Lait 86 (2006) 141–154
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DOI: 10.1051/lait:2006001

Original article

Antimicrobial activity and stability of partially purified bacteriocins produced by *Propionibacterium freudenreichii* ssp. *freudenreichii* and ssp. *shermanii*

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Received 19 April 2005 – Accepted 18 November 2005

Abstract – In the last decade a few bacteriocins synthesized by *Propionibacterium* were described. Almost all of them are produced by *P. thoenii* and *P. jensenii* species and only a limited number of reports is available on the antimicrobial activity of other species. In the present work bacteriocin preparations obtained from two strains of *P. freudenreichii* ssp. *freudenreichii* and one of *P. freudenreichii* ssp. *shermanii* were partially characterized, including their antimicrobial spectrum and stability. All preparations were active against the examined bacteria of *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. delbrueckii* and *L. helveticus*) and some strains of *Propionibacterium*. Crude bacteriocins also showed bacteriostatic activity against Gram-negative bacteria and fungistatic activity against moulds belonging to the *Alternaria* and *Fusarium* genera. Bacteriocin preparations were heat-stable and did not lose their activity in the range of pH from 4.5 to 7.0. They were also stable in the presence of organic solvents such as ethanol, isopropanol, hexane and chloroform at the concentration of 1 mg·mL^{-1}; however, under the influence of some solvents an increase in bacteriostatic activity was observed. It could indicate a formation of aggregates by active proteins. The activity of all crude bacteriocins was significantly reduced when SDS at concentrations of 1 to 20 mg·mL^{-1} was used.

bacteriocin / propionibacteria / antimicrobial activity / stability

摘要 – 由 *Propionibacterium freudenreichii* ssp. *freudenreichii* ssp. *shermanii* 产生的部分纯化细菌素的抗菌活性和稳定性。据报道，在过去的十年里只有几种细菌素是由丙酸菌合成的，并且这几种细菌素几乎都是由 *P. thoenii* 和 *P. jensenii* 产生的，而关于其它丙酸菌亚种产生的具有抗菌活性的细菌素的报道非常少。本研究利用两株费氏丙酸菌费氏亚种 (*P. freudenreichii* ssp. *freudenreichii*) 和一株费氏丙酸菌谢氏亚种 (*P. freudenreichii* ssp. *shermanii*) 菌株制备细菌素，并且对部分纯化细菌素的一些特性如抗菌谱和稳定性进行了研究。实验结果表明所有部分纯化细菌素对所试验的乳杆菌 （嗜酸乳杆菌、干酪乳杆菌、德氏乳杆菌和瑞士乳杆菌）和某些丙酸菌菌株具有明显的抗菌活性。粗制细菌素也具有抑制细菌（革兰氏阴性菌）以及部分真菌如链格菌属 (*Alternaria*) 和镰孢霉菌属 (*Fusarium*) 的作用。所有部分纯化的细菌素都具有热稳定，且在 pH 4.5~7.0 范围内能够保持活性。部分纯

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化的细菌素在 1 mg·mL^{-1} 乙醇、异丙醇、正己烷和氯仿等有机溶剂中具有很好的稳定，并且有些溶剂能够提高细菌素的抗菌活性，这可能是由于形成活性蛋白聚集物的原因。但是在 1~20 mg·mL^{-1} 的十二烷基磺酸钠溶液中，所有粗制细菌素的活性都明显降低。

细菌素 / 丙酸菌 / 抗菌活性 / 稳定性

Résumé – Activité antimicrobienne et stabilité des bactériocines partiellement purifiées produites par *Propionibacterium freudenreichii* ssp. *freudenreichii* et ssp. *shermanii.* Au cours de la dernière décennie, quelques bactériocines synthétisées par *Propionibacterium* ont été caractérisées. Presque toutes ces bactériocines sont produites par les souches: *P. thoenii* et *P. jensenii* et seul un nombre limité d'études concernent l'activité antimicrobienne d'autres souches. Ce travail porte sur des préparations de bactériocines obtenues de deux souches de *P. freudenreichii* ssp. *freudenreichii* et une souche de *P. freudenreichii* ssp. *shermanii* qui ont été caractérisées partiellement pour leurs spectres antimicrobiens et leur stabilité. Toutes les préparations étaient actives envers les *Lactobacillus* examinés (*L. acidophilus*, *L. casei*, *L. delbrueckii* et *L. helveticus*) et certaines souches de *Propionibacterium.* Elles ont aussi montré une activité bacteriostatique envers les bactéries Gram-négatives et une activité fungistatique envers les moisissures appartenant au genre *Alternaria* et *Fusarium.* Les préparations de bactériocines étaient thermostables et elles ne perdaient pas leur activité sur la plage de pH 4,5 à 7,0. Elles étaient aussi stables en présence de solvants tels que l'éthanol, l'isopropanol, l'hexane et le chloroforme à la concentration de 1 mg·mL^{-1}, cependant sous l'influence de certains solvants une augmentation de l'activité antimicrobienne était observée, suggérant la formation d'agrégats par les protéines actives. L'activité de toutes les bactériocines natives était réduite considérablement par l'utilisation de SDS à des concentrations de 1 à 20 mg·mL^{-1}.

bactériocine / *Propionibacterium* / activité antimicrobienne / stabilité

1. INTRODUCTION

Bacteriocins are antibacterial peptides or proteins produced by a wide range of microorganisms. This is a group of metabolites differing in their biochemical and physical properties, molecular mass, mode of action, activity spectrum and genetic determinants. According to the definition of Tagg et al. [37] bacteriocins are bactericidal against microorganisms closely related to the producer of bacteriocins, but many of them show antagonistic activity against bacteria belonging to genera different than the producer, including spoilage and pathogenic bacteria. Research on the molecular level showed that bacteriocins are ribosomally synthesized peptides or proteins [13], generally secreted outside the cell, although that part of activity can stay within the biomass [28, 29]. The genes coding bacteriocins are localized in plasmid or chromosomal DNA. Microorganisms which are capable of producing bacteriocins very often present immunity to this metabolite. The operon structure usually contains genes coding the active protein, immunity protein, proteins responsible for transporting bacteriocins from the cell and sometimes proteins taking part in posttranslation modification [23].

Because of the presence of the protein component in the structure of bacteriocins, they are inactivated by at least one, but usually by several different proteolytic enzymes, including pancreatic enzymes (trypsin and chymotrypsin) and stomach enzymes (pepsin) [28]. Bacteriocins containing lipid or carbohydrate components are also inactivated by lipolytic and amylolytic enzymes [11, 25, 35]. Enzymatic sensitivity is a very important property because it indicates that bacteriocins will be decomposed in the digestive tract. Therefore these metabolites are safe for human health. Besides, some research has proved that bacteriocins added to food did not change its organoleptic properties [28].

Bacteriocins also have many physicochemical properties making them useful as food biopreservatives. The majority of them are heat-stable, resistant to pasteurization without losing their activity, active in a wide range of pH and resistant to different organic and non-organic solvents [2, 5, 15, 22, 24, 26, 31, 35, 36].

Bacteriocins synthesized by bacteria naturally occurring in food are particularly interesting. The best studied are bacteriocins produced by lactic acid bacteria [6]. However, research carried out in the last two decades led to isolation and characterization of new bacteriocins, produced by bacteria belonging to other genera such as *Propionibacterium*.

Propionic acid bacteria have been known and used in the food industry for many decades. They are especially important as a secondary starter culture in the production of Swiss-type cheeses, where they are responsible for the formation of the characteristic flavor, texture and eyes. The propionibacteria are also well known for their production of bactericidal and fungistatic metabolites such as propionic acid, acetic acid, hydrogen peroxide and bacteriocins [8, 10, 14, 17, 41].

Bacteriocins of propionic acid bacteria are a very interesting group because of their physicochemical properties and unusual activity spectrum. Until now, only a few bacteriocins have been described [2, 7, 17, 18, 21, 32, 39]. The best known is propionicin PLG-1 produced by *Propionibacterium thoenii* P127. This bacteriocin shows a wide activity spectrum, including Gram (+) and Gram (–) bacteria, yeast and moulds [17, 18]. Jenseniin G, isolated from *P. thoenii* (*jensenii*) P126 inhibits some dairy propionibacteria, a few strains of *Lactobacillus* and *Lactococcus*, *Streptococcus thermophilus* and *Clostridium botulinum* types A, B and E [7, 10]. The bacteriocins: propionicin T1 synthesized by *P. thoenii* LMG [8], propionicin SM1 produced by *P. jensenii* DF1 [21], bacteriocin synthesized by *P. jensenii* B1264 [32] and recently described thoeniciin 447 isolated from *P. thoenii* 447 [39] and propionicin GBZ1 isolated from *P. thoenii* P127 [2] have a rather narrow activity spectrum. Faye et al. [9] described an antimicrobial peptide produced by *Propionibacterium jensenii* LMG 3032, secreted from the cell as an inactive proprotein and activated by proteases in the environment. This compound, named PAMP (a protease-activated antimicrobial peptide), inhibited propionibacteria and lactobacilli. The majority of described bacteriocins are produced by strains belonging to *P. thoenii* or *P. jensenii*; data on the ability of other species to synthesize bacteriocins is very limited. Only one bacteriocin produced by *Propionibacterium freudenreichii*, propionicin F, has been described and characterized on the molecular level to date [4]. The aim of this work was the isolation and partial purification of bacteriocins from *Propionibacterium freudenreichii* ssp. *freudenreichii* and *Propionibacterium freudenreichii* ssp. *shermanii* and determination of their antimicrobial activity and stability.

2. MATERIALS AND METHODS

2.1. Bacterial cultures

2.1.1. Tested strains

Propionibacterium freudenreichii ssp. *freudenreichii* 11 and *Propionibacterium freudenreichii* ssp. *shermanii* 41 were obtained from the Agricultural University of Poznan (Poland) collection. *P. freudenreichii* ssp. *freudenreichii* 83 and 111, *P. freudenreichii* ssp. *shermanii* 109 and *P. acidipropionici* 117 were obtained from the University of Warmia and Mazury (Poland).

2.1.2. Indicator strains

Bacterial and fungal strains used as indicators are listed in Table I.

2.2. Media and growth conditions

All *Propionibacterium* strains were grown in casein medium [27] or whey medium, depending on the experiment. The casein medium consisted of 10 g Casamino Acids (Difco Laboratories, Detroit, MI, USA), 20 g Pancreatic Digest Casein (Difco Laboratories, Detroit, MI, USA), 18 g glucose, 1.76 g K_3PO_4, 1.76 g NaH_2PO_4, 0.4 g $MgCl_2 \times 6H_2O$, 0.01 g $FeSO_4 \times 7H_2O$, 0.002 g $CoSO_4 \times 7H_2O$, 0.004 g calcium panthothenate and 0.0003 g biotin in 1 L of distilled water. In the experiments, liquid, semi-solid or soft (0.7% agar) and solid (1.5% agar) casein media were used. The whey medium consisted of 100 g whey

Table I. Indicator strains.

Lp.	Indicator strain	Collection[1]
	Propionic acid bacteria	
1	*Propionibacterium thoenii* 6	UA
2	*Propionibacterium jensenii* 7	UA
3	*Propionibacterium acidipropionici* 8	UA
4	*P. freudenreichii* subsp. *freudenreichii* 11	UA
5	*Propionibacterium sanquineum* 17	UA
6	*Propionibacterium ventii* 19	UA
7	*P. freudenreichii* subsp. *shermanii* 22	UA
8	*P. freudenreichii* subsp. *shermanii* 38	NCFB
9	*P. freudenreichii* subsp. *shermanii* 41	UA
10	*P. freudenreichii* subsp. *freudenreichii* 111	UWM
	Lactic acid bacteria	
11	*Lactobacillus acidophilus*	UA
12	*Lactobacillus casei*	UA
13	*Lactobacillus delbruecki*	UA
14	*Lactobacillus helveticus*	UA
	Other Gram-positive bacteria	
15	*Bacillus cereus*	NCFB 1771
16	*Bacillus subtilis*	NIPH
17	*Bacillus coagulans*	UA
18	*Listeria innocua*	DSMZ
19	*Listeria monocytogenes*	DSMZ
20	*Staphylococcus aureus*	DSMZ
21	*Staphylococcus epidermidis*	ATCC 12228
	Gram-negative bacteria	
22	*Campylobacter jejuni*	ATCC 33291
23	*Escherichia coli*	NCFB 745
24	*Pseudomonas aeruginosa*	ATCC 27853
25	*Salmonella typhimurium*	NIH 57/90
26	*Salmonella enteritidis*	NIH 60/90
27	*Yersinia enterocolitica*	ATCC 9610
	Fungi	
28	*Alternaria alternata*	IPP
29	*Aspergillus flavus*	DSMZ
30	*Geotrichum candidum*	UA
31	*Fusarium avenaceum*	IPP
32	*Fusarium culmorum*	IPP
33	*Fusarium graminearum*	IPP
34	*Fusarium nivale*	IPP
35	*Penicillium viridicatum*	UA

[1] ATCC - American Type Culture Collection, USA; NCFB - National Collection of Food Bacteria, Great Britain; DSMZ - Germany Collection of Microorganisms and Cell Cultures, Germany; IPP - Institute of Plant Protection, Poznan, Poland; NIH - National Institute of Hygiene, Warsaw, Poland; NIPH - National Institute of Public Health, Warsaw, Poland; UA - University of Agriculture, Poznan, Poland; UWM - University of Warmia and Mazury in Olsztyn, Poland.

powder (PPHU Lactopol, Suwałki, Poland), 15 g yeast extract (BTL, Łódź, Poland), 0.02 g $MgCl_2 \times 6H_2O$, 0.005 g $FeSO_4 \times 7H_2O$, 0.002 g $CoSO_4 \times 7H_2O$, 0.002 g $ZnCl_2$ and 0.01 g $MnCl_2$ in 1 L of distilled water. Lactic acid bacteria were propagated in de Man Rogosa Sharp (MRS) broth (BTL, Łódź, Poland) for 24 h at 37 °C. Other Gram (+) and Gram (–) bacterial strains used as indicators were grown in nutrient broth (BTL, Łódź, Poland) for 24 h at 37 °C. In the experiments, liquid, semi-solid (with 0.7% agar) and solid (with 1.5% agar) MRS and nutrient broth were used. Fungi were propagated in PDA-potato dextrose agar (BTL, Łódź, Poland) at 25 °C for 7 to 14 d, depending on the strain.

2.3. Biosynthesis of bacteriocin

The tested strains of propionibacteria (10% v/v inoculum) were grown in casein or whey medium in Erlenmayer flasks at 30 °C for 10 d. The pH of cultures was corrected every day to 7.0 ± 0.1 by 25% ammonia.

2.4. Preparation of crude bacteriocin

Cultures of propionibacteria were centrifuged at 3800 ×*g* for 15 min. Supernatants were heated at 80 °C for 15 min for inactivation of peptidases and cooled. Proteins were precipitated from the supernatants by ammonium sulfate to 75% saturation at 4 °C. Ammonium sulfate was added slowly to the supernatant for 4 h with constant stirring and held overnight at 4 °C. Precipitated proteins were collected by centrifugation at 5500 ×*g* for 30 min at 4 °C, resuspended in K-phosphate buffer (pH 7.0) and dialyzed overnight at 4 °C against the same buffer in dialysis tubing (molecular mass cutoff of 12000, Sigma, St. Louis, MO, USA). Before the assay of antagonistic activity, preparations were filter-sterilized with 0.45-μm low-protein binding membrane (Durapore Low-Protein Binding, Millipore, Bedford, MA, USA).

2.5. Enzymatic hydrolysis

The suspensions of the examined proteolytic enzymes (catalase, pepsin, trypsin and pronase) in phosphate buffer (PBS) pH 7.0 were added to bacteriocin preparations at a concentration of 0.5 $mg \cdot mL^{-1}$ of preparation. Hydrolysis lasted 2 h at 37 °C. After inactivation of the enzymes by heating at 80 °C (3 min) and cooling, the antibacterial activity against *Lactobacillus casei* was assayed.

2.6. Bacteriocin activity assay

2.6.1. Antibacterial activity assay

Antibacterial activity was determined by the critical dilution method with 24-cell plates using a modification of the method described by Toba et al. [38]. Bacteriocin activity units ($AU \cdot mL^{-1}$) were defined as the reciprocal of the highest dilution that inhibited the indicator strain. Dilutions of indicator strains were prepared by adding 0.25 mL of dilution from overnight culture (10^6 $cells \cdot mL^{-1}$) to 10 mL of the appropriate soft medium (casein medium, MRS or nutrient broth, depending on the strain). Two hundred μL of agar medium were poured into each cell, then 200 μL of indicator strain solution and 100 μL of serial two-fold dilutions of partially purified bacteriocin were added. In the case of the activity assay against relative anaerobic bacteria, the indicator strain dilution was poured into the cell first, than agar medium and bacteriocin. After the diffusion of the bacteriocin preparations into the agar (4 h at 4 ºC), the plates were incubated at 30 or 37 ºC, depending on the indicator strain, for 24 h. All assays were performed in three trials and the results presented are means of all trials.

2.6.2. Antifungal activity assay

The antifungal activity was tested by the measurement of the growth inhibition of indicator moulds due to partially purified bacteriocins. The bacteriocin preparations were added in the ratio of 2:10 to sterile medium (PDA), cooled to 50 ºC. Liquid medium with preparations was overlaid on the Petri dishes. The 9-mm disks of the examined moulds were placed in the center of the Petri dish. On the control plates,

moulds were grown on PDA without preparations. The plates were incubated until the control reached the edge of the Petri dish. Then the diameter of the mould's growth was measured and the percent of inhibition of mould growth was calculated using the following formula [3]:

$$\text{Percent of growth inhibition} = [(K_0 - F)/K_0] \times 100,$$

where K_0: control diameter; F: diameter of fungal growth with bacteriocin preparation.

All assays were performed in three trials and the presented results are means of all trials.

2.7. Chromatography analysis

The concentration of lactose, propionic acid and acetic acid was detected by liquid chromatography (HPLC) using a Merck-Hitachi chromatograph (Merck, Darmstadt, Germany). HPLC analysis was performed on an Animex HPX-87H 300 × 7.8 mm column (BIORAD, Philadelphia, USA). The eluent was 0.005 M H_2SO_4 at a flow rate of 0.8 mL·min^{-1}. Analysis was conducted at 30 °C. The samples were filtered by Millex-GP 0.22-μm membranes (Millipore, Bedford, MA, USA). Standard solutions of lactose (8 g·L^{-1}), propionic acid (4 g·L^{-1}) and acetic acid (2 g·L^{-1}) were included in the analysis. The amounts of sugar and acids were quantified by computer integration of the peak areas.

2.8. Stability tests of bacteriocin preparations

2.8.1. Sensitivity to heat

Crude bacteriocin preparations were treated by holding in a water bath at 80, 100 and 121 °C for 10, 20 or 30 min. After cooling, the antagonistic activity was assayed using *Lactobacillus casei* as an indicator strain.

2.8.2. pH stability

pH of bacteriocin preparations was set at the range of 2.0 to 9.0 by 20% HCl solution or 1 N NaOH solution. After 24 h of incubation at room temperature, pH was changed to 7.0 and the activity against *L. casei* was examined.

2.8.3. Stability in the presence of organic and non-organic solvents

The organic solvents hexane, chloroform, ethanol and isopropanol at concentrations of 1, 10, 100 and 500 mg·mL^{-1} were added to the preparations. The mixtures were incubated at 30 °C for 1 h. After incubation, the solvents were evaporated at 45 °C for 2 h and then activity against *L. casei* was assayed. The anionic detergent SDS-sodium dodecyl sulphate was added to the crude bacteriocins at concentrations of 0.1, 1, 10 and 20 mg·mL^{-1} and antagonistic activity was examined.

3. RESULTS

3.1. Confirmation of protein nature of bacteriocin preparations

Six strains of propionibacteria were examined for bacteriocin production. After 10 d of growth in casein medium, crude preparations were prepared. Since bacteriocins were supposed to be proteins or peptides, hydrolysis with the proteolytic enzymes pronase, pepsin and trypsin was carried out. The influence of hydrogen peroxide was tested in the reaction with catalase. The results of this experiment are shown in Table II. All preparations were protease-sensitive. Antibacterial activity was reduced or completely lost after hydrolysis with proteolytic enzymes, depending on the strain. Preparations obtained from *P. freudenreichii* ssp. *freudenreichii* 83 culture were the most resistant to proteases, whereas bacteriocin preparations obtained from *P. freudenreichii* ssp. *freudenreichii* 11 and 111 and *P. freudenreichii* ssp. *shermanii* 41 cultures lost their activity. However, only three of six preparations isolated from the cultures *P. freudenreichii* ssp. *freudenreichii* 11 and 111, and *P. freudenreichii* ssp. *shermanii* 41 were catalase-insensitive and their antibacterial activity after hydrolysis was the same as the control. The other

Table II. Sensitivity of bacteriocin preparations to catalase and proteolytic enzymes.

Tested strain	Activity of control preparations ($AU \cdot mL^{-1}$)	Activity of crude preparations after enzymatic hydrolysis ($AU \cdot mL^{-1}$)			
		catalase	pepsin	trypsin	pronase
P. acidipropionici 117	853	20	20	0	0
P. freudenreichii ssp. *freudenreichii* 11	1280	1280	0	0	0
P. freudenreichii ssp. *freudenreichii* 83	1067	0	20	40	40
P. freudenreichii ssp. *freudenreichii* 111	640	640	0	0	0
P. freudenreichii ssp. *shermanii* 41	640	640	0	0	0
P. freudenreichii ssp. *shermanii* 109	1067	0	0	20	20

preparations were catalase-sensitive and their activity was significantly reduced or completely lost. On the basis of this experiment, the three strains *P. freudenreichii* ssp. *freudenreichii* 11 and 111, and *P. freudenreichii* ssp *shermanii* 41 were selected for the next investigations. In the crude bacteriocin preparations isolated from the selected strain cultures propionic and acetic acids were determined by HPLC. Chromatographic analysis showed no volatile acids in the preparations, thus confirming the protein nature of the active metabolites.

3.2. Antimicrobial activity of crude bacteriocins

The antibacterial activity of the preparations against 6 strains of propionibacteria, 4 strains of lactic acid bacteria, 7 other species of Gram (+) and 6 species of Gram (–) bacteria including food spoilage bacteria and food pathogens was tested (Tab. III). Bacteriocin preparations were obtained from propionibacteria cultures on whey medium, which was better for the production of active metabolites in parallel research (unpublished data).

Partially purified bacteriocins were active against some species of *Lactobacillus* (*L. casei, L. acidophilus, L. helveticus* and *L. delbrueckii*) and some related strains of propionibacteria. Bacteriocin preparations did not inhibit their own producers. The lactic acid bacteria were much more sensitive towards bacteriocin preparations than the related strains of propionibacteria. The most sensitive were *Lactobacillus casei* and *Lactobacillus delbrueckii*, which confirmed the right choice of *L. casei* as indicator strain. Preparations obtained from *P. freudenreichii* ssp. *freudenreichii* 11 and *P. freudenreichii* ssp. *shermanii* 41 also exhibited a high antagonistic activity against *P. ventii* and *L. acidophilus*. Crude bacteriocin produced by *P. freudenreichii* ssp. *freudenreichii* 111 showed high activity towards *P. jensenii* 7, *P. ventii* 19 and *P. freudenreichii* ssp. *shermanii* 22.

The remaining Gram-positive bacteria were insensitive to bacteriocins produced by the tested propionibacteria. However, the preparations were bacteriostatic against the Gram-negative bacteria *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Yersinia enterocolitica*.

The antifungal activity of the bacteriocin preparations against 8 different species of moulds was also tested (Tab. IV). All of these preparations were active against *Alternaria alternata* and three of four species of *Fusarium*. The bacteriocins did not inhibit moulds belonging to the *Aspergillus*, *Penicillium* and *Geotrichum* genera.

Table III. Antibacterial activity of bacteriocin preparations.

Species	Activity of bacteriocin preparations ($AU \cdot mL^{-1}$)		
	P. freudenreichii ssp. *freudenreichii* 11	*P. freudenreichii* ssp. *freudenreichii* 111	*P. freudenreichii* ssp. *shermanii* 41
Lactic acid bacteria			
Lactobacillus acidophilus	4266	1280	5120
Lactobacillus casei	6826	10240	10240
Lactobacillus delbrueckii	5120	10240	8533
Lactobacillus helveticus	1280	1706	1280
Propionic acid bacteria			
Propionibacterium thoenii 6	320	0	320
Propionibacterium jensenii 7	640	2560	640
Propionibacterium acidipropionici 8	133	0	320
P. freudenreichii ssp. *freudenreichii* 11	0	13	0
Propionibacterium sanquineum 17	13	0	0
Propionibacterium ventii 19	6826	8533	8533
P. freudenreichii ssp. *shermanii* 22	1280	8533	853
P. freudenreichii ssp. *shermanii* 41	0	160	0
P. freudenreichii ssp. *freudenreichii* 111	0	0	80
Other Gram-positive bacteria			
Bacillus cereus	0	0	0
Bacillus subtilis	0	0	0
Bacillus coagulans	0	0	0
Listeria innocua	0	0	0
Listeria monocytogenes	0	0	0
Staphylococcus aureus	0	0	0
Staphylococcus epidermidis	0	0	0
Gram-negative bacteria			
Escherichia coli	0	0	0
Pseudomonas aeruginosa	±	±	±
Salmonella typhimurium	±	±	±
Salmonella enteritidis	0	0	0
Yersinia enterocolitica	±	±	±

+: bactericidal activity; ±: bacteriostatic activity; 0: no activity.

The highest antifungal activity was elicited by the preparation obtained from *P. shermanii* 41 culture and the lowest by the preparation from *P. freudenreichii* 11. The most sensitive mould was *Alternaria alternata*. The percent of growth inhibition of this mould by bacteriocin isolated from *P. shermanii* 41 was almost 47%. The preparation obtained from *P. freudenreichii* 111 culture also showed high activity. Less inhibition

Table IV. Antifungal activity of bacteriocin preparations.

Species	Percent of growth inhibition by bacteriocin preparations (%)		
	P. freudenreichii ssp. *freudenreichii* 11	*P. freudenreichii* ssp. *freudenreichii* 111	*P. freudenreichii* ssp. *shermanii* 41
Alternaria alternata	18.88	36.11	46.66
Aspergillus flavus	0	0.51	1.55
Geotrichum candidum	0	0	0
Fusarium avenaceum	8.33	9.11	17.11
Fusarium culmorum	1.88	6.66	16.44
Fusarium graminearum	8.33	5.00	10.00
Fusarium nivale	0.22	0	0.11
Penicillium viridicatum	0	0	0.50

was observed for crude bacteriocin produced by *P. freudenreichii* 11 (18%). Partially purified bacteriocins also inhibited three species of *Fusarium*: *F. avenaceum*, *F. culmorum* and *F. graminearum*. The level of inhibition depended on the kind of mould. The highest activity was shown by the bacteriocin preparation obtained from *P. shermanii* 41 culture. *F. avenaceum*, *F. culmorum* and *F. graminearum* were inhibited by 17, 16 and 10%, respectively. Two of the remaining preparations exhibited lower inhibition, i.e. from 1.88 to 9.11%, depending on the tested strain and species of *Fusarium*.

3.3. Stability of crude bacteriocins

Heat and pH stability and sensitivity of partially purified bacteriocins to organic and non-organic solvents were studied.

All preparations were heat-stable (Figs. 1, 2, 3). Preparations obtained from *Propionibacterium freudenreichii* ssp. *freudenreichii* 11 and 111 cultures completely lost antibacterial activity only after heating at 121 °C. However, the activity of crude bacteriocin isolated from *P. freudenreichii* ssp. *freudenreichii* 11 was reduced to about 17% after 10 min of heating at 100 °C. Longer exposure to this temperature caused a significant reduction of inhibitory activity of this bacteriocin. The preparation obtained from *P. freudenreichii* ssp. *shermanii* 41 culture was only stable up to 80 °C. Heating at 90 °C reduced the activity by 50%, while the activity was completely lost during exposition to 100 °C.

Bacteriocin preparations were stable in the pH range 4.0–7.0 (Figs. 4, 5, 6). Below and above this range, a reduction or complete loss of antimicrobial activity of the preparations was observed. The most stable bacteriocin preparation was that obtained from P. *freudenreichii* ssp. *shermanii* 111 culture; its activity was reduced only at extreme values of pH, at 2.5 and 8.0. The most pH-sensitive preparation was that obtained from *P. freudenreichii* ssp. *shermanii* 41 culture, which showed full activity in a relatively narrow range of pH, from 4.5 to 7.0.

Partially purified bacteriocins were also stable in the presence of different organic solvents such as ethanol, isopropanol, and hexane. The stability of the preparations depended on the concentration of the solvents used and the tested strain of propionibacteria (Tab. V). Antagonistic activity did not change when the solvents were used at the concentration of 1 $mg \cdot mL^{-1}$, with the exception of SDS – sodium dodecyl sulphate. Also, in the presence of isopropanol, hexane and chloroform at the concentration of 10 $mg \cdot mL^{-1}$, the antibacterial activity of the preparations was stable. In a few cases, under the influence of some organic solvents (ethanol, isopropanol and hexane), an increase in the antagonistic activity was observed. It depended on the solvent,

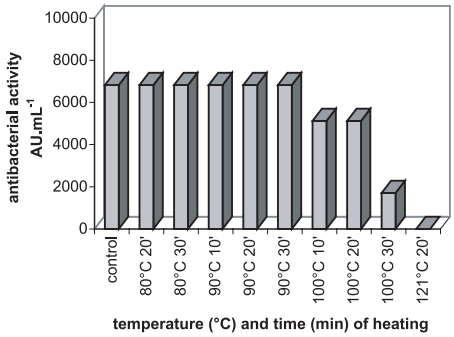

Figure 1. The influence of temperature and time of heating on the activity of crude bacteriocin produced by *P. freudenreichii* 11 against *L. casei.*

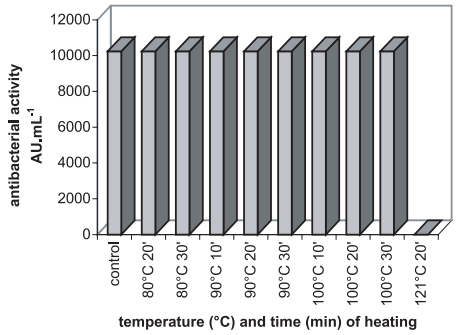

Figure 2. The influence of temperature and time of heating on the activity of crude bacteriocin produced by *P. freudenreichii* 111 against *L. casei.*

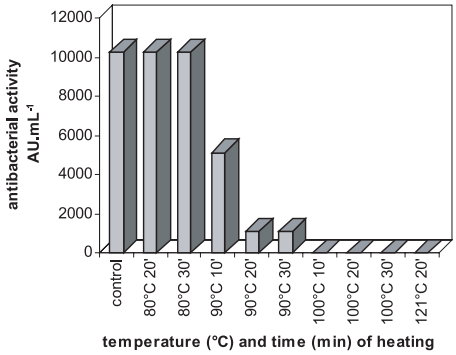

Figure 3. The influence of temperature and time of heating on the activity of crude bacteriocin produced by *P. shermanii* 41 against *L. casei.*

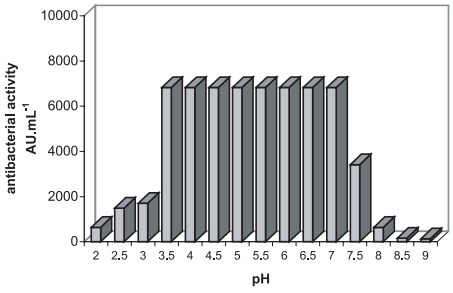

Figure 4. The influence of pH on the activity of crude bacteriocin produced by *P. freudenreichii* 11 against *L. casei.*

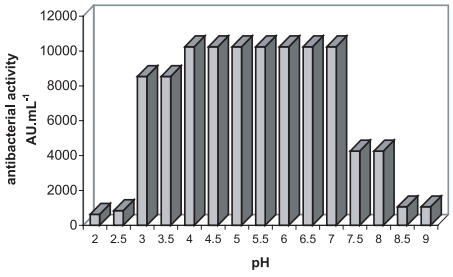

Figure 5. The influence of pH on the activity of crude bacteriocin produced by *P. freudenreichii* 111 against *L. casei.*

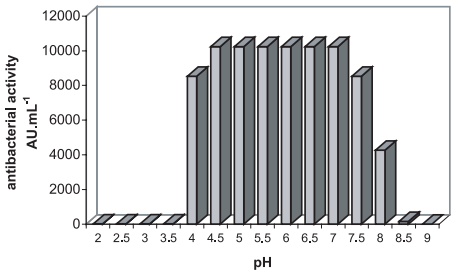

Figure 6. The influence of pH on the activity of crude bacteriocin produced by *P. shermanii* 41 against *L. casei.*

Table V. Antibacterial activity of bacteriocin preparations in the presence of some solvents (AU·mL^{-1}).

solvent	concentration (mg·mL^{-1})	Antibacterial activity of bacteriocin preparations (AU·mL^{-1})		
		P. freudenreichii ssp. *freudenreichii* 11	*P. freudenreichii* ssp. *freudenreichii* 111	*P. freudenreichii* ssp. *shermanii* 41
control		6826	10240	10240
ethanol	1	6826	10240	10240
	10	6826	13653	13653
	100	27307	68267	34133
	500	640	0	0
isopropanol	1	6826	10240	10240
	10	6826	10240	10240
	100	17067	10240	17067
	500	0	8533	4267
hexane	1	6826	10240	10240
	10	6826	10240	10240
	100	20480	34133	54613
	500	40960	68267	81920
chloroform	1	6826	10240	10240
	10	6826	10240	10240
	100	3413	5120	8533
	500	213	3413	8533
SDS	0.1	5120	6826	6827
	1	2560	4267	213
	10*	213	640	160
	20*	80	533	40

* SDS at the concentrations of 10 mg·mL^{-1} and 20 mg·mL^{-1} inhibited the growth of *L. casei*. The activity of bacteriocin preparations was diminished for these values.

its concentration and the kind of bacteriocin preparation. An increase in the activity of all preparations was observed after using hexane at the concentrations of 100 and 500 mg·mL^{-1} and ethanol at the concentration of 100 mg·mL^{-1}. The antibacterial activity of crude bacteriocins was even three- to eight-fold higher than the control. However, sometimes a decrease in antibacterial activity was found. A significant reduction of the activity was observed when the bacteriocins were treated with SDS at concentrations from 1 to 20 mg·mL^{-1} and isopropanol and ethanol at the concentration of 500 mg·mL^{-1}.

4. DISCUSSION

It was found in our research that the inhibitory substances produced by *P. freudenreichii* ssp. *freudenreichii* 11 and 111, and *P. freudenreichii* ssp. *shermanii* 41 are of protein nature. Crude bacteriocin preparations were protease-sensitive and catalase-insensitive and were also free of volatile acids, probably because all small molecules were removed by dialysis. Excluding the effect of acids is important for the identification of antimicrobial substances. Lortie et al. [16] suggested that only a part of bacteriocin-like interactions described in the literature

are caused exclusively by bacteriocins and the combined action of bacteriocins and some metabolites such as organic acids is more probable. The neutralization techniques are used quite often but, unfortunately, they can reduce the activity of bacteriocins usually active in acidic pH and unstable above pH 7.0.

Preparations obtained from the selected propionibacteria cultures were characterized, including antimicrobial activity and stability of crude bacteriocins. The preparations were active against all the examined strains of *Lactobacillus* and some strains of *Propionibacterium*. Almost all the described bacteriocins of propionibacteria showed antagonistic activity against lactic acid bacteria and propionic acid bacteria. Jenseniin G and bacteriocin synthesized by *P. jensenii* B1264 were found to be more active towards *Lactobacillus* than *Propionibacterium* [10, 32]. Similar results were noted in the present work. Bacteriocin preparations exhibited the highest activity against *L. casei* and *L. delbrueckii*. Literature data indicated that bacteriocins synthesized by *P. thoenii*: propionicin PLG-1 [17] and propionicin T1 [8], and by *P. jensenii*: jenseniin G [10] and jenseniin P [32], did not exert antibacterial activity against bacteria belonging to *P. freudenreichii* species. The authors explained this fact by attaching these species to different phylogenetic groups [19], so they are less closely related to *P. thoenii* and *P. jensenii* species. In the present work, low activity towards *P. thoenii* and *P. jensenii* was also observed, probably as a result of the attachment of the tested and indicator strains to different phylogenetic clusters.

Bacteriocin preparations were bacteriostatic against some Gram-negative bacteria: *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Yersinia enterocolitica*, and fungistatic against some species of *Alternaria* and *Fusarium*. Activities of bacteriocin preparations against both Gram-negative bacteria and moulds are rarely reported. The inhibition of Gram-negative bacteria is shown, for example, by bacteriocins from *Pediococcus* spp. Bacteriocins produced by *P. damnosus* and *P. pentosaceus* inhibited microorganisms such as *Yersinia enterocolitica*, *Salmonella infantis* and some strains of *Pseudomonas* [34]. Some strains of *Lactobacillus acidophilus* also produced bacteriocins active against Gram-negative bacteria. For example, acidophilin 801 elicited the inhibition of pathogenic strains of *Escherichia coli* and *Salmonella panama* [42], whereas plantaricin 35 d was active towards *Aeromonas hydrophila* [20]. Literature data also mentions the ability of bacteriocins synthesized by *Propionibacterium* to inhibit Gram-negative bacteria. Propionicin PLG-1 was antagonistic against *Campylobacter jejuni*, *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* [17]. Also, Warmińska–Radyko and Łaniewska–Moroz [41] observed the antibacterial activity of bacteriocin-like metabolites synthesized by *Propionibacterium acidipropionici* towards Gram-negative bacteria belonging to *Enterobacteriaceae*. Mould inhibition by bacteriocin preparations obtained from *P. freudenreichii* ssp. *freudenreichii* and *P. freudenreichii* ssp. *shermanii* cultures is particularly interesting. Until now, the ability to inhibit fungi has been reported only for propionicin PLG-1 [17] and acidocin CH5 [30]. Propionicin PLG-1 was active against *Aspergillus ventii*, *Apiotrichum curvatum*, *Fusarium tricinctum*, *Phialophora gregata* and yeasts belonging to the *Candida*, *Saccharomyces* and *Scopularopsis* genera [17]. Similarly to the presented work, fungistatic activity towards selected species and maybe even against selected strains was found.

The stability of crude bacteriocins was also investigated and some differences, depending on the tested strain, were observed. Generally, all preparations were heat-stable, did not lose their activity within the range of pH from 4.5 to 7.0 and were also stable in the presence of some solvents such as ethanol, isopropanol and hexane, depending on their concentration. All preparations lost their activity in the reaction with SDS. Probably, the reduction of antibacterial activity of the bacteriocin preparations was caused by partial denaturation. Anionic detergents such as SDS often destroy the structure of proteins by bonding to their hydrophobic parts [40]. In some

cases an increase in activity was noted, sometimes even eight-fold. The increase in the activity was probably due to the dissociation of high molecular mass proteins from small molecules and the disclosure of the active centers. It suggests that the obtained crude bacteriocins form large protein aggregates in their native form. Literature data confirm the possibility of the increase in bacteriocins' activity under the influence of some chemical reagents such as alcohols or detergents [1, 12, 23, 26, 33]. It concerns bacteriocins forming aggregates, which dissociate in the presence of these substances. For example, helveticin J and lactacin F were dissociated after the treatment with SDS [12, 23]. Oscariz and Pisabarro [26] observed an increase in cerein 7 activity in the presence of Triton X100. Sip and Grajek [33] described an increase in divercin activity by its treatment with the alcohols ethanol and isopropanol, and the detergents Tween 80, Nonidet P40 and SDS. In the preparations of partially purified bacteriocins, samples were dialyzed in dialysis tubing with a MW cutoff of 12000 $g \cdot mol^{-1}$. The active substances retained in the dialysis bag suggest that active proteins produced by tested strains of *P. freudenreichii* could have a molecular mass higher than 12000 $g \cdot mol^{-1}$. However, it is possible that this is the mass of aggregates, not single proteins. It is necessary to continue research on the structure of the obtained bacteriocin preparations, and this is in progress.

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