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RESEARCH NOTE

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Antimicrobial Substance against *Escherichia coli* O157:H7 Produced by *Lactobacillus amylovorus* ME1

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Abstract A lactic acid bacterium producing an antimicrobial substance against *Escherichia coli* O157:H7 was isolated from raw milk and identified as *Lactobacillus amylovorus* ME-1. In addition to *E. coli* O157:H7, the antimicrobial substance also inhibited the growth of *Bacillus cereus, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus pyrogenes, and Yersinia enterocolitica*. The antimicrobial substance was stable at pH 2-12 and 121°C for 15 min and insensitive to proteinase K, protease, amylase, and catalase. Purification of the antimicrobial substance was conducted through methanol and acetonitrile/ethylacetate extraction, ultrafiltration with a 500 Da cutoff, thin layer chromatography (TLC) with silicagel 60, and high performance liquid chromatography (HPLC) with a C_{18} reverse phase column. The λ_{max} of the purified antimicrobial substance was determined as 192 nm by ultra violet (UV) scanning, while the molecular weight was estimated as 453 Da based on the mass spectrum. Accordingly, the current results suggest that the antimicrobial substance from the *L. amylovorus* ME-1 was not a bacteriocin, but rather a new non-proteinaceous substance distinct from acidophilin, acidolin, diacetyl, and reuterin.

Keywords: antimicrobial substance, *Lactobacillus amylovorus*, *Escherichia coli* O157:H7

Introduction

Lactic acid bacteria are widely distributed in many fermented foods, such as yoghurt, cheese, fermented milk, and the intestinal and virginal tract of humans and animals. In addition to the production of fermented foods, lactic acid bacteria play an important role against pathogenic microorganisms (1,2). The antimicrobial activities of lactic acid bacteria are mainly due to organic acids, various kinds of bacteriocin (3), acidophilin (4), acidolin (5), diacetyl (6), the lactoperoxidase system (7), hydrogen peroxide (8), and reuterin (9). Among these antimicrobial substances, the proteinaceous antimicrobial substance, bacteriocin is mostly produced by lactic acid bacteria and has been extensively studied for its use as a biological food preservative recently. However the study of non-proteinaceous antimicrobial substances produced by lactic acid bacteria has not been considered in comparison with bacteriocins. As such, the current study describes the isolation, purification, and characterization of the non-proteinaceous antimicrobial substance produced by a *Lactobacillus* sp. isolated from raw milk.

Materials and Methods

Isolation of antimicrobial lactic acid bacteria Samples from various sources were homogenized and streaked on a MRS agar (Merck, Darmstadt, Germany), Streptococcus selective agar (Merck), and Rogosa agar (Merck). After incubation at 37°C, the colonies on the agar plates were transferred to an Elliker broth (Difco Lab., Detroit, MI, USA) containing bromocresol purple and further incubated at 37°C. Finally, a yellow colored culture was selected and assayed for its antimicrobial activity against *Escherichia coli* O157:H7.

Culture of isolate and indicator strain The isolate, *Lactobacillus* sp. ME-1, was cultured in a MRS broth (Merck) with a 2%(v/v) inoculum for 36 hr at 37°C. The indicator strains, *Bacillus cereus* ATCC11778, *E. coli* O157:H7 ATCC35150, *Pseudomonas aeruginosa* ATCC27853, and *Salmonella typhimurium* ATCC19585 were all cultured in a nutrient broth (Difco Lab.) at 37°C, *Listeria monocytogenes* ATCC15313, *Staphylococcus aureus* ATCC25923, *Streptococcus agalactiae* ATCC14928, *Streptococcus pyrogenes* ATCC19615, and *Yersinia enterocolitica* ATCC27729 were cultured in a brain heart infusion (BHI, Difco Lab.) at 37°C.

Measurement of antimicrobial activity The antimicrobial activity was measured using the paper disk method and expressed as 1 arbitrary unit (AU) when the area of the clear zone without the paper disk area was 1 mm^2.

Identification of isolate The isolate was identified by Gram-staining, catalase test, motility test, and carbohydrate fermentation test using an API 50 CHL kit (bioMerieux, Marey l'Etoile, France). Furthermore 16S ribosomal RNA sequencing was also carried out.

Enzyme sensitivity of the antimicrobial substance Proteinase K (Sigma-Alrich Chem., St. Louis, MO, USA), protease (Sigma-Alrich Chem.), α-amylase (Sigma-Alrich

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Chem.), and catalase (Sigma-Alrich Chem.) were all dissolved in a 50 mM phosphate buffer (pH 7.0) at 500 unit/mL and mixed with the crude antimicrobial substance. After incubation for 2 hr at 37°C, the antimicrobial activity of each treated sample was measured against *E. coli* O157:H7.

Stability of antimicrobial substance at various temperatures and pHs The heat stability of the antimicrobial substance was tested after 1 hr of treatment at 100°C and 15 min at 121°C. The pH stability was tested after 24 hr of treatment with the antimicrobial substance in each pH solution preadjusted with 0.1 N HCl and NaOH. After the heat and pH treatment, the antimicrobial activity of each treated sample was measured against *E. coli* O157:H7.

Purification of antimicrobial substance The antimicrobial substance was purified from cultures of *Lactobacillus* sp. ME-1 grown in a MRS broth at 37°C to the late exponential phase. The purification was performed according to a 5-step procedure: (i) methanol extraction, (ii) acetonitrile: ethylacetate (1:1) extraction, (iii) ultrafiltration (Amicon, 500 Da cutoff), (iv) thin layer chromatography (TLC, silicagel 60), and (v) high performance liquid chromatography (HPLC, C_{18} reverse phase).

The culture supernatant was obtained by centrifugation at 10,000×g for 15 min at 4°C. The supernatant was concentrated at 45°C using a rotary vacuum evaporator. The resulting concentrate was then subjected to extraction with methanol and acetonitrile:ethylacetate (1:1). The extract was ultrafiltered through a 500 Da cutoff membrane filter (Amicon Corp., Lexiton, MA, USA). The ultrafiltrate was then applied to a TLC plate (silicagel 60, Merck) and developed with butanol : methanol : water (4:1:2). After development, the TLC plate was examined under 254 nm of ultra violet (UV) light and each spot detected on the TLC plate extracted separately with methanol. The methanol extract was evaporated to remove the methanol, then each residue was dissolved in water and the antimicrobial activity was measured. In addition, another TLC plate was also stained with a bromocresol green solution [0.03%(w/v) in 80%(v/v) methanol] to identify lactic acid. The active fraction obtained from the TLC was further purified through HPLC (Jasco, Tokyo, Japan) with C_{18} reverse phase column, where the mobile phase was water and the flow rate was 0.3 mL/min.

Analysis of UV spectrum and mass spectrum (MS) The purified antimicrobial substance was scanned from a wavelength of 300 to 190 nm using UV-visible recording spectrophotometer (Shimadzu, Kyoto, Japan) and the maximum absorbance wave length was examined. The MS was also analyzed using a Platform II LC-MS (Micromass, Manchester, UK).

Results and Discussion

Isolation and identification of *Lactobacillus* sp. ME-1 From the various sources, 7,399 lactic acid bacteria were isolated and 40 strains exhibited an antimicrobial activity against *E. coli* O157:H7. Among these antimicrobial strains, ME-1 isolated from raw milk showed the strongest inhibition against *E. coli* O157:H7. The isolate was Gram-positive, rod shaped, a facultative anaerobe, catalase negative, non-motile, and non-spore former. Plus, a carbohydrate fermentation test (data not shown) and 16S ribosomal DNA sequencing revealed the isolate to be a *Lactobacillus* sp. (Fig. 1).

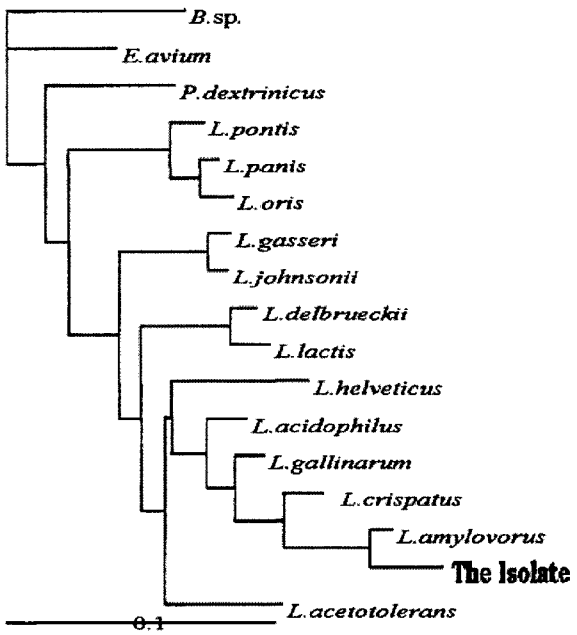

Fig. 1. The phylogenetic tree of 16S rDNA genes of genus, *Lactobacillus*.

Table 1. Enzyme sensitivity and stability of temperature and pH of antimicrobial substance

Treatment	Activity (AU)
Control	163.3
Protease	160.7
Proteinase K	163.2
Amylase	161.5
Catalase	165.6
Heat (100°C, 60 min)	163.5
Heat (121°C, 15 min)	162.7
pH 2 (25°C, 24 hr)	161.1
pH 12 (25°C, 24 hr)	163.2

Characteristics of antimicrobial substance An enzyme sensitivity test of the antimicrobial substance was carried out with proteinase K, protease, α-amylase, and catalase, plus the temperature and pH stability were also tested. The antimicrobial substance was insensitive to all the enzymes tested, stable at a pH range from 2 to 12, and no loss of antimicrobial activity was observed for 1 hr of incubation at 100°C and 15 min at 121°C (Table 1). Shahani *et al.* (4) previously reported that the non-proteinaceuos antimicrobial substance produced by *L. acidophilus* was unstable at an alkaline pH, while Brughton (10) reported that the proteinaceous antimicrobial substance, nisin, was inactivated by heat treatment at 121°C for 15 min. However, the antimicrobial substance in the current study was very

Table 2. Antimicrobial spectrum of the ME1 isolated from raw milk

Gram-positive strain	
Bacillus cereus ATCC11778	+
Listeria monocytogenes ATCC15313	+
Staphylococcus aureus ATCC25923	+
Streptococcus agalactiae ATCC14928	+
Streptococcus pyrogenes ATCC19615	+
Gram-negative strain	
Escherichia coli O157:H7 ATCC35150	+
Pseudomonas aeruginosa ATCC27853	+
Salmonella typhimurium ATCC19585	+
Yersinia enterocolitica ATCC27729	+

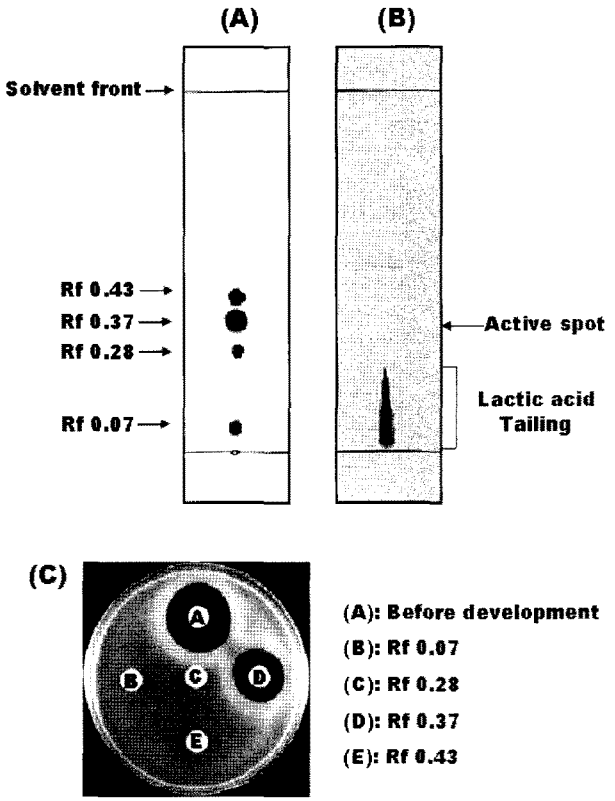

Fig. 2. TLC pattern and the antimicrobial activity of each spot. A, UV absorption spot at 254 nm; B, bromocresol green staining; C, antimicrobial activity against *E. coli* O157:H7.

stable against pH and heat, plus the substance was not proteinaceous in nature, as the antimicrobial activity was not due to hydrogen peroxide.

Antimicrobial spectrum of antimicrobial substance Table 2 shows the antimicrobial spectrum of the antimicrobial substance against Gram-positive and Gram-negative bacteria. The antimicrobial substance inhibited the growth of *E. coli* O157:H7. Also inhibited *B. cereus, L. monocytogenes, S. aureus, S. agalactiae, S. pyrogenes, P. aeruginosa, S. typhimurium, and Y. enterocolitica.* These results are similar to acidophilin (4, 11) and acidolin (5). However, the antimicrobial spectrum of the current substance was broader than that of the bacteriocin produced by *L. acidophilus* (12-14).

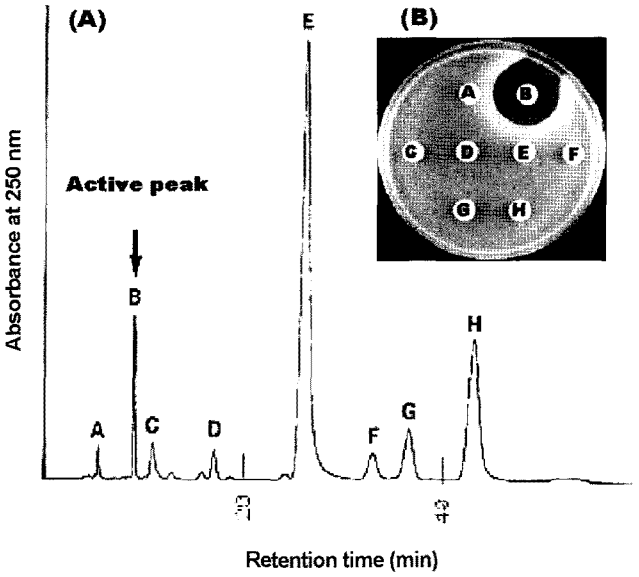

Fig. 3. HPLC chromatogram and the antimicrobial activity of each peak. A, HPLC chromatogram; B, antimicrobial activity against *E. coli* O157:H7.

Purification of antimicrobial substance For the purification of the antimicrobial substance, the culture supernatant of *Lactobacillus* sp. ME-1 was extracted with methanol and acetonitrile:ethylacetate, and then concentrated by evaporation. This substance was then dissolved in water and ultrafiltrated with a 500 Da cutoff. The ultrafiltrate was concentrated and applied to a silicagel 60 TLC plate and developed with a solution of butanol : methanol : water (4:1:2). As shown in Fig. 2A, 4 spots were detected under a UV light of 254 nm and the Rf values were 0.07, 0.28, 0.37, and 0.43, respectively. Antimicrobial activity was only exhibited by the Rf 0.37 spot (Fig. 2C). Another plate stained with a bromocresol green solution (Fig. 2B) showed that lactic acid was tailed from Rf 0.05 to 0.25, indicating that the antimicrobial activity was independent of lactic acid. The TLC was repeated to obtain a large amount of the antimicrobial substance, which was then analyzed with HPLC. On the HPLC chromatogram (Fig. 3A), 8 peaks were detected and each peak was individually fractionated and bioassayed. Only peak B exhibited antimicrobial activity (Fig. 3B) and this fraction showed a single peak on the HPLC rechromatogram. Therefore, it was concluded that the antimicrobial substance was completely purified through the series of purification steps. In a UV spectrum analysis, the maximum absorbance of the purified antimicrobial substance was 192 nm. This data differs from that for acidophilin (4) and acidolin (5), at 280 and 255 nm, respectively. Plus the mass spectrum revealed a mother peak at 453 nm, which also differs from that for acidophilin and acidolin at 284 and 198 nm, respectively. Therefore, the antimicrobial substance in the current study would appear to be a new substance distinct from acidophilin and acidolin.

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