Improvement of antioxidant properties of egg white protein enzymatic hydrolysates by membrane ultrafiltration

Jelena R. Jovanović[1], Andrea B. Stefanović[1], Milena G. Žuža[1], Sonja M. Jakovetić[1], Nataša Ž. Šekuljica[3], Branko M. Bugarski[2], Zorica D. Knežević-Jugović[1]

[1]*Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia*
[2]*Department of Chemical Engineering, Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia*
[3]*Innovation Center, Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia*

Abstract

The production of bioactive peptides from egg white proteins (EWPs) and their separation are emerging areas with many new applications. The objective of this study was to compare antioxidant activity of three distinct EWP hydrolysates and their peptide fractions prepared by membrane ultrafiltration using membranes with 30, 10 and 1 kDa molecular weight cut-off. The hydrolysates were obtained by thermal and ultrasound pretreated EWPs hydrolyzed with a bacterial protease, Alcalase. It appeared that the pretreatment significantly affected peptide profiles and antioxidant activity of the hydrolysates measured by ABTS, DPPH and FRAP methods. The hydrolysate prepared using alcalase and ultrasound pretreatment at 40 kHz – 15 min has shown to be the most effective in scavenging both DPPH and ABTS radicals (28.10±1.38 and 79.44±2.31%, respectively). It has been noticed that this hydrolysate had a nutritionally more adequate peptide profile than the other hydrolysates with a much lower amount of peptides <1 kDa (11.19±0.53%) and the greatest content of the peptide fraction in the molecular weight (MW) range of 1–10 kDa (28.80±0.07%). This peptide fraction has shown the highest DPPH and ABTS antioxidant activity compared to all other fractions having a potential to be used as a functional food ingredient.

Keywords: antioxidant derived egg white peptides, membrane ultrafiltration, ultrasound pretreatment, hydrolysis, alcalase.

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The importance of oxidation processes in the body and foodstuffs has been widely recognized. It is well known that the formation of free radicals and other reactive oxygen species (ROS), which can induce oxidative damage, is a consequence of the oxidation metabolism which is indispensable for the survival of cells [1]. An important role in the preservation of human health has inhibition of oxidative reaction and scavenging of free radicals which take place under the influence of antioxidants. Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which were ordinarily applied for radical scavenging in biological systems pose a risk for human health. Accordingly, the contribution of many studies was aimed to isolate natural antioxidants such as tocopherols, catechin, phenolic compounds and peptides [2–5].

Correspondence: Z.D. Knežević-Jugović, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Republic of Serbia.
E-mail: zknez@tmf.bg.ac.rs
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The bioactive peptides derived from food proteins with low molecular weight and useful bioactivities which are easily absorbed have attracted more attention, because they are safer and healthier than synthetic ones [6]. The evaluation and characterization of bioactive peptides, released after enzymatic hydrolysis, have been widely investigated demonstrating that besides its nutritional value, they might have pharmacological activities. These bioactive peptides, obtained from animal and plant proteins have indicated antioxidant, antitumoral, antithrombic, antihypertensitive or antimicrobial activities opening the opportunity of having therapeutically functional food [7]. In the last decade, a number of studies have dealt with the isolation of bioactive peptides with high antioxidant activity. These peptides have been produced by enzymatic hydrolysis of different protein from plant and animal sources like rice bran [8], peanut [9], sunflower [10], corn gluten meal [11], casein [12], yam [13], milk-kefir and soymilk-kefir [14], mackerel [15], curry leaves [16], egg-yolk [17], cotton leaf worm [18] and several others.

Due to its nutritional quality, egg white proteins (EWPs) are suitable choice for the production of bioactive peptides intended for human consumption. 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. Furthermore, enzymatic hydrolysis of EWPs is considered to be one of the possibilities to exceed problem with allergenicity and high viscosity of the native proteins, since these are the main constraints for more extensive usage of EWPs in food formulations [19].

Many recently published studies attempt to fractionate proteins, protein hydrolysates and peptides with membrane separation processes and isolate the fractions with the most antioxidant active peptides with the aim of enhancing their biological and/or functional properties. The separation processes are based upon selective permeability of one or more of the liquid constituents through the membrane according to the pressure difference. The ultrafiltration membrane system separates the protein hydrolysates into defined molecular weight ranges and represents the best technology available for the enrichment of peptides with a specific molecular weight range [20]. In particular, fractions with a molecular weight between 0.1–0.5 kDa and 1–3.5 kDa would be the most interesting bioactive peptides for nutritional and pharmaceutical uses. The extraction and recovery of these fractions from the hydrolysate are the key issues. Compared to other bioseparation methods such as gel chromatography, membrane technology offers several advantages such as higher productivity, lower capital investment, high throughput of products and while maintaining product purity under ambient conditions, ease of translation to large-scale commercial production and easy equipment cleaning [5].

We recently reported on the impact of ultrasound pretreatment on the enzymatic hydrolysis of EWPs with a range of commercially available proteases and on the functionalities of the resultant hydrolysates [21]. In the present study, the antioxidant activities of the above mentioned EWP hydrolysates obtained with Alcalase and of the fractionated hydrolysates prepared using membrane ultrafiltration were investigated by multiple assays, including the ability of the scavenging effect on free radicals and the ferric reducing ability. Hence, the aim was to identify the most antioxidant peptide fractions hydrolysate for application as a functional ingredient in food products production.

EXPERIMENTAL

Materials

Chicken egg white obtained from a local supermarket was separated from the yolk and gently stirred without foam formation to provide homogeneous mixture. Alcalase 2.4L (protease from *Bacillus licheniformis*) was obtained from Sigma Aldrich (St Louis, MO, USA). The enzyme activity was ≥2.4 U/g Anson units, where 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 min, giving the same color with Folin–Ciocalteu phenol reagent as one milliequivalent of tyrosine at 25 °C and at 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) and complex ferric ion-TPTZ (2,4,6--tri(2-pyridyl)-*s*-triazine) were purchased from Sigma––Aldrich. All other chemicals used in this research were of analytical grade.

Ultrasound and thermal protein denaturation

Prior to the enzymatic hydrolysis, a series of 10 mass% egg white solution samples was exposed to ultrasonic waves in ultrasonic water bath with a frequency of 40 kHz (EI-NIS-RO-VEP, Serbia) and 35 kHz (Bendelin electronic, sonorex digitec, DT 102 H, Germany) during 15 and 30 min, respectively. At the same time, the samples were subjected to the thermal pretreatment at high temperature (75 °C) for half an hour. The samples were prepared in a beaker of 600 cm^3 capacity where the working volume (~360 cm^3) was kept constant for all pretreatments. For the ultrasound control, 10 mass% egg white solution was left in the ultrasonic bath at a frequency of 0 kHz at the same time and thermal control was kept out at the ambient temperature for half an hour. Each treatment was conducted in triplicate.

Preparation of protein hydrolysates

Before the enzymatic reaction catalyzed by alcalase, egg white solution samples were pre-incubated in a glass reactor at optimum conditions for protease (50 °C and pH 8.0) during 20 min. The hydrolysis reaction was initiated by adding the enzyme in the amount of 2 mass% with stirring at 240 rpm. During the course of the reaction, pH was kept at a constant value by adding 0.2 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 12,000×*g* for 10 min. The supernatant was finally collected as the hydrolysate and stored at 4 °C for subsequent studies.

Fractionation of protein hydrolysates using membrane ultrafiltration

The hydrolysates obtained after ultrasound and thermal pretreatments were subjected to ultrafiltration

using membranes with 30, 10 and 1 kDa molecular weight cut-off (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. During the ultrafiltration process, the pressure was applied with nitrogen, as indicated by the manufacturer of the membranes. The experimental setup and diagram of the ultrafiltration system for separation of antioxidant peptides from EWP hydrolysate using membranes with various molecular weights cut-off are presented in Scheme 1.

A sample of 50 cm^3 hydrolysates was first ultrafiltered through a 30 kDa membrane to obtain two fractions: retentate (Fraction 1, 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, representing hydrolysates between 10 and 30 kDa) and the second permeate (MW <10 kDa). Permeate was also further subjected to ultrafiltration through an 1 kDa membrane to obtain the third retentante (fraction 3, representing hydrolysates between 1 and 10 kDa) and permeate (fraction 4, representing hydrolysates <1 kDa). Each retentate or permeate was collected and stored in the freezer. After ultrafiltration, protein content was determined by Lowry method [22].

Determination of antioxidant properties

DPPH radical scavenging activity

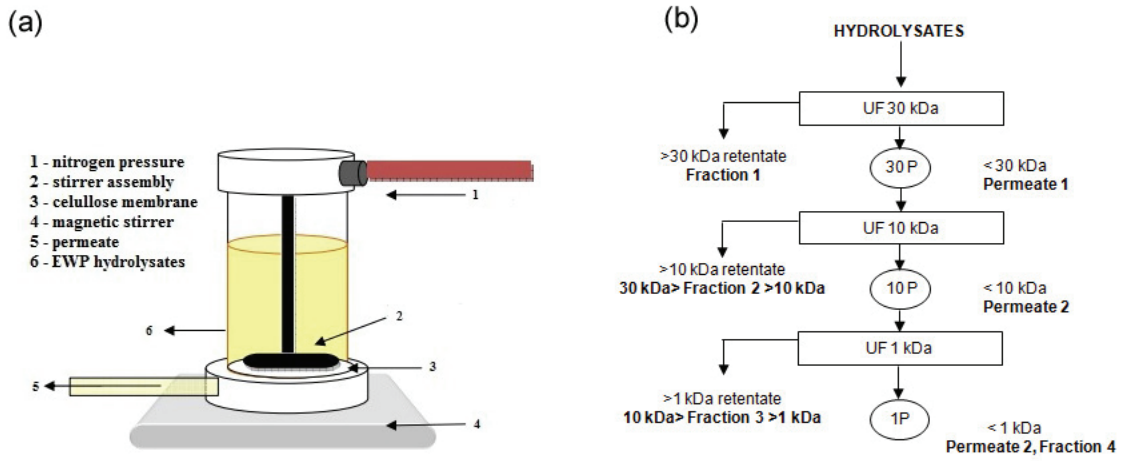
The scavenging activity of egg white protein hydrolysate and its fractions against the DPPH radical was determined based on the method described previously [23] with only slight protocol modification. Briefly, a 0.5 cm^3 aliquot of each obtained hydrolysate was mixed with 0.5 cm^3 of 0.15 mM DPPH solution 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, Amerscham Bioscience). DPPH radical scavenging activity (*RSA*, %), expressed as the percentage of inhibition, was calculated according next equation:

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

where A_s is the absorbance of the tested egg white hydrolysate (EWH), A_b is the absorbance of the EWH in methanol and A_c is the absorbance of the DPPH solution without the sample. All experiments were carried out in triplicate.

ABTS radical scavenging activity

The ABTS radical scavenging activity of prepared hydrolysates and their fractions was measured using the decolorization assay [24]. This method is based on the ability of antioxidant molecules to quench the stable bisradical cation, $ABTS^{\bullet+}$, a blue-green chromophore with characteristic absorption at 734 nm. In this research, the $ABTS^{\bullet+}$ solution was prepared by reaction of 5 cm^3 of a 7 mM aqueous ABTS solution and 0.088 cm^3 of a 140 mM (2.45 mM final concentration) potassium-persulfate solution. After storage in the dark for 16 h, the radical cation solution was further diluted with 5 mM phosphate-buffered saline (PBS, pH 7.4) until the initial absorbance value of 0.7±0.05 at 734 nm was reached. Solutions of each sample under study were prepared in water so that their final concentration after the addition of 0.01 cm^3 the radical solution (2 cm^3) was 0–15 μM and a 20–80% decrease in the initial absorbance of the reaction solution was achieved. Absorbance was measured at 734 nm after 5 min and ABTS radical scavenging activity (%) was calculated using following equation:



Scheme 1. a) Experimental set-up unit; b) diagram of the ultrafiltration system applied for separation antioxidant peptides from EWP hydrolysate using membranes with various molecular weights cut-off.

$$ABTS = 100\frac{A_s - A_b}{A_c} \quad (2)$$

where A_s represents the absorbance of the sample solution in the presence of the $ABTS^{\bullet+}$, A_b is the absorbance of the sample solution without $ABTS^{\bullet+}$ and A_c is the absorbance of the control solution with $ABTS^{\bullet+}$. The assay was standardized using 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*). All experiments were carried out in triplicate.

Ferric reducing antioxidant power (FRAP) assay

The reducing ability of peptide fractions from EWH was determined by FRAP assay of Benzie and Strain with slight modifications [25]. FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue Fe (II)–TPTZ compound from colorless oxidized Fe(III) form by the action of electron donating antioxidants. The working FRAP reagent was prepared by mixing 10 volumes 0.3 M acetate buffer pH 3.6, with 1 volume 10 mM TPTZ (2,4,6-tri(2-pyridyl)-*s*-triazine) in 40 mM hydrochloric acid and with 1 volume 20 mM ferric chloride. A 0.15 cm^3 EWH was added to 4.5 cm^3 freshly prepared FRAP reagent and mixed thoroughly and reading taken at 593 nm. Absorbance readings were taken after 30 min. The aqueous solutions of known Fe(II) concentration, in the range of 0.1–1.0 mM $FeSO_4 \times 7H_2O$ were used for calibration. All solutions were used on the day of preparation. In the FRAP assay, the reducing ability of the hydrolysates under the test was calculated with reference to the reaction signal given by a Fe(II) solution of known concentration. The values were expressed as micromole of Fe(II) equivalents per mg of proteins. All measurements were done in tree repetitions for each EWH.

Statistical analysis

All experiments were performed in triplicate and the data are presented in average of triplicates and standard deviation (*SD*). Statistical differences between antioxidant activity of enzymatic hydrolysates and their respective ultrafiltration fractions were determined by one-way analysis of variance (ANOVA). A Tukey test was applied as a test a posteriori with a level of significance of 95%. All the tests were considered statistically significantly at $p < 0.05$. Statistical analyses were performed using the Origin Pro 8 software package.

RESULTS AND DISCUSSION

Effect of pretreatments on the antioxidant activity of EWPs

Heat pretreated and ultrasound pretreated EWPs were hydrolyzed in the batch reactor using an endopeptidase, namely alcalase. This endopeptidase was chosen as the biocatalyst for the EWP hydrolysis on the basis of preliminary results and literature survey [21,26]. The *in vitro* antioxidant activities of the obtained hydrolysates were measured by ABTS, DPPH and FRAP methods since each assay reflected a different aspect of the antioxidant behavior of the hydrolysates. Namely, when discussing the antioxidant activity of a food product, it is strongly recommended to use at least two methods due to the differences between the test systems [27]. The results of antioxidant activity of hydrolysates obtained after different pretreatments and measured by three methods are presented in Table 1.

It seemed that the pretreatment significantly affected the antioxidant properties of the hydrolysates ($p < 0.05$). The DPPH and ABTS activity of the hydrolysates generally decreased in the order: ultrasound pretreated at 40 kHz – 15 min > ultrasound pretreated at 35 kHz – 30 min > thermal pretreated at 75 °C – 30 min. It is worth noting that all hydrolysates had better antioxidant properties than the native EWP solution, which is in agreement with similar studies [28,29].

As a result of the enzymatic hydrolysis, a complex mixture of peptides and amino acids produced in which individual effect of each molecule in the subsequent fractionation process is difficult to reveal and quantify. Molecular weight is an important parameter reflecting the hydrolysis of proteins, which further correlates with the bioactivity of protein hydrolysates. Thus, it is attempted in this study to design of an efficient fractionation methodology for peptides separation with the aim of enhancing their antioxidant properties.

Table 1. Antioxidant activities and total protein content of EWP hydrolysates prepared by ultrasound and thermal pretreatment; m_p – total protein content determined by Lowry method. DPPH and ABTS radical scavenging activities of untreated egg white were 15.8±2.04 and 7.69±1.59 %, respectively. Concentration of reducing Fe^{2+} for untreated egg white was 0.062±0.01 μM mg^{-1} proteins

Parameter	Ultrasound pretreatment		Thermal pretreatment
	40 kHz – 15 min	35 kHz – 30 min	75 °C – 30 min
m_p / mg	591.7±3.67	578.6±3.02	574.9±6.02
DPPH / %	28.10±1.38	25.20±1.75	23.50±1.12
ABTS / %	79.44±2.31	77.34±2.46	76.17±2.59
Fe^{2+}, mM mg^{-1} proteins	0.097±0.01	0.109±0.009	0.064±0.01

Therefore, the obtained hydrolysates have been separated into four fractions depending on their molecular size by successive UF using membranes with molecular cut off sizes of 30, 10 and 1 kDa. The values of the separated fraction (< 1, 1–10, 10–30 and >30 kDa) and its impact on the peptidic population in terms of molecular weight were presented in Figure 1.

Significant differences concerning the content and the molecular weight distribution profiles of obtained peptides occurred among pretreatments. It was apparent that the hydrolysis after thermal 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 was 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. Although the mechanism of ultrasound-induced protein unfolding is not completely understood, important differences in protein unfolding have been suggested for egg white proteins caused after heat or ultrasound pretreatments [30].

DPPH radical scavenging activity

The antioxidant properties of different EWPs hydrolysates fractions have been chosen as the most relevant criteria to choose the most appropriate hydrolysates for the food application. The DPPH radical scavenging activity assay is one of the *in vitro* methods for the measurement of the capacity of an antioxidant to reduce free radicals. The degree of color changes appears to be correlated with the sample antioxidant activity. The obtained ultrafiltration fractions from Alcalase hydrolysates were tested for DPPH radical scavenging activity, and results are depicted in Figure 2.

The results showed that enzymatic hydrolysis and ultrasound pretreatment enhanced antioxidant properties of hydrolysates and fractions. The highest DPPH scavenging activities were achieved for fraction 3 induced by both ultrasound and thermal pretreated hydrolysates which outcomes have been statistically significant ($p < 0.05$) and values were 33.57±0.07, 21.17±2.01 and 30.30±2.65 %, respectively. These results indicated that the ultrasound pretreatment could improve inhibition of DPPH radical of EWP hydrolysates compared with conventional thermal pretreatment. Consequently, it can be deduced that ultrasound released peptides and isolated fractions, especially fraction 3, possibly contained peptides which acts as electron donors and could react with free radicals to. Unhydrolysed EWPs showed a rather low DPPH radical scavenging activity (15.8±2.04%) and the values increased significantly with the course of hydrolysis for both pretreatments. The antioxidant activity of all obtained Fractions 3 varied significantly between the different pretreatment used ($p < 0.05$). The appropriated EWPs pretreatment prior to hydrolysis has resulted in different alterations in the protein's tertiary structure affecting profile of produced peptides and consequently their DPPH radical scavenging activity. The results of this research are in agreement with results obtained by several authors who reported that the antioxidant activity of the defatted soybean meal was improved after sonication [31]. It can be noticed that the frequency of

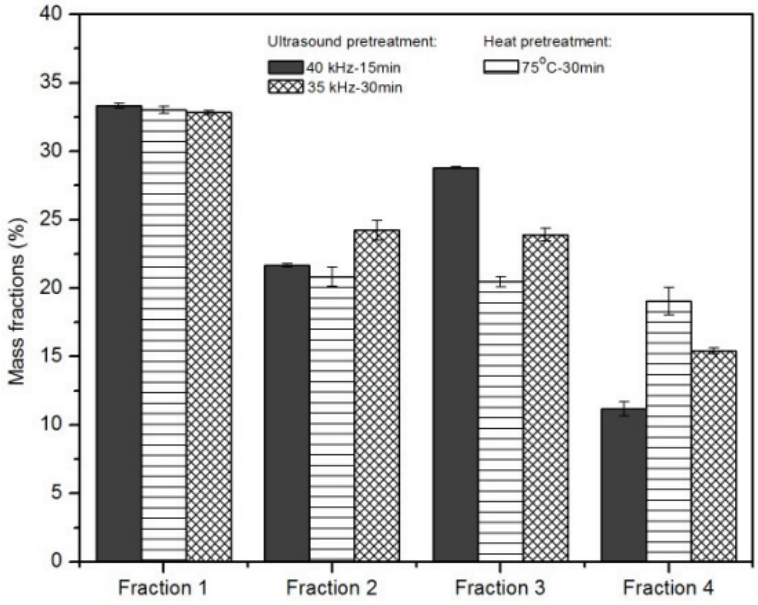


Figure 1. The molecular weight distribution profile of EWP hydrolysates and their ultrafiltered fractions obtained by thermal and ultrasound pretreatment (Hydrolysis conditions were as follows: 50 ℃, pH 8.0; 2.12 U of alcalase, 10 mass% aqueous solution of egg white (E/S mass ratio 0.02)).

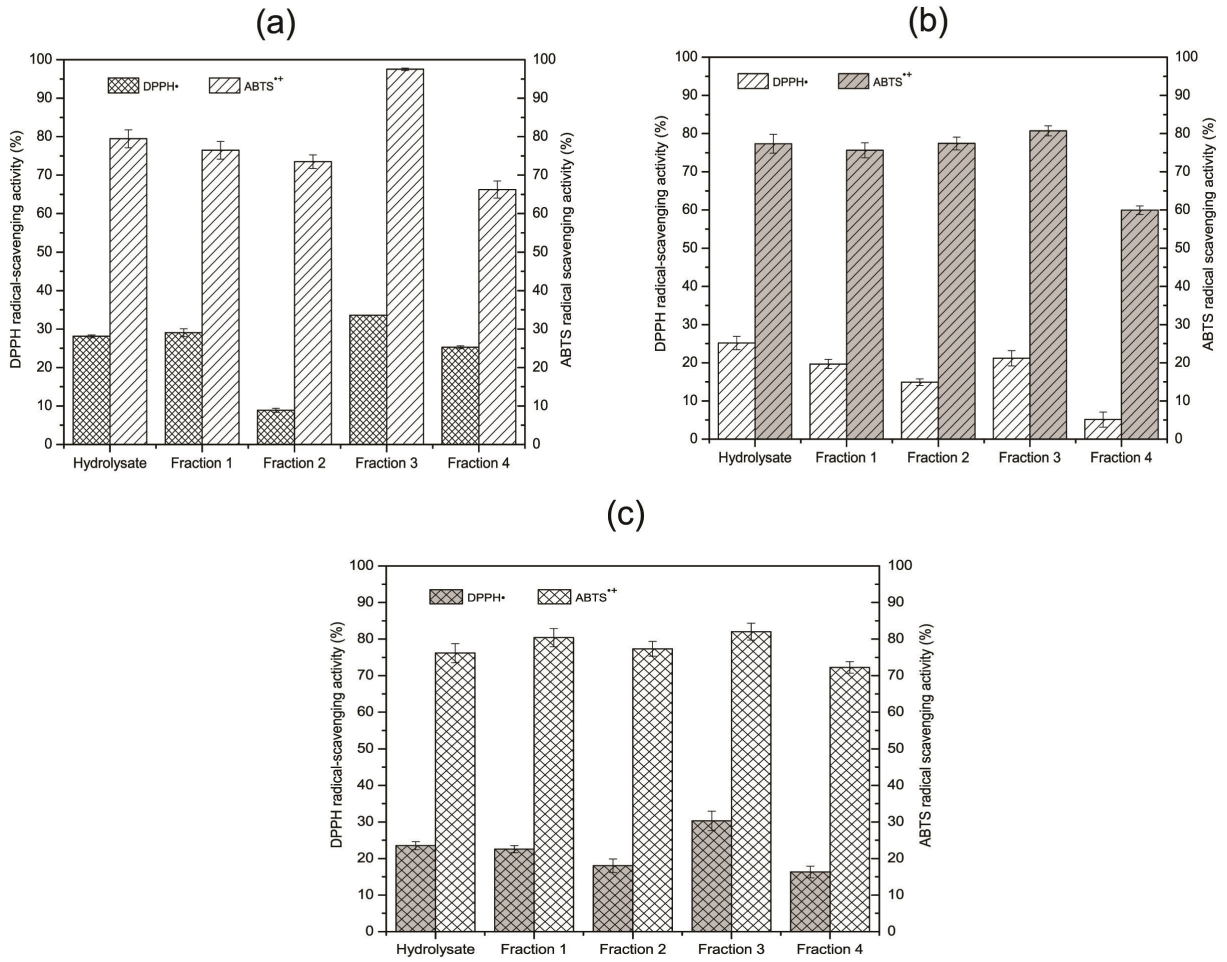


Figure 2. DPPH and ABTS radical scavenging activity of EWP hydrolysates and their ultrafiltered fractions: a) ultrasound pretreatment at 40 kHz during 15 min, b) ultrasound pretreatment at 35 kHz during 30 min and c) thermal pretreatment at 75 ℃ during 30 min.

ultrasound did not significantly affect ($p > 0.05$) on the DPPH scavenging activities, but it is evident the results disclose the presence of antioxidant peptides in all isolated ultrasound pretreated fractions and the fraction 3 was the most abundant with the highest DPPH values for both pretreatments. Very little attention in the literature was devoted to the influence of ultrasound on the isolation of antioxidant peptides from EWPs but some researches were investigated antioxidant activity of EWPs hydrolysates. Thus, Chenand and colleagues [28] have been separately hydrolysed EWPs with several commercially available proteases and it has been noted that antioxidant activity increased with the increasing time of hydrolysis. Also, they reported that papain hydrolysate had the scavenging activity of DPPH and hydroxyl radicals more effective than superoxide anion radical ($p < 0.05$) and a greater DPPH radical scavenging activity (78.74% at 5 mg/ml) was noted with low molecular-weight peptides (<3 kDa). Other authors showed that alcalase EWPs hydrolysates possessed the strong antioxidant ability compared with the other hydrolysates (pepsin and tripsin), particularly for the fraction with peptides <1 kDa [29].

ABTS radical scavenging activity

ABTS radical is relatively stable but readily reduced by antioxidants. The scavenging activity against cationic ABTS radical indicates the ability of peptide fractions to act as electron donors or hydrogen donors in free radical reactions [32]. To assay the ABTS radical scavenging of peptide fractions, cationic ABTS radical decolorization was carried out. The results obtained from this experimental set are presented in Figure 2. Taking into account these results it has been shown that fraction 3 with MW between 1 to 10 kDa revealed the highest scavenging activity. Hence, ABTS scavenging activity induced by ultrasound (35 kHz – 30 min) and thermal pretreated hydrolysates of fraction 3 have not been statistically significant ($p > 0.05$) and values were 80.72±1.32 and 82.01±2.29%, respectively. However, fraction 3 obtained by membrane ultrafiltration of ultrasound pretreated hydrolysate (40 kHz – 15 min) has shown a significantly higher ABTS radical scav-

enging assay of 97.54±0.3%. The ABTS scavenging activity of eleven amaranth peptide fractions has been reported, and among them, two fractions exhibited higher antioxidant activity than the other fractions. Their activities were 66.8 and 83% (both at 310 μg cm^{-3}), but the other fractions (at concentration lower than 200 μg cm^{-3}) registered less than 37% [33].

For easy comparison with literature data, results of ABTS radical scavenging activity were expressed as Trolox and ascorbic acid equivalents antioxidant capacity and presented in Figure 3.

Between hydrolysates and their fractions, except for the fraction 3 obtained after ultrasound pretreatment (40 kHz – 15 min), no substantial difference in the values of Trolox and ascorbic acid equivalents. The fraction 3 has shown the notably values for both Trolox and ascorbic acid equivalents: 1.569±0.007 and 1.852±0.008 μmol g^{-1} peptides, respectively. As well, the ABTS radical scavenging activity for fraction 3 was approximately two times lower than the peptide fraction with small peptides (fraction 4, < 1 kDa). Generally, the results showed that fraction with a small molecular weight exerted better ABTS inhibition ability.

It was found that hen egg white lysozyme (HEWL) hydrolysate possessed ABTS radical inhibition assay 1.91±0.13, 2.57±0.19 and 2.82±0.14 μmol trolox equivalents (TE) mg^{-1} protein for trypsin, papain and trypsin–papain hydrolysates, respectively [34]. For HEWL hydrolysate obtained by alcalase, TEAC value was 1.69 μmol trolox equivalents (TE) $mg^{-1}$of protein [35].

Ferric reducing antioxidant activity (FRAP)

The FRAP is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen, and some researchers have indicated that there is a direct correlation between antioxidant activities and reducing power of peptide [36]. The FRAP activity of protein/ /peptide components in enzymatic hydrolysates and their ultrafiltration fractions are represented in Figure 4.

Hydrolysates prepared after ultrasound pretreatment frequency 40 and 35 kHz and with thermal pretreatment had no detectable activity and/or the values were not statistically significant. Therefore, the FRAP activity of the EWP hydrolysates was concentrated in the peptides with 1–10 kDa and <30 kDa size for thermal and ultrasound pretreatment, respectively, meanwhile presence of the other peptides (>10 and >30 kDa) could be responsible for the lack of the activity. On the basis of this antioxidant assay, the concentration of reduced iron was 3.79±0.60 μM per milligram of proteins for Fraction 3 obtained by hydrolysate prepared after thermal treatment. The fractions 1, for both ultrasound pretreatment 35 and 40 kHz, had a significantly lower value of Fe (II) concentration amounted 1.79±0.13 and 2.05±0.34 μM per milligram of proteins, respectively. According to the available literature data, it can be said that protein hydrolysates from plant source possess higher ferric reducing antioxidant activity than hydrolysates acquired from animal proteins. The researchers found that the phenolic and indolic groups of tyrosine and tryptophan play important roles as hydrogen donors in redox systems [37].

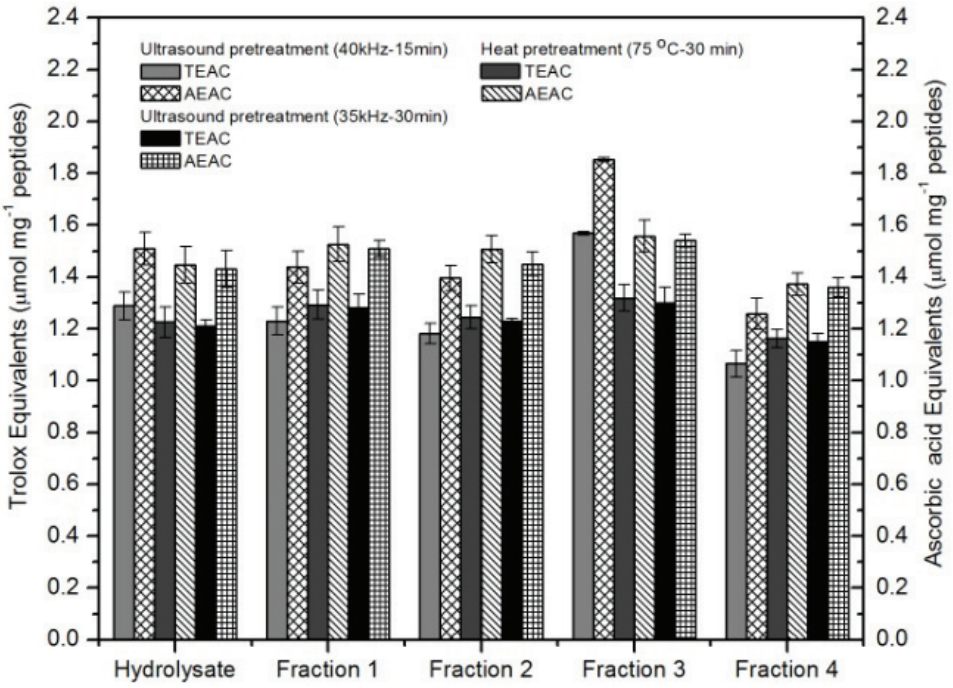


Figure 3. ABTS scavenging activity for EWP hydrolysates and their ultrafiltrated fractions obtained with ultrasound and thermal pretreatments expressed in Trolox and ascorbic acid equivalents per gram of dry proteins.

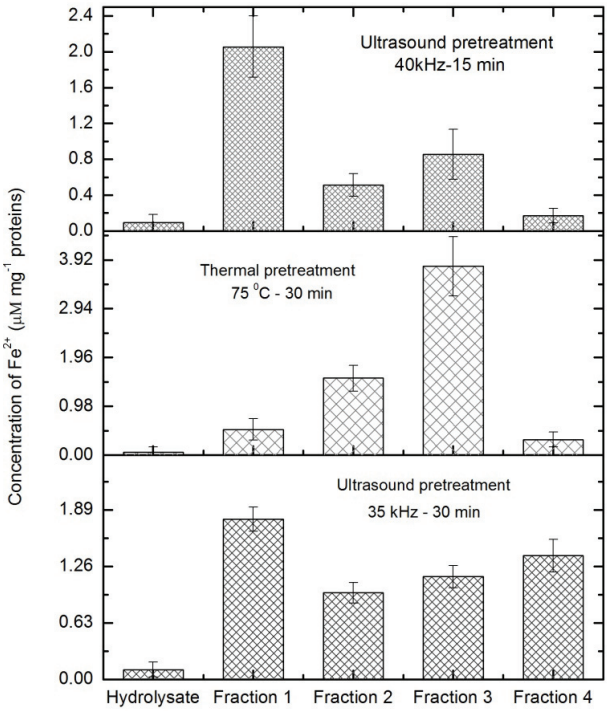


Figure 4. Ferric reducing power assay of EWP hydrolysates and their ultrafiltered fractions expressed as concentration of Fe(II).

Generally, based on the all represented results, fraction 3 with low molecular weight peptides possessed relevant antioxidant activity and these results are in agreement with literature data which indicates that antioxidant peptides from food proteins contain 5–16 amino acid residues. Namely, the 1–10 kDa fractions was the most abundant after membrane ultrafiltration of the protein hydrolysates after ultrasound pretreatment which suggests that the protease alcalase and ultrasound pretreatment (40 kHz – 15 min) were efficient in reducing the native egg white proteins into low molecular weight peptides.

CONCLUSION

The impact of ultrasound and thermal pretreatments of EWPs on enzymatic hydrolysis and antioxidant properties of the obtained hydrolysates and their fractions was assessed. The hydrolysate prepared using Alcalase and ultrasound pretreatment at 40 kHz – 15 min has shown to be most effective in scavenging both DPPH and ABTS radicals (28.10±1.38 and 79.44±2.31 %, respectively). The release of bioactive peptides from intact proteins has been shown to be significantly affected by the pretreatment type. The fraction produced from the ultrasound pretreated EWPs by enzymatic hydrolysis and ultrafiltration containing antioxidant peptides of MW ranging from 1 to 10 kDa showed the highest DPPH and ABTS radicals quenching capability compared to other fractions. The outcomes showed that this fraction possibly contained some effective antioxidant peptides, which could convert free radicals to more stable products and force out the radical chain reaction. These findings suggest that Alcalase hydrolysis of ultrasound pretreated egg white protein hydrolysates combined to ultrafiltration fractionation of hydrolysate could provide new opportunities for the development of health-promoting ingredients.

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REFERENCES

[1] K. Cui, X. Luo, K. Xu, M.R. Murthy, Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants, Prog. Neuro-psychoph. **28** (2004) 771–799.

[2] B. Hernandez-Ledesma A. Davalos, B. Bartolome, L. Amigo, Preparation of antioxidant enzymatic hydrolysates from alpha-lactalbumin and beta-actoglobulin. Identification of active peptides by HPLC–MS/MS, J. Agric. Food Chem. **53** (2005) 588–593.

[3] J.T. Hancock, R. Desikan, S.J. Neill, Role of reactive oxygen species in cell signaling pathways, Biochem. Soc. Trans. **29** (2001) 345–50.

[4] B. Halliwell, Free radicals, antioxidants, and human disease: curiosity, cause, or consequence, Lancet **344** (1994) 721–724.

[5] S. Saidi, A. Deratani, M.P. Belleville, R.B. Amar, Antioxidant properties of peptide fractions from tuna dark muscle protein by-product hydrolysate produced by membrane fractionation process, Food Res. Int. (2014), doi: 10.1016/j.foodres.2014.09.023.

[6] B.H. Sarmadi, A. Ismail, Antioxidative peptides from food proteins: A review, Peptides **31** (2010) 1949–1956.

[7] N.P. Moller, K.E. Scholz-Ahrens, N. Roos, J. Schrezenmeir, Bioactive peptides and proteins from foods: indication for health effects, Eur. J. Nutr. **47** (2008) 171–182.

[8] E. Revilla, C.S. Maria, E. Miramontes, J. Bautista, A. Garcia-Martinez, O. Cremades, R.Cert, J. Parrado, Nutraceutical composition, antioxidant activity and hypocholesterolemic effect of a water-soluble enzymatic extract from rice bran, Food Res. Int. **42** (2009) 387–393.

[9] G.T. Chen, L. Zhao, L.Y. Zhao, T. Cong, S.F. Bao, In vitro study on antioxidant activities of peanut protein hydrolysate, J. Sci. Food Agric. **87** (2007) 357–362.

[10] C. Megias, J. Pedroche, M.M. Yust, J. Giron-Calle, M. Alaiz, F. Millan, J. Vioque, Production of copper-chelating peptides after hydrolysis of sunflower proteins with pepsin and pancreatin, Food Sci. Technol. **41** (2008) 1973–1977.

[11] X.X. Li, L.J. Han, L.J. Chen, In vitro antioxidant activity of protein hydrolysates prepared from corn gluten meal, J. Sci. Food Agric. **88** (2008) 1660–1666.

[12] K. Suetsuna, H. Ukeda, H. Ochi, Isolation and characterization of free radical scavenging activities peptides derived from casein, J. Nutr. Biochem. **11** (2000) 128––131.

[13] T. Nagai, N. Suzuk, Y. Tanoue, N. Kai, T. Nagashima, Antioxidant and antihypertensive activities of autolysate and enzymatic hydrolysates from yam (*Dioscorea opposita* Thunb.) ichyoimo tubers, J. Food Agric. Environ. **5** (2007) 64–68.

[14] J.R. Liu, M.J. Chen, C.W. Lin, Antimutagenic and antioxidant properties of milk-kefir and soymilk-kefir, J. Agric. Food Chem. **53** (2005) 2467–2474.

[15] C.H. Wu, H.M. Chen, C.Y. Shiau, Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*), Food Res. Int. **36** (2003) 949–957.

[16] M. Ningappa, L. Srinivas, Purification and characterization of ~35 kDa antioxidant protein from curry leaves (*Murraya koenigii* L.), Toxicol. In Vitro. **22** (2008) 699––709.

[17] S. Sakanaka, Y. Tachibana, Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates, Food Chem. **95** (2006) 243–249.

[18] L. Vercruysse, G. Smagghe, T. Beckers, J. Van Camp, Antioxidative and ACE inhibitory activities in enzymatic hydrolysates of the cotton leafworm, Spodoptera littoralis, Food Chem. **114** (2009) 38–43.

[19] G. Martos, R. López-Fandiño, E. Molina, Immunoreactivity of hen egg allergens: Influence on in vitro gastrointestinal digestion of the presence of other egg white proteins and of egg yolk, Food Chem. **136** (2013) 775––781.

[20] L. Najafian, M. Jafarzade, M. Said, A.S. Babji, Biochemical properties and antioxidant activity of myofibrillar protein hydrolysates obtained from patin *(Pangasius sutchi)*, Int. J. Food Sci. Tech. **48** (2013) 2014–2022.

[21] A.B. Stefanović, J.R. Jovanović, S.Ž. Grbavčić, N.Ž. Šekuljica, V.B. Manojlović, B.M. Bugarski, Z.D. Knežević-Jugović, Impact of ultrasound on egg white proteins as a pretreatment for functional hydrolysates production, Eur. Food Res. Technol. 239 (2014) 979–993.

[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. **193** (1951) 265–275.

[23] S. Jakovetić, B. Jugović, M. Gvozdenović, D. Bezbradica, M. Antov, D. Mijin, Z. Knežević-Jugović, Synthesis of aliphatic esters of cinnamic acid as potential lipophilic antioxidants catalyzed by lipase b from *Candida antarctica*, Appl. Biochem. Biotehnol. **170** (2013) 1560––1573.

[24] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. **26** (1999) 1231–1237.

[25] I.F. Benzie, J.J. Strain,The ferric reducing ability of plasma (FRAP) as a measure of bantioxidant power Q the FRAP assay, Anal. Biochem. **239** (1996) 70-76.

[26] S. Jakovetić, N. Luković, B. Jugović, M. Gvozdenović, S. Grbavčić, S.J. Jovanović, Z. Knežević-Jugović, Production of Antioxidant Egg White Hydrolysates in a Continuous Stirred Tank Enzyme Reactor Coupled with Membrane Separation Unit, Food Bioprocess Technol. **8** (2015) 287–300.

[27] K. Schlesier, M. Harwat, V. Bohm, R. Bitsch, Assessment of antioxidant activity by using different in vitro methods, Free Radical Res. **36** (2002) 177–187.

[28] J. Xu, Q. Zhao, Y. Qu, F. Ye, Free radical scavenging activity of peptide fractions from defatted soybean meal hydrolysates evaluated by electron spin resonance, Food Sci. Technol. Int. **19** (2013) 557–566.

[29] C. Chen, Y. Chi, M. Zhao, L. Lv, Purification and identification of antioxidant peptides from egg white protein hydrolysate, Amino Acids **43** (2012) 457–466.

[30] İ. Gülseren,D. Güzey, B.D. Bruce, J. Weiss, Structural and functional changes in ultrasonicated bovine serum albumin solutions, Ultrason. Sonochem.**14** (2007) 173–183.

[31] S. Lin, Y. Jin, M. Liu, Y. Yang, M. Zhang, Y. Guo, G. Jones, J. Liu, Y. Yin, Research on the preparation of antioxidant peptides derived from egg white with assisting of high-intensity pulsed electric field, Food Chem. **139** (2013) 300–306.

[32] R.L. Prior, X. Wu, K. Schaich, Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements, J. Agric. Food Chem. **53** (2005) 4290–4302.

[33] V.A. Tironi, M.C. Añón, Amaranth proteins as a source of antioxidant peptides: Effect of proteolysis. Food Res. Int. **43** (2010) 315–322.

[34] M. Memarpoor-Yazdi, A. Asoodeh, J.K. Chamani, A novel antioxidant and antimicrobial peptide from hen egg white lysozyme hydrolysates, J. Funct. Foods **4** (2012) 278–286.

[35] S.J. You, C.C. Udenigwe, R.E. Aluko, J.P. Wu, Multifunctional peptides from egg white lysozyme, Food Res. Int. **43** (2010) 848–855.

[36] X. Tang, Q.P. Wu, G.W. Le, J. Wang, K.J. Yin, Y.H. Shi, Structural and antioxidant modification of wheat peptides modified by the heat and lipid peroxidation product malondialdehyde, J. Food Sci. **77** (2012) 16–22.

[37] Y. Xia, F. Bamdad, M. Gänzle, L. Chen, Fractionation and characterization of antioxidant peptidesderived from barley glutelin by enzymatic hydrolysis, Food Chem. **134** (2012) 1509–1518.

IZVOD

BIOLOŠKI AKTIVNI PEPTIDI SA POBOLJŠANOM ANTIOKSIDATIVNOM AKTIVNOŠĆU DOBIJENI ULTRAFILTRACIONOM SEPARACIJOM HIDROLIZATA PROTEINA BELANCETA

Jelena R. Jovanović[1], Andrea B. Stefanović[1], Milena G. Žuža[1], Sonja M. Jakovetić[1], Nataša Ž. Šekuljica[3], Branko M. Bugarski[2], Zorica D. Knežević-Jugović[1]

[1]*Katedra za biohemijsko inženjerstvo i biotehnologiju, Tehnološko–metalurški fakultet, Univerzitet u Beogradu, Karnegijeva 4, 11000 Beograd, Srbija*
[2]*Katedra za hemijsko inženjerstvo, Tehnološko–metalurški fakultet, Univerzitet u Beogradu, Karnegijeva 4, 11000 Beograd, Srbija*
[3]*Inovacioni centar, Tehnološko–metalurški fakultet, Univerzitet u Beogradu, Karnegijeva 4, 11000 Beograd, Srbija*

(Naučni rad)

U ovom radu određena je antioksidativna aktivnost utrafiltracionih frakcija hidrolizata proteina belanceta ispitivanjem sposobnosti neutralizacije 2,2'-difenil--1-pikrilhidrazil (DPPH) i 2,2'-azinobis(3-etilbenzotiazolin-6-sulfonska kiselina)--diamonijum so (ABTS) radikala, kao i sposobnost redukcije 2,4,6-tri(2-piridil)-*s*--triazina (TPTZ). Proteini belanceta, korišćeni kao supstrat, neposredno pre enzimske hidrolize pretretirani su ultrazvučnim talasima frekvencije 35 i 40 kHz ili termički na 75 °C kako bi se postigla delimična denaturacija nativnih proteina i time omogućila olakšana dostupnostproteaze unutrašnjim peptidnim vezama u molekulu. Bakterijska endopeptidaza izolovana iz *Bacillus lichenoformis*, komercijalnog naziva alkalaza, korišćena je kao biokatalizator u reakciji hidrolize. Radi lakšeg literaturnog poređenja sa konvencionalnim termičkim postupkom, osim prethodno navedenim ultrazvučnim talasima visoke frekvencije, proteini belanceta su termički tretirani 30 min na 75 °C. Ultrafiltracionim frakcionisanjem dobijenih hidrolizata, korišćenjem celuloznih membrana različitih veličina pora, izolovane su četiri frakcije (>30, 10–30, 1–10 i <1 kDa) koje su okarakterisane sa aspekta sadržaja proteina i antioksidativne aktivnosti u cilju dobijanja bioaktivnih peptida. Dokazano je da je frakcija 3, koja sadrži peptide molekulske mase 1–10 kDa, u poređenju sa ostalim frakcijama svih pretretmana, imala najveće vrednosti za sva tri ispitivana antioksidativna testa. Značajno je istaći da je najveći doprinos izolovanju peptida sa visokom antioksidativnom aktivnošću dao ultrazvučni pretretman frekvencije 40 kHz. Naime, stepen inhibicije DPPH i ABTS radikala iznosio je 28,10±1,38 and 79,44±2,31%, redom. Na osnovu dobijenih rezultata, može se zaključiti da se ultrazvučnim pretretmanom i enzimskom hidrolizom proteina belanceta uspešno mogu izolovati bioaktivni peptidi, čija primena u prehrambenoj industriji kao dodataka ishrani predstavalja izuzetan doprinos.

Ključne reči: Antioksidativni peptidi belanceta • Membranska ultrafiltracija • Ultrazvučni pretretman • Enzimska hidroliza • Alkalaza