Comparative analysis of the degree of hydrolysis and antioxidant activity of milk and whey hydrolysates

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Abstract. The degree of hydrolysis and antioxidant activity of protein hydrolysates from fresh cow's milk and whey obtained by the action of the proteolytic enzymes papain, bromelain and chymosin were compared. The lowest degree of hydrolysis in fresh milk hydrolysates was reported for sample MP1 (10 min reaction time, treatment with 0.1 mg/ml papain), and the highest percentage was obtained at hydrolysate MB12 (at 60 min reaction time, treatment with 1.0 mg/ml bromelain). For the whey samples in sample WC1 (10 min reaction time, treatment with 1.0 µl/ml chymosin), the percentage of hydrolysis was the lowest. The highest percentage was achieved at WP12 hydrolysate using papain at a concentration of 1 mg/ml and a 60-min reaction time. The obtained values for the antioxidant capacity of the hydrolysed products show a higher activity compared to the starting substrates. The highest activity in the milk hydrolysates of 11.32 mg TE/100 ml was found in variant MB3, and in the whey hydrolysates of 7.83 mg TE/100 ml - in variant WP7. Hydrolysates treated with chymosin had lower TE values compared to the hydrolysate's variants, treated with papain and bromelain.

1 Introduction

In recent years it has been observed ever increasing interest to natural products, healthy foods, as well as to the useful substances they contain. Particular attention is paid to bioactive peptides derived from food proteins. Bioactive peptides are defined as specific protein fragments, with a positive effect on the physiological and metabolic functions of the body and a beneficial impact on human health [1, 2, 3, 4].

Through the process of hydrolysis (chemical, enzymatic or microbial), peptide bonds in proteins are broken down, releasing peptides of different sizes and free amino acids [5, 6, 7].

The sources of protein can be of plant and animal origin, and the generated peptides are defined as bioactive peptides because, in addition to high nutritional value, they also exhibit biological activity. The most common foods containing bioactive peptides are eggs, meat, fish, cereals and soy, but the best precursors of bioactive peptides remain milk and milk products [8, 9, 10].

In the primary structure of protein molecules, peptides are characterized by latent biological activity. Their overall activity is manifested at proteolytic enzyme treatment or protein hydrolysis process, i.e. degradation, release and activation of encoded bioactive peptides from the original protein [11, 12].

Enzymatic hydrolysis takes place under gentle conditions - pH (6 - 8) and temperature (40 - 60°C), and thus side reactions are minimized. Another advantage of enzyme hydrolysis is that the nature and extent of treatment can be controlled due to the inherent specificity of the different proteases. The process of releasing peptides from casein and whey proteins occurs in three ways: through digestive enzymes, through the fermentation process or through the action of proteolytic enzymes, obtained from microorganisms or plants [13, 14, 15, 16].

A number of scientists prove, that milk hydrolysates exhibit a wide range of biological activities, including antimicrobial, antihypertensive, antioxidant, anticytotoxic, immunomodulatory, opioid, etc. [18, 19, 20].

Dairy products are one of the most interesting and promising foods in terms of their antioxidant potential. Dairy products and their fractions (milk, whey, casein and lactoferrin) have been found to exhibit weak antioxidant properties. Hydrolysates obtained after hydrolysis of milk proteins exhibit higher antioxidant activity. During the hydrolysis process, the tertiary structure of the protein is disrupted and solvent accessibility to the released amino acids increases, leading to an increase in its overall antioxidant activity. The active amino acid sequences released by the enzymes exhibit antioxidant activity. Bioactive peptides with antioxidant properties are also released during hydrolysis.

Milk proteins exert various antioxidant effects. They trap ROS (reactive oxygen species), chelate metals, modulate enzymes, involved in the production and elimination of ROS. Peptides composed of tryptophan, tyrosine, histidine, proline are powerful antioxidants. Hydrophobic amino acids (leucine or valine), present in

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the peptide structure, influence the antioxidant activity of the peptides and their ability to inhibit lipid peroxidation. It has been found that peptides derived from αs-casein can reduce free radicals and inhibit enzymatic and non-enzymatic lipid peroxidation [21, 22, 23, 24, 25].

Mann et al. [26] prove antioxidant activity of flavored milk, enriched with whey protein hydrolysate. Whey protein concentrate is hydrolysed using proteolytic enzymes. The antioxidant activity of all hydrolysates is higher than the output whey protein.

Many epidemiological studies have reported, that frequent consumption of foods, containing natural antioxidants can reduce the incidence of certain types of cancer, hypertension, diabetes and cardiovascular diseases [27, 28, 29].

The aim of the present study is to compare the degree of hydrolysis of the hydrolysates obtained from cow's milk and whey, through the action of three proteolytic enzymes - papain, bromelain and chymosin. The plant enzymes papain and bromelain are preferred over enzymes of microbial origin mainly due to safety, lack of pathogenicity and other adverse effects [30, 31]. The third enzyme, that will be used for hydrolysis of the starting protein substrates is chymosin - a proteolytic enzyme secreted in the stomach of small ruminant mammals. It is the main enzyme in milk curdling [32].

The other main objective is to compare the antioxidant effect of the starting products and the resulting protein hydrolysates from fresh cow's milk and whey, obtained by the action of the three proteolytic enzymes.

2 Materials and methods

2.1 Materials

Protein substrates - fresh milk and whey provided by the Experimental Base of Institute of Mountain Animal Husbandry and Agriculture – Troyan

Proteolytic enzymes - papain (Papain, Technical Powder) from MP Biomedicals, LLC, cat. No. 102566; bromelain (Bromelain from pineapple steam) from SERVA Electrophoresis GmbH, cat. #15250 and chymosin (CHY-MAX EXSTRA chymosin yeast) from Admix LTD, lot 3478838.

2.2 Technological experiments

Enzymatic hydrolysis. The starting substrates fresh milk and whey are hydrolysed with three different proteolytic enzymes - papain, bromelain and chymosin. Each of the enzymes was dissolved in 0.1 M Universal buffer with the corresponding pH. The following enzyme concentrations are added to 100 ml of milk/whey: papain and bromelain 0.1, 0.5 and 1.0 mg/ml, and chymosin 1, 5 and 10 µl/ml. The hydrolysis process was carried out in a water bath at a temperature of 45°C and in four-time intervals: 10, 20, 30 and 60 min. The process was terminated by heating to 85°C for 5 min, after which the samples were rapidly cooled. The following variants of milk and whey hydrolysates are obtained:

1) *Milk hydrolysates when using a papain concentration of 0.1 mg/ml:* MP1 (reaction time 10 min), MP2 (reaction time 20 min), MP3 (reaction time 30 min), MP4 (reaction time 60 min);

2) *Milk hydrolysates when using a papain concentration of 0.5 mg/ml:* MP5 (reaction time 10 min), MP6 (reaction time 20 min), MP7 (reaction time 30 min), MP8 (reaction time 60 min);

3) *Milk hydrolysates when using a papain concentration of 1.0 mg/ml:* MP9 (reaction time 10 min), MP10 (reaction time 20 min), MP11 (reaction time 30 min), MP12 (reaction time 60 min);

4) *Milk hydrolysates when using bromelain concentration 0.1 mg/ml:* MB1 (reaction time 10 min), MB2 (reaction time 20 min), MB3 (reaction time 30 min), MB4 (reaction time 60 min);

5) *Milk hydrolysates when using bromelain concentration 0.5 mg/ml:* MB5 (reaction time 10 min.), MB6 (reaction time 20 min), MB7 (reaction time 30 min), MB8 (reaction time 60 min);

6) *Milk hydrolysates when using a bromelain concentration of 1.0 mg/ml:* MB9 (reaction time 10 min), MB10 (reaction time 20 min), MB11 (reaction time 30 min), MB12 (reaction time 60 min);

7) *Milk hydrolysates when using a chymosin concentration of 1 µl/ml:* MC1 (reaction time 10 min), MC2 (reaction time 20 min), MC3 (reaction time 30 min), MC4 (reaction time 60 min);

8) *Milk hydrolysates when using a chymosin concentration of 5 µl/ml:* MC5 (reaction time 10 min), MC6 (reaction time 20 min), MC7 (reaction time 30 min), MC8 (reaction time 60 min);

9) *Milk hydrolysates when using a chymosin concentration of 10 µl/ml:* MX9 (reaction time 10 min), MX10 (reaction time 20 min), MX11 (reaction time 30 min), MX12 (reaction time 60 min);

10) *Whey hydrolysates when using a papain concentration of 0.1 mg/ml:* WP1 (reaction time 10 min), WP2 (reaction time 20 min), WP3 (reaction time 30 min), WP4 (reaction time 60 min);

11) *Whey hydrolysates when using a papain concentration of 0.5 mg/ml:* WP5 (reaction time 10 min), WP6 (reaction time 20 min), WP7 (reaction time 30 min), WP8 (reaction time 60 min);

12) *Whey hydrolysates when using a papain concentration of 1.0 mg/ml:* WP9 (reaction time 10 min), WP10 (reaction time 20 min), SP11 (reaction time 30 min), SP12 (reaction time 60 min);

13) *Whey hydrolysates when using a bromelain concentration of 0.1 mg/ml:* WB1 (reaction time 10 min), WB2 (reaction time 20 min), WB3 (reaction time 30 min), WB4 (reaction time 60 min);

14) *Whey hydrolysates when using a bromelain concentration of 0.5 mg/ml:* WB5 (reaction time 10 min), WB6 (reaction time 20 min), WB7 (reaction time 30 min.), WB8 (reaction time 60 min);

15) *Whey hydrolysates when using bromelain concentration 1.0 mg/ml:* WB9 (reaction time 10 min),

WB10 (reaction time 20 min), WB11 (reaction time 30 min), SB12 (reaction time 60 min);

16) *Whey hydrolysates when using a chymosin concentration of 1 µl/ml:* WC1 (reaction time 10 min), WC2 (reaction time 20 min), WC3 (reaction time 30 min), WC4 (reaction time 60 min);

17) *Whey hydrolysates when using a chymosin concentration of 5 µl/ml:* WC5 (reaction time 10 min), WC6 (reaction time 20 min), WC7 (reaction time 30 min), WC8 (reaction time 60 min);

18) *Whey hydrolysates when using a chymosin concentration of 10 µl/ml:* WC9 (reaction time 10 min), WC10 (reaction time 20 min), WC11 (reaction time 30 min), WC12 (reaction time 60 min).

2.3 Biochemical studies

Degree of hydrolysis of the obtained hydrolysates - determined by a modified Adler-Nissen method using ninhydrin reagent [33]. The method is based on the reaction of amino acids with ninhydrin hydrate ($C_9H_6O_4{\times}H_2O$) at pH 5, temperature 100°C and a certain period. The obtained hydrolysates are centrifuged under the following conditions: time interval of 10 min, temperature 8°C and revolutions 4000 rpm. 0.4 ml of ninhydrin solution was added to 2 ml of the supernatant. The samples are heated to a temperature of 100°C for 10 min and cooled. Their absorption is measured spectrophotometrically at a wavelength of λ= 570 nm against a blank sample.

Antioxidant activity - the antioxidant effect of the starting products - fresh cow's milk and whey and of their hydrolysates was measured by the Brand-Williams method [34]. It is one of the most commonly used methods for screening antioxidant activity of foods. The method is based on the analysis of radical scavenging activity with DPPH (1,1-diphenyl-2-picrylhydrazyl) against a Trolox (6-hydroxy-2,5,7,8-tetramethylchro man-2-carboxylic acid) standard. For this purpose, a solution of DPPH radical (0.004%, w/v, Sigma Aldrich) in 95% ethanol was prepared. A volume of 2 ml DPPH in ethanol was added to 2 ml samples, mixed well and incubated for 30 min in a dark room at room temperature (30°C ± 0.5). The absorbance of each sample was measured at 517 nm using a UV-visible spectrophotometer (Biochrom Libra S22, Biochrom UV/Vis, USA). Ethanol was used as a blank sample, while DPPH solution in ethanol was used as a control. Trolox (Acros, USA) with a concentration of 0.045 to 1.5 mmol was used in the standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) curve to quantitative determination of antioxidant activity. Determination of the antioxidant effect was performed in triplicate. Antioxidant activity was expressed in mg TE/100 ml.

2.4 Statistical analysis

The results were processed using the MS Office Excel 2016 program product.

3 Results and discussion

3.1 Determination of degree of hydrolysis of the obtained hydrolysates

The hydrolysis of fresh cow's milk and whey was carried out by the indicated proteases at 3 different concentrations, resulting in different hydrolysates are obtained. The changes, that occur in the degree of hydrolysis during the process, are determined for each separated variant. The obtained values of the degree of hydrolysis (DH) for the two substrates studied are presented in Fig. 1 - 6.

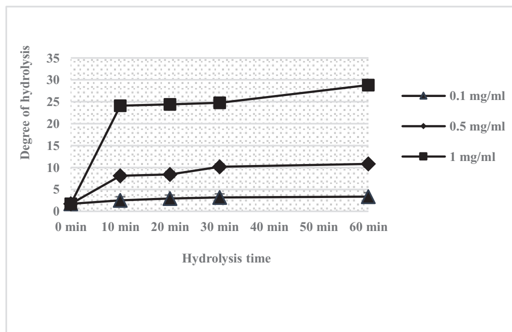

Fig. 1. Degree of hydrolysis of milk hydrolysates treated with papain enzyme (n=3)

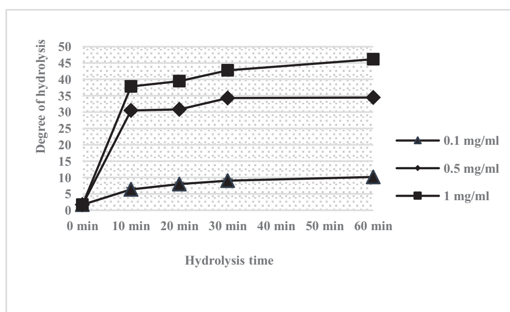

Fig. 2. Degree of hydrolysis of milk hydrolysates treated with bromelain enzyme (n=3)

The lowest degree of hydrolysis of the milk hydrolysates was reported in sample MP1, when treated with papain (2.52%), and the highest percentage was obtained in variant MB12, when treated with bromelain at a high concentration of 1.0 mg/ml (46.17 %). For the whey samples, the lowest percentage of hydrolysis was found for variant WC1 - 13.18%. The highest percentage was achieved with WP 12 hydrolysate using papain at 1 mg/ml (75.62%).

The comparative analysis of the obtained results shows, that the hydrolysis percentage depends to a greater extent on the concentration of the enzyme used and to a lesser extent on the treatment time factor.

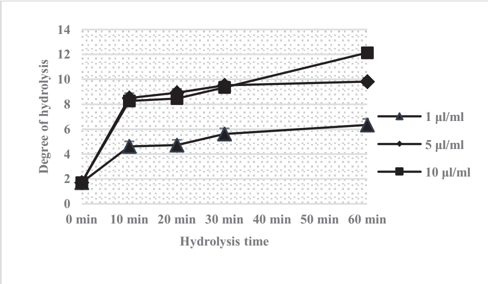

Fig. 3. Degree of hydrolysis of milk hydrolysates treated with chymosin enzyme (n=3)

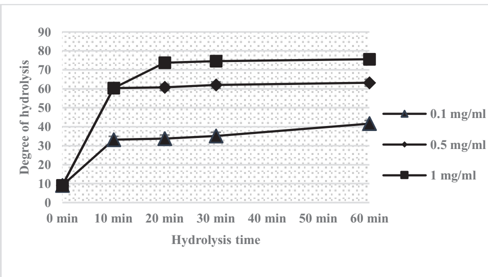

Fig. 4. Degree of hydrolysis of whey hydrolysates treated with papain enzyme (n=3)

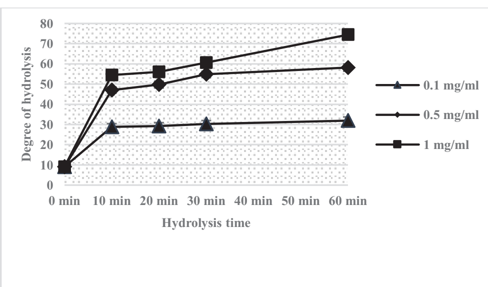

Fig. 5. Degree of hydrolysis of whey hydrolysates treated with bromelain enzyme (n=3)

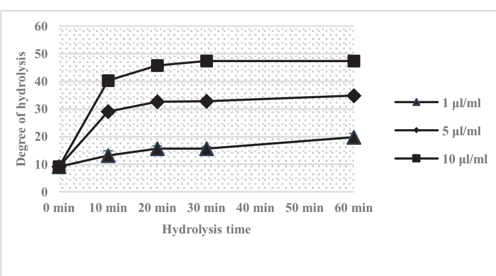

Fig. 6. Degree of hydrolysis of whey hydrolysates treated with chymosin enzyme (n=3)

All lines increased rapidly during the first 10 min and the rate of hydrolysis was highest for all three proteases in this time interval. For both fresh milk and whey samples, a rapid increase in hydrolysis percentage was reported in the initial 10 min, and with increasing processing time, the values remained stable. The rate of hydrolysis decreases, due to a reduced number of peptide bonds, and as a result proteases and their substrates reach a saturation state. The obtained results are comparable to those of other authors [35; 36].

Hydrolysates obtained from cow's milk, after treatment with the enzyme bromelain, showed higher DH values, compared to those treated with papain and chymosin. The degree of hydrolysis of the whey hydrolysates obtained after treatment with bromelain is similar to the variants treated with papain – 75.62% (WP12), compared to 74.45% obtained with bromelain (WB12). The lowest ability to hydrolyze milk and whey samples was reported for the enzyme chymosin.

DH values differ, when treated with different enzymes, which is due to the difference in substrate specificity and in the catalytic sites of action [37]. The lower degree of hydrolysis of the hydrolysates obtained with chymosin can also be explained by the fact, that the enzyme has a lower starting activity.

Another factor, influencing the amount of enzyme hydrolysates, is the concentration of protein in the starting substrate and its ratio relative to the hydrolyzing enzyme. In advance research conducted on the substrates we used, was found 3.48% protein in fresh milk and 0.84% in whey. The probable reason for the higher DH values in whey, compared to those obtained with the same parameters from fresh milk is the different amount of protein substrate.

3.2 Evaluation of the antioxidant activity of milk and whey hydrolysates

A comparative analysis of the antioxidant effect of the starting products and the obtained protein hydrolysates was performed. Results are presented as Trolox equivalents antioxidant capacity (TE) mg /100 ml product in Tables 7-12.

The obtained values for antioxidant capacity of the hydrolysed products from fresh milk show a higher activity, compared to the starting substrate. The highest activity of 11.32 mg TE/100 ml was found in variant MB3 (milk hydrolysed with bromelain with a concentration of 0.1 mg/ml for 30 minutes). In the variants treated with concentrations of 0.5 mg/ml and 1 mg/ml of this enzyme, the highest antioxidant activities (9.36 mg TE/100 ml and 7.31 mg TE/100 ml, respectively) were found in the samples MB7 and MB11, which show their antioxidant capacity also at the 30th minute. The TE value decreases in the variants treated with the higher concentrations of bromelain (0.5 mg/ml and 1 mg/ml), and this is particularly well seen in the experimental samples hydrolyzed with this enzyme with a concentration of 1 mg/ml.

The variants hydrolyzed with the papain enzyme followed the same trend to manifest most strongly their antioxidant capacity at the 30th minute from the start of hydrolysis. Here, the greatest increase in TE values was reported in the variants treated with 0.5 mg/ml papain.

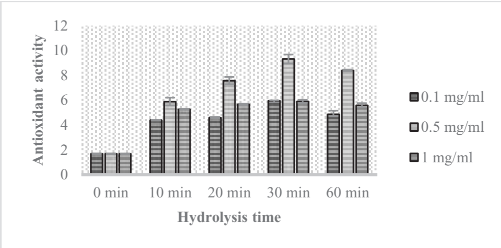

Fig. 7. Antioxidant activity of milk hydrolysates treated with papain enzyme (n=3)

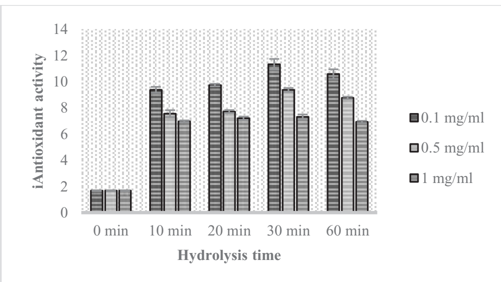

Fig. 8. Antioxidant activity of milk hydrolysates treated with bromelain enzyme (n=3)

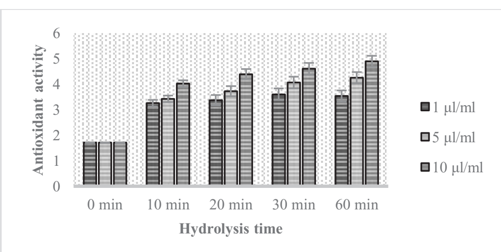

Fig. 9. Antioxidant activity of milk hydrolysates treated with chymosin enzyme (n=3)

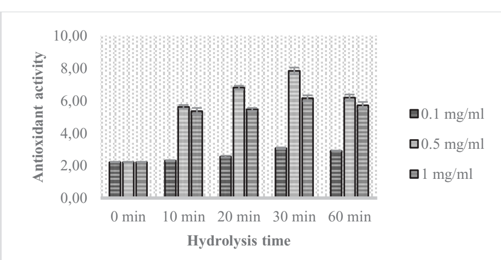

Fig. 10. Antioxidant activity of whey hydrolysates treated with papain enzyme (n=3)

The maximum manifested antioxidant activity in the samples treated with the enzyme chymosin, was recorded at the 60th min from the start of hydrolysis (for MC12, treated with an enzyme concentration of 10 µl/ml – 4.90 mg TE/100 ml). For the samples treated with an enzyme concentration of 5 µl/ml, a similar trend was observed in variant MC8, where antioxidant activity was recorded at the 60th min from the start of hydrolysis. A lower activity is reported in the chymosin hydrolysates, compared to the variants treated with papain and bromelain.

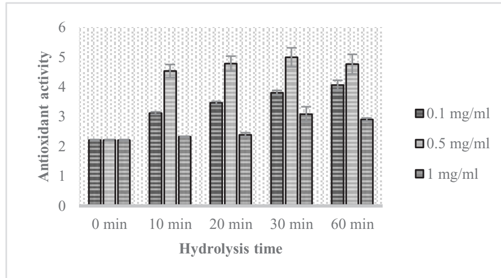

Fig. 11. Antioxidant activity of whey hydrolysates treated with bromelain enzyme (n=3)

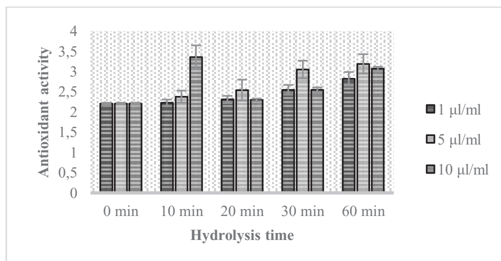

Fig. 12. Antioxidant activity of whey hydrolysates treated with chymosin enzyme (n=3)

Too greater antioxidant activity is reported in hydrolyzed whey products, compared to the starting substrate. The highest antioxidant activity 7.83 mg TE/100 ml has variant WP7. In the variants hydrolyzed with papain and bromelain, an increase in the antioxidant index was observed until the 30th minute of hydrolysis and then the level decreased slightly. The only exception is seen in the experimental samples treated with 0.1 mg/ml bromelain, where the maximum value of the antioxidant effect has sample WB4.

Here, in the whey hydrolysates, after treatment with the chymosin enzyme, the same trend can be noted, as in the milk hydrolysates, treated with chymozyme - lower TE values compared to the other variants of the whey hydrolysates treated with papain and bromelain.

The data from the experiments conducted on the antioxidant activity of milk and whey hydrolysates show, that the level of TE at 60 min of treatment with papain and bromelain decreases, as a result of the complete degradation of protein components in milk to amino acids, that do not have such strong expressed antioxidant properties, like bioactive peptides.

All three enzymes, used for the hydrolysis process are endopeptidases, but the mechanism of cleavage of peptide bonds is different for each of them. As a result, peptides of different size and quantity with antioxidant activity are obtained.

Based on the obtained results for antioxidant capacity, variant hydrolysates can be selected to be experimented as fermentation media with selected strains of lactic acid bacteria to obtain new biologically active dairy products. For fresh milk substrate, these are variants MB3, MB7 and MB11 - hydrolysed with the enzyme bromelain. For whey substrate, the fermentation variants are hydrolysates WP3, WP7 and WP 11, prepared with papain.

5 Conclusion

As a result of the conducted experiments, several conclusions can be drawn. When determining the degree of hydrolysis of the resulting hydrolysates obtained from fresh cow's milk, the highest percentage was hydrolysate MB12 (46.17%), treated with bromelain, and the lowest percentage - hydrolysate MP1 (2.52%), treated with papain. Hydrolysates obtained from cow's milk, after treatment with the enzyme bromelain, showed higher DH values, compared to those treated with papain and chymosin.

When determining the degree of hydrolysis of the whey hydrolysates, with the highest value was WP12 hydrolysate (75.62%) using papain at 1mg/ml. Hydrolysate WC1 (with chymosin treatment) has the lowest percentage - 13.18%. The degree of hydrolysis of the whey hydrolysates obtained after treatment with bromelain is similar to the variants treated with papain – 75.62% (WP12), compared to 74.45% obtained with bromelain (WB12).

The degree of hydrolysis of whey hydrolysates ranged from 13.18 to 75.62% and was higher than, that reported for fresh cow's milk hydrolysates, which ranged from 2.52 to 46.17%. Furthermore, the percent of hydrolysis depends to a greater extent on the concentration of the enzyme used and to a lesser extent on the factor of treatment time.

The obtained values for antioxidant capacity of the hydrolysed products from fresh milk and whey show a higher activity compared to the starting substrates. The highest activity of 11.32 mg TE/100 ml was found in variant MB3 (with bromelain with a concentration of 0.1 mg/ml). The TE value decreases in the variants treated with the higher concentrations of bromelain, and this is particularly well seen in the experimental samples, hydrolysed with the enzyme with a concentration of 1 mg/ml.

In the whey hydrolysates, the highest antioxidant activity of 7.83 mg TE/100 ml was accounted for variant WP7 (with 0.5 mg/ml papain). In the variants hydrolysed with papain and bromelain, an increase in the antioxidant index was observed until the 30th minute of hydrolysis and then the level decreased slightly. An exception is the hydrolysates treated with 0.1 mg/ml bromelain, where the maximum value of the antioxidant effect is observed in sample WB4.

The data from the experiments conducted on the antioxidant activity of milk and whey hydrolysates show that the level of TE at 60 minutes of treatment with papain and bromelain decreases, as a result of the complete degradation of protein components in milk to amino acids that do not have such strongly expressed antioxidant properties, like bioactive peptides.

Based on the obtained results for the antioxidant effect of the studied models, the hydrolysates with the greatest antioxidant activity - those based on fresh cow's milk MB3, MB7 and MB11, hydrolysed with the enzyme bromelain and whey hydrolysates WP3, WP7 and WP11, obtained with papain, would could be used as fermentation media with selected strains of lactic acid bacteria, for bio fermentative experiments to obtain new biologically active dairy products.

References

1. Tsv. Tsvetkov, I. Nacheva, L. Georgieva, Food Proc. Ind. Magaz., **01**, 28 (2006) [In Bulgarian].
2. J. Tondt, H. E. Bays, Obesity Pillars. **2**, 100017 (2022)
3. P. Nicolás, M. L. Ferreira, V. Lassalle, J. Food Eng. **246**, 7 (2019)
4. D. J. Daroit, A. Brandelli, Curr. Opin. Food Sci. **39**, 120 (2021)
5. A. S. Sandberg, In: *Functional Foods* (Woodhead Publishing, Sawston, 2011)
6. M. Memarpoor-Yazdi, H. Mahaki, H. Zare-Zardini, J. Funct. Foods. **5**, 62 (2013)
7. G. Krasteva, E. Lakova, Sci. Diet. **1**, 21 (2009) [In Bulgarian].
8. Y. Hou, Z. Wu, Z. Dai, G. Wang, G. Wu, J. An. Sci. Biotech. **8**, 24 (2017)
9. Zh. K. Atanasova, *Study of the probiotic capacity of lactic acid bacteria included in the composition of starter cultures for different types of cheese*, (PhD Thesis, Department of General and Industrial Microbiology, Faculty of Biology, Sofia University "St. K. Ohridski", 2015) [In Bulgarian]
10. H. Fan, H. Liu, Y. Zhang, Sh. Zhang, T. Liu, D. Wang, J. Future Foods. **2**, 143 (2022)
11. I. Ricci-Cabello, M. Olalla, R. Artacho, Nutr. Rev. **70**, 241 (2012)
12. A. Pihlanto, T. Virtanen, H. Korhonen, Int. Dairy J. **10**, 3 (2010)
13. G. K. Pal, P. V. Suresh, Int. J. Biol. Macromol. **101**, 304 (2017)
14. L. Egger, O. Ménard, Curr. Opin. Food Sci. **14**, 116 (2017)
15. H. Korhonen, A. Pihlanto, Curr. Pharm. Des. **9**, 1297 (2003)
16. H. Korhonen, A. Pihlanto, Bioactive Peptides from Food Proteins. In: Y. H. Hui ed, *Handbook of Food Products Manufacturing* (John Wiley & Sons, Inc., New York 2007)
17. Y. W. Park, M. S. Nam, Korean J. Food Sci. Anim. Res., **35** (6), 831 (2015)
18. J. Giacometti, A. Buretić-Tomljanović, Food Chem., **230**, 91-98 (2017)
19. A. Dullius, M. I. Goettert, C. F. V. de Souza, J. of Funct. Foods, **42**, 58-74 (2018)
20. I. S. Magalhães, A. D. B. Guimarães, A. A. L. Tribst, E. Basílio de Oliveira, B. R. de C. Leite Júnior, F. Research Int., **157**, 111310 (2022)
21. N. Estévez, C. Fuciños, A. Rodríguez-Sanz, M. L. Rúa, Food Chem., **394**, 133459 (2022)

22. M. Bielecka, G. Cichosz, H. Czeczot, Int. Dairy J., **127**, 105208 (2022)
23. S. G. Rival, C. G. Boeriu, H. J. Wichers, J. Agr. Food Chem., **4**, 295-302 (2001)
24. M. Bottani, S. Cattaneo, V. Pica, M. Stuknytė, I. De Noni, A. Ferraretto, J. Funct. Foods, **64**, 103673 (2020)
25. O. Power, P. Jakeman, R. J. FitzGerald, Amino Acids, **44**, 797-820 (2013)
26. B.Mann, A. Kumari, R. Kumar, R. Sharma, K. Prajapati, S. Mahboob, S. Athira, J. of Food Sci. Tech., **52** (6), 3235-3241 (2015)
27. A. M. Rychter, S. Hryhorowicz, R. Słomski, A. D. I. Krela-Kaźmierczak, Clin. Nutr., **41**, 1557-1565 (2022)
28. A. P. Azevedo de Carvalho, C. A. Conte-Junior, Trends Food Sci. Technol., **111**, 534-548 (2021)
29. R. Lv, Y. Dong, Zh. Bao, S. Zhang, S. Lin, N. Sun, Trends Food Sci. Technol., **122**, 171-186 (2022)
30. Jr. A. J. Cichoke, Textbook of Natural Medicine (Fifth Edition), 458-464, e3 (2020)
31. F. G. Corazza, J. V. Ernesto, F.A.N. Nambu, L.R.de Carvalho, V. R. Leite-Silva, G.H.C. Varca, L.A. Calixto, D. P. Vieira, N. Andréo-Filho, P. S. Lopes, J. Drug Deliv. Sci. Tech., **55**,101413 (2020)
32. F. Ersöz, M. İnan, Prot. Expres. Purif., **54**, 126-133 (2018)
33. M. A. Navarrete del Toro, F. L. García-Carreño, Cur. Prot. Food Anal. Chem., **10**, B2-2 (2003)
34. W. Brand-Williams, M. E. Cuvelier, C. L. W. T. Berset, LWT - Food Sci. Tech., **28**, 25-30 (1995)
35. G. Shu, J. Huang, C. Bao, J. Meng, H. Chen, J. Cao, Biomolecules, **8**, 101 (2018)
36. Y. Yu, F. Fan, D. Wu, C. Yu, Zh. Wang, M. Du, Molecules, **23**, 1189 (2018)
37. M. Abdel-Hamid, J. Otte, C. de Gobba, A. Osman, E. Hamad, Int. Dairy J., **66**, 91-98 (2017)