Folia Microbiol. 47 (4), 391–400 (2002) http://www.biomed.cas.cz/mbu/folia/

Isolation and Partial Characterization of an Antibacterial Substance Produced by *Enterococcus faecium*

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Received 6 February 2002

ABSTRACT. A strain of *Enterococcus faecium* isolated from Bulgarian yellow cheese "kashkaval" produced a bacteriocin-like substance named enterococcin A 2000. The antibacterial substance had a low molar mass (<2 kDa), was relatively stable toward heat but was sensitive to selected proteolytic enzymes. It was active against Gram-positive bacteria including enterococci, such as *Listeria*, *Bacillus* and *Streptococcus*, and also against Gram-negative *E. coli*. Production of enterococcin A 2000 has a maximum near the end of the exponential phase of producer growth. The peptide was purified by ammonium sulfate precipitation, butanol extraction, followed by cation-exchange chromatography and reversed-phase chromatography. A partial sequence of purified enterococcin A 2000 indicated that this substance does not belong to the class IIa of bacteriocins presenting the consensus anti-*Listeria* motif YGNGV.

Lactic acid bacteria (LAB) are an abundant source of diverse antibacterial substances including organic acids, hydrogen peroxide and bacteriocins, which play an important role in food preservation. Bacteriocins are extensively studied from both scientific and industrial point of view due to the possibility to use them as safe natural food additives for elimination of spoilage and of pathogenic microflora, and for an extension of the shelf life of multiple food products.

A large number of well-characterized bacteriocins with different properties has been described and characterized with respect to their biochemical properties and structure, activity spectrum, genetic determinants and mechanism of action. Four different classes of bacteriocins in LAB have been established: I – lantibiotics, II – small heat-stable nonlantibiotic bacteriocins, III – large heat-labile bacteriocins, and IV – complex of bacteriocins with a lipid and a saccharide moiety (Piard *et al.* 1993; Klaenhammer 1993; Nes *et al.* 1996).

The genus *Enterococcus* is classified among LAB often associated with foods that produce bacteriocins. Numerous reports describing the potential of different *Enterococcus* species to produce bacteriocin-like antibacterial substances were published in recent years (Giraffa 1995; Nes *et al.* 1996; Holo *et al.* 1997; Neviani *et al.* 1997; Smid *et al.* 1998; Belicová *et al.* 1999; Lauková *et al.* 1999, 2001; Lauková and Mareková 2001; Morovský *et al.* 2001). Most of the reported bacteriocinogenic strains were isolated from foods – cheese (Maisnier-Patin *et al.* 1996; Nunez *et al.* 1997) and other dairy products (Olasupo *et al.* 1994; Torri Tarelli *et al.* 1994; Vlaemynck *et al.* 1994; Faryas *et al.* 1996). An interesting phenomenon was found for the bacteriocinogenic strain *E. faecium* L50 since biochemical and genetic studies showed that the strain features a temperature regulation of bacteriocin production (Cintas *et al.* 2000).

Here we report on the characterization of a small hydrophobic antibacterial protein, named enterococcin A 2000, produced by the strain *E. faecium* A 2000 isolated from Bulgarian yellow cheese, with an anti-*Listeria* and anti-*E. coli* activity.

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MATERIALS AND METHODS

Bacterial strain and growth conditions. The bacteriocinogenic strain *Enterococcus faecium* A 2000 was successfully cultivated at 37 °C in modified MRS broth (*Merck*, Germany) containing 5 g/L yeast extract as the source for organic nitrogen (without Tween, pH not regulated).

Phenotypic identification. The strain (producer of the enterococcin A 2000) was identified to the species level by the Gram-positive identification (*Vitek Systems*, USA), API Rapid Strep System 20 and API 50 CH (*bioMérieux*, France) and conventional biochemical tests: phase-contrast microscopy of cell shape and arrangement, catalase and oxidase tests, mobility, production of gas from glucose by the hot loop test (Sperber and Swan 1976), growth in anaerobic conditions, production of ammonia from arginine, acid formation in mannitol, arabinose and raffinose broth, growth in MRS broth at 4, 10, 28, 37, 45 and 50 °C, growth in MRS broth adjusted to pH 5.0 and 9.6, growth in MRS broth with 3 or 6.5 % NaCl, production of dextran from sucrose, growth on acetate agar, presence of *meso*-2,6-diaminopimelic acid (Dap) in the cell wall, lactic acid isomer produced from glucose (*Boehringer*, Germany), growth in the presence of sodium azide and 2,3,5-triphenyltetrazolium chloride (TTC).

Molecular identification of the producer strain was performed by PCR-amplification of the 16S–23S rRNA intergenic spacer region (ISR) and following restriction fragment length polymorphism (RFLP) analysis. Bacterial DNA was extracted according to Saruta *et al.* (1995) and approximately 50 ng of DNA templates was subjected to PCR amplification. Identification at the genus level was obtained by amplification of the 16S–23S rRNA ISR with primers 16S/p4 (5′-GCT GGA TCA CCT CCT TTC T-3′), which anneals to positions 1526–1542 of the 16S rRNA gene, and 23S/p7 (5′-GGT ACT TAG ATG TTT CAG TTC-3′), located to positions 209–189 of 23S rRNA gene from *E. coli* numbering (GenBank accession no. V00331), respectively. The primers were designed to amplify almost the full length of ISR. Identification at the species level was performed by ISR-RFLP analysis. The PCR products for ISR-RFLP analysis were obtained after amplification with primer $tRNA^{Ala}$-23S/p10. The reverse primer 23S/p10 (5′-CCT TTC CCT CAC GGT ACT G-3′) was designed from the flanking terminal sequences of the 23S gene conserved among various bacteria (Gürtler *et al.* 1996). The forward $tRNA^{Ala}$ primer (5′-TAG CTC AGC TGG GAG AGC-3′) was chosen inside the 16S–23S ISR of *Oenococcus oeni*. This primer corresponds to a conserved sequence of the $tRNA^{Ala}$ gene. All PCR amplifications were performed in a PTC-100 thermocycler (*MJ Research*, Watertown, USA). PCR mixture in a total volume of 50 μL contained 5 μL 10× PCR buffer, 2 μL of each 0.25 mmol/L dNTP, 0.45 μmol/L of each primer, 2.5 mmol/L $MgCl_2$ and 1 U of Taq DNA polymerase (5 U/μL; *Ampligene*). The temperature profile was as follows: an initial extensive denaturation step (94 °C, 5 min), 35 reaction cycles, each cycle consisting of denaturation (94 °C, 1 min), annealing (60 °C, 75 s), extension (72 °C, 75 s) and final extension (72 °C, 7 min). The PCR products were examined by horizontal electrophoresis in 1.5 % agarose gels (high resophor agarose; *Eurobio*) in 1× TBE buffer. Gels were stained with 0.5 mg/L ethidium bromide and visualized under UV light. PCR products were purified with the QIAquick PCR purification kit (*Qiagen*) according to the manufacturer's protocol.

Restriction enzyme analysis was carried out with the following enzymes: *Hin*fI, *Hin*pI and *Taq*I. Digestions were performed as described by the supplier with an excess of restriction enzymes (10 U per reaction mixture) in a final volume of 25 μL at an optimum temperature according to the manufacturer's protocols (*Ampligene*) for 3 h. The resulting digested products were separated by electrophoresis on 2 % agarose gel in 1× TBE buffer and photographed.

Computer-assisted analysis of rRNA restriction patterns. Gel images were digitized with a charged-coupled device video camera (*Sony*) and stored as TIFF files. They were converted, normalized with molar size markers and analyzed with Bio-Profil Bio-1D^{++} Software (*Vilbert Lourmat*, France). For ISR-RFLP analysis, a band-matching algorithm was selected to calculate pair-wise similarity matrices with the Dice's coefficient. A band-matching tolerance of 5 % was chosen. Cluster analysis of similarity matrices was performed by the group method using arithmetic averages UPGMA (unweighted pair-group method). The PCR and RFLP patterns of the strain A 2000 were compared to type strains of *E. faecium* ATCC 19434^T, CIP 103 014, LC11, LC15, LP68, *E. faecalis* ATCC 19433^T, CIP 103015, *E. durans* TH 481^T, *E. gallinarum* TH 479T, *Streptococcus thermophilus* NCDO 573^T, ATCC 19258^T, *Lactococcus lactis* subsp. *lactis* ATCC 11454^T and *L. lactis* subsp. *cremoris* CNRZ 117^T.

Bacteriocin isolation and purification. After 1-d cultivation (at 37 °C without regulation of pH), the culture (600 mL) was centrifuged (12000 ***g***, 30 min). The supernatant was precipitated overnight by ammonium sulfate (final concentration 80 %, *W/V*) at 4 °C and then centrifuged (12000 ***g***, 1 h). The pellet was resuspended in MilliQ water and bacteriocin was extracted 3-times with 60 mL 1-butanol for 2 h. The extract was left to separate overnight, then organic phase was evaporated and residue was tested for its antimicrobial activity.

Further purification of enterococcin A 2000 was performed by cation-exchange chromatography on a 1 mL Resource S column (*Pharmacia Biotech*, Sweden). Crude substance suspended in 10 mmol/L KCl buffer (pH 1.5) was applied on the column previously equilibrated with the same buffer. Proteins were eluted with 0.1–1.0 mol/L NaCl step-gradient in KCl buffer (pH 1.5; flow-rate of 0.5 mL/min). Fractions of 0.5 mL were collected and tested for anti-*Listeria* activity.

The active fractions were further purified by RP-HPLC system (PerSeptive Biosystem; *BioCAD*), on a C_{18} column Eurosil Bioselect 300 (250 × 8 mm, 5 μm; *Knauer*, Germany). Products with antibacterial activity were eluted with mobile phases A (0.1 % trifluoroacetic acid in water) and B (0.1 % trifluoroacetic acid in acetonitrile) at a flow rate of 0.8 mL/min; peptides were monitored at 220 nm. The fractions with the highest bacteriocin activity were pooled and evaporated on a Speed-Vac concentrator (*Savant*).

The fractions showing an antimicrobial activity were resuspended in 500 μL 0.1 % trifluoroacetic acid in 0.1 % sodium dodecyl sulfate (SDS) and analyzed by RP-HPLC (*BioCAD*), on a C_{18} analytical column (Eurospher, 250 × 4.5 mm, 5 μm; *Knauer*). After equilibration of the column with solvent A (flow rate of 0.6 mL/min) the peptides were eluted by using a gradient from solvent A to solvent B; peptides were monitored at 220 nm. All the peaks were collected manually in vials, evaporated and assayed for their bacteriocin activity.

Bacteriocin assay and spectrum of activity. Bacteriocin screening was performed by the agar spot-test method (Schillinger and Lucke 1989); 1.5 % agar was used (for overlay 0.75 %).

In order to eliminate the action of lactic acid on the test organisms, the pH of the tested supernatants was adjusted to 6 with NaOH.

The activity was expressed in arbitrary units (AU/mL); 1 AU was defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator strains *Listeria innocua* F and *E. coli*.

Effect of heat, pH and hydrolytic enzymes on enterococcin activity. Partially purified bacteriocin was heated at different temperatures, at various pH values and in the presence of some surfactants. The substance was also incubated (37 °C, 2 h) in the presence of the following enzyme solutions (1 g/L): prolidase, α-chymotrypsin, trypsin, rennin, proteinase X, pronase E (all in 50 mmol/L Tris, 0.1 mol/L NaCl; pH 8.0); α-amylase (in 50 mmol/L MOPS, 0.1 mol/L NaCl; pH 7.0); papain (in 0 mmol/L MES, 0.1 mol/L NaCl; pH 6.0); pepsin (in 50 mmol/L phosphate buffer, 0.1 mol/L NaCl; pH 2.0) and then boiled for 2 min. The same buffers without enzymes were used as controls.

Surfactants tested (final concentration of 1 %) were *N*-laurylsarcosine, SDS, Triton X-100, Tween 20, Tween 80 and urea; controls made of either active supernatant or detergents were used. All samples and controls were incubated (37 °C, 5 h) and tested for activity.

The sensitivity of enterococcin to pH was estimated by adjusting the pH of culture supernatant in the range of 2–10 (with HCl or NaOH); the solutions were incubated for 30 min and 2 h, neutralized and tested against the indicator strains.

The sensitivity to heat was evaluated by heating partially purified samples of bacteriocin to 50, 60, 70, 80 and 100 °C and estimating of residual activity after 5, 10, 15, 20 and 30 min by the agar diffusion assay.

Protein concentration was determined by the BCA method according to the manufacturer's instructions (*Pierce*, USA); bovine serum albumin was used as standard.

Determination of the amino acid composition. Purified bacteriocin was hydrolyzed in 6 mol/L HCl (*Pierce*) (110 °C under vacuum, 1 d) in a Pico-Tag station (*Waters*). The amino acids were derivatized with phenyl isothiocyanate according to Bidlingmeyer *et al.* (1984) and separated by RP-HPLC on a Pico-Tag C_{18} column (150 × 3.9 mm). The column was equilibrated with solvent C: 94 % of 0.14 mol/L sodium acetate and 0.5 mL/L triethylamine (pH 6.4) + 6 % acetonitrile; elution was performed by using a gradient from solvent C to solvent D (water–acetonitrile, 2 : 3). Both the column and solvents were maintained at 38 °C, the flow rate was 1.0 mL/min; A_{254} was monitored.

Tricine–SDS-PAGE was performed according to Schagger and von Jagow (1987) at room temperature on a 16.5 % acrylamide running gel and 4 % acrylamide stacking gel. The silver staining of the gel was done according to Nesterenko *et al.* (1994); staining with Schiff reagent was done according to Groves *et al.* (1992).

MS analysis. The molar mass of RP-HPLC purified enterococcin A 2000 was determined by HPLC–electrospray ionization (ESI)-MS on a PE Sciex Api III$^+$ triple–quadruple mass spectrometer (*Sciex*, Canada) equipped with an atmospheric pressure-ionization source.

The *N-terminal amino acid sequence* of RP-HPLC purified enterococcin A 2000 was determined using an *Applied Biosystem* model 477A sequencer with on-line identification of 3-phenyl-2-thiohydantoin derivatives.

RESULTS AND DISCUSSION

Identification of the strain. The morphology and mobility of the strain A 2000 were determined by phase-contrast microscope examination. The cells were small ovoid cocci, occurring singly, in pairs or in short to long chains. Isolated strain was catalase negative, Gram-positive, facultative anaerobe, did not have *meso*-Dap in the cell wall and produced only L-(+)-lactic acid. The strain grew at 10 and 45 °C but not at 50 °C, fermented glucose to L-lactic acid but did not produce gas, gave a positive Voges–Proskauer reaction, and produced ammonia from arginine. The final pH in glucose broth was 4.6; acidic pH was produced from ribose but not from raffinose, arabinose, glycogen, arabinitol, tagatose, glucitol or gluconate. The strain did not exhibit urease activity; also the hemolytic tests were negative. Based on the saccharide fermentation pattern, ability to grow at pH 9.6, in the presence of 6.5 % NaCl or in the presence of 400 ppm sodium azide and TTC, this isolate was identified as *Enterococcus faecium*.

The PCR amplification of the 16S–23S rRNA-ISR resulted in two amplification bands (ca. 550 and 650 bp). All tested strains of *E. faecium* (including type strains ATCC 19434^T, LC11, LC15 and LP68) showed two amplification bands migrating at approximately 550 and 650 bp. By contrast, all strains of *E. faecalis* (ATCC 19433^T, CIP 103015) displayed two smaller bands (ca. 450 and 600 bp). A single amplified fragment was revealed in the case of *Lactococcus* strains (ca. 500 bp) and *Streptococcus thermophilus* (ca. 550 bp) (*data not shown*). Limitations of the PCR-ISR method for identification at the species level were resolved by RFLP analysis. Primers tRNA^{Ala} and 23S/p10 amplified a single fragment of about 900 bp. This fragment was an alternative genetic target in addition to the ISR and was further analyzed by digestion with endonucleases (*Taq*I, *Hin*fI, *Hin*pI). These restriction enzymes were selected according to the knowledge on amplicon size and sequence polymorphism of the ISR (Naimi *et al.* 1997). The observed individual RFLP patterns generated true probabilities for differentiation at the genus and species levels. Digestion with *Taq*I generated four genotypes with well-resolved bands. In this case, a polymorphism was found between *E. faecium*, *E. faecalis*, *L. lactis* and *S. thermophilus* (Fig. 1). The tested strains corresponding to the group of *E. faecium* showed identical RFLP patterns with two bands of ca. 200 and 320 bp. The strains of *E. faecalis* exhibited digestion fragments located at 180 and 400 bp; however, all strains from *L. lactis* group showed two bands at 200 and 500 bp. The identification of strain A 2000 as *E. faecium* was supported by comparing of its PCR and PCR-RFLP patterns with database of PCR-RFLP profiles from other lactic acid bacteria according to Naimi *et al.* (1997) and Moschetti *et al.* (1998, 2001).

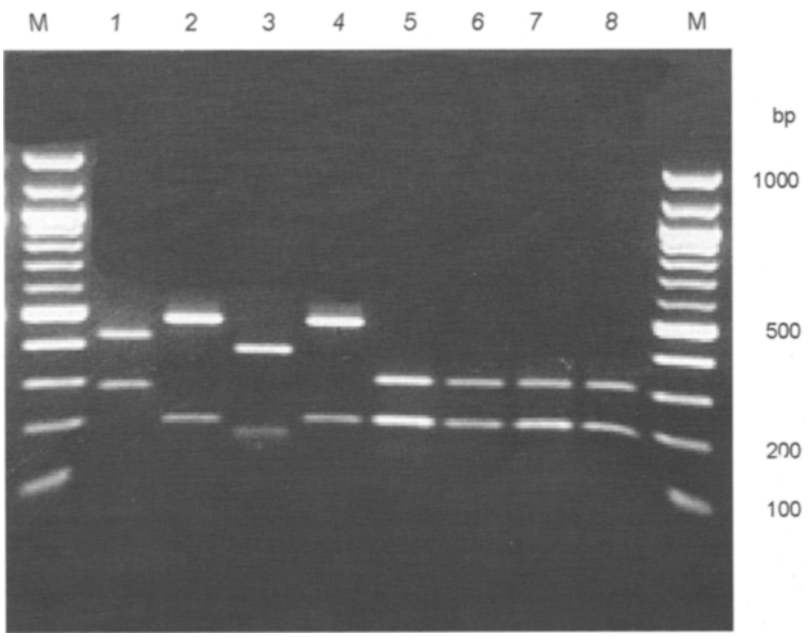


Fig. 1. PCR-based RFLP patterns obtained by *Taq*I digestion of ISR-PCR products; *1* – *Streptococcus thermophilus* NCDO 573, *2* – *Lactococcus lactis* subsp. *lactis* ATCC 11454, *3* – *Enterococcus faecalis* ATCC 19433, *4* – *Lactococcus lactis* subsp. *cremoris* CNRZ 117, *5* – *Enterococcus faecium* A 2000, *6* – *Enterococcus faecium* ATCC 19434, *7* – *Enterococcus faecium* CIP 103 014, *8* – *Enterococcus faecium* LC1, M – DNA molar-mass markers.

Production of antibacterial activity. Fig. 2 shows the cultivation profile of *E. faecium* A 2000 at 37 °C in modified MRS broth, without Tween, containing 5 g/L yeast extract as a source of organic nitrogen. The bacteriocin-like activity was extracellulary produced at the end of the exponential phase. A maximum yield (1600 AU/mL) was measured after a 1-d incubation.

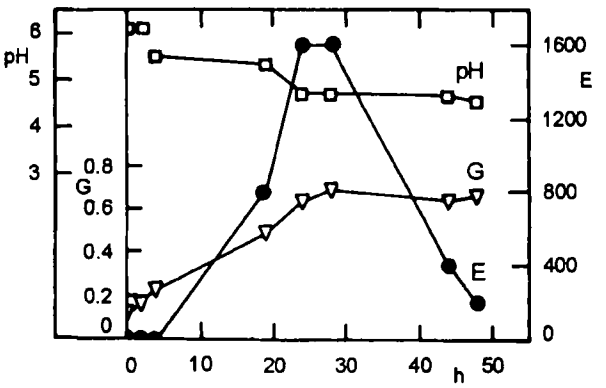


Fig. 2. Culture profile and activity of *Enterococcus faecium* A 2000 cultivated at 37 °C on modified MRS broth (without Tween, with 5 g/L yeast extract); G – growth, A_{600}; E – enterococcin A 2000 activity, AU/mL; pH.

Inhibitory activity and spectrum of inhibition. Enterococcin A 2000 showed antibacterial activity against Gram-positive bacteria including enterococci such as *Listeria*, *Bacillus* and *Streptococcus* and also against some Gram-negative bacteria such as *E. coli*, *Salmonella typhymurium*, *Yersinia pseudotuberculosis* and *Y. enterocolitica* (Table I).

Table I. Inhibitory spectrum of enterococcin A 2000

Indicator species	I/T[a]	Indicator species	I/T[a]
Bacillus cereus	1/1	*Lactococcus lactis* 3931	1/1
Bacillus subtilis	2/2	*Lactococcus lactis* ATCC 11454	1/1
Carnobacterium piscicola	2/2	*Lactococcus lactis* subsp. *cremoris*	0/1
Enterococcus faecalis	1/1	*Leuconostoc mesenteroides*	0/2
Escherichia coli	4/6	*Listeria innocua* F	1/1
Lactobacillus acidophilus	1/1	*Listeria monocytogenes*	4/4
Lactobacillus brevis	1/1	*Pediococcus domnosus*	0/1
Lactobacillus casei	0/2	*Salmonella typhymurium*	1/1
Lactobacillus delbrueckii subsp. *bulgaricus*	1/31	*Staphylococcus aureus*	0/1
Lactobacillus delbrueckii subsp. *lactis*	0/1	*Streptococcus thermophilus*	3/8
Lactobacillus helveticus	0/4	*Yersinia enterocolitica*	1/1
Lactobacillus plantarum	0/1	*Yersinia pseudotuberculosis*	1/1
Lactococcus lactis 14	1/1		

[a]Number of strains inhibited/tested.

Characterization of the inhibitory agent (Table II). The stability of enterococcin A 2000 activity was determined upon treatment with various effectors. Complete inactivation was observed after treatment with pronase E and prolidase, which indicates the protein nature of the active agent. At the same time, trypsin, chymotrypsin, rennin, proteinase X, papain, pepsin and α-amylase had no effect. Among the surfactants studied, only *N*-laurylsarcosine suppressed the bacteriocin activity. The pH stability of the antibacterial substance (studied in the pH 2–10) was not changed in a wide pH range of 3–8. The substance both in the neutralized active-culture supernatant and partially purified appeared to be heat labile as it was completely inactivated above 60 °C (after 15 min at 60 °C the activity was lost).

Purification of enterococcin A 2000 from the culture supernatant was performed by ammonium sulfate precipitation, 1-butanol extraction, cation-exchange chromatography and reversed phase chromatography (Table III). Fig. 3 and 4 show typical cation-exchange and reversed phase chromatography elution profiles. The antibacterial activity was found in fractions eluting with approximately 0.3 mol/L NaCl in case of cation-exchange chromatography and with 80 % acetonitrile in case of RP-HPLC.

Table II. Effect of enzymic treatment, surfactants, pH and temperature on enterococcin A 2000 activity[a]

Treatment	Bacteriocin activity	Treatment	Bacteriocin activity
Enzymes		**pH**	
Trypsin (*Sigma*, no. T 8253)	+	2	+/–
α-Chymotrypsin (*Serva*, no. 17160)	+	3	+
Prolidase (*Sigma*, no. P 6675)	–	4	+
Rennin (*Sigma*, no. R 2761)	+	6	+
Proteinase X (*Sigma*, no. P 1512)	+	8	+
Pronase E (*Sigma*, no. P 5147)	–	9	–
α-Amylase (*Sigma*, type VIIA)	+	10	–
Papain (*Sigma*, no. P 4762)	+		
Pepsin (*Merck*, no. 7189)	+		
Surfactants		**°C**	
N-Laurylsarcosine	–	50	+
SDS	+	60	+
Triton X-100	+	70	–
Tween 20	+	80	–
Tween 80	+	100	–
Urea	+		

[a](+) – positive, (–) – negative; specific activity of enterococcin A 2000 without treatment 1600 AU/mL.

Table III. Purification of enterococcin A 2000

Purification step	Volume mL	Activity AU/mL	Total activity AU × 10^{-3}	Protein g/L	Specific activity AU/mg protein	Recovery %	Purification fold
Culture supernatant	600	1600	966	4.5	355	100	1
Ammonium sulfate	30	3200	96	3.8	842	10	2.4
Butanol extract	10	3200	332	2.3	1391	3.3	4.0
Cation-exchange chromatography	5	3200	16	0.8	4000	1.7	11.3
RP-HPLC (first run)	1.5	6400	9.6	0.2	48000	1.0	135
RP-HPLC (second run)	0.5	12800	6.4	0.1	64000	0.7	180

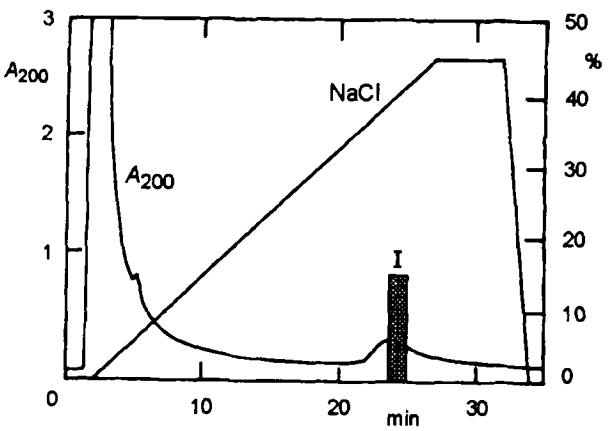


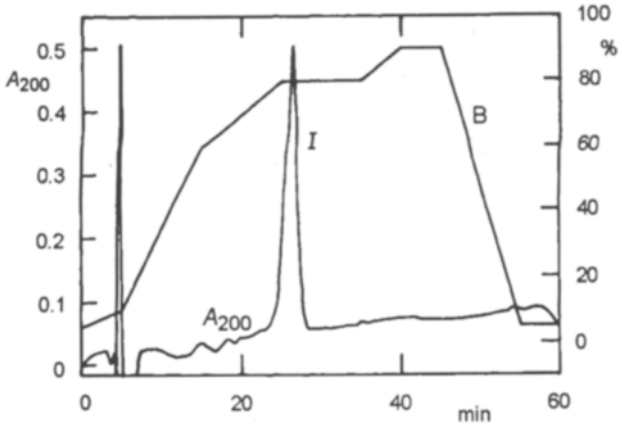
Fig. 3. Purification of enterococcin A 2000 by cation-exchange chromatography using Resource-S 1-mL column; I – inhibitory activity; A_{200} – detector response; NaCl (%) – NaCl gradient; for further details *see Materials and Methods.*

Molar mass determination. The substance obtained after reversed phase chromatography gave a smudge band between the bands representing insulin β-chain (3496 Da) and bacitracin (1423 Da) (Fig. 5). Confirmation of the molecular size of enterococcin A 2000 was obtained by analyzing the purified peptide by LC–MS and MS–MS that revealed the presence of one peptide with a molar mass of 1342 Da (*data not shown*).

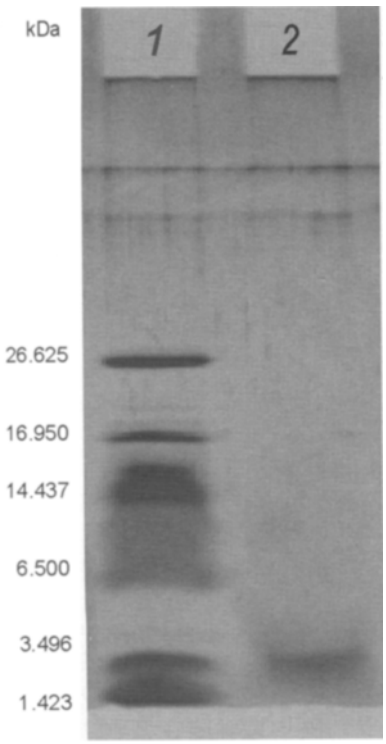
Amino acid composition and N-terminal sequence analysis. Amino acid composition of purified enterococcin A 2000 obtained after final chromatography purification step is shown in Table IV. On the

basis of one phenylalanyl residue per molecule it could be stated that the active peak issuing from RP-HPLC contains a majority (80 %) of hydrophobic and uncharged amino acids and that it is particularly rich in prolyl residues, which explains the inactivation of enterococcin A 2000 after action of prolidase (Table II)

The proposed N-terminal region of enterococcin A 2000 obtained by Edman degradation and MS–MS analysis is **Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile/Leu-X-X---X-X**.



➢**Fig. 4.** Purification of enterococcin A 2000 by reversed-phase chromatography on Eurospher C_{18} (250 × 4.5 mm), 5 μm, 8 nm; I – inhibitory activity; A_{200} – detector response; % of solvent B in solvent A (for further details *see Materials and Methods*).



➢**Fig. 5.** Silver-stained Tricine–SDS-PAGE of enterococcin A 2000; *1* – molar mass markers, *2* – enterococcin A 2000 purified by reversed phase chromatography on Eurospher C_{18} column; insulin β-chain (3496 Da) and bacitracin (1423 Da) used as relevant standards (both *Bio-Rad*).

During the last few years, a large number of bacteriocins from LAB has been described (Klaenhammer 1993). Enterococci produce a wider variety of antimicrobial peptides than it has been described for strains of most of other LAB genera. Enterococcins are generally active against other enterococci as well as against strains of *L. monocytogenes* (Giraffa 1995). The anti-*Listeria* activity may be explained by the fact that enterococci and listeriae are phylogenetically closely related (Devriese *et al.* 1993). Some enterococcins are active against other LAB as well as *Clostridium* spp., including *C. botulinum*, *C. perfringens* and *C. tyrobutyricum* (Torri Tarelli *et al.* 1994; Franz *et al.* 1996). Bacteriocinogenic enterococci were isolated from a variety of sources, including fermented meat (Aymerich *et al.* 1996; Casaus *et al.* 1997), dairy products (Olasupo *et al.* 1994; Torri Tarelli *et al.* 1994; Vlaemynck *et al.* 1994; Faryás *et al.* 1996), and vegetables (McKay and Baldwin 1990; Villani *et al.* 1993; Franz *et al.* 1996).

Bacteriocinogenic enterococci may be used as anti-*Listeria* agents in the dairy industry, particularly in certain types of soft cheeses (*e.g.*, Camembert or Taleggio) where pH in the rind increases to a level that allows growth of *L. monocytogenes*. Enterococci often predominate during ripening of cheeses and could produce bacteriocins at levels sufficient to inhibit *Listeria* (Giraffa 1995; Lauková *et al.* 2001).

Strains of enterococci, including *E. faecium* and *E. faecalis*, are known to produce bacteriocins called enterocins or enterococcins; they generally belong to class II. Examples of the best characterized enterocins are enterocin A, a class IIa, pediocin-like bacteriocin (Aymerich *et al.* 1996), and enterocin B (Casaus *et al.* 1997), a bacteriocin that is not pediocin-like, but is similar to the class IIa bacteriocins because of its chemical characteristics, heat stability and anti-*Listeria* activity. Enterocin P belongs to class IIc because its

secretion occurs by the sec-pathway (Cintas *et al.* 1997). Enterocins L50A and L50B are novel bacteriocins. Each has an antimicrobial activity of its own; together they show synergistic activity. They are not post-translationally modified and do not require the presence of a leader or signal peptide for secretion (Cintas *et al.* 1998).

Table IV. Amino acid composition of enterococcin A 2000

Amino acid	AAR_{100}[a]	Molar ratio per fraction
Hydrophobic		
Ile	5.8	1
Phe	6.3	1
Leu	19.6	3
Val	9.0	1–2
Charged		
Glx	11.6	2
Neutral		
Gly	6.0	1
Tyr	5.0	1
Pro	24.9	3–4

[a]Amino acid residues per 100 residues.

Most enterococcal bacteriocins are small (2–6 kDa), thermostable (still active after 1 h at 100 °C) and hydrophobic peptides. Enterococcin A 2000 fits into this category, except for its thermal stability at temperatures higher than 50 °C. Our results also indicate that enterococcin A 2000 does not contain modified amino acids. The molar mass (1342 Da) was smaller than that deduced from amino acid composition. This difference can be ascribed to difficulties to obtain complete amino acid composition for such a small peptide. Despite their physico-chemical similarities, there is a remarkable lack of sequence similarity among most of the bacteriocins obtained from lactic acid bacteria (Klaenhammer 1993). The pediocin family is the only group that has been identified by sequence similarities. All the bacteriocins of this family contain the motif YGNGVXC in their N-terminal part (Aymerich *et al.* 1996). Enterococcin A 2000 does not contain such a motif. However, its N-terminal sequence is identical to that of plantaricin SA6 from *Lactobacillus plantarum* (Rekhif *et al.* 1995). The same sequence homology has already been described for other non-lantibiotic bacteriocins such as enterocin B (Holo *et al.* 1997) and carnobacteriocin A (Worobo *et al.* 1994). Surprisingly, N-terminal sequence of enterococcin A 2000 is also identical to the fragment 59–66 of milk β-casein which is known to possess opioid (fragment 60–66; Meisel 1998) and immuno-stimulating activities (fragment 63–68; Smacchi and Gogetti 2000). Stimulation of phagocytic activity of human and murine macrophages and protection against *Klebsellia pneumoniae* infection in mice have been ascribed to β-casein fragment 63–68 (Migliore-Samour *et al.* 1989).

Bacteriocins are synthesized in ribosomes as precursor peptides with an N-terminal leader sequence or a signal peptide, which is cleaved concomitantly with export across the cytoplasmic membrane by dedicated ATP-binding cassette transporters and their accessory proteins or by the general secretory pathway. The effect of enterococcin A 2000 on liposomes indicated that a receptor of a protein nature is not absolutely essential for bacteriocin action; the broad antimicrobial spectrum of enterococcin A 2000 also supports the assumption that a protein receptor is not required for the activity (Pantev *et al.* 2001). However, *in vivo* activity enhancement by some protein components of the membrane or cell surface should be involved.

Enterococcin A 2000 described here is different from other enterococcal bacteriocins. It is not a bacteriocin *sensu stricto* but it possesses several advantages over bacteriocins produced by Gram-positive bacteria. Since *E. faecium* is used in the surface-ripening flora of cheese, metabolites secreted by this bacterium should be acceptable in food. The spectrum of activity of enterococcin A 2000 is interesting (among the bacteria tested, the pathogens *Helicobacter pylori* and *Campylobacter* were sensitive) but its further characterization, *i.e.*, complete structure, toxicity and efficacy *in situ* will be necessary for its possible application in cheese production. If not used in food, prospective application of enterococcin A 2000 can be seen in antibiotic human therapy.

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