УДК 579.22

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ИЗУЧЕНИЕ БАКТЕРИОЦИНОВ ШТАММА *ENTEROCOCCUS FAECALIS* Т23, ИЗОЛИРОВАННОГО ИЗ ИРАНСКОГО СЫРА

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STUDY OF BACTERIOCINS PRODUCED BY THE STRAIN ENTEROCOCCUS FAECALIS T23 ISOLATED FROM IRANIAN CHEESE

Аннотация. В ходе исследования бактериоцинов, продуцируемых штаммом Enterococcus faecalis T23, установлены свойственные им особенности. Спектр антимикробной активности данного штамма определяли против различных патогенных и условно-патогенных бактерий. Штамм проявил ингибирующую активность против Listeria monocytogenes, Salmonella typhimurium и близкородственных штаммов Lactobacilli. Изучение влияния различных ферментов на антимикробную активность выявило, что активный компонент имеет белковую природу. При помощи ПЦР анализа, со специфичными праймерами для различных классов бактериоцинов, были амплифицированы два бактериоцин-кодирующих гена в геноме исследуемого штамма: entB and entA. Изучено влияние различных факторов среды, а также различных химических соединений на активность бактериоцинов. Бактериоцины проявили высокую термостабильность и сохраняли активность в широком диапазоне pH (3–10). Тритон X-20, Тритон X-80, Тритон X-100, β-меркаптоэтанол, Na-EDTA, SDS и NaCl не влияли на активность бактериоцинов.

Ключевые слова: бактериоцины, энтерококки, антимикробная активность, антимикробные пептиды, энтероцины.

Abstract. We have partially characterized the bacteriocin activity of the strain Enterococcus faecalis T23. The antimicrobial activity of this strain was determined against Listeria monocytogenes, Salmonella typhimurium and closely related Lactobacilli strains. Treatment with different enzymes revealed that the active substance has a protein nature. PCR amplification resulted in detection of two bacteriocin genes: entB and entA. Bacteriocins of the studied strain were heat stable and active over a wide range of pH (3–10). Triton X-20, Triton X-80, Triton X-100, β-mercaptoetanol, Na-EDTA, SDS and NaCl did not influence the bacteriocin activity.

Key words: bacteriocins, enterococci, antimicrobial activity, antimicrobial peptides, enterocins.

Preservation of food by exploiting the antimicrobial potential of the endogenous microbiota is known as biopreservation, and has raised considerable interest in the last years. The application of specific proteinaceous antimicrobial compounds, called bacteriocins, to inhibit growth of many pathogenic and spoilage micro-organisms in food has set a new platform to develop novel and effective biopreservation strategies. These bacteriocins may be applied directly or produced during the manufacture of fermented products by bacteriocinogenic starters or adjunct cultures. Bacteriocin production is a widespread trait among LAB, including enterococci. Enterococci are frequently associated with fermented foods including cheeses and fermented milk. They produce powerful bacteriocins named enterocins [2, 3]. Enterocins represent antimicrobial compounds not only active against strains closely related to the producer micro-organisms but also displaying large spectra of inhibition against food-spoiling or pathogenic bacteria such as *Listeria* sp., *Staphylococcus aureus* or *Bacillus* sp [6, 9].

The aim of this study was the characterization of antimicrobial compounds produced by strain *Enterococcus faecalis* T23 isolated from Iranian cheese.

Materials and methods

Isolation and identification of bacteriocin-producer strain. The strain investigated in this study was isolated from white Iranian cheese manufactured from cow's milk. The preliminary determination of antimicrobial activity was performed by modified deferred antagonism test [11]. Phenotypic identification was performed by microscopic examination of cell morphology, Gram staining and catalase production. Genotypic identification was performed by 16S rDNA fragment sequencing. Genomic DNA purified with DNeasy purification kit (Qiagen, Hilden, Germany) was used as a template for PCR amplification of 16S rDNA fragment with universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5' TAA GGA GGT GAT CCA GGC-3') according to Weisburg et al. [12]. Nucleotide sequencing was analysed and the blast algorithm (http://blast.ncbi. nlm.nih.gov/Blast) was used to determine the most related sequence relatives in the NCBI nucleotide sequence database.

Antimicrobial activity. Antimicrobial activity in AU/ml was calculated according to spot-on-lawn method [13]. Cell-free supernatants of overnight (16-18h) cultures were obtained by centrifugation at 10000 x g for 15 min at 4°C and adjusted to ph 6.5 with 1N NaOH. The resulting sample was serially diluted twofold with 100 mM Na-phosphate buffer (pH 6.6). Soft nutrient agar was solidified in a sterile Petri dish after addition of 100 µl of an overnight culture of indicator strain. After 30 min of drying, 10 µl of each diluted sample was spotted onto the plate. The plates were incubated at 37°C overnight, and the titer was defined as the reciprocal of the highest dilution (2^n) that resulted in inhibition of the indicator lawn. Thus, the AU of antimicrobial activity per milliliter was defined as 2^n Ч 1000 µl 10 μl^{-1}.

Effect of pH, temperature, enzymes and different chemicals on antimicrobial activity. To evaluate the heat stability of the active substances, neutralized CFS had been incubated at 60 and 80 °C 30 min, in boiling water at 100^0C for 5, 15 and 30 min. The residual activity was then tested as described above. Untreated supernatants were used as control.

The pH sensitivity of the active substances was estimated by adjusting the pH of supernatants between 3 and 10 by using 1 N NaOH and 1 N HCl. After 2 h of incubation at 37^0C, the pH was adjusted to 6.5 and the residual activity was tested as described earlier. Nutrient media (MRS) adjusted to pH between 3 and 10, and untreated supernatants were used as controls.

In order to determine the biochemical nature of the active substance, the CFS of the strain was treated separately with proteolytic, amylolytic and lipolytic enzymes at a final concentration of enzyme of 1 mg/mL. After incubation of CFS with enzymes at 37 °C for 2 h the enzymatic activity was stopped by heating at 80 °C for 10 min and the remaining activity was tested as described earlier. Untreated CFS and reaction mixtures, where supernatant was replaced by MRS media, were used as controls.

The effect of different chemicals on antimicrobial activity was tested by treating CFS with SDS (sodium dodecyl sulfate) at a final concentration of 1% (w/v), Triton X-20, Triton X-80, Triton X-100, β-mercaptoethanol at a final concentration of 1% (v/v), Na-EDTA (ethylene diamine tetra acetic acid) at a final concentration of 1 mM and NaCl at a final concentration of 6.5% (w/v). Reaction mixtures were incubated for 2 h at 37 °C and the residual activity of treated CFS was tested as described before. Untreated CFS and the same chemicals re-suspended in MRS media were used as controls. All described experiments were performed in duplicate.

PCR amplification of bacteriocin genes. The PCR amplification was performed with the primers specific for different bacteriocins of *Enterococci* (Table 1). For all sets of primers, a reaction mixture, in a final volume of 50 µL, containing 30 ng of bacterial DNA, 1 x PCR reaction buffer, 1.5 mM MgCl2, 0.5 U of Taq DNA polymerase, 200 µM of dNTPs mix and 1 µM of each primer was prepared. Amplifications were carried out in Veriti® 96-Well Thermal Cycler (Applied Biosystems) using a program consisting of the initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at an appropriate temperature depending on Tm of primers for 1 min, elongation at 72 °C for 1 min and a final extension step of 7 min at 72 °C. Agarose gel (2%, w/v) in 0.5 x TAE buffer was used to visualize the amplification product by UV trans-illumination after staining with ethidium bromide (0.5 mg/mL).

Table 1

Primers used for detection of bacteriocin genes

Target gene	Primer (5' — 3')	Product size (bp)	Refer.
Ent A	F: GAG ATT TAT CTC CAT AAT CT R: GTA CCA CTC ATA GTG GAA	542	[1]
Ent B	F: GAA AAT GAT CAC AGA ATG CCT A R: GTT GCA TTT AGA GTA TAC ATT TG	159	[6]
Ent P	F: ATG AGA AAA AAA TTA TTT AGT TT R: TTA ATG TCC CAT ACC TGC CAA ACC	216	[8]
Ent L50A	F: CCA TGG GAG CAA TCG CAA AA R: AAG CTT AAT GTT TTT TAA TCC ACT CAA T	135	[2]
Ent L50B	F: ATG GGA GCA ATC GCA AAA TTA R: TAG CCA TTT TTC AAT TTG ATC	252	[4]
Ent 31	F: CCT ACG TAT TAC GGA AAT GGT R: GCC ATG TTG TAC CCA ACC ATT	130	[5]

Results and discussion

The strain investigated in this study was isolated from Iranian white cheese base on its antimicrobial activity profile. Preliminary identification by phenotypic methods showed that it was a Gram-positive, catalase-negative coccus forming short chains. Results of genotypic identifications revealed that it belongs to the *E. faecalis* species.

E. faecalis T23 presented antimicrobial activity against *Listeria monocytogenes* EGDe107776 (Fig.1), *Salmonella typhimurium* and also closely related *Lactobacilli* strains, such as *Lb. brevis* F145, *Lb. bulgaricus* L340. No inhibition of tested gram negative bacteria or yeasts was observed. The inhibitory activity of enterococci against *Listeria monocytogenes* were described previously by other authors [9, 10]. The absence of inhibitory activity against Gram-negative bacteria is in agreement with the data about enterococcal bacteriocins. Most of the bacteriocins show narrow spectra of antimicrobial activity and activity against Gram-negative bacteria is very rare [7].

The antimicrobial agent was heat stable and was active over a wide range of pH (3–10) (Table 2). Generally bacteriocins are heat stable peptides and high level of stability after heating and exposure for autoclaving was already reported for enterococcal bacteriocins [10]. The thermostability and pH stability of bacteriocins is a very useful characteristic for their application as food preservative, because many food-processing procedures involve strong heating steps, as well acidic conditions.

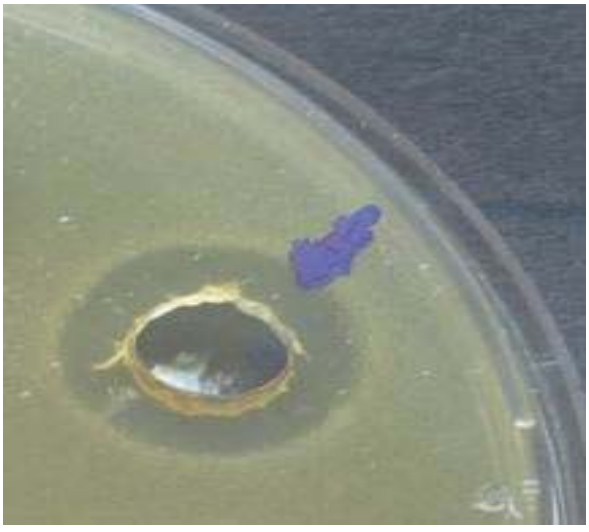

Fig. 1. Antimicrobial activity of *E. faecalis* T23 against *Listeria monocytogenes* EGDe107776

Table 2

Biochemical characterization of bacteriocins produced by strain E. faecalis T23

Treatments	**Antimicrobial activity in AU / ml**
Heating	
60°C 30 min	3200
80°C 30 min	3200
100°C 5 min	3200
100°C 15 min	3200
100°C 30 min	1600
pH	
3-10	3200
Enzymes	
protease K	200
pronase E	0
Trypsin	0
α-Chymotrypsin	0
catalase	3200
lipase	1600
α-amylase	3200
Chemicals	
SDS	3200
Triton X-20	3200
Triton X-80	3200
Triton X-100	3200
β-mercaptoethanol	3200
Na-EDTA	3200
NaCl 6.5%	3200

Activity was completely eliminated after treatment with proteinase K and α-chymotrypsin, and did not decrease in the presence of catalase, which excluded inhibition by hydrogen peroxide. These indicate the proteinaceous nature of the antimicrobial compound and thus we can almost surely conclude that active compound(s) produced by *E. faecalis* T23 is/are bacteriocin(s).

Effect of different chemicals on bacteriocin activities in cell-free supernatants of studied strain revealed, that Triton X-20, Triton X-80, Triton X-100, β-mercaptoetanol Na-EDTA, SDS and NaCl didn't influence antimicrobial activity, as we observed inhibition of the test organism with the treated supernatants

PCR amplification with specific primers resulted in detection of two bacteriocin genes in *E. faecalis* T23: *entB* and *entA* (Fig. 2). Both of identified enterocin genes encode bacteriocins, which belong to class II bacteriocins. Class II bacteriocins are small (<10 kDa), heat-stable, non-lanthionine-containing peptides. Their biosynthesis does not undergo extensive post-translational modification. The bacteriocins of this large group are classified in four subclasses (II a — d). Enterocin A is chromosomly-located bacteriocin and belongs to subclass IIa or pediocin-like bacteriocins, produced as pre-peptides with a leader sequence and containing the YGNGVXC motif at their N-terminus [1]. Enterocin B belongs to class IIc bacteriocins. This subclass contains the cyclic unmodified peptides, including sec-dependently secreted bacteriocins.

In the present study it was shown, that strain *E. faecalis* T23 produce antimicrobial compound with strong anti-*Listerial* activity. These compounds are most probably bacteriocins, as they are heat-stable and sensitive to proteolytic en-

zymes. Moreover the evidence of enterocin A and B encoding genes in the genome of the studied strain support this hypothesis. However further analysis, such as the mass spectrometric identification of peptide(s), study of expression, etc. should be performed. But at the present moment it is evident that due to its antilisterial activity, *E. faecalis* T23 could represent new adjunct culture for the dairy industry.

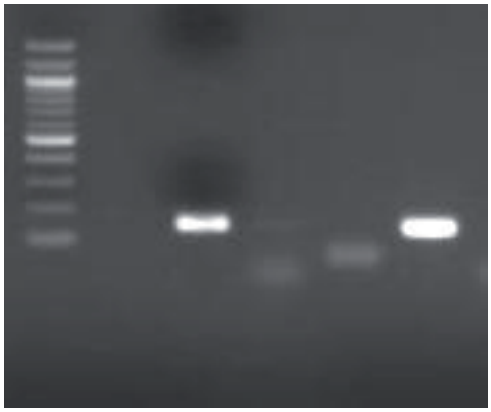

Fig. 2. PCR amplification of *entA* and *entB* genes in *E. faecalis* T23

REFERENCES:

1. Aymerich T., Holo H., Hevarstein L., Hugas M., Garriga M., Nes I.F. Biochemical and genetic characterization of enterocin A from Enterococcus faecium, a new antilisterial bacteriocin in the pediocin family of bacteriocins // Applied and Environmental Microbiology. — 1996. — № 62. — P.1676-1682.
2. Batdorj B., Dalgalarrondo M., Choiset Y., Pedroche J., Métro F., Prévost H., Chobert J.-M., Haertlé T. Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag // Journal of Applied Microbiology. -2006. — № 101. — P. 837-848.
3. Ben Belgacem Z., Abriouel H., Ben Omar N., Lucas R., Martinez-Canamero M., Galvez A., Manai M. Antimicrobial activity, safety aspects and some technological properties of bacteriocinogenic Enterococcus faecium from artisanal Tunisian fermented meat // Food Control. — 2010. — № 21. — P. 462–470.
4. Cintas L.M., Casaus P., Holo H., Hernàndez P.E., Nes I.F., Hevarstein L.S. Enterocins L50A and L50B, two novel bacteriocins from Enterococcus faecium L50, are related to staphylococcal hemolysins // Journal of Bacteriology . — 1998. — № 180. — P. 1988–1994.
5. De Vuyst L., Moreno M., Revets, H. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins // International Journal of Food Microbiology. — 2003. — № 84. — P. 299-318.
6. Du Toit M., Franz C., Dicks L., Holzapfel W. Preliminary characterization of bacteriocins produced by Enterococcus faecium and Enterococcus faecalis isolated from pig faeces // Journal of Applied Microbiology. — 2000. — № 88. — P. 482-494.
7. Gong H.S., Meng X.C., Wang H. Plantaricin MG active against Gram negative bacteria produced by Lactobacillus plantarum KLDS1 isolated from “Jiaoke”, a traditional fermented cream from China // Food Control., 2010, № 21. — P. 89-96
8. Gutiйrrez J., Criado R., Citti R., Martнn M., Herranz C., Nes I.F., Cintas L.M., Hernбndez P.E. Cloning, production and functional expression of enterocin P, a sec-dependent bacteriocin produced by Enterococcus faecium P13, in Escherichia coli // International Journal of Food Microbiology. — 2005. — № 103. — P. 239–250.
9. Leroy F., Foulquiй Moreno M.R., De Vuyst L. *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation // International Journal of Food Microbiology. — 2003. — № 88. — P. 235–240.
10. Rehaiem A., Martínez B., Manai M., Rodríguez A. Production of enterocin A by *Enterococcus faecium* MMRA isolated from ‘Rayeb’, a traditional Tunisian dairy beverage // Journal of Applied Microbiology. — 2009. — № 108. — P. 1685–1693.
11. Tagg J., Dajani A., Wannamaker L. Bacteriocins of Gram-positive bacteria // Bacteriological Reviews. — 1976. — № 40. — P. 722–756.
12. Weisburg W., Barns S., Pelletier D., Lane D. 16S ribosomal DNA amplification for phylogenetic study // Journal of Bacteriology. — 1991. — № 173. — P. 697-703.
13. Yamamoto Y., Togawa Y., Shimoska M., Okazaki M. Purification and characterization of novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11 // Applied and Environmental Microbiology. — 2003. — № 69. — P. 5746-5753.