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**Properties of protein isolates extracted by ultrasonication from
soybean residue (okara)**

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Abstract

Soybean protein extraction was evaluated using conventional (alkaline phosphate buffer) and ultrasonication assisted methods. The impact of the extraction method on protein yield, chemical composition, and structural properties of the protein isolates was assessed. In conventional alkaline extraction, increasing pH values (from 9 to 12) led to an increase in the protein yield, with pH 12 favouring protein extraction, with yields up to 36 % (w/w). Ultrasonication process at the same pH, led to maximum protein extraction yield of 84 % (w/w). Secondary structural changes in ultrasonicated OPI samples were linked to cavitation effects and the duration of the extraction, rather than the intensity of ultrasonication process. Extracted proteins exhibited increased β -sheet content, improved zeta-potential and smaller particle size of ultrasonicated proteins, rendering them suitable ingredients for tailored food applications.

Keywords: Protein isolate, extraction, ultrasonication, okara, soybeans, FTIR

1. Introduction

Plant-based sources of protein are considered more sustainable for the increasing global population compared to animal protein. Among all vegetable sources of protein, soybeans have gained much popularity due to their rich content in all eight essential amino acids, as well as exhibiting similar digestibility to that of cow's milk, meat and egg proteins (Soderberg, 2013). Soybean seeds are principally used to produce soymilk and tofu. Soymilk is increasingly becoming more popular by consumers, although, it is mostly consumed by lactose intolerant individuals, and those conscious of cholesterol. Currently, the demand for soymilk is high with the global soymilk market value amounted to about 15.33 billion US dollars in 2018 and estimated to be 23.2 US dollars in 2025 (Statista, 2021a). In the UK, the sale volume of soybean milk rose to 85.7 million litres in 2016 (AHDB, 2017) and to about 92.6 million litres in 2018 (Statista, 2021b). The main market drive for soymilk production and consumption is its association with health benefits, especially following the FDA health claim approval on soy protein effectiveness in reduction of coronary heart disease risk. Consequently, the accumulation of soymilk by-product (okara) is expected to increase; it is estimated that for every 1000 litre of soymilk produced via commercial process (the soya technology systems process) or traditional process (cold extracted and no treatment to remove off-flavour), 250 kg or 398 kg of okara are generated, respectively (Gavin & Wettstein, 1990). Okara contains notable amounts of protein (26.8 %-37.5 % w/w) (Ma et al., 1997; Vishwanathan et al., 2011), since a significant proportion is left in the residue following soymilk production (O'Toole, 1999) due to the complexity of soybean structure. However, this residue is scarcely utilised and as such, it currently has little market value. Recently, okara has caught the interest of some researchers for its potential application in the food industry, as a raw material for soy protein isolate extraction, with potential applications in beef burger production, cookies, and sausage

formulations. Soybean-based protein isolates are reported to demonstrate useful functional properties, that would enable their application in a variety of food systems (Singh et al., 2008). Therefore, okara could be converted into a valuable starting for commercial production of protein isolates and this approach could, in turn, minimise waste, in-line with the cradle-to-cradle concept for sustainability (Eze, 2017).

Soybean protein isolate has been produced commercially, using soybean meal or soybean flakes as feedstocks and previous studies have evaluated the extraction of proteins in soybean flakes or soybean meal in aqueous-alkaline (NaOH) conditions (conventional method), via enzymatic routes or through the use of ultrasonication-assisted methods (Ma et al., 1997; Vishwanathan et al., 2011). According to Ma et al. (1997), okara protein extraction in aqueous-alkaline (NaOH) conditions at 25°C resulted in low protein recovery (14.1%, w/w); when temperature was elevated 80 °C, 53.4 % (w/w) of okara proteins were extracted. However, the latter extraction conditions may cause protein denaturation and aggregation as the denaturation temperatures of the two major soybean proteins, (glycinin and β -conglycinin) are approximately 82 °C and 68 °C, respectively (Riblett et al., 2001). It is also expected that most of the soluble proteins are removed during soymilk production, leaving the residue (okara) with mostly water-insoluble proteins. Heating at elevated temperature of about 80 °C is an indispensable process step during soymilk production to get a final product with desirable quality, but leaves the residue (okara) with aggregated proteins in the intact cotyledon cells that are not easily extracted (Preece et al., 2015). Moreover, protein fractions that have been extracted under high temperature conditions could have low solubility and decreased thermal stability (Ma et al., 1997).

Ultrasonication technology has recently attracted much research interest as a technique to assist protein extraction processes from a variety of raw materials (Zhang et al., 2018). Ultrasonication allows the development of sustainable extraction processes by increasing

extraction efficiency and at the same time reducing solvent and energy utilisation (Chemat et al., 2017). These advantages seem to be more prominent on lab-scale studies, whereas for industrial scale applications, further aspects of the process still need to be optimised (e.g. reactor design, energy consumption reduction, solid-liquid separation post-extraction) to establish ultrasonication's industrial prospects (Chemat et al. 2020; Preece et al 2017; Vernes et al. 2019). The mechanism of extraction by ultrasonication is based on the cavitation phenomenon which leads to particle or cell disintegration (Khanal et al., 2007). The disintegration of cell walls by cavitation exposes hidden compounds in the cells to the extracting medium, hence promoting higher extraction yields at shorter times (Mason et al., 1996). However, this effect can alter the native conformational structure of proteins with resultant changes in their functional properties (McClements, 1995). Various studies have reported the exposure of hydrophobic groups and redistribution of the secondary structure (Li et al., 2016), as well as promoting the unfolding and dissociation of protein isolates extracted via ultrasonication (Huang et al., 2017). As such, ultrasonication can employed not only for extraction processes but also for the enhancement of functional properties of proteins.

The aim of this study was to develop a protein extraction process via ultrasonication from soybean residue (okara) under alkaline conditions. It is hypothesised that ultrasonication could enhance the release of proteins located in the protein bodies of palisade-like cells in soybean cotyledon within a short time and could possibly preserve the functional properties of the protein isolates. To this end, detailed chemical analysis of okara protein isolates was carried out, together with the assessment of their structural properties.

2. Materials and methods

2.1 Raw material and chemical reagents

Yellow soybean seeds (*Glycine max*) sourced from a local shop in Nigeria (year of harvest 2017) was used to produce okara (soymilk residue). All chemicals used in this research were of analytical grade and were purchased from Sigma-Aldrich (UK) and Fisher Scientific (UK).

2.2 Preparation of defatted okara flour

Yellow soybean seeds (*Glycine max*) were soaked in water (1:5 w/v) for 8 hours and hulls were removed manually by washing. Dehulled, washed beans were ground with a hammer mill. Milk was separated from ground soybean slurry using a clean linen cloth and the obtained wet okara was dried in an oven dryer (60 ± 2 °C) for 4 hours. The dried okara flakes were ground into a fine flour of particle size less than 1.0 mm. The okara flour was defatted using hexane (Sigma-Aldrich, UK) in the ratio of 1:10 (w/v) for 30 min while continuously stirring in a beaker prior to its use.

2.3 Alkaline extraction of okara proteins

Defatted okara flour was mixed with 0.1 M phosphate buffer (pH 9-12) (Fisher Scientific, UK) in ratio of 1:20 (w/v). The sample slurry was placed in a 60 °C water bath with stirring for 1 h. The protein extract/supernatant was recovered by vacuum filtration using Buchner funnel and proteins were precipitated at pH 4.6 with 2 N HCl (Fisher Scientific, UK) and left at 4 °C overnight to enhance the precipitation. The precipitate was recovered after centrifugation at $10,000 \times g$ for 10 min. The obtained protein pellet was washed twice and re-dissolved in distilled water (Purite reserve osmosis system, Oxon, UK), its pH was adjusted to 7.0 using 2 N NaOH (Fisher Scientific, UK) and was freeze dried to obtain the protein isolate (Virtis 2KBTES, Warminster, Pennsylvania).

2.4 Ultrasonication-assisted alkaline extraction of okara proteins

The ultrasonic system used was a high intensity ultrasonic process system (model: P100/6-20) with typical titanium process horn configuration which operates at a nominal frequency of 20 KHz. The system was submerged to a depth of 1-2 cm in the sample. The ultrasonicator maximum amplitude and power were 16 $\mu\text{m-pp}$ and 100 W, respectively. Defatted milled okara flour was mixed with 0.1 M phosphate buffer (pH 12) (Fisher Scientific, UK) in the ratio of 1:20 (w/v); the slurry was transferred into a double walled flow cell for ultrasonication extraction (total volume 70 mL, Celbuis Ltd., UK) through which water was circulated to maintain the temperature of extraction constant (60° C). Three different sonication amplitudes were monitored representing intensities, and these are referred to as low (5 $\mu\text{m-pp}$), medium (10 $\mu\text{m-pp}$) and high (15 $\mu\text{m-pp}$) amplitude. The extraction was carried out for 50 min under continuous pulse mode. The obtained slurry was centrifuged at $10,000 \times g$ for 10 min at 4 °C. The pH of the supernatant was reduced to 4.5 using 2 N HCl (Fisher Scientific, UK) to isolate the proteins, refrigerated at 4 °C overnight, and centrifuged at $10,000 \times g$ for 10 min at 4 °C to recover the isolated protein precipitate. The precipitate was washed twice with distilled water, centrifuged after each washing at the same conditions and was dispersed in distilled water with its pH adjusted to 7.0 with 2 N NaOH (Fisher Scientific, UK). The neutralized protein was frozen and then freeze-dried (Virtis 2KBTES, Warminster, Pennsylvania), ground and packaged in an air-tight container and stored in a desiccator until further use.

2.5 Determination of protein content in okara extracts

The protein content of okara extracts was measured according to Bradford (Bradford, 1976). An aliquot of 1.5 mL of the Bradford reagent (Sigma-Aldrich, UK) was mixed with 50 μL of appropriately diluted protein solution (based on the concentration of the protein solution)

and the absorbance of the mixture was read at 595 nm with a spectrophotometer (BioMate 3, Madison, WI 53711 USA). Bovine serum albumin (BSA), (Sigma-Aldrich, UK) was used as standard for the calibration curve. Samples were measured in triplicate and the protein yield of the extraction was estimated according to Equation 1 below:

$$\text{Protein yield (\%, w/w)} = \frac{\text{protein concentration in the extract}}{\text{protein concentration in the defatted okara}} \times 100 \quad (\text{Eq. 1})$$

2.6 Chemical composition determination of okara protein isolates

Moisture, fat, ash and protein contents of okara flours and protein isolates were determined according to the methods of AOAC (2000). For protein content, a nitrogen to protein conversion factor of 6.25 was used.

2.7 Amino acid profile determination of protein isolates

Lyophilised protein isolates (0.1 g) were mixed with 6 M HCl (Fisher Scientific, UK) in a sealed container and nitrogen was flushed into it to prevent oxidation reactions and then the suspension was hydrolysed for 24 hours at 110 °C. The hydrolysate was neutralised with CaCO₃ powder (Sigma-Aldrich, UK) prior to derivatization. The neutralised hydrolysate was analysed for amino acid content using the EZ-Faast amino acid derivatization kit for GC-MS (Phenomenex, Torrance, CA) (Hushek 2000). The derivatization was carried out on 100 µL of each sample using the EZ-Faast amino acid analysis kit for free amino acid analysis by GC-MS. The same procedure was carried out on the standards. The derivatised amino acids were analysed in electron impact mode using Agilent 5975 system (Agilent, Palo Alto, CA). An aliquot (1 µL) of the derivatised amino acid solution was injected in split mode (40:1) at 280 °C onto a zebron ZB-AAA capillary column (10 m × 0.25 mm; 0.25 µm film thickness), with

the flow rate of the carrier gas held at 1.5 mL per min, throughout the run. The oven temperature was kept at 110 °C for 1 min and then increased at 30 °C per min to 310 °C, while the transfer line and ion source were kept constant at 320 °C and 230 °C respectively. Samples and standards were analysed in duplicate and the retention time of the standards were used to identify the respective amino acids peaks.

2.8 Fourier transform infrared spectroscopic analysis (FTIR)

Okara protein isolate samples were measured in a Perkin-Elmer Spectrum 100 FTIR spectrophotometer at room temperature by placing the samples on the crystal cell and the cell was clamped into the pin hole of the FTIR spectrophotometer. The spectra from the samples in the range of 600 – 4000 cm⁻¹ wavenumbers, averaged from 16 scans at a resolution of 4 cm⁻¹ were automatically recorded against a background spectrum from the clean empty cell at room temperature. The amide 1 spectrum was deconvoluted and then curve fitted at 100% Gaussian to calculate the percentages of the secondary structures in it using WIRE 4.0 software. Samples were analysed in triplicate.

2.9 Sulphydryl content of protein isolates

Total and free sulphydryl content of okara protein isolates and the commercial soybean protein isolate was determined using the modified method of Tang et al (2009). In brief, free sulphydryl (FSH) content was determined by dissolving 50 mg of protein isolate samples in 10 mL of Tris-glycine buffer (86 mM Tris, 90 mM glycine, 4 mM ethylenediaminetetraacetic acid, pH 8.5) (Sigma-Aldrich, UK) containing 8 M urea (Sigma-Aldrich, UK) and then kept overnight at room temperature with gentle mixing using an orbital shaker. The protein solution was centrifuged in 50 mL centrifuge tube at 10 000 x g for 10 min at room temperature and the supernatant was collected. Protein concentration in the supernatant was determined by

209 Bradford method and then diluted to 0.1 mg/mL with Tris-glycine buffer (Sigma-Aldrich, UK).
210 A 1 mL aliquot of the sample reacted with 10 μ L of Ellman's reagent (0.4% 5,5'-dithiobis-[2-
211 nitrobenzoic acid] (Sigma-Aldrich, UK) in 10 mM Tris-glycine buffer, pH 8.5] (Sigma-
212 Aldrich, UK) for 10 min at room temperature and another 1 mL aliquot of the same sample
213 was taken without Ellman's reagent (used as blank), and absorbance was read at 412 nm using
214 ultraviolet-visible (UV) spectrophotometer (BioMate 3, Madison, WI 53711, USA) and plastic
215 cuvettes (1 cm path length). For total SH (TSH) content determination, 50 mg of protein isolate
216 was dissolved in Tris-glycine buffer (pH 8.5) (Sigma-Aldrich, UK) containing 8 M urea
217 (Sigma-Aldrich, UK) and kept overnight at room temperature with continuous gentle mixing.
218 A 1 mL aliquot of the protein solution was diluted with 4 mL of Tris-glycine buffer (pH 8.5)
219 (Sigma-Aldrich, UK), then 50 μ L of 2-mercaptoethanol (Sigma-Aldrich, UK) was added and
220 the mixture was left to stand for 1 h at room temperature. Then, 10 mL of 12 % (w/v)
221 trichloroacetic acid (TCA) (Sigma-Aldrich, UK) were added to the mixture, left for 1 h at room
222 temperature, and centrifuged at $10,000 \times g$ for 10 min at 4 $^{\circ}$ C to collect the precipitate. The
223 precipitate was re-suspended in 5 mL of 12 % (w/v) TCA with subsequent centrifugation at
224 $10,000 \times g$ for 10 min to remove residual 2-mercaptoethanol. The washed pellet was re-
225 dissolved in 2 mL of Tris-glycine buffer (pH 8.0) containing 8 M Urea and protein
226 concentration determined using the method of Bradford with BSA (Sigma-Aldrich, UK) as a
227 standard and afterwards, diluted to final concentration of 0.1 mg/mL. A 1 mL aliquot of the
228 sample was reacted with 10 μ L of Ellman's reagent (0.4% 5,5'-dithiobis- [2-nitrobenzoic acid]
229 in 10 mM Tris-glycine buffer, pH 8.5) for 10 min at room temperature and another 1 mL aliquot
230 of the same sample was taken without Ellman's reagent (used as the blank to zero the
231 instrument). The absorbance was read at 412 nm using ultraviolet-visible (UV)
232 spectrophotometer and plastic cuvettes (1 cm path length). Disulphide bonds content (SS) for
233 each sample was determined based on the equation below (Eq. 2):

$$SS (\mu\text{mol/g}) = \frac{(TSH-FSH)}{2} \quad \text{Eq. 2}$$

All samples were analysed in triplicate.

2.10 Zeta potential and particle size distribution of protein isolates

The zeta potential of all protein isolates was determined by a laser doppler velocimetry and phase analysis light scattering technique using a Malvern Zetasizer Nano-ZS (model: ZEN3600) instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The protein samples were dispersed in distilled water (0.5 % w/v), allowed to stand for five minutes and 1 mL was collected without shaking the protein dispersion and transferred into an electrophoresis cell (model: DTS 1060C, Malvern Instruments Ltd., Malvern Worcestershire, UK). The analysis was run at 25 °C and average values of three measurements of each sample were generated and each sample was prepared in triplicate. The z-average diameter of all the protein isolates was determined by a dynamic light scattering technique using a zetasizer Nano-ZS (model: ZEN3600) instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The protein samples were dispersed in distilled water (0.5 % w/v) and diluted further in distilled water to obtain an appropriate concentration index in the zetasizer and to obtain polydispersity index (PDI) below 0.5 which indicates accurate measurement. All measurements were carried out in triplicates.

2.11 Statistical analysis

OPI extractions (alkaline and ultrasonication-assisted) were carried out in triplicate. Statistical analysis was carried out by ANOVA using IBM SPSS statistics version 25 (SPSS Inc, Chicago, USA) and means compared with Tukey's HSD test at $P < 0.05$ level of significance.

3. Results and discussion

3.1 Protein extraction from okara

Initially, aqueous extractions at varying pH values were carried out to investigate the influence of pH on protein recovery from okara. A range of alkaline pH values was studied (from 9 to 12) using 0.1 M phosphate buffer. The protein recovery during conventional aqueous alkaline extractions of defatted okara at different pH values is shown in **Fig.1**. Protein recovery in phosphate buffer ranged from 4.3 % (w/w) at pH 9 to 35.9 % (w/w) at pH 12 ($p < 0.05$). The low protein recovery at pH values between 9 and 11 indicated that phosphate buffer had weak buffer capacity within that pH range, while pH 12 exhibited a stronger buffer capacity, having with pKa at pH 12.3. These findings are also in agreement with the report of other researchers indicating that high pH can increase protein solubility and extractability in okara (Ma et al., 1997). Karki et al. (2010) reported lower protein yields of about 27 % (w/w), using aqueous-alkaline extraction at pH of 9. The low extractability of soybean protein by conventional methods could be as a result of the complex nature of soybean cell wall matrix. The major soybean proteins co-exist with other proteins and non-protein components which result into protein-protein interactions or protein-carbohydrate interactions that may hinder protein solubility in aqueous alkaline media. Repeated extractions with fresh media at high temperature of 80 °C has been shown to enhance the protein extraction yield (Vishwanathan et al., 2011) as it may assist in overcoming issues related to solvent saturation. However, there some limitations to this method including accumulation of more wastewater, long processing time, higher energy utilisation (higher temperature of 80 °C). To overcome these limitations and still achieve high protein recovery, ultrasonication method was assessed as an alternative process for protein extraction.

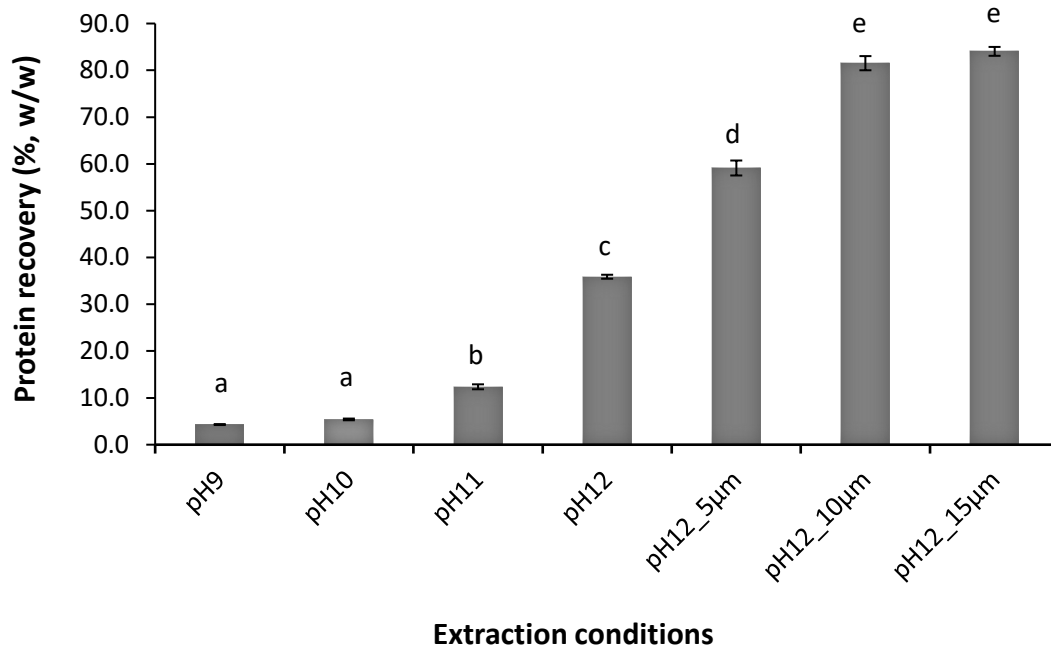


Fig.1. Protein recovery (% w/w) from defatted okara using 0.1 M phosphate buffer at different pH values (9, 10, 11 and 12), and ultrasonication-assisted extraction (5 µm, 10 µm and 15 µm amplitude using 0.1 M phosphate buffer pH 12). Significant difference exists between bars with different letters as determined by Tukey HSD test ($p < 0.05$). Values are mean \pm SD of triplicate measurements.

Okara proteins were extracted via ultrasonication utilising alkaline phosphate buffer as the extraction medium at pH 12, at three different amplitudes (5, 10, and 15 µm-pp, peak to peak amplitude in µm, representing low, medium and high intensity) (**Fig. 1**). It was noted that ultrasonication improved the protein extraction yield by 2.5-fold in the case of medium and high intensities (10 µm-pp and 15 µm-pp) reaching up to 84% (**Fig. 1**). In terms of the rate of protein extraction yield during ultrasonication (**Fig. 2**), application of low amplitude (5 µm-pp) during extraction exhibited a slower rate, due to the lower intensity of the cavitation applied. Medium amplitude (10 µm-pp) exhibited an almost linear extraction rate, reaching 80% (w/w) after 45 min of extraction. High ultrasonication amplitude (15 µm-pp) exhibited higher

extraction rate, with 77% (w/w) of the protein yield reached after 25 min and declined thereafter, improving the protein yield by only 7% (w/w). Worth mentioning is the fact that during extraction at high intensity, the extraction temperature rose to higher than 70°C (denaturation temperature for soy proteins), due to excess heat dissipated. As such, all subsequent structural properties were studied only for ultrasonicated samples at low and medium intensities (extraction temperature 60°C, for 50 min).

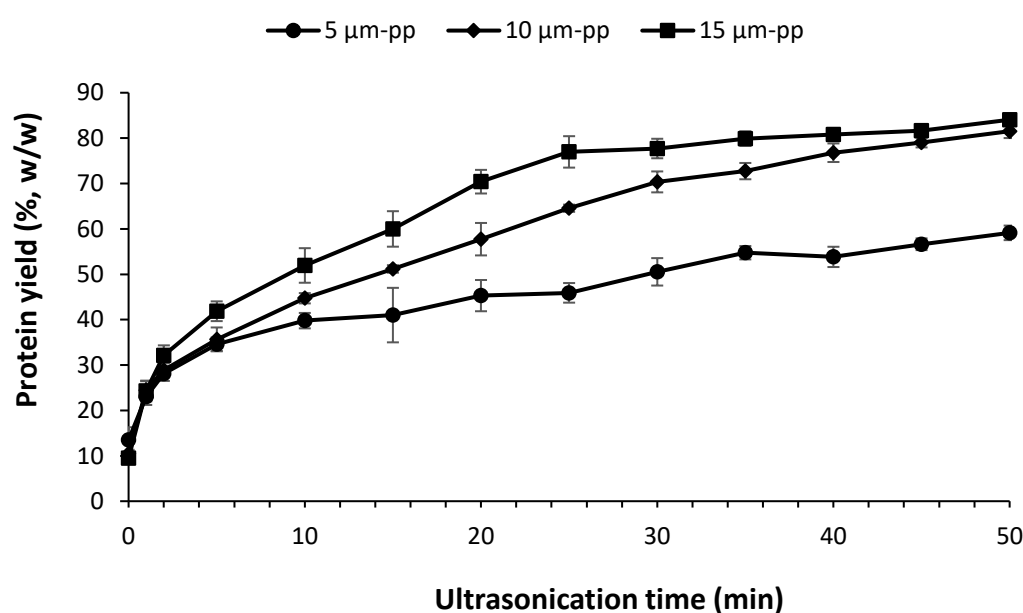


Fig. 2 Protein yield rate during ultrasonication-assisted extraction of okara at different amplitudes (5, 10 and 15 $\mu\text{m-pp}$). Values are mean \pm SD of triplicate extractions.

3.2 Amino acid composition of okara protein isolates (OPIs)

OPIs obtained by ultrasonication exhibited similar chemical composition, with high protein content (85.5%, w/w), and low amounts of carbohydrate (3.5%, w/w) and ash (3.8%, w/w). The amino acid composition of okara protein isolates (OPI) is shown in **Fig.3** and is compared to that of commercial soy protein (CSP). All OPI contained most of the essential amino acids such as valine, leucine, isoleucine, threonine, tyrosine, phenylalanine, and

histidine above the FAO scoring pattern (FAO/WHO, 1991). Like in other legumes, tryptophan and methionine were the limiting amino acids while glutamic acid and aspartic acid were the most predominant amino acids in all isolates. No significant changes were observed between the amino acid profile of OPIs obtained by ultrasonication at different intensities. Comparing amino acid profiles between commercial protein and ultrasonication derived isolates, it was noted that the content of hydrophilic amino acids dropped in ultrasonicated protein samples (~314 mg/g) compared to CSP (~350 mg/g), primarily due to the reduction of lysine and secondarily to glutamic acid (**Fig. 3**).

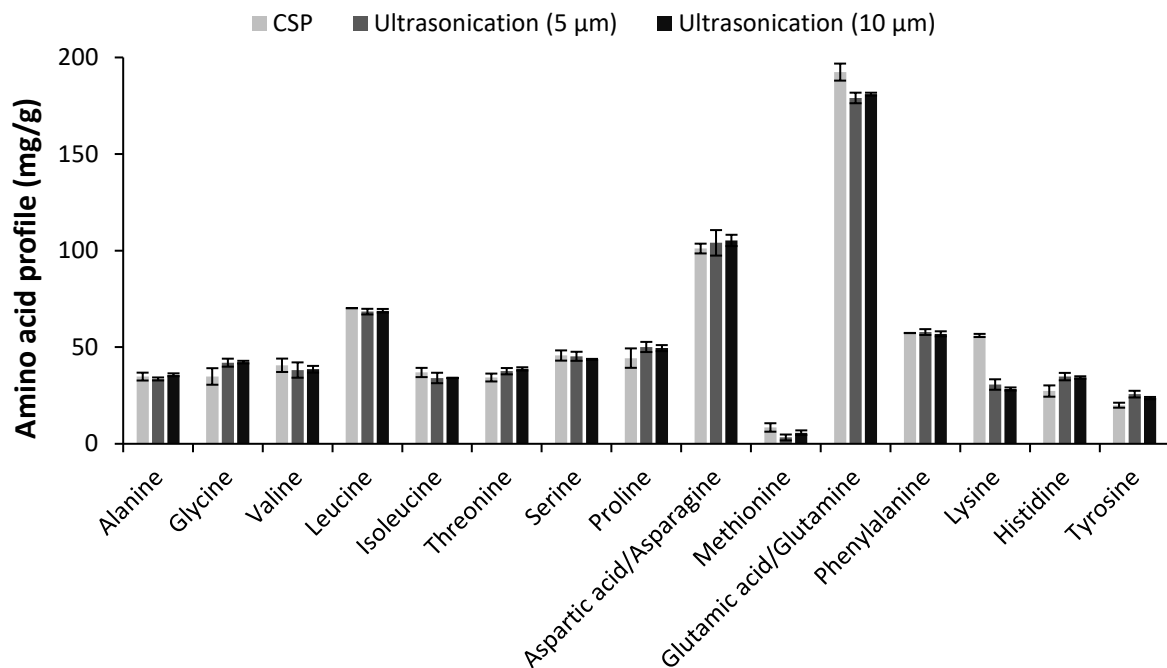


Fig. 3 Amino acid profile of commercial soy protein (CSP) and okara protein isolates (OPIs) extracted with ultrasonication (5 and 10 μm, pH 12). Values are mean ± SD of triplicate determinations.

3.4. Sulfhydryl content of okara protein isolates (OPI)

The free sulfhydryl (FSH), total sulfhydryl (TSH) and disulphide bond (S-S) contents of OPIs extracted using ultrasonication method are depicted in **Fig. 4** below. OPIs derived from ultrasonication had similar FSH values (92 and 94 $\mu\text{mol/g}$ respectively) ($p>0.05$), indicating similar levels of partial protein denaturation and oxidation of the SH groups, leading to aggregation reactions (Montero and Lopez-da Silva, 2018). However, total sulfhydryl content increased in ultrasonicated OPIs, mainly due to the increase of disulphide bond content, compared to CSP ($p<0.05$). The increase in total sulfhydryl contents and S-S bonds in ultrasonicated samples (**Fig. 4**) could be attributed to structural changes that might lead to dissociation from one form of sedimentation coefficient to another (such as from 11 S glycinin to 7 S β -conglycinin or vice versa). However, this could be referred to as cavitation induced conformation changes. Previous research has confirmed the possible shift from one sedimentation coefficient of soy globulins to another especially between the two major globulins (7 S and 11 S) caused by factors such as ionic strength and pH (Lakemond et al. 2000). The application of sonication (especially in high power) can generate more free SH groups and hydroxyl radicals in soy proteins, which react with themselves to convert into SS bonds and thus reduce the number of free SH groups (Rahman and Lamsal, 2021); this could explain our findings in terms of sulfhydryl content in okara protein isolates.

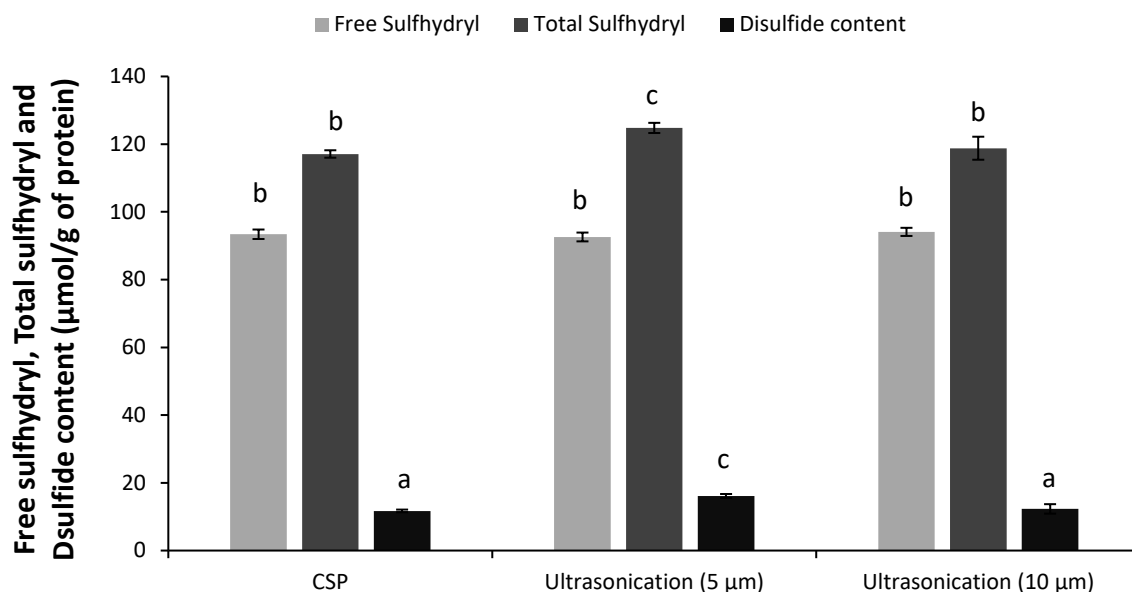


Fig. 4 Free sulfhydryl (FSH), total sulfhydryl (TSH) and disulphide bond (SS) contents of commercial soy protein (CSP) and OPI extracted by ultrasonication at 5 and 10 μm intensity. Means and standard deviation are from triplicate determinations; Means with different letters on the top of the bars with the same colour were significantly different ($p < 0.05$).

3.4 Fourier transform infrared (FTIR) spectroscopic analysis of okara protein isolates (OPIs)

The FTIR spectroscopic analysis of the OPIs obtained with ultrasound-assisted process was carried out and was compared to commercial soy protein samples. The region that is most sensitive to slight changes in the protein structure is that of 1600 – 1700 cm^{-1} frequency band. It is referred to as the amide I region and is associated to greater extent with C=O stretching vibration (70 – 85 %) and to a lesser extent with C-N groups (10-20%). Bands in this region typically overlap and in order to evaluate any changes in the secondary structural components of the protein samples, the original spectra were deconvoluted to the second derivative spectra.

Ultrasonication caused the β -sheet content to increase ($p<0.05$) compared to CSP, while α -helix and random coil decreased (**Table 1**). The reduction in the random coil that occurred in ultrasonication, suggested that the proteins reordered slightly to give rise to more α -helix structure. Ultrasonicated OPI samples exhibited higher amount of β -sheet than α -helix and β -turn, similar to other plant globulins such as buckwheat and rice globulins (Choi & Ma, 2005; Ellepola et al., 2005). Our results indicate the occurrence of decomposition of α -helices and random coils and subsequent conversion to β -sheets during ultrasonication, whereas the increase in the intensity of the ultrasonication conditions did not have a pronounced effect on structural changes. Since the secondary structure of protein depends on both the local sequence of amino acids and the interactions between different parts of a molecule (Montero and Lopez-da Silva, 2018), the above results indicated that cavitation as means of extraction could disrupt these interactions, leading to secondary structure changes. Worth mentioning is the fact that although literature suggests that ultrasonication treatment can alter the secondary structure of soy protein isolates, these changes vary due to different modification technologies, variations among commercial soy protein isolates as well as due to variations in protein fractions analysed (Hu et al., 2013; Yang et al., 2018).

Table 1 Secondary structure contents of commercial soy protein and OPI extracted via ultrasonication at different intensities

Sample	α -Helix (%)	β -Sheet (%)	β -Turn (%)	Random coil (%)
Commercial soy protein	25.8 \pm 0.2	31.0 \pm 0.4	19.2 \pm 0.1	11.3 \pm 0.3
Ultrasonication (5 μ m)	22.7 \pm 0.3	37.7 \pm 0.3	20.7 \pm 0.1	9.4 \pm 0.1
Ultrasonication (10 μ m)	23.0 \pm 0.2	38.2 \pm 0.2	19.5 \pm 0.0	9.8 \pm 0.2

From a functionality point of view, higher β -sheet content can improve the emulsion stability properties of proteins, as the β -sheet structure is considered more stable than the α -helix structure (Yang et al., 2018).

3.6 Zeta potential and particle size distribution of OPIs

Zeta potential is the difference that exist between the surface of a solid particle and the liquid where the particle is immersed. An emulsion can be electrically stable if it has high zeta potential (either positive or negative), provided it is further away from zero point, and unstable (tendency to coagulate or flocculate easily) if it has low zeta potential (Lu & Gao 2010). The pH of the medium is the most important factor that affect the zeta potential. The zeta-potential was carried out at pH 7, away from the isoelectric pH (pH 4) of soy proteins and the pH for most food applications. The values of the zeta potential of the CSP and OPIs obtained by alkaline extraction and ultrasonication are presented in **Fig. 5A** below. CSP had the lowest zeta value of -27.73 mV, comparable to the zeta value (-28 mV) obtained by Zhang et al. (2016) for the native soy protein isolate. Ultrasonication improved the zeta potential of the OPI from -38.8 (obtained with alkaline extraction) to -44.1 mV and or -42.8 mV, at low and medium intensity, respectively.

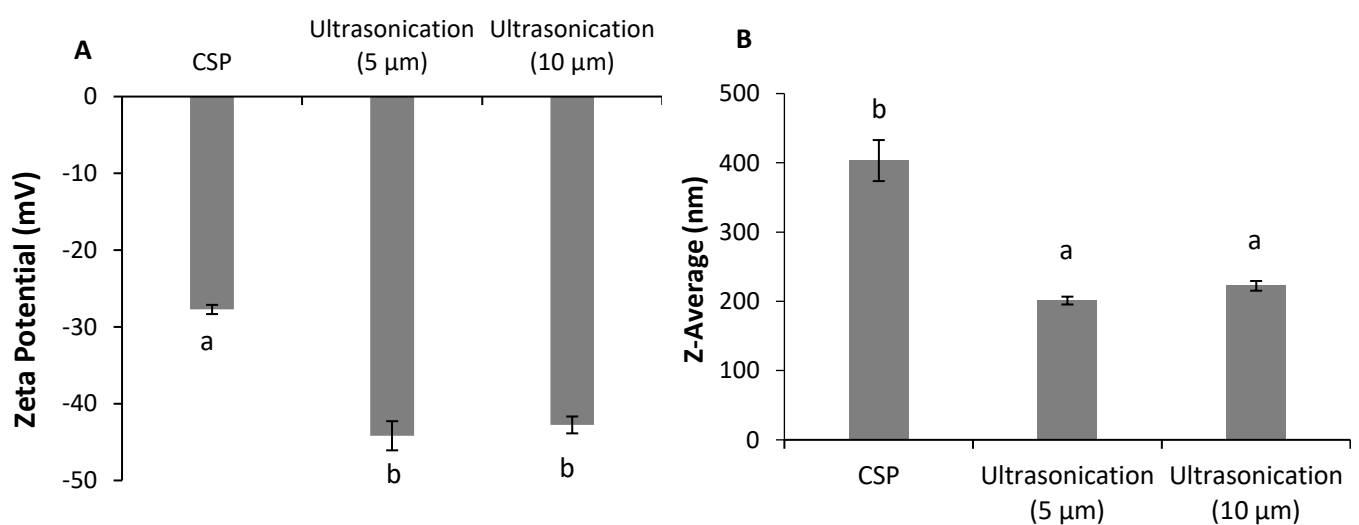


Figure 5 (A) Zeta potential and (B) Z-average diameter of commercial soy protein (CSP) and OPI extracted at different intensities (5 and 10 μm). Means and standard deviation are from triplicate determinations. Different letters denote significant difference ($p < 0.05$) as determined by Tukey HSD test ($p < 0.05$).

These results are linked with changes in the amino acid profile of ultrasonicated samples and more specifically with the decrease in lysine (positively charged amino acid), as well as with the increased β -sheet structure of ultrasonicated OPIs. The improvement of zeta potential indicates that ultrasonication-derived OPI would form emulsions and disperse with longer stability, which is a desired property in beverage formulations, salad dressings and mayonnaise formulations. Moreover, the particle size of the samples (**Fig. 5B**) further supports these findings. CSP had the highest particle size (403.3 nm), significantly different ($p < 0.05$) from the particle size of ultrasonicated OPIs (ranging from 201.1 nm – 222.4 nm) and this property could also contribute to lower zeta-potential of CPS samples.

The intensity of the ultrasonication applied in this study did not seem to affect the particle size of the protein isolates; however, cavitation has been reported to lead to smaller protein structures, as in our study, especially under low intensities (Tian et al., 2020; Bernardi et al., 2021). On the contrary, high ultrasonication intensity and extended extraction duration lead to increase in protein particle size, indicating the formation of small aggregates (Zhao et al., 2019).

4. Conclusion

Ultrasonication was proven an efficient tool for protein extraction from soybean residues. The cavitation process did not affect the macronutrient content of the OPI nor the amino acid profile of the proteins but caused alterations in their secondary structure and size. Structural changes in OPI samples were linked to cavitation effects and the duration of the extraction, rather than the intensity of ultrasonication process. The combination of increased β -sheet content, improved zeta-potential and smaller particle size of ultrasonicated proteins, could render them as suitable ingredients for food applications.

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