Slovak Society of Chemical Engineering

Institute of Chemical and Environmental Engineering

Slovak University of Technology in Bratislava

PROCEEDINGS

42nd International Conference of Slovak Society of Chemical Engineering

Hotel Hutník

Tatranské Matliare, Slovakia

May 25 – 29, 2015

Editor: prof. Jozef Markoš

HONORARY COMMITTEE

Prof. Jozef MARKOŠ
Chairman of Slovak Society of Chemical Engineering, SK
Prof. Jozef DUDÁŠ
Head of Institute of Chemical and Environmental Engineering, SUT in Bratislava, SK
Prof. Ľudovít JELEMENSKÝ
Slovak University of Technology in Bratislava, SK
Prof. Lucie OBALOVÁ
Technical University Ostrava, Ostrava, CZ
Prof. Jiří HANIKA
Institute of Chemical Process Fundamentals, Czech Academy of Science, Prague, CZ
Prof. Jiří KLEMEŠ
University of Pannonia, Veszprém, Hungary
Prof. František ŠTĚPÁNEK
Prague Institute of Chemical Technology, Prague, CZ

Proceedings of the 42nd International Conference of SSCHE
Tatranské Matliare, May 25 – 29, 2015

ISBN: 978-80-89475-14-8
EAN: 9788089475148

Editor: prof. Jozef Markoš

PROGRAM COMMITTEE

Prof. Jozef MARKOŠ
Slovak University of Technology in Bratislava, SK
Prof. Milan POLAKOVIČ
Slovak University of Technology in Bratislava, SK
Assoc. Prof. Juma HAYDARY
Slovak University of Technology in Bratislava, SK
Dr. Edit CSÉFALVAY
Budapest University of Technology and Economics, Budapest, H
Assoc. Prof. Michal PŘIBYL
Institute of Chemical Technology, Prague, CZ
Dr. Pavel IZÁK
Institute of Chemical Process Fundamentals, Czech Academy of Science, Prague, CZ

ORGANIZING COMMITTEE

Prof. Jozef MARKOŠ
Ing. Barbora DUDÁŠOVÁ
Assoc. Prof. Pavel TIMÁR
Prof. Milan POLAKOVIČ
Ing. Mário MIHAĽ
Ivan ČERVEŇANSKÝ
Viliam RIZMAN
Marta ORDOGOVÁ

ADDRESS OF THE ORGANIZING COMMITTEE

Institute of Chemical and Environmental Engineering
Faculty of Chemical and Food Technology
Slovak University of Technology in Bratislava
Radlinského 9, 812 37 Bratislava
Slovak Republic
email: sschi@stuba.sk

ACKNOWLEDGEMENT

This conference was organized with the support of the Ministry of Education, Science, Research and Sport of the Slovak Republic within the Research and Development Operational Programme for the project "University Science Park of STU Bratislava", ITMS 26240220084, co-funded by the European Regional Development Fund.

Slovak Society of Chemical Engineering
Institute of Chemical and Environmental Engineering
Slovak University of Technology in Bratislava

PROCEEDINGS

42nd International Conference of Slovak Society of Chemical Engineering

Hotel Hutník
Tatranské Matliare, Slovakia
May 25–29, 2015

Editor: prof. Jozef Markoš

ISBN: 978-80-89475-14-8, EAN: 9788089475148

Jovanović, J., Stefanović, A., Grbavčić, S., Šekuljica, N., Elmalimadi, M., Bugarski, B., Knezevic-Jugovic, Z.: Peptides with improved antimicrobial activity screened by membrane ultrafiltration from egg white protein hydrolysates, Editor: Markoš, J., In *Proceedings of the 42nd International Conference of Slovak Society of Chemical Engineering*, Tatranské Matliare, Slovakia, 732–739, 2015.

Peptides with improved antimicrobial activity screened by membrane ultrafiltration from egg white protein hydrolysates

Jelena Jovanović [1], Andrea Stefanović [1], Sanja Grbavčić [2], Nataša Šekuljica [2], Mohamed Elmalimadi [1], Branko Bugarski [3], Zorica Knežević-Jugović [1]

[1]Department of Biotechnology and Biochemical Engeneering, University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, Belgrade; Serbia, *e-mail*: zknez@tmf.bg.ac.rs; *Tel:* +381 11 303776
[2]Innovation Center, Faculty of Technology and Metallurgy, University of Belgrade
[3]Department of Chemical Engeneering, University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, Belgrade, Serbia

Key words: antimicrobial derived egg white peptides, radial diffusion assay, membrane ultrafiltration, hydrolysis, Alcalase

Abstract

This contribution was aimed at the fractionation and identification of peptides with improved antimicrobial activity from egg white protein hydrolysates, obtained by membrane ultrafiltration. For this purpose, the thermal treated egg white proteins were intensively hydrolysed with a commercial food-grade bacterial endopeptidase from *Bacillus licheniformis*, namely Alcalase. Thus, obtained hydrolysates were further separated by sequential ultrafiltration into four peptide fractions *viz*. fraction I (> 30kDa), II (10 - 30 kDa), III (1 - 10 kDa) and IV (< 1kDa) which were investigated in terms of their antimicrobial activity. The antimicrobial activity was tested against Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Escherichia coli* ATCC 25922) and against yeast *Candida albicans* (ATCC 24433) by fractions' susceptibility of agar diffusion. Our results showed that these peptide fractions have an intense inhibitory activity on Gram-positive bacteria, poor on Gram-negative bacteria and none inhibitory activity on growth of *C. albicans*. The results showed interesting antimicrobial potentials versus the tested microorganisms, especially fractions with peptides molecular weight of 10-30 kDa and 1-10 kDa. It can be concluded, that the controlled enzymatic hydrolysis of egg white proteins and their subsequent membrane ultrafiltration is considered to be a suitable way for production of biocative peptides with exhibit antimicrobial efficiency.

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]. 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 [2].

Antimicrobial peptides have acquired heightened concern due to the occurrence of bacteria resistance and prospective toxicity of chemical food preservatives. Considerable numbers of antimicrobial peptides have been screened and identified from a wide variety of source or generated by protein hydrolysates [3]. The separation and purification of antimicrobial peptides is essential for

examining their features and further applications, but identification of them in complex samples is often challenging. The mechanism of the antibacterial effect has not been obviously entrenched. Nevertheless, it has been suggested that cellular membrane lysis caused by the direct action of the peptides is responsible for bacterial death [4]. In the past decade, research on antimicrobial agents has been widely accomplished and attention has mainly focused on the antimicrobial peptides which could be generated during the digestion of food proteins in the gastrointestinal tract. Some antimicrobial peptides were released upon the digestion or breakdown of protein such as ovotransferrin [5], ovalbumin [6] and lysosyme [7].

Between broad ranges of diverse protein sources, egg white proteins (EWPs) are widely used as a functional and nutritional ingredients in food products and their hydrolysates obtained by enzymolysis are also an excellent source of biological active peptides and have a high nutritional value. Most of the EWPs appear to possess antimicrobial properties or certain physiological functions to interfere with the growth and spread of invading microorganisms [8]. In order to improve and upgrade the bioactive features of EWPs and obtain value-added egg products, enzymatic hydrolysis is frequently used. The 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. It should be emphasized that researches verify peptides released from lysozyme, ovalbumin and ovotransferrin, during proteolytic action of enzymes, possessed antimicrobial activity against Gram-positive and Gram-negative bacteria [9].

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 [10]. Especially, fractions of molecular weight between 100 – 500 Da and 1000 – 3500 kDa would be the most interesting bioactive peptides for nutritional and pharmaceutical uses. The extraction and recovery of these fractions from the hydrolysates are the main issues. Compared with other bioseparation methods such as gel chromatography, membrane technology provides several important advantages such as higher productivity, lower capital investment, high flow of products and while keeping product purity under ambient conditions, ease of translation of large-scale commercial production and easy equipment cleaning [11].

This paper was focus on determining the antimicrobial activity of peptidic fractions obtained by enzymatic hydrolysis and ultrafiltration with molecular weight cut off membranes (1, 10 and 30 kDa) from egg white.

Matrials and methods

Materials

The EWP samples were prepared using commercial eggs which were kindly provided from Jata Emona d.o.o (Ljubljana, Slovenia) and stored at 4 °C. After separation from the yolk, EWs were leisurely stirred without foam formation to ensure homogeneous mixture. The enzyme complex used in this research for determination the susceptibility of thermal treated EWPs to enzymolysis was Alcalase 2.4 L from *Bacillus licheniformis* which purchased from Sigma Aldrich (St. Louis, USA). The claimed enzyme activity was $\geq$ 2.4 U g^{-1} Anson Units solid for Alcalase whereby the one Anson Unit is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute which gives the same color with the Folin–Ciocalteu phenol reagent as one milliequivalent of tyrosine at 25 °C and pH

7.50. All other chemicals were of analytical reagent grade and they were used without any further purification.

Thermal treatment and enzymolysis of EWPs

Thermal treatment of EWPs was carried out by heating EW solution at 75 °C for 30 min. Upon completion of treatments the treated 10 % w/w EWP solutions (11.9 mg mL^{-1}, protein content determined according to the standard Kjeldahl method, $N \times 6.25$) were 20 min preheating at optimal temperature 50 °C for Alcalase. Thereafter, the solution was adjusted to optimum pH 8.0 and the enzymolysis was initiated by addition of the appropriated amount of protease (enzyme to substrate ration was 2.0 % w/w). The progress of the enzymolysis was followed by monitoring the degree of hydrolysis (DH, %) using pH-stat method. The DH was calculated according the Eq. 1 [12]:

$$DH\ (\%) = \frac{h \cdot 100}{h_{tot}} = \frac{N_b \cdot B \cdot 100}{\alpha \cdot m_p \cdot h_{tot}} \quad (1)$$

where h is the number of equivalents of peptide bonds hydrolyzed at the time per weight unit; h_{tot} is the total amount of peptide bonds per weight unit of a protein and can be calculated from its amino acid composition (for EWPs h_{tot} is 7.67 mmol g^{-1} protein), N_b is the normality of the base, B is the consumption of the base in mL, α is the degree of dissociation of the α-amino groups (1/α=1.13 at 50 °C and pH 8.0) and m_p is the mass of protein in g. The enzymolysis was terminated by boiling the mixtures for 15 min, then centrifuged at 3,500×g for 20 min and after cooling at room temperature. The supernatant was collected and stored at 4 °C for further antimicrobial assay.

Microbial susceptibility by diffusion in agar

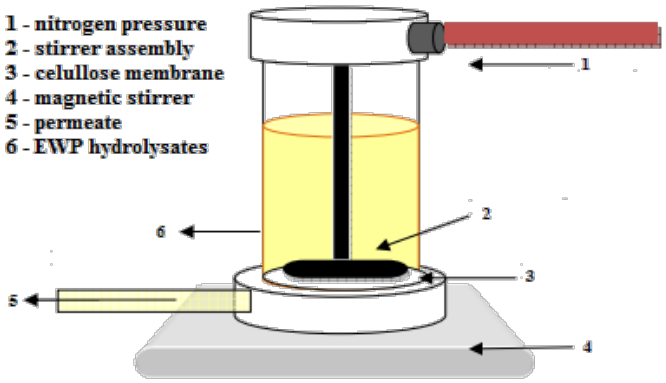
The antimicrobial activity was determined by susceptibility of agar diffusion and it was carried out according to *Aničić et al*. [13]. Briefly, 9 mm tubes were placed on a layer of suitable agar media (Tryptic soy agar (TSA) supplemented with yeast extract (0.6% w/v)). This media was overlaid with a layer of soft agar medium (TSA containing 0.6 % of agar) inoculated with tested microorganisms (*E. coli* ATCC 25922, *S. aureus* ATCC 25923, *C. albicans* ATCC 24433). Tested microorganisms were part of culture collection of Microbological laboratory of the Fuculty of Technology and Metalurgy, Belgrade. Upon soft agar firming, tubes were aseptically removed, forming wells such way. In each well, 30 µL of sample was added (untreated EWPs, Alcalase hydrolysate and peptidic fractions of various size). Petri dishes were incubated at 37 ^{o}C for 48 h and size of inhibition zones (in mm) were measured thereafter.

Minimum inhibitory concentration (MIC) assay

The minimum inhibitory concentration (MIC) of the selected peptides was determined using a micro-well dilution method. This survey implied mixing 50 µL of each bacterial suspension (concentrations adjusted to $1x10^5$ CFU/mL with TSB) with 50 µL of serially diluted samples in 96-well microplates and incubated at 37 °C by 24 h. Only samples that initially presented highest antimicrobial activity according to agar diffusion method were tested for MIC. The microbial growth was evaluated after 24 h by reading the absorbance at 600 nm (OD 600) using the Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, USA). The lowest concentration of Alcalase hydrolysate fractions where no observable growth was noted by the change of initial absorbance reading was established to be the MIC. Obtained results were further verified by plating 30 µL of the samples from the wells on the TSA agar medium. All experiments were done in triplicate to ensure reproducibility.

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. Scheme 1 exhibits the illustration of the ultrafiltration set up used in this research.



Scheme 1. Schematic overview of an ultrafiltration cell unit

Statistical analysis

All experiments were performed in triplicate and the data are presented in average of triplicates and standard deviation (SD). Statistical differences between antimicrobial activity of Alcalase hydrolysate and his 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

The enzymolisis of thermal treated EWPs and membrane ultrafiltration

Molecular weight represents 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 fraction (< 1, 1-10, 10-30, >30 kDa) and its impact on the peptidic population in terms of molecular weight and hydrolytic curve of thermal treated EWPs with Alcalse were presented on Figure 1.

The results obtained in the enzymolysis of untreated and thermal treated EWPs (Figure 1) implied that thermal treatment improved susceptibility of EWPs to enzymolysis by Alcalase. On the other hand, DH achieved in the hydrolysis of EWPs varied from 7.5-35 % over the 105 min time period. The hydrolysis proceeded at a rapid rate during the initial 15 min of the reaction. Afterwards, enzymolysis proceeded with a slow increase in hydrolysis rate for the next 75 min, and then entered the steady-state. This was confirmed that thermal treated EWPs as substrate has the considerable susceptibility by Alcalase and thus prepared hydrolysate may be used to isolate bioactivity peptides.

Some differences between the molecular weight distribution profiles of obtained hydrolysate were observed. As shown in Figure 1, four fractions with diverse yields were obtained by membrane ultrafiltration. Different protein contribution of prepared fractions with different peptide molecular weigth could influence on antimicrobial activity of EW peptides.

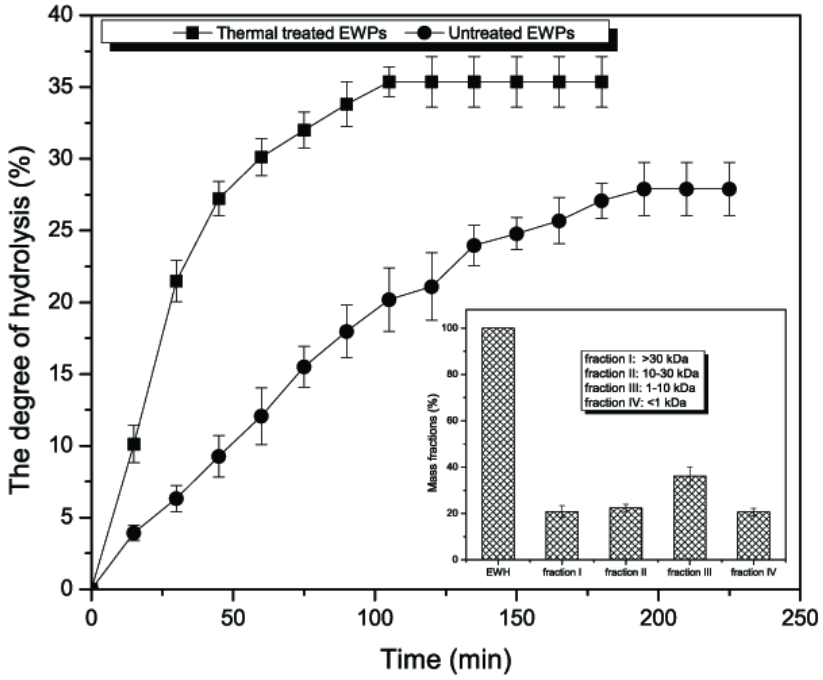


Figure 1. Profile and degree of hydrolysis of thermal treated EWPs. *Insert* The molecular weight distribution profile of EWP hydrolysate and its ultrafiltered fractions obtained by thermal treatment. Treatment conditions: 75 °C during 30 min. Hydrolysis conditions were as follows: 50 °C, pH 8.0; 2.12 U of Alcalase, 10 % (w/w) aqueous solution of egg white (E/S ratio 2.0 % w/w).

Antimicrobial activity assay of prepared EW peptide fractions

To test antimicrobial activity of Alcalase hydrolysate and four peptidic fractions two strain of bacteria and yeast were used and obtained results were presented in Table 1. The minimum inhibitory concentration (MIC) of the peptide fractions which provided the largest values of inhibition zone was determined using a micro-well dilution method and MIC values are presented in Table 2.

Table 1 Antimicrobial activity of peptidic fraction against bacterial (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922) and yeast Candida albicans (ATCC 24433) using the radial diffusion agar method

Target strains	Inhibition zone diameter (mm)*					
	EW	EWH	fraction I	fraction II	fraction III	fraction IV
Staphylococcus aureus	15.0±0.10	19.0±0.15[b]	18.0±0.22[b]	22.0±0.24[b]	19.0±0.12[b]	15.0±0.14[b]
Escherichia coli	11.5±0.22	12.3±0.14[a]	12.7±0.24[a]	12.3±0.15[a]	12.3±0.22[a]	12.5±0.12[a]
Candida albicans	NIA	NIA	NIA	NIA	NIA	NIA

EW: egg white; EWH: egg white hydrolysate; fraction I: >30 kDa; fraction II: 10-30 kDa; fraction III: 1-10 kDa; fraction IV: <1 kDa.

[a] statistically not significant differences among samples and control ($p > 0.05$)

[b] statistically significant differences compared with the control ($p < 0.05$)

NIA - no inhibition activity or below available limit.

Antibiotic which was used in this research as a control was neomycin. The inhibition zone values of neomycin were 14.1±0.19 and 16.4±0.12 mm against *E. coli* amd *S. aureus*, respectively.

*The inhibition zone values presented the average of three individual trials

The results of radial diffusion agar assay showed that the all peptidic fractions have statistically significant antibacterial effects against *S. aureus*. Can be noted that peptidic fractions have poor inhibitor activity on *E. coli* and none inhibitory activity on growth of *C. albicans*. Likewise, the results showed interesting antimicrobial potentials versus the tested microorganisms, especially fractions II and III with peptides molecular weight of 10-30 kDa and 1-10 kDa, respectively. The MIC values of the fraction II peptide were 28.28±2.2 and 70.71±2.8 μg/ml against *S. aureus* and *E. coli*, respectively. It should be emphasized that antimicrobial activities of all fractions were lower than neomycin as antibiotic. *Mine et al.* [7] isolated antimicrobial peptides from HEWL hydrolysate using peptic and tryptic digestion. They found that the peptide sequences of IVSDGDGMNAW (residues 98–108 of HEWL) and HGLDNYR (residues 15–21 of HEWL) were the most active peptides against *E. coli* K-12 and *S. aureus* 23-394, respectively. For comparison, it can merely be noted that these peptides showed a 70 % bacterial growth inhibition at the concentration 400 μg/ml, while the fraction III peptide (molecular weight of 1-10 kDa) at 29.16 and 58.32 μg/ml exhibited 70 % of inhibition effect against *S. aureus* and *E. coli*, respectively. Review of literature data can be emphasized that peptide OTAP-92, cationic fragment of hen ovotransferrin showed strong bactericidical activity aganst both Gram-positive *S. aureus* and Gram-negative *E. coli* strains [15].

Table 2 Antimicrobial activity of EWP hydrolysate and its fraction expressed as minimal inhibitory concentration (MIC) determined by the radial diffusion agar method

Samples	MIC (mg/ml)	
	Staphylococcus aureus	*Escherichia coli*
EWH	26.99 ± 1.23	53.98 ± 1.98
fraction II (10 – 30 kDa)	28.29 ± 1.11	70.71 ± 2.16
fraction III (1 – 10 kDa)	29.16 ± 0.98	58.32 ± 1.43

Presented results suggest that EW peptides don't possess inhibitor activity against yeast *C. albicans*. Obviously can be observed that enzymolysis EWPs with Alcalase and subsequent membrane ultrafiltration significantly increase antimicrobial activity. Generally, it can be concluded that EW peptidic fractions II and III presented antibacterial activity in both bacterial Gram-positive and Gram-negative strains because it is possible that peptides have easier diffusion through the cellular membrane

of bacteria in liquid media. These results suggest that short peptide chains possess the capacity to penetrate easier the bacterial cellular membrane facilitating the mode of action of antibacterial peptides.

Conclusions

Following the results from this research can be reasoned that ultrafiltration process increased the antibacterial activity of the fractions comparing to the hydrolysate and EW. From the results of the radial diffusion assay, it could be noticed that the antibacterial activity of the fraction II and III (peptides with molecular weight 10-30 and 1-10 kDa, respectivaly), prepared with thermal treated EWPs prior hydrolysis, exerted excellent inhibitor effects. The outcomes showed that these fractions possibly contained some effective antibacterial peptides, which have possess the capacity to penetrate easier the bacterial cellular membrane facilitating. 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.

Acknowledgment

This work was supported by EUREKA Project E!6750, Innovative project “Production of new dietary formulations based on natural protein with antioxidant and antitumor effect” and III 46010 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

References

1. Sarmadi, B.H., Ismail, A. (2010). Antioxidative peptides from food proteins: A review, Peptides, 31, 1949-1956.
2. Moller, N.P., Scholz-Ahrens, K.E., Roos, N., Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: indication for health effects. European Journal of Nutrition 47, 171-182.
3. Pellegrini, A. (2003). Antimicrobial peptides from food proteins. Current Pharmaceutical Design, 9, 1225-1238.
4. Wong, J.H., Ng, T.B. (2005) Lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*Phaseolus lunatus* L.). Peptides 26, 2086-2092.
5. Ibrahim, H.R., Sugimoto, Y., Aoki, T. (2000). Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through amembrane damagemechanism. Biochimica et Biophysica Acta, 1523, 196-205.
6. Pellegrini, A., Hulsmeier, A.J., Hunziker, P., Thomas, U. (2004). Proteolytic fragments of ovalbumin display antimicrobial activity. Biochimica et Biophysica Acta 1672, 76-85.
7. Mine, Y., Ma, F., Lauriau, S. (2004). Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. Journal of Agricultural and Food Chemistry, 52, 1088-1094.
8. Chen, C., Chi, Y., Zhao, M., Lv, L. (2012). Purification and identification of antioxidant peptides from egg white protein hydrolysate. Amino Acids, 43, 457-466.
9. Mine, Y., Kovacs-Nolan, J. (2006). New insights in biologically active proteins and peptides derived from hen egg. World’s Poultry Science Journal, 62, 87-95.
10. Najafian, L., Jafarzade, M., Said, M., Babji, A.S. (2013). Biochemical properties and antioxidant activity of myofibrillar protein hydrolysates obtained from patin *(Pangasius sutchi)*. International Journal of Food Science and Technology, 48, 2014-2022.
11. Saidi, S., Deratani, A., Belleville, M.P., Amar, R.B. (2014) Antioxidant properties of peptide fractions from tuna dark muscle protein by-product hydrolysate produced by membrane fractionation process. Food Research International, 65, 329-336.

12. Adler-Nissen, J. (1986). Enzymic Hydrolysis of Food Proteins, Elsevier Applied Science Publishers.
13. Aničić, N.V., Dimitrijević, S., Ristić, M., Petrović, S.S., Petrović S.D. (2005). Antimikrobna aktivnost etarskog ulja *Mellisa officinalis L., Lamiaceae*. Hemijska industrija, 59, 243-247.
14. Chen, J.R., Suetsuna, K., Yamauchi F. (1995). Isolation and characterization of immunostimulative peptides from soybean. The Journal of Nutritional Biochemistry, 6, 310-313.
15. Ibrahim, H.R., Iwamori, E., Sugimoto, Y., Aoki, T. (1998). Identification of a distinct antibacterial domain within the N-lobe of ovotransferrin. Biochimica et Biophysica Acta, 1401, 289-303.