 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 behaviour. The viscosities of SPF 1 and SPF 4 were similar, while the shear thinning behaviour 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 0.001–0.01 Pa s, almost approaching the reported dynamic viscosity of water (0.89 mPa s, 25 °C) (https://wiki.anton-paar.com/nl-en/water/).

### 3.10. Gelation behaviours

The gelation behaviours of all the SPFs upon and after heating treatment were measured and are shown in **Fig. 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 cross-over temperature where the $G'$ exceeds $G''$ (Table 3). The $T_{gel}$ of SPF 1 was around 64.54°C, while the highest $T_{gel}$ value was observed for SPF 4, which was approximately 94.93°C. By contrast, the $G'$ of SPF 6 and SPF 7 was constantly higher than $G''$, indicating that a solid-like structure was formed before heating already. In addition, a phase separation was observed with a sludge layer at the bottom and a watery layer above, this could be a result of the low solubility of SPF 6 and SPF 7, leading the sedimentation over time during the measurement. Therefore, $T_{gel}$ was not reported in Table 3. For SPF 5 which also had a NSI lower than 30%, a cross point of $G'$ and $G''$ was observed after around 6 min of heating, which is an indication of gel formation at 40.22°C. A thin watery layer was observed after the measurement, but it was less obvious than the layers presented above SPF 6 and SPF 7. One possible explanation could be that the fast gel formation of SPF 5 did not allow the sedimentation to take place. Besides, upon cooling, the gel firmness for all the SPFs increased.
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

Fig. 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.
disrupted as compared to other SPFs, while SPF 1 gave the strongest gel.

4. Discussions

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 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 1), which provides the possibility to control the Ca and Na content precisely in the ingredients during 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 is known that both Ca and Na ions can influence the electrostatic interactions, while Ca ions can also cross-link negatively-charged protein molecules forming a matrix (Mulvihill & Kinsella, 1988). Therefore, the Ca content may be 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. 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 & Rha, 1978; Zhang, Liang, Tian, Chen, & Subirade, 2012). 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, Su, & Lee, 2003). This change may be related to the formation of larger aggregates during the neutralization step. With the increase of the Ca concentration, the Ca bridges progressively became dominating, which could result in the protein aggregation into large and dense aggregates surrounded by the aqueous medium (Mulvihill & Kinsella, 1988). During freeze drying, noncontinuous structures formed, and as a result, a more porous and compact structure was observed after drying and milling steps. In contrast, at low Ca concentration, most of the proteins were highly solubilized in the dispersions after the neutralization step. In that case, a continuous structure was shaped during freeze drying, and a glass-like structure was observed after milling.

FT-IR is a powerful technique used to analyze the secondary structure of proteins. Early studies reported that native SPI showed strong absorptions at 1692, 1678, 1630 and 1619 cm$^{-1}$, indicating that $\alpha$-sheets dominated the secondary structures in native SPI. After thermal treatment, only minor absorptions remained, and a loss of most protein secondary structures occurred during the denaturation of SPI protein (Zhang et al., 2012). In this study, the strong band around 1635 cm$^{-1}$ confirms a predominant $\beta$-sheet conformation of all the SPFs (Fig. 4). Also, all the SPFs are still in their native state with the present of their secondary structures. This is also confirmed by the results of DSC analysis (Table 2). The overall decreasing amount of helix elements for 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 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. 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 hypothesised 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, Murphy, 2006, 2007; Teng et al., 2009).

In addition, the change of Ca(OH)$_2$ and NaOH ratio modified the thermal behaviour of 7S and 11S differently. A stabilization of 7S and 11S (increase in $T_g$) in SPF was induced by the increase of the Ca content, but a lower Ca content is required to change the $T_g$ of 11S than that of 7S. The $T_g$ 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 $T_g$ 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 & Anión, 1996). The addition of Ca ions in SPI dispersions produced higher $T_g$ of both 7S and 11S, and
increased the enthalpy of denaturation, mainly the 11S fraction (Sci-lingo & Anión, 2004). These findings can be linked to the protein ag-
grgregation discussed above, and the cross-linking effects probably
increase the thermal stability of the SPFs.

The viscosity of the SPF dispersions increased sharply when the Ca
content in the SPF changed from 0.57 to 1.90 mg/g protein (SPF 1 to SPF
2) and reached a maximum. When the Ca content increased further, the
viscosity of the mixture decreased. The viscosities of SPF 5, SPF 6, and
SPF 7 measured at high shear rates were similar and much lower than
SPF 4. These changes may be related to the soy protein-Ca complex
formation. It was reported that Ca salt has been used as a thickening
agent in soy protein suspensions (Lee & Rha, 1977). When Ca salts were
added to the soy protein with low concentration without heating, the
primary effect of Ca ions was likely to diminish repulsive forces. Protein:
protein association occurred to form the matrix. By further Ca addition,
the soluble soy proteins were bonded to a network and therefore,
the primary effect of Ca ions was likely to diminish repulsive forces. Protein:

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, WHCp 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. Future research could
investigate the Ca bioavailability of soy protein ingredients, and how
this would be affected by the use of different types of Ca salts.

Author statement

Yu Peng: Conceptualization, Methodology, Formal analysis, Inves-
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Declaration of competing interest

None.

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Appendix A. Supplementary data

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