DETECTION, PURIFICATION AND PARTIAL CHARACTERIZATION OF A NOVEL BACTERIOCIN SUBSTANCE PRODUCED BY *LACTOCCOUS LACTIS* SUBSP. *LACTIS* B14 ISOLATED FROM *BOZA*—BULGARIAN TRADITIONAL CEREAL BEVERAGE

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Introduction

Cereals are subjected to fermentation in almost all regions of the world where cereals are consumed as stable food and therefore variety of raw materials and conditions of perspective fermentations are quite large. In these fermentations lactic bacteria are the predominant organisms together with yeasts.

Boza is a Bulgarian traditional drink based on cereals widely consumed by people of all ages in the Balkan Penninsula. It is a viscous non-alcoholic beverage, produced from different cereals(or mixture of them) and slightly fermented. Little information is available about its microflora [4].

The aim of using bacteriocins to improve the microbial quality and safety of food has stimulated intensive research efforts in recent years. The best characterized inhibitor to date is nisin, a bacteriocin produced by certain strains of *Lactococcus lactis*. A number of lactococci exhibit antimicrobial activities different from nisin were isolated from different products [3, 6, 13]. The lactococcal bacteriocins examined so far are all small heat stable proteins that kill closely related bacteria. Some of them desighed as Lactococcin Gal, Lactococcin Gb, Lactococcin A, Lactococcin B [5, 12, 17]. The sequence analysis showed that they belong to different subgroups,of class 2 characterized by their heat stability and dimensions less than 10 kDa [10].

The antagonistic activity of lactic acid strains isolated from boza was performed. As a result of these screening several strains were found to produce bacteriocin like substances. The strain identified by physiological and biochemical techniques and *Lactococcus. lactis* subsp. *lactis* B14 was used as producer of antimicrobial substance designed as Bozacin 14.

Here we report the characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* with wide inhibitory spectrum including a group of foodborne pathogens.

1. Materials and Methods

1.1. Strains and media

Lactococcus lactis subsp. *lactis* B14, the producer strain, was isolated from *boza* (traditional Bulgarian cereal drink, produced from "Belgrano", Sofia, Bulgaria) using MRS and Elliker broth (Biokar, Beauvais, France) and agar (Biokar, Beauvais, France). The strain was indentified by physiological and biochemical techniques and API 50 carbohydrate galleries (Biomérieux, Marcy-l'Etoile, France). Before experimental use, culture of the producer strain was cultivated successively in MRS medium at 30 °C. The microorganisms used for the tests were propagated in appropriate media as indicated in Table 1. The strain of *Lactococcus lactis* subsp. *lactis* B14 was stored at −80 °C in MRS broth containing 15% (v/v) glycerol. Before use, the strain was cultivated for 24 h at 30 °C in MRS broth.

1.2. Plasmid isolation

Plasmid isolation was done according to the method of Anderson and Kay, 1983.

1.3. Bacteriocin activity assay

Bacteriocin sreening was performed by two methods - the agar spot test and the well diffusion method as described by Schillinger and Lucke (1989) and Tagg and McGiven (1971). Normally 1.5% agar was used. For overlay 1.0% soft agar was prepared. In order to eliminate the action of lactic acid bacteria on the test organisms, the pH of the tested supernatants was adjusted to 6.0 with NaOH. The activity was expressed in arbitary units (AU ml^{-1}). One AU was defined as the reciprocal of the highest serial two fold dilution showing a clear zone of growth inhibition of the indicator strain. (Barefoot and Klaenhammer, 1983). *Listeria innocua* F (ENITIAA) and *Esh. coli* HB1O1(IMB) were used as sensitive strains.

The inhibitory spectrum was studied by using test microorganisms belonging to different species as listed in Table 1.

1.4. Production studies

Tween 80-free MRS medium prepared from basal ingredients was sterilized by autoclaving (15 min , 121 °C) and was aseptically transferred to a bioreactor connected to an automatic pH and temperature controller (Set 2M; SGI Toulouse, France). It was then inoculated with 10% (v/v) of an overnight culture of *Lactococcus lactis* subsp. *lactis* B14. pH was maintained at 5.5 with 6N NaOH. The temperature was held at 30 °C, and agitation was set at 100 rpm. Samples were removed at different time intervals

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Table 1

Inhibitory activity of neutralized, cell free supernatant of *Lactococcus lactis* 14 against lactic acid bacteria and other indicator strains

Indicator Strain	Inhibitory activity	Source	Media
Lactobacillus plantarum 1011	++	SD PC	MRS
Lactobacillus plantarum 1033	–	SD PC	MRS
Lactobacillus plantarum 1060	–	SD PC	MRS
Lactobacillus plantarum 1095	++	SD PC	MRS
Lactobacillus plantarum 1175	++	SD PC	MRS
Lactobacillus plantarum 1189	+	SD PC	MRS
Lactobacillus plantarum 1220	–	SD PC	MRS
Lactobacillus plantarum 1254	++	SD PC	MRS
Lactobacillus plantarum 1287	–	SD PC	MRS
Lactobacillus plantarum 1341	+	SD PC	MRS
Lactobacillus plantarum 1361	–	SD PC	MRS
Lactobacillus plantarum 1383	++	ENITIAA	MRS
Lactobacillus plantarum 1390	–	Ldc Boll	MRS
Lactobacillus plantarum 1397	–	Ldc Boll	MRS
Lactobacillus plantarum 1408	++	ENITIAA	MRS
Lactobacillus plantarum 1409	–	IP	MRS
Lactobacillus plantarum 14917	–	ENITIAA	MRS
Lactobacillus plantarum 73	+++	ENITIAA	MRS
Lactobacillus casei subsp. casei 1374	+++	ENITIAA	MRS
Lactobacillus casei subsp. casei 1038	–	SD PC	MRS
Lactobacillus casei subsp. casei 1416	++	SD PC	MRS
Lactobacillus casei subsp. pseudoplantarum 1125	+++	SD PC	MRS
Lactobacillus casei subsp. pseudoplantarum 1127	+++	SD PC	MRS
Lactobacillus casei subsp. pseudoplantarum 1128	+++	SD PC	MRS
Lactobacillus casei subsp. pseudoplantarum 1131	+++	SD PC	MRS
Lactobacillus casei subsp. pseudoplantarum 1141	–	SD PC	MRS
Lactobacillus brevis 1078	–	SD PC	MRS
Lactobacillus brevis 1104	++	SD PC	MRS
Lactobacillus brevis 1142	–	SD PC	MRS
Lactobacillus curvatus 1307	++	SD PC	MRS
Lactobacillus curvatus 1371	–	SD PC	MRS
Lactobacillus delbrueckii ssp. delbrueckii 1280	+++	SD PC	MRS
Leuconostoc mesenteroides ssp. dexranicum 1185	+++	LdC	MRS
Leuconostoc mesenteroides ssp. dexranicum 1414	+++	SD PC	MRS
Leuconostoc mesenteroides 1044	+++	SD PC	MRS
Leuconostoc mesenteroides 8293	–	ATCC	MRS
Leuconostoc mesenteroides 1000	–	SDPC	MRS
Leuconostoc mesenteroides 1324	–	SDPC	MRS
Leuconostoc mesenteroides 1228	–	SDPC	MRS
Lactococcus lactis ssp. cremoris 117	+++	INRA CNRZ	MRS
Lactococcus lactis subsp. lactis ATCC 11494	++	IP	MRS
Lactococcus lactis subsp. lactis CNRZ 1075	++	INRA	MRS
Lactobacillus alimentarius 1404	++	IP	MRS
Listeria innocua F	+++	ENITIAA	MRS
Listeria innocua I	+++	ENITIAA	Elliker
Carnobacterium piscicola 2762	–	NCDO	Elliker
Leuconostoc dextranicum 1055	+++	SDPC	Elliker
Leuconostoc dextranicum 1414	+++	LdC	MRS
Leuconostoc dextranicum 1185	+++	SDPC	MRS
Pediococcus pentosaceus 1164	+++	SDPC	Elliker
Pediococcus pentosaceus 1272	–	SDPC	Elliker
Clostridium butyricum	–	ENITIAA	Elliker
Clostridium sporogenes	–	ENITIAA	Elliker
Listeria monocytogenes	++	ENITIAA	Elliker
Citrobacter freundii	–	ENITIAA	Elliker
Yersinia enterocolitica	–	ENITIAA	Elliker
Klebsiela pneumoniae	–	ENITIAA	Elliker
Proteus vulgaris	–	ENITIAA	Elliker
E. coli HB101	+++	IMB	Luria
E. coli	++	ENITIAA	Elliker
P. fluorescens	–	ENITIAA	Elliker

Notes. ATCC—American Type Culture Collection, Rockville, MD; ENITIAA—Ecole Nationale des Ingnieurs des Techniques des Industries Agricoles et Alimentaires, Nantes, France; INRA—Institut National de la Recherche Agronomique; SDPC—Sourdough Private Collection; LdC—Levain de Cracker USA; IP—Institut Pasteur; NCDO—National Collection of Dairy Organism, Reading, UK IMB—Collection of the Institute of Microbiology, Bulgarian Academy of Science, Sofia, Bulgaria;
+++ inhibitory activity 10 mm; ++ inhibitory activity 5 mm.

for determination of optical density at 600 nm and antimicrobial activity.

1.5. Effect of enzymes, pH, detergents, and heat treatment

The sensitivity of the active substance to proteolytic and other enzymes was tested on cell-free supernatant (pH 6.0) of 24 h cultures incubated at 30 °C. Samples of 100 μl were treated for 2 h with 0,1 mg ml^{-1} and 1,0 mg ml^{-1} final concentration of the following enzymes: pronase E, proteinase K, pepsin, trypsin, α-chymotrypsin and rennine.

The surfactants tested were sodium dodecyl sulphate (SDS), Tween 20, Tween 80, N-laurilsarcosine, Tritone X-100, EDTA, PMSF and urea at final concentration 0.1, 1, 2 or 5%. Controls, consisted of either active supernatant or detergents used. All samples and controls were incubated at 37 °C for 5 h and tested for activity. The sensitivity of the active substance to different pH was estimated by adjusting the pH of supernatant samples to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 with NaOH or HCl and testing against the indicator strain after 30 min and 2 h incubation. The sensitivity to heat was tested by heating cell free supernatant samples to 30, 40, 50, 60, 70, 80, 90, 100, and 121 °C and testing the residual activity after 5, 10, 15, 20, 30, and 40 min by the agar diffusion assay.

1.6. Cell lysis

To study the effect of the antibacterial compound on sensitive cells, 2 ml of culture supernatant (pH 6.0) was added to 10 ml ml growing cells of *Listeria innocua* F (ENITIAA) in Elliker broth medium in early exponential phase. The absorbance was determined at appropriate intervals.

1.7. Adsorption studies

The method of Yang et al. (1992) was used. After 18 h of cultivation 300 ml cultures were adjusted to pH 6.0. Following centrifugated and washing in sodium phosphate buffer, pH 6.5, the cells were resuspended in 10 ml 100 mM NaCl (pH 2.0) and stirred for 1 h at 4 °C. Cell suspensions were centrifugated and the supernatants were tested for activity after neutralization.

1.8. Bacteriocin purification

The bacteriocin was purified from a culture of *Lactococcus lactis* subsp. *lactis 14* grown in MRS medium at 30 °C at pH 5,5. After 8 h incubation the culture was centrifuged for 30 min at 12 000 g, 4 °C. The proteins were precipitated with 80% ammonium sulphate for 24 h at 4 °C and centrifuged for 50 min at 17 400 g. The pellet was resuspended in 10 m of 3 M urea and loaded on a Sep-Pack C 18 cartridge (Waters, Millipore). The cartridge was washed with 10%, 40%, and 80% acetonitrile. After drying under reduced pressure (Speed-Vac; Savant) the bacteriocin fraction was dissolved in 10% acetonitrile, 0,1% trifluoroacetic acid (TFA) in water. This fraction was used for final purification by reversed-phase perfusion liquid chromatography on a column PrepLC Universal Base Waters, RP-18 (120×25 mm, 15–20 μm) on chromatography system (Biocad Sprint system, PerSeptive Biosystem, Voisins les Bretonneux, France). Bacteriocin was eluted with the following mobile phases: A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile). Peptides were monitored spectrophotometrically at 220 and 280 nm, at a flow rate 20.0 ml/min. The fractions with highest bacteriocin activity were mixed and evaporated on a Speed-Vac concentrator (Savant). Eluted peaks were dried under vacuum, dissolved in deionized water and store at -20 °C. Their protein content was estimated by the BCA Protein Assay Kit and their antagonistic activity was determined at each step of the purification process.

1.9. Determination of the amino acid composition

The purified bacteriocin was first hydrolysed in 6 N HCl (Pierce) for 24 h at 110 °C under vacuum in a Pico-Tag station (Waters). The amino acids were derivatised with phenylisothiocyanate (PITC) according to the method of Bidlingmeyer *et al.* (1984) and separated by RP-HPLC on a Pico-Tag C_{18} column (3.9 mm $\times$ 15 cm). The column was equilibrated with solvent A (94% 0.14 M CH_3COONa, 0.5 ml TEA/L, pH 6.4 / 6% acetonitrile) and elution was performed by using a gradient from solvent A to B (40–60% H_2O / acetonitrile). Both the column and solvents were maintained at 38 °C, the flow rate was a 1.0 ml/min and absorbance was monitored spectrophotometrically at 254 nm.

2. Results

2.1 Inhibitory activity and spectrum

Lactococcus lactis subsp. *lactis* B14 was isolated from a cereal fermented product boza (traditional Bulgarian beverage) using MRS and Elliker broth and agar. The strain was identified by physiological and biochemical techniques and API 50 carbohydrate galleries. The analysis of plasmid content revealed the presence of one big plasmid with molecular weight of 8,4 kb approximatively (data not shown).

It was found to possess antibacterial activity both by the agar spot method and the well diffusion method. It inhibited only wide range of strains from the group of closely related LAB species. Activity of neutralized culture filtrates was displayed against 9 strains of *Lactobacillus plantarum*, two of *Lactobacillus casei* subsp. *casei* and four of *Lactobacillus casei* subsp. *pseudoplantarum*. One strain of *Lactobacillus brevis* and two of *Lactobacillus curvatus* were sensitive to the studied antibacterial substance too. *Leuconostoc mesenteroides* and *Leuconostoc mesenteroides* subsp. *dextranicum* were sensitive to the cell free extract too. The studied substance was active against strains of the food pathogen Listeria. No activity was detected to other studied pathogens with one exception *E. coli* (Table 1).

2.2. Characterization of the inhibitory agent

The activity of the inhibitory agent was tested under conditions which eliminate the possible effect of organic acids by adjusting the pH of the cells free supernatant to pH 6,5 and of hydrogen peroxide by catalase treatment. In the both cases the activity was not affected.

Table 2

Purification of bacteriocin Bozacin 14 produced by *Lactococcus lactis* subsp. *lactis* B14

Purification stage	Volume (ml)	Activity (AU ml^{-1})	Protein (mg/ml)	Specific activity (AU/mg protein)	Purification factor	Recovery (%)
Culture supernatant	500	6400	13.6	470	1	100
Ammonium sulphate precipitation, 80%	10	6400	9.3	688	1.5	68
Sep-pack RP-18	1	12800	1.3	9850	20.9	9.55
HPLC reverse phase RP-18	0.200	12800	0.7	18285	47	5.14
HPLC reverse phase rechromatography	0.200	12800	0.6	21333	45	4.41

The effect of various enzymes on the inhibitory agent was studied. Complete inactivation or significant reduction in activity was observed after treatment of the cell free supernatant or purified inhibitoty substance with proteinase K, pronase E, pepsin which indicated of the proteinaceous nature of the active agent. At the same time trypsin, chymotrypsin and rennine had no effect. The other enzymes tested in our study (amylase and lipase) did not cause inactivation . This confirmed that carbohydrate and lipid moieties were not required for the inhibitory activity (Table 3).

The exposure to surfactants showed they did not affect the activity of the bacteriocin in the concentration used.

The antimicrobial substance in neutralized active culture supernatant appeared to be relatively heat labile as it was inactivated partially at 80 °C for 10 min and completely at 100 °C for 10 min (Table 3).

The pH stability of the culture supernatant was studied in range from 2 to 12. The was not changed under a wide pH range from 3 to 10.

Storage of the active substance at 4 °C for more than two months and in frozen state over six months did not influence the activity.

2.3. Production studies

The production studies were performed in different media at different temperatures and both in buffered and no-buffered system. Under conditions of unregulated pH, activity was detected at the very beginning of the exponential phase at 30 °C. At 30 °C the producer strain *Lactococcus lactis* subsp. *lactis* B14 had maximum activity (1600 AU/ml) at the 6 h of the beginning of cultivation. This maximum level was about 2–3 h and after that the activity declined over 22 h coinciding with the low pH reached at that time (Fig. 1). Similar results were obtained in the conditions of regulated pH (5,5). The production of the bacteriocin started at the early exponential phase and the activity reached its maximum at ? h of cultivation (12 800 AU/ml) (Fig. 2). The investigation of the influence of the inoculum showed that the amount of 2% to 4% led to higher bacteriocin production.

2.4. Mode of action

The mode of action of the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* B14 as studied may be consider as bacteriostatic. The optical density of the test culture remain constant after the addition of the bacteriocin (Fig. 3).

Table 3

Effect of enzymes, pH, detergents and heat treatment on inhibitory activity of *Lactococcus lactis* subsp. *lactis* B14 cell-free supernatant

Treatment	Activity (AU ml^{-1})
Untreated	6400
Enzymes	
α-chymotripsin (Serva n.17160)	+
trypsin (Sigma, n. T-8253)	+
pepsin (Merck, n. 7189)	−
renin (Sigma, n. R2761)	+
proteinase K (Sigma, n. P-0390)	−
pronase E (Fluka, n 81750)	−
α-amylase (Sigma, type VIIA)	+
lipase (Sigma, n. L-1754)	+
Heat	
30°C	+
40°C	+
50°C	+
60°C	+
70°C	+
80°C	−/+
90°C	−
100°C	−
121°C	−
pH	
2	−
3	+
4	+
5	+
6	+
7	+
8	+
9	+
10	−
11	−
12	−
Agent	
Tween 20 (1%)	+
Tween 80 (1%)	+
Urea (1%)	+
N-laurilsarcosine (1%)	+
SDS (1%)	+
Triton X-100 (1%)	+
EDTA (0.1%)	+
EDTA (2%)	+
EDTA (5%)	+
PMSF (0.1%)	+
PMSF (2%)	+
PMSF (5%)	+

2.5. Adsorption studies

Our experiment reveal significant adsorption of the active substance on the producer strain cells. The released activity after treatment of the cells at pH 2 was much lower compared to the activity of the initial supernatant (data not shown). This suposed that the bacteriocin substance

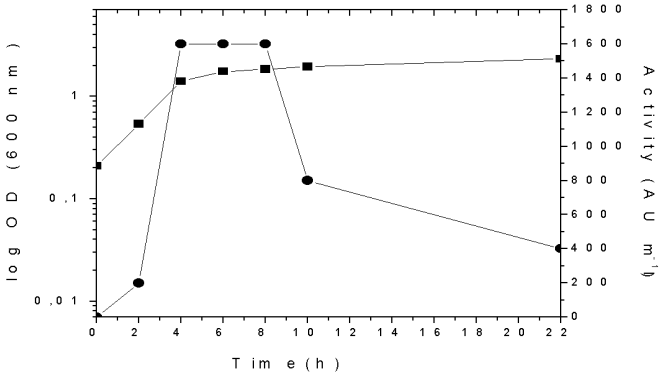

Fig. 1. Growth (■) and activity (●) of *Lactococcus. lactis* subsp. *lactis* B14 in MRS at 30 °C under nonbuffered conditions. Bacteriocin activity was measured by spotting 10 μl cell free supernatant on agar plates seeded with *Listeria innocua* F.

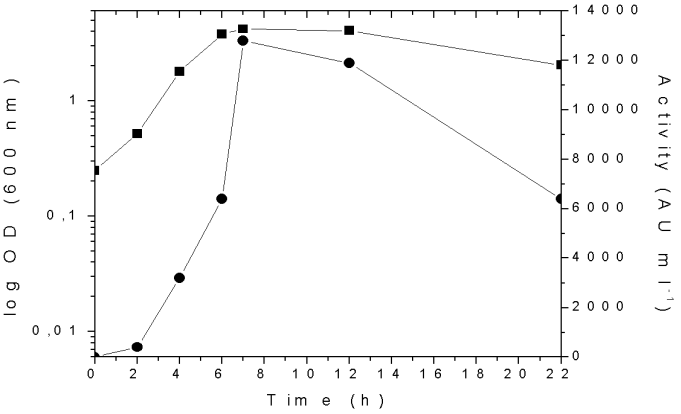

Fig. 2. Growth (■) and activity (●) of *Lactococcus. lactis* subsp. *lactis* B14 in MRS at 30 °C under buffered pH 5,5. Bacteriocin activity was measured by spotting 10 μl cell free supernatant on agar plates seeded with *Listeria innocua* F.

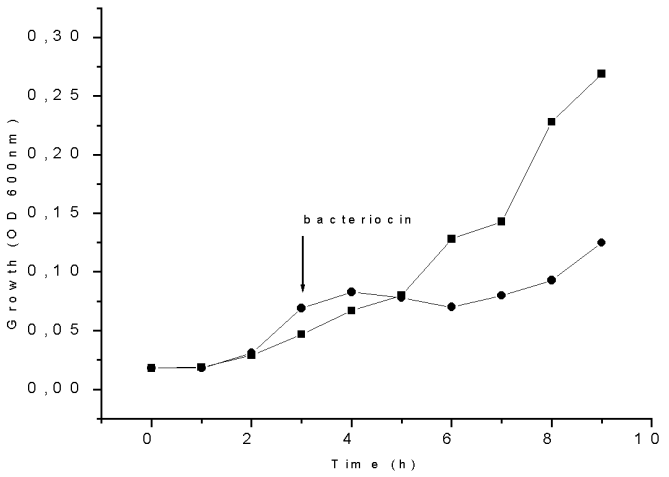

Fig. 3. Mode of action of bozacin 14 on *Lactococcus. lactis* subsp. *lactis* B14, growth of *Listeria innocua* F without bacteriocin (■) and with bacteriocin (●). The arrow shows the addition of bacteriocin containing supernatant.

produced by *Lactococcus lactis* subsp. *lactis* B14 was poorly adsorbed on the producer cells.

2.6. Bacteriocin purification

The studied bacteriocin was purified from 8 hour culture in MRS at pH 5,5. The first step in the purification protocol was to concentrate the activity from the growth medium by ammonium sulfate precipitation. Approximately 1.5-fold concentration was achieved. The recovery was 68%. Further the precipitate was subjected to a Sep-Pack 18 cartridge. The active fraction was eluted with 80% acetonitrile. At this stage of purification, the recovery was 9.55% and the specific activity increased to 9850 AU/ml. This fraction was chromatographed on an HPLC RP-18 reversed phase column. The eluted peaks were collected and checked for bacteriocin activity. At this stage the purification factor reached 47 and the recovery was 5.14%. The active fraction was purified by a subsequent reverse phase chromatography and its amino acid sequence was determined (see Table 2 and Fig. 4).

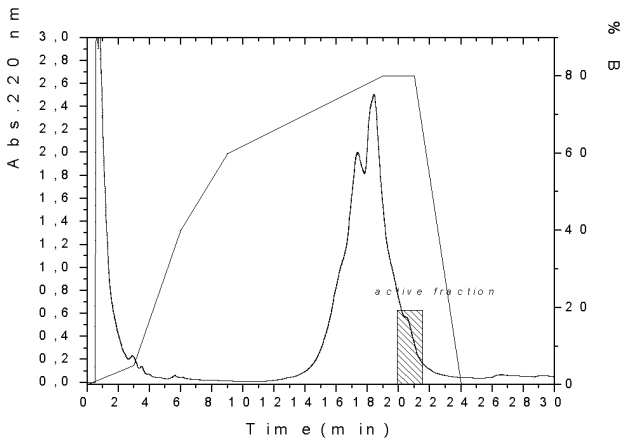

Fig. 4. RP-HPLC chromatogram of the active fraction from Sep-Pack, a PrepLC Universal Base Waters, RP-18 column (120×25 mm, 15–20 μm). The active fraction was noted in the figure.

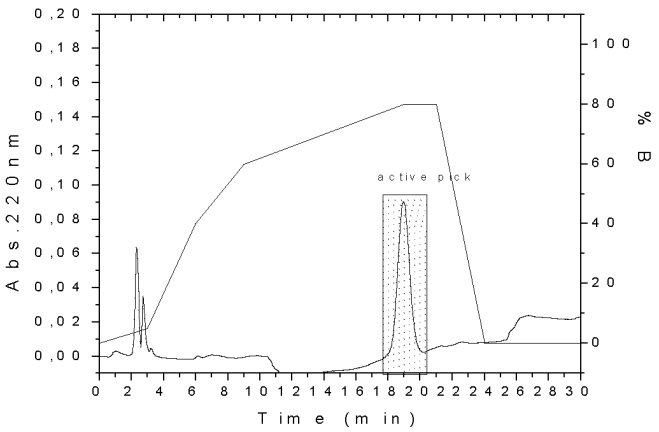

Fig. 4a. Rechromatographied active fraction recovered from RP-HPLC eluted from PrepLC Universal Base Waters, RP-18 column (120×25 mm, 15–20 μm) in same conditions. The active pick was noted in the figure.

2.7. Amino acid composition

The amino acid composition of the studied bacteriocin obtained after the final reverse-phase chromatography was determined. The calculation of the number of amino acid residues in the bacteriocin molecule revealed that it contained 49 amino acids. The molecular mass, based on amino acid content was 6200 Da (Table 4).

Table 4

Amino acid composition of bacteriocin, produced by *Lactococcus lactis* subsp. *lactis* B14

Amino acid	Amino acid residues/100
Hydrofobic:	
Ala	1
Ile	3
Phe	5
Leu	6
Met	1
Va	6
Prol	17
Charged:	
Asp	1
Glu	3
Neutral:	
Gly	2
Ser	1
Thr	1
Tyr	2
Molecular weight	6206 Da (49 a.a.)

2.8. Electrophoretic studies

Tricine-SDS polyacrylamide electrophoresis was used for detection of the all stages of the purification protocol (Fig. 5). From the received results it could be concluded that the molecular mass of the purified bacteriocin was about 5000 Da.

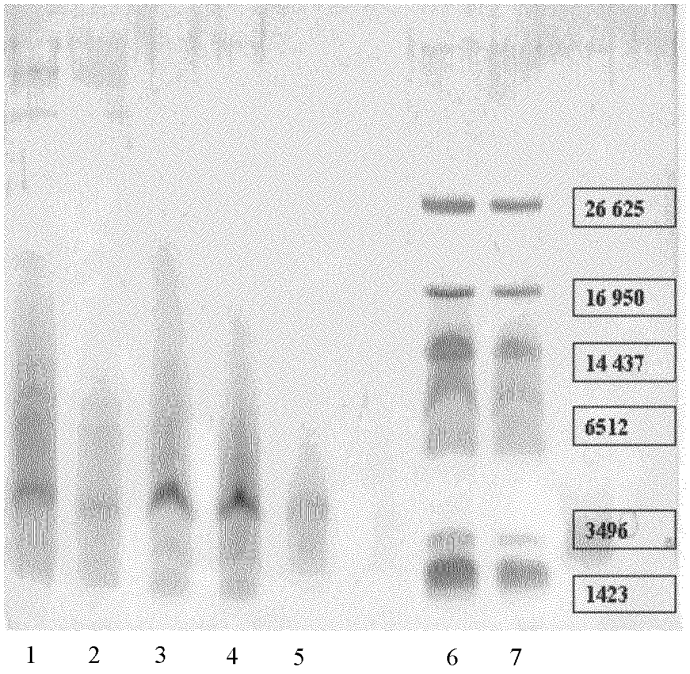

Fig. 5. Tricine PAGE of bacteriocin isolated from *Lactococcus lactis* 14. Heated, SDS—treated sample (approximately 5 μg) were separated on SDS—polyacrylamide gels (16.5%) [14] that were silver stained [11]. Lanes 1 and 2, culture supernatant of the strain *Lactococcus* 14; lanes 3 and 4 fractions of bozacin 14 after ammonium sulfate precipitation; lane 5—purified peptide bozacin 14 after HPLC separation; lanes 6 and 7—molecular mass markers (Da) are indicated on the right.

3. Discussion

In this study a bacteriocin from *Lactococcus lactis* sbsp *lactis 14* was isolated and characterised. It was found that it inhibit Gram-positive and one Gram-negative bacteria (E. coli HB101). The best characterized inhibitor to date is nisin produced by certain strains of *Lactococcus lactis*. In comparison with the isolated in this study bozacin 14 nisin is effective against gram negative only if used in combination with chelating agents.

The sensitivity of the found substance to proteinase K, pronase E and pepsin is a proof of its proteinaceous nature, which allows to consider it a bacteriocin. The purified substance was not sensitive to trypsin and chymotrypsin. As its known nisin is sensitive to trypsin. This provides additional indirect evidence that bozacin 14 is not closely related to nisin. The other enzymes tested in our study—amylase and lipase did not cause any inactivation. It could be explained with the lack of lipid or carbohydrate moiety in the studied bacteriocin. In respect to temperature sensitivity the isolated bacteriocin bozacin 14 strongly differ from nisin. It appeared to be relatively heat labile. The only report of small heat sensitive lactococcal bacteriocin was published by Martinez et al. (1996). The bacteriocin of *Lactococcus lactis* 14 was stable over a wide pH range, which is common feature of many bacteriocins. But it must be underlined that the highest activity is associated with pH values in the range of 5 to 7, which is not characteristic to nisin.

The mode of action of bacteriocin of *Lactococcus lactis* 14 as studied here may be suposed as bacteriostatic. A higher concentrations could be necessary in order to achieve a bactericidical effect. Further experiments on the mode of action of the purified substance not only on growing cells but on buffered test cells will be needed.

The optimal cultivation conditions for bacteriocin production were estimated. From the received results it could be concluded that the optimal pH in the regulation production is 5.5.

The effect of detergents on different bacteriocins gives information about the structure of the active molecules. Anionic detergents often unfold proteins by complexing to the interior hydrophobic core of their native structure, which may affect their three dimensional conformation. The observed stability of bozacin 14 indicated that denaturation or disruption did not occur.

During purification several different protocols were applied (data not shown).Optimal recovery was achieved by including ammonium sulphate precipitation and HPLC reversed -phase chromatography. The used protocol resulted in increase in the specific activity and 5.14% recovery.

The amino acid composition analysis of the purified bacteriocin revealed no presence of unusual amino acids. The bacteriocin has an estimated molecular weight 6000 Da The electrophoretic analysis was very successful and during the whole purification procedure was used as control. Received data from Tricine-SDS electrophoresis for the molecular weight of the purified substance indicated that it was in the range 5000 Da.

It's interesting to underline the found plasmid in *Lactococcus lactis* subsp. *lactis* 14 will play a role in the pro-

duction of bozacin 14. Additional studiy on the peptide sequence of bozacin 14 and nucleotide sequence of isolated plasmid will contribute to better understanding production of this substance.

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