Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706

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(Received 14 April 1992; revised 19 July 1992; accepted 26 August 1992)

Sakacin A, a bacteriocin produced by *Lactobacillus sake* Lb706 and which inhibits the growth of *Listeria monocytogenes*, was purified to homogeneity by ammonium sulphate precipitation and ion-exchange, hydrophobic-interaction and reversed-phase chromatography. The complete amino acid sequence of sakacin A was determined by Edman degradation. The bacteriocin consisted of 41 amino acid residues and had a calculated M_r of 4308.7, which is in good agreement with the value determined by mass spectrometry. The structural gene encoding sakacin A (*sakA*) was cloned and sequenced. The gene encoded a primary translation product of 59 amino acid residues which was cleaved between amino acids 18 and 19 to yield the active sakacin A. Sakacin A shared some sequence similarities with other bacteriocins.

Introduction

Bacteriocins are proteinaceous compounds produced by bacteria. Bacteriocins normally inhibit the growth of closely related species. However, some bacteriocins may also inhibit the growth of pathogens and spoilage organisms during food processing and food fermentations, and may thus be of interest to the food fermentation industry.

Sakacin A, a bacteriocin produced by *Lactobacillus sake* Lb706, has been shown to inhibit lactic acid bacteria and *Listeria monocytogenes* (Schillinger & Lücke, 1989). Inhibitory action against *Listeria monocytogenes* has also been observed in minced meat and comminuted cured raw pork, where viable counts were reduced by about one log cycle (Schillinger *et al.*, 1991).

A number of bacteriocins of lactic acid bacteria have been identified, but few have been purified and

characterized. These bacteriocins are often heat-stable, small polypeptides with an M_r of a few thousand. The best known is nisin, a 3400 Da polypeptide produced by *Lactococcus lactis*. Its structure was established by Gross & Morell (1971). Recently, the amino acid sequences of some other bacteriocins from lactic acid bacteria have been determined. Three bacteriocins, lactococcins A, B and M, have been identified in *Lactococcus lactis* subsp. *cremoris* and their genes sequenced (Holo *et al.*, 1991; Van Belkum *et al.*, 1991a, 1992). Hastings *et al.* (1991) have cloned the gene for leucocin A-UAL 187 of *Leuconostoc gelidum*. The gene of *Lactobacillus acidophilus* encoding lactacin F has been sequenced by Muriana & Klaenhammer (1991). Two bacteriocins from *Lb. sake*, lactocin S and sakacin P, have been purified and sequenced by N-terminal amino acid sequencing (Mørtvedt *et al.*, 1991; Tichaczek *et al.*, 1992) and a partial amino acid sequence of curvacin A from *Lactobacillus curvatus* has been obtained (Tichaczek *et al.*, 1992). The gene for the larger 37 kDa helveticin J from *Lactobacillus helveticus* has also been sequenced (Joerger & Klaenhammer, 1990).

Lactococcin A appears to function by permeabilizing the cytoplasmic membrane (Van Belkum *et al.*, 1991b). Information on the mode of action of other bacteriocins is scarce. Here we describe the purification and primary

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Abbreviations: BU, bacteriocin unit; TFA, trifluoroacetic acid.

The amino acid and nucleotide sequence data reported in this paper have been submitted to EMBL and have been assigned the accession numbers P80097 (sakacin A) and Z14233 (*sakA* gene).

structure of sakacin A and the cloning of the corresponding structural *sakA* gene.

Methods

Bacterial strains. The sakacin A producer, *Lactobacillus sake* Lb706, and its non-producing and susceptible derivative *Lb. sake* Lb706-B were obtained from Friedrich-Karl Lücke (Fachhochschule, Fulda, Germany). The indicator organism, chosen on the basis of its susceptibility, was identified as a *Lactobacillus brevis* strain by microscopy and its API sugar fermenting pattern. *Lactobacillus sake* L45D, a variant of *Lb. sake* L45 which does not produce lactocin S (Mørtvedt & Nes, 1990), was a laboratory strain. *Lactococcus cremoris* LM0230 was obtained from the Laboratory of Microbial Gene Technology, Ås, Norway. *Lb. sake* Lb706 was grown in Lactobacilli MRS broth (MRS) (de Man *et al.*, 1960) at 25 °C.

Bacteriocin assay. Bacteriocin activity was quantified in a microtitre plate assay system (Geis *et al.*, 1983). Twofold dilutions of bacteriocin fractions in MRS were prepared in the microtitre plates. Approximately 5×10^3 cells of the indicator organism were then added (10 µl; OD₆₀₀ = 0.00005), giving a final volume of 110 µl per well. After incubation (18 h, 25 °C) culture turbidity was measured spectrophotometrically at 600 nm in a Titertec Multiskan microplate reader. When necessary, bacteriocin fractions were adjusted to pH 6.5 and sterilized by filtration. One bacteriocin unit (BU) was defined as the amount of bacteriocin which inhibited growth of the indicator organism by 50% as compared to a control culture without bacteriocin.

Gel filtration. Analytical gel filtration was carried out by using a FPLC Superose 12 column (Pharmacia) with a separating range of 1×10^3 – 3×10^5 Da. The column was equilibrated with 50 mM-sodium phosphate buffer (pH 6.4). Gel filtration standards (Bio-Rad) ranging from 1350 to 6.7×10^5 Da were employed for calibration of the column.

Bacteriocin purification. The bacteriocin was purified by a modification of the method of Nissen-Meyer *et al.* (1992). A two-litre culture of *Lb. sake* Lb706 was grown to the early stationary phase and cells removed by centrifugation (4000 g, 15 min, 4 °C). The bacteriocin present in the supernatant fraction was concentrated by ammonium sulphate precipitation (400 g l⁻¹). The precipitate was pelleted by centrifugation (7000 g, 20 min, 4 °C) and dissolved in 250 ml 20 mM-sodium acetate, pH 4.2 (buffer A) (fraction I). Fraction I was applied to a 10 ml S-Sepharose Fast Flow ion-exchange chromatography column pre-equilibrated with buffer A. The column was washed with 2 vols buffer A and subsequently the bacteriocin was eluted in 26 ml 20 mM-sodium phosphate/1 M-NaCl buffer, pH 5.7 (fraction II). Fraction II was made 10% (w/v) with respect to ammonium sulphate and applied to a 4.5 ml Octyl-Sepharose CL-4B column equilibrated with 20 mM-sodium phosphate buffer/10% ammonium sulphate, pH 5.7 (buffer B). After washing with 2 vols buffer B, the bacteriocin was eluted with ethanol/20 mM-sodium phosphate buffer (70/30, v/v), pH 5.7 (fraction III). The eluate was diluted fivefold in aqueous 0.1% trifluoroacetic acid (TFA) and applied to a PepRPC HR 5/5 C₂/C₁₈ reversed-phase chromatography column, equilibrated with 2-propanol/H₂O (10/90, v/v) containing 0.1% TFA. Bound bacteriocin was eluted with a linear gradient of 20–50% 2-propanol containing 0.1% TFA (fraction IV). The diluted bacteriocin was rechromatographed on the same column. The purified bacteriocin was stable in 30% (v/v) 2-propanol containing 0.1% TFA at –20 °C.

Mass analysis. Mass analysis of the purified bacteriocin was carried out on a Biolon 20 mass analyser (Applied Biosystems) as described by Sørensen *et al.* (1990). Approximately 10 µg of purified, vacuum-evaporated bacteriocin was dissolved in 50 µl TFA containing 20%

acetonitrile, and 5 µl of this solution was used in the experiment. Data were accumulated by the mass analyser for 2 h at 16 kV.

Amino acid sequencing. A gas-phase 477A automatic microsequencer (Applied Biosystems) with an online 120A phenylthiohydantoin amino acid analyser was used (Cornwell *et al.*, 1988).

Recombinant DNA techniques. The basic cloning techniques of Sambrook *et al.* (1989) were used. Oligonucleotide probes were synthesized on an Applied Biosystems synthesizer 381A. The probes were end-labelled with ³²P using a terminal transferase end-labelling kit (Amersham). Plasmid DNA from *Lb. sake* Lb706 was digested with various restriction endonucleases and the resulting fragments separated on agarose gels, blotted onto nylon filters and hybridized with sakacin A probes. The 1.4 kb *EcoRI* fragment hybridizing to the oligonucleotide probes was purified from agarose gels using DEAE nitrocellulose filter strips (Dretzen *et al.*, 1981) and cloned in *E. coli* DH5α using the *Lb. plantarum*/*Lb. sake*/*E. coli* shuttle vector pLPV106. This vector is composed of the erythromycin resistance gene of *Lb. reuteri* (Axelsson *et al.*, 1988), replication determinants obtained from the cryptic *Lb. plantarum* plasmid p256 (Cosby *et al.*, 1989) and the pUC19 origin of replication. Both strands of the 1.4 kb *EcoRI* fragment were sequenced by the dideoxy chain-termination method of Sanger *et al.* (1977), using synthetic oligonucleotide primers (primer walking) and the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corporation).

Computer analyses were carried out on an IBM personal computer employing the DNASIS sequence analysis program (Pharmacia) and on a microVAX 3400 computer employing the GCG program package (Devereux *et al.*, 1984).

Results

Production of sakacin A

The production of sakacin A was investigated during growth of *Lb. sake* Lb706 in MRS (Fig. 1). The results indicated that sakacin A production was constitutive during exponential-phase growth and ceased as the cells entered the stationary phase, with subsequent loss of activity. Similar results were obtained when the growth medium was buffered to pH 6.5 with 0.1 M-potassium phosphate or to pH 6.0 with 0.2 M-MOPS.

Consequently, the bacteriocin was harvested as the cells entered the stationary phase.

Purification of sakacin A

Sakacin A was purified to homogeneity by ammonium sulphate precipitation, and sequential cation-exchange, hydrophobic-interaction and reversed-phase chromatography (Table 1). Overall, a more than 9000-fold increase in specific activity to a final value of 250 BU (µg protein)⁻¹ was obtained. Approximately 2.6×10^5 molecules per indicator cell thus represented 1 BU in the microtitre plate assay. The purified bacteriocin appeared homogeneous when subjected to FPLC reversed-phase chromatography. Likewise, a single band was observed

Fig. 1. Production of sakacin A by *Lb. sake* Lb706 grown in MRS. ●, Bacteriocin activity; ○, OD₆₀₀; □, pH.

1 Ala-Arg-Ser-Tyr-Gly-Asn-Gly-Val-Tyr-Cys- 10
 11 Asn-Asn-Lys-Lys-Cys-Trp-Val-Asn-Arg-Gly- 20
 21 Glu-Ala-Thr-Gln-Ser-Ile-Ile-Gly-Gly-Met- 30
 31 Ile-Ser-Gly-Trp-Ala-Ser-Gly-Leu-Ala-Gly- 40
 41 Met-***

Fig. 2. Amino acid sequence of sakacin A.

when sakacin A was subjected to SDS gel electrophoresis.

The complete amino acid sequence of the purified bacteriocin was determined by Edman degradation (Fig. 2). Uncertain amino acid determinations in positions 2, 10, 15 and 40 were verified by sequencing of the cloned gene (see below). Sakacin A consisted of one polypeptide chain of 41 amino acid residues with a calculated M_r of 4308.7. No unusual amino acid residues such as lanthionins were found. The calculated M_r was almost identical to the value of 4309.6 determined by plasma desorption mass spectrometry. The extra 1 Da in the latter analysis probably originated from protonation of the polypeptide during desorption (Tsarbopoulos, 1989).

To determine whether sakacin A existed as an aggregate in solution, the bacteriocin was subjected to gel filtration. Supernatant after growth in MRS was precipitated with ammonium sulphate and the bacteriocin was dissolved in 50 mM-sodium phosphate buffer pH 6.4 and

Fig. 3. M_r of sakacin A determined by gel filtration. Ammonium-sulphate-concentrated growth supernatant was dissolved in 50 mM-sodium phosphate buffer pH 6.4 and subjected to Superose 12 gel filtration. The elution positions of the reference proteins (○) are superimposed on the activity profile of sakacin A (●).

subjected to FPLC Superose 12 gel filtration chromatography at the same pH (Fig. 3). The bacteriocin eluted at an M_r of approximately 2400. This suggested that it exists as a monomer in the medium. Similar results were obtained when the gel filtration chromatography was performed at pH 4.5 (results not shown).

Cloning and DNA sequencing of the sakacin A structural gene

Two degenerate 29-mer oligonucleotide probes, deduced from the amino acid sequence of sakacin A, both hybridized with a 1.4 kb *Eco*RI fragment, originating from the larger (approx. 60 kb) of the two plasmids present in *Lb. sake* Lb706. The *Eco*RI fragment was cloned in *E. coli* DH5α using the shuttle vector pLPV106 and sequenced. The structural gene, *sakA*, consisted of an open reading frame of 177 bp encoding a primary

Table 1. Purification of sakacin A

Purification stage	Total A_{280}	Total activity (BU)	Specific activity*	Yield (%)
Culture supernatant	61 050	259 000	4.2	100
(NH ₄) ₂ SO ₄ conc (Fr I)	2725	248 400	91	96
S-Sepharose chrom. (Fr II)	73	36 500	500	14
Octyl-Sepharose chrom. (Fr III)	27	131 500	4830	51
Rev.-phase FPLC (Fr IV)	5.6	214 500	38 300	83

*Specific activity = total activity/total A_{280} .

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1   CTTCACTCGTACAAAAATAATAACAGAGGAGATTCTTAGTTATGAATAATGTAAAGAA   60
-18                               M N N V K E                               -13

61   TTAAGTATGACAGAATTACAAACAATTACCGGCGGTGCTAGATCATATGGCAACGGTGT   120
-12 L S M T E L Q T I T G G A R S Y G N G V                               8

121  TACTGTAATAATAAAAAATGTTGGGTAAATCGGGGTGAAGCAACGCAAAGTATTATTGGT   180
9    Y C N N K K C W V N R G E A T Q S I I G                               29

181  GGTATGATTAGCGGCTGGGCTAGTGGTTTAGCTGGAATGTAAATAGAGGGTCTAATTGA   240
30   G M I S G W A S G L A G M *                               41

241  AGGCAGATTATAAAAAATAAATTCAATACTAACTTACA   279

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Fig. 4. Nucleotide sequence of the *sakA* gene. Only the antisense strand (mRNA-like) is shown. The putative ribosome-binding site is underlined. The downward-pointing arrowhead indicates the cleavage site to yield the mature sakacin A.

Fig. 5. Effect of sakacin A on different lactic acid bacteria. The cells (10^4) were incubated with 480 BU of purified bacteriocin for various times and the number of colony-forming units was determined. $\square$, *Lb. sake* Lb706 + sakacin A; $\blacksquare$, *Lb. sake* Lb706B + sakacin A; $\blacktriangle$, *Lb. brevis* + sakacin A; $\triangle$, *Lb. brevis* minus sakacin A; $\bullet$, *Lb. sake* L45D + sakacin A; $\circ$, *L. cremoris* LM0230 + sakacin A.

translation product of 59 amino acid residues (Fig. 4). Cleavage of the polypeptide chain after amino acid 18 would yield the mature sakacin A as determined by amino acid sequencing. A putative Shine–Dalgarno sequence was located upstream of the gene.

Effect of sakacin A

To investigate the effect of sakacin A, different lactic acid bacteria were exposed to a fixed amount of bacteriocin for various time (Fig. 5). No effect on survival of cells was observed for short incubations with sakacin A. However, when cells were incubated with the bacteriocin for 16 h, different effects were clearly visible. For the most sensitive strain, *Lb. brevis*, which was used

as the indicator organism, a nearly 100-fold decrease in viable cell numbers was observed, whereas for *Lb. sake* Lb706-B and *Lb. sake* L45D, a bacteriostatic effect was observed. *Lactococcus cremoris* LM0230, which in the microtitre assay was sensitive to sakacin A, was apparently capable of overcoming growth inhibition during longer periods of incubation.

Discussion

In contrast to what has been stated earlier (Schillinger & Lücke, 1989), sakacin A activity was present throughout the exponential phase of growth and not solely as the cells entered the late exponential growth phase or early stationary phase. In this respect sakacin A differs from the very similar bacteriocin curvacin A from *Lb. curvatus* LTH1174. Curvacin A is only produced in the late exponential growth phase (Tichaczek *et al.*, 1992).

Many of the bacteriocins of lactic acid bacteria are polypeptides of M_r in the range 3000–6000, with a surplus of positively charged amino acid residues and showing some hydrophobic characteristics. Consequently, purification schemes often involve ion-exchange chromatography and hydrophobic-interaction chromatography. During purification, an increase in bacteriocin activity was observed after Octyl Sepharose (hydrophobic-interaction) chromatography and after reversed-phase chromatography. The reason for this increase is unknown.

Sakacin A was purified more than 9000-fold to homogeneity and sequenced. The M_r calculated from the amino acid sequence was almost identical to that determined by plasma desorption mass spectrometry and confirmed the correctness of the proposed amino acid sequence. This also seems to preclude the possibility of post-translational covalent modifications of the polypeptide such as glycosylation, although non-covalent interactions may occur. In addition, the similarity between

(a)

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Curvacin A      ACSYGNGVYCINNKKSWVNRGEATQXXIXXG...
                  * * * * *
Sakacin A        ARSYGNGVYCINNKKCWVNRGEATQSIIGGMISGWASGLAGM
                  : * * * * * : * * : * * : * : * :
Sakacin P        KYYGNGVHCGKHSC TVDWGT AIGNIGNNAAANXATGXNAXX
                  * * * * * : * * * * * * * : * * * :
Pediocin PA-1    KYYGNGVTCGKHSCSVDWGKATTCI INNGAMAXATGGHQGNHK...
                  * * * * * * * : * * * * * : * * * :
Leucocin A       KYYGNGVHCTKSGCSVNWGEA----FSAGVHRLANGGNGFW

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(b)

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Sakacin A        ARSYGNGVYCINNKKCWVNRGEATQSIIGGMIS----GWASGLAGM
                  : * : * * : : * * : * : : * * : * *
Lactococcin A    KLTFIQSTAAGDLYYNTNTHKYVYQQTQNAFGAAANTIVNGWMGGAAGGFGGLHH

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Fig. 6. Comparison of the amino acid sequence of sakacin A with (a) curvacin A, sakacin P, pediocin PA-1 and leucocin A, and (b) lactococcin A. Identical amino acids are indicated by asterisks and conservative changes are shown by colons. Putative amphiphilic α -helices are underlined.

the calculated and the determined M_r values indicated that the cysteine residues existed in the reduced state and were not engaged in intramolecular cystine bridging. Furthermore, when sakacin A was heated with 1 mM- β -mercaptoethanol at 80 °C for 10 min to ensure that the thiol residues existed in the reduced state, no change in activity was observed (results not shown). This indicates that the oxidation state of the cysteine residues may not be important for the bacteriocin activity. In contrast, the two cysteine residues of leucocin A-UAL 187 are thought to form a cystine bridge, although reduction without loss of activity can occur (Hastings *et al.*, 1991).

The active sakacin A is apparently processed from a longer primary translation product, with the precursor sequence sharing some similarities with precursor sequences from other bacteriocins (Kok *et al.*, 1992).

The charged amino acid residues were all located in the N-terminal hydrophilic part of the polypeptide chain, whilst the more hydrophobic C-terminal part consisted of pairs of hydrophobic amino acid residues regularly spaced by pairs of the hydrophilic amino acid residues glycine and serine. No significant homology was found with other proteins. However, sakacin A shared some homology, especially in the N-terminal region, with the newly sequenced bacteriocins leucocin A-UAL 187 (Hastings *et al.*, 1991), pediocin PA-1 (Nieto Lozano *et al.*, 1992) and sakacin P (Tichaczek *et al.*, 1992) and appeared to be very similar to the partly sequenced curvacin A (Tichaczek *et al.*, 1992) (Fig. 6). The N-terminal similarities between sakacin A, sakacin P and curvacin A may be reflected in their common antagonistic spectrum (Scillinger & Lücke, 1989; Tichaczek *et al.*, 1992). These sequence similarities indicate that bacteriocins may be grouped into families as was suggested by Nieto Lozano *et al.* (1992). The mechanism of action of this group of bacteriocins is at present not known.

Sakacin-A-susceptible strains appear to vary widely in their degree of sensitivity to the bacteriocin. In some cases only a bacteriostatic effect was observed, whereas in other cases a drop in viable cell counts was detected. When lactococcin A was mixed with a susceptible strain and the fluorescent dye propidium iodide, influx of fluorescent dye into the sensitive cells was observed by flow cytometry (H. Holo, personal communication). This indicated permeabilized and dead cells. No influx of propidium iodide was observed when similar experiments were carried out with sakacin A (results not shown).

Amphiphilic α -helices often exist as transmembrane regions in pore-forming proteins (Ojcius & Young, 1991). Putative amphiphilic α -helix structures were detected in sakacin A between amino acids 26 and 41 (Rao & Argos, 1986) (Fig. 6b), the region encompassing the regularly spaced hydrophobic and hydrophilic amino acids. In this respect sakacin A showed similarities with lactococcin A, which is a cytoplasmic membrane-permeabilizing molecule (Van Belkum *et al.*, 1992) spanning the cytoplasmic membrane by an amphiphilic α -helix (Kok *et al.*, 1992). Since it was not possible to demonstrate pore formation with sakacin A, the significance of the putative amphiphilic transmembrane helix A is unknown.

We want to thank Dr U. Schillinger and Dr F.-K. Lücke for supplying the bacteriocin-producing *Lb. sake* Lb706 and the corresponding cured variant *Lb. sake* Lb706-B, Dr K. Sletten at the Department of Biochemistry, University of Oslo, for carrying out the automatic amino acid sequencing, J. Thomsen, Department of Structural Chemistry, Novo Nordisk A/S, Denmark, for the use of the Biolon 20 mass analyser, and S. Bayne, Applied Biosystem Division of KEBO Lab, Ballerup, for analysing the peptide samples on the Biolon 20 mass analyser. Also we want to thank Dr I. F. Nes and Dr J. Nissen-Meyer for fruitful discussions. Finally, we are grateful to J. Reseland

for help with flow cytometer experiments and Birgitta Baardsen for technical assistance.

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