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Effect of high-power sonication pretreatment on extraction and some physicochemical properties of proteins from chickpea, kidney bean, and soybean

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Abstract

Impact of high-power sonication (HPS) as pretreatment in extraction and some physicochemical properties of proteins from soybean flakes, flour of soybean, chickpea, and kidney bean was evaluated. Soybean flakes and flours from soybean, chickpea, and kidney bean were dispersed in distilled water (1.10 w/v) and sonicated at two power densities (PD) of 2.5 and 4.5 W/cm3 for 5 min continuously. Proteins were extracted at pH range 8–8.5. PD 2.5 and 4.5 W/cm3 significantly increased protein extraction yields from soy flakes to 29.03% and 25.87%, respectively, compared to 15.28% for unsonicated controls, but did not increase for flours. Freeze-dried spent substrates at higher PD sonication aggregated in size. Free sulfhydryl content for both sonicated and unsonicated soy flakes and flour were similar but increased in chickpea and kidney bean when HPS of 4.5 W/cm3 was applied, indicating the unfolding of protein structure. The protein band patterns for sonicated and unsonicated legumes proteins were found to be similar, indicating no peptide profile alterations by HPS. However, circular dichroism analysis showed changes in secondary structure composition in extracted kidney bean protein causing unfolding and destabilizing the native structure. The secondary structure composition for soy flakes and flour protein and chickpea protein remained unchanged.

Keywords

Legume proteins, High power sonication, Ultrasonic-assisted extraction, Protein extraction, Protein secondary structure

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Abstract

Impact of high-power sonication (HPS) as pretreatment in extraction and some physicochemical properties of proteins from soybean flakes, flour of soybean, chickpea, and kidney bean was evaluated. Soybean flakes and flours from soybean, chickpea, and kidney bean were dispersed in distilled water (1:10 w/v) and sonicated at two power densities (PD) of 2.5 and 4.5 W/cm^3 for 5 min continuously. Proteins were extracted at pH range 8-8.5. PD 2.5 and 4.5 W/cm^3 significantly increased protein extraction yields from soy flakes to 29.03% and 25.87%, respectively, compared to 15.28% for unsonicated controls, but did not increase for flours. Freeze-dried spent substrates at higher PD sonication aggregated in size. Free sulfhydryl content for both sonicated and unsonicated soy flakes and flour were similar but increased in chickpea and kidney bean when HPS of 4.5 W/cm^3 was applied, indicating the unfolding of protein structure. The protein band patterns for sonicated and unsonicated legumes proteins were found to be similar, indicating no peptide profile alterations by HPS. However, circular dichroism analysis showed changes in secondary structure composition in extracted kidney bean protein causing unfolding and destabilizing the native structure. The secondary structure composition for soy flakes and flour protein and chickpea protein remained unchanged.

Keywords: Legume proteins, High power sonication, Ultrasonic-assisted extraction, Protein extraction, Protein secondary structure.

1. Introduction

Plant-based food proteins are studied for its better and cheaper source of nutrients including essential amino acids. Legumes, for example, peas, lentils, soybeans, and dry beans are rich source of proteins, fibers, and carbohydrates making them valuable as food ingredients. They can also be incorporated as meat replacers to lower the energy density while providing important nutrients [1]. Extraction, isolation, and purification of proteins is the first step in utilizing them as ingredients; however, presence of cell wall polysaccharides and other cell polymers and the location of proteins inside the cell-matrix limit their extraction [2].

Conventional extraction methods for plant proteins include alkali extraction with pH modulation in the range of 8.5-9, where the solubility of proteins plays a major role in extraction. This method extracts approximately half of the available proteins from defatted soy flakes [3]. Several other extraction methods like, microwave heating, enzymatic modifications and chemical modifications of soy substrate were carried out to improve protein extractability from plants [4,5]. In addition to conventional methods, enzyme-assisted extraction (Endoprotease protex 5L) at pH 9.5 of soybean and rapeseed resulted in the increased protein extraction yields by 10% and 40%, respectively but both enzymes and process are expensive [6]. On the other hand, Mu et al [7] reported that ultrasonication as pretreatment substantially increased the protein yield from soy flakes and reduced the cost of producing the proteins, which demonstrated potential alternative to existing conventional methods. Karki et al [3] also reported the use of high-power sonication as one of the pretreatment methods with improved protein extraction yield of 40-46% from defatted soy meal.

Ultrasound with low frequency (16-100 kHz) and power intensity of 10-1000 W/cm ^{3} is

known as high power sonication (HPS). When HPS is applied to the aqueous medium, cavitation bubbles form and collapse violently leading to extreme temperatures (5000 K) and pressures (1000 atm) that produces high shear energy and turbulence in the cavitation zone [8]. Cavitation disintegrates cellular matrices and aids in the extraction of protein, sugar, oils, isoflavones, polyphenols, and saponins from plant cells [3,9]. HPS can decrease particle size by approximately 10-fold resulting in more protein extraction from substrate due to increased surface area [3].

Apart from enhanced extraction, HPS, however, can alter protein molecular configuration by breaking hydrogen bonds, and hydrophobic interactions; HPS may also induce the dissociation and/ or aggregation of subunits. The interaction among polar, non-polar, acidic, and basic groups within the polypeptide chain create a complex three-dimensional structure. As sonication can break down the interaction between proteins, structural integrity is lost, and hence the altered functionality. Thus, the secondary structure of proteins is important in evaluating the changes in functionality. Functional properties of protein obtained from sonicated soy flakes like solubility and emulsion capacity are reported in the literature: the solubility for sonicated soy flakes increased by 34% and emulsion capacity decreased by 12% [10]. Ultrasound has also been used to alter structural, physical, chemical, and functional properties of protein isolates prepared from various sources, for example, rice, soybean, pea, black bean, and sunflower [11–14]. High-intensity ultrasonication induced changes in free sulfhydryl content, particle size and secondary structure, which in turn resulted in alteration of surface hydrophobicity and activity [15]. Hu et al [16] reported partial unfolding and reduction in intermolecular interactions of soybean protein isolate based on increased free sulfhydryl groups and surface hydrophobicity, which in turn

improved the solubility of soy protein isolates (SPI) dispersion.

HPS has been used in a limited way as a pretreatment for extraction of proteins from legumes. In the sonic-assisted extraction of oil from chickpea, extraction yield increased by 10.45% when using ultrasonic power of 230 W was used [17]. Extraction of oil from soybean [18] and glucose release from corn slurry obtained from dry-ground ethanol plants was done using ultrasonication [19]. Use of high-power sonication as a pretreatment in maximizing legume protein extraction can benefit the industry, but has not been reported for various types of legumes, for example, chickpeas and kidney beans, nor has its effect on their protein secondary structure been evaluated. This study investigated the effects of HPS on the extraction yields and physical and structural properties of proteins from important legumes like chickpea and kidney bean and compared with soybean. The specific objectives of this study were to 1) evaluate the effect of sonication power densities on extraction and yield of plant-based protein preparations, and 2) evaluate the comparative changes in the secondary molecular structure of extracted plant proteins as affected by high power sonication.

2. *Materials and methods*

2.1 Materials and reagents

Defatted soy flakes (20 PDI, protein dispersibility index) were obtained from Cargill Inc., Cedar Rapids, IA. Defatted soy flour was obtained from Archer Daniels Midland Company, Decatur, IL. Chickpea and red kidney beans were obtained from Dr. Chibuike Udenigwe, University of Ottawa, Ottawa, Canada and were prepared into flours as described in Sec 2.2.

The Pierce BCA protein assay kit, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), urea, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), and β mercaptoethanol were purchased from Fisher Scientific (Waltham, MA, USA). Bovine serum

albumin (BSA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Milli-Q water (Millipore, Billerica, MA, USA) had a resistivity of 18.2 MΩ-cm at 25ºC. All other chemicals were of analytical grade.

2.2 Preparation of legume flours

Chickpea and kidney beans were soaked in water for 12 h and manually de-hulled followed by oven drying at 50 °C for 24 h. Milling was done by passing beans through 0.03-inch gap with corrugation of 1/8 inch followed by 0.02-inch gap and 1/16 inch corrugation using a Witt corrugated roller mill (Witt Corrugating Inc., Wichita, KS) then ground using Nutri mill (Pleasant Hill Grain, NE, USA). The particle size of ground soy flakes (100 g) was analyzed using a Ro-Tap sieve shaker (W.S. Tyler Industrial Group, Mentor, OH) fitted with graded U.S standard mesh sieves 12 (1.7 mm), 16 (1.18 mm), 25 (0.71 mm), 30 (0.6 mm), 35 (0.5 mm), and 60 (0.25 mm). Sieve used for chickpea flour, kidney bean flour, and soy flour were mesh 25 (0.71 mm), 35 (0.5 mm), 60 (0.25 mm), 120 (0.125 mm), 170 (0.09 mm), and 200 (0.075 mm).

2.3 Proximate analyses

The proximate analysis of all legume samples was carried out using standard methods in Plant Polymer Research Unit Lab (USDA-ARS, Peoria, IL). Moisture, crude protein (Dumas combustion % N x 6.25), crude oil, and crude fiber contents were analyzed according to AOCS standard methods Ba 2a-38, Ba 4e-93, Am 5-04, and Ba 6-05, respectively [20]. Ash contents were analyzed according to AOAC method 942.05 [21] and carbohydrate content was calculated by difference (100 - sum of other components). Soluble or insoluble carbohydrates were not identified.

2.4 High-power sonication-based extraction of legume proteins

Ultrasonic treatments (model VCX 750, Sonics & Materials, Inc., CT, USA) were carried out in temperature-controlled centrifuge tubes at frequency of 20 kHz with 750 W maximum power output. A 13-mm (1/2 inch) probe of titanium alloy threaded to a 3 mm tapered micro tip generated ultra-high intensity by sonication for 5 min at 160μm peak-to-peak. The ultrasonic power density (PD) was defined as the input power of the ultrasound per volume of the slurry and varied at 2.5 W/cm^3 and 4.5 W/cm^3 by adjusting amplitude (20-40%) and volume of the samples. 40% was the highest amplitude supported by the horn. Power density 2.5 W/cm^3 (intensity~315 W/cm^2) and 4.5 W/cm^3 (intensity~390 W/cm^2) were designated as lower and higher PD in this research. These two PD was determined as PD 2.5 is the power that is relevant to the industrial application [22] which was compared to PD 4.5 to see how protein will behave. Also, the highest PD limitation of the sonication unit at the given volume was PD 4.5.

Protein products were prepared according to modified protocols of the standard methods [23]. The schematic diagram of the process is shown in Figure 1. Sample to water ratio of 1 g: 10 mL were treated at PD 2.5 W/cm^3 and 4.5 W/cm^3 for 5 min by placing the centrifuge tubes in a temperature-controlled ice bath (below 45 °C). These sonicated samples were then used for the extraction of the proteins at pH 8.5, 60 °C, and 30 min stirring using a magnetic bar on a stir plate. The samples were centrifuged at 14,000 x *g* at 15 °C for 10 min. The supernatants were collected to measure total volume with a graduated cylinder and protein content quantified for extraction yield. The supernatant pH was adjusted to 4.5 by adding 2 N HCl and then refrigerated at 4 °C for 1 h to facilitate the formation of larger and stronger curds. Centrifugation was done again at 14,000 x *g* at 15 °C for 10 min to segregate curd and whey where curd was neutralized using distilled water of pH 7.0 and then freeze-dried at -20

°C. The mass of the freeze-dried sample was measured, and the percent yield of protein preparation was calculated. In addition, spent solids were also collected and freeze-dried to measure particle size distribution.

2.5 *Protein content of extracted supernatant*

Protein content of extracted supernatant was quantified using bicinchoninic acid (BCA) protein assay kit (Pierce™ Rockford, IL 61105, USA). An aliquot (0.1 mL) of supernatant was mixed with 2 mL of working reagent, incubated at 37 °C for 30 min, and then cooled down for 10 min. The absorbance was measured using spectrophotometer (Shimadzu UV 160) at 562 nm and converted to protein concentration using BSA standard curve.

2.6 Scanning electron microscope

Freeze-dried spent solids were placed onto aluminum stubs with metallic backed adhesive tape and sputter-coated with platinum (30 nm) using a Cressington HR208 sputter coater. Images were captured using a Hitachi SU-4800 field emission scanning electron microscope (Hitachi High Technologies in America, Schaumburg, IL) at 10kV.

2.7 Particle size distribution after sonication

The freeze-dried spent solids from protein extraction were passed through a 1-mm pore size then analyzed for particle size distribution using particle size analyzer (Mastersizer 2000 S, Malvern Inc., Worcestershire, UK). The particle size distribution was measured using a refractive index ratio of 1.520. The sonicated and unsonicated spent solids were then dispersed into distilled water to obtain the obscuration of 12-16%. Analyses were done in triplicate and averaged.

2.8 Effect of sonication on protein secondary structure

2.8.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The sonicated and unsonicated protein solutions were subjected to SDS-PAGE with slight modification [24]. Thirteen percent resolution gel (Acryl-Bisacrylamide) at the bottom and 4% percent stacking gel at the top were prepared. The protein concentration of 2 mg/mL was prepared in sample buffer (15.1 g/L Tris, 300 g/L urea, 2 g/L SDS, 20 mL/L glycerol, and 0.1 g/L bromophenol blue) and incubated at 80 °C for 5 min. The protein standard (6,500 – 66,000 Da, Product number M3913-SigmaMarker™) and samples were loaded onto gel at equal volume (10µL) and electrophoresed at a constant voltage of 200V for 45 min using standard SDS buffer (25mM Tris, 191 mM glycine and 1 g SDS per liter). The gels were stained with Coomassie blue for 1 h and de-stained with methanol: acetic acid: deionized water in ratio 10:2:8 until the gels were clear and transparent.

2.8.2 Free sulfhydryl content of final protein products

The free sulfhydryl (SH) content of the soluble fraction of freeze-dried samples was determined using Ellman's Reagent DNTB (5, 5'-dithio-bis- [2-nitrobenzoic acid]) [25]. Reaction buffer containing 6 M guanidine hydrochloric acid and 1.27 mm ethylenediaminetetraacetic acid (EDTA) of pH 8.6 was prepared. Two hundred fifty mg of protein product was dissolved in 25 mL of reaction buffer overnight followed by centrifugation at 12,000 x *g* for 10 min. The supernatant was collected and 250 µL of native and sonicated protein solutions from the supernatant was added to 2.5 ml of reaction buffer, followed by the addition of 50 µL of Ellman's reagent. Samples were then vortexed and incubated at room temperature for 15 min. Absorbance at 412 nm was measured using a UV–visible spectrophotometer. The free SH is presented in µmol/g protein.

2.8.3 Secondary structure analysis by circular dichroism

Circular dichroism (CD) spectra were scanned at the far- UV range (260-180) with a CD spectropolarimeter (Jasco 715, Jasco Corp) in a 0.1 cm quartz CD cuvette at 25 °C. Freeze-dried protein samples (0.03 mg/mL) were dissolved in 0.01 M sodium phosphate buffer (pH 7.0) and centrifuged at 12,000 x g for 10 min to remove any insoluble residue. The values of scan rate, response, and bandwidth were 50 nm min^{-1}, 0.25 s, and 1.0 nm, respectively [26] Three scans were averaged to obtain one spectrum. The mean residue ellipticity (θ) was expressed as deg cm^2 $dmol^{-1}$. The protein concentration was determined by BCA protein assay kit as mentioned in section 2.5. The secondary structure was estimated using computer program SELCON3 originated by the method of Sreerama & Woody [27] and reference dataset 6 was used. Four secondary structures were estimated, i.e., α-helix, β-sheet, β-turn, and unordered using the method of Whitmore & Wallace [28].

2.9 Protein extraction yield and protein yield in product

The extraction yield and protein preparation (product) yield are calculated by the equations given below:

$$Extraction\ yield\ (\%) = \frac{Total\ protein\ in\ extracted\ supernatant}{Total\ protein\ in\ initial\ substrate} * 100$$

$$Protein\ yield\ in\ product\ (\%) = \frac{Total\ protein\ in\ freeze\ dried\ product}{Total\ \ protein\ in\ initial\ substrate} * 100$$

2.10 Statistical analyses

The experimental design was randomized complete block design (RCBD) with two replications. Statistical analyses were performed using the JMP® statistical methods (100 SAS Campus Drive;

Cary, NC). A two-way analysis of variance (ANOVA) was performed to assess the effect of sonication on extraction yield and structural characteristics. Treatment means were compared within each substrate for sonication effect on protein extraction yield, Protein yield in product, free sulfhydryl content, and secondary structures. Results having different superscript letters within each substrate group show a significant difference ($p<0.05$). Graphs were prepared using GraphPad Prism software (GraphPad Software, San Diego, CA, USA) and OriginPro 8.5® (Northampton, MA, USA).

3. ***Results and discussion***

3.1 Proximate analyses

The final particle size of soy flakes, soy flour, chickpea flour, and kidney bean flour used for further analyses was 0.85 mm, 0.07 mm, 0.22 mm, and 0.10 mm respectively. The composition of legumes for proximate analysis is given in Table 1. Soy flakes and flours had similar proximate contents and are a rich source of proteins, as flours are prepared by milling the flakes. These legumes are an excellent source of proteins that are essential for the synthesis and repairing of body tissues. The proximate composition of defatted soy flour in our research agrees with Rosset et al [29] with slight variation in protein content (47.79%) which might be due to the defatting and processing conditions of soy flour and flakes preparations. The proximate composition for kidney beans is comparable except for the carbohydrate composition which is 70.9% for our sample and 53.02% for Hayat et al [31] which might be due to the different varieties of kidney beans. Similarly, the composition of chickpea is comparable to values reported by Boye et al [32]. Crude fibers observed were less than 2%, as dehulling or seed coat removal might be a possible factor for the reduced crude fiber content [33]. The carbohydrate contents observed were higher in the samples, which were not differentiated between soluble and

insoluble fractions.

3.2 *Protein extraction yield*

The ultrasound-assisted extraction yields of the proteins for different legume substrates are provided in Fig 2. The protein extraction yield was greater for soy flakes when exposed to high power sonication both at higher and lower PD, compared to unsonicated soy flakes. Extraction yield of proteins from sonicated soy flakes increased significantly ($p < 0.05$) by 90% and 68.5% for higher and lower PD respectively compared to unsonicated soy flakes. This increase in extraction yield was also observed in Karki et al [3] where two minutes sonication at 84 μm_{pp} gave 46% greater yield. This sonication induced increase in protein extraction yield might be due to the structural damage, as was corroborated by the extensive cellular disruptions seen in SEM analysis (Section 3.4) and resulting in the release of cell constituents into the aqueous system [34]. However, the increase was not linear with the increase in power density. Similarly, there was no significant ($p>0.05$) change in extraction yield for soy flour after sonication possibly due to the smaller particle size than the flakes. The yield increased significantly ($p<0.05$) by 16.39% when higher PD sonication was applied to kidney bean flour. On the other hand extraction yield for chickpea reduced after sonication. This decrease may have been due to the higher fat content in chickpea (7.03%), which can form protein-lipid interaction that inhibits the dissolution of proteins and limits the isolation of proteins [35]. Also, the carbohydrates which are present at higher levels of ~66% contain cellulose and non-cellulosic polymers in chickpea which lowered the free water to extract proteins; made the gel viscous preventing the accessibility of proteins to be extracted. Overall, our finding indicates that HPS could be used in efficient way as extraction pretreatment when substrate particle size are bigger, leading to decrease in particle size and facilitating the extraction of proteins as in soy flakes.

3.3 Protein yield in product

The protein product yields for various legumes are presented in Fig 3. Soy flakes protein product yield increased significantly ($p < 0.05$) from 8.4% (unsonicated) to 33.45% for lower PD and up to 30.6% for higher PD respectively. For soy flour, protein yield increased slightly from 43% to 50% with sonication but the values were not significantly different. Similarly, protein product yield of kidney beans increased from 44.5% (unsonicated) to 51.4% (lower PD), but the change was not significant ($p > 0.05$). Chickpea, when exposed to the two PD, showed a reduction in the protein product yield, which was also seen in extraction yields, possibly due to protein-lipid interactions forming viscous slurry-like appearance. The protein content of the final product is shown in Table 2; the purity of the proteins in product for all legumes decreased as the sonication power density increased. The highest protein content is that of unsonicated soy flakes followed by soy flour, kidney bean and chickpea. Due to sonication, several other compounds like oils, sugar and iso-flavones might have been extracted along with the protein, lowering the purity of protein [9,17].

3.4 Scanning electron microscopy of spent substrate

SEM for unsonicated flakes and flour showed intact cells for all the samples and presence of intracellular materials (Fig. 4 A, B, C, D left column), which are comparable to SEM studies by other researchers [3,36]. Several micro-fractures appeared in sonicated soy flakes and soy flour samples. There was a deposition of debris on the surface (Fig. 4 middle and right column) which suggested cell breakdown and layer formation. The sonicated samples looked like an aggregation of fragmented parts. The lower and higher PD seemed to disintegrate the cell (Fig. 4 A and B middle & right column) and caused the deposition of cell-matrix in both soy flakes and flour. Formation of larger aggregates was observed in a dry state after freeze-drying of ultra-sonicated

legumes which was also observed by Hu et al [16].

SEM examination for chickpea and kidney beans without sonication (Fig. 4C and 4D left column) showed the presence of starch granules that are embedded in the matrix of protein bodies and surrounded by the fiber-rich cell wall [37]. Large oval and small spherical granules of starch having a smooth surface without cracks were observed in unsonicated chickpea [38] and kidney beans (Figure 4C and 4D left column). On the other hand, the sonicated chickpea (Fig. 4C, 4D middle and right column) had an irregular structure with cracked granules along with deposition of cell debris and aggregation of cell-matrix with embedded starch granules. The cracks/fissures that are due to sonication helps in the release of the proteins and several other biological components. Similarly, in the unsonicated kidney bean, starch granules were regular in shape. At lower PD, there was protein layer embedding starch granules, whereas higher PD sonication degraded the cell-matrix resulting in the aggregation of the fragmented cell materials.

3.5 *Sonication effect on the particle size of spent substrate*

All the samples after treatment were freeze-dried, which led to the aggregation of particles. The samples were gently ground with mortar pestle. The particle size distribution is shown in Fig 5. Soy flour treated at lower PD showed a bimodal distribution with major and minor peaks and reduced particle size upon sonication. Also, because of the aggregation of the cell-matrix and starch granules as seen in SEM (Fig. 4B middle and right column), there was an apparent enlargement particle size.

The particle size of chickpea apparently increased from approximately 20 μm to 110 μm at higher PD which is supported by SEM studies (Fig. 4C right column) where there is an aggregation of proteins and cellular matrix that was fragmented during the sonication process.

Similar aggregation was seen in ultrasonicated soy protein isolates [16]. Similarly, Jiang et al [13] reported the higher particle size of sonicated (150W - 450W, 12-24 min) black bean protein compared to untreated samples which might be due to the formation of unstable aggregate. The particle size for kidney bean spent substrate decreased from approximately 120 μm to 105 μm for lower PD and increased from approximately 20 μm to 130 μm at higher PD compared to unsonicated sample, which had been attributed to the re-polymerization of aggregates through noncovalent interaction such as hydrophobic interactions [39].

3.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Figure 6 depicts the electrophoresis pattern of proteins subunits obtained from flakes and flours of soybean, kidney beans, and chickpeas. Soybean protein consists of two major proteins namely 7S (β-conglycinin) and 11S (glycinin). β-conglycinin is a trimeric protein and consists of three subunits (namely, α'~72, α ~68, and β- ~52 kDa) (Fig. 6 Left). Glycinin consists of acidic and basic subunits with ~ 35 and ~20 kDa, respectively [31]. The unsonicated soy proteins and ultra-sonicated proteins at higher and lower PD had generally similar protein subunit electrophoretic patterns, suggesting that sonication did not change the protein profiles for soy protein regardless of sonication conditions. Similar results were observed by Wang et al [41] and Karki et al [10] who also reported no modification in soy protein profiles. Kidney bean protein consists of phaseolin and chickpea protein consists of legumin and vicilin as a major protein subunit; these can be seen in the gel as major bands (Fig. 6 Right). These gels show that sonication conditions did not alter the primary structure of protein subunits in kidney bean and chickpea protein, however, secondary structures may have been altered (Sec 3.8). Comparing protein bands with the standard (6.5 kDa-66 kDa), suggested no changes in molecular weight of the protein after sonication at both PD.

3.7 Free sulfhydryl content of final protein products

The free sulfhydryl content of protein product had no significant changes between unsonicated and sonicated soy flakes and flour (Table 3). This result is similar to Arzeni et al [11], who studied the effect of high-intensity ultrasound on free and total sulfhydryl content of SPI and reported no significant modification. However, Hu et al [16] reported an increase in free sulfhydryl content of soluble SPI from 9.13 ± 0.44 μmol g^{-1} soluble protein to 18.08 ± 0.39 μmol g^{-1} upon sonication at 600 W for 30 min. Such differences in free sulfhydryl content might be due to the sonication conditions and preparation methods of protein products.

Higher and lower PD increased the free SH in kidney beans protein products. Free SH increased significantly ($p < 0.05$) from 3.95 ± 0.87 μmol g^{-1} (unsonicated) to 11.81 ± 1.46 μmol g^{-1} at lower PD and 13.67 ± 3.85 μmol g^{-1} at higher PD. There was significant ($p < 0.05$) increase of free SH in chickpea protein from 6.76 ± 0.39 μmol g^{-1} to 20.40 ± 4.94 μmol g^{-1} at higher PD sonication. This finding suggests that HPS could break the disulfide bonds (i.e., can cause the reduction of S-S linkage to form free -SH groups), which exposes the sulfhydryl group to the surface of proteins [16,41]. The buried sulfhydryl group of proteins when exposed to high pressure and sheer force of cavitation phenomenon might lead to an increase in the free sulfhydryl group.

3.8 Secondary structure composition of legume by circular dichroism

The contents of α-helix, β-strands, β-turns, and unordered groups are shown in Table 4. The distribution of these attributes of protein secondary structure from both unsonicated soy flour protein and soy flakes protein seems to be similar. The results suggested that α-helical structure is not the main structure in protein; instead, the β- structure is the main secondary structure for all the unsonicated legumes [16,42]. Hu et al [16] reported that HPS (400 W and 600W)

combined with a longer time decreased the β-strands in soy protein isolates. Soy flakes protein at higher PD tended to an increase in β- structure and decrease in α-helix which was also reported by Stathopulos et al [43] for BSA, myoglobin, lysozyme, and black bean protein [44].

The α-helical structure seemed to decrease for proteins from sonicated chickpea and kidney beans. The β-strands decreased significantly in kidney beans when high and low PD sonication was applied, and unordered form increased from 33.22 ± 3.55% to 52.35 ± 4.54% and 50.73 ± 5.14% for kidney beans significantly during sonication (Table 4). The unordered structure ranged at lower and higher PD respectively. CD spectra of kidney bean protein are shown in Figure 7. As the secondary structure of proteins is stabilized by hydrogen bonding and electrostatic interactions, it is reasonable to infer that sonication might cause the disruption of these interactions leading to changes in secondary structure [45]. In the current work, ultrasonication likely destabilized the native structure of proteins, and therefore, changed the secondary structure composition by increasing the unordered structure. HPS induced partial unfolding and intermolecular interactions as indicated by an increase in free sulfhydryl content in chickpea and kidney beans. The variation among the literature for the secondary structure may be due to the various reference spectra, algorithm and software used for analyses. Furthermore, the protein isolation technique also contributes to differences in the conformation of proteins.

4. Conclusions

High power sonication of defatted soy flakes resulted in higher protein extraction yields when exposed to higher power sonication compared to unsonicated soy flakes, for example, 90% and 68.7% increment with lower and higher PD sonication in the study. However, sonicated chickpea flours resulted in a decrease in the protein extraction yield, possibly due to high carbohydrate and fat reducing the access to proteins in cell matrices. Protein subunit bands for all the substrate

were not altered by sonication; significant changes in the secondary structure of kidney bean protein were observed and indicated by circular dichroism analyses. Also, an increment of the free sulfhydryl contents in sonicated kidney bean protein and chickpea protein suggests an alteration in the structure of native protein due to partial unfolding. Our study indicated that HPS has the potential to improve the extraction of various plant proteins with altered molecular structure. This will have an impact on how these proteins will be utilized in various food applications.

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Fig. 1. Schematic diagram and analysis

Fig. 2. Effect of high-power sonication on protein extraction yield of some plant-based proteins at two power densities.

Fig. 3. Effect of sonication on protein yield in product of some plant-based proteins at two power densities.

Fig. 4. Scanning electron microscopy of spent substrate before and after sonication of A: soy flakes, B: soy flour, C: chickpea, and D: kidney bean. Left (Unsonicated), Middle (Lower PD) and Right (Higher PD)

Fig. 5. Particle size distribution of (a) Soy flakes; (b) Soy flour; (c) Kidney bean; (d) Chickpea at unsonicated, Lower PD and Higher PD.

Fig. 6. (Right) Kidneybean protein (Lane 1-3)1: control, 2: PD 2.5, 3: PD 4.5, chickpea protein(Lane 4-6)4:control, 5: PD 2.5, 6: PD 4.5

(Left) Soy flour protein 1: unsonicated, 2: PD 2.5, 3: PD 4.5, 4: soy flour protein-control 5: soy flakes protein control, 6: PD 2.5, 7: PD 4.5.

Fig. 7. Representative CD spectra of unsonicated and sonicated kidney bean proteins measured at 185-240 nm.

Table 1 Proximate analysis (% dry basis) of legumes before sonication treatment

Sample	% Moisture	% Protein	% Fat	% Crude Fiber	% Ash	% Carbohydrate
Soy flakes	8.52 ± 0.06	54.95 ± 1.28	0.79 ± 0.09	1.77 ± 0.15	6.29 ± 0.05	36.21
Soy flour	8.99 ± 0.18	53.11 ± 0.95	0.62 ± 0.19	1.24 ±0.26	6.18 ± 0.02	38.86
Kidney bean	7.34 ± 0.12	23.84 ± 0.72	1.31 ± 0.12	0.35 ± 0.07	3.60 ± 0.03	70.90
Chickpea	5.89 ± 0.09	23.66 ± 0.64	7.03 ± 0.17	0.57 ± 0.06	2.74 ± 0.01	65.99

Table 2 Percent protein in powder product prepared after sonication at two power densities

Substrate	Treatment	% Protein in product
Soy flakes	Unsonicated	65.76 ± 0.46^{a}
	PD 2.5	64.08 ± 0.47^{a}
	PD 4.5	57.00 ± 1.21^{b}
Soy flour	Unsonicated	60.04 ± 1.28^{a}
	PD 2.5	59.32 ± 1.65^{a}
	PD 4.5	59.48 ± 3.90^{a}
Kidney bean	Unsonicated	59.55 ± 2.66^{a}
	PD 2.5	48.66 ± 4.28^{a}
	PD 4.5	39.91 ± 2.47^{b}
Chickpea	Unsonicated	54.17 ± 4.77^{a}
	PD 2.5	34.17 ± 0.94^{b}
	PD 4.5	35.44 ± 1.62^{b}

Mean % protein in product were compared within each substrate for sonication effect. Results having different superscript letters within each substrate group show significant sonication effect ($p<0.05$).

Table 3 Free sulfhydryl in unsonicated and sonicated plant-based protein preparations at two power densities

Substrate	Treatment	Free SH (µg/gram of protein)
Soy flakes	Unsonicated	4.31 ± 0.21[a]
	PD 2.5	4.54 ± 0.02[a]
	PD 4.5	4.85 ± 0.94[a]
Soy flour	Unsonicated	6.41 ± 0.43[a]
	PD 2.5	5.50 ± 0.60[a]
	PD 4.5	5.89 ± 0.26[a]
Kidney bean	Unsonicated	3.95 ± 0.87[a]
	PD 2.5	11.81 ± 1.44[b]
	PD 4.5	13.67 ± 3.85[b]
Chickpea	Unsonicated	6.76 ± 0.55[a]
	PD 2.5	8.38 ± 3.52[a]
	PD 4.5	19.30 ± 2.0[b]

Mean free sulfhydryl content were compared within each substrate for sonication effects. Results having different superscript letters within each substrate group show significant sonication effect ($p<0.05$).

Table 4 Secondary structure composition of unsonicated and sonicated legume from CD in far UV region (180-260nm)

Substrate		**α-Helix**	**β-Strands**	**β-Turns**	**Unordered**
Soy flakes protein	Unsonicated	25.2 ± 2.2[a]	21.8 ± 4.4[a]	20.5 ± 0.8[a]	32.4 ± 2.9[a]
	PD 2.5	26.0 ± 1.9[a]	20.1 ± 1.4[a]	20.3 ± 0.4[a]	33.3 ± 0.9[a]
	PD 4.5	13.0 ± 5.2[a]	37.1 ± 5.0[a]	25.7 ± 5.4[a]	24.0 ± 4.7[a]
Soy flour protein	Unsonicated	26.2 ± 0.6[a]	19.9 ± 0.6[a]	19.9 ± 2.0[a]	33.8 ± 3.3[a]
	PD 2.5	28.1 ± 1.0[a]	20.7 ± 0.0[a]	21.8 ± 0.4[a]	29.2 ± 0.6[a]
	PD 4.5	25.4 ±4.9[a]	25.1 ± 4.5[a]	20.4 ± 1.5[a]	29.8 ± 1.8[a]
Kidney bean protein	Unsonicated	23.5 ± 0.8[a]	22.4 ± 1.1[a]	20.5 ± 1.6[a]	33.2 ± 3.5[a]
	PD 2.5	16.2 ± 1.8[a]	16.6 ± 1.06[b]	14.7 ± 1.5[a]	52.3 ± 4.5[b]
	PD 4.5	14.3 ± 2.8[a]	19.5 ± 0.4[b]	15.3 ± 2.1[a]	50.7 ± 5.1[b]
Chickpea protein	Unsonicated	21.6 ± 1.4[a]	17.6 ±1.3[a]	17.1±2.[a]	43.6 ± 4.8[a]
	PD 2.5	18.4 ± 4.4[a]	20.7± 6.3[a]	18.5 ±1.0[a]	42.1 ± 2.9[a]
	PD 4.5	16.4 ± 1.8[a]	18.8 ±1.7[a]	16.0 ±1.7[a]	48.6 ±1.7[a]

Mean secondary structure composition were compared within each substrate for sonication effect. Results having different superscript letters within each substrate group show significant sonication effect ($p<0.05$).

Highlights

- High-power sonication (HPS) efficient in breaking plant cell walls for extraction
- HPS did not alter legume proteins electrophoretic bands
- HPS produced partial protein unfolding and changes in secondary structure
- HPS treatment increased protein yield from flakes but not from legume flours

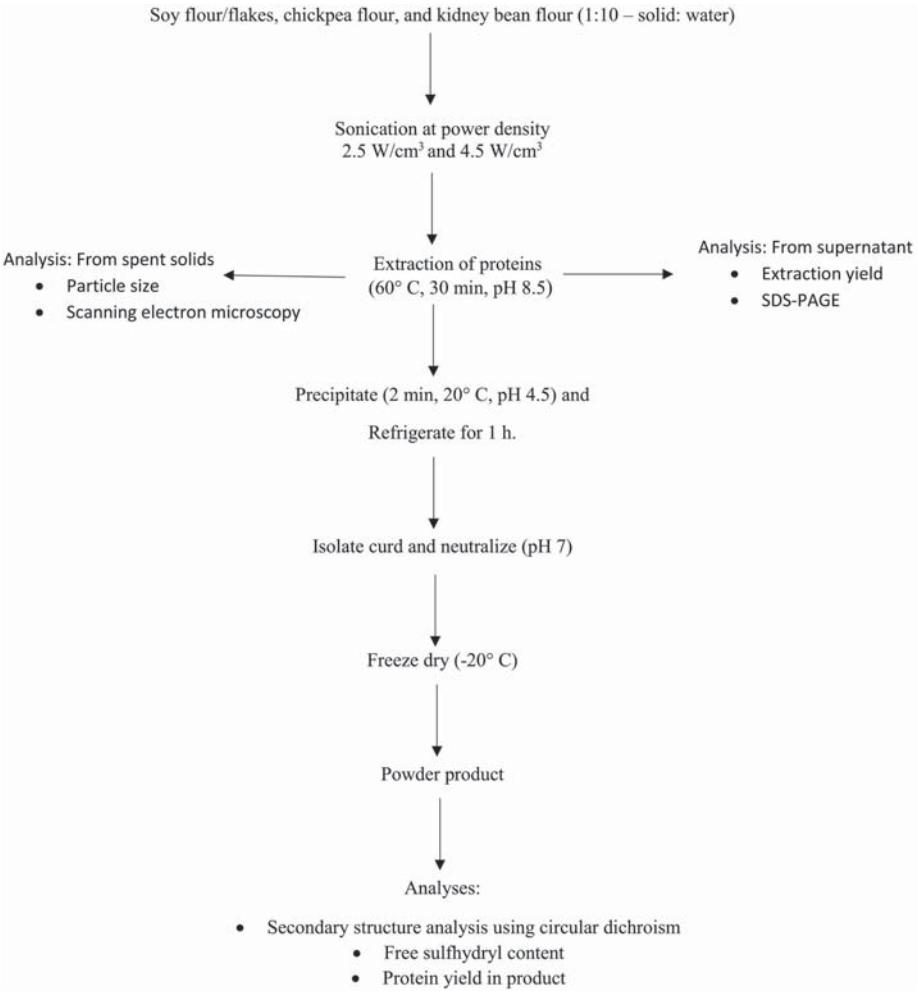
Soy flour/flakes, chickpea flour, and kidney bean flour (1:10 – solid: water)
Sonication at power density
2.5 W/cm3 and 4.5 W/cm3
Extraction of proteins
(60° C, 30 min, pH 8.5)
Analysis: From spent solids
Particle size
Scanning electron microscopy
Analysis: From supernatant
Extraction yield
SDS-PAGE
Precipitate (2 min, 20° C, pH 4.5) and
Refrigerate for 1 h.
Isolate curd and neutralize (pH 7)
Freeze dry (-20° C)
Powder product
Analyses:
Secondary structure analysis using circular dichroism
Free sulfhydryl content
Protein yield in product

Figure 1

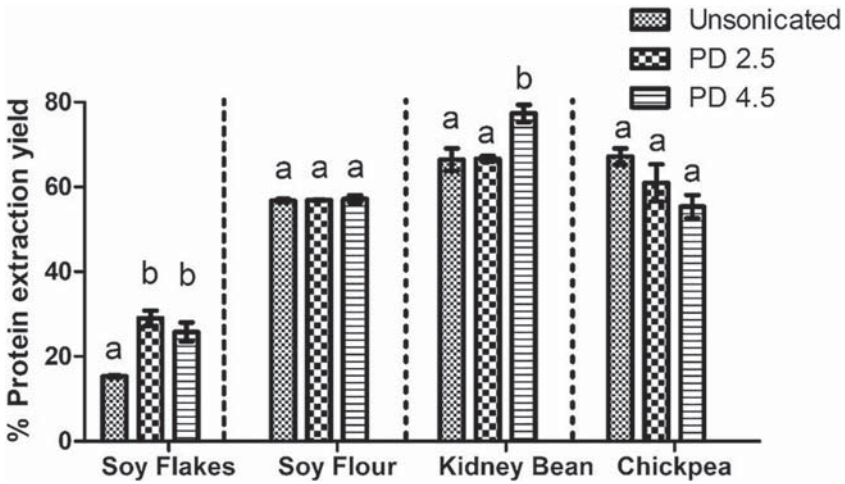
Unsonicated
PD 2.5
PD 4.5
% Protein extraction yield
80
60
40
20
0
a
b b
a a a
a a b
a a a
Soy Flakes
Soy Flour
Kidney Bean
Chickpea

Figure 2

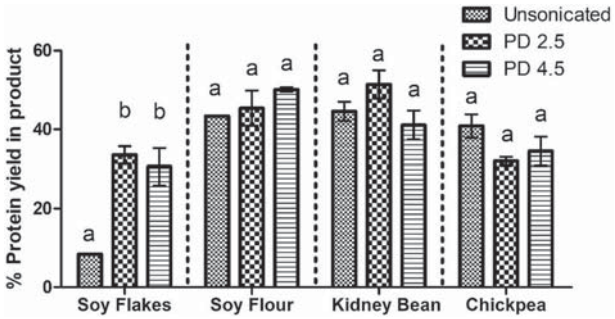
Unsonicated
PD 2.5
PD 4.5
% Protein yield in product
60
40
20
0
a
b
b
a
a
a
a
a
a
a
a
a
Soy Flakes
Soy Flour
Kidney Bean
Chickpea

Figure 3

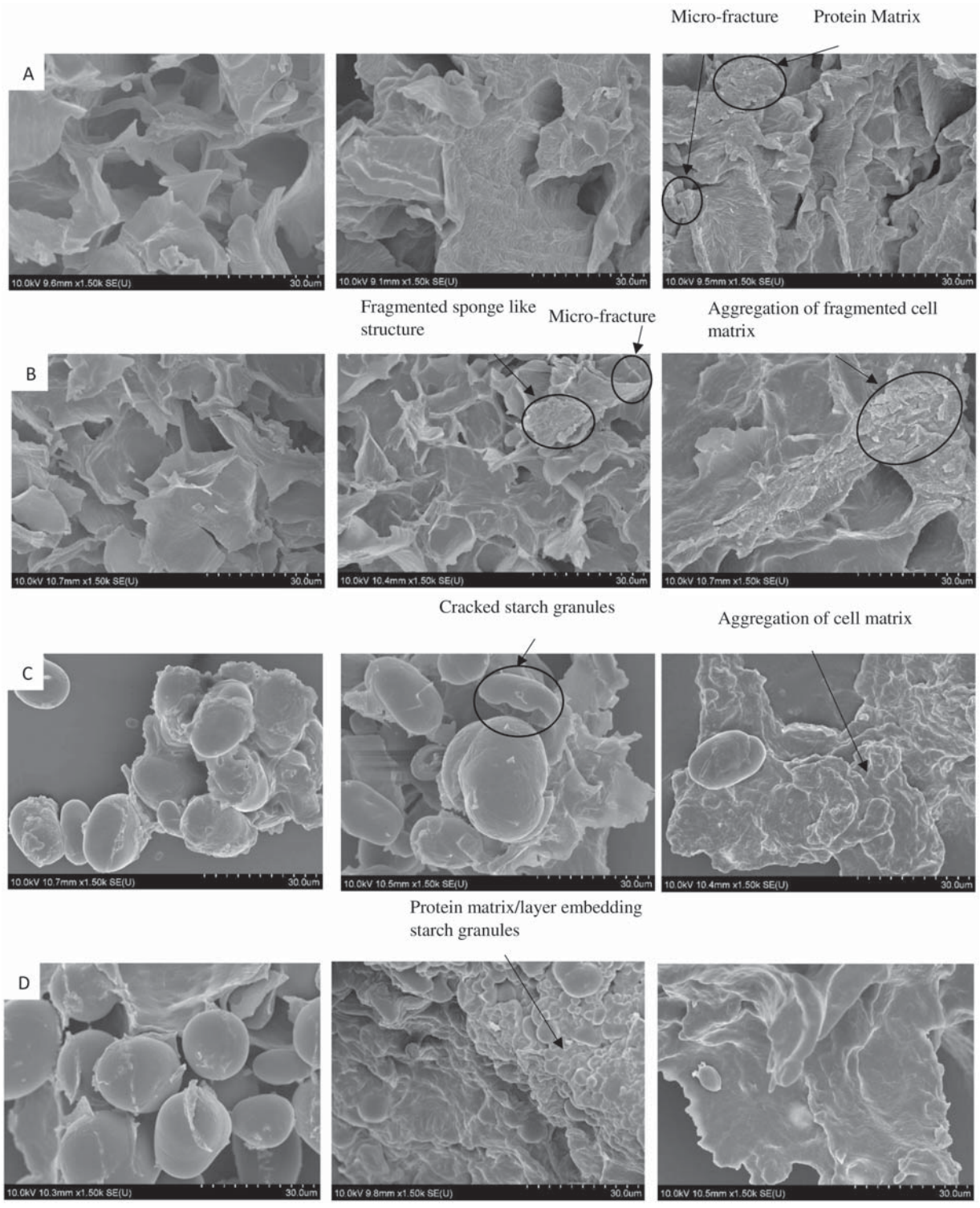
Micro-fracture
Protein Matrix
A
10.0kV 9.6mm x1.50k SE(U)
30.0um
10.0kV 9.1mm x1.50k SE(U)
30.0um
10.0kV 9.5mm x1.50k SE(U)
30.0um
Fragmented sponge like structure
Micro-fracture
Aggregation of fragmented cell matrix
B
10.0kV 10.7mm x1.50k SE(U)
30.0um
10.0kV 10.4mm x1.50k SE(U)
30.0um
10.0kV 10.7mm x1.50k SE(U)
30.0um
Cracked starch granules
Aggregation of cell matrix
C
10.0kV 10.7mm x1.50k SE(U)
30.0um
10.0kV 10.5mm x1.50k SE(U)
30.0um
10.0kV 10.4mm x1.50k SE(U)
30.0um
Protein matrix/layer embedding starch granules
D
10.0kV 10.3mm x1.50k SE(U)
30.0um
10.0kV 9.8mm x1.50k SE(U)
30.0um
10.0kV 10.5mm x1.50k SE(U)
30.0um

Figure 4

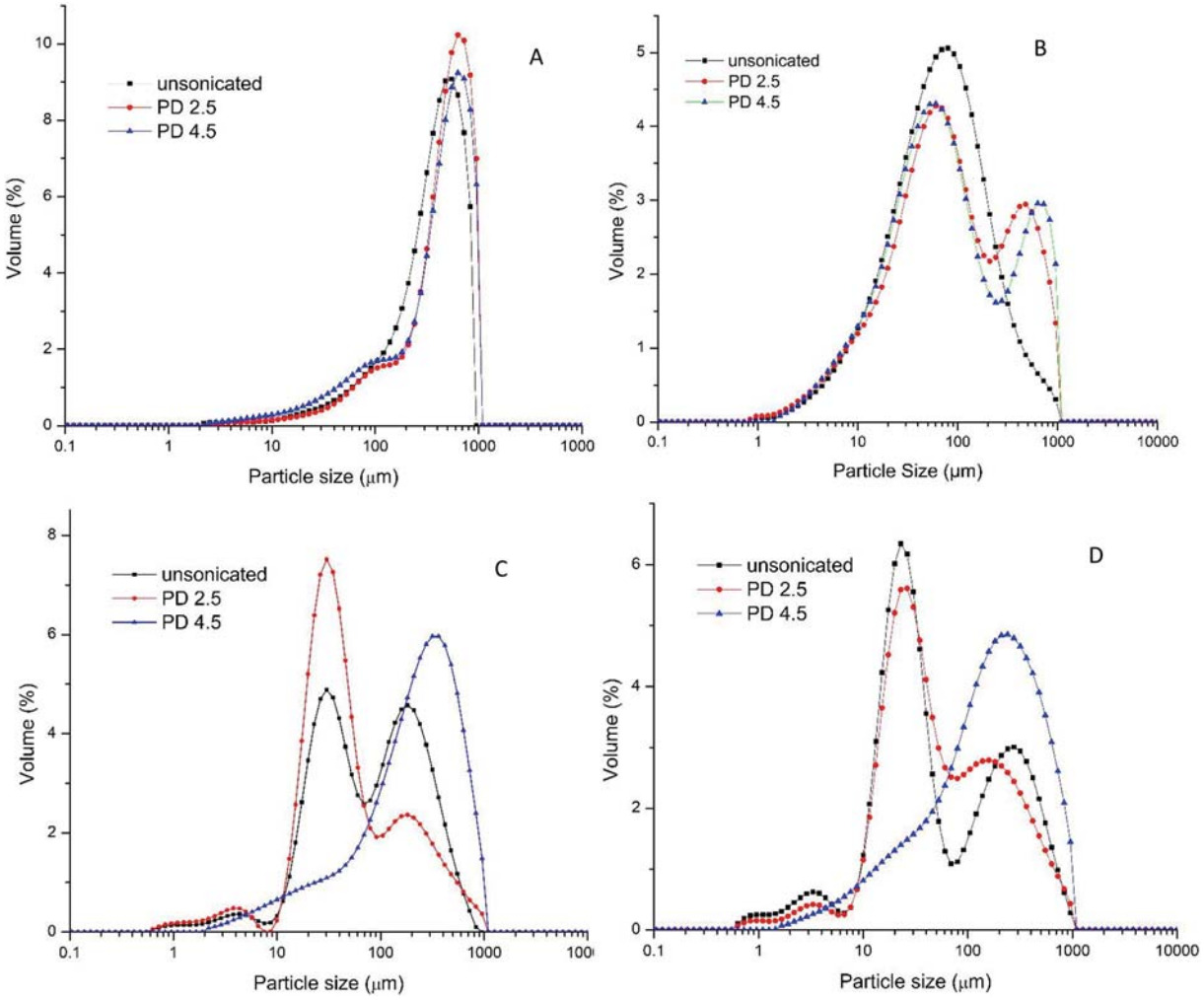
A
unsonicated
PD 2.5
PD 4.5
Volume (%)
Particle size (μm)
B
unsonicated
PD 2.5
PD 4.5
Volume (%)
Particle Size (μm)
C
unsonicated
PD 2.5
PD 4.5
Volume (%)
Particle size (μm)
D
unsonicated
PD 2.5
PD 4.5
Volume (%)
Particle size (μm)

Figure 5

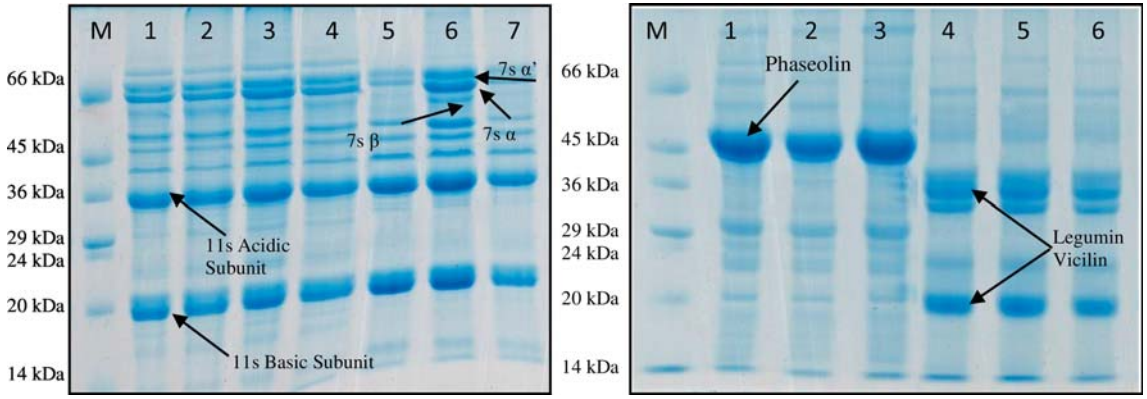
M
1
2
3
4
5
6
7
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa
7s α'
7s β
7s α
11s Acidic Subunit
11s Basic Subunit
M
1
2
3
4
5
6
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa
Phaseolin
Legumin
Vicilin

Figure 6

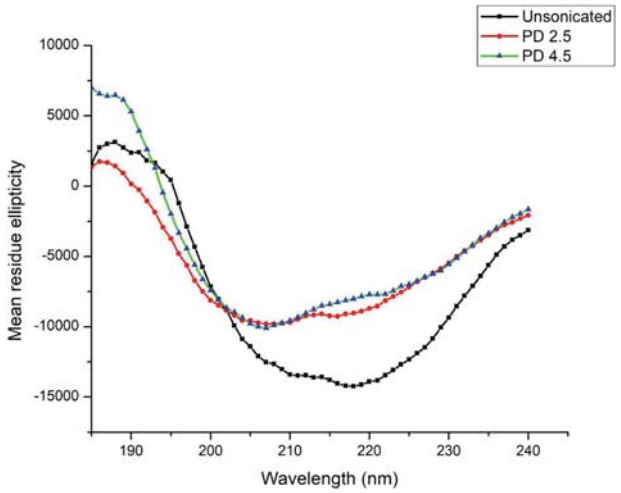
Unsonicated
PD 2.5
PD 4.5
10000
5000
0
-5000
-10000
-15000
Mean residue ellipticity
190
200
210
220
230
240
Wavelength (nm)

Figure 7