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A Novel High-Molecular-Mass Bacteriocin Produced by *Enterococcus faecium*: Biochemical Features and Mode of Action

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Abstract
Discovery of a novel bacteriocin is always an event in sciences, since cultivation of most bacterial species is a general problem in microbiology. This statement is reflected by the fact that number of bacteriocins is smaller for tenfold comparing to known antimicrobial peptides. We cultivated *Enterococcus faecium* on simplified medium to reduce amount of purification steps. This approach allows to purify the novel heavy weight bacteriocin produced by *E. faecium* ICIS 7. The novelty of this bacteriocin, named enterocin-7, was confirmed by N-terminal sequencing and by comparing the structural-functional properties with available data. Purified enterocin-7 is characterized by a sequence of amino acid residues having no homology in UniProt/SwissProt/TrEMBL databases: NH2 - Asp - Ala - His - Leu - Ser - Glu - Val - Ala - Glu - Arg - Phe - Glu - Asp - Leu - Gly. Isolated thermostable protein has a molecular mass of 65 kDa, which allows it to be classified into class III in bacteriocin classification schemes. Enterocin-7 displayed a broad spectrum of activity against some Gram-positive and Gram-negative microorganisms. Fluorescent microscopy and spectroscopy showed the permeabilizing mechanism of the action of enterocin-7, which is realized within a few minutes.

Keywords Bacteriocin · Enterocin · *Enterococcus* · Class III · Antimicrobial peptides · Liquid chromatography

Introduction

During evolution, microorganisms acquire an effective chemical arsenal that regulates inter-microbial relationships in communities. Bacteriocins are biologically active protein moieties, which determine antagonistic activity of bacteria and play a significant role in the ability to widespread [1]. According to their structural and functional characteristics, bacteriocins can be related to antimicrobial peptides (AMPs) produced by eukaryotes. Meanwhile bacteriocins are a relatively small group compared to other AMPs, since they are included in about 10% of the total known AMPss [2]. This disparity is due to the fact that most bacterial species are uncultivated in laboratory conditions [3]; in addition, separation of bacteriocins from microbial metabolites is an another problem [4]. Partial solution of stated problems is provided by searching for new bacteriocins using genome database mining approach [5, 6]. Nevertheless, purification of novel bacteriocins is a significant event in microbiology and related sciences. Every finding expands the knowledge about the microbial world and can be used in biotechnological or medical sectors [7].

The classification of bacteriocins undergoes changing and updating, which led to the existence of several classification schemes [8]. In our opinion, the most objective classification includes three groups of bacteriocins [9]. The first group includes bacteriocins that are subjected to enzymatic modifications during biosynthesis, as the mature peptide contains unusual amino acids. The second group of bacteriocins is the most diverse, characterized by molecular mass of less than 10 kDa. The third group is represented by proteins with molecular mass of more than 30 kDa. Significant numbers of novel bacteriocins were classified to the first and second groups, but few bacteriocins were classified in the third group [8].

We report on the purification and structural-functional features of enterocin-7, which is a novel bacteriocin produced by

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Enterococcus faecium ICIS 7 strain isolated from human feces. This strain is characterized by the presence of antagonistic activity against Gram-positive and Gram-negative strains.

Achievement of the stated goal was realized through complex approaches. Initially, we performed optimization of medium composition to reduce chromatographically steps. At the next stage, the one-step liquid high-performance chromatography was successfully used. The mode of action was assessed using the fluorescent spectroscopy and routine bacteriological methods.

Materials and Methods

Cultivation Condition

Investigations of growth and bacteriocin production were performed using the bacteriocin-producing strain *E. faecium* ICIS 7. Bacteria were incubated in 50-ml flacons at 37 °C for 24–36 h on a simplified media (Table 1). The dynamic of bacterial growth was assessed by reading and plotting the absorbance data at 620 nm obtained by spectrophotometer IEMS MF (Labsystems, Finland). Evaluation of the bacteriocin content was performed by isolating an aliquot of the culture medium followed by centrifugation (20 min, 4 °C, 9000 g) and then filtering through a Durapore PVDF filter with a pore size of 0.22 μm (Millipore). The activity of the supernatant was evaluated by agar diffusion assay (described below) against *Listeria monocytogenes* EGDe and was expressed in arbitrary units per milliliter, calculated according to formula (1) [10]:

$$AU = (1000/V)*D$$

where,

V supernatant volume
D dilution factor

Purification of Bacteriocin

The chromatography manipulation was performed as described earlier [11]. In the next step, desalting of the culture medium was performed using reversed-phase low-pressure chromatography on an FPLC column completed with a Synchroprep RP-P C_{18} phase (SynChrom Inc., USA) equilibrated with solvent A (10% acetonitrile; Merck, Germany) in ultra-purified water (18 Mohms, Milli-Q, Millipore) with 0.1% trifluoroacetic acid (TFA). Elution was performed using solvent B (80% acetonitrile in ultra-purified water with 0.1% TFA) followed by lyophilization to withdraw a residual quantity of TFA and concentration. The obtained desalted extract was tested to reveal antibacterial properties. The lyophilized mass was resuspended in ultrapure water and applied to semi-preparative column C_{18} Luna (250 × 10 mm, Phenomenex, USA) integrated into a high-performance liquid chromatography (HPLC) system (Knauer SmartLine 200, Knauer, Germany). Elution was performed using solvent B (80% acetonitrile in ultrapure water with 0.1% TFA) in a linear gradient according to the following scheme: 5–80% for 60 min at a flow rate of 2.0 ml/min. Absorbance was detected at 214 nm.

Table 1 Composition of media used for enterocin-7 production

Components	Content
Yeast extract (g L)	3.0
Minerals (g L)	
Dipotassium hydrogen phosphate	7.7
Potassium dihydrogen phosphate	2.2
Magnesium sulphate	0.1
Ferrous sulphate $7H_2O$	0.008

Structural Analysis of Enterocin-7

SDS-PAGE of the Active Fraction

The electrophoretic separation of the active fraction obtained by RP-HPLC was conducted according to the protocol described [12]. To an analyzed sample an equal volume of buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol was added. 1 ml of a 0.2% solution of bromophenol blue was mixed with the sample and applied to the wells of the polyacrylamide plate consisting of 4% in the stacking gel and 10% in the separating gel. The phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (Amersham Biosciences, USA) were used as the markers of molecular mass of the proteins.

After the electrophoresis (1.5–2.0 h) gel was transferred to PVDF membrane "Immobilone," the membrane was stained with 0.1% solution of Coomassie R-250 in 50% aqueous methanol for 3–5 min, and then washed with water.

N-Terminal Sequencing

The proteins were sequenced by automated Edman degradation on a model PPSQ-33A protein sequencer (Shimadzu Corp., Japan) according to the manufacturer's protocol. Cysteine residues were determined as pyridilethylated derivatives. Homology search was performed using databases with a BLASTP algorithm.

Effect of Enzymes and Temperature Treatment on Activity

The protease susceptibility was carryout by incubation of enterocin-7 with 10 mg mL of protease K and 20 mg mL of

trypsin (biotechnology grade, Sigma-Aldrich, USA) at 37 °C for 1 h in buffer (5 mM HEPES, pH 7.5) as described in [13]. Enterocin-7 in buffer without enzymes was used as positive control; enzyme in buffer solution and buffer alone were used as negative controls. Evaluation of thermostability of enterocin-7 was performed by heating in the solid-state thermostat "Thermit" designed for microfuge tubes ("DNK-Technologiya," Russia). The inhibitory activity of active fraction after treatments were determined using the agar diffusion assay.

Determination of Antimicrobial Activity by Agar Diffusion Assay

Determination of bacterial metabolites' bactericidal properties, HPLC fractions, and purified enterocin-7 was performed using the agar diffusion assay [14]. Briefly, the microorganisms (Table 3) were cultured for 18 h in LB broth (HiMedia, India), after which 50 μL of the bacterial suspension (containing ~ 10^7 CFU) was mixed with 10 mL of soft (0.6%) LB agar and placed immediately over a Petri dish, which was previously overlaid with a 1.5% agar plate. The test samples were transferred on the upper layer of soft agar. After incubation at 37 °C overnight, inhibitory areas were observed.

Characterization of Mode of Action of Enterocin-7

Determination of Minimal Inhibitory Concentration

To evaluate the minimal inhibitory concentration of enterocin-7, *L. monocytogenes* EGDe was incubated in 96-wells microtiter plate with twofold dilutions of the enterocin-7 according to protocol [15]. Dynamic of bacterial growth was assessed by reading and plotting the absorbance data at 620 nm obtained by spectrophotometer IEMS MF (Labsystems, Finland). Antimicrobial activity was expressed by the minimal inhibitory concentration (MIC), which was defined as the lowest peptide dose at which no visible growth were detected.

Permeabilization of the Plasma Membrane

The LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, USA) was used to evaluate the cytoplasmatic membrane integrity of *L. monocytogenes* EGDe according to the manufacture's protocol. The fluorescence microscopy of stained bacteria was performed using a Mikromed-3-Lum fluorescent microscope (Lomo, Russia) equipped with filter sets useful for simultaneous viewing of SYTO 9 and PI stains.

Measurement of SYTO 9 fluorescence kinetic was performed using a plate reader Infinite F200 pro (Tecan, Austria) at 485 nm emission and 535 nm of excitation wavelength as described earlier [16]. Indolicidin and pure water were used as positive and negative controls, respectively.

Statistical Analysis

All experiments were preformed using three independent series.

Results

Purification of Enterocin-7 and Structure Determination

E. faecium ICIS 7 was quickly adapted to the simplified nutrient medium (Table 1) since the exponential phase was finished at 6 h of cultivation. Bacteriocin production was observed in the middle of the stationary phase of bacterial growth (Fig. 1). After cultivation, the growth medium was desalted by low-pressure liquid chromatography and, after evaporation of acetonitrile, applied on a semi-preparative C_{18} reverse-phase HPLC column. The chromatographic profile obtained was characterized by 23 main peaks (Fig. 2a) among which antimicrobial activity was demonstrated for the most hydrophobic fractionwith a retention time of 34–37 min. The active fraction demonstrates thermostability when heated at 90 °C for 30 min (Table 1). Enzymatic treatments with proteinase K and trypsin led to complete loss of activity, which is preliminarily suggested about a proteinaceous nature of the active substance (Table 2).

PAAG electrophoresis of the concentrated fraction in native denaturated and denaturated/reduced conditions revealed two separated stained bands characterized by similar mobility in polyacrylamide gel (Fig. 2b). The upper band have comparable electrophoretic mobility with the bovine serum albumin, and its molecular mass was determined to be about 65 kDa, whereas the bottom band was approximately 50–55 kDa. A preparative PAAG electrophoresis, followed by electroblotting to Immobilon PVDF hydrophobic membrane, was carried out for structure identification of detected proteins (named Ent-1 and Ent-2 according to Fig. 1b). Both bands were cut and analyzed separately by Edman sequencing. As a result, for Ent-1, we could estimate 15 N-terminal amino acid residues: NH2 - Asp - Ala - His - Leu - Ser - Glu - Val - Ala - Glu - Arg - Phe - Glu - Asp - Leu-Gly; for Ent-2, only eight amino acid residues were identical to Ent-1. Thus, both isolated proteins are homologous, but differences in molecular mass hypothetically can be explained by partial absence of amino acid residue at the C-terminal region of the Ent-2. Concerning homology analysis using a BLASTP algorithm, there was no identity among *E. faecium* deposited in UniProt/SwissProt/TrEMBL primary structure databases; the same result was obtained for the whole *Enterococcus* genus. When result had analyzed, no translated amino acid sequences (including ESTs) were found. We tentatively designated this bacteriocin enterocin-7.

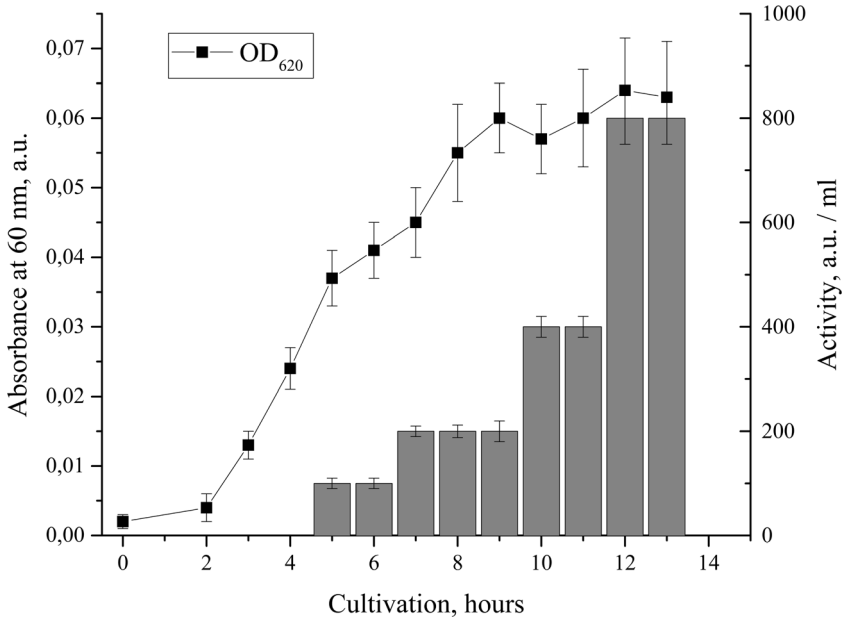

Fig. 1 The growth curve of *E. faecium* ICIS 7 and the accumulation of enterocin-7 in the medium, expressed in relative units

Biological Activity of Enterocin-7

Qualitative evaluation revealed the ability of enterocin-7 to inhibit growth of some Gram-positive and Gram-negative microorganisms (Table 3). Agar diffusion assay revealed a more pronounced inhibitory effect of enterocin-7 on Gram-positive than on Gram-negative strains. The most sensitive strain of *L. monocytogenes* EGDe was selected for further studies as a model. The addition of enterocin-7 to the culture medium inoculated by *L. monocytogenes* EGDe allowed to record of the minimal inhibitory concentration (MIC), which was 1.75 μg mL (Fig. 3).

Effects of enterocin-7 treatment on bacterial membranes were evaluated with fluorescent spectroscopy and microscopy

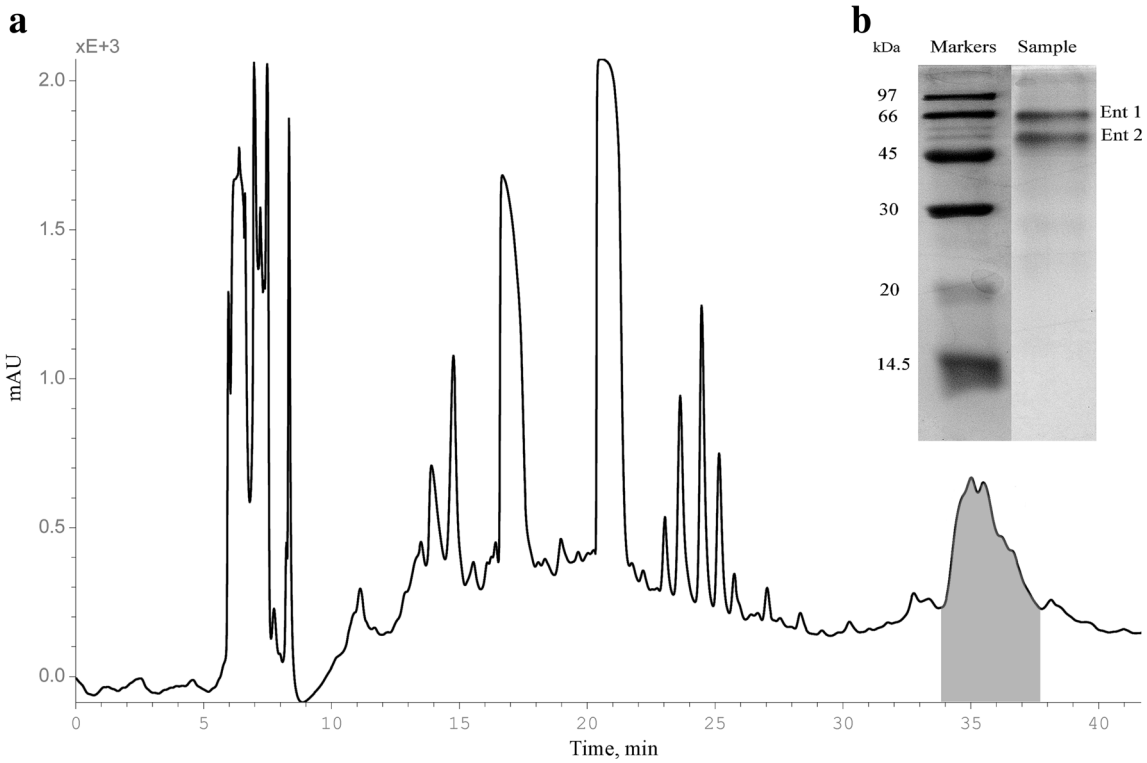

Fig. 2 **a** The chromatographic profile of purification of the *E. faecium* ICIS 7 metabolites using a semi-preparative RP-HPLC. The active fraction is marked in gray color; **b** the separation profile of the active fraction by 2-ME SDS-electrophoresis in 10% polyacrylamide gel

Table 2 Characteristics of the enterocin-7 produced by *Enterococcus faecium* ICIS 7

Treatment	Diameter of inhibition zone (mm)
Enzyme treatment	
Proteinase K	0
Trypsin	0
Positive control	1.5
Negative controls	0
Heat treatment	
60 °C/30 min,	1.20
80 °C/30 min	1.05
95 °C/30 min	1.25
Control	1.21

imaging. Using a commercial staining kit containing DNA-intercalating dyes, we visualized treated bacterial cells with a permeabilized membrane (Fig. 4b). Moreover, we traced the kinetic of fluorophore accumulation within bacterial cells and found that enterocin-7 was able to disturb cell' membranes very quickly (Fig. 4c). The lightweight green fluorescence SYTO 9 (~400 Da) freely penetrates even through integral bacterial membrane, in contrast heavier red fluorescence PI (668 Da) is accumulated inside only through disordered plasma membrane. Membrane disordering was spectroscopically recorded as quenching of the green fluorescence since the SYTO 9 replaces by PI.

Discussion

Enterococci are among of the most important intestinal microbiota of humans and animals [17]. The significance of Enterococci for human is controversial, because while known to cause infectious disease, but they are still actively used in the food industry. Benefits which are provided by enterococci partially are

Table 3 The spectra of strains susceptible to enterocin-7 action, assessed by agar diffusion assay

	Strains	Halo of inhibition zone, cm
Gram-negative	*E. coli* MG1655	0.4
	E. enterica ATCC 14028	0.3
	S. typhimurium ATCC 140289	0.25
	P. aeruginosa ATCC 27853	0.5
Gram-positive	*B. cereus* ATCC 14893	0.63
	S. aureus FDA 209 P	0.3
	L. monocytogenes EGDe	1.66
	E. faecium ICIS *5*	1.2

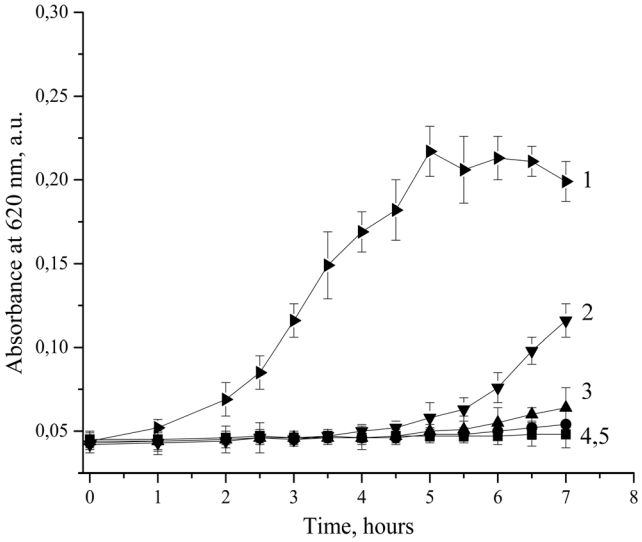

Fig. 3 The effect of enterocin-7 on the growth of *L. monocytogenes* EGDe. Enterocin-7 was added at a starting point of cultivation at 1–0 μg mL (negative control), 2–0.22 μg mL, 3–0.44 μg mL, 4–0.88 μg mL, and 5–1.75 μg mL

determined through bacteriocin's production. For example, bacteriocin-producing enterococcus introduced in the gut can able to replace indigenous enterococci including vancomycin-resistant strains, which are lacking bacteriocins [18].

Unlike peptides of animal origin, bacteriocins are much more difficult to study, because bacteria usually synthesize bacteriocins in small amounts, while their extraction and purification from microbial metabolites is a non-trivial task. The main problems of purification are related to the fact that bacteria grow and produce bacteriocins in multi-component media saturated with various proteins and peptides (for example, beta-casein hydrolyzate). To obtain a homogeneous form of bacteriocins, a variety of isolation and purification methods needs to be used such as precipitation, ion-exchange chromatography, and multiple reversed-phase HPLC (semi-preparative and analytical) [8, 19].

Effectiveness of liquid chromatography may be increased due to cultivation on media with a minimum content of proteins and other hydrophobic components that impede purification of bacteriocins [20, 21]. For preparation of the simplified medium, we used only inorganic salts and yeast extract. According to the manufacturer (Becton Dickenson, BBL), the used yeast extract consisted of 70% peptides with molecular masses of about 250 Da and 20% with 0.5–2.0 kDa. Thus, using the yeast extract as a one component for cultivation medium reduces loading of chromatographically column by proteins and peptides. Respectively, this approach decreases number of purification steps and increases the efficiency of bacteriocin purification from *E. faecium* metabolites. Of course, not all strains grow and produce bacteriocin on media with a minimal nutrient content. Moreover, using of specialized rich media such as MRS or Shaedler increase production of bacteriocins, but ultimately it

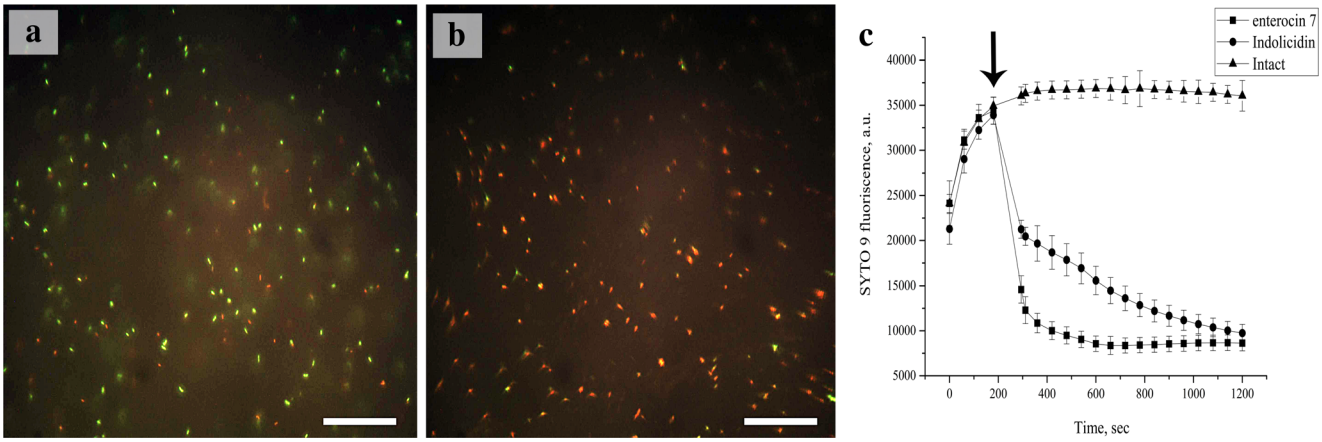

Fig. 4 The fluorescence microscopy images of intact (**a**) cells of *L. monocytogenes* EGDe and treated with enterocin-7 (**b**). Dynamics of permeation of SYTO 9 into bacterial cells treated with AMPs (**c**): enterocin-7 at 2 MIC; indolicidin at 2 MIC (positive control). Arrows show the time when test substances were added. If bacterial membranes are permeabilized, PI penetrates into the cell after displacing the SYTO 9 from DNA, which leads to decreasing fluorescence intensity in a green region of the spectrum (emission at 535 nm). The scale bar - 10 μm

complicates purification and reduces the bacteriocin content at each step of chromatography.

Today, performed enquiry to the antimicrobial peptide database (APD) resulted in 36, entries, which are corresponded to various annotated enterocins [2]. The greatest number of enterocins belongs to class II, only a few large proteins related to class III have been revealed [22, 23]. According to the existing classification of bacteriocins, large-molecular- mass and heat-labile antimicrobial proteins are classified as class III [8, 9]. If compared with existing representatives, enterocin-7 has significant differences. Apparently, purified active fraction is represented by large protein with a molecular mass of about 65 kDa. Currently, only bacteriocin 41 (Bac41), produced by *E. faecalis* have a similar molecular mass > 60 kDa (GenBank ID: BAH02371.1) [22]. It is important to note that enterocin-7 had no homologue with previously isolated bacteriocins and predicted sequences encoded with potential bacteriocin-like proteins. Comparison of the first 15 amino acids of enterocin-7 with existing protein sequences from the *Enterococcus* genus and Bacteria kingdom revealed no sequence similarity to any known proteins or peptides.

Secondly, enterocin-7 is a heat-stable protein. Only a few high-molecular-mass bacteriocins are known, but they are characterized by heat-labile property [22, 24–28]. Previously, we isolated a novel heat-stable bacteriocin-like protein, which is secreted by the *E. faecium* ICIS 8 into culture liquid. This protein has a molecular mass of about 14 kDa, and consists two Cys residues located in the N-terminal part of the molecule [11].

In addition, enterocin-7 is active against some Gram-negative bacteria, which is intrinsic to bacteriocins of low molecular mass, for example enterocin E-760 [19]. The ability to inhibit bacteria with a Gram-negative cell wall type is a property of a relatively small fraction of known bacteriocins. According to the APD database, enterocins which are inhibited both Gram-positive and Gram-negative bacteria constitute the minor group (< 15%). It is difficult to identify any physico-chemical features of enterocins that determine their ability to kill Gram-negative bacteria. Whereas anti-Gram-negative activity revealed for various enterocins including lantibiothics [29], circular [30], and two-chain peptide [31].

Another feature of enterocin-7 is rapid permeabilization of bacterial membranes like peptides, but not proteins. For example, interaction of lacticin 3147 with Gram-positive bacteria resulted in potassium efflux immediately after addition [32]; enterococcal cytolysin interact in a similar way [33]. In turn, known proteins related to class III have endopeptidase enzymatic activity that is realized within several hours [24, 34]. The time-killing dynamic similar with enterocin-7 was described for zoocin A that stopped growth of *S. mutans* within 10 min [35].

Thus, the isolated enterocin-7 has some differences from known enterocins; moreover, it differs from other bacteriocins too.

Conclusion

In the present work, according to the results of amino acid sequencing, we report firstly about a novel bacteriocin. On the one hand, a molecular mass of 65 kDa allows to classify enterocin-7 as a class III bacteriocin. On the other hand, a number of features are not in accordance with existing data. However, the small number of bacteriocins of class III does not allow us to speak unambiguously about distinctive or non-distinctive features. Further study will be focused on revealing the complete primary structure of enterocin-7 and detailed molecular characteristics of interaction with cellular targets.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no competing interests.

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