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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF PEPTIDES FORMED DURING THE HYDROLYSIS OF CASEIN BY PROTEASES OF THE *ENTEROCOCCUS FAECALIS* AN1

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Abstract. Much attention is paid to the study of biologically active peptides from milk caseins, which are usually obtained by partial hydrolysis by the proteolytic system of lactic acid bacteria. These peptides have a wide range of beneficial properties, including mineral binding, antidiabetic, satiating, immunomodulating, opioid, antimicrobial and antioxidant properties. In this work, we investigated the antimicrobial and antioxidant activity of peptides obtained during the hydrolysis of milk caseins by *Enterococcus faecalis* AN1 proteases. SDS PAAG electrophoretic analysis of peptide fractions after 8 hours of milk casein proteolysis showed that proteases of this strain cleave all casein fractions (αs1-, αs2 and β-casein). RP-HPLC profile elution revealed a large amount of small, medium and long cationic and anionic peptides. Some fractions of these peptides have antimicrobial activity against *Listeria monocytogenes* EGDe 107776, *Bacillus cereus, Escherichia coli* CCM 4517 and Aspergillus niger CCM 8189. Peptide fractions obtained during the hydrolysis of caseins possessed strong antioxidant activity and surpassed native casein by 4.5 times. These data stimulate further research in the field of biomedicine and food technology with the aim of developing new and effective preparations and products for maintaining the health and safety of consumers.

Keywords: *casein, Enterococcus, proteolysis, biologically active peptides, antimicrobial activity, antioxidant activity*

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Abbreviations:
ABTS - 2,2/-azinobis (3-ethylbenzothialine 6-sulfonate)
AOA - antioxidant activity
BAP - biologically active peptides
RP-HPLC - reversed-phase high-performance liquid chromatography
SDS - PAAG
TEAP - Trolox Equivalent Antioxidant Potential
TFU - trifluoroacetic acid

1. Introduction

Modern studies of biologically active peptides (BAP), obtained from various sources of proteins, are of significant interest in the context of their potential use in biomedicine and the food industry. Particular attention is paid to peptides formed during the hydrolysis of casein by lactic acid bacteria, as this process not only improves the

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functional properties of protein products, but also contributes to the formation of biologically active compounds with potential antimicrobial and antioxidant properties (Hayes *et al.,* 2006; Raveschot *et al.,* 2018). According to literature data, in the composition of various native proteins, including caseins and whey proteins, there are many BAPin encoded form, which are stored in the form of pro-peptides or mature C-terminal peptides (Gobbetti *et al*., 2002; Hill *et al*., 1974). Such peptide fragments are inactive in the composition of the native protein, but they can be released after its hydrolysis during digestion, in vitro enzymatic hydrolysis or microbial fermentation (Raveschot *et al.,* 2018; Korhonen, 2009).

In addition to antimicrobial and antioxidant properties, BAP have other useful biological functions, such as inhibition of angiotensin-converting enzyme, mineral binding, antidiabetic, satiating, immunomodulating and opioid (Nongonierma & FitzGerald, 2015; Park & Nam, 2015; Caron *et al.,* 2017; Nielsen *et al*., 2017; Gobbetti *et al.,* 2002).

BAP studies provide valuable information about the effect of food proteins on health. In addition, there are industrial applications, such as the production of functional food products (Korkhonen & Pikhlanto, 2006; Nongonierma & FitzGerald, 2015; Park & Nam, 2015) or the production of peptides that will serve as active ingredients of pharmaceutical preparations or food additives (Caron *et al*., 2017).

In this article, we present the results of our research aimed at studying the antimicrobial and antioxidant activity of peptides formed during the hydrolysis of casein by proteases of *Enterococcus faecalis* AN1, isolated from the traditional Azerbaijani cheese sample "Motal".

2. Materials and methods

The strain Enterococcus faecalis AN1 was isolated from the traditional Azerbaijani cheese "Motal". Proteolytic enzymes synthesized by this strain hydrolyze αs1- and β-casein milk (Ahmadova *et al*., 2010). The strain was stored at -80°C in MRS medium containing 30% glycerol. Before use, the strain was cultivated twice in MRS medium at a temperature of 37°C. Test-organisms of the genus Listeria were incubated in BHI medium at a temperature of 37 °C, and the same organisms of the genus Escherichia were incubated in a BH medium at 30 °C.

Proteolysis of caseins was carried out according to the modified method by Fira et al. (2001). The cells obtained by centrifugation (8000 rpm) of the fresh culture of the strain were washed with physiological solution (0.8% NaCl) in the presence of 5 mM Ca2+ ions and diluted in phosphate buffer (100 mM, pH 7.0) to OD 600 nm, equal to 10. The cell suspension was mixed in equal proportions with the substrate (Na-caseinate 12 mg/ml) dissolved in the same buffer and incubated for 8 hours at a temperature of 37°C. After incubation, the cells were removed (centrifugation, 12000 rpm) and the supernatant was checked for the presence of hydrolysis using SDS-PAAG-electrophoresis and RP HPLC. This same supernatant was used for further experiments.

The analysis of the results of native protein proteolysis was carried out using SDS-PAAG-electrophoresis and reversed-phase high-performance liquid chromatography (RP-HPLC). SDS-PAAG-electrophoresis with acrylamide concentration of 15% was carried out in the Mini Protean II Gel Electrophoresis apparatus (Bio-Rad Hercules, California, USA) according to Laemmli (1970). The hydrolyzates 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 carried out on a liquid chromatograph "Waters system" (Alliance system, Milford, MA) equipped with a column "Symmetry C18" (5 μm, 2.1 mm x 150 mm). Elution was carried out under the conditions of a linear gradient of solution B (80% acetonitrile, 20% deionized water, 0.09% trifluoroacetic acid (TFU)) from 0 to 100% at an eluent flow rate of 0.2 ml/min. The column was pre-washed with solution A (deionized water, 0.11% TFU). Determination was carried out at a wavelength of 220-330 nm using a diode spectrophotometer (model 996, Waters).

Determination of the antimicrobial activity of the obtained peptide fractions. Antimicrobial activity of hydrolysates obtained after each stage of hydrolysis was checked by the well-diffusion method (Tagg *et al.,* 1976). The hydrolysates were collected at every 5 min elution (v/v 1 ml) after the appearance of the first traces of peptide fractions and was continued until the end of the chromatography, which lasted approximately 40 min. Thus, 5 portions of peptides with different compositions were obtained. *Listeria monocytogenes* EGDe107776 was used as a test organism. Solvents were removed from the collected fractions by evaporation, and protein concentration was determined using a Bio-Rad protein assay (Macart & Gerbaut, 1982).

Determination of antioxidant activity of obtained peptide fractions. Antioxidant activity (AOA) of hydrolysates was determined spectrophotometrically with Tlolox Equivalent Antioxidant Potential (TEAP) (Pellegrini, 2003). This method is based on the capture of ABTS (2,2/-azinobis (3-ethylbenzothialine 6-sulfonate) radical cation and its reaction with an antioxidant. A solution containing ABTS + radical (Sigma-Aldrich) was prepared by this method: ABTS 7mM aqueous solution 2.45 mM was oxidized in the presence of potassium persulfate (molar ratio 1:0.5) in the dark for 12-16 s. At the beginning of the experiment, the solution of ABTS+ radical was dissolved with the 5mM phosphate buffer (pH 7.4) until OS 0.70±0.2 wavelength λ=734 nm was obtained. 10 μl of the sample was added to the obtained solution (1 ml) and incubated at 250C for 2 hours. The AOA of the samples was determined by measuring the decrease in OD at a wavelength of 734 nm using a spectrophotometer (Shimadzu, model UV-3100, Kyoto, Japan). Trolox (Sigma-Aldrich), a hydrophilic analog of vitamin E, was used as an AOA standard. The concentration range of Trolox 0-25 μmol/ml was used to establish the calibration curve. AOA was expressed as milliliters of micromole of Trolox equivalent and calculated from a standard curve. The experiment was repeated three times and the average value was calculated.

3. Results and discussion

The results of electrophoretic analysis of the products of hydrolytic breakdown of milk caseins are presented in Figure 1. This figure shows that under the action of proteolytic enzymes of the *E. faecalis* AN1 strain, all casein fractions (αs1-, αs2 and β-casein) were cleaved to form numerous peptides.

It was previously noted that the proteolytic system of this strain cleaves only αs1- and β-casein in milk. However, in previous studies, the co-incubation time of proteases from the studied strain and native caseins lasted only 3 hours. This time we extended the proteolysis time to 8 hours. The observed difference in the substrate specificity of the proteases of the active strain is apparently due to the fact that in short periods of time

proteolytic enzymes do not have time to cleave all the polypeptide chains of native milk caseins (Ahmadova *et al*., 2010; Mustafayeva *et al*., 2012).

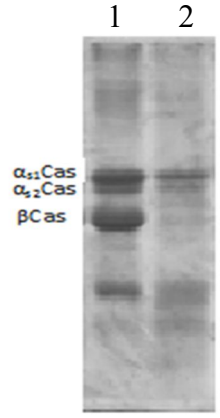

Figure 1. SDS PAAG analysis of milk casein proteolysis products by *Enterococcus faecalis* AN1: 1- native caseins, 2- products of proteolysis

Figure 2 shows the chromatographic profile of peptides formed during the hydrolysis of caseins by proteases of the *Enterococcus faecalis* strain AN1. The first traces of eluted peptides were detected after 15 min from the start of chromatography and ended after 40 min. As a result of the hydrolysis of caseins, the formation of a large number of oligopeptides of medium size and hydrophobicity, as well as a small number of smaller, more hydrophilic peptides, was observed.

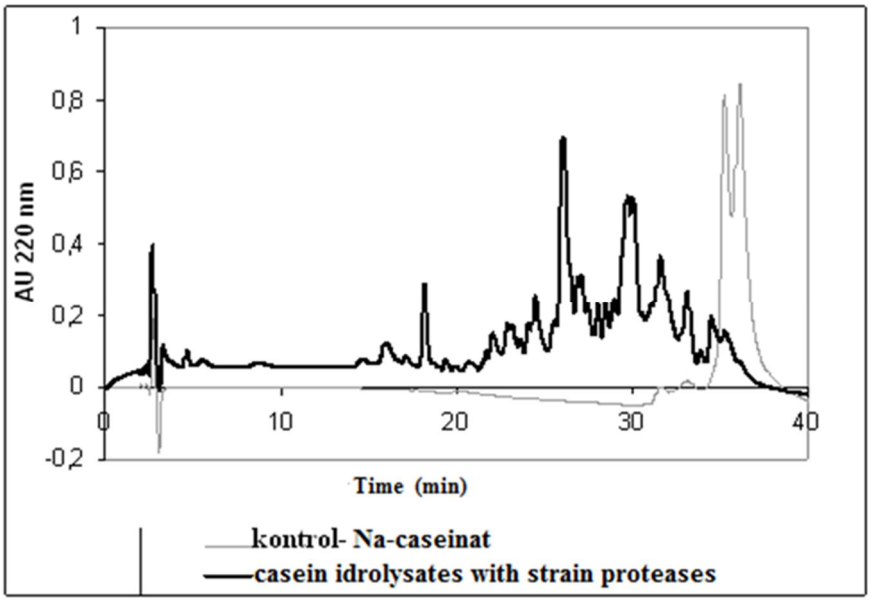

Figure 2. RP-HPLC profile of peptides formed during the hydrolysis of caseins by proteases of the *Enterococcus faecalis* AN1

In the next part of our experiments, we tested the antimicrobial activity of all 5 collected peptide fractions against 3 gram-positive (*L. monocytogenes* EGDe107776, *B. cereus* and *S. aureus* CCM 4516), 1 gram-negative (E. coli CCM 4517) bacteria and

Aspergillus niger CCM 8189. The results are summarized in the table 1. As can be seen from the table, the main part of the antimicrobial peptides was found in the third fraction of peptides. They showed activity against 3 test organisms - 2 gram-positive (*L. monocytogenes* EGDe 107776, *B. cereus*) and 1 gram-negative (*E. coli* CCM 4517) bacteria. The fourth fraction also had weak anti-listeria activity. However, the peptide composition of this fraction did not affect the growth of the other four test organisms. The table also shows that only the first fraction of shorter peptides had fungicidal activity against the *Aspergillus niger* CCM 8189.

Table 1. The inhibitory spectrum of peptide fractions synthesized following fermentation of *Enterococcus faecalis* AN1 in native sodium caseinate

Peptides fractions / Test organisms	I	II	III	IV	V
L.monocytogenes EGDe107776,	-	-	++	+	-
B. cereus	-	-	++	-	-
S. aureus CCM 4516	-	-	-	-	-
E. coli CCM 4517	-	-	+	-	-
Aspergillus niger CCM 8189	+	-	-	-	-

The diameters of the zones of inhibition were as follows: , ++, 6-10 mm; +, 2-5 mm; -, no zone detected.

According to literature data, caseicidins are the first antimicrobial peptides identified by Hill et al. (1974) from milk casein, which are cationic antibacterial glycopeptides. One of its representatives - isracidin consists of 23 amino acid residues and has a wide spectrum of activity against gram-positive bacteria (Lahov & Regelson, 1996). *Lactococcus lactis* (Kunji *et al.,* 1998; Pritchard & Coolbear, 1993) and *Lactobacillus helveticus* (Martin-Hernandez, 1994; Zevaco, 1988) have high proteolytic activity. The proteolytic systems of these bacteria were studied as microbial catalysts for the generation of bioactive peptides (Meisel, 2005; Kunji *et al.,* 2009). Antimicrobial and antioxidant characteristics of peptides formed during casein degradation are described for *L. helveticus* and partially for *Lactobacillus casei* (de Palencia, 1997). In addition, proteinase associated with the cell wall of Lactobacillus delbrueckii subsp. lactis ACA-DC 178 was characterized and documented for its specificity to β-casein. Three peptides produced by a Lactobacillus acidophilus DPC6026 fermentation of sodium caseinate and showing antibacterial activity against pathogenic strains *Enterobacter sakazakii* ATCC 12868 and *Escherichia coli* DPC5063 were characterized. These peptides were all generated from bovine αs1-casein (Hayes *et al*., 2006).

One of the important groups of BAPs obtained from the hydrolysis of caseins are peptides that have antioxidant properties (Raveschot *et al.,* 2018). Therefore, the next series of experiments was devoted to studying the antioxidant activity of the products of proteolysis of milk caseins by the *Enterococcus faecalis* AN1 strain. The TEAP of the resulting peptides was consistently determined depending on the time of proteolysis of native milk caseins. The results obtained are summarized in Figure 3.

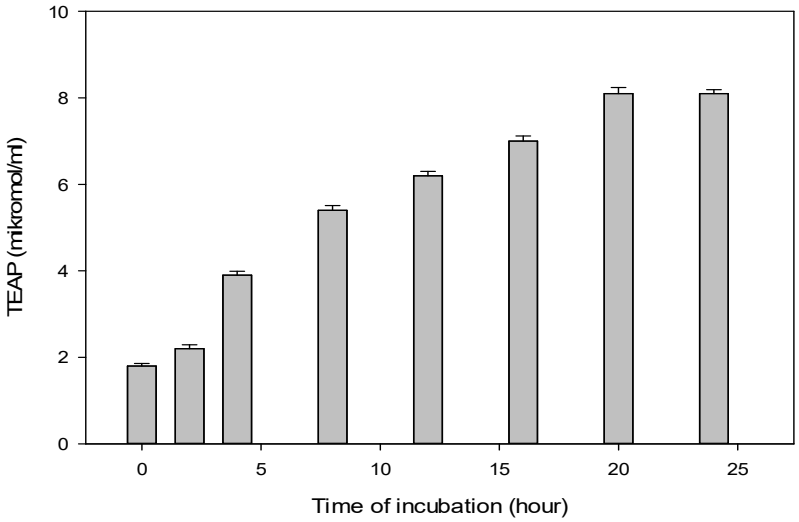

Figure 3. Changes in the antioxidant activity of peptides depending on the time of incubation with proteases of the Enterococcus faecalis AN1

From this figure it can be seen that native milk caseins themselves collectively have weak AO activity. The TEAP of intact caseins was 1.8 μmol/ml. However, this indicator began to increase after 2 hours of proteolysis and reached up to 2.5 μmol/ml. This increase was continued. For example, after 4 hours of incubation, TEAP was at a level of 4 μmol/ml, 8 hours – 5.8 μmol/ml, 16 hours – 7.2 μmol/ml. After 20 hours of proteolysis exposure, TEAP reached its maximum value at the level of 8.2 μmol/ml. Further incubation of caseins with proteases did not change this level.

The increase in the level of TEAP by bacterial proteases is apparently due to the fact that as the incubation time increases, a deeper hydrolysis of intact proteins and peptides occurs, as well as an increase in their quantity in the medium. Antioxidants exert their activity by neutralizing or preventing the formation of hydrogen peroxide and other peroxides. Milk proteins are considered an important source of natural antioxidants, antioxidant peptides, which are released in the process of proteolytic cleavage of casein and milk whey proteins (Quiroga *et al.*, 2020; Yun *et al.*, 2015). The dependence of these peptides on their structure and amino acid content has been actively studied in recent years. Data obtained during many studies showed that the antioxidant activity of milk protein hydrolysates depends on the amino acid sequence of the peptides and, accordingly, on the specificity of the proteases involved in the hydrolysis process (Hernandez-Ledesma, 2005).

Thus, the results of our studies confirm the potential of peptides obtained during hydrolysis of casein by proteolytic enzymes of *Enterococcus faecalis* strain AN1 as valuable biologically active compounds with pronounced antimicrobial and antioxidant activity. These data stimulate further research in the field of biomedicine and food technology with the aim of developing new and effective preparations and products for maintaining the health and safety of consumers.

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