Influence of ultrasound probe treatment time and protease type on functional and physicochemical characteristics of egg white protein hydrolysates

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ABSTRACT The objective of this study was to discover the relationship between the ultrasound probe treatment (UPT) on egg white proteins (EWPs) before EWPs hydrolysis by different proteases, and the functional properties of the obtained hydrolysates. To fulfill this goal, the protein solubility, foaming, and emulsifying properties were studied as a function of the UPT time and then related to the surface characteristics and structural properties. The changes in the hydrolysates microstructures and macromolecular conformation, induced by the UPT, were followed using scanning electron microscope analyzis (SEM) and Fourier transforms infrared spectroscopy (FTIR). The results showed that UPT influenced ($P < 0.05$) the proteolysis of egg white proteins for all examined treatment times. Alcalase hydrolysates (AHs) and papain hydrolysates (PHs) were found to have a higher solubility, as a consequence of their relatively higher foaming, and emulsifying properties compared to the untreated hydrolysates. The changes in surface hydrophobicity, sulfhydryl content and surface charge of AHs and PHs indicated unfolding of EWPs affected by ultrasound. SEM analyzis showed that UPT destroyed the microstructures of AHs and PHs, while FTIR spectra indicated remarkable changes in the macromolecular conformation of AHs and PHs after UPT. This study revealed that by combining ultrasound pre-hydrolysis treatment under controlled conditions with thoughtful proteases selection, hydrolysates with improved functional properties could be produced, enhancing utilization of EWPs in food products.

Key words: functionality of egg white, hydrolysates, ultrasound probe treatment, structural characterization, surface characteristics

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INTRODUCTION

Innumerable investigations to create new protein ingredients and processes, which would contribute to the development of the functional foods, have been performed in recent years. In the area of food science and technology, protein functionality has always been among the most inconsistently and ambiguously defined topics. Development of "new" or "emerging" technologies to monitor possible changes in the structure of proteins and the structure-function relationships, such as microwaves, high hydrostatic pressures, and high-intensity ultrasound has been the target research of food scientists (Pour-El, 1981).

In addition to their contribution to the human nutrition, proteins are functional ingredients that can affect sensory, kinesthetic, hydration, surface and rheological/textural properties of foods, pharmaceuticals, and healthcare products. Therefore, it can be rightly said that the protein functionality is a direct manifestation of protein structure and molecular interactions (Phillips et al., 1994; McClements, 2002). Among a wide range of different protein sources, egg white proteins (**EWPs**) possess properties that make them attractive as a protein source in human nutrition due to the high bioavailability, high content of essential amino acids and unique functional characteristics such as foaming, gelling and, emulsification (Mine, 1995; Chen and Chi, 2012; Lunow et al., 2013; Arzeni et al., 2015). Enzymatic hydrolysis is widely employed to improve and upgrade the functional and nutritional properties of EWPs and obtain value-added egg products (Knežević-Jugović et al., 2012; Stefanović et al., 2014; Jovanović et al., 2016). EWP hydrolysates (**EWPHs**) provide the number of benefits as the protein source in human nutrition, in regard to the native EWPs, having a positive

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impact on body functions and may ultimately influence health. Moreover, EWPHs obtained by protease treatment have shown antihypertensive, antioxidant and antimicrobial activities (Pellegrini et al., 2004; Liu et al., 2010). Enzyme type and degree of hydrolysis (**DH**) are substantial factors affecting the hydrolysates' performance, so as the costs associated with the enzymatic hydrolysis are relatively high, an appropriate selection of protease for EWP hydrolysis will result in the high-quality hydrolysate providing a low-cost process with high productivity. Proteases of microbial origin, such as alcalase, neutrase, and flavourzyme, are the most common enzymes used in producing protein hydrolysates. Nevertheless, protease from the plant (e.g., papain) and animal sources (digestive enzymes, e.g., pepsin and trypsin) are also intensively used (Eckert et al., 2013; Lin et al., 2013; Singh and Ramaswamy, 2014). Therefore, proteases derived from cheap by-products and microorganisms have recently drawn increased interest for the production of protein hydrolysates on a commercial scale due to their unique functions and specificity, broad accessibility, and cost-effectiveness.

An essential tool in generating peptides with desired functionality is the usage of the substrate pretreatment before enzymatic hydrolysis due to insufficient release of functional peptides from their parent proteins by enzymatic hydrolysis (Zhang et al., 2015). Thermal treatment is often applied to modify the hydrolysates' performance, but emerging technologies like high hydrostatic pressure and ultrasound are considered to be more environmentally friendly while demonstrating satisfactory results (Knežević-Jugović et al., 2012; Chao et al., 2013; Zhang et al., 2015). It is considered that the utilization of high-intensity ultrasound (**HIU**) can modify protein conformation by affecting hydrogen bonds and hydrophobic interactions due to the cavitation phenomenon allowing more hydrolysis sites to be accessible by the enzyme, causing an increase in the degree of hydrolysis and functionality (O'Sullivan et al., 2016). Numerous studies have shown that ultrasound pretreatment can change the functional and structural properties of proteins by altering their molecular characteristics (Arzeni et al., 2012; Yanjun et al., 2014; O'Sullivan et al., 2016). The effects of HIU on proteins are increasingly being studied, showing it could induce macroscopic changes like reduced viscosity and turbidity or improve some functional properties such as gelling, solubility, foaming, and emulsifying properties (Arzeni et al., 2012; O'Sullivan et al., 2016; Zhou et al., 2016). However, the HIU treatment has to be controlled because prolonged treatments often cause aggregation with diminishing functional properties when applied to complex biological systems. Recently, Abadia-García et al. (2016) investigated the effect of ultrasound pretreatment (750 W and 20 kHz) on the whey protein hydrolysis and observed the DH increase between 26 and 63% compared to control treatment without ultrasound. Regarding other protein sources, Li et al.(2016) studied the effect of ultrasound pretreatment (58 W/L, 28 kHz, 15 min) on the proteolysis and structural characteristics of rice protein. According to the results shown in this study, ultrasound pretreatment significantly improved the DH, destroyed the microstructures, and reduced the particle size of rice protein. In contrast to these works, Lei et al. (2011) did not find an ultrasound effect (20 and 60 kHz) on the overall DH in ovotransferrin pretreated from 5 to 480 s.

There is a lack of information regarding the effects of ultrasound probe treatment (**UPT**) time on the enzymatic hydrolysis, surface characteristics, functional and structural properties of EWPHs. Based on our knowledge, there are no studies on the effect of the length of UPT and subsequent enzymatic hydrolysis with alcalase and papain, on the sulphydryl content, hydrophobicity and surface charge of the ultrasound probe treated EWPHs. Therefore, this study aimed at evaluating the physicochemical and functional properties of EWPHs obtained from alcalase and papain hydrolysis at different pretreatment times and to assess the changes in the secondary structure and surface morphology.

MATERIALS AND METHODS

Materials

Freshly laid chicken eggs (pH 7.6–8.6), kindly provided from PKB "Inshri", Padinska Skela, were stored at 4°C and used within 24 h after collection. Egg white was separated manually from the yolk (chalaza was removed) and gently stirred without foam formation to provide the homogeneous mixture. The enzymes used in this research to determine the susceptibility of ultrasound treated samples to proteolysis were alcalase 2.4 L (EC 3.4.21.14), a non-specific bacterial endopeptidase from *Bacillus licheniformis* and papain from papaya latex (EC 3.4.22.2), provided by Sigma Aldrich (St. Louis, MO). The claimed enzyme activities were $\geq$ 2.4 U Anson Units/g solid and 1.5–10 U/mg solid for alcalase and papain respectively. The deionized water (18.2 MΩ) used for the experiments was produced using the Thermo Scientific Barnstead Smart2Pure water purification system. All other chemicals were of analytical reagent grade, and they were used without any further purification.

Ultrasound Probe Treatment of EWPs

The series of 10 wt.% egg-white aqueous solutions were exposed to the ultrasound waves using a probe-type sonicator (frequency of 20 ± 0.2 kHz) and treated for different time (2, 5, 10, 15, and 20 min) with an amplitude of 40% (Dojčinović and Volkov-Husović, 2008). A 13 mm high-grade titanium tip was immersed in the liquid, and the liquid was irradiated with an ultrasonic wave directly from the horn tip. The samples were prepared in a custom made glass reaction water-jacketed vessel of 300 mL capacity where the working

volume (∼180 mL) was kept constant for all experiment sets. The constant temperature (25 ± 1°C) was maintained by circulating thermostated water through a water jacket (ordinary water flow: from 5 to 10 mL/s).

In order to determine the power of ultrasound probe the samples were subjected to the ultrasonication for different time (2, 5, 10, 15, and 20 min) and the temperature change was monitored by evaluating the temperature over a specified period (e.g., 1, 2, and 5 minutes) using a thermocouple with digital display and then calculated the average increase in temperature after several treatments. The power of ultrasound probe was designated calorimetrically according to the following equation (Tiwari and Mason, 2012):

$$P = mC_p\left(\frac{dT}{dt}\right)_{t=0} \quad (1)$$

where m is the mass, C_p is the specific heat capacity and (dT/dt) is the initial rate of change of temperature during ultrasonication. This was determined by fitting the data obtained for temperature rise against time to a polynomial curve and extrapolating to time.

The value of actual ultrasonic power dissipated in the liquid under adiabatic conditions has been calculated to be 34.11 ± 1.46 W. The pH values of protein model solutions were also checked before and after ultrasound treatment at 20 ± 0.2 kHz for 5 to 20 min by pH meter Eutech instrument, Netherlands. Values of pH did not change significantly ($P > 0.05$) upon the treatment.

The Enzymatic Hydrolysis of EWPs

The susceptibility of ultrasound treated EWPs to enzymatic hydrolysis was detected by incubation of 180 mL of treated samples (containing 10.7±1.8 mg/mL, protein determined according to the standard Kjeldahl method, $N \times 6.25$, pH 8.0) with a proper amount of enzymes (E/S ratio was 0.22) at 50°C. The enzymatic hydrolysis were performed in a mechanically stirred batch reactor at a speed of 240 rpm with temperature and pH control. The pH was maintained by continuous addition of 0.2 M NaOH during the enzyme reaction. All hydrolysis were performed during a 240 min and after the end of reaction mixtures were inactivated by heating for 15 min at 95 °C and then centrifuged at 12,000 × g for 10 min after cooling at room temperature. The supernatant was collected and stored at 4°C for further analyzis. Enzymatic hydrolysis, without ultrasound probe treatment was used as the control.

The progress of the reaction was followed by monitoring the degree of hydrolysis (DH, %) using the pH-stat method. The DH was calculated according to the following equation (Adler-Nissen, 1986):

$$DH\ (\%) = \frac{h \cdot 100}{h_{tot}} = \frac{N_b \cdot B \cdot 100}{\alpha \cdot m_p \cdot h_{tot}} \quad (2)$$

where h is the number of equivalents of peptide bonds hydrolyzed at the time per weight unit; h_{tot} is the total amount of peptide bonds per weight unit of a protein and can be calculated from its amino acid composition (for EWPs h_{tot} is 7.67 mmol/g protein), N_b is the normality of the base, B is the consumption of the base in mL, α is the degree of dissociation of the α-amino groups ($1/\alpha = 1.13$ at 50°C and pH 8.0) and m_p is the mass of protein in grams.

Functional Properties of Ultrasound Probe Treated EWPHs

Protein Solubility To determine protein solubility of EWPHs, samples were diluted in water to final concentration of 1 mg/mL and pH was adjusted to 2, 4, 6, 8, 10, and 12 using 0.5 M NaOH or 0.5 M HCl while stirring at room temperature for 1 h. The samples were then centrifuged at 7,889 × g and 4°C for 15 min. The protein contents in the supernatant were determined using the Sigma Procedure No. TRPO-562 using bovine serum albumin as the standard (Lai et al., 2010). Solubility was expressed as the percentage of protein remaining in the supernatant as compared to the untreated samples.

Determination of Emulsion Activity and Stability Emulsion activity index (**EAI**) and emulsion stability index (**ESI**) were determined according to the method by Pearce and Kinsella (1978) except for the noted changes in each section. Emulsions of each EWPHs dispersion (3% w/w) were prepared with sunflower oil in ratio 2:1 and mixing for 90 s using a homogenizer (Yellowline, DI 25 basic, Ica Works Inc., Wilmington, 600 W, 50 V) at 8,000 rpm at 25 °C. The absorbance of the diluted emulsions was measured at 500 nm immediately after emulsion formation by UV/Vis spectrophotometer (Amersham Bioscience, Ultrospec 3300 pro). The turbidity was calculated by the following formula:

$$T = 2.303\frac{A}{l} \quad (3)$$

where T is the turbidity, A is the absorbance at 500 nm and l is the path length (m). The emulsion activity index (EAI) was then calculated as:

$$EAI = 2 \cdot T\frac{r}{c \cdot \theta} \quad (4)$$

where θ is the volume fraction (mL), c is the weight of protein per unit volume of aqueous phase before emulsion is formed (g) and r is the dilution factor.

For determining the emulsion stability, the EWPH dispersions were kept at 4°C for 24 h and analyzed for emulsifying activity as described previously. The emulsion stability index (ESI) was calculated by the following formula:

$$ESI = \frac{T \cdot \Delta t}{\Delta T} \quad (5)$$

where T is turbidity value at 0 h, ΔT is a change in turbidity during 24 h period and Δt is the time interval (24 h).

Determination of Foaming Properties For assessing foaming properties, foam capacity (**FC**) and foam stability (**FS**) were determined. The analyzis was according to the method as described previously (Stefanović et al., 2014). An aliquot (50 mL) of EWPHs solution (8 mg protein/mL solvent) was placed in a graduated glass cylinder (internal diameter 72.0 mm) in a water bath at 20°C and whipped for 4 min with a laboratory homogenizer at a speed of 9,500 rpm (Yellowline, DI 25 basic, Ica Works Inc., Wilmington, 600 W, 50 V, 8,000–24,000 rpm). For all tested samples, pH values were 8.0. After whipping, the propeller was immediately removed and the glass cylinder sealed with parafilm to avoid the foam disruption. The FC was determined by measuring the foam volume after whipping (mL) at 0 min and expressed as the percentage of the initial volume before the whipping (mL). The FS was measured by determining the foam volume after 30 min of standing (mL) and expressing it as the percentage of the FC (Stefanović et al., 2014).

Measurement of Surface Characteristics

Surface Charge (Zeta Potential) The zeta potential of untreated (control) and ultrasound probe treated EWPHs were measured with a Zetasizer Nano ZS (Malvern Instruments, UK) with automatic titrator unit using a universal dip cell in disposable cuvettes. The titrator unit was equipped with a sample container, which was connected through a capillary system, and with a peristaltic pump with a folded capillary cell. Before the automatic titration, freshly prepared protein solutions were filtered using a 0.45 μm filter. pH values of the samples were between 7.6 and 8.0. The Smoluchowski approximation was used to convert the electrophoretic mobility to a zeta potential using the Smoluchowski equation (Shaw, 1992).

$$\xi = \mu\eta/\varepsilon \tag{6}$$

where ξ is the zeta potential, μ is the mobility, η is the viscosity of the solution, and ε is the dielectric constant of the solvent. Experiments consisted of 30 runs per measurement, and all experiments were carried out in triplicate. The mean of each triplicate measurement is shown. The average value and standard deviation of 30 measurements per sample were reported.

Surface Hydrophobicity (H_0) Measurements Surface hydrophobicity of EWPHs was determined with the fluorescence probe 1-anilino-8- naphthalenesulfonate (**ANS**) according to the method of Mu et al. (2010), with minor modifications. Ultrasound probe treated and control samples were diluted (0.0025 to 0.5% v/v) with phosphate buffer (0.1 M, pH 7). Dilutions were poured into quartz cuvettes, and the fluorescence intensity (**FI**) was measured at 25°C using a Horiba FluoroMax-4 spectrofluorometer at 365 nm (excitation wavelength) and 480 nm (emission wavelength) with a constant excitation and emission slit of 2.5 nm and 10 nm/s of scanning speed. Then 7 μL of ANS (8.0 mM in phosphate buffer 0.01 M, pH 7) was added to 1.393 mL of protein solutions, and the fluorescence intensity was read. Surface hydrophobicity was expressed as the initial slope of the plot of fluorescence intensity as a function of protein concentration. All the determinations were conducted in triplicate.

Determination of Sulfhydryl (SH) Groups The content of SH groups of EWPHs upon treatment with the ultrasound probe was determined spectrophotometrically by Ellman's procedure using 5,5′-(dithiobis-2-nitrobenzoate) (**DTNB**), which reacted with free SH groups to yield a product with a maximum absorbance at 412 nm. The analyzis was according to the method as previously described (Shimada and Cheftel, 1988; Stefanović et al., 2017).

Spray Drying Conditions and Field Emission Scanning Electron Microscopy The samples (pretreated and untreated EWPHs) were spray-dried using a pilot scale spray dryer (BÜCHI Dryer B-290, Büchi, Switzerland). The EWPHs solutions were fed by a peristaltic pump at a fixed rate of 0.3 L/h. Inlet and outlet air temperatures were set to 120°C and 90°C, respectively. The obtained powder was collected at the cyclone. The products were stored in glass bottles at room temperature, in a dry place, in the absence of light.

The surface morphology of the obtained pretreated and untreated EWPHs spray dried powders were studied by field emission scanning electronic microscopy (**FESEM**) Mira 3 XMU (Czech Republic) at an accelerating voltage of 5 kV. Prior to the FESEM analyzis, the powder samples were coated with gold in an argon atmosphere using a spatter coater.

Molecular Structure Characterization Measured by FTIR Spectroscopy Fourier transforms infrared spectroscopy (**FTIR**) absorbance spectra of EWPHs (spray-dried powder) were acquired using an ATR-FTIR spectrometer IRAffinity-1 (Schimadzu, Japan). Spectra (100 accumulated scans at 4 cm^{-1} resolution) were collected over the frequency range of 500 to 3,500 cm^{-1}. The FTIR spectra of each sample were performed in triplicate and the results were reported as the averages of these replicates (relative standard deviation <5%).

Statistical Analysis

In this research, all experiments were carried out at least in triplicate and expressed as means with standard error. Analysis of variance (**ANOVA**) followed by the Tukey test was performed to compare the effects of ultrasound probe under the significance level of $P < 0.05$. All statistical analysis, including calculations were conducted using OriginPro 9.0 (OriginLab Corporation, Northampton, MA).

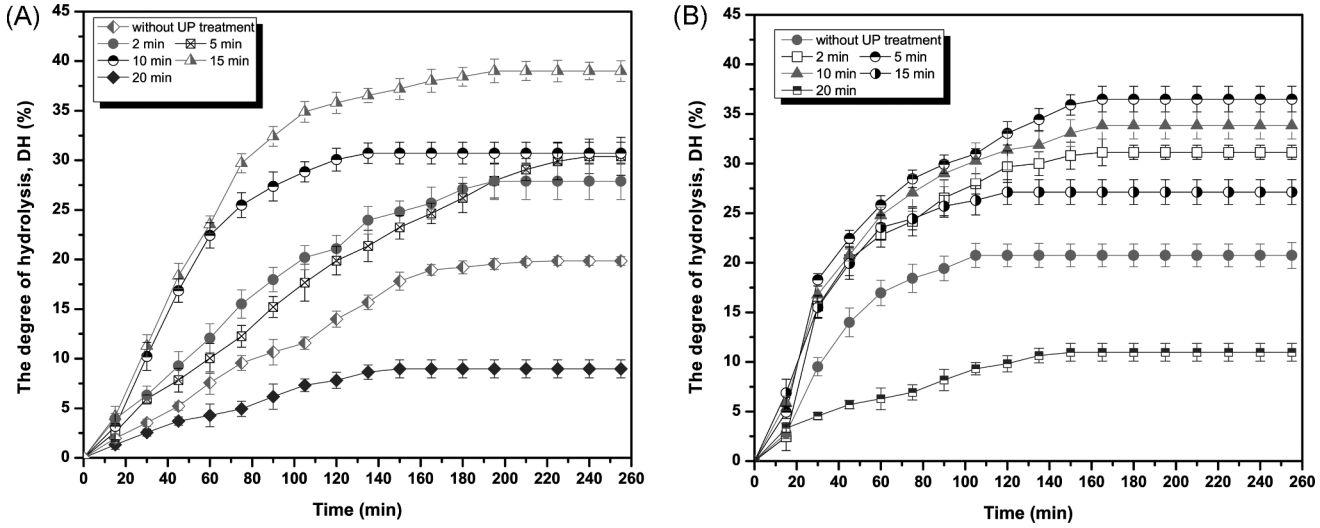

Figure 1. The time-course of hydrolysis by alcalase (A) and papain (B) as a function of ultrasound treatment time. Hydrolysis conditions were as follows: 10% (w/w) egg white; 50°C and pH 8.0 for alcalase and papain, E/S ratio 0.22.

RESULTS AND DISCUSSION

The Effect UPT on EWP Hydrolysis

Owing to the link between structural integrity and susceptibility to enzymatic hydrolysis for many proteins cited in the literature (Liu et al., 2014), the protein structural changes associated with ultrasound application were studied regarding their susceptibility to hydrolysis by the commercially available food-grade proteases alcalase and papain. It is apparent from Figure 1 that the UPT changed the proteolytic pattern of EWPHs ($P < 0.05$) for all examined treatment times. As shown, the initial rate and DH increased with the increase of the pretreatment time up to 15 min followed by the gradual decrease with further ultrasonication for both enzymes. A higher degree of hydrolysis was observed in ultrasound probe (**UP**) pretreated alcalase-hydrolyzed samples than UP pretreated papain-hydrolyzed samples. This result can be expected due to the random cleavages by alcalase, which preferentially hydrolyzes peptide bonds containing aromatic amino acid residues in comparison to the selective cleavages by papain with a preferred cleavage at basic amino acids, particularly arginine, lysine, and phenylalanine (Waglay and Karboune, 2016).

Changes in Degree of Hydrolysis of EWPHs

Because of the endo-type mode of action of alcalase and papain and due to the presence of the desirable cleavage sites, the DH values for both enzymes without UPT are nearly the same, but after applying UPT, different final levels of DH were observed after the various duration of UPT (Figure 1). The highest DH values of 36.5% and 39.01% were detected after 5 and 15 min UPT for alcalase and papain, respectively, indicating the lesser exposure to ultrasound waves for papain probably due to his small ability to act also as an exopeptidase, causing a more efficient hydrolysis after only 5 min pretreatment. The incipient increased susceptibility to enzymatic hydrolysis could be ascribed to ultrasonically induced conformational changes, which can induce full or partial unfolding of polypeptides, leading to the exposure of buried peptide bonds and making them more accessible to the enzyme attack. However, HIU treatment for 20 min decreased the susceptibility of EWPs remarkably to enzymatic hydrolysis, suggesting protein aggregation, which in turn protected the internal bonds of the proteins.

Changes in Functional Properties of EWPHs

Solubility The increase in protein solubility is the most notable effect on the protein functional properties after the hydrolysis process presented in the large number of articles in the literature, and is also an important aspect that needs to be considered due to its considerable effect on other techno-functional properties and quality of the end product (Pour-El, 1981).

The impact of UPT on the solubility of the EWPHs prepared by alcalase and papain in the pH range of 2 to 12 as a function of UPT time is presented in Figure 2. Both hydrolysates were soluble over a wide pH range with more than 72% solubility for all treatment times applied, except for 20 min (results are not presented in Figure 2). The solubility of hydrolysates was comparatively lower (54.2%) at pH 4, whereas solubilities above 80 and 70% were noticeable at other pH values and for longer a UPT time for alcalase and papain, respectively. From these results, it can be assumed that some protein and peptides with high molecular weight and capable of precipitating at this pH, which is close to the isoelectric point (pI) of egg white proteins (pH 5), remained present after hydrolysis. The increase in solubility as a function of UPT time was positively correlated with

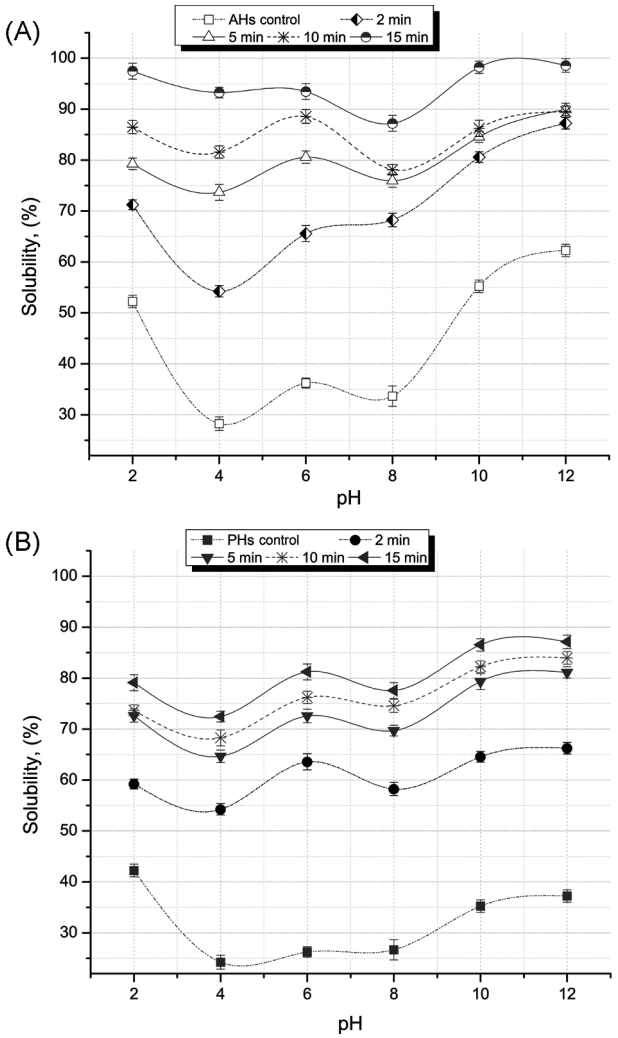

Figure 2. Solubility profiles of (A) alcalase hydrolysates (AHs) and (B) papain hydrolysates (PHs) as a function of pH. Data are expressed as means ± standard error from triplicate determinations.

DH ($P < 0.05$) among all tested hydrolysates. These results further supported the finding of Liu et al. (2014) and Chalamaiah et al.(2010) who reported that hydrolysates with high DH had excellent solubility. Understanding the impact of UPT is probably hidden in the fact that after UPT hydrophilic parts of amino acids from inside open toward water molecules causing the reorganization of water molecules and increased solubility. Enhancement in EWPHs solubility could be explained by the fact that UPT improved the enzymatic accessibility of egg white proteins. Thus more egg white proteins can be readily hydrolyzed and become soluble.

Impact of UPT on Solubility of EWPHs

Foaming Properties Numerous researchers have found that the ultrasound treatment improved FC and FS of native proteins due to the homogenization effect of ultrasound or increased protein surface hydrophobicity and flexibility, allowing a more efficient adsorption of the protein molecule onto the air-water interface (Jambrak et al., 2009; Mirmoghtadaie et al., 2016). However, less is known about the mutual effect of UPT and enzymatic hydrolysis to enchantment foaming properties of protein hydrolysates.

FC and FS of the hydrolysates prepared by alcalase and papain as a function of UPT time are shown in Figure 3a. As UPT time increased, the hydrolysates prepared by both enzymes displayed higher FC and FS ($P < 0.05$) compared to the control (hydrolysis without UPT). The hydrolysate prepared by alcalase exhibited better foaming properties among all applied treatment times compared to papain hydrolysates ($P < 0.05$), which can be explained by the higher peptide content of AHs. Possibly, the differences of the surface hydrophobicity and charge of peptides could make the divergence in the foam properties between AHs and PHs understandable.

The enhancement in foaming properties of UP pretreated AHs and PHs might be explained by the homogenizing effect of UPT manifested by equal dispersion of protein and air particles, which tends to improve the foaming properties. During UPT EWPs became partially unfolded, which followed the increase in foam's ability when creating foam afterward with the homogenizer. This trend in foaming properties was in agreement with data reported by Bellerini et al.(2016) and Liu et al. (2014) for whey and fish protein hydrolysates. Compared to the findings of Chalamaiah et al. (2010) who reported foam capacities of alcalase and papain protein hydrolysates of meriga fish egg protein, which were 70 and 25%, respectively, the results presented here showed significantly higher values (81 and 64%), and this could be ascribed to the UPT applied before enzymatic hydrolysis. An important notice of this research is the finding that the effect of enhanced foaming properties of EWPHs caused by enzymatic hydrolysis is strongly dependent on the applied UPT before hydrolysis and this improvement is noted for all tested pretreatment times.

Emulsifying Ability The emulsifying ability of the hydrolysates prepared by alcalase and papain as influenced by UPT time is presented in Figure 3b. The better emulsification properties, in terms of both, EAI and ESI, can be noticed for AHs compared to the hydrolysate prepared by papain, irrespective of pretreatment time. AHs pretreated for 15 min produced the maximum EAI and ESI with the values: 169.9 m^2/g and 172.37 h, respectively, while those values for PHs were: 153.16 m^2/g and 71.78 h respectively, but after 5 min of UPT. The enzyme choice is very important because hydrolysis with different proteases leads to differences in emulsifying ability, which is presented by studies performed by Betancur-Ancona et al. (2009) on lima bean protein. Also, in our research, it was observed that enzymatic hydrolysis of UPT EWPHs significantly enhanced emulsifying ability. Nevertheless, literature data concerning these improvements are inconstant. Some authors reported improved emulsification properties of proteins by limited hydrolysis due to the exposure of hydrophobic amino acid residues that may interact with

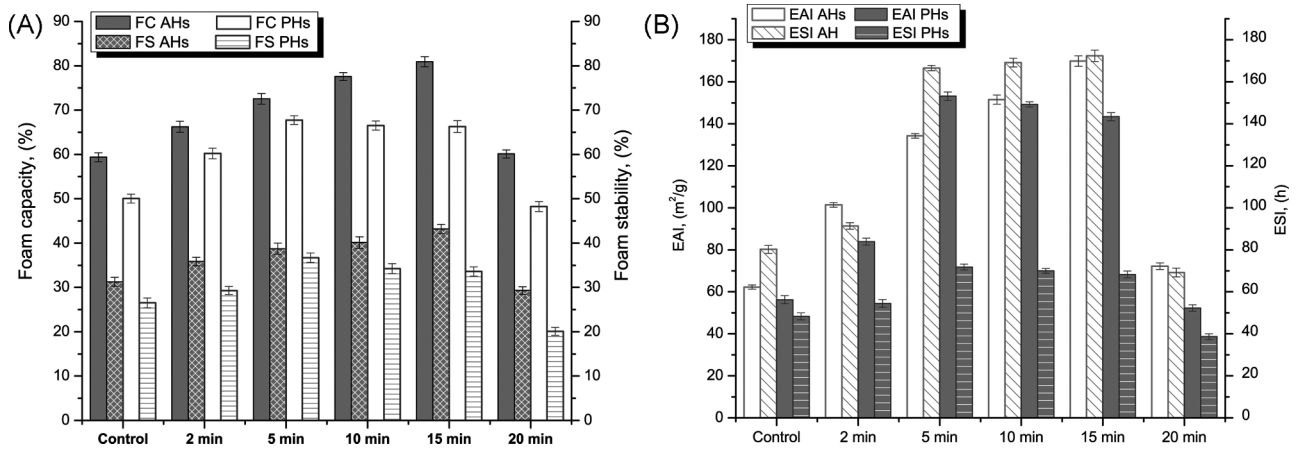

Figure 3. Foam (A) and emulsifying ability (B) of ultrasound probe-treated alcalase hydrolysates (AHs) and papain hydrolysates (PHs) as a function of ultrasound treatment time. Data are expressed as means ± standard error from triplicate determinations.

the oil phase, whereas the hydrophilic residues interact with water (Betancur-Ancona et al., 2009; Zheng et al., 2015). In comparison with un-hydrolyzed treated egg white proteins (Stefanović et al., 2017), the emulsification properties (EAI and ESI) of ultrasound treated alcalase and papain egg white hydrolysates are significantly different. Namely, the emulsification properties depended on the ultrasound probe treatment time, and it seemed to be evident that for treatment time less than 15 min (2, 5, and 10 min) both hydrolysates indicated approximately the same values of EAI and ESI, while for treatment time longer than 15 min (15 and 20 min), the un-hydrolyzed ultrasound-treated egg white proteins demonstrated significantly higher values. These results are in accordance with the literature data because it is generally consider that the enzymatic hydrolysis leads to the loss of emulsification properties because of the formation of smaller size peptides, which cannot form the same molecular interaction as proteins of higher molecular weight (such as un-hydrolyzed egg white proteins). This may be due to their low amphiphilicity that is not enough to evince the good emulsification properties (Jambrak et al., 2009; Zheng et al., 2015). Due to the high degree of hydrolysis, alcalase and papain hydrolysates mainly consist of short peptides, free amino acids and only a limited concentration of surface-active large peptides. Although peptides with lower molecular weight can diffuse quickly and adsorb at the interface, they are less effective than proteins in reducing the interfacial tension, because they cannot unfold and re-orientate at the oil/water interface.

In addition, taking into account the accordance between emulsification properties and solubility, it can be assumed that the higher emulsifying ability of both EWPHs was accompanied by their higher solubility (Figure 2). These findings can be explained by rapid diffusion and adsorption of high soluble hydrolysates at the interface. Results are in accordance with bibliographic data, and a similar phenomenon was also found by Klompong et al. (2007). Likewise, the improved emulsifying ability for both ultrasound probe

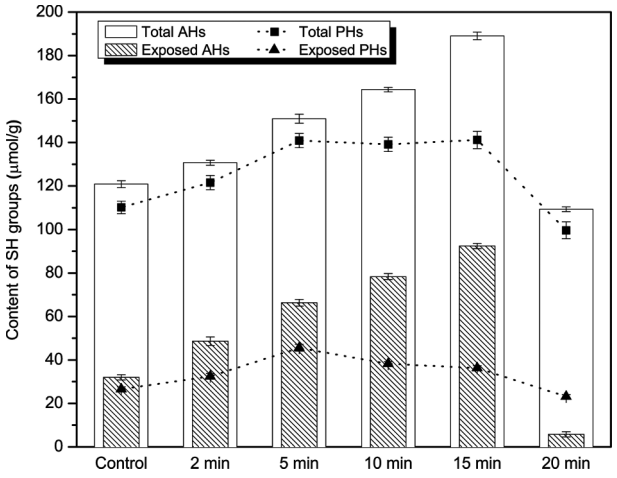

Figure 4. Changes in the sulfhydryl content of ultrasound probe treated alcalase hydrolysates (AHs) and papain hydrolysates (PHs) as a function of ultrasound treatment time. Data are expressed as means ± standard error from triplicate determinations.

pretreated hydrolysates can be correlated with a higher availability of hydrophobic groups after applying a UPT (Figure 5).

Total and Reactive (Surface) SH-group Measurements Results of total and reactive SH-group measurements are presented in Figure 4. As shown, the amount of total and reactive sulfhydryl groups of the both EWPHs showed an increase ($P < 0.05$) up to 15 min, but started to decrease with further ultrasonication. These results uncovered that the UPT induced some degree of unfolding, which is consistent with improved solubility and emulsifying properties (Figure 2 and Figure 3b). The sulfhydryl groups content of both hydrolysates was found to be higher in comparison with control ($P < 0.05$), especially for AHs. Results suggest that mutual effect of UPT and subsequent hydrolysis by different proteases may encourage proteolytic cleavage of the existing protein aggregates until SH groups may take part in this phenomenon by reducing of S-S bonds, which are responsible for maintaining aggregates' structure. Videlicet, in the course of 2 to 15 min of UPT most

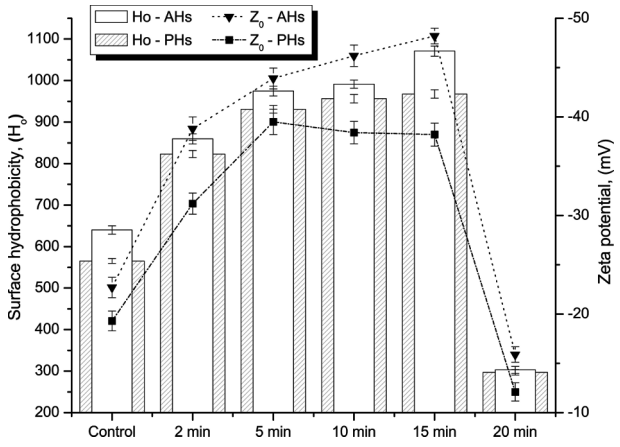

Figure 5. Surface hydrophobicity (H_0) and zeta potential (Z_0) of the alcalase hydrolysates (AHs) and papain hydrolysates (PHs) as a function of the length of ultrasound probe treatment at 20 ± 0.2 kHz. Data are expressed as means ± standard error from triplicate determinations.

sulfhydryl residues in EWPHs existed in the interior of protein molecules were exposed with ultrasound denaturation, but they formed S-S bonds by SH oxidation or SH/S-S interchange reaction after 20 min of UPT. To our knowledge, there are no studies that have investigated the effect of UPT on the sulfhydryl content of EWPHs, but generally, there are many contradictory reports in the literature. Opposite to our results, Zhao et al. (2011) and Surówska et al. (2004) found that the hydrolysis of peanut protein isolate and soy protein concentrate by alcalase led to a decrease of the exposed/total sulfhydryl groups. On the other hand, Lei et al.(2011) found that the content of the total SH group of ovotransferrin solution showed a significant increase as a function of treatment time up to 30 or 60 s at both 20 and 60 kHz, respectively, and then started to decrease. This difference may arise due to the differences in the intensity and duration of the applied ultrasound or the complexity of the EWPs solution, which contains a mixture of proteins rather than the pure ovotransferrin used by Lei et al. (2011).

Figure 4

Surface Characteristics of EWPHs As a prerequisite for satisfactory surface activity, protein should possess both charge and patches of hydrophobicity on its surface. A plot of both surface hydrophobicity and zeta potential as a functionof ultrasound treatment time is presented in Figure 5. As can be seen, both characteristics of UPT EWPHs were affected ($P < 0.05$) by ultrasound treatment time and enzyme type; the highest values were reached after 15 and 5 min of UPT for alcalase and papain, respectively. AHs can uncover more buried hydrophobic groups while differences were not observed between PHs for prolonged UPT time. Alcalase has broad specificity in cleavage of peptide bonds. Thus, it can be concluded that the highest surface hydrophobicity is primarily due to cleavage of the peptide bonds in which the carboxyl side contains hydrophobic residues such as tyrosine, leucine, and valine (Amaral do Evangelho et al., 2017). A possible explanation could lay in a fact of reducing the intermolecular associations caused by the cavitations' phenomenon, leading to more hydrophobic regions, which were located in the interior of the protein molecules being exposed to the surface of EWPHs enhancing the enzymatic accessibility. The results may explain the detected increase in the emulsifying ability of UPT AHs and PHs since the molecular unfolding of pretreated EWPHs may lead to more release of hydrophobic amino acids during hydrolysis.

Figure 5

The values of zeta potential were negative (Figure 5) meaning the stability and electrokinetic potential of the EWPHs solution could be maintained well during UPT. It can be justifiably concluded that the observed increase of electrostatic repulsive forces between both hydrolysate molecules would favor an increase of their solubility and concentration of total and exposed SH groups in regard to the control (Figure 2 and Figure 4). Comparing the synergistic influence of UPT and the specificities of proteases can be evidently remarked that the differences in the values of hydrolysates' zeta potential are considerable ($P < 0.05$). The exhibited results were in agreement with the above results (Figure 1) where the UPT proved to be one which notably improves the DH for both proteases, as compared to the control (without UPT), thus increasing the DH increased the molecular charge or the value of absolute zeta potential, which was in accordance with the results reported by Liu et al. (2014).

Molecular Structure Characterization Measured by FTIR Spectroscopy FTIR analyzis was conducted to investigate further the difference between the secondary structure of alcalase and papain hydrolysates with the highest reached DH, AHs-15 min, and PHs-5 min, respectively. The FTIR spectra of EWPHs in the region of 500 to 3,500 $cm^{-1}$are shown in Figure 6. The absorbing peak in the region from 3,100 to 3,500 cm^{-1}, which was ascribed mainly to the N-H and O-H stretching vibrations of hydroxyl groups and Amide A of proteins, became wide and strong in UPT AHs and PHs, due to the O-H stretching vibration of water, combined with N-H stretching during enzymatic hydrolysis. UPT caused the changes in the intensity of the vibrations of the C–H_2, C–H, and =C–H stretching bands near 1,450, 1,456, 2,933 and 3,060 cm^{-1}, which may be connected to hydrophobic interactions and backbone conformational changes originating from the UPT. The stronger absorbance bands for EWPHs compared to the control in the range of 1,300 to 1,700cm^{-1} (Amide I and II) resulting in structural changes in the amide groups. Lower band intensity near 1.620 cm^{-1} indicated a less presence of aggregated proteins after applying the UPT for both EWPHs reflecting in a small share of

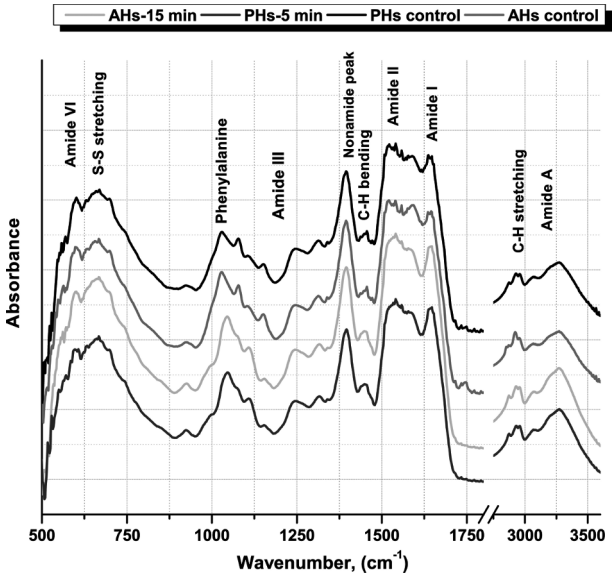

Figure 6. Fourier transform infrared spectroscopy spectra of untreated and ultrasound probe-treated alcalase hydrolysates (AHs) and papain hydrolysates (PHs).

intermolecular β-sheets (Barth, 2007). The intensity of the N-H stretching at 1,546 cm^{-1} (Amide II) also increased after UPT due to the presence of a greater number of amino groups, which can also be evidencedby the presence of α-helix conformation (Adochitei and Drochioiu, 2011). In the Amide III region (1,229 to 1,301 cm^{-1}), minimal differences were found. The intensity of the S-S stretching band around 560 cm^{-1} within Amide VI region (from 537 to 606 cm^{-1}) were decreased by UPT of EWPHs for both hydrolysates, compared to the control, inferring the reduction of S-S bonds arising from the UPT. These results confirmed previous findings in terms of the highest sulfhydryl groups content of EWPHs (Figure 4). It showed that UPT had disrupted the interactions between the local sequences of amino acids and between the different parts of the protein molecule causing the unfolding of EWPHs with the presence of less ordered structure leading to the conclusion that structure and functionality were highly correlated.

FESEM Characterization

The influence of the structure on the effectiveness of the ultrasound pretreated enzymatic hydrolysis was observed by FESEM analyzis (Figure 7), because of the well-known impact of protein microstructure on some functional properties, such as emulsifying and foaming ability. The analysis was performed for hydrolysates that showed the best functional properties in previously discussed trials, and those were AHs and PHs prepared after 15 and 5 min UPT, respectively.

Figure 7

Based on the FESEM micrographs, it is obvious that the particle morphology of UPT AHs and PHs were completely different. It was found that the powder particles of UPT EWPHs showed a drastic modification in surface morphology compared to the hydrolysates without UPT. In the case of untreated AHs (Figure 7a), large aggregates of protein can be seen, whereas with UPT AHs these are notably reduced, while structure with smaller sub-associates can be recognized (Figure 7b). Compared to the AHs, where more particles from UPT were spherically shaped with some dents, no pore was observed on the surface of PHs (Figure 7d). During UPT, interconnected primary structure (Figure 7c) was broken up into separate spherical sub-associates without any cracks. According to the Figure 7, we hypothesized that UPT could prevent the protein aggregation due to the breakage of the disulfide bond, hydrogen bond, covalent/non-covalent bond and facilitate the enzymatic hydrolysis providing the more efficient binding sites for alcalase and papain to attack the interior of EWP aggregates lightly. Previously discussed findings, an increase in intensity of FTIR spectra, surface hydrophobicity and SH groups content can be attributed to these changes in structures of UPT EWPHs and this adds evidence to the hypothesis that UPT destroyed the cross-link between the protein molecules due to the formation of localized hot spots upon the collapse of bubbles and shear forces produced by micro streaming and shock waves (Wang et al., 2012).

CONCLUSIONS

The length of ultrasound probe treatment displayed the significative effect on the enzymatic hydrolysis of EWPs with alcalase and papain, also affecting the surface characteristics, protein conformation and microstructure, and determining functional properties. The UPT at 20 kHz for 2–15 min significantly changed the proteolytic pattern of alcalase and papain assisted enzymatic hydrolysis. Greater susceptibility to enzymatic hydrolysis was observed with alcalase compared to the papain. Throughout the 15 min of UPT and subsequent alcalase hydrolysis, the most significant increase in solubility by 54.4% compared to the untreated AHs was detected. Additionally, values for FC and FS rose by 36.3% and 38.1%, while EAI and ESI increased by 173% and 115%, respectively. Ultrasound papain pre-hydrolysis treatment showed also enhanced functional properties and the most considerable increase in FC, FS, EAI, and ESI values by 35.4%, 38.5%, 172.3%, and 48.6%, respectively was observed after 5 min of UPT. The prolonged treatment for 20 min, however, caused an excess denaturation of EWPs, enhancing the tendency of molecules toward aggregation, as shown by the decrease in the content of SH groups in hydrolysates molecules as well as the decrease of subsequent hydrolysis by both enzymes.

Hence, it seemed that the surface characteristics like sulphydryl content, hydrophobicity and charge and complemented changes in functional properties could be monitored merely by varying UPT time and enzyme

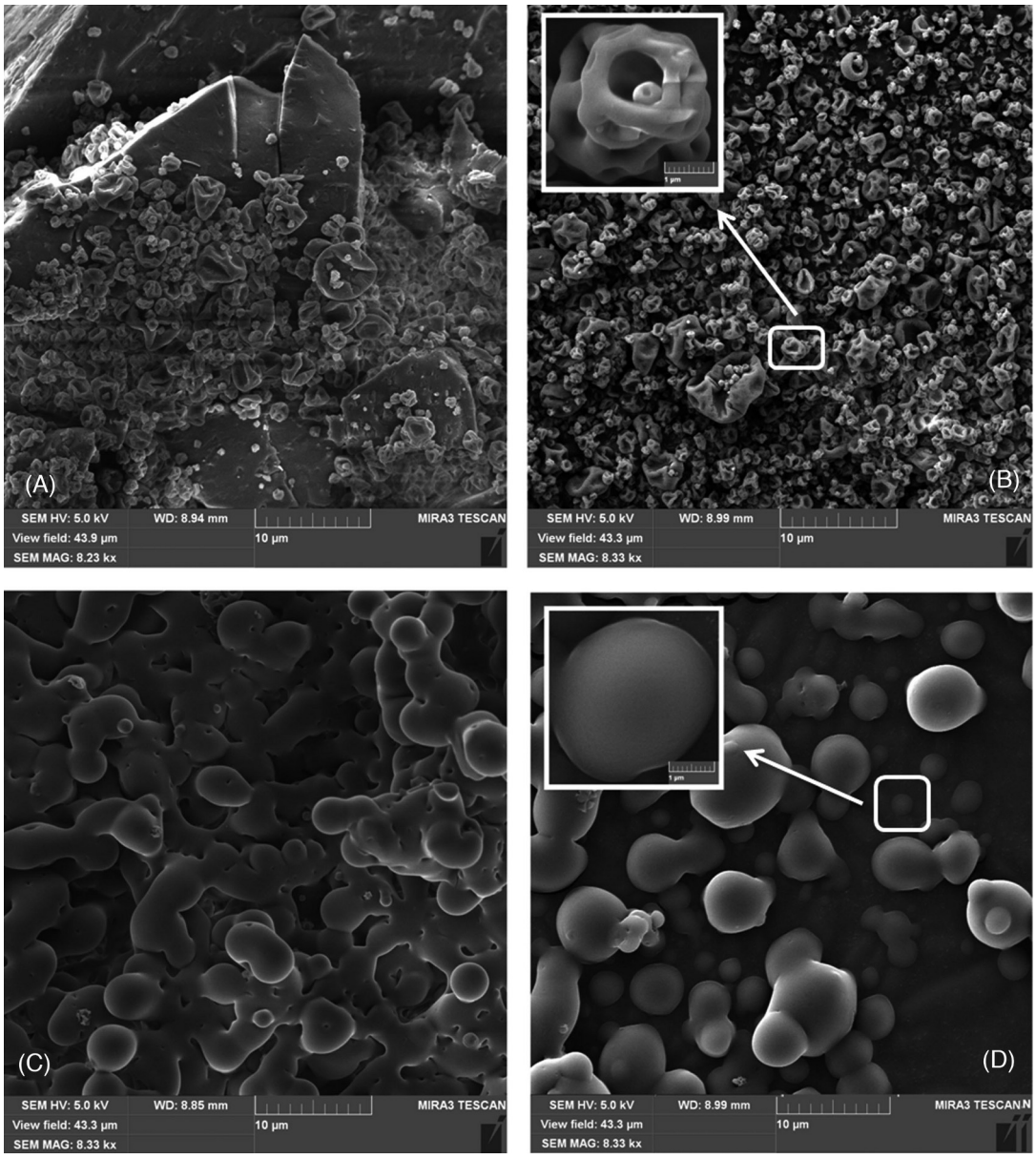

Figure 7. The FESEM (magnification × 8.33 k, bar = 10 μm) images of the untreated alcalase hydrolysates (AHs) (A), ultrasound probe treated AHs (B), untreated papain hydrolysates (PHs) (C) and ultrasound probe treated PHs (D) with frequency 20 ± 0.2 kHz. Scale bar is 10 μm in all cases.

type. Analysis of EWPHs structure by FTIR spectroscopy disclosed that the ultrasound probe treatment leads to changes in the secondary structure of AHs and PHs, which was also confirmed by SEM analyzis.

This study revealed that by combining ultrasound pre-hydrolysis treatment under controlled conditions with thoughtful proteases selection, hydrolysates with improved functional properties could be produced, enhancing utilization of EWPs in food products.

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