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EFFECT OF STRAIN *ENTEROCOCCUS FAECALIS* AN1 ON RELEASE OF BIOACTIVE PEPTIDES FROM WHEY PROTEINS IN *IN VITRO* SIMULATED GASTROINTESTINAL CONDITIONS

Summary

The aim of the study was to study the potential of the proteolytic strain Enterococcus faecalis AN1 to generate inhibition of angiotensin converting enzyme (ACE), as well as to determine the effect of subsequent hydrolysis with pepsin and pancreatin in vitro simulated gastrointestinal system on this activity. Analysis of substrate hydrolysis and peptide formation was performed using SDS-PAGE and electrophoresis by RP-HPLC. Casein hydrolyzate with proteases of the strain showed the ability to produce peptides with ACE inhibition activity, which shows the use of these strains in the development of functional dairy products with antihypertensive properties. The studied strain has the potential to produce functional dairy products.

Key words: *lactic acid bacteria, proteases, caseins, bioactive peptides, angiotensin converting enzyme*

İn vitro simulasiya olunmuş mədə-bağırsaq sistemi şəraitində *Enterococcus faecalis* AN1 ştamının südün zərdab zülallarından ayrılan bioloji aktiv peptidlərə təsiri

Xülasə

Tədqiqatın məqsədi *Enterococcus faecalis AN1* proteolitik ştamının antiotenzin çevirən fermentin (ACE) ingibirləşdirici təsirinin öyrənilməsi olmuşdur. Həmçinin də, bu aktivliyə pepsin və pankreatinlə hidrolizin *in vitro* simulyasiya edilmiş mədə-bağırsaq sisteminə təsirini təyin etmək idi. *Enterococcus faecalis AN1* ştammının proliferasiya etməyən hüceyrələri, 4 saatlıq inkubasiyadan sonra denaturasiya olunmuş zərdab zülallarını hidroliz etmək qabiliyyətinə malikdir, lakin bu zaman deqradasiya aşağı idi. *Enterococcus faecalis AN1*ştamının qeyri-proliferativ hüceyrələri tərəfindən denaturasiya məruz qalmış zərdab zülallarının hidrolizinin xromotoqrafik profilləri az miqdarda kiçik peptidlərin ayrılmasını göstərdi. Pepsinlə hidrolizdən sonra isə, xromotoqrafik profillər kontrol və hidrolizat üçün fərqli idi. Hüceyrələrlə hidrolizdən sonra 5 və 18 dəq. elüasiya ilə ayrılan peptidlər, həmçinin də, hidrolizatın pepsinlə parçalanmasından sonra da müşahidə olunurdu. Bu tədqiq etdiyimiz ştamm angiotezin çevirən fermentin aktivliyini ingibirləşdirmək qabiliyyətinə malik olan peptidlərin sintez olunmasına səbəb ola bilər. Bu da, bizim ştamların antihipertenziv xüsusiyyələrə malik funksional süd məhsullarının istehsalı zamanı istifadəsi üçün potensial ştam olduğunu sübut edir.

Açar sözlər: *süd turşusu bakteriyaları, proteazalar, kazeinlər, bioaktiv peptidlər, angiotenzin çevirən fermentlər*

Introduction

As you know, the most valuable constituent of milk is proteins, which contain all the essential amino acids. Whey proteins in terms of the content of deficient essential amino acids (lysine, tryptophan, methionine, threonine) are the most biologically valuable part of milk proteins, which is important for nutritional purposes. The main ones - lactoalbumin and lactoglobulin - have a high content of growth and protective substances. In addition, serum proteins contain immunoglobulins that act as antibodies.

In addition to their dietary value, milk proteins are able to indirectly play a more specific biological role in the body. They are a rich source of biologically active peptides with ACE (angiotensin converting enzyme) inhibiting, antimicrobial, antioxidant, immunostimulating, opioid, and other activities (Gobbetti, M., L. et. all, 2002: 223; Kamysu, W., M. et. all., 2003: 236). These biologically active peptides are embedded in the primary sequence of milk proteins, and their release occurs as a result of proteolysis, which occurs as a result of the action of milk proteolytic enzymes, starter cultures used in the fermentation process, and also continues in the gastrointestinal system of the final consumer of the fermented dairy product. It should be noted that, depending on the degree of hydrolysis, both the formation and degradation of previously released biologically active peptides can occur as a result of the cleavage of the active amino acid sequence (Lahov, E., 1996: 131). As we noted earlier, the primary formation of bioactive sequences occurs during fermentation, and starter cultures used for starter culture play an important role here. It is known that lactic acid bacteria, widely used in milk fermentation, produce proteolytic enzymes that break down milk

proteins, and thus provide them with the necessary amino acids. Primary hydrolysis of milk proteins is carried out by LAB proteases, which leads to the formation of various peptides that impart taste and aroma to the final products, as well as have biological activity (Exterkate F., et. all., 1993: 3640; Kunji E.R.S., et. all., 1996: 187). The study of proteolytic strains of LAB allows the development of functional dairy products containing biologically active peptides.

The aim of this work was to study the effect of the proteolytic strain *Enterococcus faecalis AN1* on the formation of biologically active peptides from milk whey proteins, from caseins, and also to determine the effect of subsequent hydrolysis with pepsin and pancreatin under conditions of in vitro simulated gastrointestinal system on this activity.

Materials and methods

Bacterial strains. The study used the *Enterococcus faecalis AN1* strain, which was previously isolated from traditional Azerbaijani cheese and is a producer of proteolytic enzymes (Ahmadova, A., et. all., 2010: 25). The bacterial culture was stored at – 80° C in MRS medium containing 30% glycerol and 20% skim milk. Before use, the strain was cultured twice in MRS medium at 37° C (Centeno J.A., et., all., 1999: 97).

Proteolysis of milk serum proteins. was performed according to Fira et al. (2001: 123). The bacterial culture was inoculated onto the surface of a milk-citrate agar medium containing skim milk 4.4%, Na-citrate 0.8%, yeast extract 0.1%, glucose 0.5%, agar 1.5% (w / v) and incubated for 48 h at 37° C under sterile conditions. At the end of the incubation period, bacterial cells were carefully collected from the surface of the milk-citrate agar medium and washed twice (8000 rpm) with physiological saline (0.8% NaCl) containing 5 mM Ca2 + ions. The washed cells were suspended in phosphate buffer (100 mM, pH 7.0) to OD600nm, equal to 10. A mixture of purified milk serum proteins (analytical grade) was dissolved in phosphate buffer (100 mM, pH 7.0) and heat treated at 80°C for within 20 minutes. After cooling to room temperature, the substrate solution was mixed in equal proportions with a suspension of bacterial cells of the studied strains and incubated for 24 h at 37 °C. Substrate incubated under the same conditions in the absence of cells was used as a control. The final concentration of proteins in the mixture was 5 mg / ml. After incubation, cells were removed by centrifugation (12000 rp/m) and the resulting supernatant was used for hydrolysis profile analysis by SDS-PAGE electrophoresis and RP HPLC. The same supernatant was used for further experiments.

Proteolysis in an *in vitro* simulated gastrointestinal system carried out according to Mouecoucou et al. (2004: 105). The hydrolysates obtained by hydrolysis by bacterial cells, as well as the native substrate not subject to hydrolysis by the cells of the strains (control), were further hydrolyzed with pepsin and pancreatin simulating the physiological conditions of digestion. Pepsin hydrolysis was carried out for 1 hour at pH 2.0 and 37°C. The concentration of pepsin (Sigma, 4720 U / mg protein) was calculated from an enzyme / substrate ratio of 1/400, respectively. To terminate the enzymatic reaction, the pH of the samples was adjusted to 8.0, followed by incubation at 80°C for 5 min for thermal inactivation of the enzyme. The next stage of hydrolysis was carried out with pancreatin (Sigma) in the presence of bile acids at pH 8.0 and a temperature of 37°C for 2 h. The pancreatin concentration was 0.1% (w/v). Bile acids (Ox-bile, BioChemica) were added at a concentration of 0.3% (w/v).

Analysis of substrate hydrolysis and peptide formation were performed using electrophoresis and reversed phase high performance liquid chromatography (RP-HPLC). Triscin SDS–PAGE electrophoresis with acrylamide concentration of 17% was performed according to the previously described protocol (Schägger, H., 1987: 368). The gels were stained using Coomassie Brilliant Blue G-250 (Sigma-Aldrich) according to (Devouge V., R et al, 2007: 1342). The hydrolysates were mixed in equal amounts with a solution for introducing samples into the gel (SDS 4%; Tris HCl 50 mM pH 6.8; glycerol 20%; bromophenol blue; β-mercaptoethanol) and subjected to heat treatment for protein denaturation (100°C, 3 min). RP-HPLC was performed on a Waters system liquid chromatograph (Alliance system, Milford, MA) equipped with a Symmetry C18 column (5 μm, 2.1 mm x 150 mm). The column was pre-washed with solution "A" containing 0.11% trifluoroacetic acid (TFA) in deionized water. The elution solution (solution "B") had the following composition: 80% acetonitrile, 20% deionized water, and 0.09% TFA. Elution was carried out under conditions of a linear gradient of solution "B" from 0 to 100% at a rate of 0.2 ml / min. The determination was carried out at a wavelength in the range of 220-330 nm using a diode spectrophotometer (model 996, Waters).

Results

Serum proteolysis in an in vitro simulated gastrointestinal system

Non-proliferating cells of *Enterococcus faecalis AN1* strain were able to hydrolyze denatured whey proteins after 4 hours of incubation, but degradation was low. When digestion was continued with pepsin, total hydrolysis of ALA (α-lactoalbumin) was observed only in the hydrolyzate (Fig: 1).

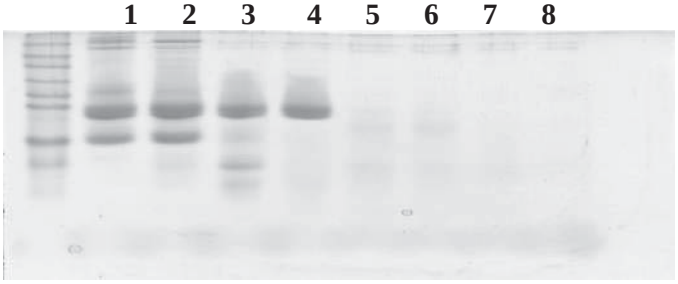

Fig. 1 Tricin-SDS-PAGE. Digestion of denatured whey proteins by gastric and pancreatic enzymes

1 - control (denatured whey proteins), 2 - hydrolyzate (denatured whey proteins hydrolyzed by cells of the Enterococcus faecalis N1 strain), 3 - control after digestion of pepsin, 4 - hydrolyzate after digestion of pepsin, 5 - control after digestion of pancreatin (1 hour), 6 - hydrolyzate after digestion of pancreatin (1 hour), 7 - control after digestion of pancreatin (2 hours) 8 - hydrolyzate after digestion of pancreatin (2 hours).

There are 2 main classes of proteases according to their substrate-selectivity: class I proteases - only break down β-casein of milk; Class III prostheses - break down S1-, S2-, and β-caseins of milk (Kamysu, W., M. et. all., 2003: 236). Based on this classification, we can conclude that the proteases produced by our strains of LAB (lactic acid bacteria) belong to class III.

β-LG (β-lactoglobulin) was resistant to digestion. However, when native whey proteins were used as a substrate, total α-LA degradation after pepsin treatment was observed in the control and in the hydrolyzate (Fig: 3). General hydrolysis of β-LG was observed for both substrates after 1 h of digestion with pancreatin.

RP-HPLC profiles of the hydrolysis of denatured serum proteins by non-proliferative cells of the *Enterococcus faecalis AN1* strain showed the release of a small amount of small peptides (Fig: 2). The peptide profiles after pepsin digestion were different for control and hydrolyzate (Fig: 2 and 3). Peptides released after 5 and 18 minutes of elution after cell hydrolysis (Fig: 2) were also observed after pepsin digestion of the hydrolyzate (Fig: 3). These peptides were not observed after pepsin digestion of the control (Fig: 3). The peptide released during the 5 minute elution was stable for the digestion of pancreatin (Fig: 4). The intensity of the other peptide increased after the digestion of pancreatin, and the same peptide appeared after the digestion of pancreatin in the control (Fig: 4).

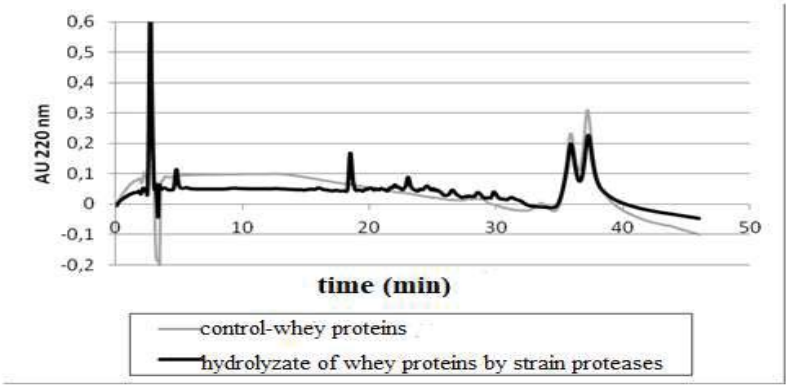

Fig. 2. Chromatographic profile of peptides formed during hydrolysis of milk serum proteins by proteases of *Enterococcus faecalis AN1* strain

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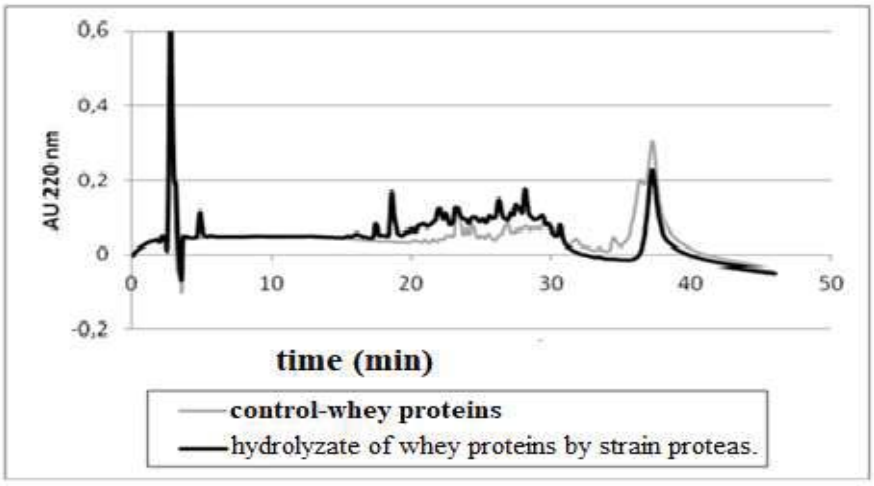

Fig. 3. Chromatographic profile of peptides formed during hydrolysis of serum proteins and hydrolysates of *Enterococcus faecalis AN1* strain by pepsin

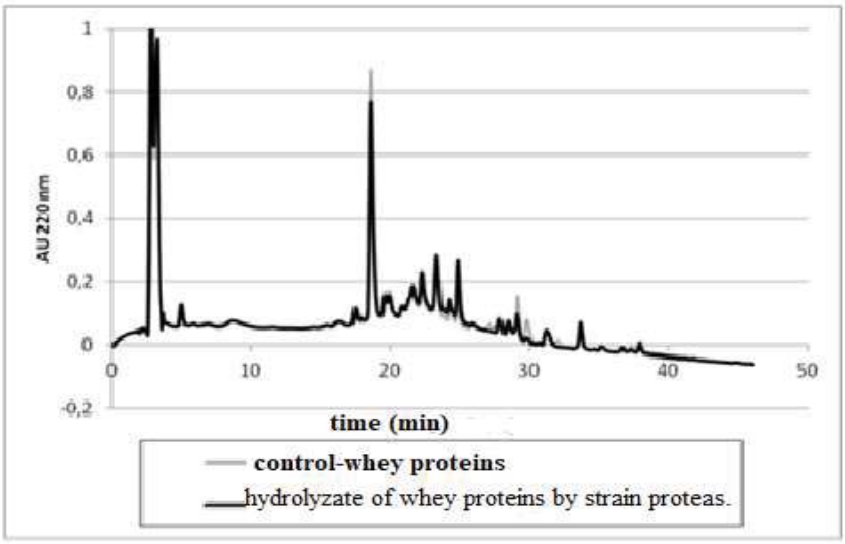

Fig. 4. Chromatographic profile of peptides formed during hydrolysis of serum proteins and hydrolysates of *Enterococcus faecalis AN1* strain by pancreatin

Our results have shown that when strains, especially *E. faecalis AN1*, are added to traditional cheeses, it plays an important role in defining the flavor and texture of the cheese. In addition, the above mentioned strain can contribute to the production of peptides with ACE (angiotensin converting enzyme) inhibition activity, which indicates the use of these strains in the development of functional dairy products with hypotensive properties. The biological activity of these peptides was retained when exposed to pepsin, but completely disappeared when hydrolyzed by pancreatin, possibly as a result of their further cleavage and degradation of the active amino acid sequence. The data obtained in this study suggests that the investigated strain has the potential to produce functional dairy products.

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