TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2014) 38: 611-618
© TÜBİTAK
doi:10.3906/biy-1312-52

Large-scale purification of a bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* using diatomite calcium silicate

Halil DÜNDAR[1,*], Ömür ÇELİKBIÇAK[2], Bekir SALİH[2], Tahsin Faruk BOZOĞLU[3]

[1]Department of Biotechnology, Middle East Technical University, Ankara, Turkey
[2]Department of Chemistry, Hacettepe University, Beytepe, Ankara, Turkey
[3]Department of Food Engineering, Middle East Technical University, Ankara, Turkey

Received: 13.12.2013 • **Accepted:** 21.05.2014 • **Published Online:** 05.09.2014 • **Printed:** 30.09.2014

Abstract: *Leuconostoc mesenteroides* subsp. *cremoris* strain W3, isolated from wine, was previously found to produce a bacteriocin with inhibitory activity towards malolactic bacteria and hence inhibit malolactic fermentation in model wine medium. The bacteriocin, which we termed mesentericin W3, was purified by multistep chromatography and found in a previous study to be similar to mesentericin Y105, albeit with a low recovery (3.2%). In this study, we aimed to achieve large-scale isolation of mesentericin W3 by adsorption of the bacteriocin from culture supernatant onto Micro-Cel (diatomite calcium silicate) and then by subsequent desorption. Water-soluble surfactants including Tween 80, Triton X-100, sodium deoxycholate, sodium dodecyl sulfate (SDS) in distilled water, organic solvents (methanol, ethanol, acetonitrile), and distilled water with a pH range of 2–10 were tested for the desorption of the bacteriocin from Micro-Cel. Mesentericin W3 was successfully desorbed from Micro-Cel with SDS, while other tested reagents were not as successful. The bacteriocin desorbed from Micro-Cel showed the same inhibitory activity as the culture supernatant. The desorbed bacteriocin was applied to an SP Sepharose Fast Flow column for final purification. MALDI-TOF mass spectrometry confirmed the identity of the bacteriocin.

Key words: *Leuconostoc mesenteroides*, malolactic fermentation, bacteriocin, bacteriocin purification, diatomite calcium silicate, MALDI-TOF

1. Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides that usually inhibit strains closely related to the producing bacteria. The bacteriocins of lactic acid bacteria (LAB) are small, heat-stable, cationic, and hydrophobic peptides (Klaenhammer, 1993). They cause cell death by forming pores in the target cells, hence disrupting membrane potentials (Oscáriz and Pisabarro, 2001). The tendency to decrease the use of chemical additives in foods has stimulated investigation into bacteriocins produced by LAB for their application in food preservation as a means to control undesirable organisms (Aymerich et al., 2000). Moreover, bacteriocins have considerable potential for human therapy as supplements to or replacements for antibiotics currently in use (Garneau et al., 2003).

Determining the chemical structures and biological activities of bacteriocins requires the application of efficient purification protocols. The purification of bacteriocins starts with removing producer cells and concentrating peptides from the supernatant by ammonium sulfate precipitation (Guyonnet et al., 2000). After this step, various combinations of cation-exchange and hydrophobic interaction chromatography with a final reversed-phase high performance liquid chromatography are applied (Parente and Ricciardi, 1999). Although these procedures provide excellent results in terms of yield and purification, they are not suitable for large-scale purification of bacteriocins due to the complex purification protocols (Guyonnet et al., 2000; Li et al., 2001). As a result, it is vital to develop more efficient, robust, low-cost procedures to purify bacteriocins on a large scale for ex situ use in inhibiting food spoilage bacteria.

Yang et al. (1992) developed a simple, pH-dependent cell adsorption-desorption method for the purification of bacteriocins produced by LAB, based on the fact that bacteriocin-producing strains of LAB adsorb their own bacteriocins around pH 6.0 and release them around pH 2.0 (Klaenhammer, 1993). This method was exploited for the purification of dextranicin 24 (Revol-Junelles and Lefebvre, 1996), pediocin AcM (Elegado et al., 1997), and leucocins A-, B-, and C-TA33a (Papathanasopoulos et al., 1998) with reverse-phase high performance liquid

* Correspondence: halildundar1@gmail.com

chromatography. Extraction with acid or alkaline rice hull ash was reported for nisin, pediocin RS2, leucocin BC2, lactocin GI3, and enterocin CS1 (Janes et al., 1998). Chloroform extraction was employed to recover lacidin, pediocin, nisin, bacillin, and subtilin (Burianek and Yousef, 2000) from fermentation broth and to purify mutacin 1140 to homogeneity (Hillman et al., 1998). Kelly et al. (2000) described the extraction of nisin, carnocin, and variacin from fermentation broth with toluene, iso-octane, paraffin, kerosene, n-butyl acetate, 2,4,4-trimethylpentane, decanol, and 1-octanol. They stated that all solvents tested resulted in similar results. Qi et al. (2001) used chloroform extraction, coupled with reverse-phase HPLC, to purify the lantibiotic mutacin I and nonlantibiotic mutacin IV produced by *Streptococcus mutans* UA140. Chloroform extraction and *n*-butanol extraction, coupled with Sephadex LH-20 size-exclusion chromatography, were used to purify brochocin A and brochocin B(10-43), which are the components of the 2-peptide bacteriocin brochocin C (Garneau et al., 2003). Food-grade diatomite calcium silicate (Micro-Cel) was applied for the large-scale isolation of nisin, pediocin PO2, brevicin, and piscicolin 126 (Coventry et al., 1996). Micro-Cel is an inexpensive food-grade anticaking agent added to foods produced for direct human consumption, and the desorption agent, SDS, has been utilized as a food-grade emulsifier at up to 0.1% (Coventry et al., 1996).

The present study describes a 2-step protocol for large-scale purification of a bacteriocin from a malolactic strain of *Leuconostoc mesenteroides* subsp. *cremoris* through adsorption and desorption properties of the bacteriocin with diatomite calcium silicate and subsequent cation-exchange chromatography.

2. Materials and methods

2.1. Bacterial strains

The bacteriocin producer *Leuconostoc mesenteroides* subsp. *cremoris* W3 was isolated from Turkish white wine during malolactic fermentation to find a competent inhibitor to control malolactic fermentation (Yurdugül and Bozoglu, 2002). *Lactobacillus delbrueckii* RSSK 498 was used as an indicator of sensitivity. All strains were maintained at –80 °C in appropriate media containing 25% (v/v) glycerol and were subcultured twice before use.

2.2. Bacteriocin activity assay

The serial 2-fold dilutions of the bacteriocin preparations (5 µL) in de Man–Rogosa–Sharpe (MRS) broth (Merck) were assayed for bacteriocin activity by the spot-on-the-lawn technique. Indicator lawns were prepared by adding 0.1 mL of overnight culture to 10 mL of MRS soft agar (0.75%). The contents of the tubes were mixed by vortexing and poured over the surfaces of prepoured agar plates. Bacterial lawns were examined for growth inhibitory zones after overnight incubation at 30 °C. The bacteriocin titer was defined as the reciprocal of the highest 2-fold dilution showing complete inhibition of the indicator lawn and was expressed in activity units (AU)/mL of culture media.

2.3. Bacteriocin production

Mesentericin W3 was produced in a 2-L culture of MRS broth (Merck) with an initial pH of 6.0 inoculated with an overnight culture of *Lc. mesenteroides* subsp. *cremoris* W3 (1% v/v) and incubated for 16 h at 30 °C, which was determined to produce the highest bacteriocin activity (3200 AU/mL). The cultures were carried out in 1-L screw-capped bottles using half volumes without shaking under uncontrolled conditions. The cells were then separated from culture fluid by centrifugation at 23,419 × *g* for 30 min (4 °C) (Sorvall RC-5C Plus; rotor code: GSA 10). Bacteriocin-containing supernatant was heat-treated for 15 min at 80 °C to inactivate proteases, cooled, filter-sterilized (pore size: 0.45 µm, Sartorius), and stored at 4 °C.

2.4. Adsorption of mesentericin W3 onto Micro-Cel

Bacteriocin-containing culture supernatant (100 mL) was mixed with Micro-Cel (Celite Corporation) at a concentration of 1% (wt./vol.) and stirred vigorously for 30 min at room temperature. Micro-Cel was pelleted from the supernatant by centrifugation (Sorvall RC-5C Plus, 16,263 × *g*, 15 min, 4 °C). The supernatant was clarified by filter sterilization (pore size: 0.45 mm; Sartorius) and assayed for bacteriocin activity. The pelleted Micro-Cel was washed with 200 mL of sterile distilled water and washing water was assayed to check any remaining bacteriocin activity.

2.5. Desorption of mesentericin W3 from Micro-Cel

Desorption of the bacteriocin from Micro-Cel was carried out by desorption agents as described, with some modifications (Coventry et al., 1996). The solutions tested to desorp the bacteriocin from Micro-Cel consisted of water-soluble surfactants (Tween 80, Triton X-100, sodium deoxycholate (SDC), and sodium dodecyl sulfate (SDS) in distilled water), organic solvents (methanol, ethanol, acetonitrile), and distilled water within the final pH range of 2–10. The pelleted Micro-Cel adsorbing the bacteriocin was resuspended in a 100-mL volume of each desorption agent. The suspension was stirred with a magnetic bar for 1 h at room temperature, and the Micro-Cel was pelleted by centrifugation (Sorvall RC-5C Plus, 16,263 × *g*, 15 min, 4 °C). The supernatant obtained was filter-sterilized (pore size: 0.45 µm) and assayed for the bacteriocin activity. During desorption with pH within the range of 2 to 10, HCl (1 M) and NaOH (1 M) were added to the Micro-Cel suspension to adjust the pH of the mixture. After pelleting the Micro-Cel, the pH of the supernatant was readjusted to pH 5.5 before assaying bacteriocin activity. Organic solvents were used without dilution and evaporated after

desorption using a vacuum system. Surfactants were used at a final concentration of 1% (wt./vol.) in sterile distilled water. Desorption solutions at the indicated concentrations and pH values containing no bacteriocin were used as control against the indicator strain.

2.6. Cation-exchange chromatography

Mesentericin W3 from 1 L of culture supernatant was extracted with adsorption onto Micro-Cel followed by desorption from it by 1% SDS (wt./vol.) as described before. Desorption was carried out in 200 mL of SDS solution. Excess SDS was removed by cold precipitation after overnight incubation of the desorbed bacteriocin solution at 4 °C and subsequent centrifugation. The pH of the bacteriocin extract was adjusted to 5.5 with HCl and half diluted using 20 mM sodium phosphate buffer (pH 5.5). This solution was mixed with 10 mL of SP Sepharose Fast Flow (GE Healthcare Biosciences) and stirred gently at 4 °C for 1 h with a magnetic bar. After packing the gel slurry into a glass column (2 × 20 cm), the column was washed with 5 column volumes (CVs) of 20 mM sodium phosphate buffer (pH 5.5) and 5 CVs of sodium phosphate buffer (20 mM, pH 5.5) containing 100 mM NaCl. Subsequently, the column was eluted with a step-wise gradient of 4 CVs of 1.0 M NaCl. Fractions of 4 mL at a flow rate of 1 mL/min were collected and assayed for bacteriocin activity.

2.7. MALDI-TOF mass spectrometry analysis

Mass spectra were acquired on a Voyager-DE™ PRO MALDI-TOF mass spectrometer (Applied Biosystems) equipped with a nitrogen UV laser operating at 337 nm. Spectra were recorded in linear mode with an average of 50 shots. α-Cyano-4-hydroxycinnamic acid (10 mg/mL in H_2O:ACN at 1:1 ratio with 0.1% trifluoroacetic acid) was prepared, and MALDI samples were prepared by mixing purified bacteriocin solution with the matrix solution (1:10 v/v) in a 0.5-mL Eppendorf microtube. Finally, 1 µL of this mixture was deposited on the sample plate, dried at room temperature, and then analyzed.

2.8. Bactericidal effect of the Micro-Cel desorbed bacteriocin

An exponentially growing culture of *L. delbrueckii* was divided into 2 equal parts. Mesentericin W3 desorbed from Micro-Cel was added to each part with a final concentration of 800 AU/mL. The control culture and bacteriocin-treated culture were incubated and samples were taken from both cultures at selected intervals for total viable colony counting. Cultures were appropriately shaken just before taking samples in order to not let the cells sediment. Total viable counts (CFU/mL) were determined by pour plating technique using the serial dilutions of indicator organism. All assays were performed in duplicate.

3. Results

3.1. Bacteriocin adsorption onto Micro-Cel

It was shown that 100% of the bacteriocin activity in the culture supernatant of *Lc. mesenteroides* subsp. *cremoris* W3 (3200 AU/mL) was adsorbed onto Micro-Cel after 30 min. The washing water applied after each desorption agent did not show bacteriocin activity.

3.2. Desorption of bacteriocin

SDS at a concentration of 1% (w/v) was the most promising agent for the desorption of adsorbed bacteriocin from Micro-Cel, the eluent with an inhibitory activity of 3200 AU/mL. The control of 1% (wt./vol.) SDS without bacteriocin did show minor activity on the lawn of the indicator microorganism (800 AU/mL). Tween 80, Triton X-100, and SDC resulted in bacteriocin desorption within the range of 200–400 AU/mL (Table 1). Methanol, ethanol, acetonitrile, and distilled water with final pH range of 2–10 did not cause desorption of the bacteriocin from Micro-Cel. However, bacteriocin activity was detected after a 1% SDS treatment of Micro-Cel, which was treated with Tween 80, Triton X-100, SDC in distilled water, methanol, ethanol, acetonitrile, and distilled water with final pH range of 2–10. The effect of SDS concentration on desorption of the bacteriocin was investigated further by using 0.5% (wt./vol.) SDS. Mesentericin W3 desorption from Micro-Cel was achieved by SDS at a concentration of 0.5% (wt./vol.), but with 1600 AU/mL of bacteriocin activity. Thus, we employed the 1% SDS as the desorbing agent when working with 1 L of culture supernatant. The excess amount of SDS was removed by cold precipitation after overnight incubation of the desorbed bacteriocin solution at 4 °C and subsequent centrifugation. In addition, it was found that more SDS could be precipitated at 1 °C than at 4 °C by holding the bacteriocin-containing eluent on a precooled rotor of Sorvall RC-5C Plus (rotor code: GSA 10) for 30 min before centrifugation. Therefore, while desorbing mesentericin W3 from Micro-Cel, centrifugation was performed at 1 °C and the centrifugation period was extended to 60 min (Sorvall RC-5C Plus, 23,419 × *g*, 60 min, 1 °C). After determination of the suitable desorption agent, bacteriocin adsorption onto Micro-Cel was carried out for 1 L of bacteriocin-containing supernatant. Bacteriocin desorption was achieved with 200 mL of 1% SDS to obtain a concentrated eluent and 95% of the initial bacteriocin activity in the culture supernatant of *Lc. mesenteroides* subsp. *cremoris* W3 was recovered after cold precipitation of SDS, indicating that the inhibitory activity was associated with the bacteriocin with 16,000 AU/mL of activity (Table 2). The eluent desorbed from Micro-Cel was applied to an SP Sepharose Fast Flow column to achieve final purification of mesentericin W3. Fractions with the highest bacteriocin activity after the SP Sepharose Fast Flow column were subjected to MALDI-TOF mass

Table 1. Desorption of mesentericin W3 from Micro-Cel with desorption agents.

Desorption agent	Mesentericin W3 activity		
	Desorption agent control (AU/mL)	Desorbed into eluent (AU/mL)	Total desorbed activity (AU)
Tween 80 (1%)	0	400	40,000
Triton X-100 -1%	0	200	20,000
SDC (1%)	0	400	40,000
SDS (1%)	800	3200	320,000
SDS (0.5%)	400	1600	160,000
Methanol (undiluted)	0	0	0
Ethanol (undiluted)	0	0	0
Acetonitrile (undiluted)	0	0	0
Distilled water with pH 2–10	0	0	0

spectrometry, which showed a peak with a m/z value of 3870 Da corresponding to $[M+H]^+$ and hence indicating a molecular mass of 3869 Da (Figure 1). The current purification protocol resulted in an active bacteriocin with 64% recovery (Table 2).

3.3. Inhibition spectrum

The eluent desorbed from Micro-Cel by SDS and the culture supernatant fluid of *Lc. mesenteroides* subsp. *cremoris* W3 were compared as to inhibition spectrum against selected bacteria. Both samples inhibited the growth of *L. plantarum*, *L. sakei*, *L. delbrueckii*, *L. cremoris*, *L. curvatus*, *Lc. mesenteroides*, *Carnobacterium divergens*, *Listeria monocytogenes*, and *Enterococcus faecalis* (Table 3). The bacteriocin desorbed from Micro-Cel was found to have the same inhibition spectrum as the culture supernatant fluid of the producer strain (Table 3).

3.4. Bactericidal effect of the Micro-Cel desorbed bacteriocin

When Micro-Cel–desorbed bacteriocin was added to the culture of *L. delbrueckii*, the organism lost its viability by 99.80% within the first 20 min; viable colony count dropped rapidly from 5.1×10^6 to 1.0×10^4 (a 2.7-log reduction) (Figure 2). After 240 min, the viable colony count of the bacteriocin-treated culture was 1200 CFU/mL, whereas the viable colony count of the control culture reached 3.4×10^8 with a 5.46-log reduction in cell viability (Figure 2). Inhibition of *L. delbrueckii* by the eluent desorbed from Micro-Cel was shown by the spot-on-the-lawn method (Figure 3).

4. Discussion

Bacteriocin-producing LAB in wine are reported to show inhibitory activity towards other LAB during vinification (Lonvaud-Funel and Joyeux, 1993; Strasser de Saad and Manca de Nadra, 1993). Oenological LAB were shown to produce bacteriocins, such as *L. plantarum* J23 (Rojo-Bezares et al., 2007), *L. plantarum* J51 (Navarro et al., 2000), *Pediococcus pentosaceus* (Strasser de Saad and de Manca de Nadra, 1993), and *L. plantarum* LMG2379 (Holo et al., 2001).

Lc. mesenteroides subsp. *cremoris* W3 was isolated from white wine in an attempt to obtain a bacteriocin-producing LAB from wines of the Cappadocia region to control malolactic fermentation (Yurdugül and Bozoglu, 2002). The freeze-dried bacteriocin-like inhibitory substance from *Lc. mesenteroides* subsp. *cremoris* W3 and its combination with nisin were shown to inhibit the growth of the malolactic strain of *L. delbrueckii* subsp. *delbrueckii* and hence its malolactic activity in model wine medium (Yurdugül and Bozoglu, 2002). The inhibitory substance was shown to be a true bacteriocin based on its protease sensitivity, characterized as well as purified in a later study by a 4-step protocol including pH-mediated cell surface adsorption-desorption, extraction with Amberlite XAD-16, cation-exchange chromatography on Macro Prep High S column, and hydrophobic interaction chromatography on Phenyl Sepharose CL-4B column with a 9.3% recovery (Dündar, 2006). The same bacteriocin that we termed mesentericin W3 was purified to homogeneity by hydrophobic interaction, cation-exchange, and reverse-phase high performance liquid chromatography with a 3.2% recovery. MALDI-TOF mass spectrometry and its partial amino acid sequence showed that it was identical to mesentericin Y105 (unpublished results), a previously characterized class IIa bacteriocin (Héchard et al., 1992). Since the bacteriocin producer *Lc. mesenteroides* subsp. *cremoris* W3 is of oenological origin, it can be given "generally regarded as safe" status. Taking into account

Table 2. Purification of the mesentericin W3 by extraction with Micro-Cel and cation-exchange chromatography on SP Sepharose Fast Flow column.

Purification stage	Vol (mL)	Activity (AU/mL)	Total activity (AU)	Yield (%)
Culture supernatant	1000	3200	3,200,000	100
Micro-Cel extract (desorption by 1% SDS)	200	15,200*	3,040,000	95
SP Sepharose Fast Flow	40	51,2	2,048,000	64

*The inhibition activity was assumed to be 15,200 AU/mL rather than 16,000 AU/mL because SDS displayed an inhibition activity of 800 AU/mL on the studied indicator organism, which was subtracted from calculated activity (16,000 AU/mL).

the safety and thermostability of mesentericin W3 over a wide pH range, mesentericin W3 has a potential for use in both high-acid and low-acid canned foods. Hence, ex situ use of mesentericin W3 can be exploited to inhibit spoilage bacteria in wine and other foods, or in in situ production it might be applied with the development of bacteriocinogenic cultures adapted to the specific food conditions through heterologous expression. With respect to ex situ use of bacteriocins, development of large-scale purification methods is necessary. Purification strategies for LAB bacteriocins from complex culture broths exploits the cationic and hydrophobic nature of bacteriocins. Laboratory purification procedures include an ammonium sulfate precipitation step, followed by various combinations of cation-exchange and hydrophobic interaction chromatography, with a final reverse-phase chromatography (Parente and Ricciardi, 1999). Although

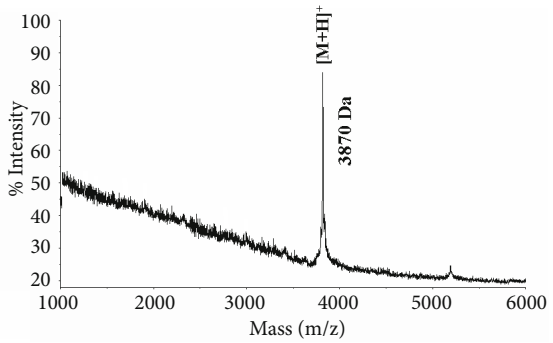

Figure 1. MALDI-TOF mass spectrometry analysis of the active peptide obtained by desorption from Micro-Cel and subsequent cation-exchange chromatography (3870 Da). A positive ion and linear mode MALDI-MS spectrum of active peptide extract was obtained in an α-cyano-4-hydroxycinnamic acid (10 mg/mL in H_2O:ACN at 1:1 ratio with 0.1% trifluoroacetic acid) MALDI matrix using nitrogen laser accumulating 50 laser shots.

the current purification methods work well for low volumes, they can be difficult to scale up when working with the large volumes needed for large-scale industrial production (Uteng et al., 2002; Cheigh et al., 2004).

In this study, the bacteriocin produced by *Lc. mesenteroides* subsp. *cremoris* W3 was isolated in large scale using Micro-Cel and purified to homogeneity with the subsequent cation-exchange chromatography without requiring time-consuming multistep chromatographic methods. The whole bacteriocin in culture supernatant fluid was adsorbed onto Micro-Cel without a loss of bacteriocin activity, a situation sometimes encountered in the applications of ammonium sulfate precipitation of bacteriocins (Xiraphi et al., 2005; Tiwari and Srivastava, 2008). The desorption conditions of mesentericin W3 from Micro-Cel were determined as described (Coventry et al., 1996) with some minor modifications. Tween 80, Triton X-100, SDC, SDS in distilled water, methanol, ethanol, acetonitrile, and distilled water with final pH range of 2–10 were investigated for their ability to desorp the adsorbed bacteriocin from Micro-Cel. Mesentericin W3 was shown to be desorbed efficiently from Micro-Cel with SDS. Cation-exchange chromatography resulted in further purification of the bacteriocin with 64% recovery. MALDI-TOF mass spectrometry of the final product showed a peak with a molecular mass of 3869 Da. The first application of Micro-Cel for bacteriocin extraction from culture medium including nisin, pediocin PO2, brevicin, and piscicolin 126 was reported successful. Adsorption of nisin, pediocin, piscicolin and brevicin onto Micro-Cel was attributed to both electrostatic and hydrophobic interactions between Micro-Cel particles and the bacteriocin molecules, but the hydrophobic interactions are more important than the electrostatic interactions (Coventry et al., 1996). These hydrophobic interactions were overcome more effectively by the use of strong surfactants like SDS and SDC (Coventry et al., 1996). In

Table 3. Inhibition spectrum of the eluent from Micro-Cel and culture supernatant.

Indicator	[a]Sensitivity: Micro-Cel eluent	[a]Sensitivity: Culture supernatant	[b]Medium	Growth temp. (°C)
L. sakei DSM 20017	+	+	MRS	30
L. casei	-	-	MRS	30
L. plantarum LP73	+	+	MRS	30
L. plantarum Z11L	+	+	MRS	30
L. plantarum RSSK 02030	+	+	MRS	30
L. plantarum RSSK 10	+	+	MRS	30
L. plantarum 80B	+	+	MRS	30
L. delbrueckii W6	+	+	MRS	30
L. curvatus DSM 20019	+	+	MRS	30
L. delbrueckii RSSK 498	+	+	MRS	30
L. cremoris RSSK 708	+	+	MRS	30
Lc. mesenteroides RSSK 1061	+	+	MRS	30
Lc. mesenteroides RSSK 923	+	+	MRS	30
C. divergens DSM 20623	+	+	MRS	30
E. faecalis LMG 2602	+	+	GM17	30
L. monocytogenes RSSK 475	+	+	GM17	30
L. monocytogenes RSSK 478	+	+	GM17	30
P. fluorescens LMG 3020	-	-	TSB	30
E. coli LMG 3083	-	-	TSB	37
S. enterica typh LMG 3085	-	-	TSB	37

[a] +, inhibition; -, no inhibition. [b] MRS, de Man–Rogosa–Sharpe broth (Merck); GM17, M17 + 0.5% glucose; TSB, Tryptic Soy Broth (Oxoid); DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; RSSK, Refik Saydam National Type Culture Collection, Ankara, Turkey; LMG, Laboratory of Microbial Gene Technology, Department of Chemistry, Biotechnology, and Food Science, Norwegian University of Life Sciences, As, Norway.

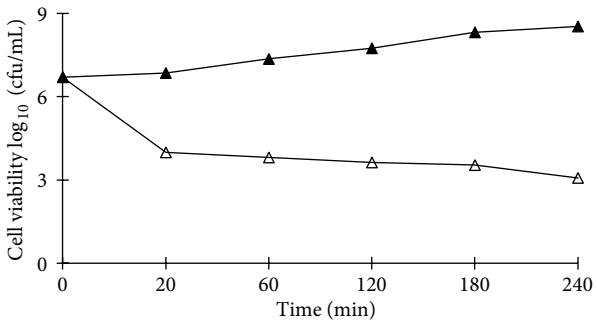

Figure 2. Effect of the bacteriocin on growing cells of *L. delbrueckii* with 800 AU/mL bacteriocin in medium. Symbols: ▲, culture without bacteriocin; Δ, culture with bacteriocin.

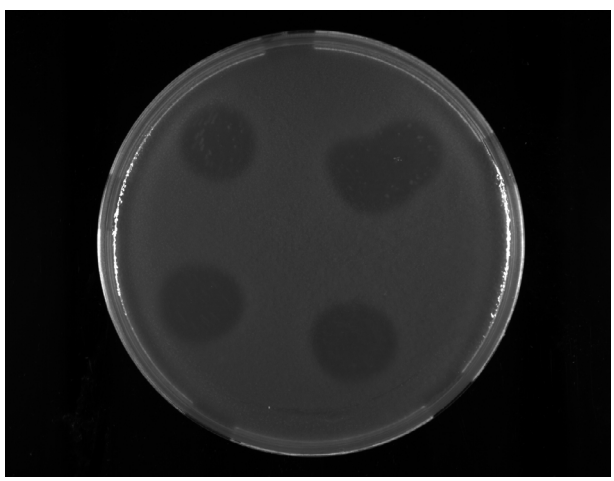

Figure 3. The bactericidal effect of the eluent desorbed from Micro-Cel on *L. delbrueckii* was shown by the spot-on-the-lawn method.

this study, SDS from the desorbed fraction was partially removed with the help of cold precipitation.

Our findings suggested that mesentericin W3 can be purified industrially using diatomite calcium silicate, without time-consuming and expensive multistep chromatographic techniques. The application of Micro-Cel for bacteriocin adsorption avoided the activity loss encountered during ammonium sulfate precipitation, because the extraction and concentration step through Micro-Cel is adsorption-based. The major problem with ammonium sulfate precipitation is the difficulty of collecting the floating precipitates after centrifugation while working with large volumes of culture fluid. Ammonium sulfate precipitation of bacteriocins from culture fluid was reported to cause variable yields: for example, 10% for plantaricin LR14 (Tiwari and Srivastava, 2008), 17% for curvaticin L442 (Xiraphi et al., 2005), 81.6% for pentocin 31-1 (Liu et al., 2008), and 500% for enterocin I (Floriano et al., 1998). Although pH-dependent cell adsorption and desorption methods can be used to extract highly pure preparations of bacteriocins, the yields are low and much of the bacteriocin activity remains in the supernatant. In our previous study, we achieved pH-dependent extraction of the mesentericin W3 from 1 L of culture supernatant with 100% recovery by collecting and using 5 L of the cell mass of producing bacteria as adsorbent (Dündar, 2006). However, it is expensive to obtain such a large amount of bacteria due to the high cost of growth media. In addition, the cells of the producer organisms can release cell wall-associated contaminating proteins and these cells cannot be repeatedly used for bacteriocin purification as other inert resins can. Another drawback to the use of this method is that bacteriocin molecules adsorbed loosely on the cells can be stripped by the centrifugation (Li et al., 2001).

The presence of both electrostatic and hydrophobic interactions between bacteriocin molecules and Micro-Cel particles not only concentrated the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* from culture fluid but also made some degree of purification possible. Application of cation-exchange chromatography provided further purification of the bacteriocin, which was identified by MALDI-TOF mass spectrometry analysis. Some bacteriocin activity was lost in the flow-through fraction of cation-exchange chromatography, which can be prevented by increasing the amount of cation-exchange resin or reducing the volume of the sample applied to the column. Although large-scale extraction and purification of the bacteriocins by diatomite calcium silicate was reported previously, this is the first study showing the extraction and purification of a bacteriocin from wine bacteria. The protocol has proven to be efficient considering the speed of the method and yield of bacteriocin needed for structural studies, as well as for obtaining large-scale preparations of the bacteriocins for use in food preservation and clinical applications.

References

Aymerich T, Garriga M, Ylla J, Vallier J, Monfort JM, Hugas M (2000). Application of enterocins as biopreservatives against *Listeria innocua* in meat products. J Food Protect 63: 721–726.

Burianek LL, Yousef AE (2000). Solvent extraction of bacteriocins from liquid cultures. Lett Appl Microbiol 31: 193–197.

Cheigh CI, Kook MC, Kim SB, Hong YH, Pyun YR (2004). Simple one-step purification of nisin Z from unclarified culture broth of *Lactococcus lactis* subsp. *lactis* A164 using expanded bed ion exchange chromatography. Biotechnol Lett 26: 1341–1345.

Coventry MJ, Gordon JB, Alexander M, Hickey MW, Wan J (1996). A food-grade process for isolation and partial purification of bacteriocins of lactic acid bacteria that uses diatomite calcium silicate. Appl Environ Microb 62: 1764–1769.

Dündar H (2006). Characterization and purification of a bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*. PhD, Middle East Technical University, Ankara, Turkey.

Elegado FB, Kim WJ, Kwon DY (1997). Rapid purification, partial characterization, and antimicrobial spectrum of the bacteriocin, pediocin AcM, from *Pediococcus acidilactici* M. Int J Food Microbiol 37: 1–11.

Floriano B, Ruíz-Barba JL, Jiménez-Díaz R (1998). Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Appl Environ Microb 64: 4883–4890.

Garneau S, Ference CA, Van Belkum MJ, Stiles ME, Vederas JC (2003). Purification and characterization of brochocin A and brochocin B (10-43), a functional fragment generated by heterologous expression in *Carnobacterium piscicola*. Appl Environ Microb 69: 1352–1358.

Guyonnet D, Fremaux C, Cenatiempo Y, Berjeaud JM (2000). Method for rapid purification of class IIa bacteriocins and comparison of their activities. Appl Environ Microb 66: 1744–1748.

Héchard Y, Derijard B, Letellier F, Cenatiempo Y (1992). Characterization and purification of mesentericin Y105, an anti-*Listeria* bacteriocin from *Leuconostoc mesenteroides*. J Gen Microbiol 138: 2725–2731.

Hillman JD, Novak J, Sagura E, Gutierrez JA, Brooks TA, Crowley PJ, Hess M, Azizi A, Leung KP, Cvitkovitch D et al. (1998). Genetic and biochemical analysis of mutacin 1140, a lantibiotic from *Streptococcus mutans*. Infect Immun 66: 2743–2749.

Holo H, Jeknic Z, Daeschel M, Stevanovic S, Nes IF (2001). Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. Microbiology 147: 643–651.

Janes ME, Nannapaneni R, Proctor A, Johnson MG (1998). Rice hull ash and silicic acid as adsorbents for concentration of bacteriocins. Appl Environ Microb 64: 4403–4409.

Kelly NA, Reuben BG, Rhoades J, Roller S (2000). Solvent extraction of bacteriocins from model solution and fermentation broths. J Chem Technol Biot 75: 777–784.

Klaenhammer TR (1993). Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12: 39–85.

Li C, Bai J, Li W, Cai Z, Ouyang F (2001). Optimization of conditions for bacteriocin extraction in PEG/salt aqueous two-phase systems using statistical experimental designs. Biotechnol Progr 17: 366–368.

Liu G, Lv Y, Li P, Zhou K, Zhang J (2008). Pentocin 31-1, an anti-*Listeria* bacteriocin produced by *Lactobacillus pentosus* 31-1 isolated from Xuan-Wei Ham, a traditional China fermented meat product. Food Control 19: 353–359.

Lonvaud-Funel A, Joyeux A (1993). Antagonism between lactic acid bacteria of wines: inhibition of *Leuconostoc oenos* by *Lactobacillus plantarum* and *Pediococcus pentosaceus.* Food Microbiol 10: 411–419.

Navarro L, Zarazaga M, Saenz JS, Ruiz-Larrea F, Torres C (2000). Bacteriocin production by lactic acid bacteria isolated from Rioja red wines. J Appl Microbiol 88: 44–51.

Oscáriz JC, Pisabarro AG (2001). Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. Int Microbiol 4: 13–19.

Papathanasopoulos MA, Dykes GA, Revol-Junelles AM, Delfour A, Von Holy A, Hastings JW (1998). Sequence and structural relationships of leucocins A-, B- and C-TA33a from *Leuconostoc mesenteroides* TA33a. Microbiology 144: 1343–1348.

Parente E, Ricciardi A (1999). Production, recovery and purification of bacteriocins from lactic acid bacteria. Appl Microbiol Biot 52: 628–638.

Qi F, Chen P, Caufield PW (2001). The group I strain of *Streptococcus mutans*, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. Appl Environ Microb 67: 15–21.

Revol-Junelles AM, Lefebvre G (1996). Purification and N-terminal amino acid sequence of dextranicin 24, a bacteriocin of *Leuconostoc* sp. Curr Microbiol 33: 136–137.

Rojo-Bezares B, Saenz Y, Navarro L, Zarazaga M, Ruiz-Larrea F, Torres C (2007). Coculture-inducible bacteriocin activity of *Lactobacillus plantarum* strain J23 isolated from grape must. Food Microbiol 24: 482–491.

Strasser de Saad AM, Manca de Nadra MC (1993). Characterization of bacteriocin produced by *Pediococcus pentosaceus* from wine. J Appl Bacteriol 74: 406–410.

Tiwari SK, Srivastava S (2008). Purification and characterization of plantaricin LR14: a novel bacteriocin produced by *Lactobacillus plantarum* LR/14. Appl Microbiol Biot 79: 759–767.

Uteng M, Hauge HH, Brondz I, Nissen-Meyer J, Fimland G (2002). Rapid two-step procedure for large-scale purification of pediocin-like bacteriocins and other cationic antimicrobial peptides from complex culture medium. Appl Environ Microb 68: 952–956.

Xiraphi N, Georgalaki M, Van Driessche G, Devreese B, Van Beeumen J, Tsakalidou E, Metaxopoulos J, Drosinos EH (2006). Purification and characterization of curvaticin L442, a bacteriocin produced by *Lactobacillus curvatus* L442. A Van Leeuw J Microb 89: 19–26.

Yang R, Johnson MC, Ray B (1992). Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl Environ Microb 58: 3355–3359.

Yurdugül S, Bozoglu F (2002). Studies on an inhibitor produced by LAB of wines on the control of malolactic fermentation. Eur Food Res Technol 215: 38–41.