Purification and characterization of plantaricin A, a *Lactobacillus plantarum* bacteriocin whose activity depends on the action of two peptides

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A *Lactobacillus plantarum* bacteriocin, plantaricin A, has been purified to homogeneity by ammonium sulphate precipitation, binding to cation exchanger and Octyl-Sepharose, and reverse-phase chromatography. The bacteriocin activity was associated with two peptides, termed α and β, which were separated upon reverse-phase chromatography. Bacteriocin activity required the complementary action of both the α and β peptides. From the N-terminal end, 21 and 22 amino acid residues of α and β, respectively, were sequenced. Further attempts at sequencing revealed no additional amino acid residues, suggesting that either the C terminus had been reached or that modifications in the next amino acid residue blocked the sequencing reaction. Judging from their amino acid sequence, α and β may be encoded by the same gene, since α appeared to be a truncated form of β. Alanine, the first amino acid residue at the N-terminal end of β was not present at this position in α. Otherwise the sequences of α and β appeared to be identical. The calculated molecular masses of the sequenced part of α and β were 2426 and 2497 Da, respectively. The molecular masses of α and β as determined by mass spectroscopy were 2687 ± 30 and 2758 ± 30 Da, respectively, indicating that (i) the only difference between α and β was the presence of the N-terminal alanine residue in β, and that (ii) in addition to the sequenced residues, two to three unidentified amino acid residues are present at the C-terminal ends of the α and β peptides. Both α and β may form amphiphilic α-helices, suggesting that they are pore-forming peptides that create cell membrane channels through a 'barrel-stave' mechanism.

Introduction

Bacteriocins are proteinaceous compounds that show bactericidal activity, often towards bacteria that are closely related to the bacteriocin-producing species (Tagg *et al.*, 1976). Due to their potential use as antibacterial agents, bacteriocins have been the subject of much interest. A number of bacteriocins produced by lactic acid bacteria have been described (Klaenhammer, 1988).

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Recently, the complete or partial primary structure has been elucidated for some of these bacteriocins. Based on their structure they may be divided into two groups. The first, termed the lantibiotic group, comprises bacteriocins consisting of one polypeptide chain that has been post-translationally modified, the modified amino acids being lanthionine and methyllanthionine as well as their precursors, dehydroalanine and dehydrobutyrine. This group contains nisin A (Buchman *et al.*, 1988; Dodd *et al.*, 1990; Gross & Morell, 1971; Hurst, 1981; Kaletta & Entian, 1989), nisin Z (Mulders *et al.*, 1991), lactocin S (Mørtvedt *et al.*, 1991), carnocin U149 (Stoffels *et al.*, 1992) and lacticin 481 (Piard *et al.*, 1992).

The second group comprises bacteriocins with no modification in their polypeptide. This group contains

curvacin A, sakacin P (Tichaczek *et al.*, 1992), pediocin PA-1 (Henderson *et al.*, 1992; Nieto Lozano *et al.*, 1992), leucocin A-UAL 187 (Hastings *et al.*, 1991), lactococcin A (Holo *et al.*, 1991; Van Belkum *et al.*, 1991), and probably lactacin F (Muriana & Klaenhammer, 1991*a*, *b*) and helveticin J (Joerger & Klaenhammer, 1990). The first four of the above-mentioned bacteriocins form a subgroup within the group of bacteriocins that consist of one unmodified peptide chain, as these four have a similar amino acid sequence (Nieto Lozano *et al.*, 1992). The second group also contains bacteriocins that require two different peptides to exert bactericidal activity. As yet only one bacteriocin of this kind has been identified (Nissen-Meyer *et al.*, 1992). This bacteriocin, termed lactococcin G, requires the complementary action of two entirely different peptides to exert bactericidal activity (Nissen-Meyer *et al.*, 1992). The N-terminal half of both of these peptides may form an amphiphilic α-helix, indicating that the peptides are pore-forming toxins (Nissen-Meyer *et al.*, 1992).

This study describes a novel bacteriocin, termed plantaricin A. Plantaricin A is produced by *L. plantarum* C-11, and in crude preparations the bacteriocin is heat stable, non-dialysable and active over the range pH 4·0–6·5 (Daeschel *et al.*, 1990). It has bactericidal activity towards some species of four genera of lactic acid bacteria, but not towards other Gram-positive or Gram-negative bacteria, or yeast (Daeschel *et al.*, 1990). The observed spectrum of activity is intermediate in breadth as compared to the narrow spectrum of lactacin B (Barefoot & Klaenhammer, 1983), lactacin F (Muriana & Klaenhammer, 1987), helveticin J (Joerger & Klaenhammer, 1990) and casecin 80 (Rammelsberg & Radler, 1990), and the broad spectrum of nisin (Buchman *et al.*, 1988) and pediocin A (Daeschel *et al.*, 1985).

Methods

Bacterial strains and media. The bacteriocin producer was *Lactobacillus plantarum* C-11 (Daeschel *et al.*, 1990) and the indicator organism used in the bacteriocin assay was *L. plantarum* 965 (Daeschel *et al.*, 1990). Both strains were grown at 30 °C in MRS broth (Oxoid).

Bacteriocin assay. The bacteriocin was quantified in a microtitre plate assay system. Each well of the microtitre plate contained 200 µl MRS broth, bacteriocin fractions at twofold dilutions and the indicator organism (OD_{600} = 0·1). The microtitre plate cultures were incubated for about 6 h at 30 °C, after which growth inhibition of the indicator organism was measured spectrophotometrically at 600 nm using a Dynatech Microplate Reader. One bacteriocin unit (BU) was defined as the amount of bacteriocin that inhibited growth of the indicator organism by 50% (50% of the turbidity of the control culture without bacteriocin).

Bacteriocin purification. The bacteriocin was purified from 1–2 litre cultures of *L. plantarum* C-11 as described for lactococcin G (Nissen-Meyer *et al.*, 1992). The cultures were grown to early stationary phase. The cells were then removed by centrifugation at 4000 *g* for 15 min at 4 °C, and 300 g ammonium sulphate per litre of culture supernatant was added. The protein precipitate was pelleted by centrifugation at 7000 *g* for 20 min and solubilized in 20 mM-sodium phosphate buffer, pH 7 (buffer A; 200 ml per 1·5 culture) (fraction I). Fraction I was applied at a flow rate of about 10 ml min^{-1} to a 7 ml S-Sepharose Fast Flow cation exchange column equilibrated with buffer A. After subsequent washing of the column with 20 ml buffer A, the bacteriocin was eluted from the column with 40 ml 1 M-NaCl in buffer A (fraction II). Ammonium sulphate was added to fraction II to a final concentration of 10% (w/v), after which the fraction was applied at a flow rate of about 4 ml min^{-1} to a 2 ml Octyl-Sepharose CL-4B column equilibrated with 10% (w/v) ammonium sulphate in buffer A. The column was then washed with 8 ml buffer A, after which the bacteriocin activity was eluted from the column with 10 ml 70% (v/v) ethanol and 30% buffer A (fraction III). Fraction III was diluted to 50 ml with water containing 0·1% (v/v) trifluoroacetic acid (TFA) and subsequently applied to a C_2/C_{18} reverse-phase column, PepRPC HR 5/5, equilibrated with 2-propanol/water (10:90, v/v), containing 0·1% TFA. The bacteriocin was eluted with a linear gradient ranging from 30 to 40% 2-propanol containing 0·1% TFA (fraction IV). The bacteriocin peptides eluting from the reverse-phase column (fraction IV) were diluted four- to fivefold with water containing 0·1% TFA and rechromatographed on the reverse-phase column. Purified bacteriocin was stored in 50–60% 2-propanol and/or ethanol containing 0·1% TFA at −20 °C.

Amino acid sequencing and mass spectroscopy analysis. Amino acid sequencing and mass spectroscopy was performed as described previously (Nissen-Meyer *et al.*, 1992).

Results and Discussion

Purification of bacteriocin

The bacteriocin was isolated from cultures in stationary phase, as maximum activity was found in the culture medium during this growth phase. The bacteriocin was purified by ammonium sulphate precipitation, binding to a cation exchanger and then to Octyl-Sepharose before the final reverse-phase chromatography purification steps (Table 1). This resulted in a 1300-fold increase in specific activity and a recovery of about 5% of the activity (Table 1, fraction III).

Bacteriocin activity depends on the complementary action of two peptides

When fraction III was applied to the reverse-phase column, one major bacteriocin activity peak (fraction 6, Fig. 1) and several absorbance peaks eluted from the column (Fig. 1). In addition, a minor bacteriocin activity peak (fraction 4, Fig. 1) was often observed (in two out of four experiments) eluting slightly ahead of the major activity peak. Judging from the absorbance profile (Fig. 1), two components were present in fraction 6, and these could be separated from each other upon rechromatography on the reverse-phase column (Fig. 2). They were termed α and β, α being the first of the two to elute from the column. Very little bacteriocin activity was associated

Table 1. *Purification of plantaricin A*

Fraction	Volume (ml)	Total A_{280}*	Total activity (BU)	Specific activity†	Increase in specific activity†	Yield (%)
Culture supernatant	1500	74000	10×10^6	135	1	100
Ammonium sulphate precipitation (fraction I)	200	1900	$3{\cdot}5 \times 10^6$	1840	14	35
Binding to cation exchanger (fraction II)	40	12	2×10^6	$1{\cdot}7 \times 10^5$	1260	20
Binding to Octyl-Sepharose (fraction III)	10	2·5	$4{\cdot}5 \times 10^5$	$1{\cdot}8 \times 10^5$	1330	5

*Total A_{280} is $A_{280} \times$ volume (ml).
† Specific activity is total activity/total A_{280}.

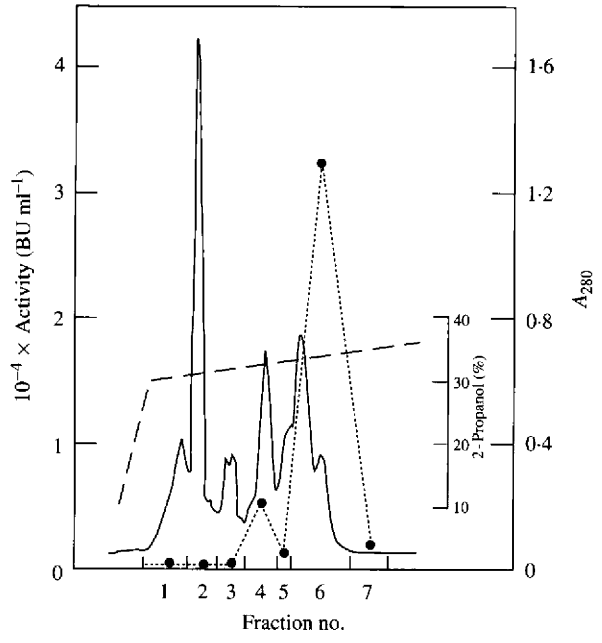

Fig. 1. Reverse-phase chromatography of plantaricin A (fraction III). The amount applied to the column was obtained from approximately 2 litres of culture. ●····●, Bacteriocin activity; ——, A_{280}; – – –, 2-propanol gradient.

with either α and β when they were assayed separately for bacteriocin activity (Fig. 2). However, bacteriocin activity was recovered upon adding β to the column fractions containing α (Fig. 2*a*), or α to the column fractions containing β (Fig. 2*b*). No significant additional increase in bacteriocin activity was seen upon adding an aliquot of the other column fractions shown in Fig. 1 (fractions 1–5, Fig. 1) to fractions containing both β and α (data not shown). Thus the complementary action of two peptides, an α and a β peptide, was necessary to obtain bacteriocin activity. The purified α and β bacteriocin activity appeared unstable during reverse-phase chromatography, since much of the activity was lost in this last purification step. The final recovery of activity after the last reverse phase chromatography purification step was in the range 0·1–1 %.

Amino acid sequence and mass spectroscopy

The N-terminal amino acid sequences of α and β peptides are shown in Fig. 3. It appears that α is identical to β except that an alanine residue is found as the first and as an additional amino acid residue at the N-terminal end of β. Otherwise the amino acid sequences of the two peptides are identical. Therefore, it is likely that α and β are encoded by the same gene, and that α is processed into a truncated form of β by post-translational cleavage. From the N-terminal end, 21 and 22 amino acid residues of α and β, respectively, were sequenced. Additional amino acid residues were not detected upon attempting further sequencing, suggesting either that the C-terminal end was reached or that modifications in the next amino acid residue blocked the sequence reaction. The calculated molecular masses of the sequenced part of α and β (Fig. 3) were 2426 and 2497 Da, respectively, whereas the molecular masses of α and β as determined by plasma disorption mass spectrometry were 2687 ± 30 and 2758 ± 30 Da, respectively, indicating that in addition to the sequenced amino acid residues shown in Fig. 3, 2–3 unidentified residues are present at the C-terminal ends of the α and β peptides. It is very unlikely that plantaricin A is a lantibiotic and contains dehydrated forms of serine or threonine which have been shown to block amino acid sequencing by Edman degradation. Amino acid composition analysis of plantaricin A has not revealed any lanthionines (data not shown). Furthermore, plantaricin A contains serine and threonine which are commonly modified to didehydroalanine and didehydrobutyrine in lantibiotics. Consequently, some unknown modification on the plantaricin A causes the blocking of amino acid sequencing. The molecular masses determined by mass spectroscopy also suggest that the only difference between α and β was the presence

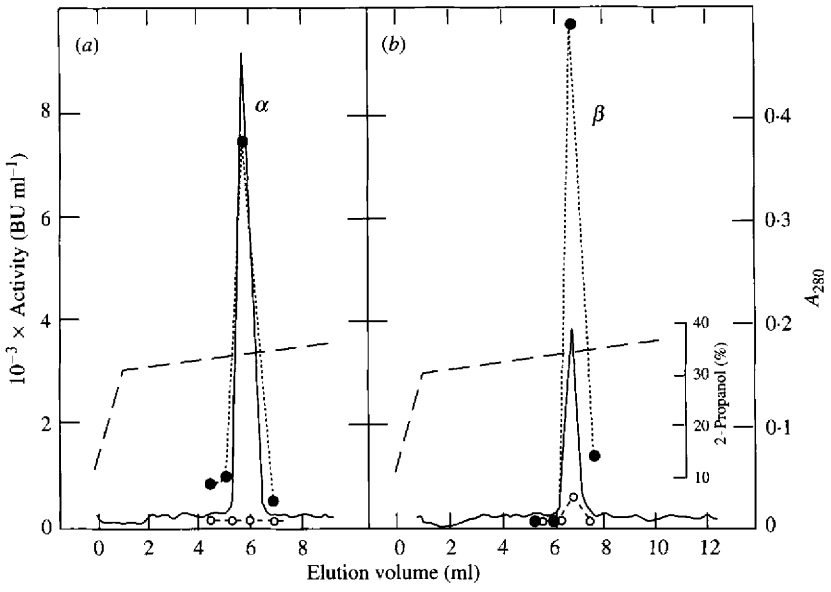

Fig. 2. Reverse-phase chromatography of (*a*) α and (*b*) β. The bacteriocin activity without (○—○) and with complementation (●····●) with (*a*) β or with (*b*) α. The amount applied to the column was obtained from approximately 2 litres of culture. ——, A_{280}; – – –, 2-propanol gradient.

	1				5					10					15					20			
α	N	Tyr	Ser	Leu	Gln	Met	Gly	Ala	Thr	Ala	Ile	Lys	Gln	Val	Lys	Lys	Leu	Phe	Lys	Lys	(Trp)	Gly	
β	N	Ala	Tyr	Ser	Leu	Gln	Met	Gly	Ala	Thr	Ala	Ile	Lys	Gln	Val	Lys	Lys	Leu	Phe	Lys	Lys	(Trp)	Gly
	1				5					10					15					20			

Fig. 3. N-terminal amino acid sequence of α and β. Uncertain amino acids are in parentheses. N indicates the N-terminal end of the peptide.

of the N-terminal alanine residue in β. It is surprising that this small difference is enough to enable the two peptides to complement each other such that bacteriocin activity may be detected in the presence of both peptides, but not when only one of the peptides is present.

The amino acid sequence of α and β suggests that these peptides may be pore-forming toxins that create cell membrane channels through a 'barrel-stave' mechanism (Ojcius & Young, 1991). The region in α starting with amino acid residue 3 and ending with residue 20, and the corresponding region in β starting with residue 4 and ending with 21, may form an amphiphilic α-helix. This is evident when the amino acid sequence in this region is displayed on an Edmundson α-helical wheel (Fig. 4; Schiffer & Edmundson, 1967). The non-polar residues are found almost completely on one side of the helix and the polar residues on the other side, the only exception being alanine (residue 7) which appears on the hydrophilic side. However, the substitution of one amino acid by one of opposite hydrophobicity may not cause a major disruption of the amphiphilic character of the peptide (Ojcius & Young, 1991). Glycine (residue 6), which appears on the hydrophobic side (Fig. 4), is relatively neutral with respect to its hydrophobic/hydrophilic character.

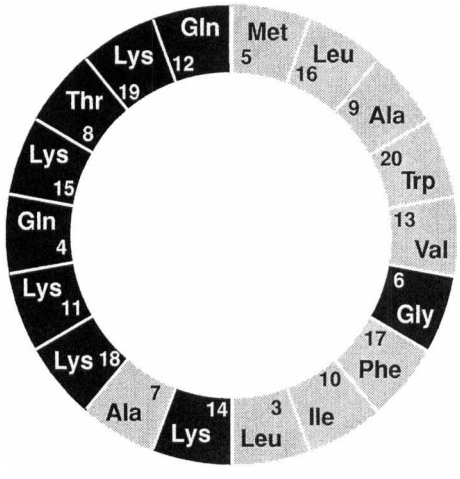

Fig. 4. Edmundson α-helical wheel representation of the amphiphilical region in α. The amphiphilical region shown starts with residue 3 and ends with residue 20. The shaded areas indicate non-polar residues. An identical α-helical wheel may be constructed for β, starting with amino acid residue 4 and ending with residue 21.

The amphiphilic region in the α and β peptides may allow the peptides to oligomerize into membrane-spanning pores such that the polar side of the helix faces towards the centre of the pore and the non-polar side faces the membrane-lipids (Lear *et al.*, 1988; Ojcius &

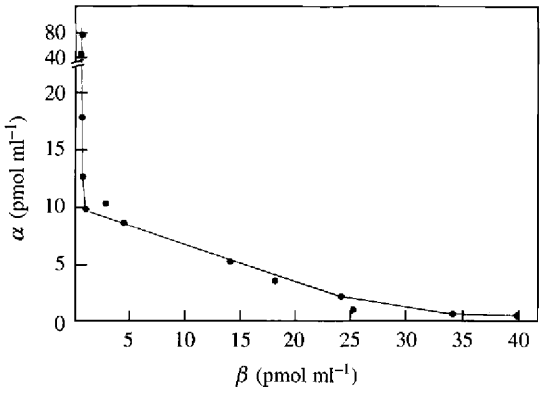

Fig. 5. The amounts of α and β which in combination inhibited growth of the indicator strain by 50%.

Young, 1991). An amphiphilic region is also found in the N-terminal half of the α and β peptides of lactococcin G, a bacteriocin produced by *Lactococcus lactis* (Nissen-Meyer *et al.*, 1992). Unlike plantaricin A, the two peptides of lactococcin G are entirely different from each other (Nissen-Meyer *et al.*, 1992). An amphiphilic distribution (although not so marked as in the peptides of plantaricin A and lactococcin G) may also be detected in other lactic acid bacteria-produced bacteriocins, such as lactocin S (Mørtvedt *et al.*, 1991) and lactococcin A (Holo *et al.*, 1991). There is good evidence that the latter bacteriocin acts in fact by permeabilizing target cell membranes (Van Belkum *et al.*, 1992), as also appears to be the case for nisin (Sahl *et al.*, 1987).

A comparison of plantaricin A amino acid sequence to other known LAB bacteriocins did not reveal any significant homology (data not shown).

No meaningful amino acid sequence was obtained when the minor bacteriocin activity peak (fraction 4, Fig. 1) was rechromatographed on the reverse-phase column and subsequently sequenced. Moreover, the bacteriocin activity was greatly reduced upon rechromatography of this fraction. One cannot exclude that the minor activity peak is a further breakdown product of the β peptide. However, this minor peak of activity could also be due to some kind of inactivation of the peptide (not degradation) as seen also with lactococcin G (Nissen-Meyer *et al.*, 1992).

Relative amounts of α and β necessary to obtain bacteriocin activity

The concentrations of α and β, which in combination inhibited growth of the indicator organism by 50%, are presented as an isobologram in Fig. 5. When α was in excess (> 10 pmol ml^{-1}), the presence of β at a concentration of 1·0–0·2 pmol ml^{-1} resulted in 50% growth inhibition (Fig. 5). Similarly, with an excess of β (> 25 pmol ml^{-1}), the presence of α at a concentration of 1·0–0·4 pmol resulted in a 50% growth inhibition (Fig. 5). Thus, about the same amounts of α and β was needed to obtain 50% growth inhibition in the presence of an excess of the complementary peptide.

To our knowledge, lactococcin G represents the only other known bacteriocin whose activity depends on the complementary action of two peptides (Nissen-Meyer *et al.*, 1992). However, whereas the α and β peptides of lactococcin G are entirely different from each other with respect to their primary structure (Nissen-Meyer *et al.*, 1992), plantaricin A is novel as its α and β peptides appear only to differ from each other in that β contains an additional alanine residue at its N-terminal end. Plantaricin A should represent an interesting model to study how two so similar peptides may complement each other to induce a bactericidal effect.

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