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Effect of Ultrasound-assisted Enzymolysis on Jackfruit (*Artocarpus heterophyllus*) Seed Proteins: Structural Characteristics, Technofunctional Properties and the Correlation to Enzymolysis

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Abstract

The aim of this study was to evaluate the effect of Ultrasound-Assisted Enzymolysis (UAE) on the techno-functional properties and structure of Jackfruit Seed Protein (JSP). Before the hydrolysis by alcalase (60 min), protein solutions (10%, w/v) were exposed to ultrasound pretreatment (200 W, 400 W, 600 W for 15 min and 30 min). Compared with the control JSP, UAE improved the proteolysis process, as confirmed by an increase in the degree of hydrolysis (DH; $p<0.05$), as well as the Oil Holding Capacity (OHC) and emulsifying stability (ES). Moreover, the UAE treatment increased the protein solubility (PS), while the Least Gelation Concentration (LGC) did not exhibit significant changes. Scanning Electron Microscopy (SEM) demonstrated that UAE disrupted the microstructure of the JSP, exhibiting larger aggregates in comparison with control JSP. Fourier transform infrared (FT-IR) spectra indicated that the UAE treatments induced molecular unfolding by increasing the α-helix, β-turn and random coil content, as demonstrated by increased surface hydrophobicity (H_0-ANS). The knowledge of this study could be selectively employed in the food industry for the development of conventional or novel foods based on jackfruit seed protein.

Keywords: Jackfruit seed protein; Ultrasound-assisted enzymolysis; Technofunctional properties; Structural properties; Surface hydrophobicity

Introduction

Proteins are the primary constituents of agricultural raw materials with two main (complimentary) functions: bio-and techno function [1]. Bio functionality of proteins is related to their nutritional and physiological properties, while techno-functionality is related to their physicochemical properties affecting appearance, texture, and stability of food products (e.g., solubility, viscosity, foaming, emulsifying and gelling ability, fat absorption capacity) [2].

In the last years, there has been increasing demand of protein sources, mainly those that are of high nutritional value, adequate functionality, and low cost and that could be used as functional ingredients in the food industry [3]. A great opportunity to recovery proteins of nonconventional sources for human consumption could be the seeds proceedings of fruit processing. An example of these is the jackfruit seeds, a byproduct from the dehydration process of edible jackfruit bulbs in Nayarit, Mexico. Jackfruit seeds are a good source of starch (22%), dietary fiber (3.19%) [4] and protein with content that is 17.8-37% depending on the variety of jackfruit [5]. In addition, the proteins of jackfruit seed have a good balance of essential amino acids considering the amino acids requirements of FAO/WHO [4].

Another important reason for the search of nonconventional protein is the need for renewable and sustainable sources of proteins and the emerging dietary preferences (e.g., vegans, vegetarians) that demand novel food ingredients and plant-based products [6]. On the other hand, protein-rich fractions, protein isolates, and concentrates offer interesting functional properties, which are usually superior to those of the flour source [7-9]. In that sense, the food industry has a growing interest in producing plant protein isolates not only due to their increasing use as food functional additives but also because they may also improve nutritive quality and functional properties of food products [9]. Hence, jackfruit seeds could be used as a nonconventional source of protein in the food industry.

To improve the functional properties of proteins, enzymatic hydrolysis is usually applied [10-12]. However, traditional enzymolysis has many disadvantages such as a low degree of hydrolysis and long enzymolysis time [13]. To overcome these drawbacks of conventional enzymatic hydrolysis method, many eco-innovative technologies such as microwave radiation assisted technology, ultrahigh pressure assisted technology and ultrasound-assisted technology has been applied [14]. According to the above, ultrasound technology, as a new nonthermal physical processing technology, has been widely applied in the food industry, especially in extraction [15] and enzymatic treatment [16]. Ultrasound pretreatment has been successfully used to improve bioactive peptides release and the enzymolysis efficiency of protein [16] such as alcalase based enzymolysis of wheat germ protein [12], corn protein [2] and wheat gluten [17]. In this context, because the jackfruit protein is mainly composed by glutelin (∑ 70%), which is alkali-soluble

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[4], ultrasound treatment prior to enzymolysis can improve mass transfer and increase the frequency of contact between the substrate and the enzyme [18]. Thus, we hypothesized that the UAE with alcalase can improve the techno-functional properties of the jackfruit seed proteins. Moreover, such treatment can change the secondary and microstructure of proteins [16,19]. In this regard, Fourier transforms infrared spectroscopy (FT-IR) is well suited to detect relative changes in protein secondary structure due to external factors, by analyzing the amide I band of proteins between 1700 cm^{-1} and 1600 cm^{-1} [20]. This band is influenced by hydrogen bonds, which are mainly affected during conformational changes in protein secondary structure (α-helix, β-sheet, β-turn, and random coil) [21]. FT-IR has been applied in the determination of structural changes of corn gluten meal hydrolysates [2], beef proteins [22] and rice protein [11]. Nevertheless, no research has been done on the effect of the UAE and its relationship between the techno-functional properties and the changes structural of jackfruit seed protein.

Therefore, the aims of this study were (i) to investigate the structural changes of jackfruit seed protein after UAE, including the comparison of degree of hydrolysis (DH), protein solubility (PS), microstructure (SEM), surface hydrophobicity (H_0), techno-functional properties and secondary structure (FT-IR); (ii) to study the relationships among H_0-ANS, PS, DH, techno-functional properties and secondary structural elements of the hydrolysates. It is also hoped that the results of this research will be of great value to support the use of jackfruit seed protein as a novel ingredient for the food industry.

Materials and Methods

Materials

The jackfruit seeds were provided by Mexican Tropical Organics S. de R.L. de C.V. located at Carretera Los Cocos-Aticama s/n San Blas, Nayarit (México). Alcalase 2.4 L with an activity of 2.4 AU/g was purchased from Novozymes Co., LTD (Tianjing, China). The Bradford reagent, 2-4-6-trinitrobenzenesulfonic acid solution (TNBS; 5%) and 1-anilino-8-naphthalene-sulfonate (ANS) were purchased from Sigma-Aldrich Corp (St. Louis, Missouri, USA). Other chemicals and solvents used in the experiment were analytical grades. All solutions were prepared with distilled deionized water.

Preparation of the jackfruit protein

The jackfruit seeds were milled using a hammer mill to pass through a 1 cm sieve and then dried in a cabinet dryer (35°C; 3.0 m/s) until they reached a constant weight. Dry seed pieces were pulverized in a mill (Cyclotec Mod. 1093, Foss Tecator, Slangerupgade, Denmark) equipped with 0.8 mm mesh. The Jackfruit Seed Flour (JSF) was sieved (150 μm) and then collected and stored in a polyethylene bag (20°C) prior to analysis. Subsequently, the slurry was prepared by mixing JSF in distilled water at a ratio of 1:20 and adjusting the pH to 12.0 using 1 M NaOH. The slurry was stirred for 30 min at 25°C and then centrifuged at 2875 g for 30 min. The pH of the supernatant was adjusted to a pH of 4.0 using 1.0 M HCl, and the slurry was stirred for 20 min at 25°C. The precipitate was separated by centrifugation at 2875 g for 20 min at 25°C. The protein precipitate was then subjected to an alcoholic extraction using 96% ethanol at a ratio of 1:2 (protein precipitate:ethanol). The protein precipitate was subsequently stirred for 10 min and separated by centrifugation at 2875 g for 10 min at 25°C. Finally, the precipitate was washed with ethyl ether (1:4; p/v) by vacuum filtration and dried (35°C; 1.5 m/s) to a constant weight, obtaining a powder of jackfruit seed protein isolate (JSPI) that was characterized according to AOAC methods [23]. The protein, fat, and ash contents were 69.62% (N × 6.25), 0.72%, and 2.34%, respectively. The percentage of carbohydrates (27.32%) was determined by the difference following the method of Lima et al. [24].

Ultrasound treatment of samples

Prior to enzymatic hydrolysis, the JSPI was pretreated by ultrasound. JSPI dispersions (10%, w/v) were prepared by adding JSPI into distilled water, gently stirring for 30 min, and adjusting to a pH of 12 using 1 M NaOH. An ultrasound processor (Model CPX750, Cole-Parmer Instruments, Vernon Hills, Illinois, U.S.A.) equipped with a 2.54 cm diameter titanium probe was used to sonicate 500 mL of the JSPI dispersions in a 1000 mL glass beaker. The solution was placed in an ice-water bath for 15 min, maintained at a temperature below 15°C, and treated at 20 kHz with power output levels of 0 W, 200 W, 400 W, and 600 W for 15 min and 30 min (pulse duration: on-time, 5 s; off-time 1 s). The final temperature of the ultrasound process was 15 ± 2°C, 23 ± 1°C and 26 ± 1°C for the High-Intensity Ultrasound (HIU) application of 200 W, 400 W, and 600 W, respectively. After ultrasound treatment, all samples were centrifuged (2875 g for 30 min). Then, the JSPI suspensions with HIU pretreatment were lyophilized and stored at room temperature in airtight containers until its use for the treatment of enzymatic hydrolysis. The ultrasonic intensity was measured by calorimetry using a thermocouple (Cole-Parmer Instruments, 04711-50, Vernon Hills, Illinois, U.S.A.) and expressed in W cm^{-2}. Using ultrasonic treatment with the 20 kHz probe at a power output of 200 W (15 and 30 min), 400 W (15 and 30 min) and 600 W (15 and 30 min), the ultrasonic intensity was 40 ± 9 W cm^{-2}, 62 ± 2 W cm^{-2} and 112 ± 1 W cm^{-2}, respectively.

Enzymatic hydrolysis

The enzymatic hydrolysis was performed according to the methods by Zhang et al. [17] with some modifications. Batches (0.3 L) of JSPI suspensions (5%; w/v) with HIU pretreatment and of a control (JSPI without HIU pretreatment) were adjusted to pH 8.0 using 1 M NaOH, and 0.075 mL of alcalase were added to the protein suspensions at an enzyme-substrate ratio [E/S] of 1:200. The enzymatic hydrolysis was performed in a thermostatic bath with agitation (Boekel Scientific, 290400, Feasterville, PA, USA) at 50°C and 80 rpm. After 60 min of reaction, the hydrolysates were boiled for 10 min to terminate the reaction. Next, the hydrolysates were stored at -20°C for further analysis.

Degree of hydrolysis (DH)

The DH, expressed as the percentage of free amino groups, was determined in triplicate using the trinitrobenzene sulfonic (TNBS) method [25] as described by Connolly et al. [26] with modifications. Samples (5%; w/v) and leucine standard solutions were prepared in duplicate aliquots (0.064 mL) and which were added to test tubes containing 1.0 mL of sodium phosphate buffer (0.2125 M; pH 8.2). TNBS reagent (0.250 mL) was then added to each tube followed by mixing and incubation at 50°C for 30 min in a covered water bath. After incubation, the reaction was stopped by the addition of 0.1 M sodium sulfite (1 mL) to each tube. The samples were then allowed to cool at room temperature for 15 min, and the absorbance values were measured at 420 nm.

The DH was calculated according to the following equation:

$$DH\ (\%) = \frac{\alpha N_t - \alpha N_0}{h_{tot} - \alpha N_0} \times 100 \quad (1)$$

where αN_t is the degree of dissociation of α-NH_2 groups at a given

time, αN_0 is the degree of dissociation of α-NH_2 groups at time 0, and h_{tot} is the total number of peptide bonds in the protein substrate. Total hydrolysis was performed by adding 0.5 mL of protein suspension (5%; w/v) to a 50 mL tube, adding 4.5 mL of 6N HCl, sealing under vacuum, and heating to 110°C for 24 h. The hydrolysis was stopped by adding 4.5 mL of 6 N NaOH. The suspension was filtered, and the α-NH_2 groups were determined as described previously.

Fourier transforms infrared spectra (FT-IR) measurement

The FT-IR spectra of the JSPI samples were scanned in the wavenumber range from 4000 cm^{-1} to 515 cm^{-1} using a Perkin-Elmer FT-IR spectrometer (LR-64912C, PerkinElmer, Inc. Norwalk, CT, USA) at room temperature (25°C). The spectra were an average of 28 scans. The data transformation, deconvolution and peak-separation analysis of the amide I band (1700 cm^{-1} to 1600 cm^{-1}) were processed using the OriginPro8 software (OriginLab Corporation, Northampton, MA 01060, USA).

Surface hydrophobicity (H_0-ANS) measurements

The H_0-ANS was determined using 1-anilino-8-naphthalene-sulfonate (ANS) as a fluorescence probe according to the method given by Kato et al. [27] as described by Jiang et al. [28] with modifications. UAE-treated and control protein dispersions (1.5 mg/mL in 0.01 M phosphate buffer at pH 9.0) were centrifuged at 8,000 g at 17°C for 20 min. After determining the protein concentration in the supernatants according to the method given by Bradford [29], each supernatant was serially diluted with the same buffer to obtain protein concentrations ranging from 0.05 to 0.0001 mg/mL. Then, 25 µL of ANS (8.0 mM in 0.01 M phosphate buffer, pH 9.0) was added to 2 mL of sample. The fluorescence intensity (FI) was measured with a fluorescence spectrophotometer (Tecan Infinite 200 Pro, Grödig, Austria) at wavelengths of 364 nm (excitation) and 475 nm (emission). The initial slope of the FI versus protein concentration (mg/mL) (calculated by linear regression analysis) was used as an index of the protein H_0-ANS. All determinations were performed in triplicate.

Scanning electron microscopy (SEM)

The microstructure of the freeze-dried JSPI samples was observed with an SEM (SEC, Mini-SEM SNE-3200 M, South Korea) at an accelerating voltage of 30 kV. Before using the SEM, the samples were coated with gold using an ion sputter coater (MCM-100, SEC).

Protein solubility measurement (PS)

For PS measurement, 60 mg of the protein sample was mixed with 40 mL 0.01 M phosphate buffer solution (pH 9.0). The solution was stirred for 60 min and then centrifuged at 8,000 g for 20 min at 17°C. The protein content in the supernatant was measured using the Bradford method [29], and bovine serum albumin was used as the standard. PS-Bradford was expressed as mg/mL.

Techno-functional properties

Water holding capacity (WHC) and oil holding capacity (OHC): A previous procedure was used with slight modification to determine the WHC and OHC [30]. Duplicate samples (1 g) were rehydrated with 10 mL of distilled water in centrifuge tubes (15 mL) and dispersed with a vortex mixer for 30 s. The dispersion was allowed to stand at room temperature for 15 min and was then centrifuged at 1238 g for 10 min. The supernatant was decanted, and the residue was weighed together with the centrifuge tube. The WHC was expressed as g of water held per g of sample. An identical method was used to measure corn oil holding capacity, and the OHC was expressed as g of oil held per g of protein.

Emulsifying activity (EA) and emulsion stability (ES): A modified version of the method described by Ulloa et al. [31] was used to determine the EA and ES of the JSPI. Suspensions were prepared by dissolving 1 g of JSPI sample in 15 mL of 0.01 M phosphate buffer solution (pH 7.0). Subsequently, 15 mL of corn oil was added to each suspension. Each mixture was stirred in a Tissue-Tearor Homogenizer (Model 985370-07, Biospec Products, Inc.) at speed setting of 20 for 1 min and centrifuged at 198 g for 5 min. The emulsion layer volume was recorded. The emulsifying activity (EA) was calculated as:

EA(%) = (height of emulsigfied layer/height of total content in tube × 100)

Finally, to determine emulsion stability, the samples were heated at 80°C for 30 min in a water bath, cooled to 25°C in running water and centrifuged as described above. The emulsion stability was expressed as the percentage of emulsifying activity remaining after heating.

Least gelation concentration (LGC): LGC was determined using 2, 4, 6, 8, 10, and 12 g/100 mL JSPI dispersions for each JSPI sample in centrifuge tubes. The pH of the dispersions was adjusted to 4. The samples were heated for 1 h in a boiling water bath, cooled rapidly under running tap water and further cooled for 2 h at 4°C. The LGC is the minimum concentration at which the cooked and subsequently cooled sample from the inverted centrifuge tube did not fall or slip from the wall of the tube [30].

Statistical analysis

Statistical analysis was performed using Statgraphics Centurion Software version XV (Statpoint Technologies, Inc. Virginia, USA). All data are shown as the mean ± standard deviation (SD). Duncan's test was used to test for significant differences between the groups analyzed, and the differences were considered to be significant at $p<0.05$ or $p<0.01$. Pearson correlation analysis was conducted to evaluate relationships between structural characteristics, DH and techno-functional properties, and $p<0.05$ or $p<0.01$ were regarded as statistically significant.

Results and Discussion

Degree of hydrolysis (DH) and protein solubility (PS)

The DH gives an initial indication of a change in the molecular integrity, and thus, large complex structured protein molecules are broken down into smaller sized peptides and specific amino acids [10]. The results of the DH of JSPI after ultrasonic treatment at different power (200 W, 400 W, 600 W) and time (15 min and 30 min) combinations are shown in Figure 1. As shown in Figure 1, JSPI-UAE (200 W to 600 W, 15 or 30 min) increased the DH significantly ($p<0.05$) when compared to the enzymolysis sample. The DH increased from an initial DH of 2.4% enzymolysis treatment to 5.3% up to 6.0% after UAE (200 W to 600 W). However, the DH did not change significantly ($p<0.05$) at different times of UAE (15 min or 30 min). This indicates that the UAE was more efficient than traditional enzymolysis.

Li et al. [18,32] evaluated the kinetics of UAE (58 W L^{-1}; 28 kHz) using rice proteins. These researchers reported that ultrasound treatment improved the enzymatic efficiency and, in turn, increased the DH ($p<0.05$) of the protein. Furthermore, Zhou et al. [33] studied the combined effect of ultrasound and/or heat on corn gluten enzymolysis. They reported that the ultrasound pretreatment (40 kHz) accelerated

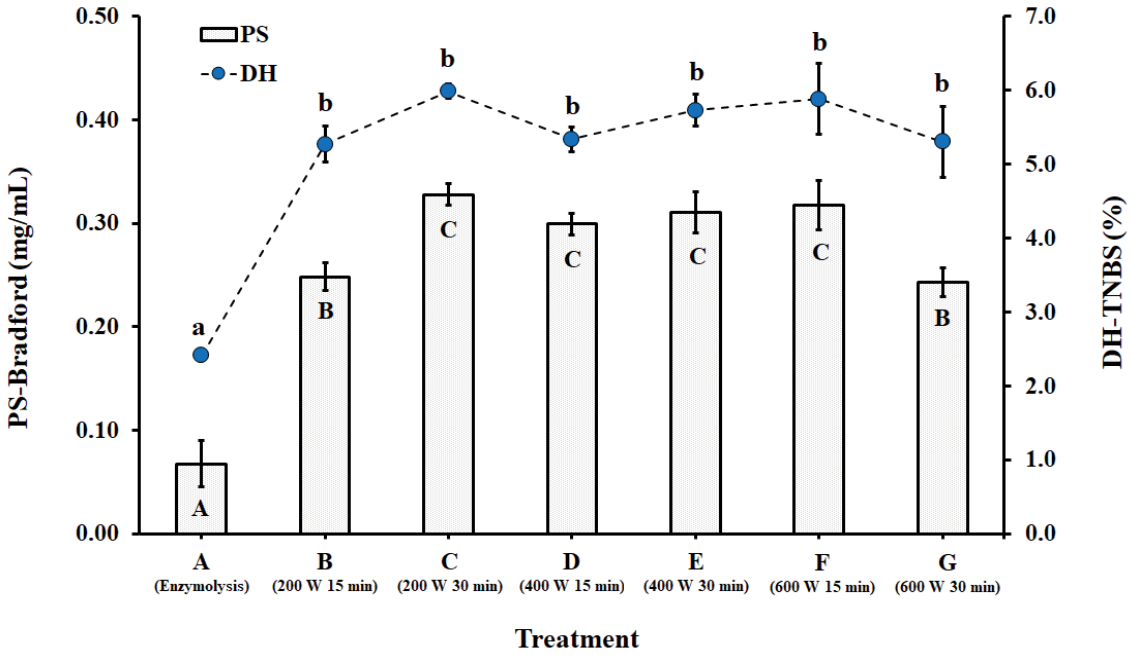

Figure 1: Effect of enzymolysis (A) and ultrasound-assisted enzymolysis (B-G) on the degree of hydrolysis (DH) and protein solubility (PS) of jackfruit seed protein isolate. Different letters mean significant differences between values with different ultrasonic power and time (Duncan; $p<0.05$).

the reaction rate of the corn gluten hydrolysis and led to an increase in the DH of the protein. The reason might be that the ultrasound and sonochemistry effects helped disrupt strong solute-matrix interactions, which involved Van de Waals forces, hydrogen bonding and dipole attractions between the molecules [18]. The foregoing results were consistent with enzymolysis of corn gluten meal [34], wheat gluten [17], oats protein [35] and potato protein [36] after ultrasound pretreatment.

The solubility is the most practical measurement of protein denaturation and aggregation and is, therefore, a reliable index of protein functionality due to its considerable effect on other technological characteristics, particularly gelation, foaming, and emulsifying, which depend on an adequate initial solubility of proteins [10,37]. The PS of JSPI increased significantly ($p<0.05$) after UAE compared with the solubility of the enzymolysis treatment (Figure 1). This might be due to that in a natural state, proteins are present in the form of aggregates, and the physical factors of cavitation might disrupt the hydrogen bonds and hydrophobic interactions, which are responsible for intermolecular association of protein aggregates [8]. The PS increased from an initial value (enzymolysis) of 0.07 ± 0.00 mg/mL to 0.29 ± 0.06 mg/mL, 0.30 ± 0.01 mg/mL, and 0.28 ± 0.05 mg/mL after UAE at 200 W, 400 W and 600 W, respectively. Therefore, this increase in solubility may be due to conformational change during ultrasonic treatment and hydrolysis of the peptide bonds of the JSPI protein molecules. Positive correlations between PS and DH ($r=0.9772$; $p<0.01$) and between α-helix and DH ($r=0.7699$; $p<0.05$) were observed after UAE. According to the above, the ultrasound-enzymolysis synergistic effect caused an increase in PS, which is directly related to the conformational changes in the secondary structure and the hydrolysis of the peptide bonds of the JSPI protein molecules, in accordance with the studies of Li et al. [14] and Yang et al. [37] who reported that the UAE increased the PS of rice proteins and wheat germ, respectively.

Secondary structure (FT-IR)

FT-IR spectroscopy is a measurement of wavelength numbers and the intensity of the absorption of infrared (IR) radiation by the sample [33]. The polypeptide and protein repeat units give rise to nine characteristic IR absorption bands, namely, amide A, B, I, II, III, IV, V, VI, and VII [38]. The amide I (1600 cm^{-1} to 1700 cm^{-1}) band represents the C=O and a small extent of the C=N stretching vibration, which provides information on the secondary structures of the proteins (α-helix, β-sheet, β-turn, and random coil) [2]. To obtain further information concerning protein structural changes, we analyzed the FT-IR spectra of the samples exposed to the UAE between wavelength numbers 1700 cm^{-1} and 1600 cm^{-1}.

The FT-IR spectra of JSPI-UAE treatments in the region of 1750 ∑ 1450 cm^{-1} are shown in Figure 2. As presented in this Figure, there was an obvious difference between the enzymolysis treatment and JSPI subjected to UAE in terms of the absorption region and the intensity of the peaks. After deconvolution and overlapping component extraction procedures, the fitted peaks are shown in Figure 3. The deconvoluted amide I bands and the content assignment of the secondary structure of JSPI are shown in Table 1 according to some previous studies [2,21,22].

The UAE treatment had a significant effect on the secondary structure compared to the enzymolysis treatment ($p<0.01$). The relative content of α-helixes, β-turns, and random coils increased from the initial value (enzymolysis treatment) of 8.68%, 27.19% and 20.81% up to 10.40%, 30.06% and 23.18% after UAE treatments, respectively. In contrast, the relative content of β-sheets decreased from an initial value of 43.32% up to 36.88%.

When UAE was applied, the β-sheet content decreased (up to 15%, 200 W-30 min) while the α-helix (up to 16.54%, 400 W-15 min), β-turn (up to 9.55%, 200 W-30 min) and random coil (up to 10.22%, 400 W, 15)

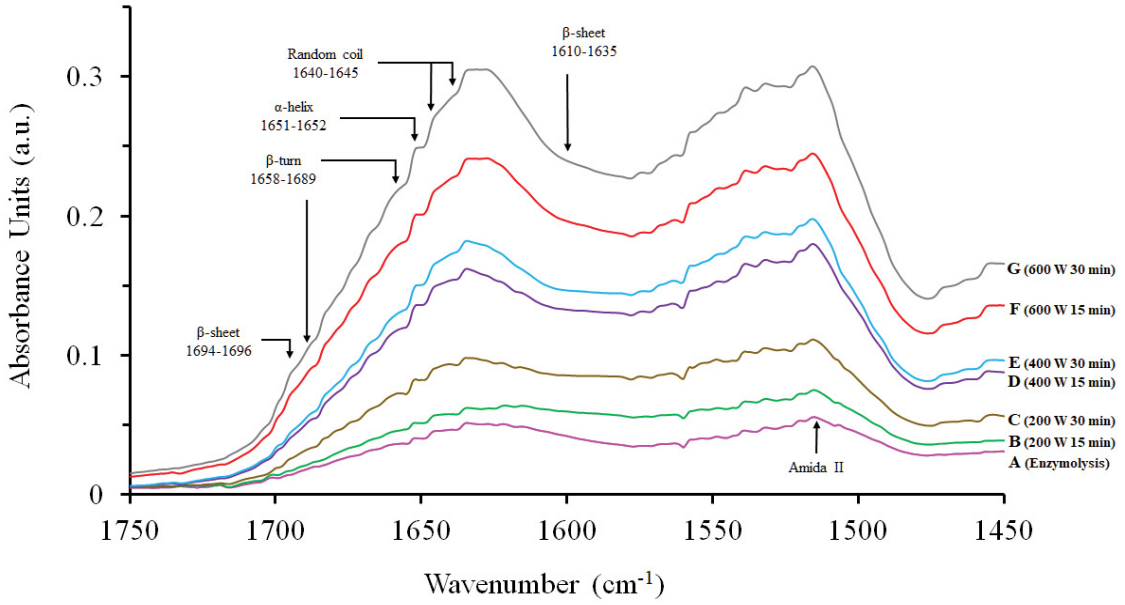

Figure 2: Effect of enzymolysis (A) and ultrasound-assisted enzymolysis (B-G) on the FT-IR absorbance spectra (amide I band) of jackfruit seed protein isolate.

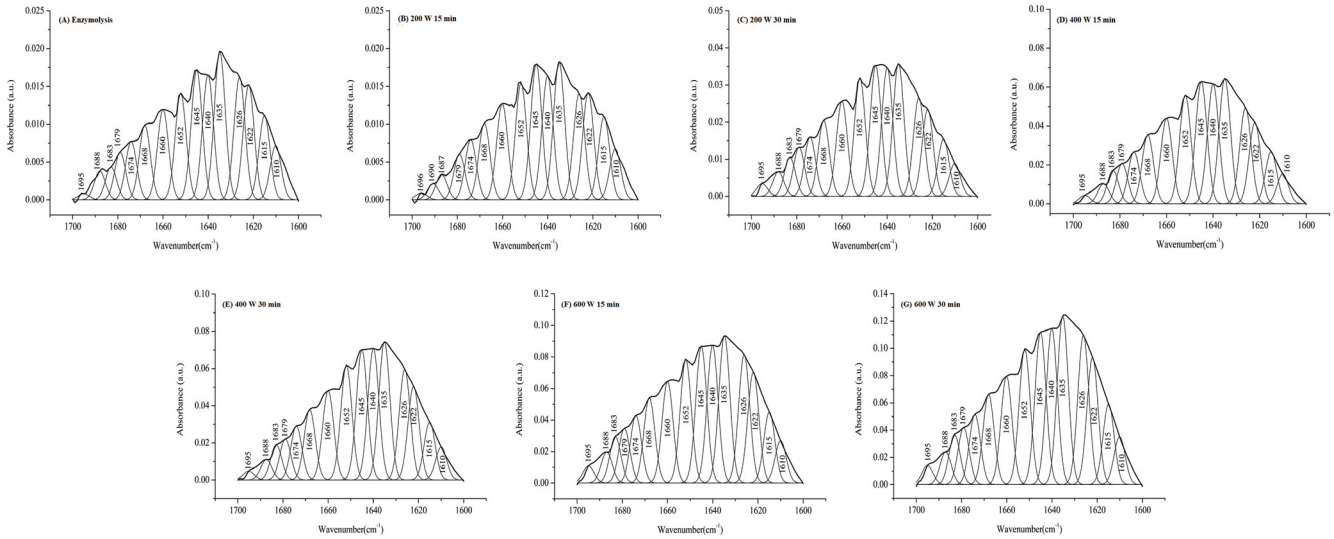

Figure 3: Deconvolution and peak-separation analysis of amide I band (1700-1600 cm^{-1}) of jackfruit seed protein isolate treated by enzymolysis (A) and ultrasound-assisted enzymolysis (B-G).

content increased. In agreement with the above, a negative correlation was observed between α-helix and β-sheet ($r=-0.9023$; $p<0.01$) and random coil and β-sheet ($r=-0.7820$; $p<0.05$) content. Furthermore, a positive correlation was observed between α-helix and random coil ($r=0.9141$; $p<0.01$).

In general, these findings suggest that UAE results in unfolding of the α-helical region followed by the formation of a β-turn and random coil as well as a decrease in the interaction of the amino acids that formed the β-sheet structure. The changes in the secondary structure content might be observed because the free radical, microjets, shear forces, shock waves, and turbulence, which were induced by ultrasound, have disrupted the interactions between the local sequences of amino acids and between different parts of the protein molecule [2].

The results of the present study are different from those of Yang et al. [11] and Jin et al. [2]. These authors reported that dual-frequency power ultrasound treatment was responsible for decreased α-helix content and an increased β-sheet and random coil content, compared with the native rice protein and corn gluten meal, respectively. Different results have also been reported by researchers who have applied sonication to other types of proteins, such as walnut protein and soy protein [37,38]. Conversely, Zhang et al. [39] utilized ultrasound-assisted alkali to pretreat rice protein, and the results of FT-IR showed that the ultrasound increased the β-turn content and decreased the β-sheet and random coil content. These contradictory results, compared to our results, might arise from the differences in sonication conditions, such as the type of ultrasound used, the frequency, the ultrasonic intensity as well as the intrinsic characteristics of the sonicated material.

Microstructure

To understand the effect of different UAE conditions applied to JSPI, the microstructure of the lyophilized JSPI was observed by SEM. Figure 4 shows a set of SEM images of different JSPI at a 450-fold magnification factor. Compared with the enzymolysis treatment, samples B-G exhibited more disordered structures and irregular fragments. In addition, the SEM images showed that on average, sample E (400 W-30 min) and G (600 W-30 min) was larger than D (400 W-15 min) and F (600 W-15 min), respectively, suggesting that a longer ultrasonic time could result in larger structures. The functional native structure of proteins is determined by the subtle balance between many non-covalent and covalent interactions; this balance can be easily disrupted by the mechanical/shear stresses from sonication leading to protein denaturation and change in the secondary structure [17]. With relatively low-power ultrasound treatment, the effect of turbulent forces and microstreaming might increase the speed of collision and aggregation; this typically results in the formation of unstable aggregates and an increase in the particle size [40,41]. When the power of the ultrasonic treatment is increased, the particles become smaller, and the particle size-distribution broadens [28]. Similar observations were reported by Malik et al. [3] for sunflower protein isolate. Kang et al. [22] showed that the exposure of the hydrophobic regions resulted in an increase in the β-sheet content, accompanied by a decrease in α-helix structures. In this context, these results might be due to the changes in the ultrasonic treatment leading to unfolding of the JSPI protein molecules and increased exposure of hydrophobic groups (see Section 3.4), and this result is confirmed by the increase in the β-sheet and random coil content (see Section 3.2), which could interact with each other and form larger aggregates during freeze drying.

Treatment	Secondary structure (%)			
	α-helix (1651-1652 cm^{-1})	β-sheet (1610-1634,1695 cm^{-1})	β-turn (1658-1689 cm^{-1})	Random coil (1640-1645 cm^{-1})
Enzymolysis	8.68^{a}	43.32^{g}	27.19^{b}	20.81^{a}
UAE at:				
200 W 15 min	9.83^{c}	41.40^{f}	26.95^{a}	21.83^{d}
200 W 30 min	10.36^{d}	36.88^{a}	30.06^{g}	22.70^{e}
400 W 15 min	10.40^{d}	37.67^{b}	28.75^{e}	23.18^{g}
400 W 30 min	10.16^{d}	39.16^{c}	27.49^{c}	23.17^{f}
600 W 15 min	9.50^{b}	39.72^{d}	29.72^{f}	21.06^{b}
600 W 30 min	9.38^{b}	40.99^{e}	28.30^{d}	21.33^{c}
Within a column, means with different superscript letters indicated significant difference (Duncan; p<0.01)				

Table 1: Effect of the enzymolysis and ultrasound-assisted enzymolysis (UAE) by alcalase on secondary structure of jackfruit seed protein isolate.

Surface hydrophobicity (H_0-ANS)

The surface hydrophobicity (H_0-ANS) of proteins is one of the structural characteristics, is important for protein stability and conformation and has an impact on protein functionality [42]. Figure 5 shows that UAE significantly increased the H_0-ANS of JSPI ($p<0.05$). This finding was consistent with previous studies that showed that UAE could cause an increase in the H_0-ANS for wheat germ protein [12] and rice protein [12,32]. Comparing the H_0-ANS of JSPI-UAE, it was observed that H_0-ANS increased with ultrasonic intensity (from 200 W to 600 W) and time (15 min and 30 min). This indicates that ultrasonic treatment induces a certain degree of the molecular unfolding of the proteins and, thereby, causes an increase in the number of hydrophobic groups and regions that are originally inside the molecules to become

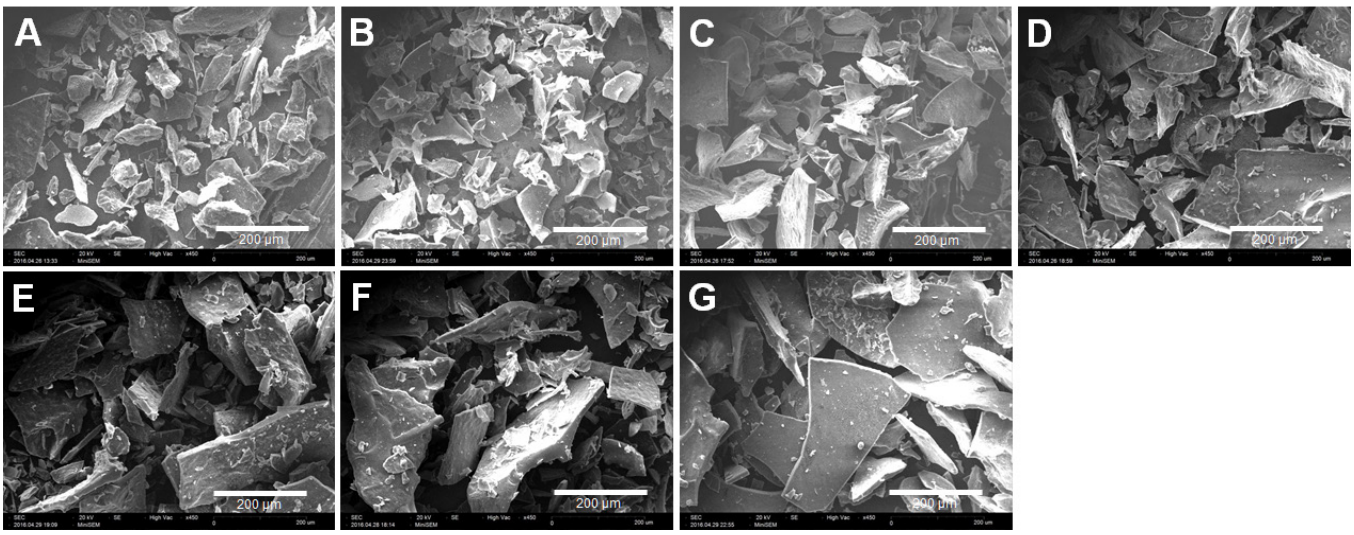

Figure 4: Effect of enzymolysis (A) and ultrasound-assisted enzymolysis B=200 W-15 min, C=200 W-30 min, D=400 W-15 min, E=400 W-30 min, F=600 W-15 min, G=600 W-30 min on microstructure of lyophilized jackfruit seed protein isolate by scanning electron microscopy. Scale bar is 200 µm in all cases.

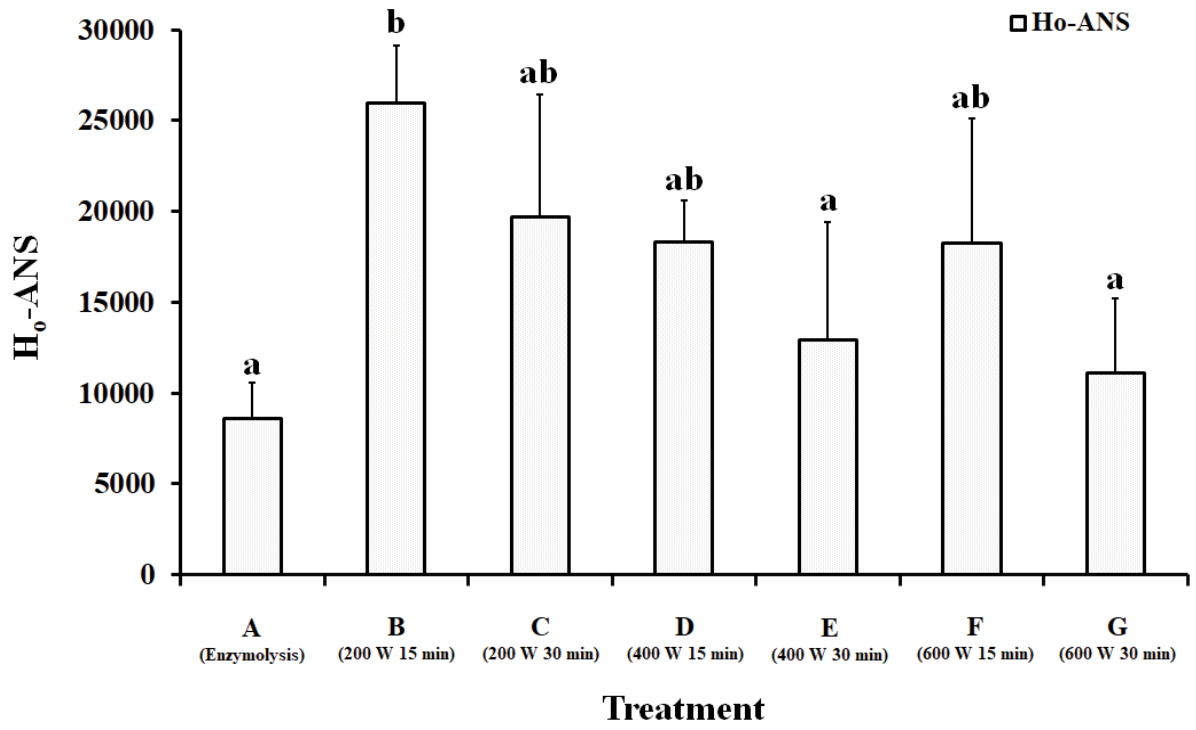

Figure 5: Effect of the enzymolysis (A) and ultrasound-assisted enzymolysis (B-G) on the surface hydrophobicity (H_0-ANS) of jackfruit seed protein isolate (mean ± SD, n=2). Mean values with different letters were significantly different (Duncan; p<0.05).

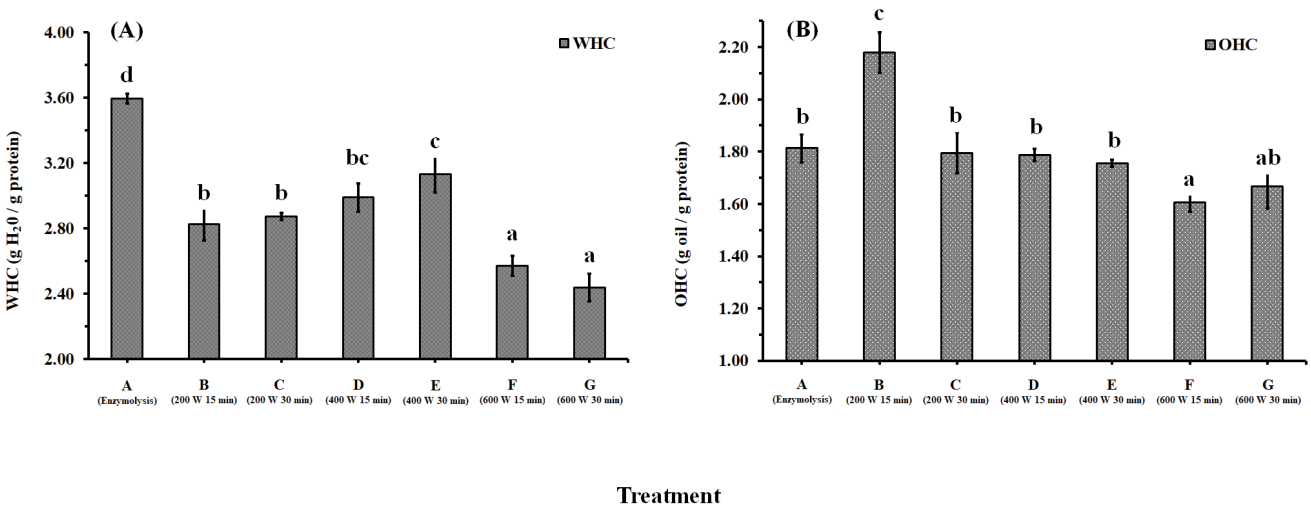

Figure 6: Effect of the enzymolysis (A) and ultrasound-assisted enzymolysis (B-G) on water holding capacity (WHC; A) and oil holding capacity (OHC; B) of jackfruit seed protein isolate. Mean values with different letters were significantly different (Duncan; p<0.05). WHC and OHC: n=4 ± SD for control; n=3 ± SD for 0 W; n=2 ± SD for 200 W to 600 W for 15 min and 30 min.

exposed to the polar surrounding environment [4]. This aggregation protects the hydrophobic regions of the proteins [28]. However, the H_0-ANS of the samples treated at an ultrasound power of 400 W and 600 W for prolonged times (30 min) decreased. High-intensity ultrasound treatment might also lead to partial denaturation of proteins, which might increase the extent of bonding and reduce the H_0-ANS [43]. Similar results were obtained by Malik et al. [42], Wu et al. [44], Zhang et al. [39] and Yang et al. [37] in sunflower protein, whey protein, rice protein, and soy protein, respectively.

Functional properties

Water holding capacity (WHC) and oil holding capacity (OHC):

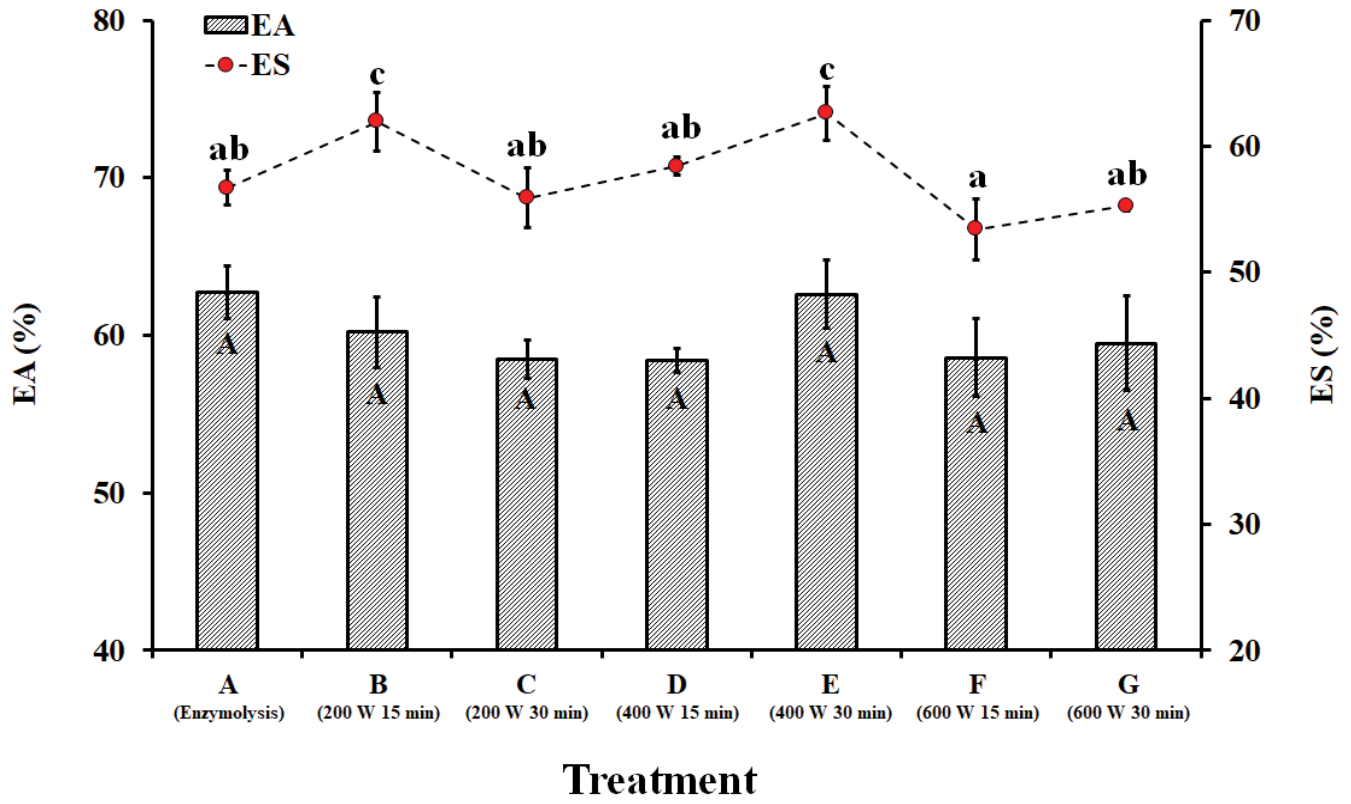

Figure 7: Effect of enzymolysis (A) and ultrasound-assisted enzymolysis (B-G) on emulsifying activity (EA) and emulsifying stability (ES) jackfruit seed protein isolate (mean ± SD, n=2). Different letters means significant differences between values with different ultrasonic power and time (Duncan; $p<0.05$).

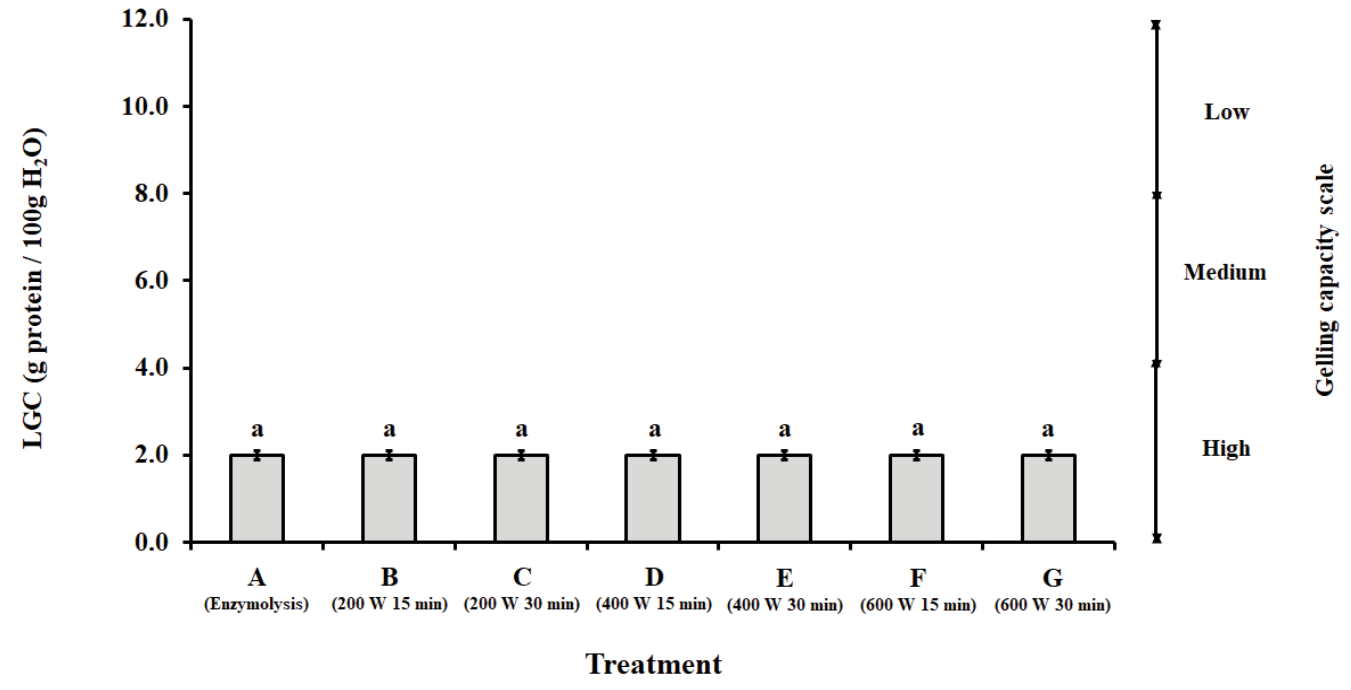

Figure 8: Influence of enzymolysis (A) and ultrasound-assisted enzymolysis (B-G) on the least gelation concentration (LGC) of jackfruit seed protein isolate (mean ± SD, n=2).

The WHC and OHC result from different JSPI samples are shown in Figure 6. The WHCs of the UAE samples were significantly ($p<0.05$) lower compared to the enzymolysis treatment (Figure 6a). The WHC decreased from an initial WHC of 3.60 g g^{-1} (enzymolysis treatment) up to 2.83 g g^{-1} after UAE at 200 W-15 min. However, at 600 W-30 min, the WHC decreased significantly ($p<0.05$) to 2.44 g g^{-1}. One reasonable explanation is that the UAE after stronger ultrasound treatment might heavily denature the molecular structure of the protein and cause an increase in the free sulfhydryl groups to the surface of the JSPI, resulting in lower levels of WHC. These results are in agreement with those reported by Zhang et al. [45] who observed an increase in the WHC of myofibrillar protein at an ultrasound power of 200 W to 600 W, but at higher ultrasound power (≥ 800 ∑ ≤ 1000 W, 15 min) the WHC decreased. A negative correlation ($r=-0.8006$; $p<0.05$) between WHC and UAE was observed.

On the other hand, the OHC of JSPI subjected to UAE at 200 W–15 min was significantly ($p<0.05$) greater compared to the enzymolysis treatment. The OHC increased from an initial OHC of 1.82 g g^{-1} up to 2.18 g g^{-1} after UAE of 200 W-15 min (Figure 6b). The presence of OHC might be because of the exposure of hydrophobic groups after the UAE allowed the physical entrapment of oil [10]. Such exposure of hydrophobic groups can be observed by the formation of large aggregates of proteins in the dry state after freeze-drying JSPI samples treated with UAE in comparison with control JSPI (Figure 4).

Emulsifying activity (EA) and emulsion stability (ES): The ability of proteins to assist in the formation and stabilization of emulsions is of critical importance for many applications, such as frozen desserts, salad dressings, comminuted meats, mayonnaise, cake batters, milk and coffee whiteners [13,46]. The EA value of the JSPI subjected to enzymolysis was not significantly different ($p<0.05$) in comparison with JSPI treated with UAE in all conditions (Figure 7). It has been reported that the emulsifying properties of hydrolysates are closely related to the degree of hydrolysis [47], with a low DH (3 ∑ 5%) increasing and a high DH (≥ 8%) decreasing emulsifying properties [10]. However, in this study, the UAE increased the DH in the range from 2.9 to 3.5 with respect to enzymolysis but without effect on EA.

The ES of JSPI at 200 W-15 min and 400 W-30 min was increased ~ 6% after UAE with respect to the enzymolysis (Figure 7). The increase in the ES can be explained by the more favorable orientation of proteins resulting from the influence of turbulent behavior produced by the ultrasound-enzymolysis [43,48]. Xiong et al. [41], Hu et al. [46], Zhou et al. [13] and Zhang et al. [49] reported that high-intensity ultrasound increased the EA and ES of ovalbumin, soy β-conglycinin, soy glycinin, and peanut protein, respectively, which is in agreement with the results of ES obtained in this study for only two treatments (UAE at 200 W-15 min and 400 W-30 min).

Partial denaturation of the tertiary and quaternary structure and formation of a more disordered structure that could provide the protein a better potential to adsorb at the oil-water interface were speculated to lead to an increase in the EA [47]. For a protein with good solubility, an increase in exposed hydrophobicity has been described to decrease the barrier for adsorption to the oil-water resulting in a greater adsorption rate [50] as well as increased activity and stability of the emulsion [46].

As in other properties, EA and ES depend upon the molecular flexibility and stability of the protein structure (such as the secondary and tertiary structure) [43]. In this sense, a negative correlation between EA and the β-turn content ($r=-0.7815$; $p<0.05$) was observed after UAE. According to these results, EA and ES improved mainly due to the increase in the β-sheet content and random coil content of JSPI after UAE.

Least gelation concentration (LGC): Gelation is often the aggregation of denatured molecules; this aggregation may be primarily driven by physical interactions in which the aggregation is random [41]. Heating a protein solution causes molecular unfolding, which leads first to aggregation and then to gelation when the amount of aggregated protein exceeds a critical concentration [51]. In general, the LGC showed no significant change ($p>0.01$) after UAE compared to the enzymolysis (Figure 8). The LGC was 2.0 g/100 g in all JSPI treated with UAE. In a previous study [8], we reported that high-intensity ultrasound modified the LGC of jackfruit seed proteins and had values of 2.0 g/100 g to 8.0 g/100 g at pH 2 ∑ pH 10, respectively. In this investigation, all JSPI had the lowest LGC value (2.0 g/100 g), which is highly desirable in food gelling agents. Nevertheless, the evaluation of the microscopic structural organization and rheological properties could lead to a greater understanding of the type of network formed by the UAE.

Conclusion

In general, the ultrasound pretreatment of JSPI improved the enzymolysis reaction by alcalase, as confirmed by an increase in DH ($p<0.05$). UAE improved the techno-functional properties in terms of the OHC and ES and modified the structure of JSPI. UAE decreased the β-sheet content and increased the β-turn content, the random coil content and the α-helix content. The effective promotion of techno-functional properties by UAE was achieved by increasing the degree of hydrolysis, the unfolding of the secondary protein structure and a reduction of intermolecular interactions, as demonstrated by an increase in H_0-ANS, leading to improved PS of JSPI. The results presented here could be used in an industrial setting to develop conventional or novel foods based on jackfruit proteins.

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