

Impact of ultrasound on egg white proteins as a pretreatment for functional hydrolysates production

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Abstract The objective of this study was to investigate the effects of different ultrasound pretreatment on enzymatic hydrolysis of egg white proteins (EWPs) by Alcalase as well as evaluating some functional and antioxidant properties of hydrolysates obtained by various proteases treatment and ultrasound technology. The effects of chosen ultrasound pretreatment parameters including frequency of ultrasonic waves (35 and 40 kHz), temperature (25 and 55 °C), time of pretreatment (15–60 min) and pH of egg white solution (7.00–10.00) were examined. It appeared that controlled ultrasound treatment can improved the hydrolysis process compared with untreated samples, but optimization of the power and length of sonication was important. The optimal ultrasound pretreatment at calorimetric power of 21.3 W and frequency of 40 kHz for 15 min at 25 °C and with naturally basic egg white (pH 9.25) resulted in increased initial rate and equilibrium degree of Alcalase hydrolysis by about 139.8 and 13.86 % compared with the control, respectively. EWP hydrolysates with ≈ 27.0 % degree of hydrolysis obtained with heat pretreatment and ultrasound pretreatments under optimal conditions were further separated by sequential ultrafiltration into 4 hydrolysate fractions (<1, 1–10, 10–30 and >30 kDa) which were investigated for protein content, peptide yield and antioxidant activity. The hydrolysis after heat pretreatment generated more peptides <1 kDa (19.04 ± 1.02 %) than ultrasound pretreatment did (11.90 ± 0.53 %), whereas the proportion of peptides <10 kDa were higher

in the second case (28.80 ± 0.07 vs. 20.46 ± 0.39 %). The fraction obtained by the ultrasound pretreatment containing peptides with a molecular weight between 1 and 10 kDa demonstrated the strongest ABTS radical scavenging efficacy among the fractions (97.54 ± 0.30) with IC_{50} value of 4.31 mg/mL. Compared with single-enzyme processes, the two-stage enzymatic processes did not significantly improve both antioxidant and functional hydrolysates' properties.

Keywords Ultrasound pretreatment · Proteases · Egg white hydrolysates · Antioxidant activity · Functional properties

Introduction

Ultrasound pretreatment as a nonthermal process for modification food ingredients has attracted more and more attention in food science and technology due to its promise in the development of novel, gentle but targeted processes to improve quality and safety of processed foods [1, 2]. Its application in food processing is based on the fact that it can bring about significant changes in some chemical, functional and physical properties of food ingredients that may be of interest as technological benefit, especially at high power (low frequency; between 20 and 100 kHz). The changes in protein molecules caused by ultrasound energy could be explained by the cavitation phenomena, heating, dynamic agitation, shear stresses and turbulence [3] and may include breaking of covalent bonds [4], generation of low molecular weight peptides [5] and fragmentation of large aggregates into smaller particles that alters the functional properties of the proteins. The parameters that are essential to cause changes of the functional properties of

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sonicated proteins seem to be intensity of ultrasonic waves, time of ultrasonic treatment and conditions in which the pretreatment was performed (pH, temperature and ionic strength of the solution) [6].

The egg white, or albumen, possesses properties that make it attractive as a protein source in human nutrition due to high bioavailability of egg white proteins (EWPs) and high content of essential amino acids [7, 8]. Furthermore, it is considered to be a multi-purpose ingredient that imparts multiple functional properties such as gelling, foaming and emulsification in many food products, in addition to their nutritional quality [9]. However, its wider commercial use as protein supplements is limited due to allergenicity, insufficient digestibility, thermolability and high viscosity [10].

Enzymatic hydrolysis is frequently used to improve and upgrade the functional and nutritional properties of EWPs and obtain value-added egg products. EWP hydrolysates provide a number of benefits as a protein source in human nutrition in regard to the native EWPs, and they also have a positive impact on body functions or conditions and may ultimately influence health. For example, the hydrolysates are more water soluble, digestible for their lower molecular weight, their allergy is decreased, and they may have potential biological functions [11, 12]. Specifically, bioactive peptides with different activities can be released by enzymatic hydrolysis enhancing the value of the hydrolysates, and, therefore, the obtained hydrolysates can be used as bioactive functional food supplements.

EWPs are thought to be a good resource to obtain bioactive peptides by protease during food processing or gastrointestinal digestion. Over 24 different proteins have been identified and characterized in egg white [13]. The most abundant protein in egg white is ovalbumin, comprising 54 % of the protein content. Other proteins include ovotransferrin (12–13.6 %), ovomucoid (11 %), ovomucin (3.5 %), lysozyme (3.4–3.5 %), ovoflavoprotein (0.8 %), ovomacroglobulin (0.5 %), ovomucoid (1.5 %), ovoglobulins G2 and G3 (8 %), cystatin (ficin-papain inhibitor, 0.05 %), and some other enzymes than lysozyme. Numerous bioactive peptides with specific benefits for human and animal health derived from egg proteins via enzymatic hydrolysis have been identified [14]. These peptides released from intact egg white as well as from purified ovalbumin, ovotransferrin or lysozyme mostly have ACE-inhibitory and antihypertensive effects [8, 15, 16] or antioxidant activity [17]. Novel antimicrobial peptides released from native lysozyme by proteolytic digestion have shown to exhibit bacteriostatic activity against both Gram-positive bacteria and Gram-negative bacteria [18]. Some egg-protein-derived peptides are multifunctional exerting both antioxidant and antimicrobial activities or strong ACE-inhibitory and antioxidant activities, making these multifunctional preparations very

attractive for the prevention and control of many chronic human diseases such as cardiovascular diseases [19].

Enzyme type and degree of hydrolysis (DH) are substantial factors which affect the hydrolysates' performances, such as amino acid distribution, molecular weight distribution and amount of residual intact protein, thus influencing their bioactivity. An appropriate selection of protease for EWP hydrolysis will result in maximum biological activity of the high-quality hydrolysate providing design of a low-cost process with high productivity. Proteases represent a very large and complex group of enzymes, which differ in their origin and properties such as substrate specificity, chemical nature of their active site and catalytic mechanism, pH and temperature activity profile. They are broadly classified into two major classes based on their ability to cleave N- or C-terminal peptide bonds (exopeptidases) or internal peptide bonds of the polypeptide chain (endopeptidases) [20]. On the bases of their active site and mechanism of catalysis, proteases can be divided into seven groups by the MEROPS database: serine proteases, cysteine (thiol) proteases, aspartic proteases, metalloproteases, threonine proteases, glutamic acid proteases and unknown proteases [21]. Proteases of microbial origin such as Alcalase, Neutrase, Flavourzyme are the most commonly enzymes used in producing protein hydrolysates, but others such as protease from plant (e.g., papain) and animal sources (digestive enzymes, e.g., pepsin and trypsin) are also used [12, 14, 17]. Alcalase (EC 3.4.21.14) and Neutrase (EC.3.4.24.28) are an alkaline and a neutral endopeptidase produced from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, respectively, and commercialized by Novozymes. Alcalase is a relatively nonspecific serine-type protease with broad specificity, characterized by the presence of an essential serine residue at its active site. A main component of the commercial Alcalase preparation is the serine protease subtilisin having capacity for cleavage various peptide bonds with some preference for terminal hydrophobic amino acids. Some studies shown that it also preferentially cleaves peptide bonds on the C-terminal side of glutamate and aspartate residues [22]. Papain (EC 3.4.22.2) is a plant enzyme extracted from *Carica papaya* also with endopeptidase activity, which is characterized by the presence of an essential cysteine and a histidine residue at the active site (cysteine protease). Flavourzyme, obtained by the controlled fermentation of *Aspergillus oryzae*, is a mixture of proteases with both exo- and endopeptidase activities, which is frequently used to reduce the bitter taste of protein hydrolysates. All these commercial microbial or plant-origin proteases have recently drawn increased interest for obtaining protein hydrolysates and/or bioactive peptides on a commercial scale due to their unique functions and specificity, wide accessibility and cost-effectiveness [7, 23]. The purpose of this investigation was to study the

impact of different ultrasound pretreatments on the EWP on hydrolysis and antioxidant activity using these commercial food-grade proteases.

Because of the presence of specific serine protease inhibitors, the hydrolysis of EWPs demands a heating process to inactivate the inhibitors before protease action and facilitate the enzymatic hydrolysis. Although the potential of heat pretreatment to improve enzymatic hydrolysis of EWPs has been explored widely in the last decade [24], such method leads to destruction of some functional properties of egg white, such as gelling and foaming, which are often desirable for food manufacture [13]. Ultrasound pretreatment has also been used to improve the hydrolysis of several proteins [25] and in turn improve the production of angiotensin-converting enzyme (ACE) inhibitory peptides, by causing unfolding of protein chains and thus release possible peptides that are responsible for ACE-inhibitory activity [26]. Despite the promising effects of sonication alone or in combination with heat or pressure on a laboratory scale, there is limited information on the hydrolysis of EWPs from different ultrasound treatments and proteolytic enzyme systems. Few studies have been carried out on the effect of ultrasound on functional properties of EWPs including surface hydrophobicity, concentration of sulfhydryl (SH) groups, gelation temperature, bulk viscosity, foaming and emulsifying properties, but research on impact of ultrasound on enzymatic hydrolysis of EWPs and biological activity of hydrolysates is a rather scarce [27, 28].

Generally, the effects of applying ultrasound pretreatment on enzymatic hydrolysis of various proteins were mostly inconsistent. For example, Lei et al. [27] showed that the application of ultrasound pretreatment on ovotransferrin as a model EWP before thermolysin hydrolysis with both, 20 and 60 kHz horn increased the reactive sulphhydryl groups in 5 % ovotransferrin solution even by 50 %, but there were no improvements in the overall DH. On the other hand, Ren et al. [23] reported that the sweeping frequency ultrasound (SFU) treatment at 40 ± 2 kHz can remarkably raise the degree of zein hydrolysis and ACE-inhibitory activity of the hydrolysates. Similarly, Qu et al. [25] reported that ultrasound significantly promoted the reaction rate of defatted wheat germ protein (DWGP) hydrolysis at various temperatures using a probe-type sonicator and an ultrasonic cleaning bath. On the contrary, the ultrasonic pretreatment reported to promote the release of ACE-inhibitory peptides from DWGP, whereas the overall DH did not significantly alter at different power intensities and at different times using both ultrasonic horn reactor and ultrasonic bath operating with single fixed and sweep frequencies (24–68 kHz) modes [29]. The differences found in these studies could be due to factors related to the type of protein substrate,

equipment design and ultrasound power, and conditions used, but also on the composition of the sonicating medium.

The aims of this study were as follows: (1) to determine the impact of the ultrasound pretreatment on the hydrolysis of EWPs under different conditions (the power and the frequency of the applied ultrasonic field, temperature and pH, time of pretreatment); (2) to investigate the effects of the ultrasound pretreatment under adapted conditions on antioxidant activity of the obtained hydrolysates as well as their fractions and compare with conventional heat pretreatment. The final aim was to provide more information about characteristics (functional properties, digestibility) of the hydrolysates obtained with the pretreatment for potential applications in industries. For this purpose, the ultrasound pretreated EWPs were subjected to hydrolysis by various combinations of exo- and endopeptidase, and some functional properties including solubility, digestibility and foaming properties have been determined and compared.

Materials and methods

Materials

Freshly laid chicken eggs (pH 7.6–8.6), kindly provided from Jata Emona d.o.o. Ljubljana, were used immediately or stored at 4 °C upon collection. Egg white was separated from the yolk and gently stirred without foam formation to provide homogeneous mixture. Four commercially available proteases used in this work were Alcalase® 2.4 L (EC 3.4.21.14) from *B. licheniformis*, Neutrase® 0.8 L (EC 3.4.24.28) from *B. amyloliquefaciens*, Flavourzyme® 500 L, and Papain® from Papaya latex (EC 3.4.22.2), providing by Sigma–Aldrich Chemie (Steinheim, Germany). The declared enzyme activities were ≥ 2.4 U/g Anson Units, ≥ 0.8 U/g Anson Units, ≥ 500 U/g Anson Units and 1.5–10 U/mg solid for Alcalase, Neutrase, Flavourzyme and papain, respectively. 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 and pH 7.50. The chemicals, bovine serum albumin (BSA), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), trypsin (from porcine pancreas, type I) and protein standards were also purchased from Sigma-Aldrich Chemie GmbH, all of analytical grade. All other chemicals used in this research were of analytical grade.

Table 1 Hydrolysis condition used for preparation of EWP hydrolysates

Type of proteases	Enzyme activity	Activity of enzyme in samples (U)	Temperature (°C)	pH
Alcalase	≥2.4 U/g	2.12	50	8
Neutrase	≥0.8 U/g	0.76	50	7
Papain	1.5–10 U/mg solid	5.00	55	7
Flavourzyme	≥500 U/g	450	50	7

The ultrasound pretreatment

Before the enzymatic hydrolysis, a series of 10 % (w/w) egg white solution samples was exposed to ultrasonic waves in ultrasonic water baths with a frequency of 40 kHz (EI-NIS-RO-VEP, Serbia) and 35 kHz (BENDELIN electronic, sonorex digitec, DT 102 H, Germany). The samples were prepared in a beaker of 600 mL capacity where the working volume (~360 mL) was kept constant for all treatments and subjected to different ultrasound treatments. Two levels of temperature (25 ± 3.5 and 55 ± 3.5 °C) were used to compare the ultrasound treatment between 40 and 35 kHz, and the samples were subjected to sonication for different time (15, 30 and 60 min). For a control, 10 % (w/w) egg white solution was left in the ultrasonic bath at a frequency of 0 kHz at the same temperatures and time. The initial temperature of double distilled water inside the ultrasonic bath was continuously monitored using a thermocouple with digital display and maintained constant during the treatment. Also, the ultrasonic pretreatment was investigated in the pH range of 7.0–10.0 at 25 ± 3.5 °C in the ultrasonic water bath at 35 kHz for 30 min. Prior to sonication, the pH of the 10 % (w/w) egg white solution was adjusted to 7.0–10.0 using 2 M HCl or 2 M NaOH.

The ultrasonic power was determined calorimetrically [30] using following equation:

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

where m is the mass, C_p is the specific heat capacity and (dT/dt) is the initial rate of change of temperature during sonication. This was determined by fitting the data obtained for temperature rise (using a standard thermocouple) against time to a polynomial curve and extrapolating to time. The values of actual ultrasonic power dissipated in the liquid have been calculated to be 65.7 and 21.3 W for water baths with frequency 35 and 40 kHz, respectively.

The heat pretreatment

To find out the effect of ultrasound on enzymatic hydrolysis of EWPs and antioxidant activity of the obtained hydrolysates, the experiments with heat-treated EWPs have also performed having the same processing conditions. Heat treatment was carried out by first heating EW solution

[10 % (w/w)] at 75 °C for 15 min and then subjecting it to the hydrolysis by Alcalase described above. The rest of the conditions were the same as those for ultrasound treatment described above.

One-stage enzymatic hydrolysis of EWPs

Enzymatic hydrolyses were carried out in a 600-mL mechanically stirred batch reactor with temperature and pH control by using three enzymes separately, namely Alcalase, Neutrase and papain according to the previously published protocol with slight modification [28]. The substrate for enzymatic hydrolysis was 10 % (w/w) aqueous solution of untreated or pretreated egg white (11.9 mg/mL, protein content determined according to the standard Kjeldahl method, $N \times 6.25$) which was adjusted to optimum pH for particular enzyme activity with 2 M HCl or 2 M NaOH, then stirred and allowed to equilibrate to the working temperature for 15 min (typically 50 °C). A preliminary assay of enzymatic hydrolysis was previously done in order to establish the suitable hydrolysis temperature and pH for each enzyme and results are presented in Table 1. The reactions were started by adding the appropriate amount of enzyme (enzyme/substrate ratio was around 2 % w/w). During the course of the reaction, pH was kept at a constant value by adding 1 M NaOH, using pH-stat method with automatic dosage of the base.

The reaction was stopped by heating the mixture at 90 °C for 15 min to inactivate the enzyme. The hydrolysate obtained was cooled down to room temperature and centrifuged at $3,500 \times g$ for 20 min. The supernatant was finally collected as the hydrolysate and stored at 4 °C for subsequent analysis or used for the following two-stage hydrolysis by selected protease. All sets of hydrolysis experiments were carried out in triplicate.

Two-stage enzymatic hydrolysis of EWPs

Alcalase Flavourzyme sequential digestions were performed as previously described in Pedroche et al. [31] with slight modifications. For the two-stage hydrolysis with Alcalase and Flavourzyme or Alcalase and Neutrase, the egg white solution (10 % w/w) was first hydrolyzed with Alcalase (2.1 % E/S ratio) at 50 °C, pH 8, until the DH of approximately 18 % was reached. Then, pH was adjusted

to 7.0 followed by adding Neutrase or Flavourzyme (2.1 % E/S ratio) and the reaction mixture was incubated at 50 °C for 60–80 min. During the reaction pH was kept at a constant value by adding 1 M NaOH, using pH-stat method. The enzymatic reaction was stopped by heat inactivation at 90 °C for 15 min.

For the two-stage hydrolysis with Neutrase and Alcalase, the egg white solution (10 % w/w) was first subjected to a partial hydrolysis with Neutrase at 50 °C, pH 7 until the DH around 18 % was reached. Then, the pH value was adjusted to 8.0 by the addition of 2 M NaOH, and 2.12 U of Alcalase was added. The rest of the protocol was the same as before. All experiments were derived in the batch reactor with continuous stirring under the appropriated reaction conditions (Table 1).

The degree of hydrolysis

The number of peptide bonds cleaved is directly proportional to base consumption. The DH was used as a parameter to measure the effect of ultrasonic waves on susceptibility of EWPs to enzymatic hydrolysis and was calculated by the following equation [32]:

$$\text{DH (\%)} = B \times N_B \times \frac{1}{\alpha} \times \frac{1}{m_p} \times \frac{1}{h_{\text{tot}}} \times 100 = \frac{h}{h_{\text{tot}}} \times 100 \quad (2)$$

where B is the consumption of the base in mL, N_B 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), α is the degree of dissociation of the α -amino groups ($1/\alpha = 1.13$ at 50 °C and pH 8.0).

The average peptide chain length (APCL) of hydrolysates was estimated using the DH [Eq. (2)], assuming that the entire hydrolysate was soluble, as only the supernatants were analyzed [32]:

$$\text{APCL} = \frac{100}{\text{DH (\%)}} \quad (3)$$

Determination of antioxidant activity

Reducing power

Reducing power of the obtained hydrolysates containing bioactive peptides was determined by the modified method of Oyaizu [33]. The sample solution (0.5 mL) was 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

3,000 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 read at 700 nm. The increased absorbance of the reaction mixture indicates increasing reducing power [34].

DPPH radical scavenging activity

DPPH has been a synthetic organic radical frequently applied to evaluate radical scavenging capacity of bioactive compounds and food extracts [35]. It is more stable than conventional natural radical and unaffected by metal ion chelation. The effect of EWP hydrolysates on the scavenging of DPPH radicals was determined as previously described by Jakovetić et al. [36] with slight modifications. Briefly, a 0.2-mL aliquot of each obtained hydrolysate was mixed with 1.8 mL of 0.1 mM DPPH solution in methanol. After mixing vigorously for 2 min, 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 (Ultrospec 3300 pro, Amersham Bioscience). DPPH radical scavenging activity (RSA, %), expressed as the percentage of inhibition, was calculated according to the method described elsewhere [36] as follow:

$$\text{RSA (\%)} = \left[1 - \frac{A_s - A_b}{A_c} \right] \times 100 \quad (4)$$

where A_s is the absorbance of the tested antioxidant (EWH), A_0 is the absorbance of the EWH in methanol and A_b is the absorbance of the DPPH solution without the sample. The protein content in all samples was determined using the Lowry method [37].

ABTS-free radical scavenging activity

The ability of EWP hydrolysates and their ultrafiltered fractions to scavenge ABTS radical was tested according to the previously described method [38]. This method is based on the ability of antioxidant molecules to quench the stable bisradical cation, $\text{ABTS}^{\bullet+}$, a blue-green chromophore with characteristic absorption at 734 nm. ABTS radical cation ($\text{ABTS}^{\bullet+}$) was produced in the reaction between ABTS (7 mM) and potassium persulfate (2.45 mM final concentration). The reaction mixture was kept in dark at room temperature for at least 12 h before use. Further, $\text{ABTS}^{\bullet+}$ solution was diluted with 5 mM phosphate-buffered saline (PBS, pH 7.4), until the absorbance of 700 (± 0.05) was reached. Diluted $\text{ABTS}^{\bullet+}$ solution (500 μL) was mixed with hydrolysates containing 2 mg/mL of protein (5 μL), and absorbance was measured at 734 nm after 5 min. The ABTS antiradical scavenging activity (%) was calculated using the following equation:

$$\text{ABTS (\%)} = 100 \times \left(\frac{A_s - A_b}{A_c} \right) \quad (5)$$

where A_s represents the absorbance of the sample solution in the presence of the ABTS, A_b is the absorbance of the sample solution without ABTS and A_c is the absorbance of the control solution with ABTS. The assay was standardized using both Trolox and ascorbic acid, a synthetic and a natural antioxidant, respectively, and results were expressed as Trolox equivalent (Trolox equivalent antioxidant capacity, TEAC) as well as ascorbic acid equivalent (ascorbic equivalent antioxidant capacity, AEAC).

Peptide fractionation by ultrafiltration

The Alcalase EWP hydrolysates obtained with ultrasound and heating pretreatments were subjected to ultrafiltration using membranes with 30, 10 and 1 kDa molecular weight cutoff (MWCO), sequentially. The ultrafiltration was performed using a Millipore ultrafiltration stirred cell unit (Model 8050 1 Unit, Millipore Corporation, Bedford, MA, USA) through cellulose membranes with different MWCO under 40 psi nitrogen gas. A sample of 50 mL hydrolysates was first ultrafiltered through a 30-kDa membrane to obtain two fractions: retentate (fraction 1, F1, representing hydrolysates >30 kDa) and permeate (MW <30 kDa). The permeate fraction was further ultrafiltered through a 10-kDa membrane to yield the second retentate (fraction 2, F2, representing hydrolysates between 10 and 30 kDa) and the second permeate (MW <10 kDa). The permeate was also further subjected to ultrafiltration through a 1-kDa membrane to obtain the third retentate (fraction 3, F3, representing hydrolysates between 1 and 10 kDa) and permeate (fraction 4, representing hydrolysates <1 kDa). Each retentate or permeate was collected and freeze-dried to determine its protein concentration. The second permeate (fraction <10 kDa) was subjected to molecular sieve chromatography.

Molecular weight distribution of EWP hydrolysates

The permeate fraction <10 kDa (before ultrafiltration through a 1 kDa membrane) was subjected to molecular sieve chromatography on a 250 × 25 mm column, packed with Toyopearl HW-40F (Tosoh Bioscience GmbH, Stuttgart, Germany) to determine the molecular weight distribution. The column was operated with a flow rate of 1 mL/min and elution was monitored at 214 and 280 nm (UV-Vis detector C-640, Buchi Labortechnik AG, Switzerland). The elution buffer was 30 mM sodium phosphate buffer (pH 7.0), while cytochrome *c* (12,400 Da), diglutathione (691.59 Da), L-glutathione reduced (307 Da) and

phenylalanine (165.19 Da) were used as the comparable standards of molecular weight.

Functional properties of EWP hydrolysates

Solubility

Solubility of all EWP hydrolysates was determined at two pH values and compared. Samples were suspended in water (10 % w/v), and pH was adjusted to 6 or 8 using 0.5 M NaOH or 0.5 M HCl while stirring at room temperature for 1 h. The samples were then centrifuged at 10,000×g for 15 min. The protein content in all samples was determined using the Lowry method [37] using BSA as the standard. Solubility was expressed as the percentage of protein remaining in the supernatant as compared to the untreated samples.

Digestibility

In vitro protein digestibility was carried out according to the method described by Elkhailil et al. [39] with slight modifications. About 2 mL of 10 % (w/w) egg white aqueous solution or hydrolysates was digested by 10 mL of trypsin solution (0.2 mg/mL in 100 mM Tris-HCl buffer, pH 7.6). The suspension was incubated at 37 °C for 2 h. Hydrolysis was stopped by adding 5 mL 50 % trichloroacetic acid (TCA). The mixture was allowed to stand for 30–35 min at 4 °C and was then centrifuged at 10,000×g for 25 min (SIGMA 2-16, rotor 12141, Germany). The resultant precipitate was dissolved in 5 mL of 0.2 M NaOH, and protein concentrate was measured using the Lowry method. Digestibility was calculated as follows:

$$\text{Protein digestibility (\%)} = \frac{A - B}{A} \times 100 \quad (6)$$

where A is total protein content (mg) in the sample and B is total protein content (mg) in the TCA precipitate.

Foaming properties

The aqueous egg white solution and the obtained hydrolysates with concentration of 10 % (w/w) were diluted to 8 % (w/w) to prepare foams. The initial volume of 50 mL (20 °C) 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, 600 W, 50 V, 8,000–24,000 rpm) [40]. Foam capacity (FC) was expressed as foam expansion at 0 min, which was calculated according to the following equation:

$$\text{FC (\%)} = \frac{A - B}{A} \times 100 \quad (7)$$

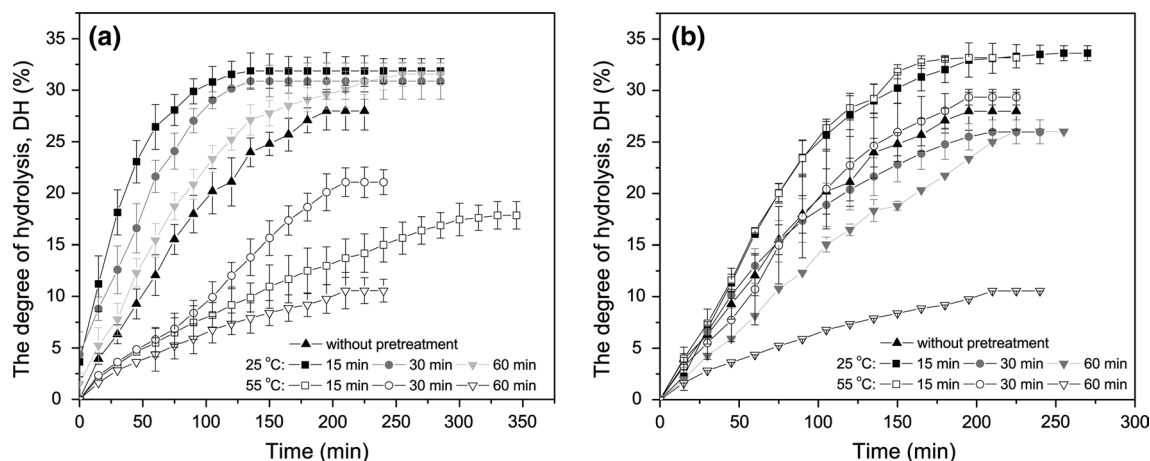


Fig. 1 Profiles and degrees of hydrolysis as a function of temperature and ultrasound pretreatment time. **a** at 40 kHz (calorimetric power of 21.3 W) and **b** at 35 kHz (calorimetric power of 65.7 W). Pretreatment conditions: closed black triangle control, closed square 25 °C,

15 min; closed circle 25 °C, 30 min, closed gray triangle 25 °C, 60 min; open square 55 °C, 15 min; open circle 55 °C, 30 min, open triangle 55 °C, 60 min. Hydrolysis conditions were: 50 °C, pH 8.0; 2.12 U of Alcalase, 10 % (w/w) egg white (E/S ratio 2.0 % w/w)

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 with the solution at 4 min after whipping:

$$FS (\%) = \frac{A - B}{A} \times 100 \quad (8)$$

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

Statistical analysis

Experiments were carried out in duplicate or triplicates and expressed as means with SD. Analysis of variance (ANOVA), followed by the Tukey test, was used to determine the significant difference between samples at $p < 0.05$ level. All analyses were done using Microsoft Office Excel 2007 software (Microsoft Corporation, WA, USA).

Results and discussion

The effect of ultrasound pretreatment of EWPs on their susceptibility to Alcalase

Because of the presence of specific serine protease inhibitors in raw egg white (for example ovomucoid) and low susceptibility to enzymatic hydrolysis, the hydrolysis of EWPs demands a pretreatment process before protease action. In this part of work, EWPs were subjected to different ultrasound pretreatments and then hydrolyzed by Alcalase. The effects of the pretreatment were studied at two

different calorimetric powers and temperatures for different periods of time. The objective was to find the conditions that would lead to irreversible denaturation of the Alcalase inhibitor, but at the same time, would not result in the formation of insoluble aggregates or precipitates, which are poor substrate for proteolysis. The results obtained from this experimental set are presented in Fig. 1.

It is apparent from Fig. 1 that the initial rate, extent and pattern of EWP hydrolysis varied according to the conditions, exposure time and intensity of the ultrasound treatment. The treatment at a calorimetric power of 21.3 W and frequency of 40 Hz at a lower temperature (25 °C) seemed to improve the hydrolysis for all treatment time (Fig. 1a). In this case, the treatment time did not significantly alter the overall DH ($X_e = 31.44 \pm 0.251$), which was higher compared with that obtained with untreated sample ($X_e = 27.99 \pm 0.925$). However, the initial hydrolysis rate was significantly lower as the pretreatment time was increased. A clear difference in the rates of reaction at a lower temperature (25 °C) was also observed when compared with that at a higher temperature (55 °C).

At lower frequency of 35 kHz and higher calorimetric power of 65.7 W (Fig. 1b), both extent and initial rate of hydrolysis for prolonged treatment showed a very large decrease when compared with short treatments. For example, when treatment time was increased from 15 to 60 min at 25 °C, a significant decrease in DH from 33.6 to 26.0 % was observed (by 22.6 %). This decrease in overall DH was even more pronounced when heating was performed during ultrasound treatment (from 33.2 to 10.6 %). It is considered that the ultrasound pretreatment caused the structural changes of EWPs beneficial to promote hydrolysis, but prolonged exposure to ultrasound of 60 min seemed to have a

negative effect. This could be due to the protein aggregates formation influencing lower accessibility of the unfolded and aggregated proteins to the appropriated protease. This is in agreement with the findings of Jambrak et al. [41], who reported that the prolonged treatment of whey protein isolate with ultrasonic bath of 40 kHz encourages the formation of aggregates of molecules.

Except for ultrasound pretreatment at 35 kHz for 30 min, it was clearly shown that the heating of the ultrasound bath did not achieve the expected improvement in hydrolysis. Namely, it was supposed that the sonication under elevated temperature, but lower than ~58 °C, at which egg white coagulation and denaturation process began would improve EWP hydrolysis due to decreased ultrasonic wave attenuation for hen's egg white at higher temperature [42]. Namely, the part of the kinetic energy of the wave has been expected to be absorbed and converted into mechanical and thermal energy that in turn may cause the structural changes in protein molecules through which the waves propagate. However, too excess ultrasound treatment in combination with heating caused aggregation of proteins preventing them from further hydrolysis. As visually apparent, an opaque suspension of protein aggregates was obtained in this case. Thus, the bath temperature of 25 °C was adopted for further investigation.

Overall, the presented results are in agreement with several previous studies showing increased subsequent proteolysis after the ultrasound treatment. Compared with the control, the optimal ultrasound pretreatment at 40 kHz and 25 °C for 15 min increased the initial rate and equilibrium DH by about 139.8 and 13.86 %, respectively. Similarly, in a related study where milk protein concentrate was treated with ultrasound at 800 W [26], an improvement in the protein hydrolysis by several proteases up to 184.9 % after pretreatment was reported, depending on the enzyme used and the pretreatment time. Likewise, an ultrasonic treatment of DWGP at 600 W and 20 °C for 10 min with the probe-type sonicator and cleaning bath has shown to significantly decrease the energy barrier required for the hydrolytic reaction, improving the reaction rate [25]. SFU and fixed frequency ultrasound pretreatment at 40 ± 2 kHz have also reported to increase the degree of zein hydrolysis by approximately 11.5 % over the control, by altering the second structure of zein and rupturing the smooth surface of protein [23].

However, our results are in conflict with several previous studies which shown that the ultrasound treatment of various proteins did not significantly alter the overall DH or the effects were minimal [43, 44]. In this light, Lei et al. [27] showed that the sonication of ovotransferrin before thermolysin hydrolysis had insignificant effect on its digestibility. Even though the ultrasound pretreatment has been reported to increase the reactive sulphydryl groups in 5 % ovotransferrin solution by even 50 %, there were no improvements in the

overall DH. However, the pretreatment was applied to a pure EWP, ovotransferrin, in the model system and thermolysin was used to measure the susceptibility to enzymatic hydrolysis. In contrast, our current research examined the effect of sonication on Alcalase hydrolysis of constituent proteins (whole egg white), which behavior is known to be strongly influenced by ovalbumin. The observed differences in the impact of pretreatment on the hydrolysis might be explained by the higher resistance of ovotransferrin to ultrasound energy compared with ovalbumin as it contains several disulphide bonds and a glycan chain attached to the C-terminal domain, stabilizing its three-dimensional structure. Ovalbumin as the major egg white protein contains only one disulphide bond and its structure is more affected by such treatment. Generally, the ultrasonic denaturation mechanisms are specific to the particular protein under investigation and depend on its amino acid composition and the conformational structure. Also, the final effect is a function on the nature of the sonicating medium, equipment and conditions used.

The effect of substrate pH during the ultrasound pretreatment on enzymatic hydrolysis

It is widely recognized that changes of the pH value of substrate solution can cause denaturation of proteins by changing the charge distribution and conformation of the molecules and facilitates the accessibility of proteases to the inner peptide bonds [28]. Therefore, the influence of the pH of EWP solution during the ultrasound pretreatment prior to hydrolysis has been tested. For this purposes, the pH values of EWP solution have been optionally adjusted to particular pH with 1 M NaOH solution (for pH 10.0) or with 1 M HCl (for pH 7.0) and the samples were subjected to sonication at 35 kHz for 30 min followed by Alcalase hydrolysis. The results are shown in Fig. 2.

It appeared that an increase in pH increased the reaction rate. Specifically, at pH 9.25, the overall DH reached 29.9 ± 1.82 %, but this value dropped to 17.6 ± 0.65 at pH 7.6. Probably, this happened because of less pronounced aggregation of protein molecules at higher pH (further away from the average pI of the acidic EWPs) at which repulsion between protein molecules was stronger. This was in agreement with the turbidity profile obtained after sonication of 10 % (w/w) solution of egg white at different pH (data not shown). Namely, sonication at pH in the range from 10 to 9.1 at 35 kHz for 15 min did not appear to change the solution turbidity, but it significantly increased when sonicating at 8.6 and 7.0. Additionally, exposure of EWPs to extreme pH conditions might cause significant structure unfolding of the molecules. Both factors would contribute to the increase in the susceptibility to Alcalase hydrolysis.

The addition of sodium hydroxide before ultrasound pretreatment had a negative impact on the subsequent

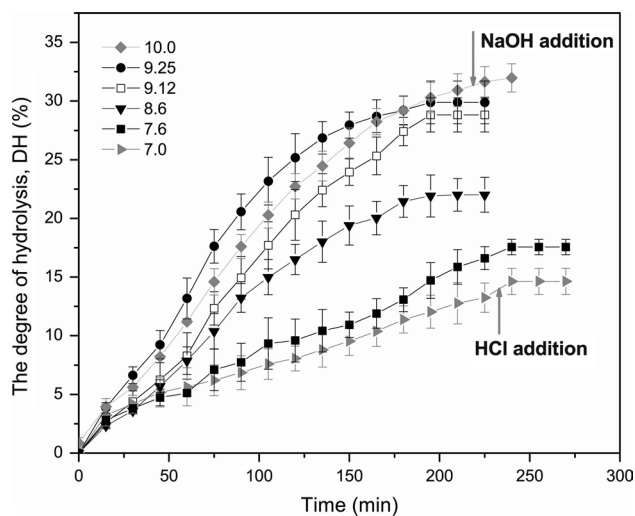


Fig. 2 The degree of hydrolysis as a function of pH of EWP solutions which were ultrasound pretreated during 30 min at 35 kHz. Hydrolysis conditions were as follows: 50 °C, pH 8.0; 2.12 U of Alcalase, 10 % (w/w) egg white (E/S ratio 2.0 % w/w)

hydrolysis of EWPs (Fig. 2). Specifically, better result has been obtained with naturally basic egg white which pH was 9.25, rather than that observed with the sample in the presence of sodium hydroxide (pH 10.0). Fresh egg white has pH of approximately 7.6–8.5 which increases to approximately pH 9.7 during storage as the CO₂ escapes [45], and the albumen pH increases at a temperature-dependent rate to a maximum value of about 9.7. After 3 days of storage at 3 °C, Sharp and Powell [45] found an albumen pH of 9.18. After 21 days of storage, the albumen had a pH close to 9.4, regardless of storage temperature between 3 and 35 °C [11]. The increase in albumen pH is due to CO₂ loss through the shell pores, and it depends on dissolved CO₂, bicarbonate ions, carbonate ions and protein equilibrium. Bicarbonate and carbonate ion concentrations are affected by the partial CO₂ pressure in the external environment. Thus, at pH of fresh egg white (7.6 and 8.6), albumen seemed to be more resistant to the hydrolysis. Present results have shown that the highest initial hydrolytic rate was achieved with egg white sample after prolonged storage (pH 9.25), almost 2.5-fold increase compared with the one of fresh egg white (pH 7.6). This pH dependence on the rate of proteolysis of EWPs is important from the practical point of view that the pH of egg white increases during storage of eggs.

Average peptide chain length (APCL) of EWP hydrolysates

The peptide chain length is related to the average molecular weight of peptides in hydrolysates. However, hydrolysates

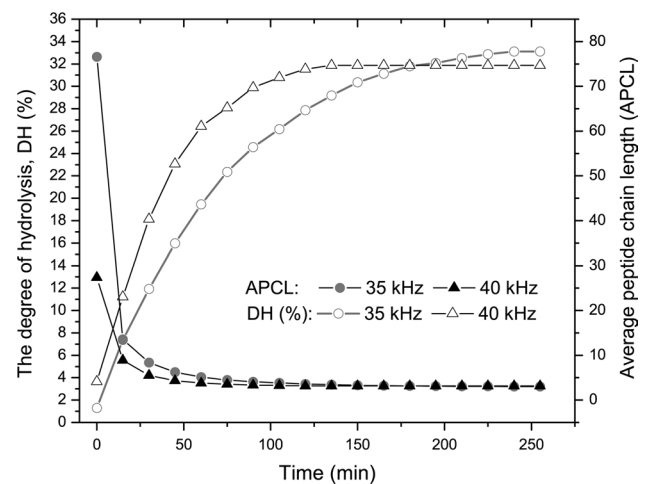


Fig. 3 The degree of hydrolysis (*open symbols*) and the average peptide chain length (*closed symbols*) during enzymatic hydrolysis of EWPs which were pretreated at two different frequencies (40 kHz triangle and 35 kHz circle). The reaction conditions: 2.12 U of Alcalase, 10 % (w/w) egg white, 50 °C, pH 8.0

with similar APCL may have substantially different peptide molecular weight distributions. Once determined the DH, the APCL of the hydrolysates could be estimated, assuming all hydrolysates are soluble. This is related to the average molecular weight of peptides in the hydrolysates and is illustrated in Fig. 3.

Figure 3 shows the changes in APCL of the ultrasound pretreated hydrolysates at two different frequencies (40 and 35 kHz) having similar processing conditions (pH 9.25, 25 °C, 15 min). It can be easily observed that the pretreatment led to changes in APCL, and it decreased from 77 to 3 amino acids after 4 h of hydrolysis, and from 28 to 3 amino acids after 2 h hydrolysis, for hydrolysates sonicated at 35 and 40 kHz, respectively. The illustrated results of APCL were in agreement with the model change of the DH, and according to that, the hydrolysis of EWPs with 15 min ultrasound pretreatment at 40 kHz has been adapted as a model reaction. The average number of amino acids in peptides generated by hydrolysis of the adopted model reaction was less than that obtained in other hydrolysis reactions, revealing that this treatment caused strongly structural changes of proteins and resulted in a higher subsequent susceptibility of EWP solutions to enzymatic hydrolysis.

Effect of ultrasound pretreatment on antioxidant activity of EWP hydrolysates

An additional aim of this research was to contribute to a number of previous studies dealing with the antioxidant activity of EWP hydrolysates most commonly prepared by the commercially available microbial food-grade protease, Alcalase, determining whether the ultrasound pretreatment

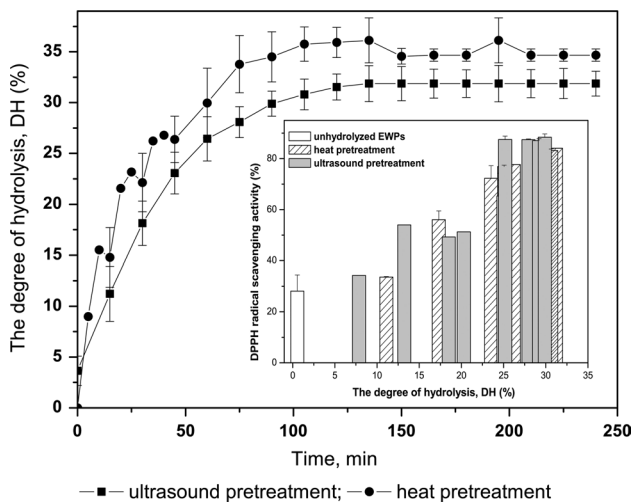


Fig. 4 Time course of EWP hydrolysis after heat (*circle*) and ultrasound pretreatments (*square*). *Insert* the radical scavenging activity of egg white hydrolysates as a function of degree of hydrolysis for heat (*sparse pattern*) and ultrasound pretreatments (*gray pattern*)

could improve antioxidant activity of these hydrolysates compared with conventional heat pretreatment. For this reason, the antioxidant activity as measured by DPPH method was detected in all EWP hydrolysates during the course of hydrolysis (results have been presented in Fig. 4).

It appeared that the antioxidant properties were enhanced by hydrolysis in both of cases. Before hydrolysis, unhydrolyzed EWPs showed a rather low DPPH radical scavenging activity (28.1 ± 6.38 %) and the values increased significantly with the course of hydrolysis for both pretreatments (84 ± 0.59 and 88.4 ± 1.31 % for heat and ultrasound pretreatment, respectively). Even though the maximum conversion for ultrasound pretreated sample was not significantly less than that achieved for the heat pretreatment, the antioxidant activity of the hydrolysates varied significantly between the different pretreatment used ($p < 0.05$). Hydrolysates prepared using the ultrasound pretreatment appeared to be more effective in scavenging DPPH radicals. This suggests that different protein treatments prior to hydrolysis may have resulted in different alterations in the protein's tertiary structure affecting profile of produced peptides and consequently their free radical scavenging activity. This is in agreement with results obtained by several authors, who reported that the ultrasound pretreatment can enhance the ACE-inhibitory activity of the obtained hydrolysates, probably due to the ability to release more potent peptides [27, 29]. Because of the mechanism of action of ultrasound on proteins denaturation, one would expect alterations in both antioxidant and ACE-inhibitory activity upon application of high-intensity ultrasound, but different claims about alterations of peptides' activity have been reported in the

literature. For example, Lei et al. [27] reported that the sonication prior the thermolysin hydrolysis of ovotransferrin enhanced the production of ACE-inhibitory peptides, whereas the antioxidant activity of hydrolysates was not significantly changed. Contrary, Xu et al. [46] reported that the antioxidant activity of defatted soybean meal was improved after ultrasound pretreatment. Generally, the ability of ultrasound to stimulate bioactive peptides' production has received very little attention. Current research concerns for the first time the impact of ultrasound pretreatment of EWPs prior the enzymatic hydrolysis on the antioxidant activity of obtained hydrolysates.

In order to confirm whether the ultrasound pretreatment prior to Alcalase hydrolysis resulted in different peptide profile compared with the heat pretreatment, EWP hydrolysates with around 27.0 % DH obtained with both pretreatments were further separated using ultrafiltration membranes with 30, 10 and 1 kDa MWCO to 4 hydrolysate fractions (<1, 1–10, 10–30 and >30 kDa) which were investigated for protein content, peptide yield and antioxidant activity in terms of ABTS radical scavenging ability. This method is very commonly used by researchers to evaluate the radical scavenging ability of antioxidants [47]. Antioxidants with higher ABTS inhibition are considered to have higher antioxidant efficacy (Table 2).

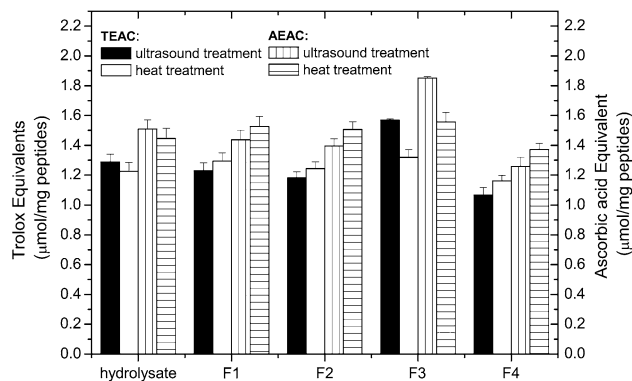
It is apparent that the hydrolysis after heat pretreatment generated more peptides <1 kDa (19.04 ± 1.02 %) than ultrasound pretreatment did (11.90 ± 0.53 %), whereas the proportion of peptides <10 kDa were higher in the second case (28.80 ± 0.07 vs. 20.46 ± 0.39 %). Different pretreatments seemed to influence the protein folding leading to different molecular weight distribution profile after hydrolysis which could influence the antioxidant activities of the hydrolysate fractions. In general, no significant difference was observed between the hydrolysates obtained with different pretreatments in their ability to scavenge ABTS•+. However, the fraction obtained by ultrasound pretreatment containing peptides with a MW between 1 and 10 kDa showed a significantly high ($p < 0.05$) ABTS radical scavenging efficacy of 97.54 ± 0.30 %. The dependence of ABTS radical scavenging activity on sample concentration (0.5–10 mg/mL) was found to be linear (6 min reading) in all samples ($R^2 > 0.97$) (data not shown). The results were also expressed as both Trolox and ascorbic acid equivalents antioxidant capacity and presented in Fig. 5.

The oxygen radical absorbance capacity of the hydrolysates and their fractions did not vary significantly between hydrolysates and/or their fractions, except for the fraction F3 obtained with the ultrasound pretreatment showing a rather high Trolox and ascorbic acid equivalents (1.569 ± 0.007 and 1.852 ± 0.008 $\mu\text{mol/g}$ peptides, respectively). The ABTS inhibitory activity of the F₃ fraction was two times lower (4.31 mg/mL) compared with the small

Table 2 Molecular weight distribution profile and antioxidant activity of the hydrolysates obtained by ultrasound and heat pretreatments and their ultrafiltered fractions

	Ultrasound pretreatment			Heat pretreatment		
	m_p (mg)	Mass fraction (%)	ABTS (%)	m_p (mg)	Mass fraction (%)	ABTS (%)
Hydrolysate	591.7 ± 3.67	100	79.44 ± 2.31	574.96 ± 6.02	100	76.17 ± 2.59
F ₁ >30 kDa	197.18 ± 2.29	33.32 ± 0.18	76.46 ± 2.32	189.90 ± 3.59	33.03 ± 0.28	80.39 ± 2.51
F ₂ 10–30 kDa	128.24 ± 1.51	21.67 ± 0.12	73.50 ± 1.77	119.76 ± 5.19	20.83 ± 0.69	77.33 ± 2.00
F ₃ 1–10 kDa	170.41 ± 1.48	28.80 ± 0.07	97.54 ± 0.30	117.61 ± 1.01	20.46 ± 0.39	82.01 ± 2.29
F ₄ <1 kDa	70.45 ± 3.59	11.90 ± 0.53	66.25 ± 2.23	109.49 ± 6.97	19.04 ± 1.02	72.25 ± 1.59

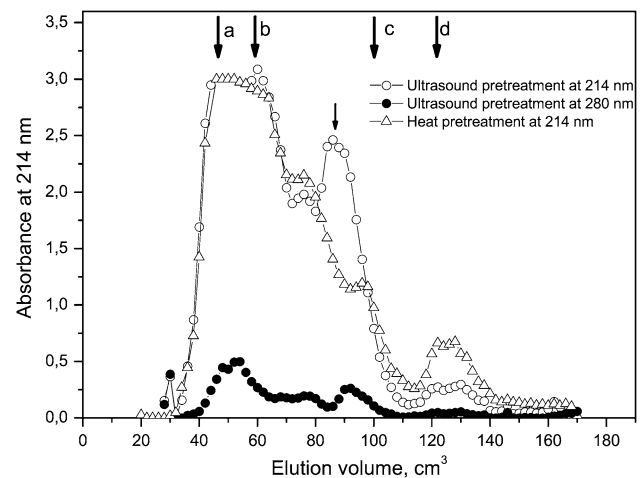
m_p : Total protein content determined by Lowry method. ABTS scavenging capacity for untreated egg white was 7.69 ± 1.59 %

**Fig. 5** ABTS scavenging capacity for EWP hydrolysate fractions obtained with heat and ultrasound pretreatments expressed in Trolox and ascorbic acid equivalents per gramme of dry weight

peptide fraction (F₄, <1 kDa) (IC₅₀ = 9.86 mg/mL), making it a good candidate for functional food production.

The fractions <10 kDa were then subjected to molecular sieve chromatography on the Toyopearl HW-40F column that has good separation efficiency in the MW range of 100–10,000 Da, and the results are presented in Fig. 6.

There is apparently very little difference in the gel-filtration pattern of the peptide fraction obtained by ultrasound and heat pretreatment. It seemed that smaller dipeptides of <1 kDa and free amino acids were produced by both pretreatments. However, after ultrasound pretreatment few additional lower molecular weight peptides were apparent (corresponding to around 1,650 and 470 Da based on standard curve). Hence, this suggested that these low molecular mass peptides from the enzymatic hydrolysis of EWPs could have a higher ABTS radical scavenging efficacy than other molecular mass peptides, contributing for higher antioxidant activity of the hydrolysate prepared after ultrasound pretreatment. Earlier studies have shown that the majority of the antioxidative peptides derived from food sources have molecular weights ranging from 500 to 1,800 Da [20, 48]. In future studies, the antioxidant properties of each fraction will be tested.

**Fig. 6** Toyopearl 40HW gel-filtration column chromatography of hydrolysates obtained by Alcalase after ultrasound (circle) and heat pretreatment (triangle). The arrows indicate the elution position of markers [a cytochrome c (12,400 Da), b diglutathione (691.59 Da), c L-glutathione reduced (307 Da), d phenylalanine (165.19 Da)]

One-stage and two-stage enzymatic hydrolysis of EWPs

The effects of protease type (endo- and exopeptidases) and manner of performing enzymatic process (one- vs. two-stage) on selected functional and antioxidant properties of obtained hydrolysates were investigated in the reactions under recommended conditions for each protease (Table 1). The ultrasound pretreatment (15 min at 25 °C and frequency 40 kHz) was performed prior the enzymatic hydrolysis in all cases. The samples were then treated with the commercial proteases Alcalase, Neutrase, papain and Flavourzyme that were added individually in the one-stage process or as a combination of two sequentially added proteases in the two-stage process. A graphical comparison of the hydrolytic curves obtained for one- and two-stage enzymatic EWPs hydrolysis is shown in Fig. 7.

It appeared that the initial rate, extent and pattern of EWP hydrolysis vary according to the protease specificity, substrate affinity and the type of enzymatic process. As regards

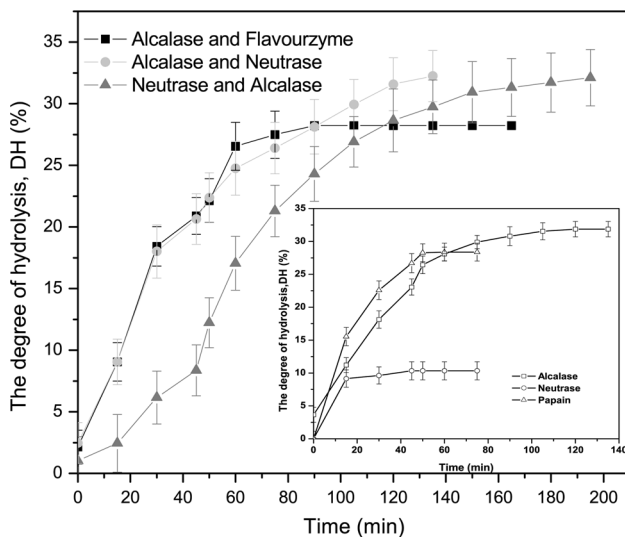


Fig. 7 Degree of hydrolysis (%DH) as a function of time for one-stage (*inset*) and two-stage enzymatic hydrolyses of EWPs with different proteases

one-stage hydrolysis process, the highest hydrolysis degree was achieved for Alcalase followed by papain and Neutrase. This result was expected due to the fact that Alcalase is endopeptidase, acting without order inside of EWPs molecules and randomly decomposed them to low molecular weight peptides. However, the initial reaction rate was slightly higher with papain than with Alcalase. In this case, a rapid reaction rate was obtained in the first 30 min, and then the rate of hydrolysis subsequently decreased, reaching a plateau after 50 min. Hydrolysis with Alcalase was more gradual approaching a plateau after 75–90 min of hydrolysis. A constant rate was also observed with Neutrase after only 15 min of reaction time, suggesting that the enzymatic reaction reached the steady state phase. Similar results were reported by Dryakova et al. [49], who studied the hydrolysis of whey protein using four microbial proteases, revealing that hydrolysates digested with Neutrase showed clearly lower DH and contained peptides with longer amino acid chain length than those prepared by Alcalase or Flavourzyme digestion.

Comparing the presented hydrolytic curves of two-stage enzymatic process, the highest initial rate and equilibrium DH were achieved by a combination of protease from *B. licheniformis*-Alcalase and protease from *B. amyloliquefaciens*-Neutrase. The order of addition of these two proteolytic enzymes did not lead to significant differences in the equilibrium DH (32.18 ± 0.04 %), but significantly affected the initial rate of reaction. The two-stage hydrolysis, in which Alcalase is followed by Neutrase, seemed to perform with the highest initial rate ($0.60 \Delta\text{DH} \% \text{ min}^{-1}$).

The functional properties of EWP hydrolysates obtained by the one-stage and two-stage enzymatic hydrolyses with various proteases

EWP hydrolysates were further tested for their antioxidant activity and functional properties. The aim was to obtain highly hydrolyzed EWPs, but also an end hydrolysate with improved antioxidant activity and functionality. Besides DPPH activity, other properties of the EWP hydrolysates important for their practical application in food products were determined such as functional properties. These properties depended also on the DH, thus protein hydrolysates with the same DH of 27 % were produced from egg white using several proteases in one- and two-stage enzymatic processes. The results are summarized in Table 3.

The DPPH radical scavenging activities of all generated EWP hydrolysates were clearly higher than non-hydrolyzed egg white. The results revealed that EWP hydrolysates potentially contain bioactive peptides which were electron donors and could react with free radicals to convert them to more stable product and terminate the radical chain reaction. Alcalase hydrolysate has shown the best antioxidant properties. It is known that the antioxidant activity of protein hydrolysates is remarkably dependent on amino acid composition and their sequences which are affected by the protein source and conditions of the hydrolysis process, including digestive enzyme, temperature, pH, reaction time, ratio of substrate/enzyme and others [50]. In addition, as previously discussed, the antioxidant activity

Table 3 The functional properties of EWP hydrolysates obtained by one- and two-stage enzymatic hydrolyses with various proteases

Parameters	Egg white	Alcalase	Papain	Alcalase and Flavourzyme	Alcalase and Neutrase
The degree of hydrolysis (DH) (%)	–	27	27	27	27
Foaming capacity (%)	75.12	73.55	74.75	75.96	77.48
The foam stability (%)	32.43	73.55	46.81	65.75	69.51
Digestibility (%)	69.29	79.17	90.72	79.3	79.83
Solubility at pH 6 (%)	70.69	89.27	95.41	100.00	77.43
Solubility at pH 8 (%)	81.85	87.34	100.00	97.73	81.19
DPPH activity (%)/c protein (mg/mL)	5.84	13.95	13.47	12.83	12.93
Reducing power at 700 nm	0.046	0.105	0.080	0.062	0.068

of bioactive peptides could be improved using ultrasound pretreatment.

A lot of research has been reported that there was a direct correlation between antioxidant activity and reducing power. Namely, bioactive peptides with higher reducing power have better abilities to donate electrons [34]. The values of the reducing power of the EWP hydrolysates which are summarized in Table 3, correlated well with those of DPPH radical scavenging assay. The hydrolysate obtained with one-stage hydrolysis by Alcalase exhibited the highest reducing power. Moreover, this result confirmed that protein hydrolysate had good abilities to donate electron, which was involved in the antioxidant activity. Likewise, Pan et al. [51] reported that the rapeseed protein hydrolysates prepared with Alcalase possessed excellent reducing power.

Generally, enzymatic hydrolysis appeared to increase solubility, digestibility and antioxidant activity of EWPs in all cases. Papain hydrolysate has been shown to have the best solubility and digestibility which could be explained by the formation of smaller, more hydrophilic and more soluble bioactive peptides in hydrolysates. It seemed that the enzymatic hydrolysis reduced molecular weight of EWPs, enhanced the solubility, increased flexibility of proteins that facilitated the formation interfacial membrane and increased foaming properties of proteins after hydrolysis. Namely, the most voluminous foams were obtained in the two-stage enzymatic process with Alcalase and Neutrase, while the most stable, dense foams were obtained in the one-stage process with Alcalase. The FS has been known to depend on the strength of the protein film and its permeability to gases [7]. Overall, the relationship between the pretreatment and application of different proteases induced changes in the physicochemical properties and the functional properties of EWPs seemed to be clear. It was reported that the application of ultrasound to wheat germ protein caused changes in protein structure, like an increase in protein surface hydrophobicity, activity and charge and decrease in SH group content that may altered its bulk functionality [52]. Also, for ultrasound pretreatments, a specificity of protease could be found to predict accurately the functional properties of EWP hydrolysates. For example, the hydrolysate with high in vitro digestibility was obtained by papain, while the hydrolysate with a rather high solubility at pH 6 and pH 8 was obtained with the combined activity of Alcalase and Flavourzyme. Compared with single-enzyme processes, the two-stage enzymatic procedures did not significantly improve both antioxidant and functional hydrolysates' properties.

Conclusions

Short-time ultrasound pretreatment of EWPs at calorimetric power of 21.3 W and frequency of 40 kHz for 15 min

conducted under lower temperature of 25 °C resulted in increased Alcalase hydrolysis, but with increasing in acoustic power, treatment time and temperature, the hydrolysis rate decreased dramatically. The pH of sonicating medium also significantly affected the hydrolysis rate. The highest initial rate was observed with egg white sample after prolonged storage (pH 9.25), almost 2.5-fold increase compared with the one of fresh egg white (pH 7.6), that could be important from the practical point of view that the pH of egg white increases during storage of eggs. The optimal ultrasound pretreatment of EWPs followed by Alcalase hydrolysis resulted in the hydrolysate with a higher antioxidant activity than that obtained with heat pretreatment, due to different hydrolytic pattern and peptide composition.

EWPs pretreated under optimal conditions have been further subjected to proteolysis in one- and two-stage processes using several proteases. Alcalase hydrolysate has shown the best antioxidant properties, whereas EWP hydrolysate obtained by papain hydrolysis had the best solubility and digestibility. DH over 30 % after 2 h hydrolysis and high yield of peptides soluble at pH 6 and pH 8 were obtained by combination of Alcalase and Flavourzyme in the two-stage process. This study showed that by combining ultrasound pre-hydrolysis treatment under controlled conditions with careful proteases selection, hydrolysates with improved functional and antioxidant properties can be produced enhancing utilization of EW in food products.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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