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Purification and Partial Amino Acid Sequence of Plantaricin S, a Bacteriocin Produced by *Lactobacillus plantarum* LPCO10, the Activity of Which Depends on the Complementary Action of Two Peptides

RUFINO JIMÉNEZ-DÍAZ,[1]* JOSÉ L. RUIZ-BARBA,[1] DECLAN P. CATHCART,[2] HELGE HOLO,[3] INGOLF F. NES,[3] KNUT H. SLETTEN,[4] AND PHILIP J. WARNER[2]

Departamento de Biotecnología de Alimentos, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, 41012 Seville, Spain[1]; Biotechnology Center, Cranfield University, Cranfield, Bedford MK43 0AL, United Kingdom[2]; Laboratory of Microbial Gene Technology, NLVF, N-1432 Ås, Norway[3]; and Department of Biochemistry, University of Oslo, 0367 Oslo, Norway[4]

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Plantaricin S, one of the two bacteriocins produced by *Lactobacillus plantarum* LPCO10, which was isolated from a green-olive fermentation (R. Jiménez-Díaz, R. M. Ríos-Sánchez, M. Desmazeaud, J. L. Ruiz-Barba, and J.-C. Piard, Appl. Environ. Microbiol. 59:1416–1424, 1993), has been purified to homogeneity by ammonium sulfate precipitation, by binding to SP-Sepharose fast-flow, phenyl-Sepharose CL-4B, and C_2/C_{18} reverse-phase chromatographies. The purification resulted in a final yield of 91.6% and a 352,617-fold increase in the specific activity. The bacteriocin activity was associated with two distinct peptides, termed α and β, which were separated by C_2/C_{18} reverse-phase chromatography. Although β alone appeared to retain a trace of inhibitory activity, the complementary action of both the α and β peptides was required for full bacteriocin activity, as judged by both the agar well diffusion and the microtiter plate assays. From the N-terminal end, 26 and 24 amino acids residues of α and β, respectively, were sequenced. Further attempts at sequencing revealed no additional amino acids residues, suggesting that either modifications in the next amino acid residue blocked the sequencing region or that the C-terminal end had been reached. The amino acid sequences of α and β show no apparent homology to each other or to other bacteriocins purified from lactic acid bacteria.

Among the variety of antimicrobial substances produced by lactic-acid bacteria (LAB), bacteriocins are one of the most promising natural food preservatives (6, 8, 23). Because of the inhibitory activity of bacteriocins against a variety of food spoilage-causing and/or competing organisms, bacteriocin-producing LAB have been widely used in dairy, meat, and vegetable fermentations (4, 5, 11, 21, 28). In this way, *Lactobacillus plantarum* LPCO10, which is a bacteriocin producer isolated from a green olive fermentation (12), has been recently shown to be useful as a starter culture to control the lactic acid fermentation of Spanish-style green olives (24).

Biochemical characterization of bacteriocins from LAB is an essential goal to study their mode of action on sensitive cells, with a view at evaluating their possible utilization as food preservatives. Many bacteriocins from *L. plantarum* have been isolated and partially characterized (7, 12–15). However, only three have been purified and sequenced: plantaricin A, plantaricin C, and plantaricin-149. Plantaricin A is a bacteriocin whose activity depends on the complementary action of two peptides (19). These peptides, named α and β, have identical amino acid sequences and differ from each other only in that β contains an additional alanine residue. Plantaricin C has been described as the first lantibiotic produced by a strain of *L. plantarum*, with a molecular mass of ca. 3,500 Da (10). Plantaricin-149 is a single-peptide bacteriocin which contains 22 amino acid residues, and its amino acid sequence differs from those of plantaricin A (14).

In this report, we describe the purification of plantaricin S (PLS), one of the two bacteriocins produced by *L. plantarum* LPCO10 (12). The partial amino acid sequences of both the α and β peptides that constitute PLS are reported, and the complementary inhibitory action of the α and β peptides which gives full PLS activity is demonstrated.

MATERIALS AND METHODS

Bacteria and media. The PLS and plantaricin T (PLT) producer *L. plantarum* LPCO10 has been described previously (12). The PLS- and PLT-sensitive *L. plantarum* 128/2 was used as the indicator strain (12). Both strains were maintained as frozen stocks at −20°C in distilled water plus 20% (vol/vol) glycerol and propagated twice at 30°C in MRS (Oxoid; Unipath Ltd., Basingstoke, Hampshire, England) broth before use. MRS agar was prepared by the addition of 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.) to MRS broth.

PLS assays. The PLS activity was quantified in a microtiter plate assay system (9). Each well of the microtiter plate contained 25 μl of twofold-concentrated MRS broth, 25 μl of PLS fractions at serial two- and threefold dilutions, and 10 μl of the indicator strain, *L. plantarum* 128/2 (A_{600} = 0.01 [ca. 10^6 CFU/ml]). As a turbidity control, *L. plantarum* 128/2 was incubated as described above, but with sterile distilled water in place of PLS fractions. The microtiter plate cultures were incubated for 7 h at 30°C, after which growth inhibition of the indicator strain was measured spectrophotometrically at 600 nm in a Bio-Rad microplate reader (model 450; Bio-Rad Laboratories, Hercules, Calif.). One PLS unit (PLSU) was arbitrarily defined as the amount of PLS that inhibited the growth of the indicator strain by 50% (50% of the turbidity of the control culture without bacteriocin). This was expressed as the reciprocal of the highest dilution exhibiting 50% inhibition of the indicator strain per milliliter (PLSU/ml). The results of the two- and threefold dilution series for every sample were averaged.

Purification of PLS. All the purification steps were carried out at room temperature, and all of the chromatographic equipment and media were purchased from Pharmacia Biotech (Uppsala, Sweden). Plantaricin S was purified from a 6-liter MRS broth culture of *L. plantarum* LPCO10. The producer strain was grown in batch culture with shaking at 30°C to the early stationary phase (A_{600} = 4.8 [ca. 10^9 CFU/ml]), and then the cells were removed by centrifugation at 10,000 × *g* for 10 min at 4°C.

(i) Ammonium sulfate precipitation. Ammonium sulfate (516 g of culture supernatant per liter) was added (80% of saturation, at 0°C). After the mixture had been stirred for 2 h at 4°C, the protein precipitate was centrifuged at 20,000

* Corresponding author. Phone: 34-5-4691054. Fax: 34-5-4691262.

TABLE 1. Purification of PLS

Fraction	Vol (ml)	Total A_{280}[a]	Total activity (BU) (10^5)[b]	Sp act (BU/A_{280})[c]	Increase in sp act[c]	Yield (%)
Culture supernatant	6,000	226,596	6	2.64	1	100
Ammonium sulfate precipitation (fraction I)	252	5,268	90.3	1,714.4	649.4	1,505
Binding to SP-Sepharose fast flow (fraction II)	104	34	23	67,647	25,623.9	383.3
Binding to phenyl-Sepharose CL-4B (fraction III)	40	6.95	9	130,000	49,051.5	150
FPLC[d] (C_2/C_{18} reverse phase) (fraction IV)	12	0.59	5.5	930,000	352,617.0	91.6

[a] Total A_{280} is the A_{280} multiplied by the volume in milliliters.
[b] BU, bacteriocin units.
[c] Specific activity is bacteriocin units (BU) divided by the A_{280}.
[d] FPLC, fast-performance liquid chromatography.

× *g* for 30 min at 4°C, and the resulting pellet was solubilized in 180 ml of citrate-phosphate buffer (50 mM; pH 5.0) (buffer C). The 180-ml sample was then desalted through PD10 gel filtration columns equilibrated with buffer C (252 ml; fraction I).

(ii) Cation-exchange chromatography. Fraction I was applied at a flow rate of about 18 ml/min to a 30-ml SP Sepharose fast-flow cation-exchange column equilibrated with buffer C. After subsequent washing of the column with 4 bed volumes of buffer C, PLS was eluted from the column with 104 ml of 2 M NaCl in buffer C (fraction II).

(iii) Hydrophobic interaction chromatography. Fraction II was applied at a flow rate of about 1.5 ml/min to a 13-ml phenyl-Sepharose CL-4B column equilibrated with 2 M NaCl in buffer C. The column was washed with 4 bed volumes of buffer C, after which PLS was eluted from the column with 40 ml of 70% (vol/vol) ethanol in buffer C (fraction III).

(iv) C_2/C_{18} reverse-phase chromatography. Fraction III was diluted fivefold with deionized, distilled water containing 0.1% (vol/vol) trifluoroacetic acid (TFA) and was then divided into four 50-ml aliquots. Each aliquot was consecutively applied to a C_2/C_{18} reverse-phase column (PepRPC HR 5/5) equilibrated with deionized, distilled water containing 0.1% TFA (buffer A). PLS was eluted with a 2-min linear gradient from 100% A to 80% A–20% B (buffer B was composed of 0.1% [vol/vol] TFA in 2-propanol), a 20-min linear gradient from 80% A–20% B to 100% B, for 20 min at 100% B, and with a 2-min linear gradient back to 100% A. The flow rate was maintained at 0.5 ml/min throughout the runs; 1-ml fractions were collected.

Fractions from the four different runs containing peaks involved in PLS activity were pooled (12 ml; fraction IV), fivefold diluted with buffer A, and rechromatographed on the reverse-phase column. PLS was eluted with a 2-min linear gradient from 100% A to 70% A–30% B, a 23-min linear gradient from 70% A–30% B to 58% A–42% B, a 1-min linear gradient from 58% A–42% B to 100% B, for 6 min at 100% B, and with a 2-min linear gradient back to 100% A. The flow rate was maintained at 0.5 ml/min throughout the run; 0.5-ml fractions were collected and analyzed for PLS activity. Purified PLS was stored in 40% 2-propanol containing 0.1% TFA at −20°C until use.

Complementation assays. Absorbance peaks resolved upon rechromatography of fraction IV were assayed for complementary inhibitory activity by both the agar well diffusion assay and the microtiter plate assay method. The 0.5-ml fractions from each absorbance peak obtained as described above were pooled, and the 2-propanol was evaporated in a rotational vacuum concentrator (Alpha-RVC; Martin Christ, Osterode, Germany) in combination with a freeze dryer (Alpha 1-4; Martin Christ) as a cooling trap. After evaporation, the initial volume of the samples was restored with buffer C.

For the agar well diffusion assays, an adaptation of the method previously described (25) was used. MRS agar (1.5% agar) was seeded with ca. 10^5 CFU of the indicator strain *L. plantarum* 128/2 per ml and poured onto individual petri dishes. After the plates were set and dried, holes (4-mm diameter) were cut from the indicator-seeded MRS agar at distances of 5 mm (from well to well edge), and their bottoms were sealed with MRS agar. To show the complementary inhibitory activity of two peaks, two opposite wells were filled up each with 50 μl of the single-peak sample in buffer C. Samples in the wells were allowed to diffuse throughout the indicator-seeded MRS agar for 20 min at 4°C, incubated at 30°C for 18 h, and then examined for inhibition zones. Alternatively, two absorbance peak samples were mixed in the same well (25 μl each in buffer C) and processed as described above. To show that the inhibition was due to protein-peptide substances, each single peak sample was treated with proteinase K or α-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) (final concentration, 1 mg/ml). After incubation for 1 h at 37°C, the protease inhibitor phenylmethylsulfonyl fluoride (Sigma) (final concentration, 1 mM) or trypsin-chymotrypsin inhibitor (Sigma) (final concentration, 1.5 mg/ml) was added, respectively, to the enzyme solutions, and then the mixtures were placed in the wells. Controls were buffer C, absorbance peak samples plus the protease inhibitors, or protease inhibitors.

For the complementation assays by the microtiter plate method, the absorbance peak samples were mixed at different ratios (each ranging from 0 to 100% in the mixtures) to a final volume of 50 μl, and then the PLS activity from each mixture was quantified as described above.

Amino acid sequencing. The NH_2-terminal amino acid sequences were determined by Edman degradation by using an Applied Biosystems (Foster City, Calif.) 477A automatic sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer, as described previously (16).

RESULTS

Purification of PLS. The purification pattern followed for isolating PLS for sequencing is shown in Table 1.

Evidence that two peptides were involved in PLS activity was obtained when fraction III was applied to the C_2/C_{18} reverse-

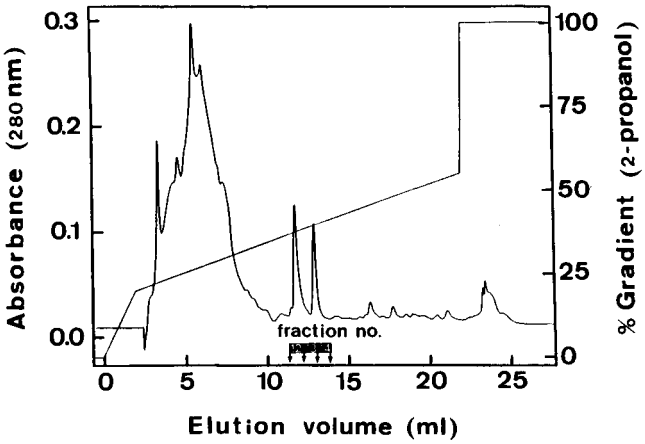

FIG. 1. C_2/C_{18} reverse-phase chromatography analysis of PLS (fraction III). Maximum bacteriocin activity was detected in fraction no. 13.

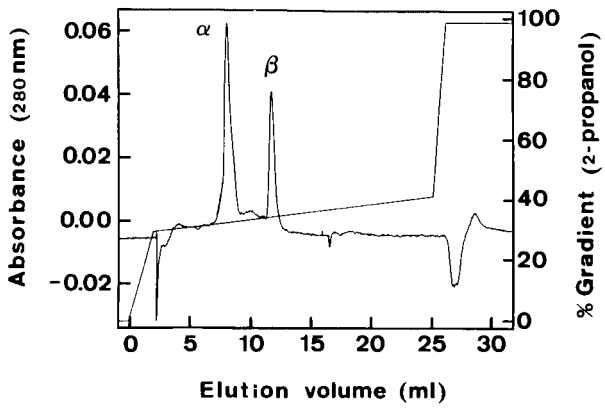

FIG. 2. C_2/C_{18} reverse-phase chromatography analysis of PLS (fraction IV). α and β refer to the absorbance peaks of each of the two peptides involved in PLS activity.

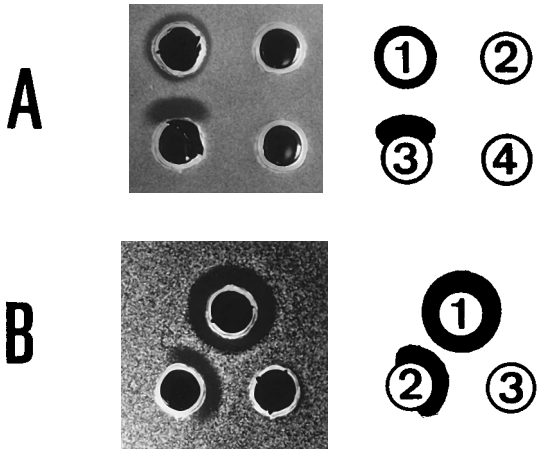

FIG. 3. Agar well diffusion assay of the α and β peaks after purification as shown in Fig. 2. (A) Well 1, peak β; well 2, peak α after treatment with α-chymotrypsin (final concentration, 1 mg/ml) for 1 h at 37°C, with subsequent treatment with trypsin-chymotrypsin inhibitor (final concentration, 1.5 mg/ml); well 3, peak α; and well 4, peak β after treatment with α-chymotrypsin (final concentration, 1 mg/ml) for 1 h at 37°C, with subsequent treatment with trypsin-chymotrypsin inhibitor (final concentration, 1.5 mg/ml). (B) Well 1, peak α plus peak β; well 2, peak α; and well 3, peak β.

phase column (Fig. 1). The bacteriocin assay of the eluted fractions revealed that most of the bacteriocin activity was present in fraction no. 13, where two absorbance peaks overlapped, whereas fraction no. 14 showed a minor inhibitory activity (Fig. 1). Neither fraction no. 12 nor other collected fractions from the same run showed any bacteriocin activity. When aliquots of each fraction eluted from the column were added to each other, no increase in the inhibitory activity was observed, except when fraction no. 12 was added to fraction no. 13 or 14. When these three fractions were pooled, an increase in the specific activity of more than 350,000-fold and a recovery of about 91% of the initial activity were obtained (Table 1, fraction IV).

Isolation of two peptides whose complementary action is necessary for full PLS activity. When fraction IV was rechromatographed and eluted in the reverse-phase column by use of a shallow 2-propanol gradient, two major absorbance peaks were isolated (Fig. 2). They were termed α and β, in the order in which they were eluted from the column.

When both the α and β peaks were assayed for inhibitory activity by the agar well diffusion assay (Fig. 3A and B), a narrow zone of inhibition surrounding the well in which the peak β had been placed was observed (Fig. 3A, well 1), whereas the α peak did not show any inhibition zone except at the α well side next to β (Fig. 3A, well 3). When the α and β peaks were placed together in the same well, a zone of inhibition surrounding the entire well was observed (Fig. 3B, well 1). This zone of inhibition was bigger (in diameter) than that exhibited by peak β alone (Fig. 3A, well 1). Again, a zone of inhibition at the α well sides next to α plus β or β wells (Fig. 3B, well 2) was observed. In this case, the concentration of β in wells 1 and 3 was calculated so that it would not show inhibition by itself (Fig. 3B, well 3). The complementary action of the α plus β fractions is shown in Table 2. The total activity increased by a maximum of 48 times with respect to that exhibited by β alone when α ranged between 40 and 80% in the mixture (Table 2).

TABLE 2. Inhibitory activity of mixtures of peaks α and β obtained as shown in Fig. 2

% in the mixtures of[a]		PLSU/ml[b]	Increase in activity (fold)[c]
α	β		
0	100	80	1
10	90	1920	24
20	80	1920	24
40	60	3840	48
50	50	3840	48
60	40	3840	48
80	20	3840	48
90	10	1920	24
100	0	0	0

[a] Total volume of the mixtures was 50 μl.
[b] PLS units per milliliter. The bacteriocin assays were carried out in microtiter plates, and *L. plantarum* 128/2 was used as the indicator strain (see the Materials and Methods).
[c] Each item is the PLSU of each mixture per ml divided by the PLSU of that of 100% β per ml.

When both the α (Fig. 3A, well 2) and β (Fig. 3A, well 4) peaks were treated with α-chymotrypsin or proteinase K, no zone of inhibition at the α well side next to β was seen (Fig. 3A, wells 2 and 3). Finally, neither the protease inhibitors nor buffer C appeared to inhibit or to increase the described complementary action of both peptides.

Amino acid sequences of the α and β peptides. The NH_2-terminal amino acid sequences of the α and β peptides are shown in Fig. 4. Further sequencing attempts did not result in detection of any additional residues, indicating either that the next amino acid of the peptide was modified and therefore blocked the reaction or that the C terminus was reached.

DISCUSSION

In this paper, the purification of PLS, which is one of the two bacteriocins produced by *L. plantarum* LPCO10 isolated from a Spanish-style green-olive fermentation (12), is described.

						5					10					15			
α:	NH_2-	Xaa	Asn	Lys	Leu	Ala	Tyr	Asn	Met	Gly	(Trp)	Tyr	Ala	Gly	Xaa	Ala	Thr	Ile	
β:	NH_2-	Lys	Lys	Lys	Lys	Gln	Ser	Trp	Tyr	Ala	Ala	Ala	Gly	Asp	Ala	Ile	Val	Ser	

					20				25		
α:		Phe	Gly	Leu	Ala	Ala	Xaa	Ala	(Leu)	(Leu)	
β:		Phe	Gly	Glu	Gly	Phe	Leu	Asn			

FIG. 4. NH_2-terminal amino acid sequences of α and β peptides. Amino acids not identified with certainty but which are probable are shown in parentheses. Xaa, unidentified amino acid; NH_2−, the amino termini of the peptide.

Our results indicate that this bacteriocin consists of two distinct peptides whose complementary action is required for full PLS activity. As demonstrated by reverse-phase chromatography and amino acid sequencing, both peptides were purified to homogeneity by the purification procedure described here.

After ammonium sulfate precipitation of the *L. plantarum* LPCO10 culture supernatant, the total bacteriocin activity recovered was about 15 times higher than that present in the initial supernatant. As well as for other described bacteriocins (3, 13, 17, 22), increased activity of PLS after ammonium sulfate precipitation has been observed previously (12), and it can be attributed to multimer dissociation of the high-molecular-weight bacteriocin aggregates into their smallest, more active forms. However, the total PLS activity recovered on treatment with ammonium sulfate was greatly reduced after the successive purification steps, although the final activity recovered by reverse-phase chromatography was similar to that present in the initial supernatant. An explanation for that loss of activity could be the presence of material in the prepurified fractions (I to IV) which did not bind to the columns.

Two absorbance peaks obtained by reverse-phase chromatography of fraction IV were associated with PLS activity. Thus, the facts that in the agar well diffusion assays the β peak showed a trace of inhibitory activity and that the indicator organism was largely inhibited by α only in a zone at its well side next to β strongly suggest that the complementary action of both the α and β peaks is required for full PLS activity. The zone of inhibition shown by α would represent the area where the α and β peaks meet each other after they diffuse throughout the agar. In addition, this zone would represent the area where the optimal concentrations of α and β required for inhibitory activity are present, as assessed by the results obtained by the microtiter plate assays. In light of these findings, it could be assumed that the diffusion of β throughout the agar was larger than that of α. Although experiments with dissociating agents are necessary, e.g., sodium dodecyl sulfate and urea, etc., this difference could be due either to a multimer conformation of α which prevents a larger diffusion of the molecules or to its own diffusion characteristics.

The described complementary action for full PLS activity was exclusively due to the interaction of two peptide substances, as demonstrated by the complementation studies of the protease-treated and nontreated α and β peaks. Thus, PLS can be considered a two-peptide bacteriocin system.

The amino acid sequences of the α and β peptides confirmed both their cationic and hydrophobic natures, as demonstrated by their behavior with regard to the purification method described here. In general, the primary structures of α and β were shown to be different from each other, although the presence in both the α and β peptides of many basic and hydrophobic amino acids would give to them very similar biochemical characteristics.

Recently, a small number of LAB bacteriocins whose full inhibitory activity depends on the complementary action of two distinct peptides have been described (1, 2, 18, 26, 27). In particular, for *L. plantarum*, only plantaricin A has been reported as a bacteriocin system defined by two heterogeneous components (19). Although the primary structures of the peptides from PLS are very different from those described for other two-peptide bacteriocin systems, they all share some characteristics. Thus, all of them are small peptides and are cationic and hydrophobic in nature. These characteristics are also very similar to those of many other antibacterial peptides for which the formation of a pore in the cytoplasmic membrane through the barrel-stave mechanism described by Ojcius and Young (20) has been proposed (10, 16, 19). Although studies on the mode of action of PLS have to be carried out with the purified peptides, the above-mentioned similarities point to the possibility of a similar inhibitory mechanism for PLS.

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