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Purification and Characterization of Enterocin 4, a Bacteriocin Produced by *Enterococcus faecalis* INIA 4

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A simple two-step procedure was developed to obtain pure enterocin 4, a bacteriocin produced by *Enterococcus faecalis* INIA 4. Chemical and genetic characterization revealed that the primary structure of enterocin 4 is identical to that of peptide antibiotic AS-48 from *Enterococcus faecalis* S-48. In contrast to the reported inhibitory spectrum of AS-48, enterocin 4 displayed no activity against gram-negative bacteria.

Several enterococcal bacteriocins have been characterized at the molecular level and identified as belonging to the lantibiotics or to the pediocin family of bacteriocins (3, 7). Enterococcal bacteriocin AS-48 has a unique cyclic structure and can therefore be considered a representative of a new class of bacteriocins (14, 17). Another remarkable property of AS-48 is its broad activity spectrum, which covers not only many gram-positive bacteria, but also gram-negative pathogens like *Salmonella* spp. and *Escherichia coli* (4, 6). To our knowledge, antagonistic activity against gram-negative bacteria has not been demonstrated for any other bacteriocin from gram-positive bacteria, and this has been attributed to the impermeability of the outer membrane to these substances (13, 18).

In previous studies it was shown that enterocin 4, a bacteriocin produced by *Enterococcus faecalis* INIA 4, is active against *Listeria monocytogenes* and histamine-producing *Lactobacillus buchneri* (9, 11). In this paper we present evidence that the primary structure of enterocin 4 is identical to that of AS-48 and that, in contrast to previous findings, this compound does not inhibit gram-negative bacteria. We also demonstrate that enterocin 4 acts only on energized cells of *Lactobacillus buchneri* St2A.

Purification of enterocin 4. *Enterococcus faecalis* INIA 4 was grown in 800 ml of double-strength tryptic soy broth (TSB, catalog no. 4311768; BBL). Ammonium sulfate was added to a final concentration of 55% to the culture supernatant at 4°C. Enterocin 4 was pelleted by centrifugation at 22,000 × *g* for 1 h and was dissolved in 20 ml of 0.1 M acetate buffer (pH 5.6). This preparation was applied to a C18 Sep-Pak Plus cartridge (Waters-Millipore), washed successively with 30 and 35% isopropanol–0.1% trifluoroacetic acid, and eluted with 50% isopropanol–0.1% trifluoroacetic acid. The eluate was dried at 50°C, and the residue was resuspended in 25 mM acetic acid and lyophilized.

Mass spectrometry. The mass of enterocin 4 was determined with a model VG Bio-Q mass spectrometer (Fisons Instruments, Altrincham, United Kingdom) as described previously for AS-48 (17), except that the sample was dissolved in acetonitrile-water-formic acid (50:49:1). A major peak was found at 7,166.1 Da, which is almost identical to the mass that was found by Samyn et al. (17) in spectra of AS-48 and attributed to the sulfoxydated form of AS-48. A minor peak was detected at 7,184.5 Da. This mass was also found in the spectra of AS-48, and it has been suggested that it could represent a second oxidation of the protein (17). A peak corresponding to the reduced form of the bacteriocin was not detected.

Elucidation of the primary structure. Peptide hydrolysis with HCl (2 h at 166°C) and a subsequent amino acid analysis were performed by workers at Eurosequence B.V., Groningen, The Netherlands. Amino acids were derivatized with orthophthalaldehyde and 9-fluorenylmethyl chloroformate and separated with a model 1090 Aminoquant instrument (Hewlett-Packard, Mountain View, Calif.). The results (data not shown) revealed only minor differences from the composition of AS-48, and these differences may well be explained by experimental error.

A sequence analysis of enterocin 4 was performed by workers at the SECU laboratory, University of Utrecht, Utrecht The Netherlands. Automatic Edman degradation and detection of phenylthiohydantoin-amino acid derivatives were carried out with a model 476A protein-sequencing system (Applied Biosystems, Foster City, Calif.). It was not possible to sequence the N terminus of enterocin 4. However, after cleavage of the peptide with cyanogen bromide (8), 35 successive amino acids could be identified (Fig. 1). Cleavage with endoproteinase Lys-C yielded a mixture of small peptides and one large, hydrophobic peptide, presumably Glu-4–Lys-52 (Fig. 1). A sequence analysis of the complete Lys-C digest of enterocin 4 yielded two main sequences. Assuming that one sequence corresponded to the N terminus of peptide Glu-4–Lys-60, the other sequence was identified as the N terminus of peptide Arg-65–Lys-3 (Fig. 1). The mixture was also analyzed by Maldi–time-of-flight mass spectrometry with a model VG Tofspec SE instrument (Fisons Instruments, Wythenshawe, United Kingdom) equipped with a reflection analyzer and an N_2 laser. One of the main peaks in the spectrum had a mass of 1,060.8 Da. This value corresponds very well to the value calculated for peptide Arg-65–Lys-3 (1,061.3), assuming that the methionine at position 1 was oxidized. The identification of this peptide also strongly suggests that enterocin 4 has a cyclic structure.

Enterocin 4 was rather resistant to chymotrypsin, but after pretreatment with CNBr various chymotryptic fragments were obtained that were analyzed by mass spectrometry. The results

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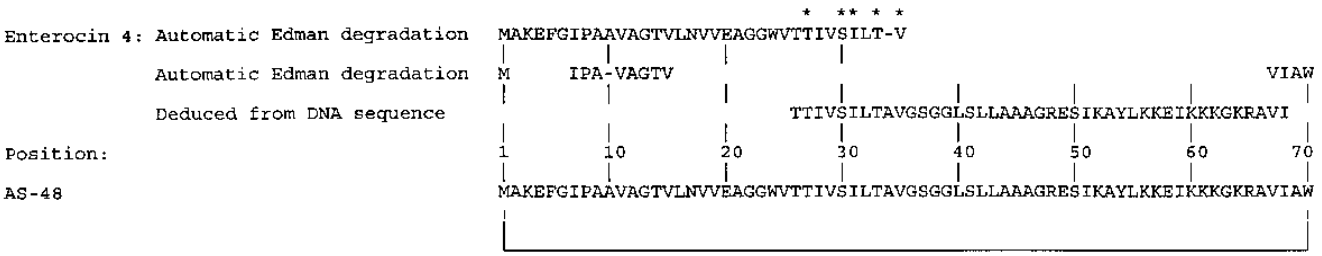

FIG. 1. Elucidation of the amino acid sequence of enterocin 4. The top line shows the sequence obtained after cyanogen bromide cleavage of enterocin 4. The presence of methionine at position 1 was inferred from the specificity of the cyanogen bromide cleavage. Asterisks indicate amino acids whose identities are uncertain. The second line is the sequence obtained after endoproteinase Lys-C cleavage of enterocin 4. The third line is the sequence obtained after PCR amplification. The sequence of AS-48 shown at the bottom is the sequence determined by Samyn et al. (17). The line connecting residues 1 and 70 of AS-48 reflects the cyclic nature of this peptide.

obtained (data not shown) were very similar to those described for AS-48 (17).

A 0.34-kb fragment of the structural gene of enterocin 4 was amplified by PCR by using oligonucleotide primers and conditions described elsewhere (12). This fragment served as the template in a cycle sequencing reaction, in which we used a fluorescein-labelled primer (5′-GCAGTTGCAGGAACTGT GCT-3′; complementary to bp 263 to 282) and Thermo Sequenase (Amersham Life Science, Buckinghamshire, United Kingdom) according to the suppliers' instructions. Direct sequencing of the PCR product revealed no differences from the corresponding part (bp 311 to 437) of the structural gene for AS-48 (EMBL accession number X79542). The deduced amino acid sequence completed the elucidation of the primary structure of enterocin 4, which is identical to that of AS-48 (Fig. 1).

Mode of action. The effects of various concentrations of enterocin 4 on growing cells of *Lactobacillus buchneri* St2A were investigated by adding purified enterocin 4 to an early-log-phase culture of St2A in all-purpose Tween broth (catalog no. 0655-01-7; Difco). Under these circumstances *Lactobacillus buchneri* St2A was very susceptible to enterocin 4; a concentration of only 0.04 μg/ml caused a decrease in the bacterial level from 6×10^6 to about 3×10^2 CFU/ml after 24 h of incubation (Fig. 2).

To obtain starved cells, *Lactobacillus buchneri* St2A was grown to the stationary phase by incubating it for 24 h at 37°C in 40 ml of all-purpose Tween broth supplemented with 0.5% L-histidine. Cells were harvested by centrifugation (10 min, 10,000 × *g*), washed with 40 ml of saline, resuspended in 40 ml of 0.1 M acetate buffer (pH 5.6), and incubated at 4°C for 24 h. Aliquots (100 μl) of the suspension of starved cells were subsequently transferred to 3 ml of 0.5 M sodium phosphate buffer (pH 5.9) with no energy source or to phosphate buffer supplemented with 1% glucose or 1% L-histidine (15). Control experiments involved adding either 1% L-leucine, 1% L-glutamic acid, or 1% L-lysine. After 1 h of preincubation at 37°C, purified enterocin 4 (0.15 μg/ml) was added. The survivors were enumerated on Rogosa agar after incubation for 1, 4, 6.5, and 24 h at 37°C (Fig. 3). Under these conditions enterocin 4 did not affect starved cells of *Lactobacillus buchneri* St2A; after 20 h the colony counts were equal to the colony counts of a control without bacteriocin. On the other hand, the decreases in the viability of the energized cells were 3 to 4 logs higher. No such decreases were found in control experiments performed with other amino acids that are not decarboxylated by *Lactobacillus buchneri* St2A (data not shown). Differences in survival cannot be explained by pH-dependent modulation of bacteriocin activity, as the pH values of the suspensions did not vary more than 0.03 pH unit. The most likely explanation is that enterocin 4, like nisin (1), needs an energized membrane to cause pore formation and that the proton motive force of the starved cells was too low. In contrast, AS-48 has been reported to act in an energy-independent way on membrane vesicles

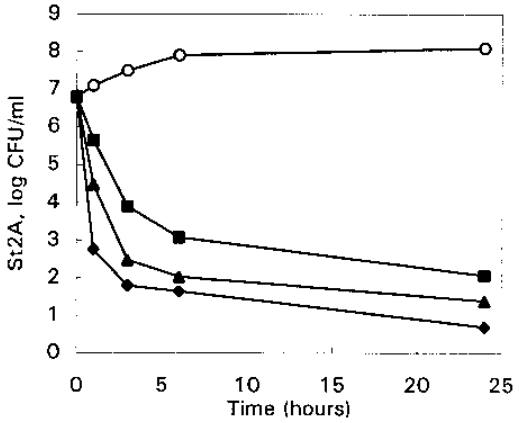

FIG. 2. Effect of enterocin 4 on actively growing *Lactobacillus buchneri* St2A in all-purpose Tween broth. Symbols: ○, no enterocin 4; ■, 0.04 μg of enterocin 4 per ml; ▲, 0.2 μg of enterocin 4 per ml; ◆, 1.0 μg of enterocin 4 per ml. Survival was monitored on Rogosa agar (catalog no. CM 627; Oxoid).

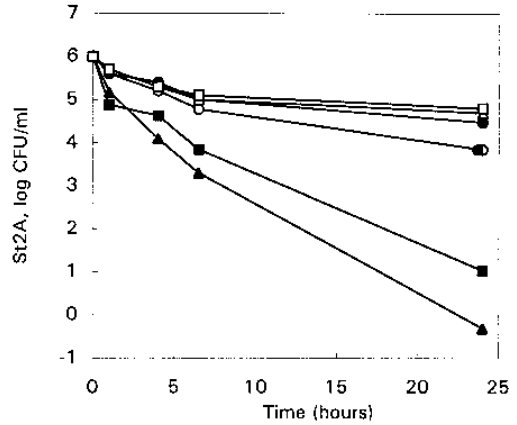

FIG. 3. Effect of enterocin 4 (0.15 μg/ml) on starved cells of *Lactobacillus buchneri* St2A suspended in 0.5 M sodium phosphate buffer (pH 5.90) (●), 0.5 M sodium phosphate buffer (pH 5.90) containing 1% glucose (■), and 0.5 M sodium phosphate buffer (pH 5.90) containing 1% histidine (▲). The open symbols indicate the results obtained with controls without enterocin 4. Survival was monitored on Rogosa agar.

TABLE 1. Inhibitory spectrum of enterocin 4

Species	Strain(s)[a]	Cultivation conditions[b]	Susceptibility[c]
Clostridium tyrobutyricum	NIZO B570, NIZO B571, NIZO B575, NIZO B577, NIZO B578, NIZO B579, NIZO B580, NIZO B584, NIZO B599, NIZO B602	RCM, 30°C, anaerobic	10/10
Lactobacillus buchneri	NIZO NZHD1, NIZO NZHD2, NIZO NZHD3, NIZO NZHD4, NIZO NZHD5, UNL St2A	APT, 37°C, aerobic	6/6
Lactobacillus brevis	NIZO 2B5B, NIZO Hem3, NIZO NZTD1, INIA 38, INIA 113, INIA 115, INIA 357	APT, 37°C, aerobic	7/7
Enterococcus faecalis	INRA EFS2, INIA HJI50, INIA HJI51	APT, 37°C, aerobic	2/3
Enterococcus faecium	INIA 66, FRI JBL 1061	APT, 37°C, aerobic	1/2
Listeria monocytogenes	INIA FLA11, INIA FLA32, INIA FLA36, INIA FLA56, INIA FLA66, INIA FLA86, INIA FLA112, INIA FLA116, INIA FLA120, INIA FLA138, INIA FLA142, INIA FLA146, INIA FLA285, INIA FLA288, INIA FLA293, INIA FLA315, INIA FLA467, INIA FLA471, INIA FLA474, INIA FLA476, INIA FLA484, INIA FLA486, INIA FLA492, FDA Scott A, FDA California, FDA V7, FDA OHIO	APT, 37°C, aerobic	27/27
Listeria innocua	INRA L35J, INRA 10	APT, 37°C, aerobic	2/2
Staphylococcus aureus	CECT 59, CECT 976, CECT 4013	APT, 37°C, aerobic	1/3
Salmonella choleraesuis	CECT 409, CECT 443	BHI, 37°C, aerobic	0/2
Escherichia coli	CECT 405	BHI, 37°C, aerobic	>200 μg/ml
Escherichia coli	CECT 434	BHI, 37°C, aerobic	>200 μg/ml
Escherichia coli	JM83	BHI, 37°C, aerobic	>200 μg/ml
Escherichia coli	K-12	BHI, 37°C, aerobic	>200 μg/ml
Bacillus laterosporus	CECT 15	APT, 37°C, aerobic	6.1 μg/ml
Enterococcus durans	CECT 411	BHI, 37°C, aerobic	0.096 μg/ml
Corynebacterium laevaniformans	CECT 445	BHI, 37°C, aerobic	0.048 μg/ml

[a] Sources: NIZO, F. Nieuwenhof and Z. Kruiswijk, Netherlands Institute for Dairy Research, Ede, The Netherlands; UNL, S. L. Taylor, University of Nebraska, Lincoln; INIA, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain; INRA, J. Richard, Station de Recherches Laitières, Jouy-en-Josas, France; FRI, J. Luchansky, Food Research Institute, Madison, Wis.; FDA, R. G. Crawford, Food and Drug Administration, Cincinnati, Ohio; CECT, Spanish Type Culture Collection, University of Valencia, Burjasot, Spain.

[b] RCM, reinforced clostridial medium (catalog no. 1808-17-3; Difco); APT, all-purpose Tween broth (catalog no. 0655-01-7; Difco); BHI, brain heart infusion (catalog no. 0037-01-6; Difco).

[c] Susceptibility to enterocin 4 was tested in an agar diffusion assay. Gram-positive organisms were exposed to a preparation containing 12.2 μg of enterocin 4 per ml (and twofold dilutions thereof). Gram-negative strains were exposed to 200 μg of enterocin 4 per ml. The results are expressed as the number of strains susceptible/number of strains tested or as the MIC.

from *Enterococcus faecalis* S47 (5). However, the latter finding was obtained by using a higher bacteriocin concentration (4 μg/ml). This leaves open the possibility that at low bacteriocin concentrations pore formation is enhanced by a high proton motive force.

Inhibitory spectrum. The susceptibilities of various gram-positive and gram-negative bacteria to purified enterocin 4 were determined with a previously described agar diffusion assay (10). The MIC was defined as the lowest concentration of the bacteriocin that gave an inhibition zone. Table 1 shows that enterocin 4 displays activity not only against *Listeria* species, but also against most of the other gram-positive genera tested. The concentration applied (12.2 μg/ml) is comparable to the concentration produced by *Enterococcus faecalis* INIA 4 in double-strength TSB broth. All of the gram-negative bacteria tested were resistant to enterocin 4.

From data of Gálvez et al., the MICs of AS-48 for *Escherichia coli* K-12, *Enterococcus durans* CECT 411, and *Corynebacterium laevaniformans* CECT 445 were calculated to be 1.2, 0.087, and 0.13 μg/ml respectively (4). The MICs of enterocin 4 for the two gram-positive strains were in the same order of magnitude (0.096 and 0.048 μg/ml), but even concentrations as high as 200 μg/ml did not inhibit *Escherichia coli* K-12 or any of the other gram-negative bacteria tested.

We have recently shown that the structural gene of AS-48 is probably shared by many other bacteriocin-producing enterococci (12). Most of the implicated bacteriocins have been tested for antagonistic activity against gram-negative bacteria, but invariably with negative results (2, 16, 19). If AS-48 kills gram-negative bacteria by pore formation in the cellular membrane, it must first pass through the outer membrane. Like many other bacteriocins, AS-48 is very hydrophobic and is not expected to diffuse through this layer. No satisfying hypothesis has been developed to explain the passage of AS-48, nor has any additional evidence been presented indicating that passage occurs. Therefore, the antagonistic activity of AS-48 against gram-negative bacteria in itself is more puzzling than the fact that similar activity could not be demonstrated for enterocin 4.

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