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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

37 ## 1. Introduction

38

39 Plant-based sources of protein are considered more sustainable for the increasing global
40 population compared to animal protein. Among all vegetable sources of protein, soybeans have
41 gained much popularity due to their rich content in all eight essential amino acids, as well as
42 exhibiting similar digestibility to that of cow's milk, meat and egg proteins (Soderberg, 2013).
43 Soybean seeds are principally used to produce soymilk and tofu. Soymilk is increasingly
44 becoming more popular by consumers, although, it is mostly consumed by lactose intolerant
45 individuals, and those conscious of cholesterol. Currently, the demand for soymilk is high with
46 the global soymilk market value amounted to about 15.33 billion US dollars in 2018 and
47 estimated to be 23.2 US dollars in 2025 (Statista, 2021a). In the UK, the sale volume of soybean
48 milk rose to 85.7 million litres in 2016 (AHDB, 2017) and to about 92.6 million litres in 2018
49 (Statista, 2021b). The main market drive for soymilk production and consumption is its
50 association with health benefits, especially following the FDA health claim approval on soy
51 protein effectiveness in reduction of coronary heart disease risk. Consequently, the
52 accumulation of soymilk by-product (okara) is expected to increase; it is estimated that for
53 every 1000 litre of soymilk produced via commercial process (the soya technology systems
54 process) or traditional process (cold extracted and no treatment to remove off-flavour), 250 kg
55 or 398 kg of okara are generated, respectively (Gavin & Wettstein, 1990). Okara contains
56 notable amounts of protein (26.8 %-37.5 % w/w) (Ma et al., 1997; Vishwanathan et al., 2011),
57 since a significant proportion is left in the residue following soymilk production (O'Toole,
58 1999) due to the complexity of soybean structure. However, this residue is scarcely utilised
59 and as such, it currently has little market value. Recently, okara has caught the interest of some
60 researchers for its potential application in the food industry, as a raw material for soy protein
61 isolate extraction, with potential applications in beef burger production, cookies, and sausage

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

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

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

87 extraction efficiency and at the same time reducing solvent and energy utilisation (Chemat et
88 al., 2017). These advantages seem to be more prominent on lab-scale studies, whereas for
89 industrial scale applications, further aspects of the process still need to be optimised (e.g.
90 reactor design, energy consumption reduction, solid-liquid separation post-extraction) to
91 establish ultrasonication's industrial prospects (Chemat et al. 2020; Preece et al 2017; Vernes
92 et al. 2019). The mechanism of extraction by ultrasonication is based on the cavitation
93 phenomenon which leads to particle or cell disintegration (Khanal et al., 2007). The
94 disintegration of cell walls by cavitation exposes hidden compounds in the cells to the
95 extracting medium, hence promoting higher extraction yields at shorter times (Mason et al.,
96 1996). However, this effect can alter the native conformational structure of proteins with
97 resultant changes in their functional properties (McClements, 1995). Various studies have
98 reported the exposure of hydrophobic groups and redistribution of the secondary structure (Li
99 et al., 2016), as well as promoting the unfolding and dissociation of protein isolates extracted
100 via ultrasonication (Huang et al., 2017). As such, ultrasonication can employed not only for
101 extraction processes but also for the enhancement of functional properties of proteins.

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

108

109 **2. Materials and methods**

110 **2.1 Raw material and chemical reagents**

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

115

116 **2.2 Preparation of defatted okara flour**

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

124

125 **2.3 Alkaline extraction of okara proteins**

126 Defatted okara flour was mixed with 0.1 M phosphate buffer (pH 9-12) (Fisher
127 Scientific, UK) in ratio of 1:20 (w/v). The sample slurry was placed in a 60 °C water bath with
128 stirring for 1 h. The protein extract/supernatant was recovered by vacuum filtration using
129 Buchner funnel and proteins were precipitated at pH 4.6 with 2 N HCl (Fisher Scientific, UK)
130 and left at 4 °C overnight to enhance the precipitation. The precipitate was recovered after
131 centrifugation at 10,000 × *g* for 10 min. The obtained protein pellet was washed twice and re-
132 dissolved in distilled water (Purite reserve osmosis system, Oxon, UK), its pH was adjusted to
133 7.0 using 2 N NaOH (Fisher Scientific, UK) and was freeze dried to obtain the protein isolate
134 (Virtis 2KBTES, Warminster, Pennsylvania).

135

136 **2.4 Ultrasonication-assisted alkaline extraction of okara proteins**

137 The ultrasonic system used was a high intensity ultrasonic process system (model:
138 P100/6-20) with typical titanium process horn configuration which operates at a nominal
139 frequency of 20 KHz. The system was submerged to a depth of 1-2 cm in the sample. The
140 ultrasonicator maximum amplitude and power were 16 μm-pp and 100 W, respectively.
141 Defatted milled okara flour was mixed with 0.1 M phosphate buffer (pH 12) (Fisher Scientific,
142 UK) in the ratio of 1:20 (w/v); the slurry was transferred into a double walled flow cell for
143 ultrasonication extraction (total volume 70 mL, Celbius Ltd., UK) through which water was
144 circulated to maintain the temperature of extraction constant (60° C). Three different sonication
145 amplitudes were monitored representing intensities, and these are referred to as low (5 μm-pp),
146 medium (10 μm-pp) and high (15 μm-pp) amplitude. The extraction was carried out for 50 min
147 under continuous pulse mode. The obtained slurry was centrifuged at 10,000 × g for 10 min at
148 4 ^{o}C. The pH of the supernatant was reduced to 4.5 using 2 N HCl (Fisher Scientific, UK) to
149 isolate the proteins, refrigerated at 4 ^{o}C overnight, and centrifuged at 10,000 × g for 10 min at
150 4 ^{o}C to recover the isolated protein precipitate. The precipitate was washed twice with distilled
151 water, centrifuged after each washing at the same conditions and was dispersed in distilled
152 water with its pH adjusted to 7.0 with 2 N NaOH (Fisher Scientific, UK). The neutralized
153 protein was frozen and then freeze-dried (Virtis 2KBTES, Warminster, Pennsylvania), ground
154 and packaged in an air-tight container and stored in a desiccator until further use.

155

156 **2.5 Determination of protein content in okara extracts**

157 The protein content of okara extracts was measured according to Bradford (Bradford,
158 1976). An aliquot of 1.5 mL of the Bradford reagent (Sigma-Aldrich, UK) was mixed with 50
159 μL of appropriately diluted protein solution (based on the concentration of the protein solution)

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

164

165 $$\text{Protein yield (\%, w/w)} = \frac{protein\ concentration\ in\ the\ extract}{protein\ concentration\ in\ the\ defatted\ okara} \times 100 \qquad \text{(Eq. 1)}$$

166

167 **2.6 Chemical composition determination of okara protein isolates**

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

171

172 **2.7 Amino acid profile determination of protein isolates**

173 Lyophilised protein isolates (0.1 g) were mixed with 6 M HCl (Fisher Scientific, UK)
174 in a sealed container and nitrogen was flushed into it to prevent oxidation reactions and then
175 the suspension was hydrolysed for 24 hours at 110 ^{o}C. The hydrolysate was neutralised with
176 $CaCO_3$ powder (Sigma-Aldrich, UK) prior to derivatization. The neutralised hydrolysate was
177 analysed for amino acid content using the EZ-Faast amino acid derivatization kit for GC-MS
178 (Phenomenex, Torrance, CA) (Hus ̌ek 2000). The derivatization was carried out on 100 µL of
179 each sample using the EZ-Faast amino acid analysis kit for free amino acid analysis by GC-
180 MS. The same procedure was carried out on the standards. The derivatised amino acids were
181 analysed in electron impact mode using Agilent 5975 system (Agilent, Palo Alto, CA). An
182 aliquot (1 µL) of the derivatised amino acid solution was injected in split mode (40:1) at 280
183 ^{o}C onto a zebron ZB-AAA capillary column (10 m × 0.25 mm; 0.25 µm film thickness), with

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

189

190 **2.8 Fourier transform infrared spectroscopic analysis (FTIR)**

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

199

200 **2.9 Sulfhydryl content of protein isolates**

201 Total and free sulfhydryl content of okara protein isolates and the commercial soybean
202 protein isolate was determined using the modified method of Tang et al (2009). In brief, free
203 sulfhydryl (FSH) content was determined by dissolving 50 mg of protein isolate samples in 10
204 mL of Tris-glycine buffer (86 mM Tris, 90 mM glycine, 4 mM ethylenediaminetetraacetic acid,
205 pH 8.5) (Sigma-Aldrich, UK) containing 8 M urea (Sigma-Aldrich, UK) and then kept
206 overnight at room temperature with gentle mixing using an orbital shaker. The protein solution
207 was centrifuged in 50 mL centrifuge tube at 10 000 *x* g for 10 min at room temperature and the
208 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 µL 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 µL 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 × *g* for 10 min at 4 ^{o}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 x 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 µL 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):

234 $$SS\ (\mu mol/g) = \frac{(TSH - FSH)}{2} \qquad \text{Eq. 2}$$

235 All samples were analysed in triplicate.

236

237 **2.10 Zeta potential and particle size distribution of protein isolates**

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

252

253 **2.11 Statistical analysis**

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

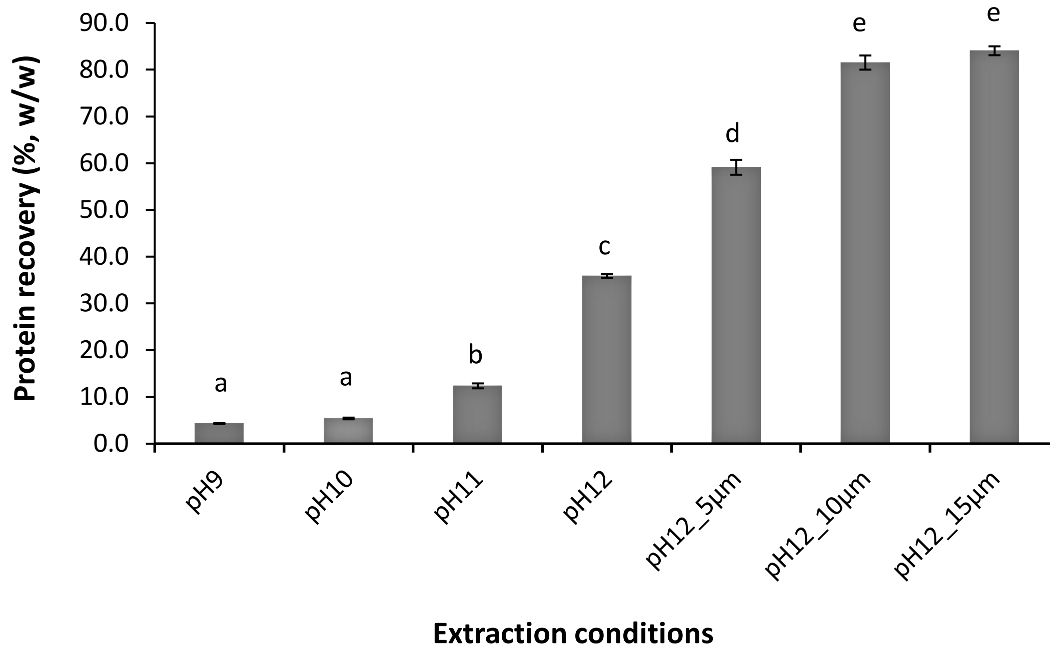
258

259 **3. Results and discussion**

260 **3.1 Protein extraction from okara**

261 Initially, aqueous extractions at varying pH values were carried out to investigate the
262 influence of pH on protein recovery from okara. A range of alkaline pH values was studied
263 (from 9 to 12) using 0.1 M phosphate buffer. The protein recovery during conventional aqueous
264 alkaline extractions of defatted okara at different pH values is shown in **Fig.1**. Protein recovery
265 in phosphate buffer ranged from 4.3 % (w/w) at pH 9 to 35.9 % (w/w) at pH 12 ($p<0.05$). The
266 low protein recovery at pH values between 9 and 11 indicated that phosphate buffer had weak
267 buffer capacity within that pH range, while pH 12 exhibited a stronger buffer capacity, having
268 with pKa at pH 12.3. These findings are also in agreement with the report of other researchers
269 indicating that high pH can increase protein solubility and extractability in okara (Ma et al.,
270 1997). Karki et al. (2010) reported lower protein yields of about 27 % (w/w), using aqueous-
271 alkaline extraction at pH of 9. The low extractability of soybean protein by conventional
272 methods could be as a result of the complex nature of soybean cell wall matrix. The major
273 soybean proteins co-exist with other proteins and non-protein components which result into
274 protein-protein interactions or protein-carbohydrate interactions that may hinder protein
275 solubility in aqueous alkaline media. Repeated extractions with fresh media at high temperature
276 of 80 ^{o}C has been shown to enhance the protein extraction yield (Vishwanathan et al., 2011) as
277 it may assist in overcoming issues related to solvent saturation. However, there some
278 limitations to this method including accumulation of more wastewater, long processing time,
279 higher energy utilisation (higher temperature of 80 ^{o}C). To overcome these limitations and still
280 achieve high protein recovery, ultrasonication method was assessed as an alternative process
281 for protein extraction.

282



283

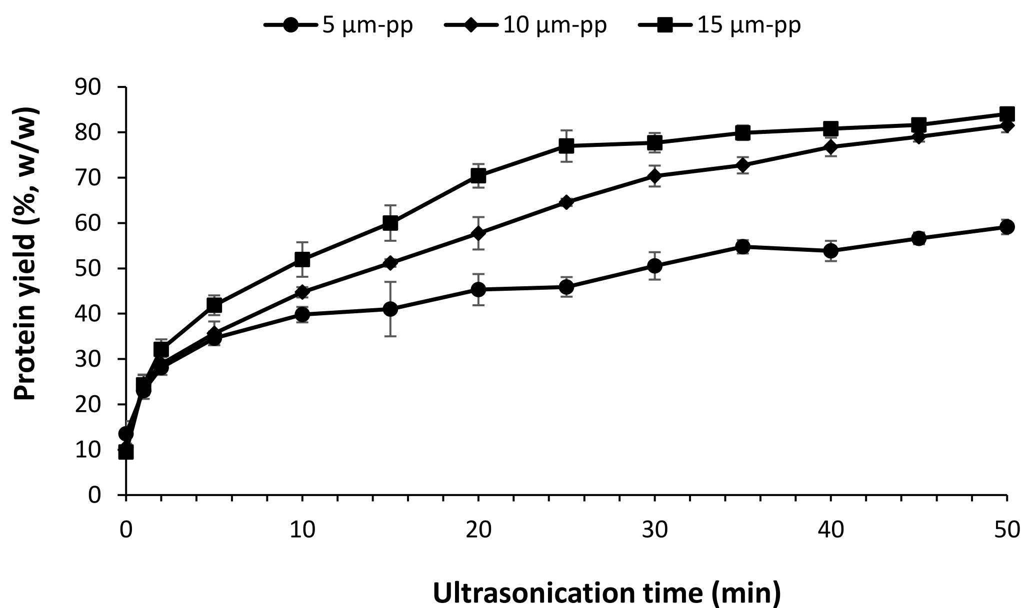
284 **Fig.1.** Protein recovery (% w/w) from defatted okara using 0.1 M phosphate buffer at different
285 pH values (9, 10, 11 and 12), and ultrasonication-assisted extraction (5 µm, 10 µm and 15 µm
286 amplitude using 0.1 M phosphate buffer pH 12). Significant difference exists between bars
287 with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

289

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

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

305



306

307 **Fig. 2** Protein yield rate during ultrasonication-assisted extraction of okara at different
308 amplitudes (5, 10 and 15 µm-pp). Values are mean ± SD of triplicate extractions.

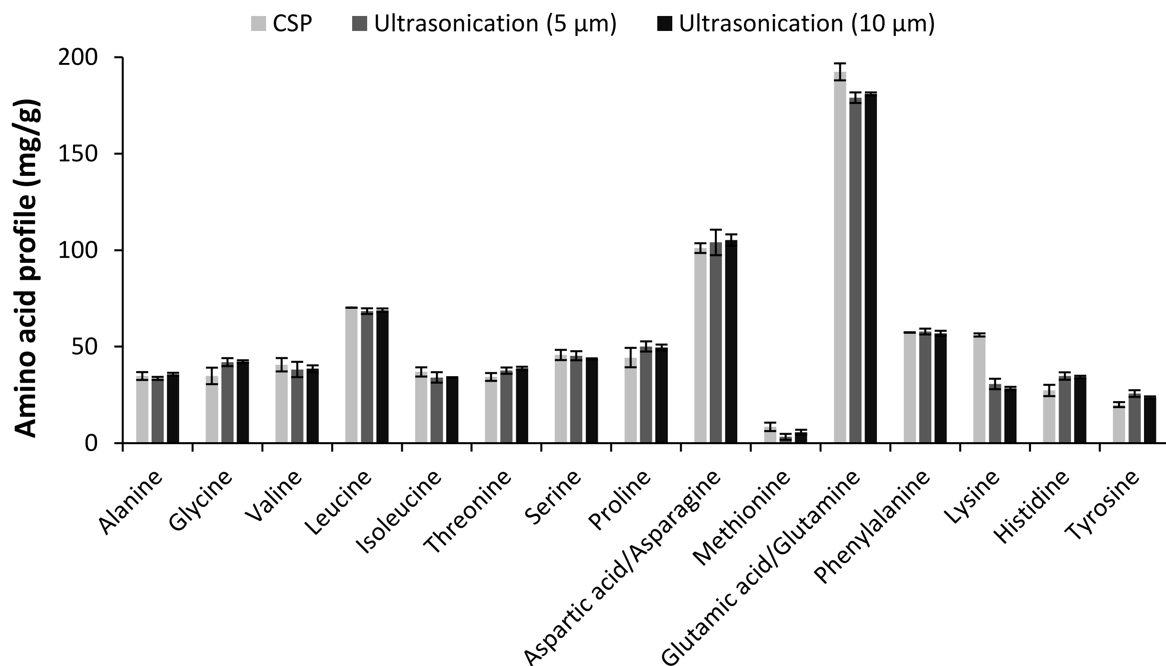
309

310 **3.2 Amino acid composition of okara protein isolates (OPIs)**

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

316 histidine above the FAO scoring pattern (FAO/WHO, 1991). Like in other legumes, tryptophan
317 and methionine were the limiting amino acids while glutamic acid and aspartic acid were the
318 most predominant amino acids in all isolates. No significant changes were observed between
319 the amino acid profile of OPIs obtained by ultrasonication at different intensities. Comparing
320 amino acid profiles between commercial protein and ultrasonication derived isolates, in was
321 noted that the content of hydrophilic amino acids dropped in ultrasonicated protein samples
322 (~314 mg/g) compared to CSP (~350 mg/g), primarily due to the reduction of lysine and
323 secondarily to glutamic acid (**Fig. 3**).

324



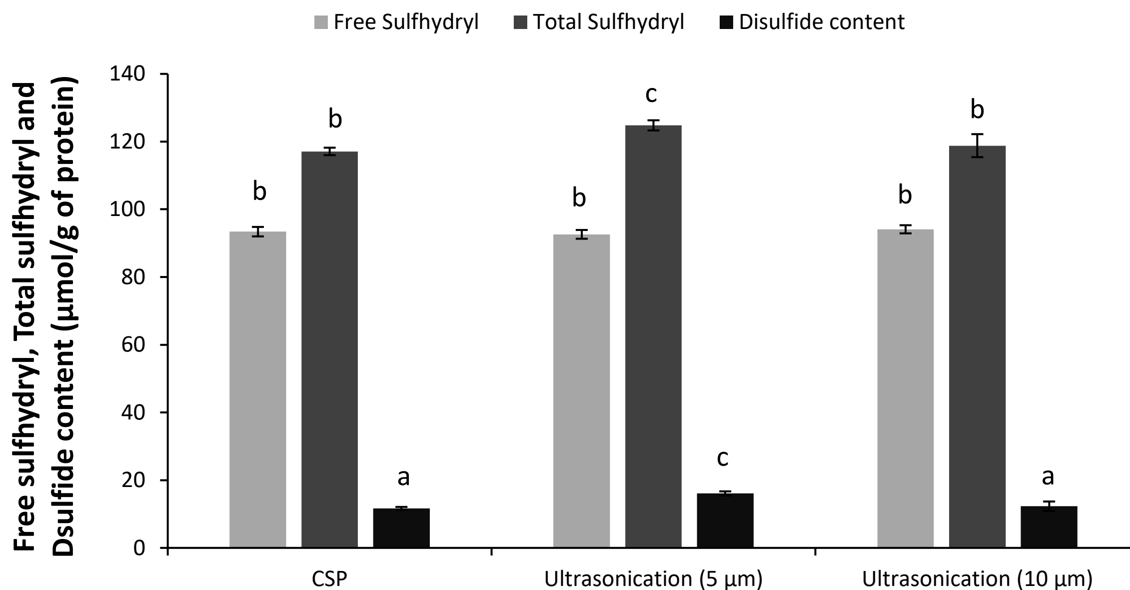
325

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

329

330 **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 μmol/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 hydroxyls 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.



349

350

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

355

356

357 3.4 Fourier transform infrared (FTIR) spectroscopic analysis of okara protein isolates 358 (OPIs)

359 The FTIR spectroscopic analysis of the OPIs obtained with ultrasound-assisted process
360 was carried out and was compared to commercial soy protein samples. The region that is most
361 sensitive to slight changes in the protein structure is that of 1600 – 1700 cm^{-1} frequency band.
362 It is referred to as the amide I region and is associated to greater extent with C=O stretching
363 vibration (70 – 85 %) and to a lesser extent with C-N groups (10-20%). Bands in this region
364 typically overlap and in order to evaluate any changes in the secondary structural components
365 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).

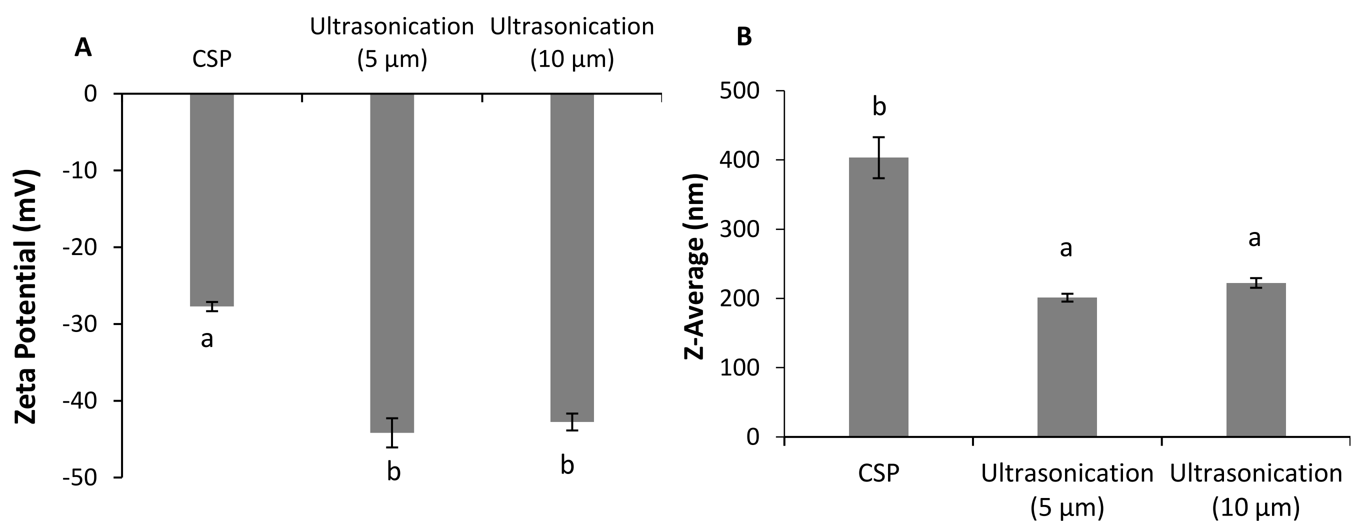
Table 1 Secondary structure contents of commercial soy protein and OPI extracted via ultrasonication at different intensities

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

386 From a functionality point of view, higher β-sheet content can improve the emulsion stability
387 properties of proteins, as the β-sheet structure is considered more stable than the α-helix
388 structure (Yang et al., 2018).
389

390 3.6 Zeta potential and particle size distribution of OPIs

391 Zeta potential is the difference that exist between the surface of a solid particle and the liquid
392 where the particle is immersed. An emulsion can be electrically stable if it has high zeta
393 potential (either positive or negative), provided it is further away from zero point, and unstable
394 (tendency to coagulate or flocculate easily) if it has low zeta potential (Lu & Gao 2010). The
395 pH of the medium is the most important factor that affect the zeta potential. The zeta-potential
396 was carried out at pH 7, away from the isoelectric pH (pH 4) of soy proteins and the pH for
397 most food applications. The values of the zeta potential of the CSP and OPIs obtained by
398 alkaline extraction and ultrasonication are presented in **Fig. 5A** below. CSP had the lowest zeta
399 value of -27.73 mV, comparable to the zeta value (-28 mV) obtained by Zhang et al. (2016) for
400 the native soy protein isolate. Ultrasonication improved the zeta potential of the OPI from -
401 38.8 (obtained with alkaline extraction) to -44.1 mV and or -42.8 mV, at low and medium
402 intensity, respectively.



403

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

408

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

418 The intensity of the ultrasonication applied in this study did not seem to affect the particle size
419 of the protein isolates; however, cavitation has been reported to lead to smaller protein
420 structures, as in our study, especially under low intensities (Tian et al., 2020; Bernardi et al.,
421 2021). On the contrary, high ultrasonication intensity and extended extraction duration lead to
422 increase in protein particle size, indicating the formation of small aggregates (Zhao et al.,
423 2019).

424

425 **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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