Comparative analysis of functionality of spray dried whey protein hydrolysates obtained by enzymatic and microbial hydrolysis

Salem M. Embiriekah[1], Maja Lj. Bulatović[1], Marija Lj. Gnjatović[2], Maja S. Vukašinović-Sekulić[1], Tanja Ž. Krunić[3], Danica B. Zarić[4], Marica B. Rakin[1]

[1]Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, Belgrade, Serbia
[2]Institute for the Application of Nuclear Energy, University of Belgrade, Banatska 31b, Belgrade, Serbia
[3]Innovation center Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, Belgrade, Serbia
[4]IHIS Techno experts, Research Development Center, Belgrade, Batajnički drum 23, Belgrade, Serbia

Abstract

The aim of this study was to examine the bioactive potential of hydrolysate powders produced by enzymatic and microbial hydrolysis of whey proteins followed by spray drying, in order to reveal which one of these processes result in a product with significantly improved functional properties. Hydrolysate powders produced by the two different biotechnological processes were compared based on their antioxidant (DPPH and FTC), antibacterial as well as erythrocyte membrane stabilizing activities. The performed tests revealed that the concentration of at least 178.4 mg mL^{-1} of the whey protein hydrolysate powder, produced by tryptic digestion, could inhibit the process of lipid peroxidation by 50 %, suppress the microbial contamination caused by *S. aureus* ATCC25923, *B. cereus* ATCC 11778 and *L. monocytogenes*, and provide the antioxidant and membrane stabilizing activities greater than 50 %. On the other hand, the hydrolysate powder obtained by whey fermentation at the concentration of at least 811.5 mg mL^{-1} achieved 50 % of all tested bioactivities, with the emphasis on the significantly more pronounced antibacterial activity against all tested strains. In that sense, tryptic hydrolysis could be highlighted as an optimal process that provides production of the whey hydrolysate with pronounced bioactive properties that could be considered as a very promising natural food supplement.

Keywords: *whey; bioactivity; hydrolysis; fermentation; food supplement*

Available online at the Journal website: http://www.ache.org.rs/HI/

SCIENTIFIC PAPER

UDC 637.34:577.112

Hem. Ind. **72 (5)** 265–274 (2018)

1. INTRODUCTION

In recent years, awareness and knowledge about health implications relating to food consumption have increased. Today's consumers express a clear tendency towards a healthy lifestyle, by choosing healthy, safe and well designed innovative products [1]. Although the use of chemical ingredients included in food production and preservation represents a comfortable solution for the food industry, recognition of chemical or synthetic ingredients as potential hazards, has led to an increase in consumer interest in completely natural foods. Therefore, research of natural bioactive compounds as alternatives to synthetic ones, and development of new healthy products that respond to both, food industry expectations and consumer demands for the healthy lifestyle, in recent years became the main objective of food industry developments with great interest among researchers [2].

The oxidative stability of food products is the result of a delicate balance between the anti- and pro-oxidative processes. Oxidation of food constituents, as a key event in food spoilage, implies free radical-mediated reactions. Free radicals are responsible for the development of rancidity and reduction of food shelf-life [3]. Therefore, delay of these oxidative processes through the use of antioxidants, especially from natural sources, is of great interest in food industry. Functional ingredients, such as whey protein hydrolysates (WPH) seem to have vast potentials as effective antioxidants [4,5]. According to Vavrusova *et al.* [6], the inclusion of relatively high amounts of WPH in products has the potential to enhance the product stability by preventing radical formation.

In addition to the fact that treatment with antioxidants offers a valuable preservation technique that can prolong the food shelf life, application of natural antioxidants as dietary supplements attracts even larger attention due to therapeutic

Correspondence: Maja Lj. Bulatovic, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, Belgrade, Serbia
E-mail: mbulatovic@tmf.bg.ac.rs
Paper received: 21 November 2017
Paper accepted: 04 September 2018
https://doi.org/10.2298/HEMIND171121019E

properties of these substances. To date, several studies have shown that whey protein hydrolysates contain a broad range of antioxidants depending on the proteases used. In general, antioxidant whey proteins or constituents have been enzymatically hydrolyzed by commercial proteases such as pepsin and trypsin [7-12], chymotrypsin [10,12], thermolysin and corolase [11], papain, protease A, P, F and N [8,12], flavourzyme [8,11,13,14], alcalase [8,9,13,15], and protamex [8].

In addition to the enzymatic hydrolysis, peptides with antioxidative potentials can be produced during microbial fermentation in the presence of strains with high proteolytic activity. Attainment of the antioxidant activity is a strain-specific characteristic. Several studies have shown the successful use of *Lactobacillus* sp. as excellent producers of antioxidants [16-18]. Strains such as *Lb. plantarum*, *Lb. kefiri*, *Lb. gasseri*, *Lb. paracasei* [19], *Lb. lactis*, *Lb. helveticus*, *Lb. jensenii*, *Lb. reuteri*, *Lb. acidophilus* [20] have been marked also as potent antioxidant producers.

Whey protein hydrolysates (WPHs) may be used in a wide variety of applications as they provide a number of benefits compared to unhydrolyzed whey proteins. WPHs are more heat stable than whey protein concentrates (WPC) at certain concentrations, pH and temperatures [21,22]. Due to abundance of bioactive peptides, lower viscosity, improved foaming and emulsification properties [23], as well as enhanced absorption and digestibility [24,25], they can be added to a wide range of functional foods, to improve the food functionality, shelf life and texture [26]. Excellent nutritional and functional properties [27,28], provide the possibility to use WPHs in a wide range of products including acid beverages/fruit juices, high protein nutritional bars, tablets and supplements, pet food, sports nutritional products, convalescence foods, meal replacement products, weight management products, and functional chocolates. Bitter tasting WPHs may be used to replace coffee or chocolate flavorings in ready to drink beverages. Those with completely degraded whey protein allergens may be used in infant formulas [29,30]. However, there are a small number of papers that comparatively analyze bioactivities of whey-based substrates hydrolyzed using lactic acid bacteria and enzymes as well as multilevel bioactive properties of produced hydrolysates. The aim of this study was to examine the bioactive potential of hydrolysate powders produced by enzymatic and microbial hydrolysis of whey proteins followed by spray drying, in order to reveal which one of these processes results in a product with significantly improved functional properties.

2. MATERIALS AND METHODS

2. 1. Media, culture, enzyme and reagents

The sweet cow's whey powder (12.11 % (w/w) of proteins, Lenic Laboratories, Belgrade, Serbia), and whey protein concentrate powder (with 80.0 % (w/w) of proteins DMV International, 5462 GE Veghel, Netherlands), were used as sources of whey proteins. The sweet cow's whey powder (W) was reconstituted to contain 8 % (w/v) of dry matter, while whey protein concentrate powder (WPC) was reconstituted to contain 5 % (w/v) of dry matter. Both suspensions were allowed to hydrate for 1h at room temperature under gentle stirring and subsequently subjected to the following processes. The strain Lactobacillus rhamnosus ATCC 7469 was supplied by American Type Culture Collection (ATCC, Rockville, USA). Stock culture was stored at -18 °C in 3 mL vials containing De Man Rogosa Sharpe (MRS) broth (Fluka, USA) and 50 % (v/v) glycerol as a cryoprotective agent. To prepare the laboratory culture, a drop of the stock culture was transferred to 3 mL of MRS broth and incubated for 18 h under anaerobic conditions at 37 °C. The working culture was pre-cultured twice in MRS broth prior to experimental use. Trypsin (porcine pancreas, EC 3.4.21.4) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The chemicals used in this research were of analytical grade purchased from Sigma-Aldrich Chemie GmbH.

2. 2. Enzymatic hydrolysis

The enzymatic hydrolysis was carried out in a 500-ml mechanically stirred batch reactor with temperature and pH control, by the commercial enzyme trypsin. The substrate for enzymatic hydrolysis was 400 mL of 5 % (w/v) aqueous solution of WPC with 44.0 ± 1.363 mg mL^{-1} of protein content [25]. Before hydrolysis, the WPC solution was adjusted to pH 8 by addition of 1 M NaOH, then stirred and allowed to equilibrate to the working temperature for 15 min. The prepared WPC solution with 0.5 % of trypsin on a protein-equivalent basis was than incubated at 37 °C for 240 min. During the course of the hydrolysis, pH of the WPC solution was kept at pH 8 by continuous addition of 0.1 M NaOH, using the pH-stat method with contentious dosage of the base. After the hydrolysis, the enzyme was inactivated by heating the solution at 90 °C for 15 min, after which period the whey protein hydrolysate (WPH) was cooled to room temperature, centrifuged at 6000 rpm for 15 min and further subjected to a spray drying process.

2. 3. Fermentation

Fermentation was conducted in 500-ml Erlenmeyer flask containing 400 mL of the reconstituted whey (W). Samples were inoculated with 2 % (v/v) *Lb. rhamnosus* ATCC 7469 strain and incubated at 37 °C for 24 h. After the fermentation, the fermented whey hydrolysate (FWH) was cooled to room temperature, centrifuged at 6000 rpm for 15 min and further subjected to a spray drying process.

2. 4. Spray drying

The produced hydrolysate solutions (WPH, FWH) as well as corresponding liquid controls (WPC, W), were spray dried in order to produce powdered hydrolysate samples (WPH-P, FWH-P) and corresponding powdered controls (WPC-P, W-P). The spray drying process was performed using a laboratory scale Mini spray dryer B-290 (BUCHI, Labortechnik AG, Switzerland), in conjunction with a peristaltic pump. According to literature findings, the outlet temperature of the spray drying process should be maintained at a low level that is in the range 60-80 °C, in order to avoid denaturation of whey proteins [31] and provide preservation of peptide bioactivity [32]. Therefore, the inlet and outlet air temperatures were maintained at 120 and 70 °C, respectively. Batches of powdered samples were stored in a desiccator (at room temperature) before the use in following assays.

2. 5. Antioxidant activity

DPPH radical-scavenging test: In order to estimate the antioxidant activity of the obtained complex biological systems that contain a wide range of antioxidants with different chemical structure, DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity of the samples was estimated according to the protocol described by McCue and Shetty [33] with minor modifications. Briefly, spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) were dissolved in distilled water at varying concentrations (50, 100, 150, 200 and 250 mg mL^{-1}). An aliquot of each sample solution (250 μL) was added to 4 mL of 0.1 mM DPPH radical dissolved in ethanol as a solvent. Mixtures were vortexed for 1 min, placed on an orbital shaker, and incubated in the dark for 60 min at room temperature. After incubation, samples were centrifuged for 15 min at 6000 rpm at room temperature. Obtained supernatants were filtered using Whatman No 40 filter paper (Sigma-Aldrich Chemie GmbH, Germany). A control sample contained DPPH radical solution and deionised water, only. Absorbances of each supernatant and the control were measured at 517 nm against ethanol as a blank. DPPH radical scavenging activity, % was calculated according to the equation:

$$\text{DPPH radical scavenging activity, \%} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

where: A_{control} is the absorbance of the control without hydrolysate, and A_{sample} is the absorbance of the sample with hydrolysate. The antioxidant activity was expressed as the inhibitory concentration (IC_{50}, mg mL^{-1}) of the hydrolysate needed to scavenge 50 % of DPPH free radicals.

2. 6. Lipid peroxidation inhibitory activity

Ferric thiocyanate assay: Oxidation of oleic acid, a monounsaturated fatty acid that is the major constituent of triglycerides, as the model compound, and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), as a free radical initiator, was monitored using the ferric thiocyanate method (FTC) for up to 5 h, as described by Azuma *et al.* [34] with slight modifications. Briefly, spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) were dissolved in distilled water at varying concentrations (100, 250, 500, 750 and 1000 mg mL^{-1}). An aliquot of each sample solution (0.30 mL) was placed in a screw-cap vial and mixed with 1.3 % (w/v) oleic acid in methanol (1.40 mL), 0.2 M phosphate buffer (pH 7.0, 1.40 mL), and water (0.70 mL). The vial was incubated at 50.0 ± 0.1 °C in the dark and sampling was carried out every hour for up to 5 h until the absorbance of the control reached the value of 0.500 ± 0.030 at 500 nm. The degree of oxidation was measured in triplicate according to the ferric thiocyanate method [35]. The reaction mixture (0.10 mL) was diluted with 75 % aqueous (v/v) methanol (9.70 mL) and mixed with 20 mM $FeCl_2$ solution in 3.5 % (w/w) HCl (0.10 mL) and 10 % (w/w) aqueous NH_4SCN solution (0.10 mL). After precisely 3 min the absorbance was measured at 500 nm, versus 75 % methanol as a blank. The results are expressed as the percentage of lipid peroxidation inhibitory activity and calculated according to the equation:

$$\text{Lipid peroxidation inhibitory activity, \%} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (2)$$

where: A_{control} and A_{sample} were the absorbance values of the control and sample, respectively, at the time at which the control sample reached its maximum absorbance. The lipid peroxidation inhibitory activity was expressed as the inhibitory concentration (IC_{50}, mg mL^{-1}) of the hydrolysate needed to suppress 50 % of lipid peroxidation.

2. 7. Membrane stabilizing activity

Membrane stabilizing activity assay [36]: Briefly, spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) were dissolved in distilled water at varying concentrations (50, 250, 500, 1000 and

2000 μg mL^{-1}). An aliquot of each sample solution (2 mL) was added to 1 mL of 10 % human red blood cells (hRBCs) suspension. The control sample was prepared in the same manner using physiological saline solution instead of the test sample solution. Diclofenac sodium (250 μg mL^{-1}, Hemofarm A.D., Serbia) was used as a reference standard drug. Centrifuge tubes were incubated at 56 ºC for 30 minutes and subsequently cooled down to room temperature. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of supernatants was measured at 560 nm. The experiment was performed in triplicate. The percentage of membrane stabilizing activity was calculated according to the equation:

$$\text{Membrane stabilizing activity, \%} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (3)$$

where: $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the test sample. The membrane stabilizing activity was expressed as the inhibitory concentration (IC_{50}, μg mL^{-1}) of the hydrolysate needed to suppress 50 % of hRBC lysis.

2. 8. Antibacterial activity

Liquid challenge method: Quantitative tests of the antibacterial activity of spray dried hydrolysate samples (WPH-P, FWH-P) and corresponding controls (WPC-P, W-P) against three Gram-positive (G+) bacteria *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), and *Listeria monocytogenes*, as well as against the Gram-negative (G-) bacteria *Escherichia coli* (ATCC 25922), were performed according to the liquid challenge method. Overnight cultures (*S. aureus* and *E. coli*) were diluted (10^{-1}) in the physiological saline solution (NaCl, 0.85 % w/v). Then, prepared cultures were used to inoculate test tubes at 2 % (v/v) that contained 2.5 mL of sterile nutrient broth (NB) and 2.5 mL of spray dried hydrolysate solution at the concentration of 100 mg mL^{-1}. The initial number of bacteria in each suspension was between 10^5–10^6 colony forming units per milliliter (CFU mL^{-1}), while the final concentration of spray dried hydrolysate was approximately 50 mg mL^{-1}. Control samples were prepared by adding 2 % (v/v) bacterial inoculum into the tubes that contained mixture of 2.5 mL of sterile nutrient broth (NB) and 2.5 mL of sterile distilled water. Thus, prepared samples were incubated for 24 h at 37 ºC. The number of bacteria was assessed at start and after 24 h of incubation. Serial dilutions were prepared and 100 μL of appropriate suspensions were plated on nutrient agar. After 24 h incubation at 37 °C, the plates containing 25–250 colonies were enumerated using a colony counter and the viable cell count was expressed as log (CFU mL^{-1}).

2. 9. Statistical analysis

All absorbance measurements were performed using a UV-Vis spectrophotometer (Ultrospec 3300 pro, Amerscham Bioscience). The IC_{50} values were calculated by the formula $Y = 100 \times A1/(X + A1)$, where A1 = IC_{50}, Y = response (Y = 100 % when X = 0), X = inhibitory concentration (linear regression analysis, $p < 0.0001$ was considered significant) using OriginPro 8 software (Origin Lab Co., Northampton, USA). The experiments were performed in triplicate and values are expressed as a mean ± standard deviation. Mean values were analyzed using One-way ANOVA. The Tukey post hoc test was performed for means comparison using OriginPro 8 software (Origin Lab Co., Northampton, USA). Differences between samples were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3. 1. Antioxidant activity of hydrolysate powders

Free radical-mediated reactions have a significant role in many biological phenomena such as cellular damage and aging by stimulating oxidation of lipids and formation of secondary lipid peroxidation products. The antioxidant activity of produced hydrolysate powders was measured by DPPH method and the results are shown in Figure 1.

As shown in Figure 1, both hydrolysate powders (FWH-P and WPH-P) expressed significantly higher DPPH radical scavenging activities as compared to the corresponding controls (W-P and WPC-P). The WPH-P showed a significantly ($p < 0.05$) higher DPPH radical scavenging activity, corresponding to the IC_{50} value of 90.5 ± 1.4 mg mL^{-1}, compared to the FWH-P that expressed a DPPH radical scavenging activity corresponding to the IC_{50} value of 123.4 ± 4.2 mg mL^{-1}. The obtained results are slightly lower than those reported in literature [37] according to which the trypsin hydrolyzed WPC expressed 67.01 % of DPPH radical scavenging activity at the concentration of 10 mg mL^{-1}. The observed difference could be explained by the significantly higher hydrolysis degree (20.17 %) induced by a higher enzyme concentration (2 %), as compared to the obtained hydrolysis degree of 5.7 % [25] induced by the enzyme concentration of 0.5 % used for the production of tested WPH-P. However, during hydrolysis and fermentation of food proteins, commercial enzymes and enzymes produced by starter cultures cleave the proteins and produce peptides of different sizes. Thus, regardless the

proteolytic activity of lactic acid bacteria, it could be assumed that enzymatic hydrolysis probably releases much smaller peptides with higher antioxidant activities, which significantly improve the antioxidant activity of produced hydrolysate.

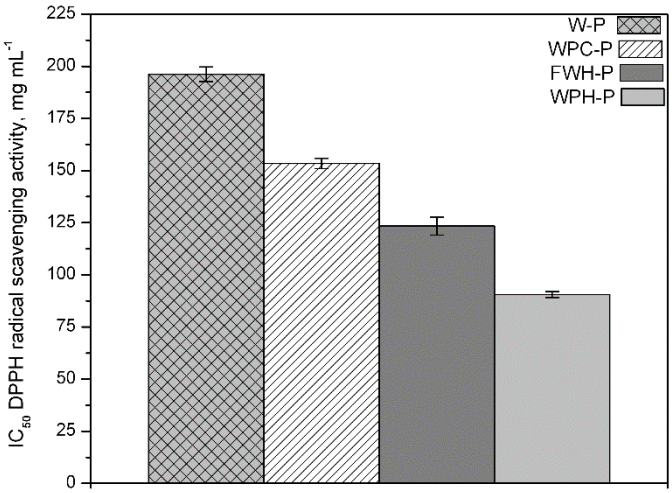

Figure 1. DPPH radical scavenging activity of whey (W-P), whey protein concentrate (WPC-P), fermented whey hydrolysate (FWH-P) and whey protein hydrolysate (WPH-P) powders, expressed as the inhibitory concentration (IC_{50}, mg mL^{-1}) needed to scavenge 50 % of the DPPH free radicals

As shown in Figure 1, both hydrolysate powders (FWH-P and WPH-P) expressed significantly higher DPPH radical scavenging activities as compared to the corresponding controls (W-P and WPC-P). The WPH-P showed a significantly ($p < 0.05$) higher DPPH radical scavenging activity, corresponding to the IC_{50} value of 90.5 ± 1.4 mg mL^{-1}, compared to the FWH-P that expressed a DPPH radical scavenging activity corresponding to the IC_{50} value of 123.4 ± 4.2 mg mL^{-1}. The obtained results are slightly lower than those reported in literature [37] according to which the trypsin hydrolyzed WPC expressed 67.01 % of DPPH radical scavenging activity at the concentration of 10 mg mL^{-1}. The observed difference could be explained by the significantly higher hydrolysis degree (20.17 %) induced by a higher enzyme concentration (2 %), as compared to the obtained hydrolysis degree of 5.7 % [25] induced by the enzyme concentration of 0.5 % used for the production of tested WPH-P. However, during hydrolysis and fermentation of food proteins, commercial enzymes and enzymes produced by starter cultures cleave the proteins and produce peptides of different sizes. Thus, regardless the proteolytic activity of lactic acid bacteria, it could be assumed that enzymatic hydrolysis probably releases much smaller peptides with higher antioxidant activities, which significantly improve the antioxidant activity of produced hydrolysate. This observation is supported by the fact that different proteolytic enzymes cleave proteins based on their specificity, and produce peptides with different sizes, sequences, and characteristics responsible for different functional properties [38]. In addition, the use of enzymatic hydrolysis to produce bioactive peptides is preferred than microbial fermentation due to the short reaction time, easier scalability and predictability [39].

3. 2. Inhibition of lipid peroxidation of hydrolysate powders

In the last years, special attention has been dedicated to searching for whey-derived peptides with lipid peroxidation inhibitory activities. The lipid peroxidation inhibitory activity of produced hydrolysate powders was measured by lipid peroxidation method and the results are shown in Figure 2.

By comparing the activities of hydrolysate powders (FWH-P and WPH-P) and corresponding controls (W-P and WPC-P) it can be noticed that fermentation significantly contributes to the lipid peroxidation inhibitory activity as compared to the process of whey protein hydrolysis. In addition, the WPH-P showed a significantly ($p < 0.05$) higher lipid peroxidation inhibitory activity corresponding to the IC_{50} value of 178.4 ± 12.4 mg mL^{-1}, as compared to that of the FWH-P that expressed a lipid peroxidation inhibitory activity corresponding to the IC_{50} value of 811.5 ± 13.7 mg mL^{-1}. By comparing the results of lipid peroxidation inhibitory activity with the results of DPPH radical scavenging activity (Figure 1), it could be assumed that the concentrations of lipid peroxidation inhibitors were substantially lower than the concentrations of hydrogen donating compounds, for both hydrolysate powders. The obtained results are in agreement with literature findings [37,40] that report similar relation of lipid peroxidation inhibitors and hydrogen donating compounds at higher degrees of hydrolysis (DH > 5 %) as well as longer times of fermentation (> 20h). However, oxidation of food constituents is a key event in food spoilage. It is well known that lipid peroxidation in food products can cause deterioration in food quality, shorten the shelf life and decrease the acceptability of processed foods. Lipid

oxidation can generate free radicals that can lead to fatty acid decomposition, which may reduce the nutritional value and safety of food by producing undesirable flavors and toxic substances [41]. Therefore, it is important to delay lipid oxidation and formation of free radicals in foods containing lipids and/or fatty acids [42]. Therefore, it could be assumed that WPH-P could be promising from a food technology perspective, since it can help to delay the peroxidation and subsequently prevent the food rancidity. On the other hand, many food allergens are stable and resistant to digestion by gastrointestinal enzymes or are processed into high molecular weight peptides which retain the Immunoglobulin E (IgE) binding and T-cell-stimulating properties as it is the case of a bovine whey protein β-lactoglobulin [43,44]. Therefore, although whey protein hydrolysates represent a promising source of bioactive peptides that can be used for production of improved functional food products, they need to be explored in more details regarding their antigenicity. Due to these facts, hydrolysate powders produced by procedures described in the present study cannot be used in food products intended for cow milk allergic persons.

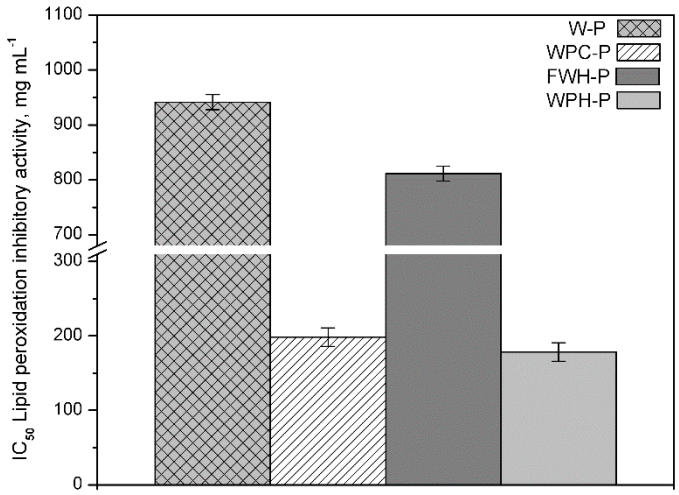

Figure 2. Lipid peroxidation inhibitory activity of whey (W-P), whey protein concentrate (WPC-P), fermented whey hydrolysate (FWH-P) and whey protein hydrolysate (WPH-P) powders, expressed as the inhibitory concentration (IC_{50}, mg mL^{-1}) needed to suppress 50 % of lipid peroxidation

3. 3. Membrane stabilizing activity of hydrolysate powders

Inflammation is the normal body response triggered by the release of chemical mediators from injured tissue and migrating cells. Stabilization of the lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils, such as enzymes and proteases, which cause further tissue inflammation and damage [45]. In-vitro membrane stabilizing activity of hydrolysate powders was screened using a membrane stabilizing model against hRBC and the obtained results are presented in Figure 3.

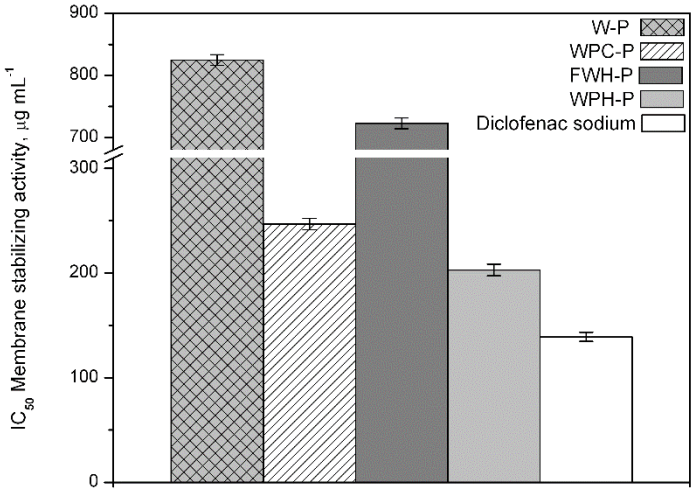

Figure 3. Membrane stabilizing activity of whey (W-P), whey protein concentrate (WPC-P), fermented whey hydrolysate (FWH-P) and whey protein hydrolysate (WPH-P) powders, expressed as the inhibitory concentration (IC_{50}, μg mL^{-1}) needed to suppress 50 % of hRBC lyses.

As shown in Figure 3, both hydrolysate powders (FWH-P and WPH-P) were effective in inhibiting the heat induced haemolysis of the erythrocyte membrane. In addition, hydrolysate powders expressed significantly higher membrane stabilizing activities as compared to those of corresponding controls (W-P and WPC-P). Membrane stabilizing activities of both hydrolysate powders were concentration dependent and comparable to the standard diclofenac sodium solution. The WPH-P powder showed a significantly ($p<0.05$) higher membrane stabilizing activity corresponding to the IC_{50} value of 202.9 ± 5.4 μg mL^{-1}, as compared to the FWH-P powder that expressed the membrane stabilizing activity corresponding to the IC_{50} value of 722.8 ± 8.6 μg mL^{-1}. The erythrocyte membrane is analogous to the lysosomal membrane [46] and its stabilization implies that the produced hydrolysate powders may also stabilize well the lysosomal membrane. Compared to diclofenac sodium, a standard anti-inflammation drug that showed 50 % membrane stabilizing activity at the concentration of 139.0 ± 4.2 μg mL^{-1}, it could be assumed that WPH-P powder exhibits a moderate membrane stabilizing activity. The obtained results are in agreement with previous studies [47,48], which reported that whey protein hydrolysates can inhibit inflammatory responses in respiratory and intestinal epithelial cells.

3. 4. Antibacterial activity of hydrolysate powders

Bacterial growth inhibition observed at the end of 24 h of incubation in the presence of WPH-P at the concentration of 50 mg mL^{-1} is shown in Table 1.

Table 1. Antibacterial activity of whey protein hydrolysate powder (WPH-P), tested at the concentration of 50 mg mL^{-1} against pathogenic bacteria

Sample after 24 h of incubation	Viable cell count - log (CFU, mL^{-1})			
	S. aureus	*B. cereus*	*E. coli*	*L. monocytogenes*
Control	8.301	6.301	8.342	8.342
WPH-P	7.361	5.301	8.361	7.342

The WPH-P powder has shown the growth inhibition of log (CFU mL^{-1}) = 1.000 for *B. cereus* and *L. monocytogenes* and 0.940 for *S. aureus*. Effects of the WPH-P powder on the Gram-negative *E. coli* were negligible. This finding is in line with previous research where WPH-P powder did not exhibit any antibacterial activity against *E. coli* [49]. Also, Pellegrini *et al.* [50] have shown that a tryptic digestion of the whey protein yielded four antibacterial peptides, which were inhibitory only to Gram-positive bacteria.

Bacterial growth inhibition observed at the end of 24 h of incubation in the presence of FWH-P at the concentration of 50 mg mL^{-1} is shown in Table 2.

Table 2. Antibacterial activity of fermented whey hydrolysate powder (FWH-P), tested at the concentration of 50 mg mL^{-1} against pathogenic bacteria

Sample after 24 h of incubation	Viable cell count - log (CFU, mL^{-1})			
	S. aureus	*B. cereus*	*E. coli*	*L. monocytogenes*
Control	8.204	7.204	8.653	7.255
FWH-P	5.477	3.477	4.301	6.204

As shown in Table 2, the antibacterial activity of FWH-P was higher than that of the WPH-P for all bacterial strains. The growth inhibition, expressed as log (CFU, mL^{-1}), was 2.727 for *S. aureus*, 3.727 for *B. cereus*, 4.342 for *E. coli*, and 1.051 for *L. monocytogenes*. Accordingly, the FWH-P has shown significantly ($p < 0.05$) higher growth inhibitions for *S. aureus*, *B. cereus*, and *E. coli* as compared to those of the WPH-P (Table 1). Several studies indicated that the lactic acid bacteria exhibit high antimicrobial activity as a result of their highly developed proteolytic system [51,52]. In addition, the inhibitory effects might due to proteins such as lactoperoxidase and lactoferrin as minor but very important whey constituents [53]. The observed findings could be also due to low pH of the fermented whey that resulted from lactic acid produced particularly by lactic acid bacteria. Based on literature findings [54,55], the *Lactobacillus* species could inhibit growth of various Gram positive and Gram negative bacteria through the production of hydrogen peroxide, bacteriocins and organic acids such as lactic and acetic acids. These substances inhibit growth of pathogenic bacteria and also improve the commensal balance between some gut flora and the human organism [56]. Also, Sagong *et al.* [57] revealed the effectiveness of lactic acid in reduction of *E. coli* and *L. monocytogenes*. Due to the low pH value of FWH, it can be adequately used as a supplement in products such as fermented beverages that are characterized by low pH.

4. CONCLUSION

Based on the results presented for different bioactivities of whey protein hydrolysate powder produced by tryptic digestion, it can be highlighted that addition of at least 178.4 mg mL^{-1} of this hydrolysate could inhibit the process of lipid peroxidation by 50 % as well as suppress the microbial contamination caused by *S. aureus* ATCC 25923, *B. cereus* ATCC 11778 and *L. monocytogenes*. In addition, the proposed concentration will surely provide antioxidant and membrane stabilizing activity greater than 50 %. On the other hand, the hydrolysate powder obtained by whey fermentation should be added in the concentration of at least 811.5 mg mL^{-1} to achieve all of tested bioactivities, while inducing a significantly more pronounced antibacterial activity against all tested strains. Therefore, that the obtained results indicate the enzymatic hydrolysis as an optimal process for production of a bioactive hydrolysate powder that could be considered as a very promising natural food supplement.

Acknowledgement: *This work was supported by the Serbian Ministry of Education, Science and Technological Development (TR 31017).*

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SAŽETAK

Uporedna analiza funkcionalnosti sprej sušenih hidrolizata proteina surutke proizvedenih enzimskom i mikrobiološkom hidrolizom

Salem M. Embiriekah[1], Maja Lj. Bulatović[1], Marija Lj. Gnjatović[2], Maja S. Vukašinović-Sekulić[1], Tanja Ž. Krunić[3], Danica B. Zarić[4], Marica B. Rakin[1]

[1]Tehnološko-metalurški fakultet, Univerzitet u Beogradu , Karnegijeva 4, Beograd, Srbija; [2]Institut za primenu nuklearne energije, Univerzitet u Beogradu, Banatska 31B, Beograd, Srbija
[3]Inovacioni centar Tehnološko-metalurškog fakulteta, Univerzitet u Beogradu, Karnegijeva 4, Beograd, Srbija
[4]IHIS Tehno eksperts, Istraživačko razvojni centar, Batajnički drum 23, Beograd, Srbija

(Naučni rad)

Cilj ovog istraživanja je bio ispitivanje bioaktivnog potencijala sprej sušenih hidrolizata proizvedenih enzimskom i mikrobiološkom hidrolizom proteina surutke praćenom sprej sušenjem, kako bi se došlo do saznanja koji od ovih procesa daje proizvod unapređenih funkcionalnih svojstava. Sprej sušeni hidrolizati proizvedeni navedenim različitim biotehnološkim procesima poređeni su na osnovu njihove antioksidativne, (DPPH i FTC) i antibakterijske aktivnosti, kao i aktivnosti stabilizacije membrane eritrocita. Dobijeni rezultati su pokazali da dodavanje najmanje 178,4 mg mL^{-1} sprej sušenog hidrolizata proteina surutke, proizvedenog hidrolizom pomoću tripsina, može inhibirati proces lipidne peroksidacije za 50 %, suzbiti razvoj mikrobiološke kontaminacije uzrokovane sojevima *S. aureus* ATCC 25923, *B. cereus* ATCC 11778 i *L. monocytogenes*, i obezbediti antioksidativnu aktivnost i aktivnost stabilizacije membrane eritrocita veće od 50 %. Sa druge strane, sprej sušeni hidrolizat dobijen fermentacijom surutke je potrebno dodati u koncentraciji od najmanje 811,5 mg mL^{-1} da bi se postiglo 50 % svih testiranih bioaktivnosti, sa naglaskom na znatno izraženiju antibakterijsku aktivnost prema svim testiranim sojevima. U tom smislu, hidroliza tripsinom može biti smatrana optimalanim procesom koji obezbeđuje proizvodnju hidrolizata surutke izraženih bioaktivnih svojstava, koji je prihvatljiv kao unapređen prirodni prehrambeni dodatak.

Ključne reči: surutka; bioaktivnost; hidroliza; fermentacija; dodaci hrani