UNIVERSITY OF EAST SARAJEVO

FACULTY OF TECHNOLOGY ŽVORNIK

PROCEEDINGS

ZBORNIK RADOVA

IV INTERNATIONAL CONGRESS

ENGINEERING, ECOLOGY AND MATERIALS IN THE PROCESSING INDUSTRY

IV MEĐUNARODNI KONGRES

INŽENJERSTVO, EKOLOGIJA I MATERIJALI U PROCESNOJ INDUSTRIJI

JAHORINA, 04.03. - 06.03.2015.

REPUBLIC OF SRPSKA

BOSNIA AND HERZEGOVINA

TEHNOLOŠKI FAKULTET ŽVORNIK

UZ PODRŠKU

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UNDER AUSPICIES OF
THE MINISTRY OF SCIENCE AND TECHNOLOGY OF REPUBLIC OF SRPSKA
THE ACADEMY OF SCIENCE AND ART OF REPUBLIC OF SRPSKA

POD POKROVITELJSTVOM
MINISTARSTVA NAUKE I TEHNOLOGIJE REPUBLIKE SRPSKE
AKADEMIJE NAUKA I UMJETNOSTI REPUBLIKE SRPSKE

WITH SUPPORT
OF UNION OF ENGINEERS
AND TECHNICIANS OF SERBIA

UZ PODRŠKU
SAVEZA INŽENJERA
I TEHNIČARA SRBIJE

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BOSNIA AND HERZEGOVINA

PUBLISHER/IZDAVAČ:
TEHNOLOŠKI FAKULTET ZVORNIK
Karakaj bb,75400 Zvornik
Republika Srpska, BiH
Telefon: +387 56 261-072
Fax: +387 56 260-190
E-mail: sekretar@tfzv.org
Web: www.tfzv.org

FOR PUBLISHER/ZA IZDAVAČA:
Prof. dr Miladin Gligorić, dean/dekan

AREA/OBLAST:
ENGINEERING, ENVIRONMENT AND MATERIALS IN PROCESSING INDUSTRY
INŽENJERSTVO, EKOLOGIJA I MATERIJALI U PROCESNOJ INDUSTRIJI

PUBLISHED/GODINA IZDANJA: 2015

PRINT/ŠTAMPA: Eurografika Zvornik
CIRCULATION/TIRAŽ: 200 copies/primjeraka
ISBN 978-99955-81-18-3

DOI: 10.7251/EEMEN1501278K
UDK: 637.413:577.1
Scientific paper

ANTIOXIDANT ACTIVITY OF PEPTIDE FRACTIONS OBTAINED BY MEMBRANE ULTRAFILTRATION OF EGG WHITE PROTEIN ENZYMATIC HYDROLYSATES

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Abstract

The objective of this research was a production of egg white protein hydrolysates with improved antioxidant properties. For this purpose, a thermal and ultrasound pretreated egg white proteins were intensively hydrolysed with a commercial food-grade bacterial protease Alcalase. Thus obtained hydrolysates were further separated by sequential ultrafiltration into four peptide fraction viz. Fraction I (> 30kDa), II (10 - 30 kDa), III (1 - 10 kDa) and IV (< 1kDa) which were investigated in terms of their antioxidant activity. The antioxidant activity of hydrolysates and peptide fractions were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical scavenging assays and measuring ferric reducing antioxidant power assay. Scavenging of 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) by Fraction III, prepared with ultrasound pretreatment (35 kHz - 30 min) was found to be significantly higher than other fractions (21.17 ± 2.01 % and 80.72 ± 1.32 %, respectively), while ferric reducing antioxidant power assay has proven to be the best for thermal pretreatment. The results show that the fractionated hydrolysates were superior to the original hydrolysate in the antioxidative activity tested and can be concluded that by combining ultrasound pretreatment hydrolysates with improved functional and antioxidant properties can be produced enhancing utilization of egg white in food products.

Key words: *antioxidant peptides, membrane ultrafiltration, egg white proteins, ultrasound pretreatment*

ANTIOKSIDATIVNA AKTIVNOST HIDROLIZATA PROTEINA BELANCETA I NJIHOVIH FRAKCIJA DOBIJENIH MEMBRANSKOM ULTRAFILTRACIJOM

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

U ovom radu određena je antioksidativna aktivnost utrafiltracionih frakcija hidrolizata proteina belanceta ispitivanjem sposobnosti neutralizacije 2,2'-difenil-1-pikrilhidrazil (DPPH) i 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonska kiselina)-diamonium 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 kHz kako bi se postigla delimična denaturacija nativih proteina i time omogućila olakšana dostupnost endopeptidaze Alkalaze peptidima. Radi lakšeg literaturnog poređenja sa konvencionalnim termičkim postupkom proteini belanceta su termički tretirani (75 °C-30 minuta). Ultrafiltracionim frakcionisanjem dobijenih hidrolizata, korišćenjem celuloznih membrana različitih veličina pora, izolovane su četiri frakcije (>30 kDa, 10-30 kDa, 1-10 kDa i <1 kDa) koje su okarakterisane sa aspekta sadržaja proteina i antioksidativne aktivnosti. Dokazano je da je frakcija III u poređenju sa ostalim frakcijama, imala najveće vrednosti za sva tri ispitivana antioksidativna testa. Značajno je istaći da je poseban doprinos izolovanju peptida sa visokom antioksidativnom aktivnošću dao ultrazvučni pretretman frekvencije 35 kHz. Naime, stepen inhibicije DPPH i ABTS radikala iznosio je 21,17 ± 2,01 % i 80,72 ± 1,32 %, 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 predstavlja izuzetan doprinos.

Ključne reči: *antioksidativni peptidi, membranska ultrafiltracija, proteini belanceta, ultrazvučni pretretmant*

Introduction

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 [1]. 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 [2], casein [3], soymilk-kefir [4], egg-yolk [5] and yam [6]. Enzymatic hydrolysis applied to this source has been suggested to transform these materials into marketable and value-added products with functional or biological properties [7]. During protein hydrolysis, protein's overall antioxidant activity isheightened because of releasing a low molecular weight peptide due to disrupting the tertiary structure of the native protein.

Among a wide range of different protein sources, egg white proteins (EWPs) are widely used as a functional and nutritional ingredients in food products and their hydrolysates obtained by protease treatment are also an excellent source of biological active substances and have a high nutritional value [8]. In order to improve and upgrade the functional and nutritional properties of EWPs and obtain value-added egg products enzymatic hydrolysis is frequently used. The egg white protein hydrolysates (EWPHs) 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.

Many studies recently published, try to fractionate proteins, protein hydrolysates and peptides with membrane separation techniques and isolate the fractions with the most antioxidant active peptides with the aim of enhancing their biological or their functional properties. 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 [9]. Thus, identifying the safe and natural antioxidant sources would be

beneficial for use in the food industry and to prevent oxidative stress related human health disorders.

In the present study, the antioxidant activities of the EWPHs 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 product production.

Experimental part

Materials and methods

All hydrolyses were performed using commercial egg-white as a substrate, which was a purchased in a local supermarket. Chemicals used for determination of hydrolysates antioxidant capacity, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were all purchased from Sigma Aldrich (St. Louis, USA). Protease from *Bacillus licheniformis*, Alcalase, (EC 3.4.21.14), with the claimed activity of 2.4 Anson unit (AU) g^{-1} was also purchased from Sigma Aldrich (St. Louis, USA), where one Anson unit represents 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 Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine at 25 °C and at pH 7.50. All other chemicals used in this research were of analytical grade.

Preparation of EWP hydrolysates

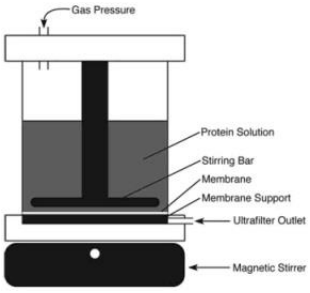
Prior to hydrolysis, 10 % (w/w) water solution of EWPs were prepared under the constant stirring and then heated at 75 ºC for 30 min and sonicated at 35 kHz for the same time in order to denature the proteins. This was followed with cooling the substrate solution to 50 °C and adjusting the pH to 8.0. For the ultrasound control, 10 % (w/w) 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. Enzymatic hydrolyses were performed in the stirred tank bioreactor (600 cm^3) with controlled temperature equipped with four-bladed propeller.

The hydrolysis reaction was initiated by adding enzyme in the amount of 2 % (w/w) 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.Each treatment was conducted in duplicate.

Fractionation of EWP hydrolysates by ultrafiltration cell unit

The hydrolysates were fractionated using a Millipore ultrafiltration stirred cell unit (Model 8050 1 Unit, Millipore Corporation, Bedford, MA, USA) under magnetic stirring with a capacity of 50 cm^3. Ultrafiltration membranes regenerated cellulose, 44.5 mm diameter (Amicon Inc., Billerica, MA, USA) with molecular weight cut-off (MWCO) of 30, 10 and 1 kDa were used. During filtration process pressure was applied with nitrogen. A portion of the supernatant containing target peptides was stored in the freezer as EWPHs while the remaining portion was passed through ultrafiltration membranes with above mentioned molecular weight cut-offs. Ultrafiltration was performed sequentially: first through the 30 kDa and permeate passed through 10 kDa whose permeate was passed through the 1 kDa membrane. The retentate from each

MWCO membrane was collected as Fraction II (10-30 kDa), III (1-10 kDa) and permeate as Fraction IV (<1 kDa) peptide fractions, respectively. The protein contents of the EWPHs and peptide fractions were determined using the Lowry method [10]. All fractions were then stored in the freezer until needed for further analysis. Sheme 1. exhibits the illustration of the ultrafiltration set up used in this research.



Scheme 1. Schematic overview of an ultrafiltration cell unit

Antioxidant activity of EWPHs and their ultrafiltered fractions

The antioxidant activity of the fractions of the EWPHs were evaluated using different tests that included the ability to sequester free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH), the sequestration ability of the ABTS free radical and ferric reducing power.

DPPH free radical scavenging activity

The scavenging activity of EWPHs and its fractions against the DPPH radical was determined based on the method described by *Stefanović et al.(2014)*[11]. Briefly, a 0.2 cm^3 aliquot of each obtained hydrolysate was mixed with 1.8 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:

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

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

ABTS radical antioxidant activity

Antioxidant capacity expressed as the ability of the hydrolysates to scavenge $ABTS^{\bullet+}$ radical was based on the $ABTS^{\bullet+}$ radical cation decolourization [12]. $ABTS^{\bullet+}$ radical was produced in the reaction between 7 mM ABTS solution and 2.45 mM potassium persulfate (final concentration), which were left in dark for 12-16 hours. Produced $ABTS^{\bullet+}$ solution was diluted with PBS, pH 7.4, until the absorbance of 0.700 (± 0.02) was reached. The 0,01 cm^3 of the hydrolysates were mixed with 1 cm^3 of prepared $ABTS^{\bullet+}$ solution and after 5 min absorbance was measured at 734 nm and ABTS radical scavenging activity (%) was calculated using following equation:

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

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 $ABTS^{\bullet+}$. All experiments were carried out in triplicate.

Ferric reducing antioxidant power assay

The FRAP assay is based on the hydrolysates ability to reduce ferric tripyridyl triazine (Fe III-TPTZ) to the blue-colored ferrous complex at low pH. FRAP solution was prepared shortly before use, according to the method previously described elsewhere [13]. Samples (buffer for blank), 0,025 cm^3 were vigorously mixed with 0,750 cm^3 of FRAP solution and after 5 min absorbance was measured at 593 nm. Standard curve was prepared using $FeSO_4*7H_2O$ (0.1-1 mmol dm^{-3}). All results were expressed as micromole of Fe (II) equivalents per miligram of proteins.

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

Preparation of antioxidant fractions

Molecular weight is an important parameter reflecting the hydrolysis of proteins, which further correlates with the bioactivity of protein hydrolysates. After different pretreatment, EWPs were separately hydrolysed by endopeptidase namely Alcalase, in order to isolate bioactive peptides with proven antioxidant activity. All hydrolysates showed the antioxidant activities and they were passed through three ultrafiltration membranes with 30, 10 and 1 kDa molecular weight cut-off because many research reported that lower molecular weight peptides are more potent as bioactive peptides [14]. The values of the separated fractions (>30, 1-10, 10-30, <1 kDa) and its impact on the peptidic population in terms of molecular weight were presented on Figure 1.

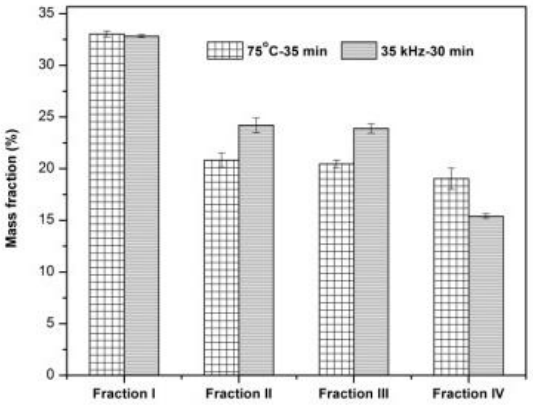


Figure 1. The molecular weight distribution profile of EWPHs and their ultrafiltered fractions obtained by thermal and ultrasound pretreatment (Hydrolysis conditions were as follows: 50 °C, pH 8.0; 2.12 U of Alcalase, 10 % (w/w) aqueous solution of egg white (E/S ratio 2.0 % w/w))

Some differences between the molecular weight distribution profiles of all hydrolysates were

observed. It is apparent that the hydrolysis after thermal pretreatment generated more peptides <1 kDa (19.04 ± 1.02 %) than ultrasound pretreatment did (15.42 ± 0.23 %), whereas the proportion of peptides <10 kDa were higher in the second case (23.89 ± 0.46 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.

Antioxidant activity of EWPHs and their ultrafiltered fractions

ABTS and DPPH radical scavenging activities

It is well known that protein hydrolysates and peptides have excellent potential as antioxidant additives in foods because they can inhibit lipid oxidation through multiple pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the properties of food systems [15]. Three methods were used for evaluation of EWPHs antioxidant capacity in the current research, namely ABTS, DPPH and FRAP methods. DPPH and ABTS radical scavenging activities are based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals by converting them to the non-radical species and the results obtained from this experimental set are presented in Figure 2.

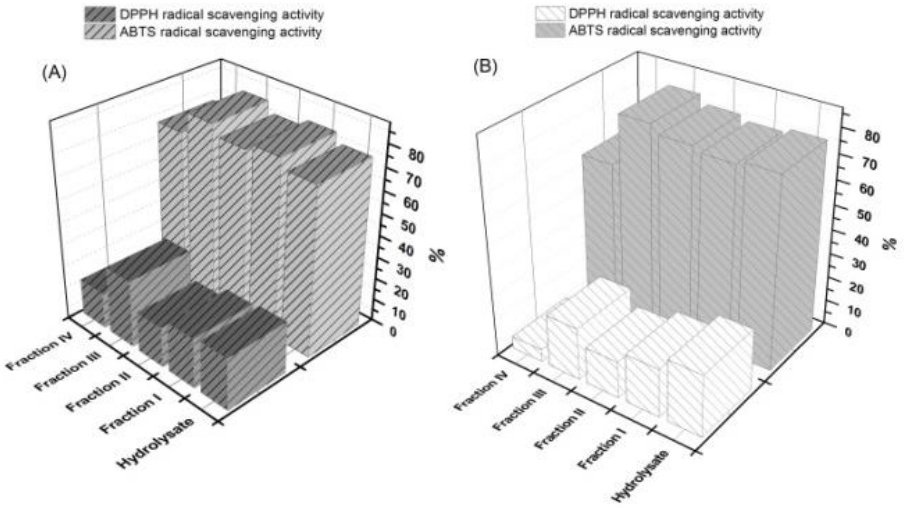


Figure 2. DPPH and ABTS radical scavenging activity of EWPHs and their ultrafiltered fractions: (A) thermal pretreatment at 75 °C during 30 min, (B) ultrasound pretreatment at 35 kHz during 30 min

Generally, the hydrolysis favored antioxidant capacity of prepared hydrolysates as compared to the control non-hydrolyzed egg-white solution, which was subjected to the same pretreatments. Beneficial effect of EWP hydrolysis on the antioxidant capacity of the obtained hydrolysates was most apparent with ABTS method, as presented in *Figure 2*, where the control sample expressed the ability to scavenge only7.69 ± 1.59 % of the aforementioned radical. The highest ABTS scavenging activities were achieved for Fraction III induced by ultrasound and thermal pretreated hydrolysates which outcomes have not been statistically significant (p>0.05) and values were 80.72 % and 82.01 %, respectively. 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 % [16]. Apparently, the results disclose the presence of antioxidant peptides in all the isolated EWPH fractions, and an expressive tendency to concentrate in the fractions with a lower molecular weight. It is well documented that, although antioxidant activity is normally widely

observed in fractionated hydrolysate, the lower most molecular weight peptide fractions usually exhibit the strongest bioactivity.

The DPPH radical is an oil-soluble free radical that becomes a stable product after accepting an electron or hydrogen from an antioxidant and show maximum absorbance at 517 nm. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavengedwhich is reflected in the decrease of absorbance values and therefore the antioxidant activity of the substance can be expressed as its ability in scavenging the DPPH radical.*Figure 2* shows results of ability of EWP and its membrane fractions to scavenge DPPH radical. EWPHs obtained by ultrasound pretreatment have higher DPPH scavenging activity (25.18%) when compared to thermal pretreated EWPHs. There was no significant difference ($p > 0.05$) between the DPPH scavenging activities of peptides with molecular weight >30 kDa and 1-10 kDa. The molecular weight peptides (1-10 kDa) exhibited the highest DPPH radical scavenging activities among all others peptide fractions for both pretreatments. This result is in agreement with the previous results reported by *Stefanović et al.(2014)* [11] which showed that peptide fractions with molecular weight 1-10 kDa have higher DPPH scavenging activities compared to all others isolated fractions. Increased hydrophobic character of peptides derived from protein sources have been shown to correlate with higher DPPH or other radical scavenging activities [17] when compared with peptide fractions of lower hydrophobic content. The highest DPPH radical scavenging activities that were found in the 1-10 kDa fractions may be due to its having the highest contents of total hydrophobic amino acid and aromatic amino acid. *Kim et al. (2007)* [18] reported that hydrophobic amino acids act as antioxidants by increasing the solubility of peptides in non-polar environments thereby facilitating better interaction with free radicals in order to terminate their activities. The results suggest that improved DPPH scavenging activities of ultrafiltered peptides could make them useful ingredients which can be used to prevent oxidative deterioration in food formulations.

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 [19]. The FRAP activity of protein/peptide components in enzymatic hydrolysates and their ultrafiltered fractions are represented in Figure 3.

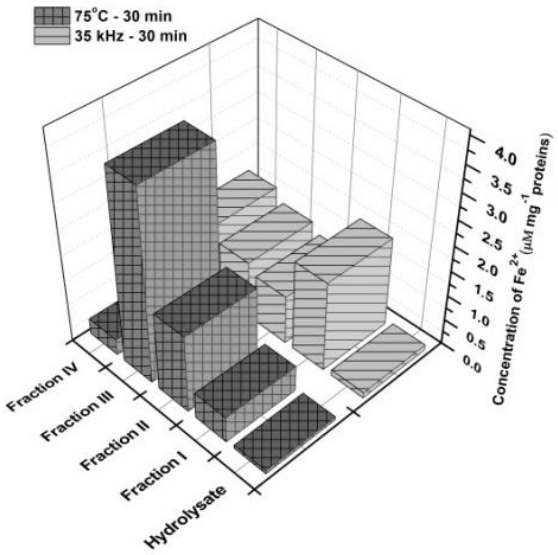


Figure 4. Ferric reducing power assay of EWPHs and their ultrafiltered fractions expressed as concentration of Fe(II)

Hydrolysates prepared with ultrasound pretreatment and with thermal pretreatment had no

detectable activity and/or the values were not statistically significant. Therefore, the FRAP activity of the EWPHs 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 kDa 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 III obtained by hydrolysate prepared with thermal treatment. The Fractions I for ultrasound pretreatment had a significantly lower value of Fe (II) concentration amounted 2.05 ± 0.34 μM per milligram of proteins. According to the availableliterature 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 [20].

Generally, based on the all represented results, Fraction III 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 were the most abundant after membrane ultrafiltration of the protein hydrolysates with both pretreatments which suggest that the combination of these pretreatments and convenient pick of suitable protease for hydrolytic reaction were efficient in reducing the native EWPs into low molecular weight peptides.

Conclusion

Following the results from this research can be reasoned that ultrafiltration process increased the antioxidant activity of the fractions comparing to the hydrolysates. From the results of the three antioxidant tests, it could be noticed that the antioxidant activity of the Fraction III (peptides with molecular weight 1-10 kDa), prepared with both pretreatment prior hydrolysis, exerted excellent radicals quenching capability. 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 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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CIP - Каталогизација у публикацији
Народна и универзитетска библиотека
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66.02-9(082)(0.034.2)
54(082)(0.034.2)
502/504(082)(0.034.2)

МЕЂУНАРОДНИ конгрес "Инжењерство, екологија и материјали у процесној индустрији" (4 ; 2015 ; Јахорина)
Knjiga radova [Електронски извор] = Proceedings / IV međunarodni kongres "Inženjerstvo, ekologija i materijali u procesnoj industriji", Jahorina, 04.03.- 06.03. 2015. = IV International Congress "Engineering, Ecology and Materials in the Processing Industry" ; [organizator] Univerzitet u Istočnom Sarajevu, Tehnološki fakultet Zvornik ; [urednici Miladin Gligorić, Milovan Jotanović, Dragica Lazić, Miomir Pavlović]. - Zvornik : Tehnološki fakultet, 2015 (Zvornik : Eurografika). - 1 elektronski optički disk (CD-ROM) : tekst, ilustr. ; 12 cm

Nasl. sa naslovnog ekrana. - Radovi na srp. i engl. jeziku. - Tiraž 200. - Bibliografija uz sve radove.

ISBN 978-99955-81-18-3
1. Технолошки факултет (Зворник)

COBISS.RS-ID 4912920



ISBN 978-99955-81-18-3