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ASSAY OF THE PEPTIDE MATTER OF CHEESE DURING RIPENING BY FAST SIZE-EXCLUSION HPLC METHOD

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Abstract

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The release of peptides from milk proteins is one of main processes during the ripening of cheese. The quality of the cheese is related to both the content of peptide matter and the distribution of molecular weight of peptides. Several bioactive functions are attributed to certain low molecular weight peptides. The aim of the present work is the development of fast method for evaluation of peptide matter of cheese together with assay of the distribution of molecular weight of the peptides. For this purpose a size-exclusion HPLC separation of peptide matter was used and fluorescence detection after post-column derivatization of peptides. The method was applied to a newly developed starter for Bulgarian white brined cheese with increased ability in production of bioactive peptides. The inhibition of Angiotensin-converting enzyme (ACE, 3.4.15.1) was the main beneficial effect of the bioactive peptides released during the cheese ripening. Using the developed method a comparison of the peptide matters was performed between cheese produced with starter with increased ACE inhibitory activity and a control cheese produced with long time used commercial starter. The difference between the contents of low-molecular weight peptides was more then twice. By help of this method it was possible not only to evaluate quantitatively the total peptide matter in the cheeses, but to distinguish between high-, middle-, and low- molecular weight peptides only within one hour. The ratio between the peptides with different molecular weight is important for the quality of the cheeses.

Key words: white brined cheese, bioactive peptides, size-exclusion HPLC

Introduction

The bioactive peptides are defined as specific protein fragments that have a positive effect on human health. These peptides are inactive in the sequence of the precursor, but can be activated by enzymatic proteolysis during digestion or food processing, by means of microbial or plant enzymes (Clare et al., 2000). Bioactive peptides encoded in the primary structure of milk proteins, may be released by the action of proteinases in the cell wall and intracellular peptidases in dairy bacteria (Bütikofer et al., 2007). The concentration of the bioactive peptides is increased significantly during the fermentation of food. Peptides with different biological activities and, in particular, antihypertensive peptides can be detected in an active form in dairy products such as fermented milks and cheeses, prepared under controlled conditions involving the selected starter cultures (Otte et al., 2007). Capacity to inhibit ACE has been detected in a number of peptides derived from food products such as milk whey, eggs, soy, corn, fish (Tavares et al, 2011). The bioactivity is encoded in the primary structure of the milk proteins which require proteolytic release of precursors (Meisel et al., 1999). Bioactive peptides derived from milk proteins may function as exogenously - regulatory substances with hormone-like activity. *In vitro* and *in vivo* studies have shown a broad spectrum biological functions attributed to bioactive peptides such as: opium-like, mineral binding, immunomodulatory, antibacterial, antioxidant, antithrombotic, hypocholesterolemic and antihypertensive activity. The release of a variety of bioactive peptides and notably ACE- inhibitory of peptides from milk proteins by proteolysis of lactic acid bacteria (LAB) is well documented (Dimitrov, 2012). One of the mechanisms through which the peptides derived from milk, can reduce the blood pressure is the inhibition of ACE. Proteinases of lactic acid bacteria such as *Lactobacillus helveticus* and *Lactobacillus casei* can produce ACE - inhibitory peptides in vitro (FitzGerald, 2004). At all,

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LAB has the potential to generate a variety of bioactive peptides. ACE - inhibitory peptides are found in different types of cheeses produced in different conditions and with different ripening starter cultures. LAB has proteolytic system necessary for their growth. Extracellular proteinases hydrolyze milk proteins, releasing peptides to the culture medium. ACE - inhibitory peptides typically comprise 2-20 amino acid residues (Mills et al., 2011). Many different antihypertensive sequences can be obtained from milk proteins such as β-, αSl-, κ- casein, β- lactoglobulin and α- lactoalbumin. Typically, in cheese the bioactive peptides are low molecular weight. The low molecular weight peptide matter is of great importance not only from the functional aspect, but also in terms of its influence on the flavor and taste of the cheese. The main objectives of this work are: evaluation of low-molecular weight peptide matter in cheese made with starter with increased ability to release bioactive peptides; and the development of a method for rapid quantification of the peptide matter with the possibility of distinguishing the high-, medium-, and low-molecular weight peptide fractions.

Material and Methods

Evaluation of peptides by size exclusion HPLC and fluorescence detection

Ten grams from the cheese samples were homogenized with 30 ml of 1.4 ml.l^{-1} trifluoroacetic acid using a stomacher (Interscience). One milliliter of the homogenizate was centrifuged in 1.5 ml tube 10 min at 10000 g. 0.5 ml from the supernatant were put onto ultrafiltration cartridge with molecular weight cut-off of 10 kDa (Sigma) for 30 min at 12000 g. 180 microlitres from the permeate were mixed with 20 microlitres of 1.0 ml.l^{-1} trifluoroacetic acid in 500 ml.l^{-1} acetonitrile. The mixture is microfiltrated through filters with 0.45 micrometers pores (Milex). Fifty microlitres were injected onto HPLC system (Shimadzu), equipped with a size-exclusion column Biobasic SEC300, 300x7.8. The eluent was 1.0 g.l^{-1} trifluoroacetic acid in 5 g.l^{-1} Acetonitrile, 0.6 ml per minute flow rate, at 35ºC temperature in the oven. The eluted peptides were subjected to post-column derivatization with ortho-phtaldialdehyde (OPA) reagent with beta-merkaptoethanol (Sigma) at temperature 45ºC. The derivatized peptides were detected by fluorescence detector (RF-10Axl, Shimadzu) using an excitation light with length of 360 nanometers and an emission light with length of 460 nanometers. The content of the peptide matter was evaluated on the base of the chromatographic peak area.

Evaluation of ACE-inhibitory activity of the cheese

Five grams from the cheese samples were homogenized with 15 ml of 1.0 ml.l^{-1} lactic acid using a stomacher (Interscience). The pH of the mixture was adjusted to 4.3 with 500 ml.l^{-1} lactic acid and the suspension was centrifuged at 5000g for 10 min. The pH of the supernatant was adjusted to 8.3 with 100 mM borate buffer and the mixture was centrifuged at the upper conditions. 0.5 ml from the supernatant was ultrafiltrated through UF-cartridge with molecular weight cut-off of 10 kDa (Sigma) for 30 min at 12000 g. The evaluation of ACE-inhibition was performed using the assay explained by Cushman & Cheung (3). Different dilutions of the permeate were used at volume of 20 microliters. The ACE solution was 40 microliters with activity 0.1 U.ml^{-1}. The substrate hipuril-histidil-leucine was dissolved in 140 mM borate buffer with pH 8.3. For the reaction 190 microliters of this buffer were used. The final concentration of the substrate was 6 mM, and the concentration of sodium chloride was 300 mM. The reaction was conducted at 37°C for 60 min and stopped with 100 microliters of 4n HCl. The extraction of the released hipuric acid was performed with 1 ml of ethylacetate. After evaporation of the ethylacetate at 105°C the residue was dissolved with 1 ml of water and the absorbance was readed at 228 nanometers using UV-VIS spectrometer (Shimadzu). The ACE-inhibitory activity in percents was calculated using the formula

$$[(B-A) / (B-C)] * 100,$$

where: A is the absorption value of the sample, B - absorption without sample, C - absorption without ACE. The sample volume corresponding to 50% of inhibition (I_{50}) was calculated graphically after the detection of ACE-inhibition of 5, 10, 15, and 20 microliters of the sample.

Results and Discussion

The starters for Bulgarian white brined cheese contain *L. bulgaricus* and *S. thermophilus* strains. In the present work a new starter, containing *L. helveticus* strain together with *L. bulgaricus* and *S. thermophilus*, was developed (starter I). The addition of specially selected *L. helveticus* strain, capable to release bioactive peptides with ACE-inhibitory activity, to the main bacterial components of the starter could change the complex proteolytic processes during the cheese ripening.

Two sets of cheese were prepared - with a traditional starter as a control, and with the developed starter containing the strain *L. helveticus* A1. The control cheese was made with one a much exploited starter, consisting of only *L. bulgaricus* and *S. thermophilus* strains (starter II). The standardized technology for production of Bulgarian white brined cheese was followed. On the base of this control starter the influence of the strain *L. helveticus* A1, added to the same *L. bulgaricus* and *S. thermophilus* strains was examined towards the released

peptides during ripening at 15, 30, 45 and 60-th day. A comparison between the content of the peptide matter of the both cheeses - the control and the one produced with the developed starter is shown in Figure 1, where the peptide profiles of both cheeses are given. The analysis of the peptide matter was done using a size-exclusion HPLC. It was analyzed the peptide matter extracted from the samples by ultrafiltration through a membrane with a cutting limit of 10 kDa. The gel filtration HPLC column Biobasic SEC300, 300x7.8, is specially optimized for peptide analysis. This way of separation arranges the peptides according to their molecular weight in descending order: from 10 kDa to the low molecular weight peptides. The duration of the HPLC run is only 15 minutes. The peptide matter on the chromatogram was divided to high molecular weight (3-10 kDa), middle molecular weight (1-3 kDa), and low molecular weight (< 1kDa). To eliminate the effect of the organic acids and other organic components non containing amine groups, a fluorescence detection was used after a postcolumn derivatization with OPA reagent. OPA can react with both peptides and amino acids. Figure 2 illustrates

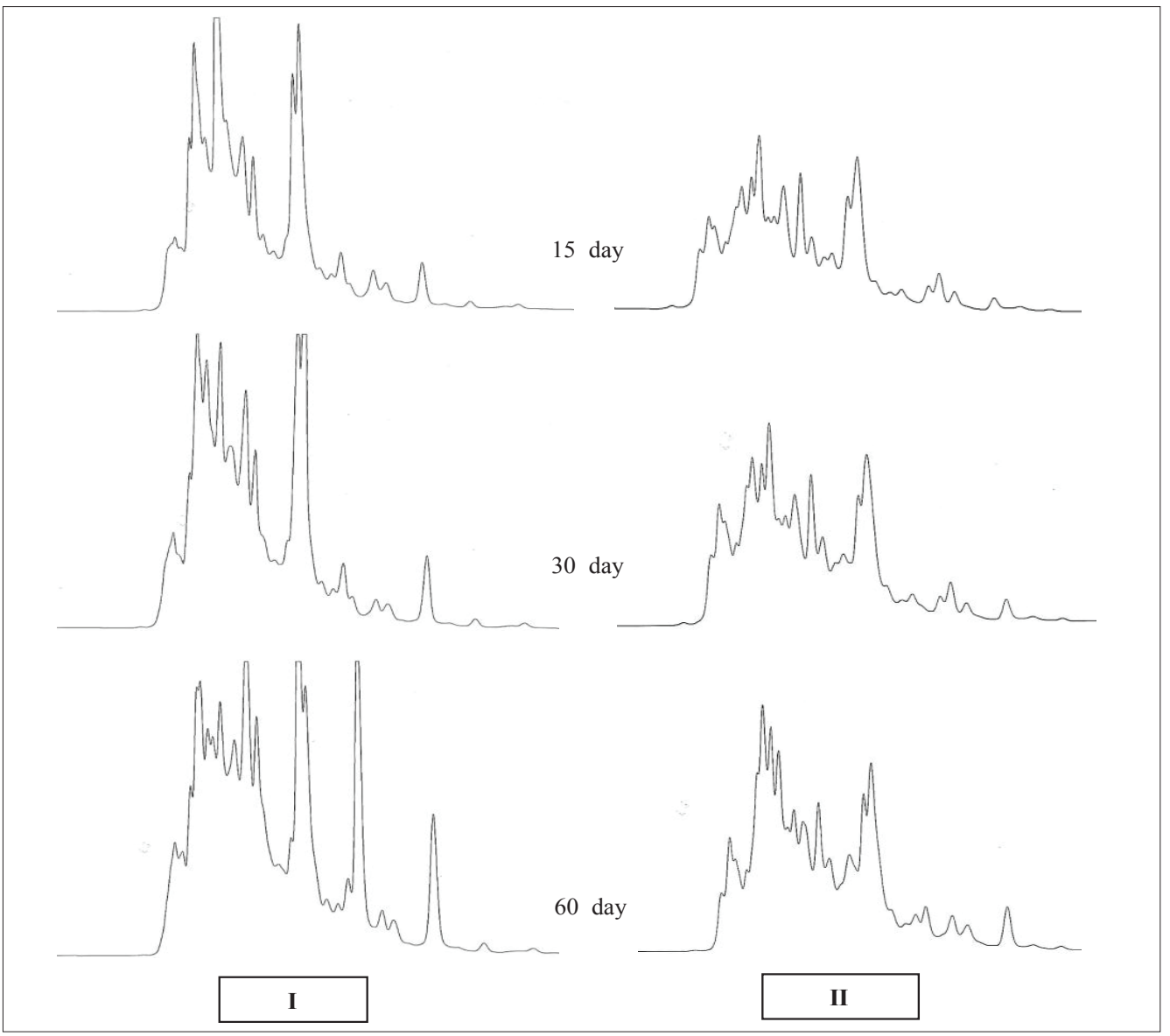

Fig. 1. Peptide profiles of the two sets of cheese - produced with a starter containing *L. helveticus* A1 (I starter) and produced with a control starter (II starter)

the change of the content of the total peptide matter of the two sets of cheese during the ripening period. The high proteolytic activity of the strain *L. helveticus* A1 leads to higher concentration of peptides in the cheese during the all ripening period. The capability of *L. helveticus* A1 to release ACE-inhibitory peptides reflects to the remarkable difference in ACE-inhibition between the two sets of cheese. The ACE-inhibitory activity of the cheese samples produced with the two starters is given on Figure 3. The ACE-inhibition of the peptides from the cheese, produced with the starter containing *L. helveticus* A1 strain, is significantly higher than the ACE-inhibition of the control cheese for every stage of the ripening. Even the ACE-inhibition of the cheese with *L. helveticus* A1 on 15-th day of the ripening is higher than the ACE-inhibition of the control cheese on 60-th day. Figure 4 presents the change in low-, medium-, and high- molecular weight peptides during the ripening of the two sets of cheese made with the control and the developed starter. It was determined the proportion in percents of each of the peptide groups in the above ranges with respect to the total peptide concentration as a function of the ripening period. The results show that the proportion of high molecular weight peptides for both series decreases with maturation, while the part of the middle molecular weight peptides is relatively unchanged during the ripening. The part of the low molecular weight peptides in both series is increased. The part of low molecular weight peptides of the developed starter (I starter, Figure 4) is significantly higher than the corresponding of the control starter (II starter, Figure 4) during the all ripening period. The increase of the relative part of the small peptides and free amino acids during the ripening is much higher in the cheese produced with the starter I than in the cheese produced with the control starter. The concentration of the total peptide matter of the cheese produced with a starter I is about 2 times higher than that in the cheese produced with the control starter II.

Conclusion

The results show that the starter containing the strain with ACE-inhibitory activity *L. helveticus* A1 leads to an increased content of low molecular weight peptides and ACE-inhibition in the cheeses. The developed method for an evaluation of the peptides allows rapid quantification of the peptide matter and determine the distribution between high-, medium- and low-molecular peptides.

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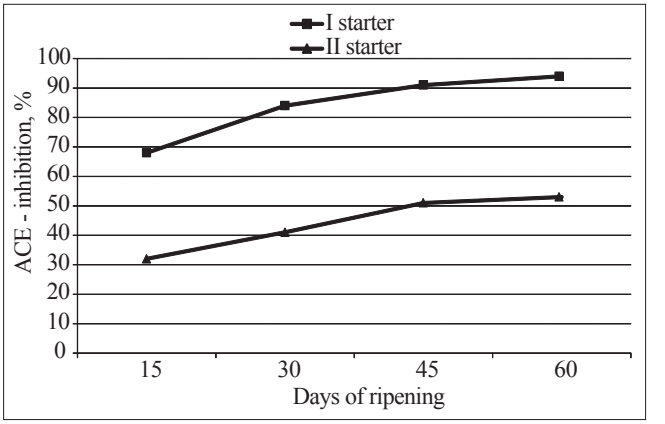

Fig. 2. Change of the total peptide matter of the two sets of cheese during the ripening

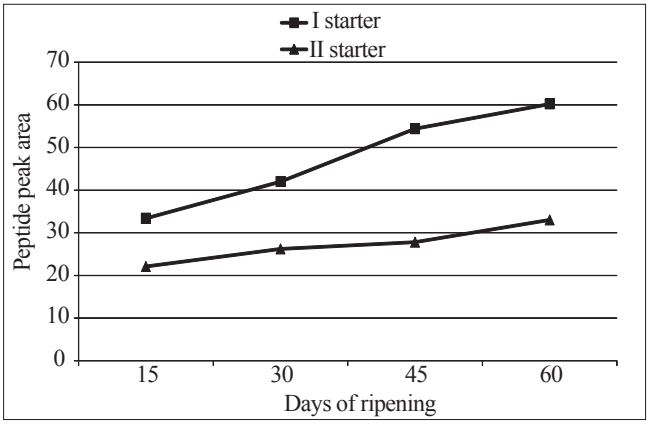

Fig. 3. ACE-inhibitory activity of the two sets of cheese - produced with *L. helveticus* A1 (starter I) and the control (starter II)

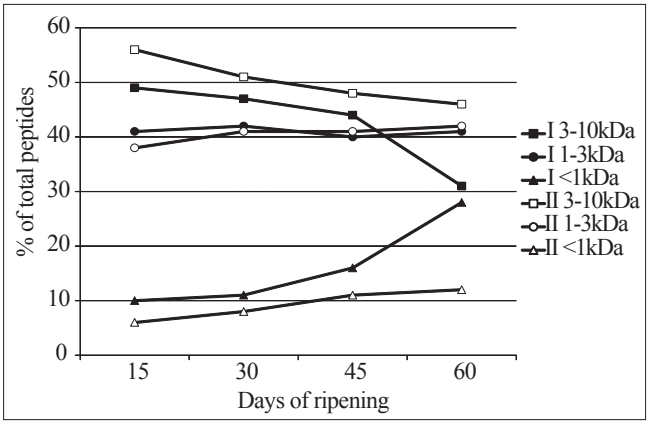

Fig. 4. Change in the ratio of the low-, medium-, and high- molecular weight peptides during the ripening of the two sets of cheese made with the developed starter (I) and the control starter (II)

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