

RESEARCH PAPER

Enhanced Emulsifying and Calcium-binding Properties of Fermented Soybean Meal

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Abstract A processed soybean meal (BF-SBM) was prepared by fermenting soybean meal with the genus *Bacillus* sp. strain for comparison with commercially prepared *Lactobacillus*-fermented soybean meal (LF-SBM), an enzyme treated soybean meal (ET-SBM) and a soy protein concentrate (SPC). Emulsifying activity and stability of the emulsions prepared from these products were investigated using typical methods in the literature with modifications and Turbiscan measurements, respectively. Calcium ion chelating ability of the SBM products was determined using a modified literature method. The resulting protein profiles, emulsifying properties and calcium-binding behaviors of the soybean products were compared. The results are discussed in terms of the influence of peptide molecular weight and the concentration of specific amino acids on emulsifying and calcium-binding properties of soybean proteins.

Keywords: soybean meal, fermentation, emulsifying properties, calcium-binding properties

1. Introduction

Soybean meals (SBM) are a low priced, nutritional and

multifunctional protein source [1]. They are used in various food industries because of their high protein content (approximately 50%), which is comprised mainly of glycinin and β -conglycinin [2].

Enzymatic treatment and fermentation of soybean protein produce bioactive peptides, which are functional in preventing lifestyle-related diseases [3,4]. Enzymes such as proteases [5], β -glucanases, or xylases [6] are used to hydrolyze soybean protein. Enzymatic treatment reduces anti-nutritional factors in SBM. Although this method has the additional advantage that the concentrations of enzyme and substrate are easy to control, it is difficult to remove salts and bitter peptides from the enzyme hydrolysate mixture in large scale processes [7]. Fermentation, one of the oldest techniques in food production, improves the nutritional value of SBM by functionalizing soybean protein with microorganisms such as *Aspergillus* species [8–10], *Bacillus* species [11–13] and *Lactobacillus* species [12,14–16]. A typical fermentation of SBM using micro-organism enzymes produces a large number of different oligopeptides, improves the amino acid profile, decreases undesirable factors, and increases antioxidant levels [12, 14–16].

Soy proteins are widely used in various food industries because of their emulsifying and gelling properties. These properties play a key role in maintaining the formulation of many food products including dairy products, beverages, soups and sauces, meat analogues, protein powders for humans, and animal feed [17]. Emulsifying activity and emulsion stability are particularly important factors for the formation and stabilization of emulsions in food products [18–20].

With the increase in ageing population, much attention has been given to the development of dietary calcium supplements to prevent and treat bone disease in this

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population segment [21]. In recent years, ionized calcium has been the primary calcium supplement for humans [22]. However, ionized calcium has the disadvantage of readily forming calcium phosphate deposits in the alkaline condition of the intestine [23] thus decreasing the bioavailability of ionized calcium. Since hydrolysates of soy protein are known to enhance calcium absorption [24], the role of organic calcium supplements, including calcium-binding peptides, in calcium absorption is an important research topic [25].

In this study, the emulsifying properties and calcium-binding properties of two fermented SBMs (one by *Bacillus* and the other by *Lactobacillus*), an enzyme-hydrolyzed SBM, regular SBM and soy protein concentrate are compared to determine the best hydrolysis method for enhancing the emulsifying and calcium-binding properties of SBM. Experimental results are discussed in terms of molecular size and structure of soy protein hydrolysates.

2. Materials and Methods

2.1. Materials

A typical soy bean meal (SBM) (CJ CheilJedang, Seoul, South Korea) fermented using bacteria of the genus *Bacillus* sp., and denoted as-BF-SBM, a *Lactobacillus*-fermented SBM (LF-SBM), an enzyme-treated SBM (ET-SBM), and a soy protein concentrate (SPC) were used in this study. The LF-SBM was obtained from DaBomb Protein Corporation (Tainan, Taiwan), the ET-SBM was purchased from Hamlet Protein A/S (Horsens, Denmark), and the soy protein concentrate (SPC) was obtained from Archer Daniels Midland Company (IL, USA). The BF-SBM was prepared according to the following procedure: *B. amyloliquefaciens* U304, isolated from the Korean traditional fermented soybean, was cultured in Nutrient Broth (Difco BD, USA) for 24 h at 37°C with shaking. The cell was adjusted to 10^9 CFU/mL with sterilized physiological saline and used as inoculum for solid-state fermentation. One hundred gram of soybean meal was mixed with 100 mL of distilled water, and the mixture was heated to 100°C for 30 min in a commercial autoclave. Then, they were cooled to room temperature inoculated with 10 mL of *B. amyloliquefaciens* U304, and incubated for 24 h at 37°C and 95% humidity in a constant temperature humidity chamber. After solid-fermentation, samples were air-dried at 60°C for 8 h and stored at 4°C.

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

SDS-PAGE was performed according to the procedure of Fling and Gregerson [26]. Briefly, protein was extracted

from SBMs and SPC in 8 M urea with a sonicator (Bandelin, GM 2070, 70 W power, BR, Germany) at 35% power. After centrifugation at 6,000 g for 10 min, the protein concentration in the supernatant was measured using the BCA assay [27]. The extracted proteins were denatured with 5× Tris-glycine SDS loading buffer, and 30 µg samples were fractionated using a 10% SDS-PAGE gel (BIO-RAD, Hercules, CA, USA) at 90 V. Protein bands were stained with Coomassie Brilliant Blue R250 stain (Sigma, St. Louis, MO, USA).

2.3. Measurement of emulsifying properties

Emulsifying activity was determined by the known method in the literature [28] with modifications. A 60 ml protein solution (0.17 mg/ml) was homogenized with 60 ml fish oil for 30 s at 3,400 g with a B-400 Mixer (BÜCHI, SWISS). The emulsion was centrifuged at 1,200 g for 30 min and the height of the emulsified layer measured. Emulsifying activity is expressed as the percent of the total content height in the tube that is occupied by the emulsified layer.

Two aspects of emulsion stability were measured: the emulsion stability at elevated temperature and the kinetic stability. Emulsion stability at elevated temperature was measured using modifications of the literature method [28]. An emulsion sample was heated for 30 min at 80°C in a water bath (HB-205 W, HANBACK Scientific Co., Korea). It was then cooled to room temperature (22°C) and centrifuged at 1,200 g for 30 min. Emulsion stability (ES) was estimated as the percent ratio of the height of the emulsified layer after heating to its height before heating.

Kinetic stability was determined by measuring the backscattered light intensity (BS) of samples scanned at hourly intervals using a near infrared laser light beam (880 nm) at 50°C using a Turbiscan Lab (Formulation, France). The BS was measured with two synchronous optical sensors. The change in BS, ΔBS , was monitored as a function of elapsed time and plotted as ΔBS (%) vs time. ΔBS (%) is defined as $\Delta BS (\%) = 100 \times (BS_t - BS_0)/BS_0$, where BS_t is the backscattered light intensity at time t and BS_0 is that at time zero. The change in BS is a measure of emulsion instability (IS), with the inverse of IS a measure of emulsion stability.

Emulsion particle size was measured by electrophoretic light scattering spectroscopy (ELS-8000, OTSUKA) using a 100 fold dilution of emulsified sample. The mean particle size was obtained from the particle size distribution data.

2.4. Measurement of calcium ion-binding capacity

The calcium ion chelating ability of a SBM sample was determined according to the modified method in the literature [29]. A processed SBM sample was dissolved in 20 mM sodium phosphate buffer solution (pH 7) to a final

Table 1. Composition of commercial and processed soybean meals

	SBM	BF-SBM	LF-SBM	ET-SBM	SPC
Composition (%)					
Moisture	9.6	6.9	6.6	7.8	8.3
Protein	50.0	55.6	50.2	54.8	65.3
Fat	1.8	0.8	0.9	1.3	0.1
Ash	6.0	7.4	6.9	6.8	4.8
Fiber	2.9	3.4	4.1	3.3	2.7
Others	29.7	25.9	31.3	26.0	18.8

Table 2. Acidic amino acid and histidine contents (%) of commercial and processed soybean meals

Amino acid	SBM	BF-SBM	LF-SBM	ET-SBM	SPC
Aspartic acid (Asp)	5.60	6.13	5.76	6.11	7.45
Glutamic acid (Glu)	9.37	10.65	9.84	10.59	12.49
Histidine (His)	1.29	1.53	1.27	1.47	1.68
Total	16.26	18.31	16.87	18.17	21.62

concentration of 1.0 mg/ml. The processed SBM-buffer solution was then mixed with a buffer solution containing excess CaCl_2 (5 mM), and stirred at room temperature for 1 h while monitoring the pH with a pH meter and maintaining a constant pH 7. The reaction mixture was centrifuged at 3,500 g at room temperature for 20 min in order to remove insoluble calcium phosphate salts. The calcium content of the supernatant was determined using a colorimetric method with ortho-cresolphthalein complexone reagent [30].

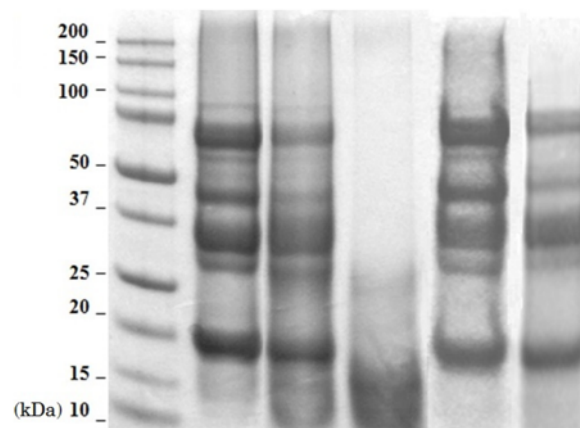
3. Results and Discussion

3.1. Composition of soybean meals

Table 1 shows the chemical composition of the SBM products and the SPC as determined using official methods recognized by the Association of the Official Analytical Chemists (AOAC). The SPC contained the highest level of protein. The next highest protein levels were in BF-SBM and ET-SBM, followed by LF-SBM. Table 2 shows the acidic amino acid and histidine contents of the SBMs and the SPC.

3.2. Protein hydrolysis and protein profile

Fig. 1 shows the protein profiles obtained by SDS-PAGE of urea extracts of the SBM samples used in this study. The protein profiles of the SBM, the LF-SBM, the ET-SBM and the SPC were nearly the same and exhibited the main bands typically found in soy proteins. These were the three major bands at MW c.a. 80 kDa, 75 kDa, and 50 kDa, corresponding to α' -, α - and β - subunits of β -conglycinin.

**Fig. 1.** SDS-PAGE profiles of commercial and processed soybean meals. Lane 1, Marker; Lane 2, SBM; Lane 3, LF-SBM; Lane 4, BF-SBM; Lane 5, SPC; Lane 6, ET-SBM.

They also yielded three major bands at MW c.a. 35 kDa, 32 kDa, and 20 kDa and one minor band at MW c.a. 39 kDa, corresponding to acidic and basic polypeptides of glycinin [31–33]. In contrast, the BF-SBM had a different protein profile with several bands only below 15 kDa, indicating that major constituents of the soy protein in this sample were degraded into smaller peptides.

Generally the *Bacillus subtilis* fermentation process degrades the three subunits from β -conglycinin and the acidic and basic polypeptides from glycinin into low molecular weight compounds at a relatively high rate [34]. Previous studies showed that prolonged fermentation can produce a 60-fold increase in the amount of free amino acids equaling about 26% of the total amino acid content [35]. Active degradation of soybean protein by *B. subtilis* fermentation can be attributed to this strain's vigorous production of extracellular enzymes such as protease. This explanation is supported by many previous studies which found an improvement in total soluble protein, elimination of allergenic proteins, and a decrease in anti-nutritional factors in *B. subtilis*-fermented soybean protein [12,36,37].

3.3. Emulsifying properties

Table 3 shows the emulsifying activity and emulsion heat stability of processed SBMs. The order of emulsifying

Table 3. Emulsifying activity and emulsion stability of commercial and processed soybean meals

	Emulsifying activity (%)	Emulsion stability (%)
BF-SBM	61.1	61.4
LF-SBM	40.8	48.8
ET-SBM	41.6	40.9
SPC	56.5	56.4

activity from highest to lowest was BF-SBM>SPC>ET-SBM>LF-SBM, and of the emulsion heat stability was BF-SBM>SPC>LF-SBM>ET-SBM. The BF-SBM sample, comprised mainly of degraded low molecular weight (MW) peptides, showed much higher emulsifying activity and emulsion heat stability than LF-SBM and ET-SBM. These results suggest that low MW peptides are responsible for the superior emulsifying properties. This suggestion is supported by a previous study of the relationships among MW, surface hydrophobicity (S_0), protein solubility (PS), emulsifying activity index (EAI), and emulsion stability index (ESI) [31]. In that study, soy protein isolates (SPI) were fractionated into controlled MW peptides by proteolytic enzymatic modification and ultrafiltration. Their measurements found that peptides with a smaller MW distribution had lower S_0 , higher PS, EAI and ESI. However, Shimizu and coworkers claimed that the high correlation between surface hydrophobicity and emulsifying activity was not true [38]. Wu and coworkers speculated that solubility and molecular size rather than surface hydrophobicity are the main factors affecting emulsifying activity [31]. However, the SPC sample used herein showed higher emulsifying

activity and emulsion heat stability than LF-SBM and ET-SBM even with a protein profile similar to that of SBM, LF-SBM and ET-SBM (Fig. 1). This apparent contradiction might be attributed to the higher protein content of SPC compared to the SBM samples, which would give rise to more low MW peptides in hydrolysates.

Backscattered light profiles of emulsions used to estimate kinetic stability are shown in Fig. 2 as plots of BS (%) vs sample height at different elapsed times. The BS (%) values for BF-SBM and SPC emulsions were about 85% over the entire sample height and were constant for the entire time of measurement. In contrast, BS (%) values of LF-SBM and ET-SBM emulsions were initially lower at about 70%. More detailed analysis of backscattered light profiles is given in Fig. 3. The Δ BS (%) values of BF-SBM and SPC emulsions remained nearly constant in the bottom zone of the sample cell (5–15 mm) for up to 6 h. However, the Δ BS (%) values of LF-SBM and ET-SBM emulsions decreased gradually over time, and that of ET-SBM emulsion decreased most significantly. Fig. 3 showed that the Δ BS (%) values of LF-SBM and ET-SBM emulsions decreased further as time elapsed, especially in the bottom

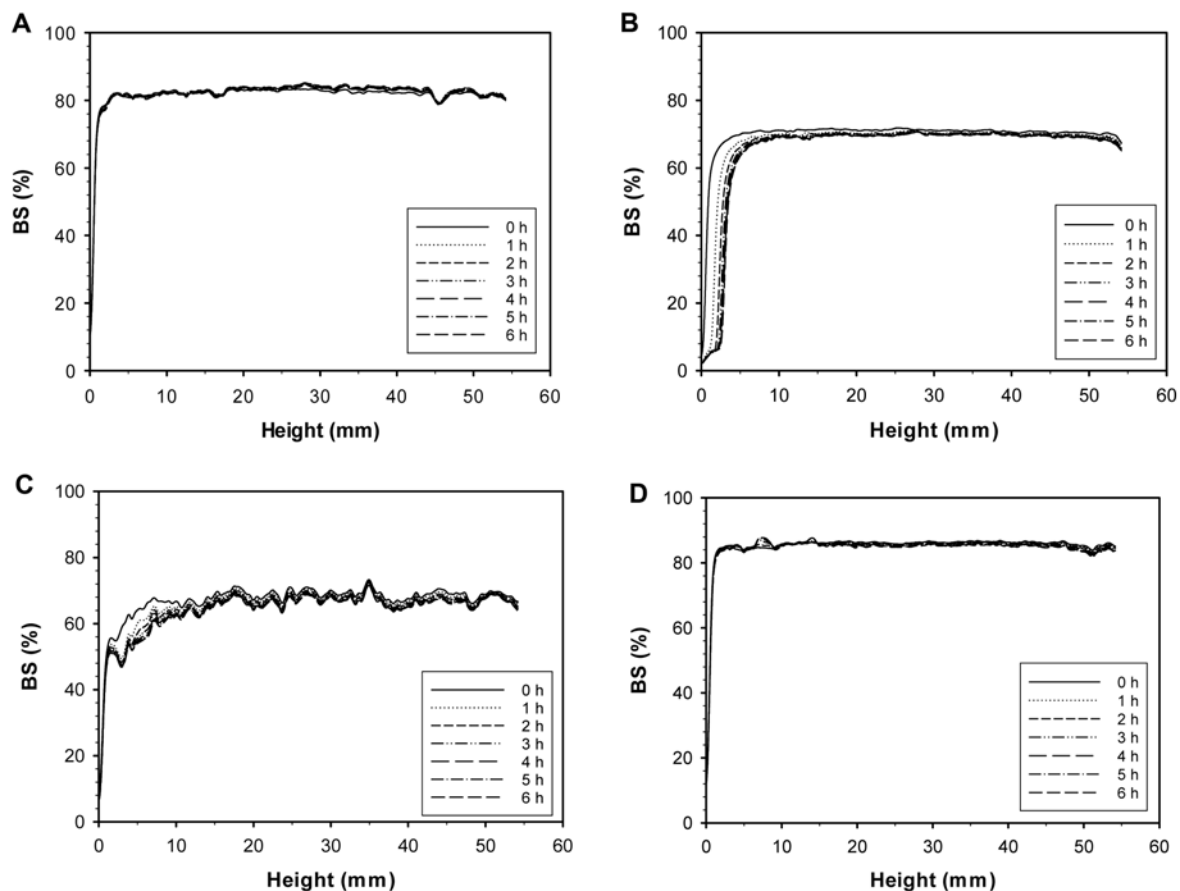


Fig. 2. Backscattering data of emulsions prepared from soybean meals (A) BF-SBM, (B) LF-SBM, (C) ET-SBM, (D) SPC.

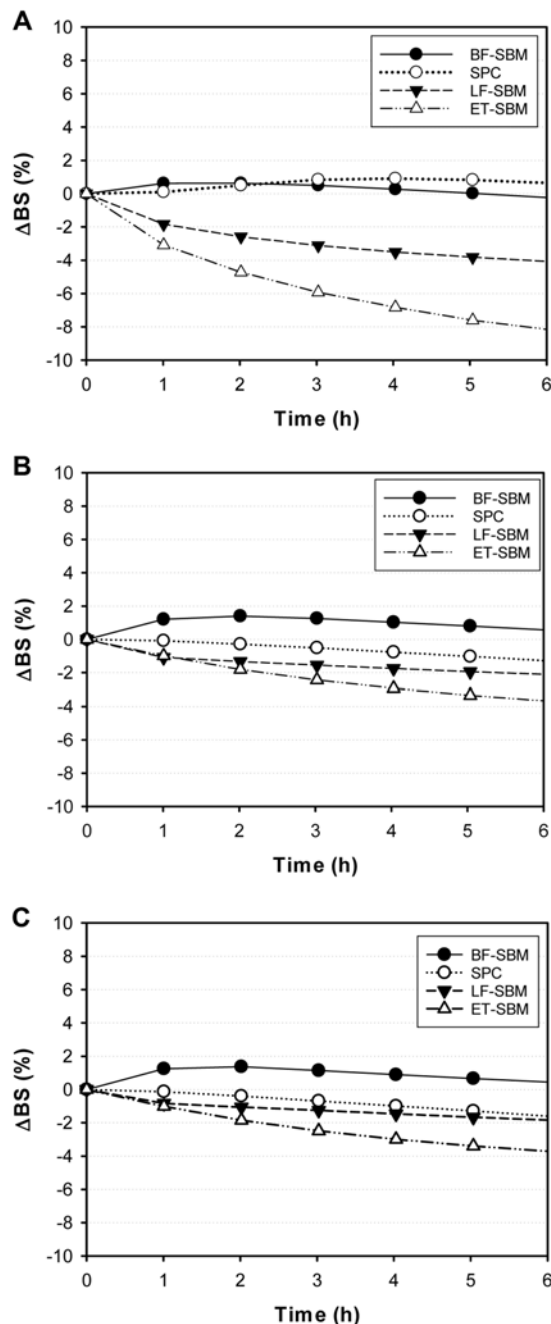


Fig. 3. Kinetic stability of emulsions prepared from soybean meals (BF-SBM, LF-SBM, ET-SBM and SPC) measured by a Turbiscan Lab for (A) zone 5 – 15 mm, (B) zone 15 – 40 mm, and (C) zone 40 – 50 mm.

zone of the sample cell (Fig. 3A). In the middle zone (15–40 mm), the BF-SBM initially showed a slight increase in ΔBS (%) followed by a gradual decrease, whereas the other emulsions showed only a gradual decrease. The ΔBS (%) values in the top zone (40–50 mm) of all emulsions were similar to those in the middle zone.

Table 4. Average particle size of emulsions of commercial and processed soybean meals

	Average particle size (μm)
BF-SBM	1.74 (± 0.09)
LF-SBM	2.86 (± 0.26)
ET-SBM	3.07 (± 0.33)
SPC	2.56 (± 0.16)

In principle, a higher BS value indicates a larger volume fraction of dispersed phase. A constant ΔBS (%) value means a constant volume fraction of dispersed phase and thus high emulsion stability, whereas a significant decrease in ΔBS (%) value indicates a reduction of volume fraction of dispersed phase and thus low emulsion stability. This instability of SBM emulsions usually stems from creaming and flocculation or coalescence of emulsion particles [39]. The decrease in ΔBS (%) values in the lower zone represents clarification due to the separation of water from the emulsion. The steep slope for the ET-SBM emulsion represents the highest clarification rate. These results of kinetic stability can be explained in conjunction with the particle size measurement results. As shown in Table 4 and Fig. 4, the BF-SBM emulsion had the smallest average particle size and the ET-SBM emulsion the largest. The creaming velocity at which a spherical particle of dispersed phase moves in a Newtonian liquid is given by Stoke's law [40]:

$$U = \frac{2g r^2 (\rho_2 - \rho_1)}{9\eta} \quad [1]$$

where U , η , r , g , ρ_2 and ρ_1 denote the creaming velocity, the shear viscosity, the particle radius, the gravitational acceleration, the densities of dispersed phase and continuous phase, respectively. This equation implies that larger emulsion particles have a higher creaming velocity, which partly explains why the ET-SBM emulsion with the largest particle size had the steepest destabilization slope. In addition, since droplet coalescence is known to be accompanied normally by a decrease in the emulsion viscosity, the coalescence accelerates the creaming process [41].

On the other hand, the high stability shown by the BF-SBM and the SPC emulsions can be attributed to the relatively higher protein content (see Table 1). According to Mohan and Narsimhan [42], the coalescence rate in emulsions stabilized by proteins decreases at high protein concentration. This rate decrease is due to the enhancement of repulsive force among colloidal emulsion droplets by the electrostatic and steric repulsions of the greater number of protein molecules.

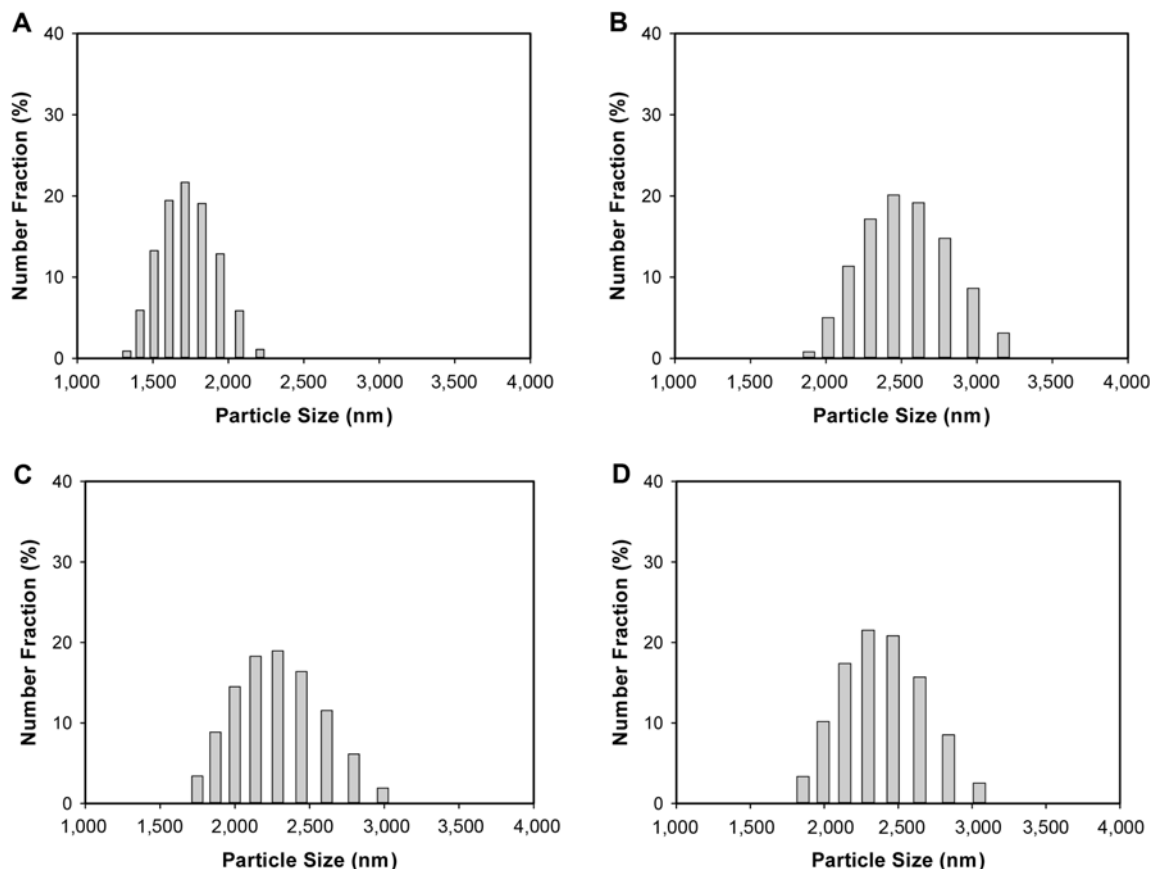


Fig. 4. Particle size distribution of emulsions prepared from soybean meals (A) BF-SBM, (B) LF-SBM, (C) ET-SBM, and (D) SPC.

Table 5. Calcium chelating activity of commercial and processed soybean meals

	Ca ²⁺ chelating (mg/g)		
	1 st	2nd	Average.
LF-SBM	4.75	5.52	5.14
BF-SBM	31.11	33.01	32.06
ET-SBM	15.17	20.61	17.89
SPC	22.78	27.64	25.21

3.4. Calcium-binding Activity

Table 5 shows the calcium chelating capacities of processed SBM products. The BF-SBM exhibited the highest calcium-binding capacity of 32.06 mg/g, while the LF-SBM had the lowest value of 5.14 mg/g. Food proteins or peptides with good metal chelation can improve mineral bioavailability and provide the body with sufficient minerals. Zhao and coworkers suggested that enzymatic hydrolysis of whey protein increased calcium and iron(II)-chelating activities [29]. Bao and coworkers showed that enzymatic hydrolysis of soybean was beneficial to calcium absorption [43]. In their study, soy protein hydrolysate fractions with average molecular weight 14.4 or 8-9 kDa had a higher calcium

binding capacity than the 24 kDa fraction. Moreover, the calcium content of the two low MW fragments in the protease M-calcium complex was much higher than in the nutrase-calcium complex. In addition, it was shown that calcium primarily binds at the carboxyl group of acidic amino acids such as aspartic acid and glutamic acid, and at the imidazole group of histidine [44]. As seen in Table 2, the order of acidic amino acids (aspartic acid and glutamic acid) and histidine concentrations in our samples was SPC>BF-SBM>ET-SBM>LF-SBM>SBM. The high calcium-binding activity for BF-SBM found in the present study can be attributed to the combination of the greater amount of low MW peptides and calcium-binding amino acids in BF-SBM compared to the other SBMs tested.

4. Conclusion

The present investigation of protein profiles, emulsifying properties and calcium-binding behaviors of BF-SBM, LF-SBM, ET-SBM, and SPC demonstrated the following important characteristics:

1. The BF-SBM showed a protein profile with smaller

peptides (< 15 kDa) than the other products tested because the *Bacillus* strain used in this study vigorously produces extracellular protease enzymes.

2. Among the tested samples, the BF-SBM showed the highest emulsifying activity and emulsion heat stability likely because of its lower MW constituent peptides. The BF-SBM emulsion had more desirable kinetic stability with a higher and nearly constant back scattering (BS) value than the other products, consistent with the stability of a high volume fraction of dispersed phase for an extended period of time. Emulsion instabilities observed in other SBMs in this study was well explained in terms of creaming and flocculation or coalescence of emulsion particles.

3. The BF-SBM had the highest level of calcium-binding activity compared to other test samples presumably because of its greater amounts of low MW peptides and calcium-binding amino acids.

This study has shown that fermentation with an appropriate strain of microorganism can enhance key functionalities such as emulsifying and calcium-binding properties of SBMs, thereby increasing SBM nutritional value and applicability as a food ingredient.

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