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Isolation, Purification, and Amino Acid Sequence of Lactobin A, One of the Two Bacteriocins Produced by *Lactobacillus amylovorus* LMG P-13139

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Lactobacillus amylovorus **LMG P-13139, isolated from corn steep liquor, produces two bactericidal peptides with respective estimated molecular masses of 4.5 and 6.0 kDa upon denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The antimicrobial activity detected in the fermentation supernatant fraction of *L. amylovorus* LMG P-13139 was heat stable (20 min, 121°C), displayed a narrow inhibitory spectrum, and was sensitive to proteinase K, trypsin, and α-chymotrypsin but insensitive to α-amylase, lysozyme, catalase, and lipase. The 4.5-kDa bacteriocin was purified and characterized and designated lactobin A. Lactobin A was isolated as a floating pellicle from culture supernatant brought to 35% saturation with ammonium sulfate. Upon this ammonium sulfate treatment, crude lactobin A was incorporated, together with Tween 80 as a major contaminant, in high-molecular-mass complexes sized at approximately 670 kDa by gel filtration chromatography. Contaminating fatty acids were removed from these micelles by a simple one-step methanol-chloroform extraction without loss of activity. Both inhibitory peptides were separated in an isocratic isopropanol gradient on a PepRPC 5/5 reversed-phase column, and both peptides retained activity towards *Lactobacillus helveticus* ATCC 15009 upon separation. Lactobin A has a molecular mass determined by electrospray mass spectrometry of 4,879 ± 0.69 Da. Its peptide chain contains 50 unmodified amino acids, of which 26% are glycine residues and 40% are hydrophobic residues (A, V, L, I, and P). It displays the highest structural homology (42% identity and 28% similarity) with the *lafX* gene product, encoded by the second open reading frame of the lactacin F operon. These data strongly indicate that lactobin A belongs to the class IIb bacteriocins according to the classification of Klaenhammer.**

Bacteriocins are antimicrobial proteins or oligopeptides displaying a spectrum of activity against strains taxonomically related to the producer organism (11, 16, 19). Many bacteriocins produced by lactic acid bacteria have been purified to homogeneity. Except for earlier purification methods used for bacteriocins such as nisin (3, 6), bacteriocin 466 (8), lactocin 27 (34), diplococcin (7), lactacin B (2), and lactostrepcin 5 (37) or a recent cell-affinity purification method (36), most current purification strategies involve a single enrichment step and one or more additional chromatographic steps (13, 14, 24, 28). A variety of purification procedures have been used successfully for the purification of bacteriocins produced by different bacterial species, including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Propionibacterium*, *Carnobacterium*, and *Enterococcus* spp. (11, 16). Due to their high hydrophobicity, class II bacteriocins have been reported to possess a tendency to interact aspecifically with medium components such as Tween 80, interfering with subsequent purification steps (13, 24).

Some of the bacteriocins produced by lactic acid bacteria are known to depend upon the complementation of two peptides for activity (1, 26, 27) and were subsequently grouped as the class IIb bacteriocins (20). Lactococcin G was the first class IIb bacteriocin that has been purified and for which complementation upon combination of the separated α and β peptides was shown (26). A comparable report has been made for the two-component bacteriocin plantaricin A (27). Complementation of lactacin F was shown upon combination of two heterologously expressed peptides, although initially only one bacteriocin was purified from the *Lactobacillus johnsonii* supernatant (1, 24).

Here we report the purification and amino acid sequence of lactobin A, one of the two peptides of a new class IIb bacteriocin produced by *Lactobacillus amylovorus* LMG P-13139. The simple three-step purification scheme included a chloroform extraction step to remove Tween 80.

MATERIALS AND METHODS

Strains and media. The strains, corresponding media, and incubation temperatures used in this study are described in Table 1. Cultures were maintained as frozen stocks at −80°C in 20% glycerol (BDH Ltd., Dorset, United Kingdom). Cultures were inoculated in broth at the 1% level and propagated at their corresponding incubation temperature as indicated in Table 1. Agar medium was prepared by the addition of 1.2% technical agar no. 3 (Oxoid) to the broth medium; soft agar was prepared with 0.7% bacteriological agar no. 1 (Oxoid).

Isolation of lactic acid bacteria. Samples of diluted fresh corn steep liquor (50°C, pH 3.8), after incubation for an additional 6 h at 45°C, were streaked onto low-glucose (0.2%) MRS agar plates supplemented with cycloheximide (0.01%). The plates were incubated anaerobically overnight at 45°C. Colonies were selected and further aerobically incubated overnight at 45°C. The developed col-

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TABLE 1. Bacterial strains used in this study

Microorganism	Cultivation medium	Incubation temp (°C)	Sensitivity to lactobin A
Gram-positive bacteria			
Lactobacillus amylovorus LMG P-13139	MRS	37	−
Lactobacillus acidophilus LMG 7943	MRS	37	−
Lactobacillus brevis LMG 6906	MRS	37	−
L. brevis LMG 7761	MRS	37	−
Lactobacillus casei subsp. *casei* ATCC 7469	MRS	37	−
L. casei subsp. *casei* LMG 6904	MRS	37	−
L. casei subsp. *casei* JAC 25	MRS	37	−
L. casei subsp. *rhamnosus* LMG 6400	MRS	37	−
Lactobacillus delbrueckii subsp. *bulgaricus* LMG 6901	MRS	37	+
L. delbrueckii subsp. *delbrueckii* LMG 6412	MRS	37	−
L. delbrueckii subsp. *lactis* LMG 7942	MRS	37	+
Lactobacillus fermentum LMG 6902	MRS	37	−
Lactobacillus helveticus ATCC 15009	MRS	37	+
L. helveticus LMG 6413	MRS	37	+
Lactobacillus hilgardii JAC 5	MRS	37	−
L. hilgardii JAC 28	MRS	37	−
Lactobacillus jensenii LMG 6414	MRS	37	−
Lactobacillus plantarum LMG 6907	MRS	37	+
L. plantarum LMG 1284	MRS	37	+
L. plantarum LMG 8155	MRS	37	−
Lactococcus lactis subsp. *lactis* ML8	MRS	30	−
L. lactis subsp. *cremoris* 1P5	MRS	30	−
Leuconostoc mesenteroides subsp. *mesenteroides* ATCC 12291	MRS	30	−
L. mesenteroides subsp. *mesenteroides* LMG 7939	MRS	30	−
Pediococcus acidilactici ATCC 8042	MRS	37	−
Bacillus cereus LMG 6924	Bacillus cereus agar	30	−
B. cereus LMG 6925	Bacillus cereus agar	30	−
Bacillus subtilis LMG 8197	Bacillus cereus agar	30	−
Enterococcus faecium LMG 8149	Slanetz & Bartley agar	37	+
Enterococcus faecalis LMG 8146	Slanetz & Bartley agar	37	+
Listeria innocua LMG 11387	Oxford agar	30	−
Listeria monocytogenes LMG 10470	Oxford agar	30	−
Listeria seeligeri LMG 11386	Oxford agar	30	−
Listeria welshimeri LMG 11389	Oxford agar	30	−
Gram-negative bacteria			
Enterobacter aerogenes LMG 2094	Nutrient agar	37	−
Escherichia coli	Nutrient agar	37	−
Pseudomonas fluorescences LMG 1794	Nutrient agar	30	−
Pseudomonas aeruginosa LMG 68029	Nutrient agar	30	−
Salmonella enteritidis LMG 10395	Brilliant green agar	37	−
Salmonella typhimurium LMG 10396	Brilliant green agar	37	−
Yersinia enterocolitica IP 383	Yersinia selective agar	37	−
Y. enterocolitica WS24/92	Yersinia selective agar	37	−
Y. enterocolitica IP 1103	Yersinia selective agar	37	−
Y. enterocolitica WA 289	Yersinia selective agar	37	−
Yeasts			
Pichia membranaefaciens 34	Yeast-peptone-dextrose agar	30	−
Saccharomyces cerevisiae IHEM 3096	Yeast-peptone-dextrose agar	30	−
Zygosaccharomyces bailii 33	Yeast-peptone-dextrose agar	30	−

onies were screened by Gram stain, spore-forming ability, catalase, and oxidase tests. Nonsporulating gram-positive, catalase-negative, and oxidase-negative bacteria were retained for further identification with the API 50 CH gallery and for antagonism tests.

Bacteriocin detection and assay. For detection of antagonistic activity, a well diffusion assay (32) and a modification of the method described by Mayr-Harting et al. (21) were used. Indicator organisms included those listed in Table 1. Bacteriocin activity was semiquantitatively determined by an adaptation of the critical dilution method used for the assay of bacteriocins (21). Activity, expressed as activity units (AU) per milliliter, was defined as the reciprocal of the highest twofold dilution showing a complete inhibitory action towards the indicator organism. Specific activity was defined as the activity units related to the amount of biomass expressed as optical density (OD) units measured at 600 nm. *Lactobacillus helveticus* ATCC 15009 displayed the highest sensitivity for the lactobin A inhibitory activity and was therefore used as the indicator strain for further routine bacteriocin assays.

The bioassay detection of antimicrobial peptides in polyacrylamide gels was accomplished as described by Bhunia et al. (4). The gels were first washed with sterile ultrapure water. After sodium dodecyl sulfate (SDS) concentrations had dropped to nontoxic levels, polyacrylamide gels were covered with a lawn of soft agar (0.7%) and seeded with an exponentially growing culture of the indicator organism, *L. helveticus* ATCC 15009.

Production of lactobin A in broth. A 50-ml inoculum of a 10-h culture of *L. amylovorus* LMG P-13139 was inoculated in 5 liters of sterile MRS or semisyn-

thetic, modified MRS (MRS without meat extract and peptone) medium in a Bioflo 2 model BF-500 fermentor (New Brunswick, Edison, N.J.). Fermentation conditions for production of lactobin A by *L. amylovorus* LMG P-13139 were evaluated at 37°C and 45°C in MRS and semidefined, modified MRS broth (5 liters) maintained at pH 4.5, 5.0, 5.5, 6.0, or 6.5 or under free pH conditions. The influence of an initial glucose concentration of 10, 15, 20, 25, and 30 g/liter was also tested.

Preliminary characterization of bacteriocin. The cells were removed by centrifugation (13,000 × *g*, 10 min, 4°C), and the resulting cell-free supernatant fraction was designated crude lactobin A. For a preliminary characterization of the *L. amylovorus* LMG P-13139 bacteriocin, cell-free culture supernatant was treated with proteinase K (1 mg/ml; 0.05 M sodium phosphate buffer [pH 7.5], 37°C), trypsin (2 mg/ml; 0.05 M sodium phosphate buffer [pH 7.0], 37°C), α-chymotrypsin (5 mg/ml; 0.05 M sodium phosphate buffer [pH 7.5], 37°C, α-amylase (1 mg/ml; 0.05 M sodium phosphate buffer [pH 7.0] plus 0.01 M NaCl, 25°C), lysozyme (1 mg/ml; 0.05 M sodium phosphate buffer [pH 7.0] plus 0.01 M NaCl, 25°C), catalase (5 mg/ml; 0.05 M sodium phosphate buffer [pH 7.0], 25°C), and lipase (1 mg/ml; 0.05 M sodium phosphate buffer [pH 7.0] plus 0.005 M $CaCl_2$, 37°C) and tested for activity as described above. Heat stability was tested by treating culture supernatant for 30 min at 60°C and 20 min at 121°C, after which the bioactivity was tested again.

Ammonium sulfate precipitation. Five-liter fermentation aliquots of crude lactobin A (adjusted to pH 6.5) were made up to a final concentration of 35% saturation by the slow addition of ammonium sulfate (VEL, Leuven, Belgium) and stored overnight at 4°C with stirring. Subsequently, the mixture was centrifuged at 13,000 × *g* at 4°C for 20 min and the supernatant fractions were decanted. Surface pellicles and bottom pellets were recovered and resuspended in 10 ml of sterile ultrapure water and designated fraction I.

Electrophoresis. Tricine-SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was performed by the method of Schägger and von Jagow (30) in 15% *T*–0.5% C_{bis} gels, where *T* is the final concentration of acrylamide and C_{bis} is the final cross-linker concentration. Polyacrylamide gels were stained with either Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.), silver stain (Bio-Rad Laboratories, Richmond, Calif.), or Oil O Red (Sigma).

Fast protein liquid chromatography FPLC. Anion-exchange chromatography was performed on a MonoQ HR 5/5 column (LKB-Pharmacia, Uppsala, Sweden) either with a phosphate (pH 6.5), Tris (pH 7.5), glycine-NaOH (pH 9.2), or NH_4OH-NH_4Cl (pH 10.0) buffer. For all buffers, a linear gradient of 0 to 100% was applied in a time interval of 30 min after the samples were injected. The eluent was monitored simultaneously at 214 and 280 nm and collected in 1-ml samples. The same elution profile was applied for cation-exchange, hydrophobic-interaction, and reversed-phase chromatographies. Cation-exchange chromatography was performed on a MonoS HR 5/5 column (LKB-Pharmacia) with a phosphate (pH 6.5 or pH 5.5), acetate (pH 4.0), or glycine-HCl (pH 2.5) buffer. Hydrophobic-interaction chromatography was performed on a phenyl-Superose HR 5/5 column (LKB-Pharmacia) with an isocratic gradient of 0.05 M sodium phosphate buffer (pH 6.5) plus 10% ammonium sulfate to 0.05 M sodium phosphate buffer (pH 6.5) or a 0.02 M NH_4OH-NH_4Cl (pH 10.0) buffer system. Reversed-phase chromatography was performed on a PepRPC HR 5/5 column (LKB-Pharmacia) with a linear methanol-isopropanol (90:1), isopropanol plus 0.1% trifluoroacetic acid (TFA), or acetonitrile plus 0.1% TFA gradient. Gel filtration chromatography was performed with a 50-min elution profile on a Superdex 75 HR 10/30 column (LKB-Pharmacia) with a 0.05 M sodium phosphate buffer (pH 6.5) or on a Superdex 200 HR 10/30 column (LKB-Pharmacia) with the same buffer.

Ethanol-diethyl ether extraction. Two volumes of a cold-water-saturated ethanol-diethyl ether mixture (1:2, vol/vol) were added to fraction I. The sample was centrifuged for 10 min at 13,000 × *g* and 4°C. The water phase was evaporated at 60°C, and the pellet was resuspended in 1 volume of sterile ultrapure water. The organic phase was washed three times with ultrapure water, evaporated at 60°C, and resuspended in 1 volume of sterile ultrapure water.

Methanol-chloroform extraction. Twenty-five volumes of a methanol-chloroform mixture (1:2, vol/vol) was added to 10 ml of lactobin A (fraction I) and stored at 4°C for 1 h with stirring. The samples were centrifuged (20 min, 13,000 × *g*, 4°C). The pellet was air dried, and the extraction was repeated. Finally, the pellet was resuspended in 10 ml of sterile ultrapure water; this partially purified lactobin A was stored at −80°C and designated fraction II.

TLC. Thin-layer chromatography (TLC) was performed by the method described by Navarre et al. (25). Briefly, 5-μl samples were loaded on the plate and the spot was immediately dried. TLC plates were placed in a saturated atmosphere of diethyl ether-toluene-ethanol-acetic acid (40:50:2:0.2) and air dried. Subsequently, the TLC plates were run in a diethyl ether-hexane (6:94)-saturated atmosphere and developed in iodine vapor. Samples were visualized by application of a 1% starch solution.

Electrospray mass spectrometry (EMS). Approximately 100 pmol of sample was dissolved in 10 μl of 50% acetonitrile–1% formic acid in water and injected into the electrospray source of a V BIO-Q triple-quadrupole mass spectrometer (VG Biotech, Altrincham, United Kingdom). The sample was pumped at a flow rate of 5 μl/min delivered by a 140-A solvent delivery system (Applied Biosystems, Foster City, Calif.). The capillary tip was set at a voltage of 4.1 kV, and the sample cone voltage was set at 51 V. The mass spectrometer, of which only the first quadrupole was used, was set to scan the mass range of 650 to 1,350 Da in 9 s. Data were collected over 2 min. The mass spectrometer was calibrated by preliminary analysis of horse heart myoglobin (Sigma).

Protein sequence analysis. The purified peptide was loaded on a precycled biobrene-coated cartridge filter and sequenced with a pulse-liquid model 477A sequenator equipped with an on-line model 120A phenylthiohydantoin analyzer from Applied Biosystems.

Protein sequence accession number. The lactobin A sequence presented in this article has been assigned Swiss Prot accession number P80696.

RESULTS

Screening. A total of 240 lactic acid bacteria, isolated from fresh corn steep liquor, were screened for antagonistic activity. Low-glucose MRS medium was used to restrict the extent of acid production. To eliminate inhibition due to hydrogen peroxide production, a first incubation was performed anaerobically. Three *Lactobacillus* strains (no. 172, 174, and 180) were found to produce inhibition zones against *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901, *L. delbrueckii* subsp. *lactis* LMG 7942, and *L. helveticus* ATTC 15009. Culture filtrates of the three positive strains, readjusted to pH 6.5 (to reduce inhibition due to lactic acid), were checked by a well diffusion assay. The culture supernatant of only two strains (no. 174 and 180) produced inhibition zones on agar against the same three indicator organisms mentioned above. All other gram-positive and gram-negative organisms tested were not inhibited (Table 1).

Identification of strain no. 174 and 180 to the species level. By use of the Analytab Products system, strain no. 180 was identified as a homofermentative *Lactobacillus* strain closely related to *L. delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus*, a conclusion corroborated by protein electrophoresis (data not shown). On the latter basis, only 60% similarity was found with *L. acidophilus*. However, no homology could be detected with these *Lactobacillus* strains via DNA-RNA hybridization. Finally, 16S rRNA sequencing revealed that strain no. 180 was an *L. amylovorus* strain (data not shown), which was further designated *L. amylovorus* LMG P-13139. Strain no. 174 was identified as *L. acidophilus* via the Analytab Products system and protein electrophoresis (data not shown). Because no bacteriocin producers were known among the former species, *L. amylovorus* LMG P-13139 was retained for further study.

Fermentative production of lactobin A. Fermentations were performed at a temperature of 37 or 45°C with initial pH and glucose concentration settings of 6.2 and 20 g/liter, respectively. A final pH value of 3.8 was reached after 12 h, whereas a maximal bioactivity level of 400 AU per ml (specific activity of 130 AU/ml OD unit) was reached after 8 h of fermentation. Bioactivity decreased drastically upon further fermentation, and maximal activity was restricted to a narrow time interval of 1 h in the exponential growth phase, at about 8 h of fermentation. Although higher bioactivity levels were reached in MRS medium up to maximum values of 800 AU per ml (specific activity of 250 AU/ml OD unit), the same fermentation profile was obtained as that obtained in semisynthetic, modified MRS medium. Fermentation runs at 45°C resulted in higher bacterial growth, as deduced from OD measurements (OD at 600 nm). At 37°C, however, a two- to fourfold increase in bioactivity was noted compared with that in fermentations performed at 45°C. Modified MRS medium, i.e., MRS without peptone and yeast extract, was preferred since the latter two components resulted in a high collagen background during N-terminal amino acid Edman degradation sequencing when lactobin A was isolated from fermented MRS broth (data not shown).

The influence of the pH on the growth pattern and lactobin

A production properties of *L. amylovorus* LMG P-13139 was examined in pH-controlled batch fermentations with constant pH settings of 4.5, 5.0, 5.5, 6.0, and 6.5. Fermentations at a constant pH of 4.5 and 6.5 showed no bacterial growth of *L. amylovorus* LMG P-13139; at pH 5.0, no bioactivity could be observed. Maximal bioactivity was obtained at a constant pH of 5.5, resulting in a fourfold increase of the specific bioactivity level expressed per unit of biomass (from 130 to 550 AU/ml/OD unit) as compared with that in a free-pH fermentation in modified MRS. A twofold decrease in OD (from 3.0 to 1.4 at 600 nm) was noted at a constant pH of 5.5, and therefore, a fermentation at pH 5.5 resulted in a twofold increase of the absolute bioactivity (400 to 800 AU per ml). No significant difference in bioactivity level (absolute or specific activity related to the biomass) could be found between a fermentation at a constant pH of 6.0 and a free-pH fermentation. These preliminary optimal conditions for lactobin A production were subsequently combined with an alteration in the initial glucose concentrations of 10, 15, 20, 25, and 30 g of glucose per liter. Maximal activity (800 AU per ml; specific activity, 550 AU/ml/OD unit) was obtained with 20 g of glucose per liter.

Preliminary characterization of bacteriocin. The antimicrobial activity detected in the fermentation supernatant fraction of *L. amylovorus* LMG P-13139 was heat stable (20 min, 121°C), displayed a narrow inhibitory spectrum (Table 1), and was sensitive to proteinase K, trypsin, and α-chymotrypsin but insensitive to α-amylase, lysozyme, catalase, and lipase.

Ammonium sulfate precipitation of lactobin A. Cell-free culture supernatant from *L. amylovorus* LMG P-13139 was adjusted to pH 6.5 with NaOH and saturated with 35% ammonium sulfate. This treatment resulted in three distinct phases after centrifugation: a surface pellicle, a bottom pellet, and the liquid supernatant fraction. The volume of the bottom pellet was negligible in comparison with that of the floating pellicle, whereas almost 95% of the total bacteriocin activity was concentrated in both of these pellets after saturation with 35% ammonium sulfate (fraction I).

SDS-PAGE of floating pellicle samples revealed that only small amounts of extraneous proteins were present in this fraction. Samples of lactobin A analyzed on denaturing SDS-PAGE could be stained with Coomassie brilliant blue R-250 or silver dye for protein detection and with Oil O Red dye for the detection of fatty acids (data not shown). Bacteriocin activity could also be localized by overlaying the denaturing SDS-PAGE gel with a lawn of indicator organisms. In all cases, however, no distinct banding pattern except for one large spot streaking from 4.5 kDa to approximately 6.5 kDa could be observed. A Coomassie brilliant blue R-250 staining of a fraction I sample is shown in Fig. 1.

Purification of fraction I lactobin samples. The purification scheme of lactobin A is shown in Table 2. Fraction I was applied to gel filtration (Superdex 75 HR 10/30 or Superdex 200 HR 10/30), cation-exchange (Mono S HR 5/5) and anion-exchange (Mono Q HR 5/5), hydrophobic-interaction (phenyl-Superose HR 5/5), and reversed-phase (PepRPC HR 5/5) chromatography columns. Fractionation of fraction I by a Superdex 75 HR 10/30 column or a Superdex 200 HR 10/30 column, simultaneously monitored at 214 and 280 nm, demonstrated a single peak that coeluted with lactobin A activity. The estimated molecular mass of the injected lactobin A samples against gel filtration standards was 670 kDa for the Superdex 200 HR 10/30 column. Lactobin A showed no ionic or hydrophobic interaction with the Mono Q HR 5/5, Mono S HR 5/5, or phenyl-Superose HR 5/5 column, and activity was detected only in the void volume of the columns although it was strongly reduced upon ion-exchange chromatography. On a PepRPC HR 5/5 column, lactobin A activity was eluted at 50% isopropanol (+0.1% TFA) as a smearing peak that did not correspond to the standard sharp peaks obtained with reversed-phase chromatography. These data obtained from chromatography, in addition to the detection of fatty acids with Oil O Red staining, suggested the formation of large lactobin A aggregates caused by the hydrophobic interaction of lactobin A with fatty acids. Further examination of fraction I proved that the distribution and chain length of the fatty acids present in these samples showed almost perfect homology with the composition of Tween 80, a nonionic detergent present in the fermentation medium as revealed by fatty acid analysis. Apparently, these micelles could not be completely disrupted by the SDS concentrations present in the denaturing gels or the conditions used in reversed-phase chromatography.

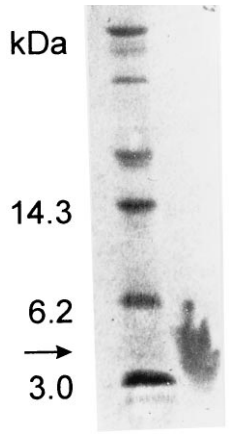

FIG. 1. Tricine-SDS-PAGE of ammonium sulfate-precipitated lactobin A. Tricine-SDS-PAGE was performed by the method of Schägger and von Jagow (30). Lanes: left, molecular mass standards with sizes given on the left; right, ammonium sulfate-precipitated lactobin A stained with Coomassie brilliant blue R-250.

Extraction of fatty acids. To disrupt lactobin A micelles, Tween 80 was extracted from the high-molecular-mass complexes by a simple single-step methanol-chloroform or ethanol-diethyl ether extraction. Upon the ethanol-diethyl ether extraction, a solvent phase and a bioactive bacteriocin-containing aqueous phase were obtained, whereas the methanol-chloroform extraction resulted in a precipitated form of the bioactive component. All phases resulting from the two extraction methods were examined by TLC and stained for the detection of fatty acids (data not shown). In both extraction methods, all fatty acids were concentrated in the organic solvent phase, whereas the bioactivity-containing aqueous (ethanol-diethyl ether extraction) and pellet (methanol-chloroform extraction) phases were free of fatty acids.

Analysis of these extracted samples on denaturing SDS-PAGE gels revealed two separate antagonistic protein bands with estimated molecular masses of 4.5 and 6.0 kDa. The methanol-chloroform extraction was preferred since no indicative loss of bioactivity was observed and, furthermore, the precipitation of the proteinaceous compounds allowed a faster and more concentrated isolation than could be obtained in the two-phase ethanol-diethyl ether extraction. These methanol-chloroform-extracted bacteriocin samples were designated fraction II. The recovery of the two bactericidal peptide molecules was not reproducible, and some fraction II samples contained only the 4.5-kDa bacteriocin. The parameters responsible for this phenomenon and the mutual relationship

TABLE 2. Purification scheme of lactobin A

Sample	Total protein (mg)	Total activity (AU)	Sp act (AU/mg)	Activity recovered (%)	Fold purification
Culture supernatant	80,000	6,760,000[a]	85	100	1
Ammonium sulfate precipitation	578	456,000[a]	788	6.7	9.5
Chloroform-methanol extraction	8.5	456,000[a]	53,647	6.7	638
PepRPC HR 5/5	0.35	6,500	18,517[b]	0.069	220[b]

[a] Two different bacteriocins were produced by *L. amylovorus* LMG P-13139, and thus only part of this activity is caused by the purified peptide lactobin A.
[b] Loss of activity upon reversed-phase chromatography gives a reduction in specific activity and an apparent decrease in fold purification.

between both peptides have not yet been completely elucidated.

Reversed-phase chromatography of fraction II lactobin A samples. Fraction II demonstrated exactly the same elution pattern as that of fraction I on a Superdex 75 HR 10/30 column, a Superdex 200 HR 10/30 column, a Mono Q HR 5/5, a Mono S HR 5/5, and a phenyl-Superose HR 5/5 column. Application of fraction II on a PepRPC HR 5/5 column with an isocratic isopropanol or acetonitrile gradient resulted in the elution of a bioactive fraction corresponding to a final concentration of 90 to 100% isopropanol (0.1% TFA) or acetonitrile (0.1% TFA). Analysis of the eluted samples on a denaturing SDS-PAGE gel showed the presence of a bioactive proteinaceous component with an estimated molecular mass of 4.5 kDa eluting at a final isopropanol or acetonitrile concentration of 90%. Once the organic solvent concentration was raised to 100% isopropanol or acetonitrile, an antimicrobial protein of 6.0 kDa was eluted. At an intermediate isopropanol or acetonitrile concentration, a mixture of both proteins was detected. The PepRPC HR 5/5 fractions containing the 4.5-kDa or the 6.0-kDa protein were designated, respectively, fraction III and fraction IV (Fig. 2).

EMS. EMS was performed on a fraction II sample that contained only the 4.5-kDa bacteriocin. Fraction II was first concentrated with a Centricon ultrafiltration unit with a membrane molecular mass cutoff of 100 kDa (Amicon, Beverly, Mass.). Almost all bacteriocin activity was retained on the ultrafiltration membrane. Approximately 10 pmol of lactobin A (fermentation equivalent of 200 ml) was applied. Only one distinct protein peak corresponding to a molecular mass of 4,879 ± 0.69 Da was detected (Fig. 3). Analysis of this sample by SDS-PAGE revealed a 4.5-kDa bactericidal protein band with an inhibitory effect towards the indicator strain. To determine the molecular mass of the 6.0-kDa protein, both bacteriocins were eluted from a PepRPC HR 5/5 column at 90 and 100% isopropanol, respectively, and separately examined by EMS. The resulting data, however, were disturbed by polypropylene peaks with an interval of 44 Da corresponding to the propylene monomer. Apparently, the high isopropanol concentrations present in the PepRPC HR 5/5 fractions resulted in the extraction of propylene polymers from the recipients used. All protein peaks present were therefore masked. Because of the high hydrophobicity of the purified bacteriocin components, they displayed a tendency to stick irreversibly to a glass matrix. Therefore, glass could not be used as an alternative to collect isopropanol-eluted PepRPC HR 5/5 fractions. Other recipient materials were not tested.

Amino acid sequence determination. Lactobin A was subjected to amino acid sequencing by the Edman degradation reaction. These experiments were performed on the same sample used for EMS. Fifty amino acids were identified, which indicated that there was no N-terminal modification in lactobin A. The amino acid sequence contained 13 glycine residues and 19 hydrophobic residues (8 alanine, 3 leucine, 7 valine, and 1 isoleucine residue), confirming the very hydrophobic properties determined during purification. The primary structure of lactobin A and its homology with the *lafX* gene product are shown in Fig. 4. The polypropylene residues registered in fraction III and fraction IV during EMS also interfered with the determination of the amino acid sequence of the proteins eluted from the PepRPC 5/5 column.

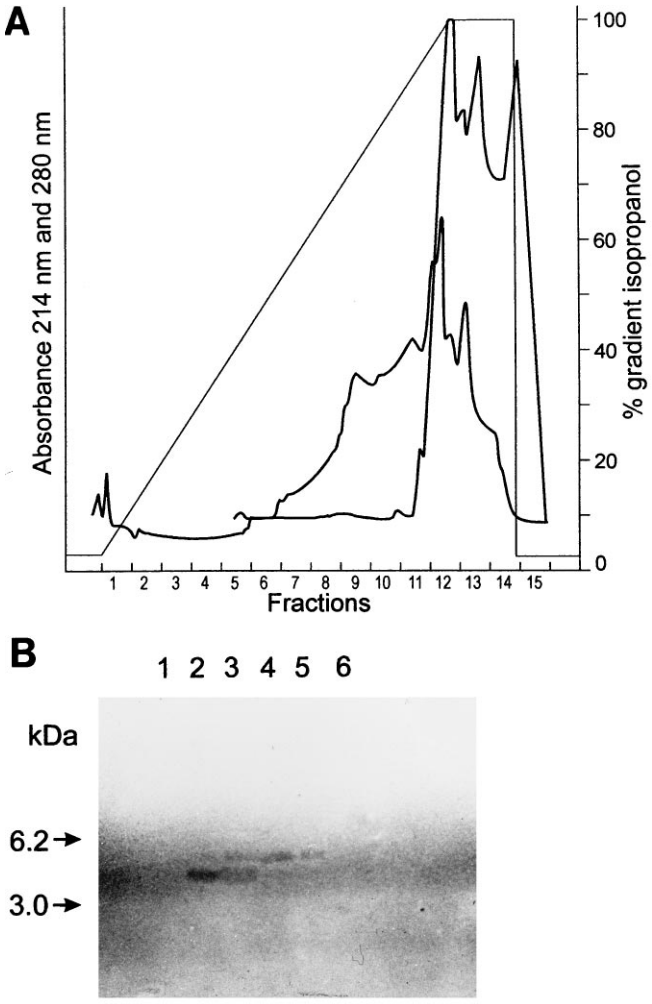

FIG. 2. (A) PepRPC HR 5/5 reversed-phase FPLC analysis of lactobin A fraction II samples obtained by ammonium sulfate precipitation and chloroform-methanol extraction. Bacteriocin activity was detected in fractions 11, 12, 13, and 14. (B) PepRPC HR 5/5 reversed-phase FPLC-purified samples containing lactobin A activity on an SDS-PAGE gel overlaid with a bacterial indicator lawn. Lanes: 1, fraction 1 to 10 from FPLC; 2, fraction 11 from FPLC; 3, fraction 12 from FPLC; 4, fraction 13 from FPLC; 5, fraction 14 from FPLC; 6, fraction 16 from FPLC.

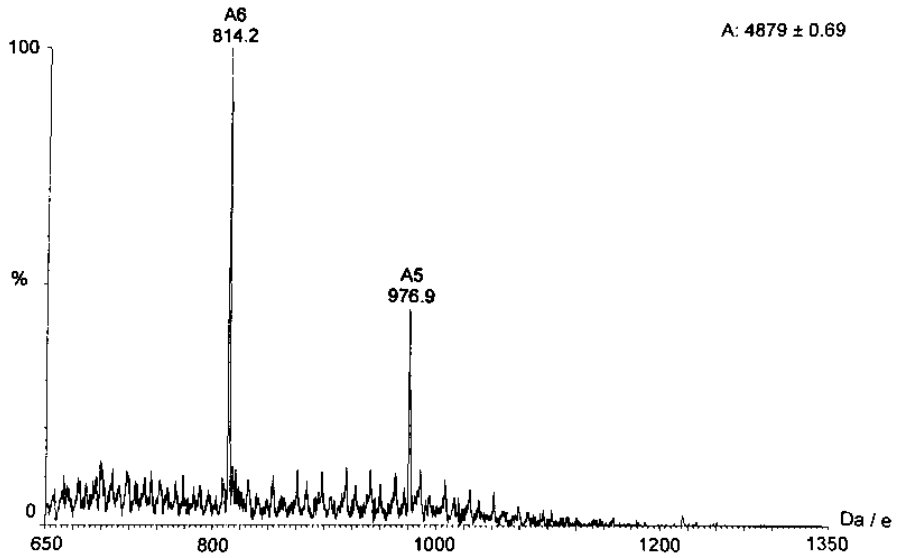

FIG. 3. EMS of a fraction II lactobin A sample. Approximately 100 pmol of sample was injected into a V BIO-Q triple quadrupole mass spectrometer.

Sequencing of both proteins separated on an SDS-PAGE gel and electrotransferred to a solid membrane support failed because both proteins were insufficiently transferred to positively charged membranes unless they were biotinylated or fluorescamine labeled prior to electrotransfer (data not shown). These modifications, however, prevented amino acid sequence determination of the N terminus or even of proteinaceous or chemically generated internal fragments. In these experiments, the hydrophobicity of the 4.5- and 6.0-kDa peptides was confirmed, since both displayed a clear repulsing effect towards aqueous solutions after electrotransfer on a solid membrane support.

DISCUSSION

In this study, three different *Lactobacillus* strains producing a proteinaceous bactericidal component were isolated from corn steep liquor. One strain, displaying the most pronounced antimicrobial effect, was identified as an *L. amylovorus* strain and designated *L. amylovorus* LMG P-13139. Its bacteriocin has been termed lactobin A. Observations made during ultrafiltration and gel filtration chromatography indicated the formation of large bacteriocin-containing complexes. The formation of globular structures resembling micelles which averaged 25 to 50 nm in diameter was also reported for lactacin F (24). In general, bacteriocins produced by lactobacilli occur as large-molecular-mass complexes (2, 13, 18, 24).

The chain length and distribution of the fatty acids found in the lactobin A complexes showed strong homology with that of Tween 80, a nonionic detergent present in the fermentation medium. The fact that the medium component Tween 80 constituted the most important component of the lactobin A micelles indicates that they result from aspecific associations rather than from specific interactions necessary for bactericidal action of lactobin A. Although not chemically confirmed, bacteriocin-Tween 80 complex formation was demonstrated as yellow-staining material upon SDS-PAGE for curvaticin FS47 and lactacin F (13, 24). Difficulties in the purification of carnocin 44A were reduced by growing *Lactobacillus carnosum* LA44A in a modified MRS medium containing only 50% of the normal peptone concentration and no Tween (35). Tween 80 could not be replaced in the fermentation medium by a detergent with a shorter carboxy chain such as SDS or Tween 20 since *L. amylovorus* LMG P-13139 failed to grow in these modified media. Production and recovery of curvaticin FS47 and lactococcin G were also shown to be dependent upon the presence of Tween 80 in the fermentation medium (13, 26).

A simple one-step methanol-chloroform extraction was used to remove most fatty acid contamination from the ammonium sulfate-precipitated lactobin A pellicle. This procedure resulted in a sufficiently pure bacteriocin isolate suitable for EMS or amino acid sequencing. Liquid-liquid extraction experiments for the purification of bacteriocins were described earlier (5, 22, 31, 34). Chloroform treatment, however, lowered or destroyed brevicin 37 and acidocin B activity (29, 33). Following the extraction of Tween 80, the antimicrobial spot streaking from 4.5 to 6.0 kDa was converted into two distinct bacteriocins of 4.5 and 6.0 kDa on SDS-PAGE. However, both these proteins were no longer stainable on an SDS-PAGE gel with Coomassie brilliant blue R-250, silver, or Oil O Red dye upon methanol-chloroform extraction and could only be visualized with a lawn of the sensitive indicator organism. In comparison, lanes containing purified lactacin B samples treated with silver stain no longer showed protein bands (2).

The 4.5- and 6.0-kDa bacteriocins mentioned above were separated on a PepRPC 5/5 column. However, the extreme

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LafX        NRWGDTVLSAASGAGTGIKACKSFGP-WGMAICGVGGAAIGGYFGYTHN- 48aa
LactobinA   NRWTNAYSAALGCAVPGVKYGKKLGGVWGAVIGGVGGAAVCGLAGYVRKG 50aa
            ***...  .* . * .*.*  *..*  ** .* ******. *  **...
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FIG. 4. Amino acid sequence of lactobin A and comparison of its primary structure with that of LafX. Residues at a well-conserved position are indicated by a period, and identical residues are marked by an asterisk. Lactobin A and LafX show an overall identity of 21 residues (42%) and an overall similarity of 14 residues (28%). The alignment was performed with the PC-gene Clustal program.

hydrophobicity of these bacteriocins causes them to stick in an almost irreversible manner to the hydrophobic C_{18} stationary phase, thereby preventing elution. A proper elution profile required saturation of the reversed-phase column with bacteriocin molecules prior to use. In contrast with lactococcin G (26) and plantaricin A (27), lactobin A displayed no additional activity towards *L. helveticus* ATCC 15009 upon combination of different PepRPC 5/5 column fractions. Furthermore, both the 4.5- and 6.0-kDa bacteriocins retained activity towards *L. helveticus* ATCC 15009 following separation on an SDS-PAGE gel. Therefore, it seems likely that lactobin A activity towards *L. helveticus* ATCC 15009 is not enhanced by the complementation of these two peptides. Initially, only one bioactive peptide (LafA) was purified from the *L. johnsonii* VPI11088 fermentation supernatant with *L. helveticus* NCK338 as an indicator organism (24). Later, it was shown that LafA is a bacteriocin that kills *L. helveticus* NCK338. Expansion of the host range to include *L. delbrueckii* and *Enterococcus faecalis* occurs only after the interaction of LafA and LafX (1). This means that two hypotheses can be advanced for lactobin A. (i) Both bacteriocins produced by *L. amylovorus* LMG P-13139 are independently lethal for *L. helveticus* ATCC 15009, and thus, complementation is not important for its activity towards *L. helveticus* ATCC 15009 but for the expansion of its bacteriocinogenic spectrum. Or, (ii) *L. amylovorus* LMG P-13139 produces two independently acting bacteriocins in the exponential growth phase. Two bacteriocins, plantaricin S and T, produced by *Lactobacillus plantarum* LPCO10 at different stages in the fermentation, have been reported by Jiménez-Diaz et al. (17).

The presence of the previously mentioned 6.0-kDa peptide was unfortunately not reproducible in all fermentations. No formation or loss of bioactive protein bands during purification could be noted. Aspecific proteolytic degradation of the 6.0-kDa peptide seemed unlikely since no other aspecific, intermediate bioactive degradation products were discovered. Furthermore, exposure of samples containing both the 4.5- and 6.0-kDa bacteriocin molecules to room temperature for 72 h, causing a 50% reduction in bioactivity, did not yield any additional bioactive peptide bands on SDS-PAGE but resulted in less pronounced activity of both peptides (data not shown). Whether the 4.5-kDa peptide concerns a single degradation product due to specific proteolytic degradation or even processing of the 6.0-kDa (precursor) peptide could not be deduced from our results. However, in normal *L. amylovorus* LMG P-13139 fermentations, bioactivity was restricted to a narrow time interval at about 8 h of fermentation and declined drastically thereafter. Small fluctuations in starter culture age and medium quality, fermentation pH, and temperature all affected the time for optimal activity, with considerable variation emerging among the different fermentations in the total amount of activity harvested. Therefore, the interval in the fermentation profile when supernatant was harvested might be critical and less favorable for the 6.0-kDa peptide. It is well known that nutritional and environmental conditions greatly influence bacteriocin production (10, 11, 23, 33). We therefore conclude that *L. amylovorus* LMG P-13139 produced two different bacteriocins, one of which seems to be more susceptible to inactivation.

Amino acid sequence analysis of purified lactobin A identified 50 N-terminal amino acid residues with a calculated molecular mass of 4,882 Da. Lactobin A contains no unusual amino acids but up to 26% glycine residues. This high glycine content has proven to be a typical characteristic among various bacteriocins (20). Structure analysis of the amino acid sequence of lactobin A indicated a hydrophobic membrane-integrated stretch extending from residues 24 to 45, whereas the extreme N- and C-terminal portions of lactobin A displayed hydrophilic tendencies. These properties also seem to be very conserved among many other bacteriocins (19). EMS indicated a molecular mass of 4,879 ± 0.69 Da, which corresponds to the 50 unmodified residues revealed by N-terminal amino acid analysis. Extraction of polypropylene polymers due to the high isopropanol concentrations necessary for bacteriocin elution in the reversed-phase chromatography experiments disturbed EMS analysis of the 6.0-kDa peptide. Plantaricin A and lactococcin G samples used for plasma desorption mass spectrometry analyses were eluted at much lower isopropanol concentrations (approximately 35%) than that of lactobin A (26, 27).

Structural analysis of lactobin A indicates that it belongs to the small heat-stable, non-lanthionine-containing membrane-active peptides (<10 kDa) characterized by a Gly^{-2}-Gly^{-1}-Xaa processing site in the bacteriocin precursor according to the classification of Klaenhammer (20). Three subgroups can be defined within these class II bacteriocins, and recently, a fourth subclass has been proposed for curvaticin FS47 (13). Lactobin A displayed no activity towards *Listeria* species and was inactivated following reduction in the presence of β-mercaptoethanol or dithiothreitol (data not shown). Therefore, lactobin A does not belong to the class IIa or IIc Klaenhammer classification (20) or a possible new class represented by curvaticin FS47 (13). Comparison of the amino acid sequence of lactobin A with those in the Swiss Prot (release 30) protein bank revealed a significant homology of lactobin A with the protein encoded by the *lafX* (*lafY*) gene in the lactacin F operon, a representative of class IIb bacteriocins (12, 20).

In conclusion, although no complementary action towards *L. helveticus* ATTC 15009 was noted, an expansion of the inhibitory spectrum upon complementation could not be excluded. The presence of two bacteriocins produced by the same strain, the structure and characteristics of lactobin A, and its homology with the LafX bacteriocin (42% identity and 28% similarity), the second bacteriocin encoded by the lactacin F operon, strongly indicate that lactobin A belongs to the class IIb bacteriocins according to the classification of Klaenhammer (20).

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