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ORIGINAL ARTICLE

Novel antilisterial bacteriocin licheniocin 50.2 from *Bacillus licheniformis* VPS50.2 isolated from soil sample

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Abstract

Aim: To isolate and characterize bacteriocin, licheniocin 50.2, from soil bacteria identified as *Bacillus licheniformis*.

Methods and Results: The strain *B. licheniformis* VPS50.2 was identified as bacteriocin producer, effective against Gram-positive bacteria, including *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *β*-haemolytic streptococci. The start of bacteriocin production coincides with the beginning of sporulation. Ammonium sulfate precipitation, chloroform extraction and ultrafiltration were used for bacteriocin purification. MALDI TOF/TOF mass spectrometry of purified sample detected the protein with molecular mass of 3253·209 Da. N-terminal sequencing recognized first 15 amino acids with the sequence: W E E Y N I I X Q L G N K G Q. We named the newly characterized bacteriocin as subclass II.3 bacteriocin, licheniocin 50·2. The bacteriocin activity was insensitive to lysozyme and proteinase K, heat stable after incubation at 100°C for 30 min and over wide range of pH (2–12). MICs of crude bacteriocin extract were determined for *L. monocytogenes* and MRSA. Time–kill study showed that licheniocin had bactericidal effect to *L. monocytogenes*.

Conclusion: A novel, thermostable, pH-tolerant bacteriocin active against Gram-positive bacteria was isolated.

Significance and Impact of the Study: Attributes of new, stable licheniocin 50.2 make it a promising agent for application as biopreservative in food industry.

Introduction

Members of the genus *Bacillus* are Gram-positive, aerobic and endospore-forming bacteria that are characterized by their rod-shaped cell morphology, catalase production and their ubiquitous distribution. They are found in diverse environments such as soil and clays, rocks, dust, aquatic environments, vegetation, food and the gastrointestinal tracts of various insects and animals (Nicholson 2002). Members of the *Bacillus* group are considered as good producers of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins (Stein *et al.* 2005). The production of antimicrobial substances and sporulation capacity confer *Bacillus* strains with a double advantage in terms of their survival in different habitats. Bacteriocins are commonly defined as antimicrobial peptides that are synthesized ribosomally by bacteria and secreted to the extracellular milieu, where they usually act against closely related species without affecting the producer strain (Klaenhammer 1993). The increasing bacterial resistance to conventional antibiotics of clinical application resulted in a growing interest to consider bacteriocins as alternative antimicrobials for the treatment of human (and possibly animal) infections (Lawton *et al.* 2007). In addition, consumer demands for minimally processed foods with no chemical preservatives have stimulated research interest in natural antimicrobial agents such as bacteriocins (Abriouel *et al.* 2010). However, most studies concerning food applications have focused on lactic acid bacteria bacteriocins, mainly nisin

and a few others (Galvez *et al.* 2008). Although nisin is the one of the bacteriocins currently licensed as a biopreservative, its applications are restricted due to its very low activity at a neutral or alkaline pH. Therefore, the search for new bacteriocins with improved biochemical properties (stability in a wide range of pH and temperature) and a broad antimicrobial spectrum is of great interest for their application in foods. Bacteriocins produced by the *Bacillus* genus *sensu lato* may be considered as the second most important after bacteriocins produced by lactic acid bacteria (Abriouel *et al.* 2010). However, in spite of the diverse array of bacteriocins produced by *Bacillus* species with attractive technological properties, the importance and industrial value of *Bacillus* bacteriocins has been largely underestimated, and only a small number of applications were reported in foods. This fact is potentiated by the lack of GRAS status of some *Bacillus* species, except for some representatives such as *Bacillus subtilis* and *Bacillus licheniformis* (Sharp *et al.* 1989).

This study was aimed at characterizing the properties of a bacteriocin produced by *B. licheniformis* VPS50.2 isolated from soil, as well as determining optimal growth condition for bacteriocin production.

Material and methods

Bacterial strains and culture conditions

To determine the antimicrobial spectrum of purified bacteriocin from *B. licheniformis* VPS50.2, 22 bacterial species were used as indicator strains (Table 1). All bacteria, including *B. licheniformis* VPS50.2, were grown aerobically in Luria–Bertani (LB) broth. Only *Lactobacillus zeae* LMG17315, *Lactobacillus plantarum* LMG92088 and *Lactococcus lactis* IL1403 were grown in MRS broth and M17 broth supplemented with 0·5% (w/v) glucose (Merck, Darmstadt, Germany), respectively, under microaerophilic conditions. Fungal species, *Aspergillus brasiliensis* and *Candida albicans,* were grown in Sabouraud medium (Torlak, Belgrade, Serbia), aerobically. Agar plates were made by adding 1·5% (w/v) agar (Torlak) to the proper liquid medium. Bacteria and fungi were incubated at appropriate temperature for 24 h.

Identification of bacteriocin-producing strain

Bacteriocin-producing strain was taxonomically identified based on phenotypic and physiological characteristics using API test system (BioMerieux, Marcy l'Etoile, France) and analysis of the partial 16S rDNA sequence. Genomic DNA from *Bacillus* strain was prepared as described earlier (Le Marrec *et al.* 2000). To obtain 16S rDNA sequences, polymerase chain reaction (PCR) was carried out using primers $P1_{16S}$ (5′-GGAATCTTCCA CAATGGACG-3′) and $P2_{16S}$ (5′-TGACGGGCGGTGTG-TACAAG-3′) and PCR protocol as follows: 94°C for 5 min, 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s and 72°C for 10 min. Purified PCR fragments were sequenced with both primers and compared with 16S rRNA gene sequences in the public database using BLAST. API CHB50 analysis was conducted according to manufacturer's instructions.

Table 1 Antimicrobial activity spectrum of bacteriocin licheniocin 50.2 produced by *Bacillus licheniformis* VPS50.2

Indicator organism	Temperature (°C)	Inhibition zone (mm)*
B. licheniformis VPS50.2	37	0
Aeromonas hydrophila ATCC 49140	37	0
B. licheniformis ATCC 12759	37	0
Bacillus subtilis ATCC 6633	37	16 ± 1
B. subtilis 168	37	25 ± 0·7
B. subtilis W23	37	25 ± 1·5
Enterobacter cloacae ATCC 49141	37	0
Enterococcus faecalis ATCC 29212	37	23 ± 2
Enterococcus saccharolyticus ATCC 43076	37	25 ± 2·5
Lactobacillus plantarum LMG92088	30	13 ± 0·8
Lactobacillus zeae	30	22 ± 1
Lactococcus lactis IL1403	30	26 ± 2
Listeria monocytogenes ATCC 19111	37	25 ± 0·6
Micrococcus luteus ATCC 7468	37	31 ± 2·5
Staphylococcus aureus ATCC 25923	37	15 ± 1
Staph. aureus† ATCC 33591	37	21 ± 1
Streptococcus agalactiae‡ ATCC 12386	37	15 ± 0·5
Streptococcus equisimillis§ ATCC 12394	37	0
Escherichia coli ATCC 25922	37	0
Proteus mirabilis ATCC 25933	37	0
Pseudomonas aeruginosa ATCC 15442	37	0
Salmonella enteritidis ATCC 13076	37	0
Salmonella typhimurium ATCC 14028	37	0
Shigella flexneri ATCC 9199	37	0
Candida albicans ATCC10231	30	0
Aspergillus brasiliensis ATCC16404	37	0

ATCC – American Type Culture Collection, Rockville, MD, LMG – Belgian Coordinated Collections of Micro-organisms/LMG Bacteria Collection.

*Diameter of the inhibition zone (mm) around the disc with standard deviations.

†methicillin-resistant.

‡β-haemolytic.

§γ-haemolytic.

Detection of antimicrobial activity and spectrum

Antimicrobial activity of *B. licheniformis* VPS50.2 was determined by agar-well diffusion assay with crude bacteriocin extract as described by Harris *et al.* (1989). In

addition, proteinaceous nature of antimicrobial compound in supernatant was tested by using pronase E (AppliChem GmbH, Darmstadt, Germany) as follows. During agar-well diffusion assay, when aliquots of tested samples were added to well, small amount of crystalline pronase E was placed on the edge of well with sterile toothpick. Radiuses of inhibition zones were measured from the edge of well and displayed in mm. Moreover, the absence of inhibition zone around the pronase E crystal was monitored (Berić *et al.* 2012). Soft agar was made by adding 0·7% agar to LB, MRS or GM17, respectively. To create an overlay, 60 μl of overnight culture of indicator strain (approximately 10^6 cells ml^{-1}) was added to appropriate soft agar. Mixture was overlaid onto LB, MRS or GM17 agar plates with positioned sterile corks of 7 mm diameter and allowed to become completely solid. The wells were made by taking out the corks. Aliquots (50 μl) of crude bacteriocin extract samples were poured in the wells, and plates were then incubated overnight at the optimal growing conditions for the indicator strain.

Kinetics of production of antimicrobial substance in different media and physical conditions

Bacillus licheniformis VPS50.2 was cultivated aerobically in 1000-ml Erlenmeyer flasks containing 200 ml of LB medium, Schaeffer's sporulating medium (Difco sporulation medium, containing, per litre, 8 g of Bacto-nutrient broth, 10 ml of 10% KCl, 10 ml of 1·2% $MgSO_4{\cdot}7H_2O$, 0·50 ml of 1 mol l^{-1} NaOH, 1·0 ml of 1 mol l^{-1} $Ca(NO_3)_2$, 1·0 ml of 0·010 mol l^{-1} $MnCl_2$, 1·0 ml of 1 mmol l^{-1} $FeSO_4$) and Spizizen's minimal medium (containing, per litre, 2 g $(NH_4)_2SO_4$, 14 g K_2HPO_4, 6 g KH_2PO_4, 1 g Na_3 citrate.$2H_2O$, 0·2 g $MgSO_4{\cdot}7H_2O$, 10 ml 50% (w/v) D-glucose, 10 ml L-tryptophan (5 mg ml^{-1})) varying physical conditions, for example temperature of incubation (30 and 37°C) and agitation (180 and 220 rev min^{-1}) for 48 h. Viable counts and spores (heat-treated counts after 80°C for 10 min) were recorded every 2 h, and supernatant was tested for activity on indicator bacterial strain *B. subtilis* 168 in agar-well diffusion assay as described in section Detection of antimicrobial activity and spectrum.

Purification of bacteriocin

Bacillus licheniformis VPS50.2 was cultivated aerobically in 500-ml Erlenmeyer flasks containing 200 ml of LB at 30°C, 200 rev min^{-1} for 38 h. Cells were harvested by centrifugation at 4500 ***g*** (Eppendorf 5804, Hamburg, Germany) for 15 min at 4°C, and the resulting supernatant was filtered through 0·45-μm Durapore membranes (Millipore, Billerica, MA). The cell-free culture filtrate was submitted to precipitation with ammonium sulfate to obtain 20, 30, 40, 50, 60, 70 and 80% of saturation. The resulting pellets were resuspended in 50 mmol l^{-1} sodium phosphate buffer, pH 7·0 and tested for antimicrobial activity. The most potent fraction was submitted to further purification by chloroform extraction as follows. Equal volumes of resuspended pellet and chloroform were vigorously shaken for 15 min, and then, phases were divided by centrifugation at 4500 ***g*** for 10 min. Obtained interphase was collected and dried in rotary vacuum evaporator (Eppendorf Concentrator 5301; Eppendorf). The total protein content was measured according to Bradford (1976). Resuspended fractions (in 50 mmol l^{-1} sodium phosphate buffer, pH 7·0) were tested for antimicrobial activity against *B. subtilis* 168. Fraction that exhibited maximum activity was then submitted to ultrafiltration trough membranes with 3 and 10 kDa molecular cut-off (Millipore), and obtained filtrates as well as retentates were collected and tested in an agar-well diffusion assay against *B. subtilis* 168.

Determination of MIC and time–kill studies

The MICs of licheniocin 50.2 for *Listeria monocytogenes* and *Staphylococcus aureus* ATCC 33591 were determined by testing the twofold dilution of crude bacteriocin extract in agar-well diffusion assay. To create an overlay of susceptible strain, 60 μl of overnight culture (approximately 10^6 cells ml^{-1}) was added to appropriate soft agar (BHI for *L. monocytogenes* and LB for *Staph. aureus* ATCC 33591). MICs were marked as last dilution that inhibited growth of indicator strain.

For time–kill studies, inoculum of *L. monocytogenes* was prepared from test organisms subcultured until exponential phase and diluted in BHI broth to approximately 10^6 CFU ml^{-1}. Aliquots (10 ml) of the culture were transferred in a separate flasks, and crude bacteriocin extract was added from a sterile stock solution to give final concentration of 0·5, 1, 2 and 4 times the MIC. A growth control flask with tested strain without bacteriocin was included in the experiment. The cultures were incubated at 37°C, and samples were obtained at every hour for 8 h following addition of bacteriocin. The samples were washed and serially 10-fold diluted in 0·5 mmol l^{-1} phosphate buffer, and 10 μl samples were spotted in triplicate onto appropriate solid media, following overnight incubation at 37°C.

SDS-PAGE and mass spectrometry

The purity, molecular weight and antimicrobial activity of active fraction obtained during purification were tested on SDS-PAGE. The electrophoresis was carried out using

16·5% polyacrylamide gel using Tris–Tricine buffer system (Schagger and Jagow 1987). The gel was fixed and one half (containing Serva Unstained SDS-PAGE protein Marker, 6·5–200 kDa; SERVA, Heidelberg, Germany) was stained with Coomassie Blue R250, then destained, and the position of the active bacteriocin was determined. To localize *in situ* bacteriocin activity, second half of the gel (containing only protein sample) was washed in 0·5% Tween-80 and MilliQ water, overlaid with 0·7% LB agar containing 10^6 cells ml^{-1} of indicator strain *B. subtilis* 168 and incubated overnight at 30°C. Mass spectrometry analysis was performed with purified sample by using a MALDI TOF/TOF mass spectrometer (Model 4800; Applied Biosystems, Carlsbad, CA) at International Centre for Genetic Engineering and Biotechnology (Trieste, Italy).

Effect of proteolytic enzymes, heat and pH on antimicrobial activity

Proteolytic enzymes were tested on purified bacteriocin by adding small amount of crystalline enzyme, with toothpick, on the edge of well while testing antimicrobial activity against *B. subtilis* 168. After overnight incubation at 37°C, appearance or absence of zones of inhibition around the spot where the enzymes were added was observed. The following enzymes were used: proteinase K; trypsin, DNase; RNase (all Sigma-Aldrich, Taufkirchen, Germany); and lysozyme (Serva). To analyse thermal stability, samples of bacteriocin were exposed to temperatures ranging from 50 to 100°C for 15 and 30 min, 121°C (103·5 kPa) for 15 min and held at 4°C for up to 6 months. The activity of bacteriocin at different pH values was estimated by adjusting the pH of purified bacteriocin to pH 2–12. After the treatment, samples were tested for antimicrobial activity against *B. subtilis* 168.

N-terminal sequencing

N-terminal sequencing of the first 15 amino acids was performed at Jozef Stefan Institute (Ljubljana, Slovenia) using a Procise protein sequence system 492A (PE Applied Biosystems, Carlsbad, CA).

Accession numbers

The partial 16S rRNA gene of *B. licheniformis* VPS50.2 has been submitted to the EMBL nucleotide Sequence Database under accession No. HE993550. The N-terminal sequence of the bacteriocin licheniocin 50.2 has been submitted to the UniProt Knowledgebase under accession No. B3EWP7.

Results

Isolation and taxonomical identification of bacteriocin-producing strain

The isolate VPS50.2 belongs to collection of 203 *Bacillus* isolates from soil, hay, straw and manure from 32 localities in Serbia. They were isolated and preliminary identified as described previously (Stanković *et al.* 2007). Many isolates from collection showed antimicrobial activity against various bacterial plant pathogens that was later attributed to lipopeptides. Only the supernatant from isolate VPS50.2, that exhibited antimicrobial activity against *B. subtilis* 168, showed sensitivity to pronase E treatment, indicating that antimicrobial substance produced by this strain has a proteinaceous nature and that most likely belongs to bacteriocins (Berić *et al.* 2012). Both taxonomical identification of *Bacillus* isolate VPS50.2 using API50CHB kit and API WEB software showed 99·9% identity with *B. licheniformis* and homology analysis of nucleotide sequence of 16S rDNA revealed 100% identity with *B. licheniformis*. The new strain was deposited in the BCCM/LMG bacteria collection under accession No. LMG 27248).

Bacteriocin production in different media and physical conditions

For the determination of the growth stage at which strain *B. licheniformis* VPS50.2 produce bacteriocin, samples of supernatants were taken every 2 h during the aerobic growth in LB medium, at 30°C for 2 days and tested for their activity using *B. subtilis* 168 as indicator strain. Changes in cell numbers (viable counts) and spores were recorded as well as the size of zones of inhibition on indicator strain, as shown in Fig. 1a. Kinetics of production of antimicrobial substance shown that synthesis and/or secretion started at the transition from exponentially to stationary phase (after 14 h of growth), slowly reaching maximum activity at the late stationary growth phase (after 36 h of incubation) and remaining at same level until the end of observation period. In addition, start of production of antimicrobial substance coincides with the beginning of sporulation (first spores were recorded at 12th hour of incubation) and with increase in spore numbers bacteriocin activity increases.

The effect of culture media on bacteriocin production by *B. licheniformis* VPS50.2 was evaluated using, apart from already described growth in LB, Spizizen's minimal medium and Schaeffer's sporulating medium. In minimal medium, very poor growth and sporulation of VPS50.2 was obtained, whereas bacteriocin production failed at all (data not shown). Growth and bacteriocin

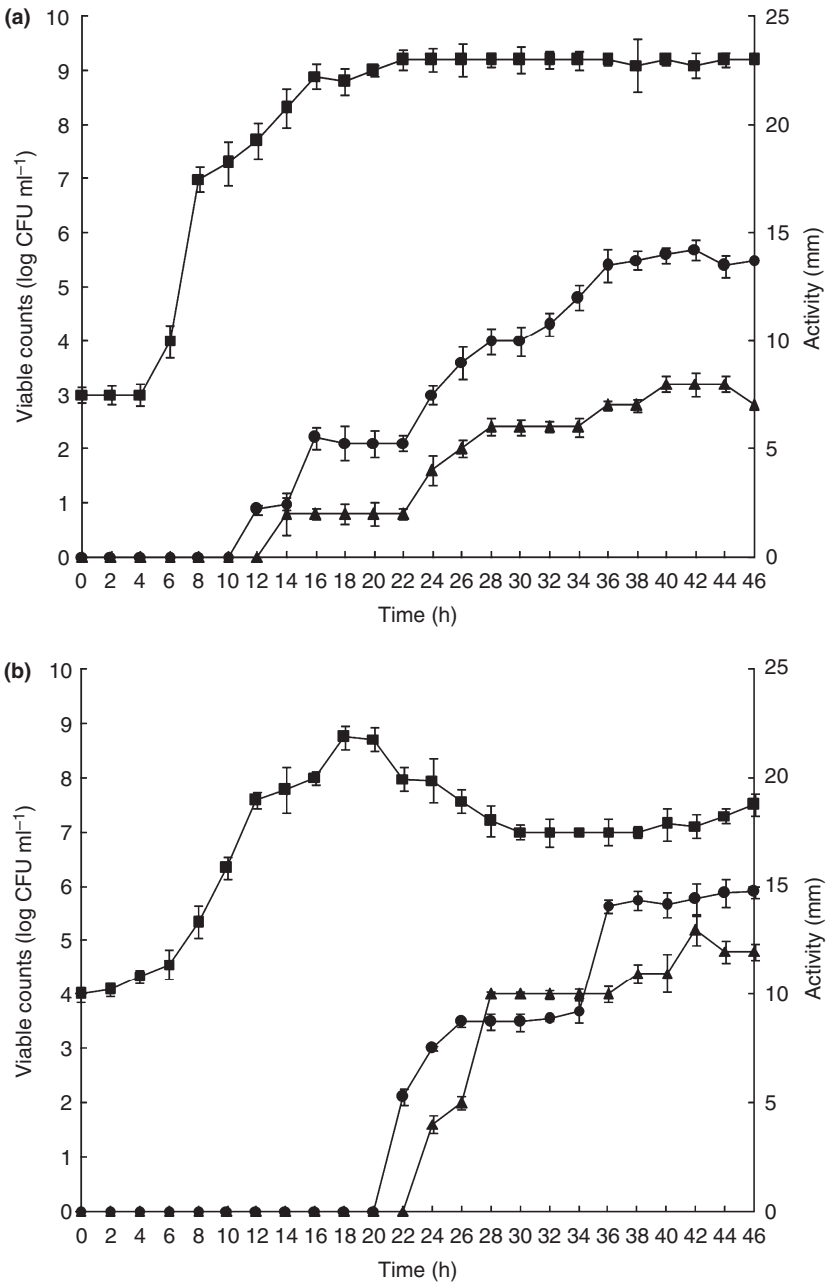

Figure 1 Antimicrobial activity during growth and sporulation of isolate VPS50.2. in: (a) LB medium and (b) Schaeffer's sporulating medium. (■) Viable counts (log cells ml^{-1}), (●) spores (log cells ml^{-1}) and (▲) antimicrobial activity (radiuses of zones of inhibition in mm). *Bacillus subtilis* 168 was used as indicator strain. Each point represents mean of three independent experiments.

production in Schaeffer's sporulation medium (Fig. 1b) were very similar to that in LB medium. The best bacteriocin production, for example, the biggest zones of inhibition on *B. subtilis* 168 lawn were obtained using following conditions: 42 h of incubation in Schaeffer's sporulating medium at 37°C with agitation at 180 rev min^{-1} (data not shown). In addition, further attempt to increase bacteriocin production by co-incubation with *B. subtilis* 168 while growing in optimal medium and physical condition did not improve the productivity (data not shown).

Inhibitory spectrum of antimicrobial substance from *Bacillus licheniformis* VPS50.2

Crude extract of bacteriocin from *B. licheniformis* VPS50.2 was tested for antimicrobial activity against wide list of different strains of bacteria and two fungal species (Table 1). Inhibitory activity was observed for considerable number of bacteria, including important pathogen *L. monocytogenes*, methicillin-resistant *Staph. aureus* and β-haemolytic streptococci. According to these results, the inhibitory spectrum recorded in this work was limited to

Gram-positive bacteria only. Antagonistic action against tested fungi was not detected.

Purification of bacteriocin licheniocin 50.2 and estimation of the molecular weight

Culture of strain VPS50.2 was grown aerobically in LB medium at 30°C for 42 h. Ammonium sulfate precipitation and chloroform purification of bacteriocin from filtrated supernatant were performed. The maximum antagonistic activity was found in the precipitate with 60% saturation of ammonium sulfate. The total protein concentration in this fraction was 2·355 mg ml^{-1}. For further purification and for determination of the approximate molecular weight of the bacteriocin, step of ultrafiltration trough membranes with 3 and 10 kDa molecular cut-off was performed. Bacteriocin was able to pass through 10 kDa membrane and was retained by 3 kDa membrane that was confirmed in agar-well diffusion test against *B. subtilis* 168.

SDS-PAGE analysis of the proteins in the purified filtrate revealed a single protein with molecular mass less than 6·5 kDa that exhibited antibacterial activity against *B. subtilis* 168 (Fig. 2). Result that is more precise was obtained with MALDI TOF/TOF mass spectrometer, which detected protein whose molecular weight was 3253·209 Da. Automated N-terminal Edman degradation of purified sample recognized first 15 amino acids and they go as follows: W E E Y N I I X Q L G N K G Q (the letter 'X' in the protein sequence stands for 'unknown component', according to the provider of N-terminal sequencing service). Using BLAST similarity search, no match with so far described proteins from NCBI database was found. Considering these findings, we named the newly characterized bacteriocin as licheniocin 50.2.

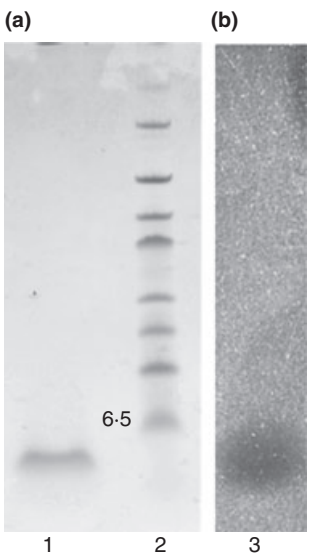

Figure 2 Tricine SDS-PAGE analysis of purified bacteriocin licheniocin 50.2 from strain VPS50.2 (a) and detection of bacteriocin activity on gel (b). a) 1, sample; 2, protein marker (kDa). (b) Gel overlaid with soft agar inoculated by *Bacillus subtilis* 168 with inhibition zone at the level of detected protein (3). Only relevant protein band (6·5 kDa) of the standard protein marker used was indicated.

Effect of proteolytic enzymes, heat and pH on antimicrobial activity

Table 2 summarizes the results obtained after different treatments of the purified bacteriocin. Antimicrobial activity of bacteriocin was insensitive to lysozyme and proteinase K, shows partial sensitivity to trypsin and was completely sensitive towards pronase E. Bacteriocin was heat stable after incubation at 100°C for 30 min and retained 70% activity after being autoclaved at 121°C for 15 min. In addition, after the storage for 6 months at 4°C, bacteriocin residual activity was 100%. The bacteriocin was stable within wide pH range (pH 2–12), remaining about 100% of its initial activity.

MIC and time–kill studies

The MIC of licheniocin for *L. monocytogenes* was 0·53 mg ml^{-1}. For the time–kill studies, *L. monocytogenes* was selected as test organism (Fig. 3). Kill profiles were characterized by a rapid and significant decline (>3 log drop) in bacterial count within the first 2 h in the presence of all bacteriocin concentrations tested. Regrowth was observed at lower concentration of bacteriocin extract (0·5× and 1× MIC) but not at concentrations 2× and 4× MIC. Time to 99·9% killing after exposure to bacteriocin sample at 0·5× and 1× MIC was about 2 h, while at 2× and 4× MIC, it was 1 h.

Table 2 Effects of enzymes and heat on licheniocin 50.2 activity

Treatment	Residual activity (%)*
Enzymes	
Lysozyme	100
Trypsin	70
Proteinase K	100
Pronase E	0
Heat	
50°C/30 min	100
70°C/30 min	100
100°C/30 min	100
121°C/30 min/103·5 KPa/15 min	70

*Residual activity compared with antimicrobial activity before the treatment.

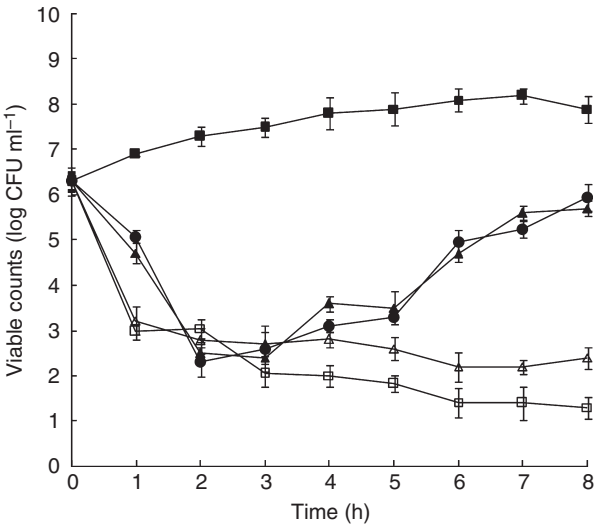

Figure 3 Bactericidal activity of licheniocin 50.2 against *Listeria monocytogenes*. (■) control; (●) 0·5× MIC; (▲) 1× MIC; (Δ) 2× MIC and (□) 4× MIC.

Discussion

A bacteriocin-producing strain was isolated from soil sample from Serbia. Strain VPS50.2 was identified as *B. licheniformis* by biochemical profiling and 16S rDNA sequence. *B. licheniformis* VPS50.2 produces an antimicrobial substance with pronounced antagonistic activities against various species of Gram-positive bacteria, including pathogenic methicillin-resistant *Staph. aureus*, β-haemolytic streptococci and *L. monocytogenes*. Sensitivity of antimicrobial substance from *B. licheniformis* VPS50.2 to pronase E indicated its proteinaceous nature. Therefore, it was affiliated to the bacteriocins. Considering other properties of this bacteriocin, that is, high tolerance to a broad range of pH and pronounced heat stability, as well as small molecular size (3·25 kDa), it could be classified to class II bacteriocins which includes small (0·77–10 kDa), ribosomally synthesized, nonmodified and linear peptides which are heat and pH stable as proposed by novel classification of *Bacillus* bacteriocins. According to Abriouel *et al.* (2010), class II of *Bacillus* bacteriocins was subdivided into four subclasses. Subclass II.1 includes pediocin-like peptides, coagulin and bacteriocins produced by *Bacillus coagulans* and *Paenibacillus polymyxa* strains. Subclass II.2 includes thuricin-like peptides produced by *Bacillus thuringiensis* strains, and cerein MRX1 produced by *Bacillus cereus* strains. Subclass II.3 bacteriocins includes other linear peptides, such as lichenin produced by *B. licheniformis* 26L-10/3RA (Pattnaik *et al.* 2005), or cerein 7A (Oscariz *et al.* 1999) and 7B (Oscariz *et al.* 2006) from *B. cereus* Bc7. Subclass III includes large proteins (>30 kDa) with phospholipase activity. Data about mode of action for class II bacteriocins of *Bacillus* are limited but indicate that putative mechanisms could correspond to that of a membrane-active compound. For class II.3 bacteriocin, cerein 7B was proposed that it may cause a membrane permeabilization (Oscariz *et al.* 2006). Another class II bacteriocin, thuricin S from *B. thuringiensis* interacts with the cytoplasmic membrane to dissipate the transmembrane potential and acts as pore-forming bacteriocin (Chehimi *et al.* 2010).

N-terminal protein sequence of bacteriocin from VPS50.2 shows no similarity with previously described proteins from *Bacillus* sp., suggesting that *B. licheniformis* VPS50.2 produces novel subclass II.3 bacteriocin licheniocin 50.2. Unlike many other previously studied bacteriocins (Martirani *et al.* 2002; Cladera-Olivera *et al.* 2004; He *et al.* 2006), where the antagonistic activity was detected at the middle exponential growth phase and the maximum activity was obtained at the start of the stationary growth phase, maximum antimicrobial activity of bacteriocin licheniocin 50.2 was coinciding with end of exponential phase and beginning of sporulation, similarly to one reported by Korenblum *et al.* (2005). It is possible that the production of this bacteriocin is the consequence of sporulation process or that some other regulatory pathways are involved in its biosynthesis, as reported for other antimicrobial compounds (Leifert *et al.* 1995; Zheng *et al.* 1999).

The use of bacteriocins to inhibit *L. monocytogenes*, food-borne pathogen involved in outbreaks linked to the consumption of contaminated dairy products or vegetables (Muriana 1996) has been already reported (O'Sullivan *et al.* 2002). Nonetheless, resistance of *L. monocytogenes* strains to conventional bacteriocins, such as nisin and pediocin, has been described (Rasch and Knöchel 1998). Crude extract of bacteriocin licheniocin 50.2 showed significant bactericidal activity, against *L. monocytogenes* starting with the first hour of application. MIC was relatively high (around 500 $\mu g\ ml^{-1}$). Literature data about MIC for class II bacteriocins of *Bacillus* are scarce. Usually, activity was given in AU, so comparison was difficult to make. Bacteriocin laterosporulin, produced by *Brevibacillus* sp. GI9, designated to class II showed broad antibacterial activity (Singh *et al.* 2012). MIC for *L. monocytogenes* was more than 20-fold lower comparing with our results, but lethal dose for *Escherichia coli* was up to 500 $\mu g\ ml^{-1}$. In addition, nisin Z, class II bacteriocin from *Lc. lactis*, inhibited *C. albicans* growth beginning at 500 $\mu g\ ml^{-1}$ (Le Lay *et al.* 2008). However, in both cases, purified sample, of laterosporulin and nisin Z, was used, whereas we tested crude extract of bacteriocin. Bactericidal potential, as well as some attributes of licheniocin 50.2, that is, high tolerance to heat, activity in broad pH

range, and prolonged shelf life, could recommend it as a natural biopreservative for control of pathogenic and spoilage micro-organisms. Varying medium composition, temperature and aeration, optimal conditions for bacteriocin production were determined, but additional work on optimization of production, as well as identification of genes involved in synthesis of bacteriocin licheniocin 50.2, is due to be done.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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