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Screening and Partial Purification of Bacteriocins by Strains of *Lactobacillus acidophilus* Isolated from Human Origin

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인체에서 분리된 *Lactobacillus acidophilus*가 생산하는 박테리오신의 선별과 정제

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ABSTART

Lactobacillus acidophilus 223, 606, and NCFM-F among 21 isolated from fecal contents of humans demonstrated inhibitory activity attributed to bacteriocin(s). The bacteriocin(s) were heat stable and nondialyzable proteinous compounds and exhibited narrow inhibitory spectra of activity. Neither hydrogen peroxide nor pH were responsible for inhibitory action. All of the producer strains were resistant to their own bacteriocin(s). The bacteriocin(s) were purified by ammonium sulfate precipitation, gel chromatography and ion exchange chromatography for further characterization. The bacteriocin(s) of human origin exhibited similar characteristics.

I. INTRODUCTION

Antagonistic effects produced by lactobacilli toward other organisms may play an important role in maintaining a proper microbial balance in intestinal tract (6) and preserving certain foods (1). Lactic acid bacteria produce a number of inhibitory substances (3). The inhibitory factors produced by lactic acid bacteria include organic acid, hydrogen peroxide, and bacteriocins. Bacteriocins are proteins or protein related complexes with bactericidal mode of action directed against species that are usually closely related to the producer bacterium (7). A number of lactobacilli including strains of *Lactobacillus acidophilus* have been shown to produce bacteriocins or bacteriocin-like inhibitory substances (4). Most bacteriocins are heat stable and sensitive to certain proteolytic enzymes.

The objectives of this study were: 1) to assay the bacteriocin activity produced by various strains of *Lactobacillus acidophilus* isolated from intestinal source of human; 2) to isolate the bacteriocin(s) and to investigate their

properties to determine whether there were differences in bacteriocins produced by various strains of *L. acidophilus*.

Ⅱ. MATERIALS AND METHODS

1. Source and maintenance of cultures

The cultures used in this study were obtained from the stock culture collection from the Dairy Food Microbiology Laboratory, Department of Animal Science at the Oklahoma State University, Stillwater. All cultures of lactobacilli were maintained by subculturing in lactobacilli MRS broth (DIFCO laboratories, Detroit, MI) using 1% inocula and 18 to 20h incubation at 37℃.

2. Preparation of cell free spent broth

Cells of L. acidophilus were removed by centrifugation (8,000×g, 10 min.) from broth cultures which had been grown at 37℃ for 18 h. The supernatant fluids (spent broths) were collected, adjusted to pH 6.5 with 10 *N* NaOH and filtered through sterile 0.45 ㎛ acrodisc filters (Gelman, Ann Arbor, MI) into sterile screw cap test tubes. The cell free spent broths were stored at 1℃.

3. Screening for bacteriocin production

Ten microliter portions of spent broths from producer cultures were aseptically placed onto the surface of an agar medium seeded with a culture sensitive to bacteriocins. The petri dishes were incubated upright for 24 h at 37℃. The presence of inhibitory material in the spent broth samples was indicated by clear inhibitory zones on the agar.

4. Effect of heat, catalase, and proteases on inhibitory action

The inhibitory samples of spent broth were heated at 121℃ for 15 min, cooled to room temperature and assayed for inhibitory activity by the agar plate assay to determine the effect of heat on the inhibitory activity. To ascertain whether or not the inhibitory activity was due to hydrogen peroxide or protein, the inhibitory spent broths were treated as described by Ferreira and Gilliland (2) with the following enzymes at the indicated pH values : A) Catalase (pH 6.0; E.C. NO. Ⅰ.Ⅱ. Ⅰ 6) from bovine liver, B) Trypsin (pH 8.0; Type Ⅱ, crude), and C) Pepsin (pH 3.0; E.C. NO. 3.4.23. Ⅰ).

5. Purification of bacteriocins

To purify the bacterocins, the proper amount of ammonium sulfate (Sigma) was added slowly to spent broth from cultures that produced bacteriocin to make the solution 50% saturated with ammonium sulfate. The precipitated fraction was recovered by centrifugation at 10,000×g for 20 min at 1℃. The collected samples were applied on Sephadex G-200 gel permeation chromatography for isolation of the bacteriocin activity. The eluent was monitored for absorbance at 280 nm. and assayed for bacteriocin activity. The active fraction from Sephadex G-200 was further purified on DE 52 anion exchange cellulose (Whatman, Clifton, NJ) column. The activity was eluted with a linear gradient from 0 to 1.5 *M* NaCl. Fractions (4 ml) were collected and measured for absorbance at 280 nm. and assayed for bacteriocin activity.

6. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS was carried out by the method of Laemmli (5) using a Mini-Protein slab cell (Bio-Rad, Richmond, CA). PAGE was conducted at a constant voltage of 200 V for 50 to 60 min. Gels were stained with coomassie brilliant blue (Bio-Rad).

Ⅲ. RESULTS AND DISCUSSION

A total of 21 strains of *L. acidophilus* isolated from fecal contents of humans were screened for the production of inhibitory activity (Table 1). Among these *L. acidophilus* NCFM-M exhibited inhibitory activity against all indicator strains whereas *L. acidophilus* 223 and 606 showed inhibitory activity only toward *L. delbrueckii* subsp. *lactis* 4797.

The sensitivity of each nonproducer strain to inhibitory action also was tested against three strains that produced inhibitory activity against the indicator culture in oder to determine the inhibitory spectrum (Table 2). *L. acidophilus* 223 and 606 inhibited 2 and 6 strains of *L. acidophilus* from human origin, respectively, whereas they inhibited none of strains from pig, *L. acidophilus* NCFM-M exhibited the inhibitory activity toward all strains tested. This shows variations in the inhibitory spectra of the inhibitor(s) produced by these strains. None of those that produced inhibitory activity were sensitive to their own inhibitor or inhibitor(s) produced by other strains.

The inhibitory action of the spent broth was destroyed by treatment with either trypsin or pepsine. Treatment with catalase had no effect on the inhibition. Heating the inhibitory broths for 15 min at 121℃ also had no effect on the inhibitory activity. These results demonstrated that the inhibitory substance(s) produced by three strains of *L. acidophilus* isolated from human origin were heat stable proteinous compounds. Neither hydrogen peroxide nor acid were responsible for inhibitory

Table 1. Screening of *Lactobacillus acidophilus* of human origin for production of inhibitory activity

Test strains	Indicator cultures		
	La-1[1]	NCFM-F[2]	4797[3]
107	−[4]	−	−
223	−	−	+
606	−	−	+
4356	−	−	−
4962	−	−	−
HM2	−	−	−
H35	−	−	−
La 1	−	−	−
La 2	−	−	−
La 3	−	−	−
La 4	−	−	−
La 5	−	−	−
La 8	−	−	−
La 11	−	−	−
La 12	−	−	−
La 14	−	−	−
La 15	−	−	−
La 20	−	−	−
NCFM-L	−	−	−
NCFM-M	+	+	+
NCFM-F	−	−	−

[1] *Lactobacillus acidophilus* La 1; [2] *L. acidophilus* NCFM-F; [3] *L. delbrueckii* subsp. *lactis* 4797; [4] + indicates the presence of zone inhibition and − indicates the absence of zone of inhibition of the agar seeded with each indicator cultures.

Table 2. The sensitivity of non-bacteriocin producing strains of *Lactobacillus acidophilus* to the inhibitory activity of producer strains of human origins

Origin	Nonproducer strains	Producer strains		
		223[1]	606[2]	NCFM-M[3]
Human	La 1	−[4]	−	+
	La 2	−	−	+
	La 5	−	+	+
	La 8	−	+	+
	La 11	−	+	+
	La 12	−	−	+
	La 14	−	+	+
	La 15	+	+	+
	La 20	+	+	+
	NCFM-F	−	−	+
Pig	149-C	ND[5]	ND	ND
	C1-3	−	−	+
	C1-5	−	−	+
	C1-6	−	−	+
	C1-5	−	−	+

[1] *Lactobacillus acidophilus* 223; [2] *L. acidophilus* 606; [3] *L. acidophilus* NCFM-M; [4] + indicates the presence of zone inhibition and − indicates the absence of zone of inhibition of the agar seeded with each indicator cultures; [5] Not determined.

activity against indicator cultures. Additional tests revealed that the inhibitory action was restricted to closely related species. The inhibitory activity of spent broth was non-dialyzable. These results show the characteristics of the inhibitor(s) to be consistent to the definiton of bacteriocins by Tagg et al. (7).

Bacteriocins produced by 3 strains were stable through purification steps, and subjected to further purification. Gel chromatography of the ammonium sulfate precipitate fraction of spent broth from *L. acidophilus* 606 in the presence of 0.1% SDS resulted in the elution of a single peak of inhibitory activity corresponding to a single absorbance peak (Fig. 1). In addition, larger portion of material absorbing light at 280 nm was fractionated as a second peak in which no inhibitory activity was detected. The elution profiles of inhibitory activity of *L. acidophilus* 223 and NCFM-M were similar to that of *L. acidophilus* 606.

The inhibitory fraction from *L. acidophilus* 606 obtained by gel filtration was subjected to anion exchange column chromatography. Two absorbance peaks at 280 nm were detected. First peak was eluted before the sodium chloride gradient was applied. The inhibitory activity was eluted with second absorbance peak at A 280 nm which corresponded to 0.4 M sodium chloride (Fig. 2). The elution profiles of *L. acidophilus* 223 and NCFM-M on ion exchange chromatography exhibited similar elution patterns.

The active fractions from the second peak of ion exchange columns were separated by electrophoresis. A 72 KDa band appeared in the active fractions from all 3 strains of *L. acidophilus*. Attempts to show inhibition zone on the indicator lawn of agar by the bands from gels were not successful, possibly because of lack of sufficient inhibitory substances to produce inhibitory zone on the agar. Because of the presence of only one band in common, it appears that this band may have been responsible for the bacteriocin activity.

Great diversity in characteristics and properties of bacteriocins produced by lactobacilli have been reported. In this study, however, the results indicated that the bacteriocins produced by three strains of *L. acidophilus* isolated human exhibited similar characteristics.

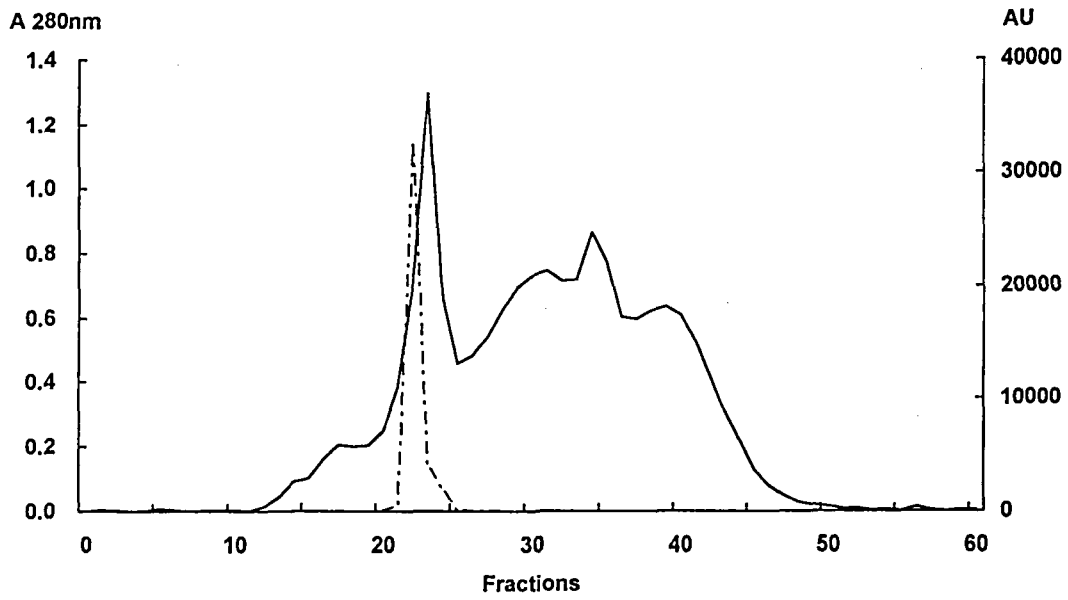

Fig. 1. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* 606 on Sephadex G-200.

Each 5 ml fraction was monitored at 280 nm(—) and was assayed for inhibitory activity(·····).

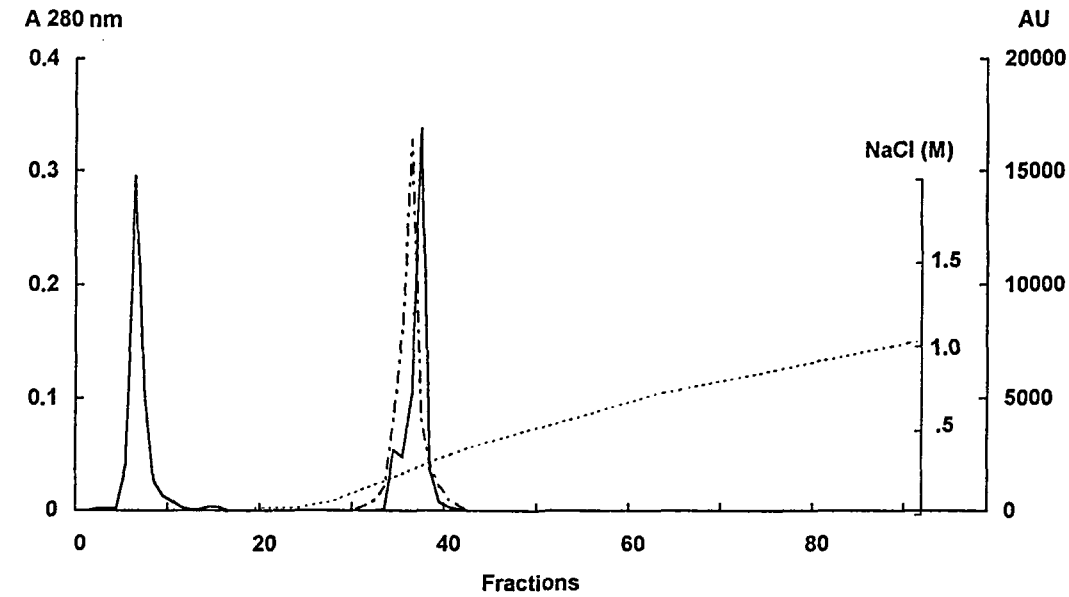

Fig. 2. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* 606 on DE 52 anion exchange.

The activity was eluted with 0 to 1.5 M NaCl(·····). Each 4 ml fraction was monitored at 280 nm(—) and was assayed for inhibitory activity(– –).

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