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Characterization and Purification of Acidocin 1B, a Bacteriocin Produced by *Lactobacillus acidophilus* GP1B

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Abstract In the present study, acidocin 1B, a bacteriocin produced by *Lactobacillus acidophilus* GP1B, exhibited profound inhibitory activity against a variety of LAB and pathogens, including Gram-negative bacteria, and its mode of action was to destabilize the cell wall, thereby resulting in bactericidal lysis. Acidocin 1B was found to be heat stable, because it lost no activity when it was heated up to 95°C for 60 min. It retained approximately 67% of the initial activity after storage for 30 days at 4°C, and 50% of its initial activity after 30 days at 25°C and 37°C. The molecular mass of acidocin 1B was estimated to be 4,214.65 Da by mass spectrometry. Plasmid curing results indicated that a plasmid, designated as pLA1B, seemed to be responsible for both acidocin 1B production and host immunity, and that the pLA1B could be transformed into competent cells of *L. acidophilus* ATCC 43121 by electroporation. Our findings indicate that the acidocin 1B and its producer strain may have potential value as a biopreservative in food systems.

Keywords: *Lactobacillus acidophilus*, bacteriocin, acidocin 1B, bactericidal lysis, plasmid, electroporation

Lactic acid bacteria (LAB) can produce a wide range of antimicrobial metabolites, which may contribute in a number of ways towards the improvement of the quality of various foods [17]. Among a number of antimicrobial compounds produced by LAB, the bacteriocins are antimicrobial peptides or proteins with a high potential for food preservation [22]. Bacteriocins have become the focus of increasing attention, and new approaches for the control of pathogenic and spoilage microorganisms have been developed [5]. Nisin is an example of a LAB bacteriocin that has been approved in over 40 countries for use as a food additive [8]. Despite the promising information and studies regarding these substances, however, LAB bacteriocins often suffer from limited effectiveness in foods, because of inactivation through the action of proteolytic enzymes and poor adaptation to food environments.

All bacteriocin producers are insensitive to their own bacteriocins. Bacteriocin production is inextricably linked to the expression of specific immunity proteins required for protection of the producing strain against the inhibitory action of its own product [1]. For all well-characterized bacteriocins, it has been demonstrated that the genes involved in immunity functions are also closely associated with the bacteriocin structural gene [16, 24].

Bacteriocins produced from LAB have been categorized into three different classes according to their biochemical and genetic properties. Many of the bacteriocins produced by *Lactobacillus* species belong to class II bacteriocins, and these are normally small hydrophobic or heat-stable peptides that harbor no unusual amino acids such as lanthionine [31]. Among the *Lactobacillus* species, *L. acidophilus* strains have been extensively utilized as probiotic cultures in dairy and pharmaceutical products and recognized as important bacteriocin-producing bacteria [29].

The purpose of this study was to characterize and purify the bacteriocin produced by *L. acidophilus* GP1B and to evaluate its potential value as a new biopreservative in food systems.

Materials and Methods

Bacterial Strains and Culture Conditions

Lactic acid bacteria and pathogens used in this study were obtained from the stock culture collection of the Food Microbiology Lab, Division of Food Science, Korea

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Table 1. Inhibitory activity of acidocin 1B produced by *Lactobacillus acidophilus* GP1B against indicator strains.

Bacterium	Medium	°C	Inhibition[a]	Source
Lactobacillus				
L. acidophilus ATCC 43121	MRS	37	–	ATCC[b]
L. acidophilus ATCC 4356	MRS	37	+	ATCC
L. acidophilus GP1B	MRS	37	–	OSU[c]
L. casei YIT 9029	MRS	37	+	YIT[d]
L. delbrueckii subsp. *lactis* ATCC 4797	MRS	37	+	ATCC
L. helveticus KU116	MRS	37	+	Lab. isolate
L. paracasei subsp. *paracasei* 910	MRS	37	–	Lab. isolate
L. paracasei subsp. *paracasei* 911	MRS	37	–	Lab. isolate
L. plantarum B-1	MRS	37	+	Lab. isolate
L. plantarum F7-1	MRS	37	+	Lab. isolate
L. rhamnosus ATCC 7469	MRS	37	–	ATCC
Pathogens				
Acinetobacter baumanii KU119	TSB[e]	37	–	Lab. isolate
Bacillus cereus ATCC 11778	TSB	37	+	ATCC
Enterobacter aerogenes KU109	TSB	37	+	Lab. isolate
Escherichia coli O157 ATCC 43889	LB[f]	37	+	ATCC
Klebsiella pneumoniae KU141	TSB	37	–	Lab. isolate
Listeria innocua KU011	TSB	25	+	Lab. isolate
Listeria ivanovii KU023	TSB	25	+	Lab. isolate
Listeria monocytogenes KU067	TSB	25	+	Lab. isolate
Pseudomonas aeruginosa KCCM11321	TSB	37	+	KCCM[g]
Pseudomonas fluorescens KU1124	TSB	25	–	Lab. isolate
Salmonella typhimurium K1186	TSB	37	–	Lab. isolate
Shigella sonnei KU101	TSB	37	+	Lab. isolate
Staphylococcus epidermidis KCCM 35494	TSB	37	+	KCCM
Staphylococcus intermedius KCCM 40149	TSB	37	+	KCCM
Staphylococcus aureus KU203	TSB	37	+	Lab. isolate
Yersinia enterocolitica KU212	TSB	37	+	Lab. isolate

[a] +, Inhibited by the cell-free supernatant; –, not inhibited.
[b] ATCC, American Type Culture Collection.
[c] Oklahoma State University.
[d] YIT, Yakult Central Institute for Microbiological Research.
[e] TSB, Tryptic Soy broth.
[f] LB, Luria Bertani broth.
[g] KCCM, Korean Culture Center of Microorganisms.

University (Table 1). *Lactobacillus acidophilus* GP1B, a bacteriocin producer strain, was isolated from pig intestine and had been identified by various biochemical tests including the API test and RFLP [13]. LAB were grown at 37°C for 18 h in MRS broth (Difco, Detroit, MI, U.S.A.) and pathogens were incubated in trypticase soy broth (TSB, Difco) or Luria Bertani broth (LB, Difco) at 25°C or 37°C for 18 h. All LAB and pathogens were subcultured at least three times prior to use.

Production of Antimicrobial Substance

Lactobacillus acidophilus GP1B was incubated in MRS broth at 37°C for 18 h and then harvested by centrifugation at 3,000 ×*g* for 15 min (4°C). The cell-free supernatant was collected, adjusted to pH 6.5 using 10 N NaOH, and filtered through a sterile 0.45-μm syringe filter (Sartorius, Göttingen, Germany) into sterile screwcap tubes.

Assays for Antimicrobial Activity

Antimicrobial activity was assessed against different LAB and foodborne pathogens by the spot-on-lawn method [3]. Antimicrobial activity was quantified by spotting 10-μl aliquots of two-fold serial dilutions of the cell-free supernatant onto the surfaces of MRS, LB, or TSB agar (10 ml). The spotted agar was then overlaid with the desired broth agar (0.8%) inoculated with 1% of the indicator strains that were cultured overnight, and the Petri dishes were then incubated upright at 37°C for 24 h. Antimicrobial activity was determined by the highest two-fold dilution, evidenced by a clear inhibitory zone on the agar, and expressed as arbitrary units (AU) per ml of cell-free supernatant.

Mode of Action

Approximately 10^7 CFU/ml of *L. delbrueckii* subsp. *lactis* ATCC 4797 in the stationary phase was inoculated in MRS

broth (100 ml) and the cell-free supernatants were added to a final activity of 32, 160, and 320 AU/ml, respectively. During the incubation at 37°C, the survivors were enumerated on MRS agar after appropriate dilutions in peptone water for up to 4 h at 1-h intervals and the colonies were counted after 48 h of incubation at 37°C.

Transmission Electron Microscopy (TEM)

MRS broth (5 ml) was inoculated with 2% of *L. delbrueckii* subsp. *lactis* ATCC 4797 in stationary phase, and after 4 h of incubation, the cell-free supernatants were added to a final activity of 1,600 AU/ml. After further incubation at 37°C for 1 h, the sample was fixed. The supernatant-treated cells were incubated overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) at 4°C, washed twice in 0.1 M cacodylate buffer, and then placed in 0.1 M cacodylate-buffered 2% osmium tetroxide for 4 h at 4°C. These preparations were then washed in 0.1 M cacodylate buffer, dehydrated in a graded series of acetone, and embedded in Spurr medium. Ultrathin sections on grids, stained with 3% uranyl acetate and lead citrate, were examined using TEM (H-600, Hitachi, Tokyo, Japan).

Heat, pH, Enzyme, and Storage Stability of Antimicrobial Substance

The cell-free supernatants were heated individually at 65, 95, and 121°C for 20, 40, and 60 min, and then tested for remaining antimicrobial activity. The cell-free supernatants were adjusted to various pH values in a range from 2 to 12 and stored at 4°C for 24 h. They were then neutralized to pH 6.5 and assessed for remaining antimicrobial activity, respectively. In order to ascertain whether the antimicrobial activity had been caused by hydrogen peroxide or protein, its enzyme susceptibility was evaluated by the incubation of the cell-free supernatant in the presence of 0.25 mg/ml of trypsin (pH 8.0; Type II, crude), pepsin (pH 3.0; E.C. No. 3.4.23.1), and catalase (pH 6.0; E.C. No. 1.11.1.6) at 37°C for 1 h, respectively. All of the enzymes were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Antimicrobial activity of the cell-free supernatant was assessed at 5, 10, 20, and 30 days after storage at 4, 25, and 37°C, respectively. All measurements were conducted in triplicate.

Purification of Bacteriocin

Lactobacillus acidophilus GP1B was incubated in MRS broth at 37°C for 18 h and the cells were removed by centrifugation (8,000 ×g, 10 min, 4°C). After the supernatant was treated with ammonium sulfate to 40% saturation, the precipitates were collected by centrifugation at 6,000 ×g for 20 min (at 4°C). The precipitate was resuspended in a 2-(4-morpholino)-ethane sulfonic acid (Fisher Biotech, U.S.A.) buffer (50 mM, pH 7.0) and was then dialyzed overnight at 4°C with stirring using dialysis tubing (Mw cutoff: 1,000 Da; Spectrum, CA, U.S.A.). The dialysed sample was then applied to a hydrophobic interaction column (2.8×20 cm) using Octyl sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden) linked to an FPLC system (Biologic system, Bio-Rad, U.S.A.). Prior to the application of the sample, the column was equilibrated with at least two column volumes at binding conditions until a stable baseline had been established. The binding buffer used was 50 mM phosphate buffer containing 1.7 M ammonium sulfate (pH 7.0). The column was then eluted with a linear gradient of decreasing ammonium sulfate and the gradient system using distilled water and ethanol (0 to 80%). The fractions (8 ml) were monitored by absorbance at 280 nm and then assayed for bacteriocin activity. Active fractions from the octyl sepharose column were pooled and applied to a C_{18} Sep-Pak cartridge (Waters Co., Milford, MA, U.S.A.), which was activated in accordance with the manufacturer's specifications. After elution with methanol in the step concentrations of 20, 40, 60, and 80%, the bacteriocin activity was assayed, respectively. The active fractions obtained were then lyophilized and applied to high-performance liquid chromatography (HPLC) gel filtration (Pharmacia SMART system) using Superdex Peptide PC3.2/30 (Pharmacia Biotech AB, Sweden). The effluents, which were monitored at 214 nm, were then eluted using 40% acetonitrile-0.1% TFA at a flow rate of 0.05 ml/min and assayed for bacteriocin activity. The active fractions were then pooled and subjected again to HPLC gel filtration.

Tricine SDS-PAGE, Mass Spectrometry, and N-Terminal Amino Acid Sequence

The active fraction purified from the HPLC gel filtration was lyophilized and electrophoresed by tricine SDS-PAGE [35]. Electrophoresis was conducted using a Mini-PROTEIN 3 cell (Bio-Rad, Hercules, CA, U.S.A.) at 100 V for 2 h. A duplicate gel was washed three times in sterile water for 30 min, placed onto MRS agar, and overlaid with MRS soft agar (0.8%) inoculated with 1% of *L. delbrueckii* subsp. *lactis* ATCC 4797. The other gel was silver-stained in accordance with the manufacturer's specifications (Bio-Rad). The active fraction was also employed for molecular mass analysis using mass spectrometry (matrix-assisted laser desorption ionization mass spectrometer system, Applied Biosystems, U.S.A.) and the sequence analysis of the N-terminal amino acid using a Procise 491 protein sequencing system (Applied Biosystems). The matrix utilized for mass spectrometry was α-cyano-4-hydroxycinnamic acid, and other experimental conditions were as follows: positive mode, room temperature, and 10-kV accelerating voltage. Approximately 600 μl of active fractions pooled from HPLC (Fig. 4) was applied for the amino acid sequence analysis.

Plasmid Curing

Plasmid curing was accomplished by growing *L. acidophilus* GP1B in MRS broth containing 25 µg/ml EtBr with three consecutive transfers every 24 h. The cells were then diluted and cultured on spread plates. Plates containing 50 to 100 colonies, which were partially picked into MRS broth, respectively, were overlaid with MRS soft agar (0.8%) seeded with sensitive strains (*L. delbrueckii* subsp. *lactis* ATCC 4797) and examined for the absence of inhibition zone. The *L. acidophilus* GP1B variants were then retested for their inability to inhibit the growth of sensitive strains, and their sensitivity to acidocin 1B was examined by adding acidocin 1B (3,200 AU) into 10 ml of MRS broth inoculated with variants (1%) and incubating for 24 h at 37°C.

The plasmids of the wild-type and variants were isolated by the method of O'Sullivan and Klaenhammer [33]. The isolated plasmids were then subjected to agarose gel (0.8%) electrophoresis for profile analysis.

Electroporation

The preparation of electrocompetent cells was performed using modified methods of Wei *et al.* [42]. Thus, stationary phase cultures of *L. acidophilus* ATCC 43121 were inoculated (2%) into MRS broth supplemented with 1% glycine. The cultures were incubated at 37°C without shaking, harvested in early log phase (OD_{600} 0.2 to 0.3), and chilled on ice for 10 min. The cultures were then washed twice in ice-cold washing buffer (5 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.4), and resuspended in ice-cold electroporation buffer (0.9 M sucrose, 3 mM $MgCl_2$, pH 7.4), Prepared electrocompetent cells were used for electroporation within 30 min. Electroporation was performed following the methods of Kim *et al.* [21]. One µl (about 25 ng/µl) of pLA1B extracted from *L. acidophilus* GP1B was mixed with 50 µl of ice-cold competent cells in a disposable Cuvette Plus (inner-electrode gap 0.2 cm) and held on ice for 5 min. This mixture was then exposed to electroporation conditions of 12.5 kV/cm pulse strength, 10 set pulse numbers, and 500-ms pulse intervals using BTX 830 (Genetronics, San Diego, CA, U.S.A.). Following the pulse, the cell suspension was diluted in 1 ml of MRS broth and incubated at 37°C for 3 h. The incubated cell suspension was again incubated in 10 ml of MRS broth containing acidocin 1B (3,200 AU) for 18 h. The bacteriocin activity of transformants was measured, and plasmids from them were isolated after two subcultures in MRS broth.

RESULTS AND DISCUSSION

Inhibitory Spectrum of Antimicrobial Substance

The twelve strains of *L. acidophilus* isolated from various origins were tested for antimicrobial activity against various pathogens and LAB of interest in the food industry as indicator strains (data now shown). Among the strains tested, *Lactobacillus acidophilus* GP1B displayed the broadest spectrum of inhibitory activity against a variety of closely related bacteria in the genus *Lactobacillus* and different pathogens (Table 1), and therefore, it was selected for further study. Protease sensitivity assays demonstrated that the antimicrobial substance produced by *L. acidophilus* GP1B was bacteriocin, since the antimicrobial activity was eliminated completely by treatment with trypsin and pepsin, but insensitive to catalase (Table 2). The bacteriocin in this study was designated as acidocin 1B.

Acidocin 1B exhibited antimicrobial activity against Gram-negative bacteria including *Escherichia coli* O157 ATCC43889, *Pseudomonas aeruginosa* KCCM11321, *Shigella sonnei* KU101, and *Yersinia enterocolitica* KU212. More interestingly, it was effective against *L. monocytogenes*, which induces severe illness, generally referred to as listeriosis, with high mortality rates between 20% and 30%, and is particularly difficult to control in foods [40].

Table 2. Effects of storage, heat, pH, and enzyme treatment on acidocin 1B produced by *Lactobacillus acidophilus* GP1B.

Treatment	Activity (%)[a]
Temperature (°C)/Duration	
4/30 days	67
25/30 days	50
37/30 days	50
65/20 min	100
65/40 min	100
65/60 min	100
95/20 min	100
95/40 min	100
95/60 min	100
121/20 min	42
121/40 min	25
121/60 min	0
pH	
2	33
3	42
4	42
5	50
6	100
7	100
8	50
9	50
10	50
11	33
12	13
Enzymes	
Trypsin	–[b]
Pepsin	–
Catalase	+

[a]Data represent the means of three independent determinations.
[b]+, Inhibited by the supernatant; –, not inhibited.

The inhibitory spectrum of the bacteriocins produced by *L. acidophilus* varies according to the strains: Lactacin B and acidocin J1229 show a narrow activity spectrum [36], whereas acidocin A exhibits a relatively broad activity spectrum against Gram-positive genera, inhibiting some foodborne pathogens such as *Listeria monocytogenes* [16]. In general, the bacteriocin activity of LAB against Gram-negative bacteria is atypical, and only a few LAB bacteriocins with a broad spectrum of activity have been reported [2, 4, 27, 39].

Mode of Action

Acidocin 1B exhibited a bactericidal mode of action on *L. delbrueckii* subsp. *lactis* ATCC 4797, with a progressive reduction of the survivors (Fig. 1), as seen with the majority of the known bacteriocins produced by LAB [34].

Acidocin 1B displayed bactericidal action with a maximum viability loss of about 2 log cycles, which was achieved in less than 60 min when it was added to final activity levels of 160 and 320 AU/ml. Fig. 2 also shows the kinetics of cell destruction in sensitive strains. Electron microscopy of the acidocin 1B-treated sensitive cells corroborated that the acidocin 1B induced cell lysis. This

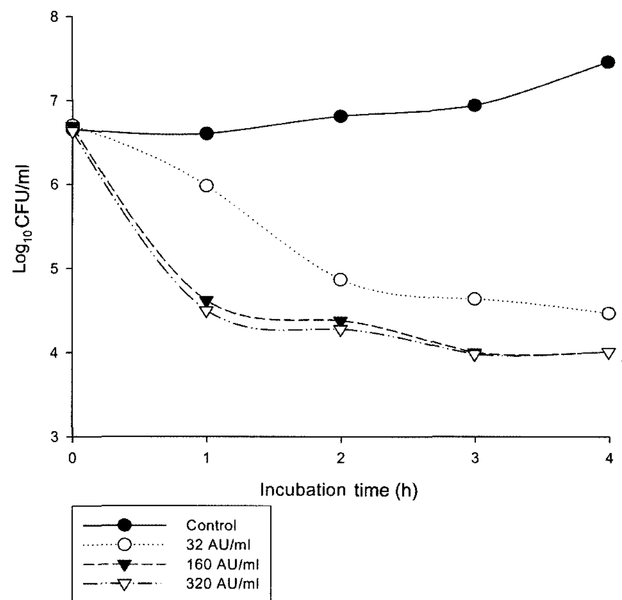

Fig. 1. Survival curves of *Lactobacillus delbrueckii* subsp. *lactis* 4797 in MRS broth.
The acidocin 1B produced by *Lactobacillus acidophilus* GP1B was added respectively to growing cultures to a final activity of 32 AU/ml (○), 160 AU/ml (▼), and 320 AU/ml (▽).

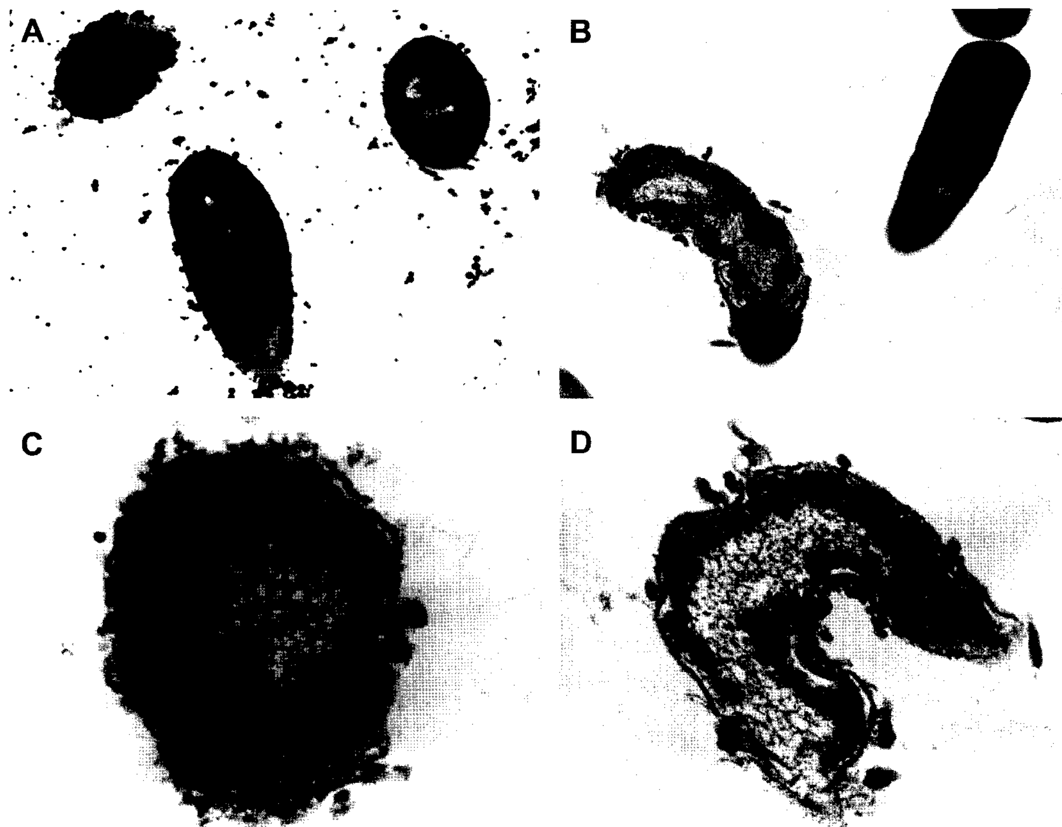

Fig. 2. Transmission electron photomicrographs of *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 after the addition of cell-free supernatant of *L. acidophilus* GP1B to a final activity of 1,600 AU/ml and then incubated for 1 h at 37°C.
A and **B**, Vesiculization of protoplasm and damaged cell wall; **C** and **D**, disintegrated cell with loss of the protoplasmic material through damaged cell wall.

was apparently caused by changes in the structure of the cell wall, resulting in the rupture of the cells in several places (Figs. 2A and 2B) and the escape of cell contents (Figs. 2C and 2D). The cell wall ultimately disintegrated, leaving debris that was apparently responsible for the granular appearance of the observation field.

The bactericidal effect of the bacteriocin produced by the *Lactobacillus* strain is generally accompanied by bacterial lysis. Bacteriocin can inhibit or eliminate the growth of target bacteria by affecting the membrane permeability [43] or by interfering with essential cell functions such as DNA replication and translation [41]. The class I and II bacteriocins are believed to form pores in the membranes of target cells through a barrel-stave mechanism, which involves the binding of monomers to the membrane, their insertion into the membrane, and finally their aggregation, leading to the formation of pores surrounding a central core [32]. A variety of antimicrobial peptides exhibit a fair degree of hydrophobicity, which may promote interaction with cell membranes of the indicator strains [11, 20].

Characterization

The acidocin 1B was found to be heat stable, because it lost no activity when heated up to 95°C for 60 min (Table 2). Bacteriocin thermoresistance can be due to either a small and low complexity structure (lacking even a tertiary structure) or a compact globular structure that is stabilized by covalent bonds, as suggested by De Vuyst and Vandamme [9].

The bacteriocins produced by *L. acidophilus*, including acidocin B [37] and acidocin J1229 [36], are highly heat stable, whereas the bacteriocin produced by *L. acidophilus* AC1 [26] and acidophilucin A [38] are found to be heat sensitive and lose their activity when heated at 50°C for 20 min and at 60°C for 10 min, respectively.

Although acidocin 1B was stable under storage of 24 h at pH 6 and 7, and its activity decreased gradually in close vicinity to acid and alkaline pHs, it retained 50% of initial activity at pH 5 and pHs from 8 to 10 and approximately 33% of initial activity even at pH 2 and 11 (Table 2). The maximal solubility and stability of nisin are at around pH 2, and nisin is inactivated at a pH above 8 [14]. Like nisin, the majority of bacteriocins and bacteriocin-like substances produced by LAB have a considerable disadvantage for use as an additive in nonacidic foods [9]. Consequently, acidocin 1B has an interesting potential as a biopreservative for foods at around neutral pH, and a stability behavior similar to acidocin 1B has also been reported for other bacteriocins [23].

Acidocin 1B remained stable for 10-day storage periods at 4°C (data not shown) and retained approximately 67% of initial activity for 30 days. Although the residual activity gradually decreased at close to higher storage temperatures, it retained 50% of initial activity under storage of 30 days

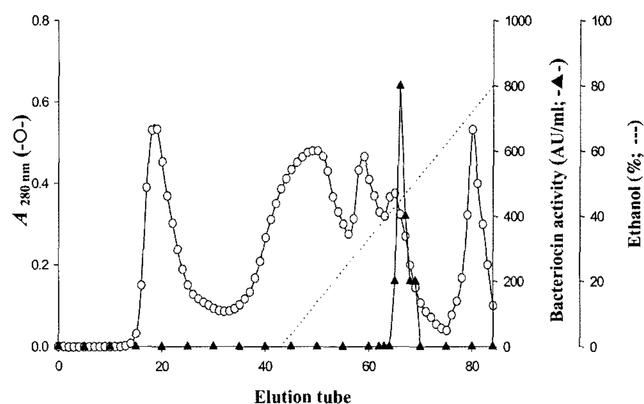

Fig. 3. Elution profile of the ammonium sulfate precipitate of crude acidocin 1B produced by *Lactobacillus acidophilus* 1B on Octyl sepharose CL-4B.

Eight ml of each fraction was monitored at 280 nm and assayed for antimicrobial activity.

at 25°C and 37°C (Table 2). It can be postulated that the gradual decrease of activity may be partially due to the action of unknown proteinases present in the cell-free supernatant.

Purification, Molecular Mass, and N-Terminal Amino Acid Sequence of Acidocin 1B

The majority of the bacteriocin activity was eluted from Octyl sepharose CL-4B at approximately 45 to 50% ethanol (Fig. 3) and then from the C_{18} Sep-Pak cartridge at 60% methanol. The final purification step by HPLC gel filtration yielded a single main peak of the acidocin 1B, and the antimicrobial activity was detected in the same peak (Fig. 4). Tricine SDS-PAGE resulted in a single band with an estimated molecular mass of approximately 3.6 kDa, and its bacteriocin activity was detected as an inhibition

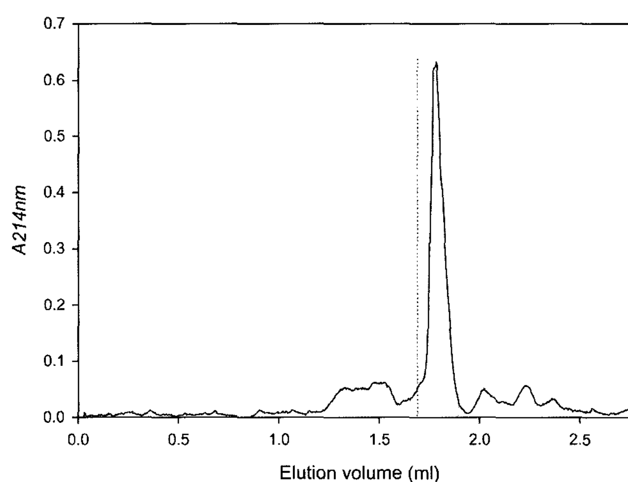

Fig. 4. HPLC gel elution profile of acidocin 1B obtained from an Octyl sepharose CL-4B column.

The effluents were monitored at 214 nm and pooled for the assay of antimicrobial activity. The dotted line indicates the acidocin 1B activity peak.

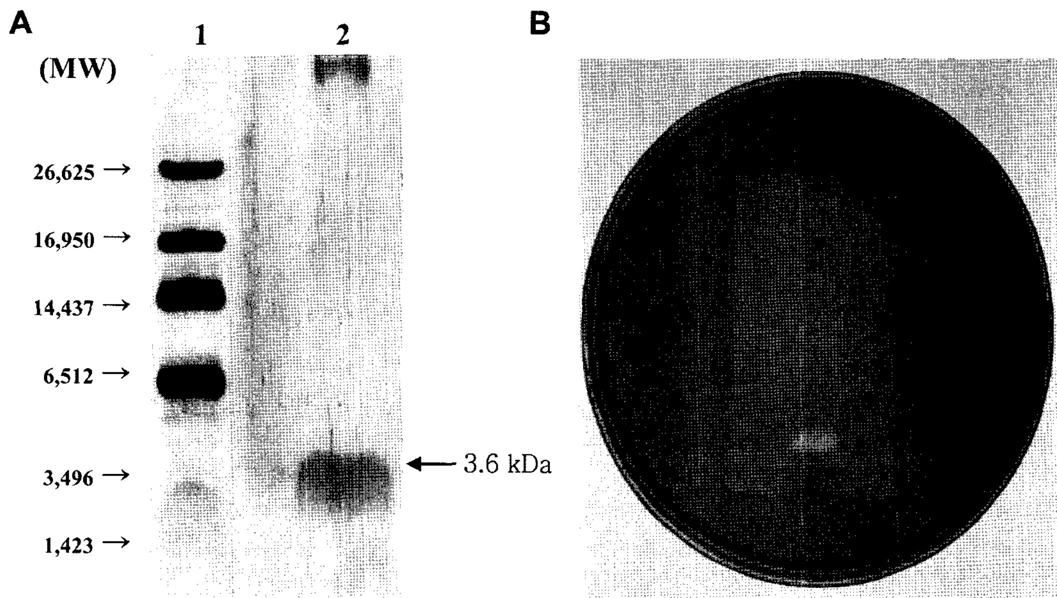

Fig. 5. Tricine SDS-PAGE of acidocin 1B obtained from HPLC gel filtration.
A. Silver-stained gel: Lane 1, polypeptide SDS-PAGE standard (Bio-Rad); lane 2, acidocin 1B activity. **B**. Gel overlaid with MRS soft agar (0.8%) inoculated with cells of *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797.

zone on the agar seeded with a sensitive strain (*L. delbrueckii* ssp. *lactis* ATCC 4797) (Fig. 5). Mass spectrophotometry indicated an estimated molecular mass of 4,214.65 Da (Fig. 6).

This difference in molecular mass between MALDI-mass spectrometry and tricine SDS-PAGE could be attributed to the nonlinear migration of small peptides on SDS-PAGE. Similarly, the molecular mass of gassericin A was estimated by SDS-

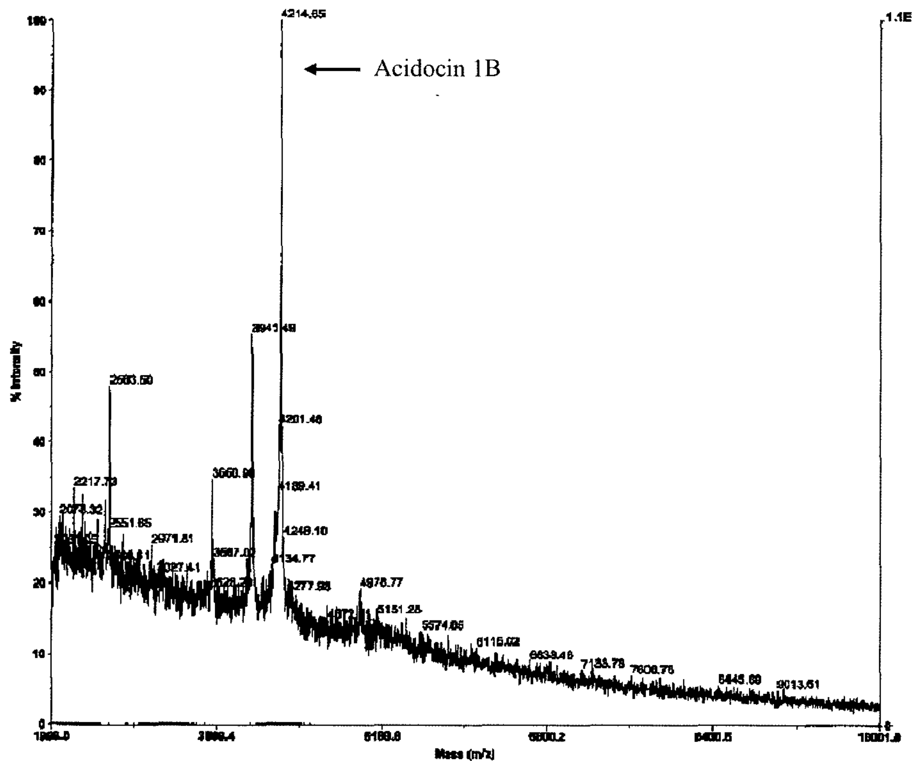

Fig. 6. Mass spectrometric analysis of acidocin 1B purified from HPLC gel filtration.

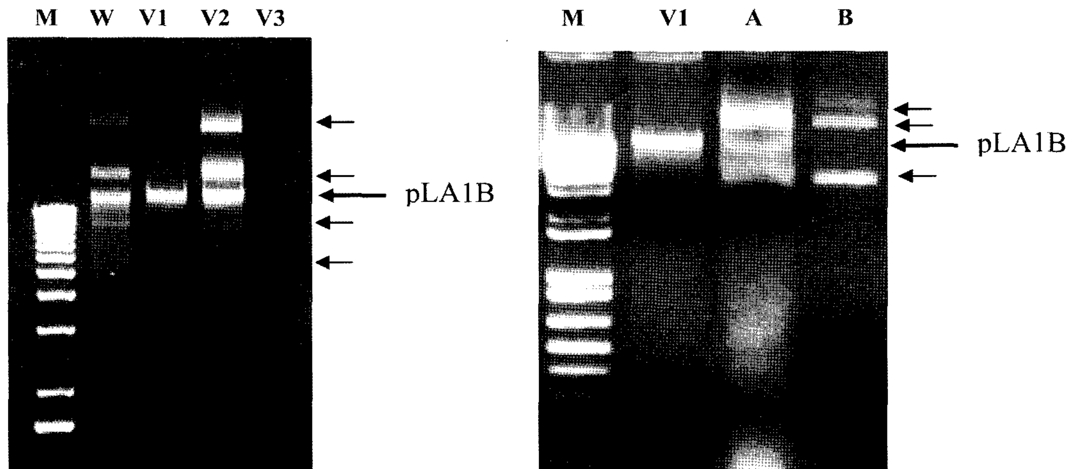

Fig. 7. Agarose gel electrophoresis of plasmids.
M, 1-kb DNA ladder marker (Gibco/BRL, U.S.A.); **W**, wild-type of *L. acidophilus* GP1B; **V1** and **V2**, acidocin 1B-producing variant of *L. acidophilus* GP1B; **V3**, acidocin 1B-nonproducing variant of *L. acidophilus* GP1B. **A**, the transformants of *L. acidophilus* ATCC 43121; **B**, wild-type of *L. acidophilus* ATCC 43121. Each plasmid is indicated by arrows.

PAGE as 3.8 kDa, but the result of mass spectrometry indicated the molecular mass of gassericin as 5,652 Da [18]. The molecular mass of acidocin 1B was smaller than that of other bacteriocins produced by the strains of *L. acidophilus*, such as the bacteriocins (5.4 kDa) from *L. acidophilus* AC1 [26], lactacin B (6.5 kDa) [22], acidocin B (5.8 kDa) [25], acidocin A (6.5 kDa) [16], and acidocin J1229 (6.3 kDa) [36].

The sequence analysis of acidocin 1B identified six consecutive N-terminal amino acid residues: NH_2-Pro-Ala-X-Leu-Met-Tyr-Arg, where X at position 3 indicates a blank cycle in which no amino acid derivative was detected, and the eighth amino acid residue was blocked. The N-terminal region has been suggested to be important for the activity and specificity of bacteriocins. Sequence alignment of many LAB bacteriocins revealed that those in the same class consisted of a highly conserved N-terminal part harboring the consensus sequence, such as the YGNGV motif of class IIa bacteriocins [10]. However, the N-terminal amino acid sequences of acidocin 1B did not exhibit a close homology to any bacteriocins of LAB that have already been reported.

Plasmid Curing

Acidocin 1B-producing strains harbored five plasmids with different molecular masses (Fig. 7). Comparison of the plasmid contents among the wild-type and variant strains indicated that the third largest plasmid DNA of the wild-type strain, designated as pLA1B, was responsible for the production of acidocin 1B in *L. acidophilus* GP1B (Table 3 and Fig. 7). In conjunction with bacteriocin production, pLA1B may include a host immunity-related gene, because the variant V3, which harbored no plasmid, proved to be sensitive to the acidocin 1B. However, there was not difference in growth rate in MRS broth between wild-type and variant strains of *L. acidophilus* GP1B (data not shown).

Plasmid-encoded bacteriocin production and host cell immunity have routinely been described for many Gram-negative and Gram-positive bacteria [6, 7, 28]. Several *Lactobacillus*, *Bifidobacterium*, *Carnobacterium*, and *Pediococcus* strains have been reported to produce bacteriocin, and the trait in all has generally been demonstrated to be plasmid linked [12, 15, 28, 44].

Curing experiments of the Plantaricin KW30-producing strain yielded derivatives that no longer produced the bacteriocin but retained immunity to it [19]. However, for the majority of the nonlantibiotics, the immunity gene codes for a single polypeptide and is located in the vicinity and in the same operon as the structural bacteriocin gene [30].

Table 3. Acidocin 1B production, host immunity, and plasmid type and variants of *Lactobacillus acidophilus* GP1B.

	Wild-type	Variant-V1	Variant-V2	Variant-V3
Acidocin 1B production[a]	+	+	+	–
Host immunity to acidocin 1B[b]	+	+	+	–
Plasmid types	5	1	4	0

[a]+, Produced; –, not produced.
[b]+, Not inhibited by acidocin 1B; –, inhibited.

Electroporation

The optimal technical condition of electroporation for *L. acidophilus* strains, which had already been developed in a previous study [21], was used to transfer the pLA1B into *L. acidophilus* ATCC 43121 that had been proven to be excellent in cholesterol reduction. When the plasmid profile of transformants of *L. acidophilus* ATCC 43121 was compared with that of wild-type strains, a new plasmid (assumed to be pLA1B) was detected in transformants in addition to the plasmids in wild-type strains (Fig. 7).

The transformants of *L. acidophilus* ATCC 43121 exhibited acidocin 1B activity (200 AU/ml) against *L. delbrueckii* subsp. *lactis* ATCC 4797 that wild-type of *L. acidophilus* ATCC 43121 did not inhibit, although their inhibitory activity appeared to be weak, compared with the original acidocin 1B producer *L. acidophilus* GP1B. A lower acidocin 1B activity in transformants suggests that plasmid replication and acidocin 1B production may be highly strain-specific. In this study, the sequential results of plasmid curing and electroporation showed that the genetic determinant for acidocin 1B production and host immunity was a plasmid (pLA1B).

At present, we are in the process of investigating the nucleotide sequences of pLA1B for molecular characterization of acidocin 1B and immunity-related genes.

Food application of LAB bacteriocins appears to be a good alternative to protect foods against pathogenic microorganisms. Therefore, acidocin 1B and its producer strain, which can endow broad antimicrobial activity against a variety of pathogens, may have potential value as a biopreservative in the various food systems during long storage periods at different preservatory temperatures and pH conditions, and may also help our further understanding of the bacteriocins produced by *L. acidophilus* strains.

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