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Effect of Ultrasound Treatment on the Physicochemical, Nutraceutical, and Functional Properties of Lupine Flour

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ABSTRACT

This study aimed to convert sweet and bitter lupine protein isolates to nano-emulsions and to develop products with the nano-emulsion and examine the stability; develop emulsification with ultrasonication; develop and characterize food protein-stabilized nanoemulsions; and to modify lupine protein properties with pH shifting and ultrasonication. The antioxidant activity of sweet and bitter isolates was decreased in the nano-particle sized samples when compared to non-nano particle-sized samples, as well as the inhibitory activity of both angiotensin-converting enzyme and alpha-amylase, while it increased in sweet and bitter lupine isolates. For both types of lupine, water holding capacity was increased, and foaming stability of nano treatment was increased in protein isolate samples. In lupine, albumins and three globulins fractions, namely, β-conglutin, α-conglutin, and γ-conglutin, were characterized as the main storage proteins. No clear differences were observed between nano and non-nano samples regarding albumins and three globulins fractions. The results of microstructure characterization showed that sonication leads to distortion/breakage of protein particles shape, which results in smaller proteins that can be used as a top-down approach for the formation of nano-sized protein particles. In this regard, sonication can be considered as a heterogeneous approach that may lead to de-aggregation, breaking of protein particles, and distortion of their shape.

Keywords: Antioxidant activity**,** Dynamic Light Scattering, Nano-emulsion**,** Sonication**.**

INTRODUCTION

Lupines belong to Leguminosea family. The species *Lupinus albus* (white lupin), *L. luteus* (yellow lupin), *L. angustifolius* (blue lupin) and) and *L. mutabilis* (Pearl lupin) have a significant role in agricultural fields (Hondelmann, 1984). The first three of these are usually planted in Australia and Mediterranean countries, while L. mutabilis is cultivated in South America (Mülayim, *et al.,* 2002).

Lupine seeds, like other legumes, are known for a higher content of protein, minerals, and dietary fiber (34.44–39.42%) (Martínez-Villaluenga *et al.,* 2006). Lupine seeds protein (33-47%) is higher than other legumes. Its seeds and flours are utilized in various cereal products such as pasta, cake, bread, cookies, and breakfast cereals (Dervas *et al.,* 1999).

Legume seed's main proteins are situated in the storage vacuoles of the cotyledonary tissues and, mostly, have a place with the group of the storage proteins, which fill in as nitrogen and carbon skeleton sources for the developing plantlet (Duranti *et al.,* 2008). Globulins are the significant protein component of lupine seed storage protein and include two significant protein types, conglutin b (vicilin like-protein or acid7S

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globulin, 43.4%) and conglutin a (legumin-like protein or 11S globulin, 33%), and two minor segments: conglutin d (2S sulfur-rich albumin, 12.5%) and conglutin g (basic7S globulin, 6%) (Duranti *et al.*, 1981).

It is essential to know the requirements for the solubilization of the oil phase and to develop a stable emulsion using food-grade components such as emulsifiers and stabilizers. Food nanomaterials, such as nanoemulsions, including nanoemulsification have gained attention (Weiss *et al.*, 2006). Nanoemulsions have different values from functional, physiochemical properties, and allergenicity that need to be investigated to determine the possibility of using these new nanoemulsions in food products (Weiss *et al.*, 2006)

Various preparation techniques have been employed to produce micro/nanoemulsion in food systems. It is well known that ultrasonication, which can produce extreme pressure and temperature variations, is a common method used for enhancing mass transfer.

Sonication treatment of proteins has been shown to reduce the size of protein aggregates, ascribed to disruption of non-covalent and electrostatic interactions maintaining the structure of these aggregates (O'connell *et al.*, 2003). This method and similar representative methods often include different reaction steps that need many hours or days, anaerobic conditions, and the existence of water to hydrolyze the protein and, then, sometimes a final treatment at high temperatures (Ling *et al.*, 2008). Non-aqueous synthetic approaches have been discovered to prepare nanoparticle with improved control over particle size, crystallinity, shape, and surface properties (Xu *et al.*, 2013).

Proteins of plant origin are of specific interest as emulsifiers in food systems because of their ability to create interfacial films and to adsorb to oil-water interfaces (Lam and Nickerson, 2013). The amphiphilic nature of the proteins, in the presence of both hydrophilic and hydrophobic groups in the peptide chain, gives the protein the surface activity property (Beverung *et al.*, 1999). For that, the legumes proteins are more popular than animal proteins due to improved nutritional value, shelf life, texture (Tsoukala *et al.*, 2006), their sustainability, low cost, high natural abundance, and functional attributes (Karaca *et al.*, 2011).

In this study, the objective was to design, characterize, and evaluate the functional properties and microstructure characterization of lupin protein in vitro to enhance the application for improving the food quality. This technology is based on knowledge and advances in legume protein chemistry such as lupine and its application in micro/nanoencapsulation of non-polar molecules. A sonication process was used in lupine protein treatment as a new endeavor that is expected to produce a lupine protein with improved functional and nutraceutical properties, when it is used as proteins-based delivery systems to improve food quality.

MATERIALS AND METHODS

In this study, two types of lupine flour (bitter and sweet) were purchased from a local source (Irbid, Jordan), Porcine pancreatic α-amylase, DPPH (2,2-DiPhenyl-1-PicrylHydrazyl), Folin–Ciocalteu's phenol reagent, Gallic acid, aluminum chloride, Sodium Carbonate (Na_2CO_3), 3,5-DiNitrosalicylic Acid (DNS), sodium hydroxide, sodium potassium tartrate, sodium metabisulfite, and methanol were purchased from Sigma (St. Louis, MO, USA). Catechine was purchased from Aldrich

Protein Isolate Extraction

Protein isolates from full-fat sweet or bitter lupine flours were extracted according to the technique described by Alu'datt *et al.* (2012) with slight modifications. The Lupine Protein Isolates (LPI) were

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suspended in 1L distilled water, adjusted to pH 9 with 1M NaOH solution, and stirred for 1 hour in a water bath. The suspension was centrifuged (15 minutes at 10,000×g) (Z32HK, Hermle Labortechnik GmbH, Germany), followed by filtration (101 FAST, 125 mm, China). After that, the suspension was acidified to 4.5 with 1M HCl. The proteins were separated by centrifugation (15 minutes at 10,000×g) followed by freeze-drying at -50°C for 48 hours (LFD-5508, Korea) and stored in a refrigerator at 4°C for further treatment and analysis.

pH-Shifting and/or Ultrasonication Processes

Lupine Protein Isolate (LPI) sample (3 g) was suspended in 100 mL distilled water for 30 min at room temperature. The LPI dispersion was then adjusted to pH 12 with 2M NaOH and sonicated for 30 minutes at room temperature using a sonicator (Sonics & Materials, Inc., Newtown, CT, USA). After that, the solution was neutralized to pH 7 using 2M HCl. The supernatant was isolated by centrifugation (15 minutes at 1,200 rpm) followed by freeze-drying at -50°C for 48 hours (LFD-5508, Korea), then stored at 4°C for further analysis. A control (sample without treatment) was used along with the tested samples (Jiang *et al.*, 2017).

Modify Lupine Protein with pH Treatments

LPI was prepared in laboratory and the content was approximately 90%. The ultrasound treatments were conducted using an ultrasound at 20 kHz. The generated acoustic energy was delivered to a probe (12.5-mm diameter) placed in the samples. Then, LPI (3 g) was added to 100 mL DI water and stirred 30 minutes at room temperature.

The LPI dispersions were treated by 4 different treatments including pH 12, ultrasound, ultrasound+pH 12, and pH 12+ultrasound. For ultrasound, the dispersion was sonicated for 5 minutes in the ice bath. For pH 12, the pH of LPI dispersion was adjusted to pH 12 with 2M NaOH. The 5-minute sonicated LPI dispersion was treated by pH 12, which was denoted as ultrasound+pH 12. For pH 12+ultrasound, the PPI dispersion treated by pH 12 was treated by sonication for 5 minutes in the ice bath. All the samples were stored at room temperature for 1 h after each treatment followed by neutralization to pH 7 with 2M HCl. Then, LPI dispersions were centrifuged at 1,200×g and 20ºC for 15 minutes. The supernatants were collected as soluble.

On the (pH 12+ultrasound) treatment with the insoluble LPI, different ultrasound treatment times (1-5 minutes) were conducted, and soluble LPIs were collected to test the recovery of soluble lupine protein with different sonication time. Basic pH 12 and pH 12+ultrasound treatments were applied followed by recovery tests and soluble lupine protein concentration. In addition, different pH levels, pH 9-11 and pH 2-4, were applied to insoluble LPI. The LPI dispersions (3 g in 100 mL DI water) were adjusted to alkaline or acid pH with 2M NaOH or HCL for (pH+ultrasound), the pH-adjusted LPI dispersions were treated by ultrasound. All treated LPI dispersions were processed by the above-mentioned methods including storage for 1 hour at room temperature, pH adjustment, and centrifugation. The final supernatants were collected as soluble LPI for further analyses.

Microstructure and Particle Size

The average mean diameters of nanoemulsion and nanocomplexes were investigated by Dynamic Light Scattering (DLS). The samples were diluted 500-fold with distilled water before measurement. The measurement was conducted at 23°C. For SEM imaging of the samples, first, the sample was diluted before the deposition to a concentration not exceeding 0.1 mg mL^{-1}. The optimal concentration depends on the substrate, imaging method, particle-substrate adsorption, etc. Second, to avoid drying of the solution drop during deposition, a drop of the solution/suspension was deposited

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onto the substrate and distributed uniformly over the substrate and let it dry for one minute to let the particles adsorb; then, the drop was removed by blowing air on to the substrate to remove the loose particles. FEI Quanta 250 FEG Scanning Electron Microscope was used for imaging.

Electrophoresis

Sample Preparation: The examined samples were mixed with sample buffer in 1:1 ratio (for 5X sample buffer: 0.6 mL of 1M Tris-HCl pH 6.8, 5 mL of 50% glycerol, 2 mL of 10% SDS, 0.5 mL of mercaptoethanol, 1 mL of 1% bromophenol blue, and 0.9 mL of H2O were mixed) and boiled for 3 min.

Gradient PAGE: Mini-Protean TGX Precast Gels (BioRad, USA) was used for gradient polyacrylamide gel electrophoresis (gradient PAGE) according to the manufacturer's instructions. Samples were loaded into ready cast gradient gels in the electrophoresis apparatus and allowed to run using a running buffer (0.025M Tris, 0.192M glycine, and 0.1% SDS) at 60 volts gradually increased to 120 volts.

PAGE Staining: Modified Coomassie was used for the visualization of proteins. Immediately after electrophoresis, the gel was removed from the electrophoresis apparatus and soaked for 1 hour in a fixing solution (10% v/v acetic acid, 10% v/v methanol, and 40% v/v ethanol). Then, the gel was transferred into a sensitization solution (1% acetic acid, 10% ammonium sulfate) and incubated for 2 hours with gentle shaking. After that, the gel was transferred into a staining solution (5% v/v acetic acid, 45% v/v ethanol, and 0.125% w/v CBB R-250) and incubated for more than 4 hours or overnight with gentle shaking. The gel was then placed in a destaining solution I (5% v/v acetic acid, 40% v/v ethanol) and stirred for 1 hour, transferred into a destaining solution II (3% v/v acetic acid, 30% v/v ethanol) until the background was clear. Gels were scanned using GS-800 Densitometer and analyzed using Quantity One Software (BioRad, USA).

Functional Properties of Lupine Flour and its Isolate

Aqueous dispersions (16% w/v) of protein isolate in water.

Foaming

The foaming stabilities were estimated according to Alu'datt *et al.* (2012) with slight modifications. The samples were mixed and shaken for 5 minutes to produce a layer of foam. The foaming stability was estimated by measuring the ratio of the initial foam volume obtained at 0 and 60 minutes.

Water Holding Capacity

Water holding capacities were determined according to the technique of (Alu'datt *et al.*, 2012). The method of gelation occurred by warming the dispersions for 30 minutes at 95°C. To avoid loss of water, the samples were secured with aluminum foil. The gels were cooled at 4°C for 24 hours. After that, they were centrifuged (15 minutes at 10,000×g) followed by measuring the supernatants.

Water Holding Capacity = {[Vol. of the distilled water (mL)–Vol. of the supernatant (mL)]/ [Vol. of the distilled water (mL)]}×100.

Emulsion Properties

Emulsion stabilities were estimated according to Alu'datt *et al.* (2012). In brief, 2 g of the sample was suspended in 20 mL olive oil and 20 mL distilled water. The suspension was mixed and shaken for 2 minutes. Then, it was centrifuged (5 minutes at 2,000×g). The suspension was heated for 30 minutes at 80°C in a shaking water bath, cooled to 25°C under running tap water for 15 min and centrifuged (15 minutes at 2,000×g). The emulsion stability is

[DOR: 20.1001.1.16807073.2021.23.4.12.1]

defined as the ratio of the volume of the emulsion to the total volume of the mixture.

Physiochemical Properties of Lupine Samples

Extract Preparation

The extracts were prepared as the following: 0.5 g of each sample was extracted with 50 mL methanol. The extraction procedure was executed by stirring for 60 minutes at 60°C, each extract was filtered (Fast Speed 101, 125 mm, Hanker International Group, China), then, the filtrate was made up to 50 mL by the addition of methanol and stored in the dark until analysis.

Determination of Radical DPPH-Scavenging Activity

Five hundred µL of methanol extract was reacted with 0.2 mL of DPPH solution (Fahmideh *et al.,* 2019). The mixture was brought to a total volume of 4.0 mL using methanol, mixed thoroughly, and allowed to stand in the dark for 30 minutes at room temperature. The absorbance was measured at 515 nm using a spectrophotometer (CELL, model CE 1020, Cecil Instruments, Cambridge, UK). Methanol was used as a blank. The radical-scavenging activity is defined as the percentage of inhibition:

Inhibition (%)= [(Abs. of blank–Abs. of sample)/Abs. of blank]×100.

IC50 is defined as the concentration of extract in mg mL^{-1} needed to scavenge 50% of the DPPH radical.

Enzymatic Assays of Lupine Flour

Angiotensin-Converting Enzyme (ACE) Inhibitory Activity

Inhibitory activity of ACE was assayed using a modified method described by Cushman and Cheung (1971). Hippuryl-Histidyl-Leucine (HHL) solution was diluted by mixing 6 µL of the solution in 2 mL of HEPES-HCl buffer (1.30 g HEPES sodium salt and 1.75 g NaCl in 100 mL distilled water). After that, 200 µL of HHL solution was mixed with 100 µL of phenolic extract. Later, 50 µL of ACE solution (333 mU mL^{-1}) was incubated with 50 µL of HEPES-HCL buffer at 37°C for 15 minutes. Then, the reaction was terminated through the addition of 250 µL of 1M HCl. The resulting hippuric acid was extracted by adding 2 mL of ethyl acetate and centrifuged (50 Hz, USA). The ethyl acetate layer was evaporated at 100ºC for 15 minutes followed by mixing with 3 mL of distilled water. The absorbance was measured at 228 nm using a spectrophotometer (UV 1800). Finally, 50 µL of ACE with 200 µL of HHL was mixed in 100 µL distilled water as the control. The following equation was applied to calculate the ACE inhibition.

ACE Inhibition (%) = [(A228 Blank-A228 Sample)/A228 Blank]×100

Inhibitory Activity of Alpha-Amylase

The inhibitory activity of a-amylase was assayed as described by McCue *et al*. (2005). The a-amylase solution was prepared by dissolving 0.03 g of a-amylase enzyme in 100 mL of distilled water. A total of 100 µL of the phenolic extract was mixed with 500 µL of a-amylase solution and 500 µL of phosphate buffer (pH 7), then incubated at 25ºC for 10 minutes. After that, 500 µL of the starch solution was added to the mixture and incubated at 25ºC for 10 minutes. The reaction was terminated by adding 1 mL of the colorimetric dinitrosalicylic acid (DNS) reagent. The mixture was placed into a 100ºC water bath for 5 min and cooled at room temperature. The reaction mixture was diluted with 7.4 mL of distilled water. Maltose concentration was measured at 540 nm using a spectrophotometer (UV 1800). The α-amylase inhibitory activity was expressed as a percent and determined using the following equations:

[DOR: 20.1001.1.16807073.2021.23.4.12.1]

Inhibition (%) = [(A540 Blank - A540 Sample)/A540 Blank]×100

Statistical Analysis

Results were reported as the mean and standard deviation based on the independent experiments. All experiments were conducted in triplicate with three independent experiments. The differences were analyzed using ANOVA with the JMP statistical package (JMP Institute Inc., Cary, NC, USA). Significant differences ($P \leq 0.05$) between means were identified by Fisher's Least Significant Difference test.

RESULTS AND DISCUSSION

Functional Proprieties of Protein Isolates from Lupine

Emulsion Properties

The emulsion activity expresses the ability of the sample to rapidly adsorb at the water-oil interphase during emulsion formation, therefore, preventing coalescence and flocculation (Subagio, 2006). The emulsifying properties are typically attributed to hydrophobic domains exposure and solute flexibility. Emulsion stability and formation are very necessary for food systems (Ahmed *et al.,* 2011; Lqari *et al.,* 2002).

Emulsion Stabilities (ES) of the lupine protein isolate samples at pH 4.5 are presented in Table 1. The emulsion stability of non-nano lupine protein isolate samples showed higher emulsion stability in sweet protein isolate than bitter protein isolates i.e. 32.50 vs. 26%, while the corresponding values for nano protein isolates were 16 and 14%. The highest significant ($P \leq 0.05$) value of emulsion stability of protein isolate was 32.50% of non-nano sweet lupine protein isolate. The result of lupine protein isolate obtained agrees with what was reported by Piornos *et al.* (2015), i.e. emulsion stability value was 12.56%. Alu'datt *et al.* (2017) also found that the stability of the emulsion was 33.8%. This may suggest that the globulin fraction, which is isolated and collected by isoelectric precipitation, is effective as an emulsifier for oil/water emulsions (Makri *et al.*, 2005).

The emulsion stability of nano protein isolate decreased. This could be related to the aggregation of denatured protein molecules while sonication. Soria and Villamie (2010) reported that denatured protein would expose more hydrophobic groups, which usually results in protein aggregation. Thus, there must be a balance between exposure of hydrophobic groups and aggregation of protein molecules (Malik *et al.*, 2017). Other study reported by Abbastabr *et al*. (2020) encapsulated curcumin with whey protein isolate had a better stability than pure curcumin.

Foaming Stability

Foam formation is an important property that depends on the ability of the protein to reduce surface tension between two phases

Table 1. Water holding stability, foaming stability and emulsion stability of LPIS (Lupine Protein Isolated Sweet), LPIB (Bitter), LPINS (Nano Sweet), LPINB (Nano Bitter).

Treatments	WHC (%)	Emulsion stability (%)	Foaming stability (%)
LPIS	58.50 ± 0.71 c	32.50 ± 3.53 a	51.00 ± 1.41 b
LPINS	67.00 ± 1.40 b	16.00 ± 1.41 c	81.65 ± 2.33 a
LPIB	51.50 ± 0.71 d	26.00 ± 1.41 b	35.15 ± 2.61 c
LPINB	77.50 ± 2.12 a	14.00 ± 1.41 c	78.50 ± 2.12 a

[a] (a-d) The same letter within the same column are not significantly different ($P \leq 0.05$). Means±standard deviation (n= 3).

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(air-water) and retain the film formed around the gas bubble. Foaming property is related to energy provided to make foam, pH of the protein solution, temperature, and nature of protein molecules (Smith and Olson, 2010).

Foaming stabilities of sweet and bitter protein flour are shown in Table 1. The results of non-nano isolates of sweet, and bitter lupine are 51, and 35.15%, which agree with what was noted by Piornos *et al*. (2015), that is, the lowest foaming stability (FS) value was registered at pH 4 (30.68%) because of the protein's PI. However, FS increased at higher and lower pH values. Alu'datt *et al*. (2017) also studied the foaming stability of LPIs and agreed with our results 50%. The results of nano protein isolate for sweet, and bitter isolate are 81.65, and 78.50%, which shows that sweet protein isolate has higher foaming stability than bitter protein isolate.

Consequently, the results showed that, among treatments for all samples, the foaming capacity revealed higher values in nano treatment, because that protein-based particles foaming and surface behavior depends on processing conditions like ionic strength modification, heating, and pH value (Wang *et al.*, 2012) These treatments will impact surface charge, and size of protein particles, therefore, surface behavior variations. Some researchers (Ruíz-Henestrosa, *et al.*, 2007; Wan *et al.*, 2016) have reported that protein modifications by physical treatment could increase their foaming properties. Also, these treatments could modify the net charge of particles, which will lead to the decrease of the energy barrier to adsorption at the air/water interface (Peng *et al.*, 2018).

Water Holding Capacity

Water Holding Capacity (WHC) is an important property for food processing and quality. Also, WHC is the ability of a protein matrix to absorb and retain bound, hydrodynamic, capillary, and physically entrapped water against gravity (Liu *et al.*, 2018).

Results in Table 1 show that the percentage of WHC of non-nano sweet LPI, and bitter LPI are, respectively, 58.50 and 51.50%, whereas, WHC for nano LPI of sweet, and bitter are 67, and 77.21%, respectively. The result concerning LPI goes with what was reported by Alu'datt *et al.* (2017), who found that WHC capacity of the lupin isolate was 33%. The significant variations in functional properties are likely due to the variation in protein content, which reflects the ability of flour to imbibe water (Shevkani *et al.*, 2014).

Antioxidant Activity

Non-nano protein isolates antioxidant values (Table 2) for sweet and bitter lupine are 2.03 and 1.61%, while for sweet and bitter nano protein isolates, the values are -1.75 and -1.40%, repectively. According to the statistical analysis, there were no significant differences between nano and non-nano protein samples in each category. Moreover, sweet lupine exhibited better antioxidant activity than bitter lupine in both phases (nano and non-nano). However, none of the samples exhibited a high total antioxidant content. These findings were in agreement with Wang *et al.* (2012) who ascribed this phenomenon to the relation between frequency and the number of cavitation bubbles. When the frequency increased, not only did the number of

Table 2. Antioxidant activity (% of inhibition) of non-nano and nano samples of LPIS (Sweet), LPIB (Bitter), LPINS (Nano Sweet), LPINB (Nano Bitter).

Trt	Antioxidant activity (%)
LPIS	2.03 ± 0.20 a
LPINS	(-1.75) ± 0.99 b
LPIB	1.61 ± 0.40 a
LPINB	(-1.40) ± 0.30 b

[a](a-d) The same letter within the same column are not significantly different (P≤ 0.05). Means±standard deviation (n=3).

[DOR: 20.1001.1.16807073.2021.23.4.12.1]

Table 3. Inhibitory Activity of Angiotensin I-Converting Enzyme (% of inhibition) of nano, non-nano LPIS (Sweet), LPIB (Bitter), LPINS (Nano Sweet), LPINB (Nano Bitter).

Trt	ACE inhibitory activity (%)	Alpha-amylase inhibition (%)
LPIS	71.93 + 0.76 b	87.28 ± 5.99 a
LPINS	68.59 + 0.46 c	78.77 ± 6.04 a
LPIB	46.59 + 0.61 d	61.84 ± 5.99 b
LPINB	75.72 + 1.06 a	87.28 ± 5.99 a

[a] (a-d) The same letter within the same column are not significantly different (P≤ 0.05). Means±standard deviation (n=3).

cavitation bubbles increased but also the size of these bubbles became smaller, thereby it may be inferred DPPH free radical scavenging rates decreased with increasing ultrasonic power, time, and temperature.

Enzymatic Assay

Alpha-Amylase

The results in Table 3 show the values in percent of alpha-amylase of sweet and bitter isolates and indicate that there is considerable percentage of inhibition activity of α-amylase in lupine samples. The results indicated that non-nano sweet, and bitter lupine protein isolate are 87.28 and 61.84%, while the nano samples of sweet and bitter lupin protein isolate are 78.77 and 87.28%, respectively. The results of all samples of lupine isolate for nano samples did not differ much than non-nano samples, though some results showed lower values. The obtained results can be explained with what was found by Barton *et al.* (1996), that is, the sonication process increases the efficiency of the mechanisms of mixing and diffusion of components, where, it enhances the rate of catalyzed enzyme hydrolysis of starch and sucrose, though ultrasound influence the intermolecular interactions of the enzyme invertase and improve the enzymatic efficiency.

ACE

The results in Table 3 show the inhibitory activity of the Angiotensin-Converting Enzyme (ACE) of nano samples of sweet and bitter LPIs. The ACE-inhibition was determined by a modified method described previously (Cushman and Cheung, 1971) and all samples exhibit a high percentage of the inhibition activity of the ACE.

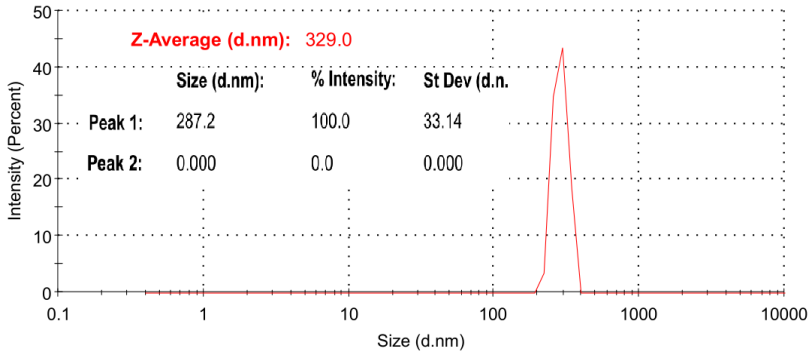
Results shown in Table 3 indicated that the nano sample of bitter LPI (75.72%) showed a higher value than non-nano (46.59%), while the nano sample of sweet LPI (46.59%) was lower than the non-nano sample (68.59%).

The results proved that the lupine samples had an effect on blood pressure, and improved the vascular function; which agrees with what was reported by Pilvi *et al. (* 2006) that the high ACE effect is related to high arginine content of lupine protein 99.3 mg g^{-1}. Arginine is a physiological substrate for endothelial Nitric Oxide Synthase (eNOS) and supplementation with L-arginine has been shown to reduce blood pressure and improve vascular function. The results proved that sonication increased the inhibitory activity of the ACE for bitter protein isolate, which agrees with what was reported by Zhou *et al.* (2013) and Daskaya-Dikmen *et al*. (2017).

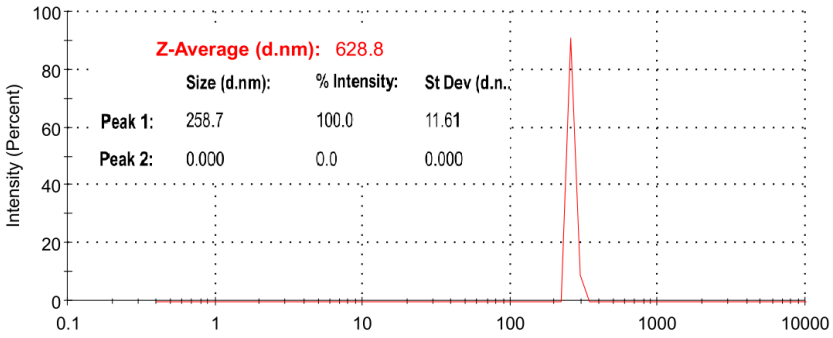
Microstructure Characterization

The size of sonicated and non-sonicated proteins was measured by dynamic light scattering in Figure 1 (DLS) using a Zeta-sizer Nano Series (Malvern Instruments, UK). The mean hydrodynamic diameter was reported as Z-average (dz). The protein size and span values are reported as the average

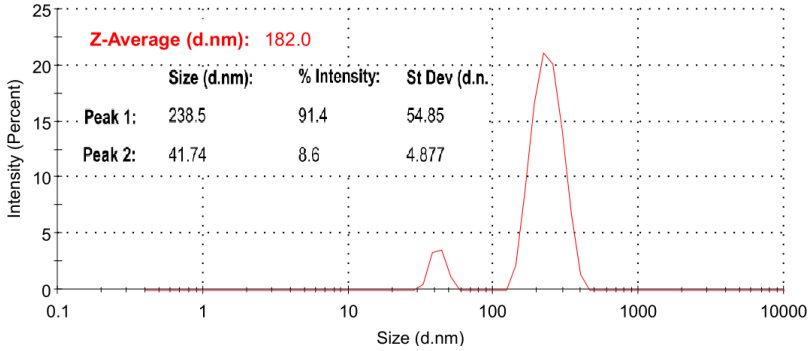
[DOR: 20.1001.1.16807073.2021.23.4.12.1]



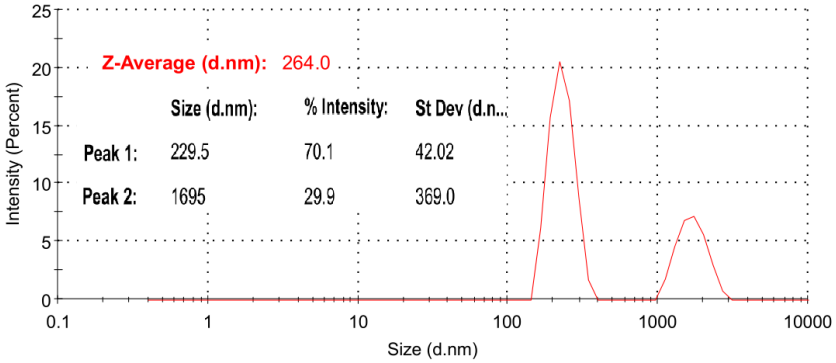
(a) LPIB



(b) LPIS



(c) LPINB



(d)LPINS

Figure 1. DLS where: (a) LPIB (Bitter), (b) LPIS (Sweet); (c) LPINB (Nano Bitter), and (d) LPINS (Nano Sweet).

[DOR: 20.1001.1.16807073.2021.23.4.12.1]

and the standard deviation of three repeat measurements. Stock samples were suspended in water at a concentration of 100 µg L^{-1}, then filtered with a 0.4 µm syringe filter, and diluted 1:10 with distilled water (10 µg mL^{-1}).

Untreated protein samples exhibit a single-mode population in the DLS patterns (Figure 1). However, after ultrasound treatment, all patterns show a bimodal population, one population having a quite similar size as the parent untreated protein, and the other population is nano-sized.

Primary results show that for all sonicated samples, an evolution of new peaks was observed with relatively small average size (dz) compared to untreated samples, in all of which just one single peak appears in the DLS patterns Figure 1 (a to h). The figure also shows that there is a significant reduction in protein size with sonication. This may suggest that increasing the time duration and the power of ultrasonic irradiation may reduce the size and change the shape of the protein aggregates. Moreover, this decrease in protein size can be attributed to disruption of the hydrophobic and electrostatic interactions, which maintain untreated protein aggregates from the high hydrodynamic shear forces associated with ultrasonic cavitation. However, the variation of size reduction of the protein agglomerates depends on the highly aggregated structure and the insolubility of the protein components.

In order to validate these hypotheses, Scanning Electron Microscopy (SEM) images were captured at room temperature for samples with and without sonication (Figures 2-a to -h).

SEM results reveal a significant change in the morphology, distortion of particles shape, and reduction of size of the proteins particles after sonication.

It can be concluded that sonication leads to distortion/breakage of protein particles shape, which results in smaller sized protein that can be used as a top down approach for formation of nano-sized protein particles. In this regard, sonication can be considered as a heterogeneous approach that may lead to de-aggregation, breaking of protein particles, and distortion of their shape.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In lupines, albumins and three globulins fractions, namely, β-conglutin, α-conglutin and γ-conglutin, have been characterized as the main storage proteins (Figure 3). β-conglutin, usually the major component, presents the greatest heterogeneity between species showing numerous polypeptide chains with molecular masses from 15 to72 kDa. The α-conglutin fraction is composed of some heavy polypeptide chains (from 31 to 63 kDa) and a lighter polypeptide chain (20 kDa). And γ-conglutin, generally the minor component, contains two polypeptide chains (one of around 17 kDa and another of around 27–30 kDa). In general, no clear differences were observed between nano and non-nano samples regarding albumins and three globulins fractions in the same type of lupine, while a clear difference was observed between bitter and sweet lupine.

CONCLUSIONS

In general, no clear differences were observed between nano and non-nano samples regarding albumins and three globulins fractions in the same type of lupine, while a clear difference was observed between bitter and sweet lupine. Functional properties showed variations in the samples, emulsion stability decreased in protein isolate samples. The sonication leads to distortion/breakage of protein particles shape, which results in smaller sized protein that can be used as a top down approach for formation of nano-sized protein particles. In this regard, sonication can be considered as a heterogeneous approach, which may lead to de-aggregation, breaking of protein particles, and distortion of their shape. This study demonstrated that the sonication

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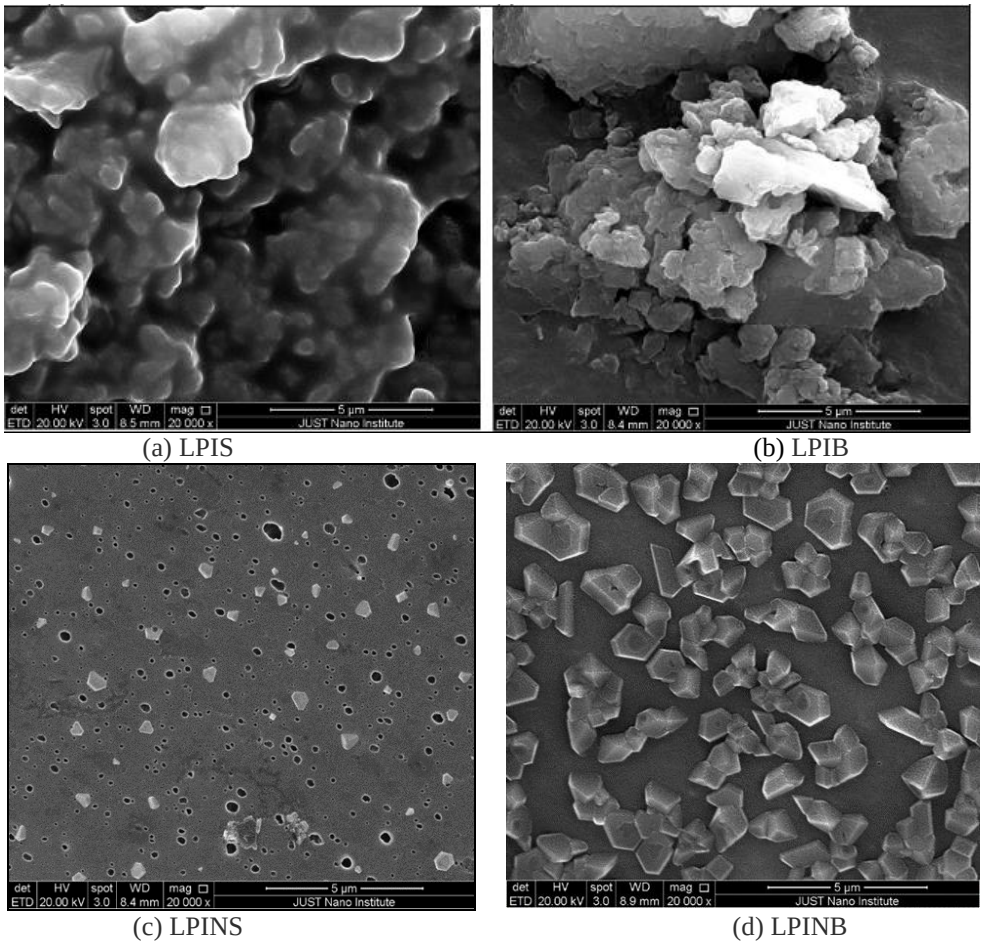

Figure 2. SEM images of Nano and non-nano lupine protein sample: (a) LPIS (Sweet); (b) LPIB (Bitter); (c) LPINS (Nano Sweet), and (d) LPINB (Nano Bitter).

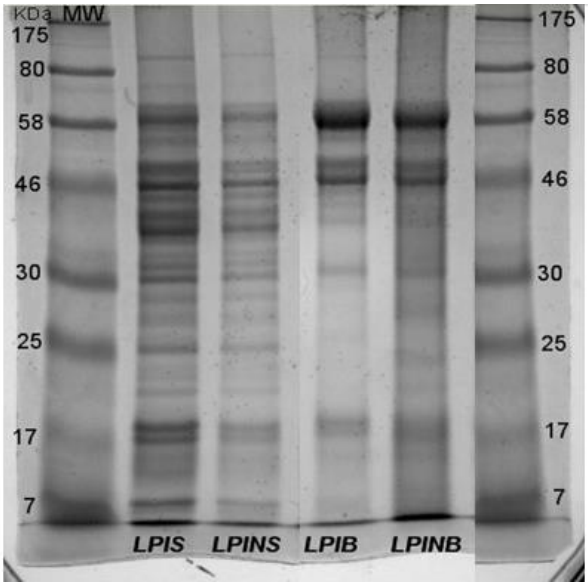

Figure 3. Sodium DodecylSulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) patterns o nano and non-nano lupine protein samples. LPIS (Sweet); LPINS (Nano Sweet); LPIB (Bitter), and LPINB (Nano Bitter).

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treatment may be a useful means to modify lupine protein for enhancing its functional properties.

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اثر تیمار فراصوتی روی خواص فیزیکوشیمیایی، غذا دارویی، و عملکردی آرد باقلای مصری (Lupine)

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چکیده

هدف این پژوهش، تبدیل جدایه های تلخ و شیرین پروتئین باقلای مصری به نانوامولسیون و تهیه محصولاتی با نانوامولسیون و بررسی پایداری آنها، ایجاد امولسیون با فرایند فراصوتی، تهیه و مشخص کردن نانو امولسیون های تثبیت شده پروتئین غذا، و نیز تغییر خواص پروتئین باقلای مصری با تغییر پی اچ و فرایند فراصوتی بود. نتایج حاکی از کم شدن فعالیت آنتی اکسیدانی جدایه های تلخ و شیرین در نمونه دارای ذرات به اندازه نانو در مقایسه با نمونه بدون ذرات نانو بود، و نیز فعالیت های بازدارندگی هردو آنزیم "تبدیل کننده آنژیوتانسین" و آلفا آمیلاز کاهش یافت در حالیکه این مووارد درجدایه های شیرین و تلخ باقلای مصری افزایش نشان داد. برای هر دو جدایه های باقلای مصری، ظرفیت نگهداری آب افزایش یافت و پایداری کف (foaming stability) تیمار نانو در نمونه جدایه های پروتئین افزایش داشت. در باقلای مصری ، البومین ها و سه بخش از گلوبولین به نام های β-conglutin، α-conglutin، و γ-conglutin به عنوان پروتئین های ذخیره سازی شناسایی شد. در ارتباط با آلبومین و این سه ماده هیچ تفاوت روشنی بین نمونه های نانو و غیرنانو مشاهده نشد. نتایج بررسی ریزساختار نشان داد که کاربرد امواج صوتی(sonication)منجر به شکسته شدن و مخدوش شدن شکل ذرات پروتئین می شود که این امر به کوچک شدن پروتئین ها میانجامد و در نتیجه می توان از رویکرد بالا به پایین برای ایجاد ذرات پروتئین هم اندازه نانو استفاده کرد. در این پیوند، کاربرد امواج صوتی(فراصوتی) را می توان به عنوان رویکردی ناهمگن در نظرگرفت که ممکن است به تجمع زدایی (de-aggregation)، شکسته شدن ذرات پروتئین، و ومخدوش شدن شکل آنها بیانجامد.

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