IMPACT OF HIGH-INTENSITY ULTRASOUND PROBE ON THE FUNCTIONALITY OF EGG WHITE PROTEINS

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Abstract

High-intensity ultrasound represents a non-thermal processing method that has been extensively researched and used in the last decade. The application of power ultrasound offers the opportunity to modify and improve some technologically important compounds which are often used in food products, such as proteins. The focus of this research was to evaluate the effect of high-intensity ultrasound on egg white proteins (EWPs) with an objective to improve their functional properties.

In this study, 10% (w/w) aqueous solution of egg white was treated with ultrasound probe which frequency was 20 kHz and treatments were performed for 5, 10, 15 and 20 min. The effect of the treatment was examined for aforementioned properties: the change of pH and temperature, solubility, foaming by a whipping method, emulsifying by turbidometric technique, sulphydryl content (exposed and total SH groups), antioxidant and antimicrobial activity and susceptibility of treated samples to enzymatic hydrolysis.

Ultrasound affected functional properties of egg white proteins and improved antioxidant and antimicrobial activity. Furthermore, the samples showed an increased concentration of the total SH groups, while the concentration of exposed SH groups was not affected. pH did not change significantly upon the ultrasound treatment, while the temperature of the egg white solutions increased.

These results suggest that high-intensity ultrasound probe can be used for improvement of the functionality of the EWPs and thus it could be potentially applied in the food industries. The trials reported here may represent relevant information to consider when attempting the use of high-intensity treatment for improving functional properties.

Key words: High-intensity ultrasound probe, Egg white protein, Functional properties, Antioxidant and antimicrobial activity.

1. Introduction

High-intensity ultrasound pretreatment represents a non-thermal processing method that has been extensively researched and used in the last decade. The application of power ultrasound offers the opportunity to modify and improve some technologically important compounds which are often used in food products, such as proteins. The modifications in protein molecules caused by ultrasound energy could be caused by the cavitations’ phenomena, heating, dynamic agitation, shear stresses and turbulence [1] and may include breaking of covalent bonds [2], generation of low molecular weight peptides [3] and fragmentation of large aggregates into smaller particles that modifies the functional properties of the proteins.

Its application is based in the fact that it can lead about significant changes in some chemical, functional and physical properties of proteins that may be of interest as technological benefit, especially at frequencies between 20 and 100 kHz. The impact of ultrasound pretreatment on functional properties of proteins largely depends on the nature of proteins. Therefore, Arzeni et al. [4] observed that the effect of ultrasound pretreatment was different on the proteins originated from whey protein concentrate, egg white (EW) or soy. In the literature can be found that the application of ultrasound pretreatment was frequently performed in ultrasound batch and with ultrasound probe. It was found that the that pretreatment of whey proteins with ultrasound probe at frequency of 20 kHz affect-
ed their solubility and foaming ability, which was attributed to sample exposure to high temperatures caused by sonication [5]. Additionally, for hydrolysates obtained during proteolysis of ultrasound pretreated wheat germ protein have been reported that their angiotensin-converting enzyme (ACE) inhibitory activity was improved [6].

Egg white proteins (EWPs) are utilized in many food products because of their good functional and physical properties like gelling, emulsification, foaming, etc. However, their wider commercial use as protein supplements is limited due to allergenicity, insufficient digestibility, thermolability and high viscosity. Still, bioactive peptides, produced by enzymatic hydrolysis have been reported to have many activities, including antimicrobial properties, antioxidant and antihypertensive activities and other significant biological and functional properties. Hence, egg-white hydrolysates became irreplaceable ingredients of food products, from the viewpoint of their functional, nutritional and immunological properties. Likewise, the functional properties of bioactive peptides can be improved by application of some pretreatment such as the ultrasound pretreatment [7, 8].

The main objective of this study was to examine the effects of the ultrasound pretreatment with an ultrasound probe at frequency of 20 kHz on functional properties and antioxidant activity of egg white proteins. The effect of the treatment on susceptibility of treated samples to enzymatic hydrolysis was also investigated. Finally, the ability of the high-intensity ultrasound pretreatment for pasteurization purposes at moderate temperature has been explored.

2. Materials and Methods

2.1 Materials

Chicken egg white obtained from a local supermarket was separated from the yolk and gently stirred without foam formation to provide homogeneous mixture.

The enzyme complexes used in this work for determination the susceptibility of different pretreated samples to proteolysis was Alcalase 2.4 L (Sigma Aldrich, St Louis, MO, USA). The enzyme activity was ≥ 2.4 U/g Anson Units whereby the one Anson Unit is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute which gives the same color with the Folin-Ciocalteu phenol reagent as one milliequivalent of tyrosine at 25 °C at the pH 7.50.

For the determination of antimicrobial assay of the pretreated samples plate count agar, Enterobacteriaceae Enrichment (EE) broth, Violet Red Bile Glucose (VRBG) agar, Buffered Peptone Water, Rappaport-Vassiliadis (RVS) broth, Mueller Kauffmann Tetrathionate Novobiocin Broth (MKTTn broth) and Xylose Lysine Deoxycholate (XLD) agar were supplied from HiMedia Laboratories Pvt.Ltd (Mumbai, India). All other chemicals were of analytical reagent grade and they were used without any further purification.

2.2 Methods

2.2.1 The pretreatment of EWP solution with ultrasound probe

The series of 10% (w/w) egg white aqueous solutions were exposed to ultrasound waves by using ultrasound probe frequency of 20 ± 0.2 kHz. Namely, in this case the test set up for conducting the laboratory testing of the cavitation resistance by using the Modified Vibratory Cavitation Test Method [9]. The samples were prepared in a beaker of 600 mL capacity where the working volume (~360 mL) was kept constant for all experiments sets. The ultrasound pretreatment was carried out at ambient (25 ± 1 °C) temperature, and the samples were subjected to cavitation for different time (5, 10, 15 and 20 minutes).

Before and after pretreatment, temperature of samples EWPs has been measured with thermometer and then calculated average increase in temperature after treatment. Additionally, during the ultrasound pretreatment temperature change was monitored by evaluating the temperature over a certain period of time (e.g. 1, 2 and 5 minutes). The pH values of protein model solutions were determined before and after ultrasound treatment for 20 ± 0.2 kHz probe by pH meter Eutech instrument, Netherlands.

2.2.2 Solubility

Solubility of pretreated samples EWPs was determined at different pH and compared. Samples were suspended in water (10% w/w) and were centrifuged at 10,000 x g for 15 minutes. The protein content in all samples was determined using the Lowry method [10]. Solubility was expressed as the percentage of protein remaining in the supernatant as compared to the untreated samples.

2.2.3 The foaming properties

The aqueous egg white solution with concentration of 10% (w/w) was diluted to 8% (w/w) to prepare foams. The initial volume of 50 mL foaming solution was placed in the plastic beaker of 700 mL (diameter 7.2 cm) and whipping for 4 min with laboratory homogenizer at a speed of 9.500 rpm and ambient temperature (Yellowline, DI 25 basic, Ica Works Inc., Wilmington) [11]. Foam capacity (FC) was expressed as foam expansion at 0 min, which was calculated according to the following equation:
where $A$ is the volume after whipping (mL) and $B$ is the volume before 4 min. of whipping (mL). The foam stability ($FS$) was defined as the percentage of liquid still present in the foam after 30 min. compared to the solution at 4 min. after whipping:

$$FS(\%) = \frac{A - B}{A} \times 100$$

$A$ is the volume of foam after 30 min. standing (mL) and $B$ is the volume before 4 min. whipping (mL).

2.2.4 Emulsification activity

Protein dispersions were analyzed by the turbidimetric technique for emulsion activity index and emulsion stability index as described by Pearce and Kinsella [12] except for the noted changes in each section. Emulsions of the each protein dispersion (3% w/w) were prepared with sunflower oil in molar ratio 1 : 2 and mixing for 90 sec. with laboratory homogenizer. The absorbance of the diluted emulsions was measured by a UV-Vis spectrophotometer (Ultraspex 3300 pro, AmeriSham Bioscience) at 500 nm in 1 cm path length cuvettes. The absorbance was read initially and turbidity and EAI were calculated by the following formula:

$$T = 2.303 \frac{A}{l}$$

where $T$ - turbidity, $A$ - absorbance at 500 nm and $l$ - a path length (m).

The emulsion activity index (EAI) was then calculated as:

$$EAI = 2 \cdot T \cdot \left(\frac{A \cdot r}{c \cdot \theta \cdot 1000}\right)$$

where $T$ - turbidity (calculated from above equation), $\theta$ - volume fraction (mL), $c$ - the weight of protein per unit volume of aqueous phase before emulsion is formed (g) and $r$ - dilution factor.

2.2.5 The content of SH groups

The content of SH groups in EWP samples before and after pretreatment with ultrasound probe was designated using Ellman’s reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) - DTNB) according to Ellman’s procedure with slight modifications [13]. The exposed SH groups were measured as follows. A solution of egg white (10% w/w, prepared as mentioned above) was diluted to a concentration of 0.05% (w/w) with a standard buffer of pH 8.0. The buffer was composed of 86 mM TRIS, 90 mM glycine and 4 mM EDTA. The samples were then centrifuged for 20 min. at 20 °C and 12,875 x g. The supernatant was used for the determination. A volume of 0.025 mL of Ellman’s reagent solution (4 mg of DTNB/ mL of standard buffer) was added to a 2.5 mL aliquot of control and ultrasound pretreated protein supernatants. After the solution was rapidly mixed and allowed to stand at room temperature for 15 min, absorbance was read at 412 nm on a UV-Vis spectrophotometer (Ultraspex 3300 pro, AmeriSham Bioscience). The total SH groups’ content was also determined following the same technique but using but using a denaturing buffer consisting of the standard buffer (86 mM TRIS) plus 8 M urea and 0.5% (w/v) sodium dodecyl sulphate. The standard and the denaturant buffers were used as reagent blanks instead of protein solutions. A protein blank was measured in which 0.025 mL of each buffer replaced Ellman’s reagent solution.

2.2.6 The susceptibility of proteins to enzymatic hydrolysis

The susceptibility of the ultrasound pretreated proteins to enzymatic hydrolysis was examined as follow. The enzymatic hydrolysis were performed with Alacalase, endo-peptidase, under its optimal conditions (50 °C, pH = 8.0) in a mechanically stirred batch reactor with temperature and pH control and working volume of approximately 360 mL. The substrate for enzymatic hydrolysis was 10% (w/w) aqueous solution of pretreated EW (11.9 mg/mL, protein content determined according to the standard Kjeldahl method, $N$´ 6.25) which was adjusted to optimum pH for enzyme activity with 2 M HCl, then stirred and allowed to equilibrate to the working temperature for 15 min. (typically 50°C). The degree of hydrolysis was determined using the pH-stat method and was calculated by the following equation:

$$DH(\%) = \frac{B \times N_p \times \frac{1}{a} \times \frac{1}{m_p} \times \frac{1}{h_{tot}} \times 100}{h_{tot} \times 100}$$

where $B$ is the consumption of the base in mL, $N_p$ is the normality of the base, $m_p$ is the mass of protein in g, $h$ 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), $a$ is the degree of dissociation of the α-amino groups (1/$a$ = 1.13 at 50 °C and pH = 8.0).

2.2.7 Antioxidant activity

The effect of the pretreated samples on the scavenging of DPPH radicals was determined as previously described by Jakovetić et al. [14] with slight modifications. In brief, a 0.2 mL aliquot of pretreated samples of EWPs was mixed with 1.8 mL of 0.1 mM DPPH solution in methanol. After mixing vigorously for 2 minutes, the mixture was allowed to stand at room temperature in the dark and after 30 min. absorbance was measured at 517 nm using UV-Vis spectrophotometer (Ultraspex 3300 pro, AmeriSham Bioscience). DPPH radical scav-
enging activity (RSA, %), expressed as the percentage of inhibition:

\[
RSA (\%) = \left[ 1 - \frac{A_{s} - A_{c}}{A_{c}} \right] \times 100
\]

where \(A_{s}\) is the absorbance of the tested antioxidant (EWPs after and before pretreatment), \(A_{c}\) is the absorbance of the EWPs after and before ultrasound pretreatment in methanol, and \(A_{0}\) is the absorbance of the DPPH solution without the sample. The protein content in all samples was determined using the Lowry method [10].

The antioxidant activity of the all samples was also determined by measuring the reducing power described by Oyaizu method [15]. EWPs samples after and before ultrasound probe treatment (0.5 mL) were mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An aliquot (2.5 mL) of 10% trichloroacetic acid was added to the mixture after cooling to room temperature, followed by centrifugation at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 2.5 mL of 0.1 % (v/v) iron (III) chloride and the absorbance was measured at 700 nm using UV-Vis spectrophotometer.

2.2.8 The total number of microorganisms

The total number of microorganisms was determined by using plate count method. A 20 mL of sample was added to 180 mL of sterile physiological solution (0.9% water solution of NaCl) and homogenized. The cells count was realized by using decimal dilutions of cells in physiological solution. From selected dilutions, a 1 mL of sample was transferred to petri dish and ~15 mL of melted agar was added (PCA Agar at ~44 °C). The samples were incubated for 48 h at 30 °C. The total number of cells was determined as CFU after period of incubation.

2.2.9 Enterobacteriaceae

A sample (20 mL) was added to 180 mL of sterile physiological solution (0.9% water solution of NaCl) followed by homogenization. A 1 mL of decimal dilution of cells in physiological solution was transferred to 10 mL of EE broth and tempered at 37 °C for 24 h. After incubation, the sample was transferred on selective agar plate (VRBG agar) by inoculating loop and tempered at 37 °C for 24 h. The positive reaction on Enterobacteriaceae was identified by characteristic color of colonies.

2.2.10 Salmonella spp.

A sample (20 mL) was added to 225 mL of Buffered Peptone Water and incubated at 37 °C for 18 h. A 0.1 mL of incubated sample was transferred to 10 mL of RVS broth and incubated at 41.5 °C for 24 h.

Also, a 1 mL of incubated sample was added to 10 mL of MKTTn broth and incubated at 37 °C for 24 h. After incubation, using inoculating loop sample from RVS broth was transferred on surface of XLD agar plate and incubated at 37 °C for 24 h. The same procedure was repeated for cells incubated in MKTTn broth. The positive reaction on Salmonella spp. was identified by characteristic color of colonies.

3. Results and Discussion

3.1 pH and temperatures changes of ultrasound probe pretreated EWPs

The influence of the ultrasound pretreatment on pH and temperature of solutions was assayed, first, and the results are presented in Table 1 and Fig. 1. It appeared that the values of pH did not change significantly (p < 0.05) upon the ultrasound probe pretreatment.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Before pretreatment</th>
<th>After pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPEW-5</td>
<td>9.1 ± 0.05</td>
<td>8.99 ± 0.01</td>
</tr>
<tr>
<td>UPEW-10</td>
<td>9.1 ± 0.05</td>
<td>8.89 ± 0.02</td>
</tr>
<tr>
<td>UPEW-15</td>
<td>9.2 ± 0.04</td>
<td>8.72 ± 0.04</td>
</tr>
<tr>
<td>UPEW-20</td>
<td>9.0 ± 0.03</td>
<td>8.73 ± 0.03</td>
</tr>
</tbody>
</table>

* statistically not significant among samples when (p < 0.05)

However, the temperatures of EWP model system increased significantly after ultrasound probe pretreatment and one can observe that the highest temperature of sample was obtained after ultrasound pretreatment during 20 min. where temperature increased up to 45 °C. This value is still significantly lower than the denaturation temperature of most proteins [16]. This can be excused by the fact that 20 min. treated samples with 20 ± 0.2 kHz probe are the most exposed to high power and consequently leads to the most excess of the energy that causes the greatest increase in temperature. From these data, the ultrasonic power was
The ultrasound pretreatment of EWPs appeared to alter their emulsifying properties and this could be explained by the increase of the percentage of adsorbed proteins after the treatment. Under turbulent conditions that are occurring during ultrasound pretreatment, like homogenization, movements have been shown to favor the adsorption of proteins and formation of aggregates predominates [17]. We can assume that this increase in emulsion activity index (EAI) lead to a better potential adsorption of EWPs at the oil-water interface. Emulsifying stability index (ESI) is estimation of protein capacity for staying at water/oil interface after emulsion storage or heating [18]. From the data, the statistically significant increase ($p < 0.05$) for all samples was evident. The increase of emulsion stability index (ESI) could be explained by better orientation of proteins, under the influence of turbulent behavior produced by ultrasound and iteration of oil bubbles in the emulsion. The improved emulsifying properties of EWPs could be attributed to changes in the conformational and surface properties of protein structures upon ultrasound pretreatment. According to Jambrak et al. [19] protein denaturation and changes on the conformational structure would affect surface hydrophobicity and subsequently lead to better adsorption of the oil-in-water emulsion system.

3.3 The influence of high-intensity ultrasound probe pretreatment on solubility, foaming properties and antioxidant activities of EWPs

Generally, solubility represents the most practical criterion of protein denaturation and aggregation and therefore a good index of protein functionality. In this work, the high-intensity ultrasound probe ($20 \pm 0.2$ kHz) was applied. Suspensions of EWPs (10% w/w) were treated with the ultrasound probe during different time (5, 10, 15 and 20 min.). Solubility was expressed as the percentage of protein remaining in the supernatant as compared to the untreated samples. As shown in Figure 2., the solubility increased significantly ($p < 0.05$) for all samples pretreated with $20 \pm 0.2$ kHz probe compared to the sample without ultrasound pretreatment. The solubility increase could be ascribed to the effect of high-intensity ultrasound probe on the proteins’ conformation and structure, causing their changes in a such manner that hydrophilic parts of amino acids from inside are opened toward water molecules. Comparing samples treated for different time with the probe, it can be said that the solubility didn’t change significantly for EW and hence, taking into account the changes of temperature during ultrasonic treatment, 15 minutes may be adopted as optimal process time for ultrasound probe. Jambrak et al. [19] obtained similar results at equivalent working conditions for solubility of soy protein isolate and concentrate. Ultrasound treatment with $20 \pm 0.2$ kHz probe has showed the largest increase in protein solubility ($p < 0.05$) of soy
protein concentrate model systems. Increase in solubility was the greatest for 20 kHz treatment for 30 min., followed by 40 kHz bath treatment for 15 min. For other treatments there were not significant changes in protein solubility.

The foaming properties of the different time treated samples (20 ± 0.2 kHz) of the EW are reported in Figure 2 in terms of foam capacity (%) and foam stability (%). The control values for both, foam capacity and foam stability, was EW without ultrasound treatment. Foam stabilities were increased statistically significant (p < 0.05) after 20 ± 0.2 kHz probe treatment for 5, 10, 15 and 20 min. comparing with control. The foam stability has been known to depend on the strength of protein film and its permeability to gases. Foam capacities were improved for all samples, but this improvement didn’t significant compared to the control. The best results for foam capacity were obtained after 5 min. treated, but the stability of this sample has been changed significantly. Still, the EW solution after 15 min. were 0.046 ± 0.001, 0.073 ± 0.002, 0.052 ± 0.001, 0.106 ± 0.001, and 0.082 ± 0.003, respectively. In this case, the increased absorbance of the EW solutions with ultrasound treatment in regard to EW solution before treatment (control) indicates increasing reducing power. Obtained differences in results are statistical significant in correlation with control (p < 0.05). The ultrasound pretreated sample of EWP for 15 min. shown the highest reducing power revealing that it has a better ability to donate electron. It may suggest that the high-intensity ultrasound probe led to changes in the structure of the EWPs and it can be used as a pretreatment in the preparation of a peptide with enhanced antioxidant activity.

An additional aim of this research was to determine the effects of ultrasound probe on antioxidant activity of EW solution. The antioxidant activity as measured by DPPH method was detected in all EW samples before and after ultrasound treatment with probe 20 ± 0.2 kHz and the results have been presented in Figure 2. It appeared that the antioxidant properties were enhanced after 5 and 15 min. of ultrasound treatment. Namely, the antioxidant activity was significantly different (p < 0.05) after 15 min. with regard to the sample without sonication (18.9% and 46.6% of without and 15 min. sonication, respectively). This suggests that different time of ultrasound protein treatments may have resulted in different alterations in the protein’s tertiary structure and consequently their free radical scavenging activity. This is in agreement with results obtained by several authors who reported that the ultrasound treatments had positive effects on changes of protein’s structure and increased the susceptibility of protein to enzymatic hydrolysis. Consequently, it made easier to obtain the bioactive peptides with antioxidant, antimicrobial and antihypersensitive activities. Further work is necessary to confirm the influence of ultrasound probe as a pretreatment on the possibility of obtaining a peptide with antioxidant properties.

It is recognized that there is a direct correlation between antioxidant activity and reducing power. Therefore, in this study the reducing power of EWP samples was measured before and after high-intensity ultrasound probe treatment. The results of the reducing power of EWPs correlated well with those of DPPH radical scavenging assay. The values of absorbance readings for runtime of treatment 0, 5, 10, 15 and 20 min. were 0.046 ± 0.001, 0.073 ± 0.002, 0.052 ± 0.001, 0.106 ± 0.001, and 0.082 ± 0.003, respectively. In this case, the increased absorbance of the EW solutions with ultrasound treatment in regard to EW solution before treatment (control) indicates increasing reducing power. Obtained differences in results are statistical significant in correlation with control (p < 0.05). The ultrasound pretreated sample of EWP for 15 min. shown the highest reducing power revealing that it has a better ability to donate electron. It may suggest that the high-intensity ultrasound probe led to changes in the structure of the EWPs and it can be used as a pretreatment in the preparation of a peptide with enhanced antioxidant activity.
3.4 The impact of runtime of ultrasound probe pretreatment on the content of SH groups in EWPs

Effect of ultrasound probe on the content of sulfhydryl groups was performed by using Ellman’s reagent and is shown in Figure 3.

As shown in Figure 3., the total free sulfhydryl groups of the high-intensity treated EWP solutions for prolonged time (15 and 20 min.) were not significantly modified upon sonication compared to control, but the significant change was occurred after 5 and 10 min. treatment. The same results were obtained for the content of exposed sulfhydryl groups. Hence, it appeared that the ultrasound energy produced by ultrasound probe at 20 ± 0.2 kHz led to the change in the molecules of EWPs. It is thought that the sonication causes the haemolytic water molecule cleavage, generating high energy intermediates such as hydroxyl and hydrogen free radicals, and therefore, causing protein structural changes. Data found in bibliography are very dissimilar. Some works reported changes in the SH groups content for food proteins after sonication. For example, Gülseren et al. [20] found that the amount of free sulfhydryl groups in BSA decreased with increasing sonication time (up to 90 min., with temperature control). Lei et al. [21] determined that the sonication of ovotransferrin at 6 kHz, under HIUS pulsed conditions, with temperature control, did not affect the total content of SH groups up to 480 s of treatment, but increased it after that time. Based on the above, the effect of ultrasound probe with frequency 20 ± 0.2 kHz on the content sulfhydryl groups should be thoroughly examined.

3.5 The impact of ultrasound probe treatment on susceptibility of EWPs to Alcalase

It is well known that proteins in EW are the best among all kinds of protein, but their widespread use has been confined for their special properties such as allergenicity, heat instability and high viscosity. This is the reason why protein hydrolysates have been used for the nutritional management of individuals who can not digest intact protein. The mentioned properties can be improved by enzymatic hydrolysis and the protein structural changes associated with ultrasound application may also enhance enzyme hydrolysis due to weakened interactions and disrupted quaternary and tertiary structures rendering more hydrolysis sites to be accessible by an enzyme [22].

In this work, EWPs were treated by high intensity ultrasound probe and then hydrolyzed by Alcalase. The effect of treatment time on the susceptibility to proteolysis was investigated.

The aim of the pretreatment time optimization was to find the conditions that would lead to irreversible denaturation of the Alcalase inhibitor, but would not result in the formation of dense and compact precipitates, which are poor substrate for proteolysis. As shown in Figure 4, it appeared that for all treatment time studied, the ultrasound probe pretreatment changed the proteolytic pattern of EWPs, but an increase in their digestibility by Alcalase was observed.
only for runtime of 10 and 15 min. Results have shown the statistically significant \((p < 0.05)\) changes in the proteolytic pattern of EWPs. It is easily observed that pretreatment of the EW with an ultrasound probe at \(20 \pm 0.2\) kHz for 10 to 15 minutes had a better effect than the hydrolysis treatment in the course of 5 and 20 minutes. Pretreatments for 5 and 20 minutes even adversely affected the course of the enzymatic reaction and the initial reaction rate as compared to the control sample. The prolonged exposure to ultrasound of 20 min. seemed to have a negative effect on the EWPs hydrolysis because under this condition, a turbid suspension of protein aggregates could be observed, which might lower the accessibility of the unfolded proteins to the Alcalase. It can also be seen that the pretreatment time led to changes in the equilibrium degree of hydrolysis and the greatest equilibrium degree of hydrolysis of \(40.5 \pm 1.03\%\) was achieved for the pretreatment for 15 minutes. Accordingly, the treatment of the model protein egg white with \(20 \pm 0.2\) kHz ultrasound probe during 15 min. can be adopted as the optimal. This ultrasound probe-dependent increase in the susceptibility to enzymatic hydrolysis is in accordance with the results of Su et al. [23], who observed an increase of susceptibility to Alcalase hydrolysis of EW after 120 min. treatment at 40 kHz.

In order to verify the impact of the ultrasound probe pretreatment on the susceptibility of EWPs to proteolytic hydrolysis these results were compared with previously results obtained by applying the ultrasound bath and thermal treatment as pretreatments prior hydrolysis (Figure 5).

The ultrasound pretreatment at \(20 \pm 0.2\) kHz for 15 min by using an ultrasound probe has been shown to accelerate the hydrolysis process and generate more reproducible results, compared with both, heat treatment or treatment in an ultrasonic water bath at 40 kHz under similar conditions. This can be explained by the fact that suspension were treated with 20 kHz probe are the most exposed to high power that those treated with 40 kHz bath and also, the way of treatment is different. At ultrasound probe pretreatment horn is inserted in suspension which favors contact between tip and sample, whereas at baths suspension were inserted in baths with flask so there was not direct contact with irradiating surface.

The results showed that the ultrasound technology could improve susceptibility of EWPs to Alcalase under the controlled conditions and thus, the combination of ultrasound pretreatment with enzyme hydrolysis offers a potential way for improving the proteolytic pattern of EWPs and producing new bioactive peptides from proteins.

### 3.6 The impact of ultrasound probe pretreatment on the total number of microorganisms

To analyze in more detail the potential of using the ultrasound pretreatment with probe at frequency of \(20 \pm 0.2\) kHz for EWPs hydrolysis, the influence of pretreatment on the total number of microorganisms was investigated. Based on previously reported results, the treatment of the model protein EW with \(20 \pm 0.2\) kHz ultrasound probe with runtime 15 min. has been adopted as the optimal and these samples EWPs were used for antimicrobial analysis. Received results were compared to previous results obtained by applying the ultrasound bath at frequency of 35 kHz and thermal treatment and were summarized in Table 3.

Comparing the results in Table 3, it obviously may be noticed that the statistically significant values of number aerobic bacteria to form colonies \((p < 0.05)\) were reduced for samples which were subjected to thermal treatment and 15 min. pretreated with high-intensity ultrasound probe. Namely, the number of microorganisms in EWPs was reduced using the ultrasound and thermal treatment by the 92% and 100%, respectively. This analysis confirmed the positive effect of ultrasound from a microbiological point of view.
Table 5. The impact of different pretreatment (thermal, ultrasound bath and ultrasound probe) on the total number of microorganisms

<table>
<thead>
<tr>
<th>Samples of EWPs with different pretreatments</th>
<th>The number of aerobic bacteria to form colonies</th>
<th>Enterobacteriaceae</th>
<th>Salmonella spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^1$</td>
<td>$10^2$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>EW</td>
<td>327</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>319</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>UBEW-15 min</td>
<td>426$^a$</td>
<td>45$^a$</td>
<td>6$^a$</td>
</tr>
<tr>
<td></td>
<td>405$^a$</td>
<td>41$^a$</td>
<td>4$^a$</td>
</tr>
<tr>
<td>UBEW-30 min</td>
<td>443$^a$</td>
<td>51$^a$</td>
<td>6$^a$</td>
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<tr>
<td></td>
<td>451$^a$</td>
<td>53$^a$</td>
<td>1$^a$</td>
</tr>
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<td>UBEW-60 min</td>
<td>337$^a$</td>
<td>35$^a$</td>
<td>4$^a$</td>
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<td></td>
<td>365$^a$</td>
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</tr>
<tr>
<td>TEW-30 min</td>
<td>-$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UPEW-15 min</td>
<td>34$^b$</td>
<td>6$^b$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>23$^b$</td>
<td>4$^b$</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ statistically not significant difference vs control ($p > 0.05$)
$^b$ statistically significant difference vs. control ($p < 0.05$)

EW-untreated egg white (control); UBEW-15 min – ultrasound bath at 35 kHz, 15 min pretreated egg white; UBEW-30 min. – ultrasound bath at 35 kHz, 30 min. pretreated egg white; UBEW-60 min. – ultrasound bath at 35 kHz, 60 min. pretreated egg white; TEW-30 min. – thermal treatment at 75°C during 30 min.; UPEW-15 min. – ultrasound probe at 20 kHz, 15 min. pretreated egg white.

4. Conclusions
- In this paper, the influence of runtime of the ultrasound pretreatment with probe at 20 ± 0.2 kHz on egg white protein model system is investigated and obtained results indicated that the high-intensity ultrasound probe has a major effect on egg white proteins’ functional properties.

- Novel and interesting findings regarding the effects of a new emerging technology on the physicochemical attributes of one of the most used food protein is offered. In this study, the ultrasound pretreatment caused significant changes in functionalities of egg white proteins and showed great influence on all examined properties, suggesting the protein denaturation caused by cavitation effects.

- Within this framework, it was found that the sonication led to an increase of antioxidant activity and solubility of egg white proteins. Values of pH did not change significantly ($p > 0.05$) upon ultrasound pretreatment. The sulphhydryl content (exposed and total SH groups) of control and high-intensity pretreated egg white protein solutions were not significantly modified upon sonication, but the most significant change was occurred after 5 and 10 min. pretreatment.

- On the other hand, emulsion activity, emulsion stability and foam stability were increased after sonication, while the foam capacity was improved, but this difference was not significant compared to the control sample. Susceptibility of egg white proteins to Alcalase under the controlled conditions has been greater after the ultrasound probe pretreatment and the highest equilibrium degree of hydrolysis has been observed after 15 min pretreatment. Likewise, it may be remarked that the statistically significant values of number aerobic bacteria to form colonies ($p < 0.05$) were obtained for 15 min. pretreated sample.

- These results suggest that the high-intensity ultrasound treatment with probe at 20 kHz can be used for improvement of the functionality of the EWPs and thus it could be potentially applied in the food industries. The trials reported here may represent relevant information to consider when attempting the use of high-intensity ultrasound probe treatment for improving functional properties of other proteins.

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5. References


