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Antibacterial Effect of Antibacterial Substance Produced by *Lactobacillus amylovorus* IMC-1 against Food Spoilage Bacteria

Jong-Soo Mok†, Poong-Ho Kim, Hyen-Duk Yu, Ji-Hoe Kim*, Hee-Jung Lee* and Young-Mog Kim**

Tongyoung Laboratory, South Sea Fisheries Research Institute, National Fisheries Research & Development Institute, Tongyoung 650-940, Korea

**Sanitation & Processing Research Division, National Fisheries Research & Development Institute, Pusan 619-900, Korea*

***Department Food Science & Technology, Pukyong National University, Pusan 608-737, Korea*

Lactobacillus amylovorus IMC-1에 의해서 생산되는 항균성 물질의 식품 오염세균에 대한 항균 효과

목종수† · 김풍호 · 유현덕 · 김지회* · 이희정* · 김영목**

*국립수산진흥원 남해수산연구소 통영분소, *국립수산진흥원 위생가공연구실, **부경대학교 식품공학과*

ABSTRACT – To develop a lactic starter to produce antimicrobial substance for inhibiting the growth of a variety of foodborne spoilage bacteria in fermented foods, we investigated the antibacterial effect of the antibacterial substance, produced by *Lactobacillus amylovorus* IMC-1, against foodborne spoilage strains, and its sensitivity on the treatment of proteolytic enzymes. *L. amylovorus* IMC-1, which was isolated from a traditional cheese in Inner Mongolia, produced a maximum amount of antibacterial substance in the skim milk medium after 72 h incubation at 37°C, and further incubation resulted in the same activity. The substance obtained from gel filtration inhibited all strains used such as *Bacillus subtilis* IFO 3025, *Staphylococcus aureus* IAM 1011, *Listeria monocytogenes* VTU 206, *Escherichia coli* RB, and *Pseudomonas fragi* IFO 3458 at the concentration of 20 units/ml. This substance was found to show bactericidal action against *B. subtilis*, *E. coli*, and *Ps. fragi*, and bacteriostatic activity against both *Staph. aureus* and *L. monocytogenes*. The bactericidal action was due to cellular lysis. The substance is not organic acid, hydrogen peroxide and proteinaceous compound.

Key words □ *Lactobacillus amylovorus*, Antibacterial substance, Spoilage bacteria, Antibacterial effect, Lactic starter

Many lactic acid bacteria have important roles in the production of fermented foods, and some of these bacteria have been shown to be capable of inhibiting the growth of a wide variety of food spoilage organisms.[8,14,17] Although lactic acid bacteria are now also recognized for human health and nutritional benefits,[3] they are industrially important organisms that are extensively used for the preservation of highly perishable foods of plant or animal origin.[18] The antimicrobial substances produced by these bacteria play an essential role in ensuring the safety and extending the shelf-life of these products. Increasing consumer demand for natural and additive-free products has led to greater interest in the application of natural antimicrobials as food preservatives which could replace or reduce the use of chemical additives.[19]

Other applications of lactic acid bacteria have been directed towards probiotics. Their GRAS (generally recognized as safe) character has encouraged many lines of research to develop new food and probiotic products.[8,10] Probiotics involve the prophylactic use of microorganisms to help protect the host animals and humans from disease.[19] The behavior of some strains as probiotics have

†Author to whom correspondence should be addressed.

been useful at promoting bacterial interference by the production of antimicrobial substances.

Lactobacillus acidophilus (*L. acidophilus*) group lactic acid bacteria (*L. acidophilus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum*, *L. gasseri*, and *L. johnsonii*) are one of the predominant lactobacilli in the normal flora of the human intestine, and are believed to play an important role in controlling undesirable bacteria in the digestive tract.[6,7] One possible effect of *L. acidophilus* group strains in preventing an increasing of pathogenic bacteria in the intestinal tract may be the production of antimicrobial substances. *L. acidophilus* group strains are notably used in acidophilus milk products as a source of dietary lactobacilli.[8,13,16] Therefore, these substances are especially important because of their roles in lactic fermented foods and intestinal tracts.

We have isolated *Lactobacillus amylovorus* IMC-1, an antibacterial substance-producing strain, from Inner Mongolian cheese. This substance was stable following heat treatment, had greater activity at relatively low pH, and showed a wide spectrum of inhibitory activity.[12] To develop a lactic starter to produce antimicrobial substance for inhibiting the growth of a variety of foodborne spoilage bacteria in fermented foods, we investigated the antibacterial effect of the antibacterial substance on foodborne spoilage strains, and its sensitivity on enzyme treatment was determined.

MATERIALS AND METHODS

Bacterial strains and media

Lactobacillus amylovorus IMC-1 (*L. amylovorus* IMC-1), an antibacterial substance-producing strain, has been isolated from a traditional cheese in Inner Mongolia. The producer was maintained in skim milk medium (10% skim milk and 0.5% yeast extract) at 4°C, and was subcultured monthly. The strain was propagated in TYLG broth (1% tryptone, 0.5% yeast extract, 0.5% lactose, 0.5% glucose, 0.1% Tween 80 and 0.02% L-cystein HCl) at 37°C. The indicator strains used in this study were maintained on slant of Mueller Hinton agar (Difco), which was used for antibacterial activity assay, and were subcultured monthly. The indicator strains were inoculated at the 1% level in Mueller Hinton broth (Difco), grown overnight at their optimum growth temperature.

Antibacterial activity assay

Antibacterial activity and activity units were determined by the paper disc assay as described by Mok et al.[12] 0.1 ml of the samples (pH 4.5) was applied to a sterile paper disc (ϕ13 mm) which has been placed on the surface of 5 ml of agar medium seeded with culture of indicator strains. The plates were incubated for 24 h at their optimum growth temperature, and the clear zone surrounding the disc was measured.

A standard curve for the antibacterial substance was designed by a linear relationship between the size of inhibitory zone and the concentration of the substance by using the ethanol soluble fraction adjusted to pH 4.5. One unit was defined as the amount of antibacterial activity required to produce an inhibitory zone of 17.9 mm in diameter when tested by using 0.1 ml of the ethanol soluble fraction against *Pseudomonas fragi* IFO 3458. Units/ml={the diameter of inhibitory zone (mm)−13}/0.49.

Production of antibacterial substance

To examine the acid production and the production of antibacterial substance in the skim milk medium containing 10% skim milk and 0.5% yeast extract, the medium were inoculated with the 1% of *L. amylovorus* IMC-1 and incubated at 37°C. Samples were withdrawn at 0, 24, 48, 72, 96 and 120 h, and the pH values, the acidities, and the antibacterial activities were determined. *Pseudomonas fragi* IFO 3458 (*Ps. fragi*) was used as the indicator strain.

Preparation of crude antibacterial substance

The crude antibacterial substance from *L. amylovorus* IMC-1 was prepared according to the method as shown in Fig. 1. Producer was propagated for 3 days at 37°C in the skim milk medium containing 10% skim milk and 0.5% yeast extract. The culture supernatant was obtained by centrifugation at 12,500 × g for 20 min after adjusting to pH 4.5. Ten volumes of cold ethanol were added to the culture supernatant with constant stirring for 2 h at 4°C, and precipitates were removed by centrifugation at 5,400 × g for 20 min. Ethanol soluble fraction was evaporated at 40°C, and adjusted to 1/5 volumes of culture supernatant with distilled water. Ethanol soluble fraction was applied to gel filtration column (Sephadex G-15, Pharmacia Fine Chemicals, 1.6 × 100 cm) equilibrated with distilled water. The active fractions were collected at the rate of 30 ml/h, and evaporated at 40°C. Re-gel filtration was performed again under the same conditions. The active fractions were readjusted to pH 4.5, concen-

10% Skim Milk containing 0.5% yeast extract
| *Lactobacillus amylovorus* IMC-1
| Incubated at 37℃ for 72 h
Cultured Milk
| Adjusted to pH 4.5
| Centrifuged at 12,500 xg for 20 min
Culture Supernatant
| Added ten volumes of cold ethanol to culture supernatant
| Centrifuged at 5,400 xg for 20 min
| Evaporated at 40℃
| Resuspended with distilled water
Ethanol Soluble Fraction
| Gel filtration by Sephadex G-15
| Concentrated to 1/5 volumes of culture supernatant by evaporation at 40℃
| Adjusted to pH 4.5
Gel Filtrated-Fraction

Fig. 1. Preparation scheme of antibacterial substance produced by *Lactobacillus amylovorus* IMC-1.

trated to 1/5 volumes of culture supernatant by evaporation at 40℃, and used as crude antibacterial substance for inhibitory, bactericidal and lytic activity assay against indicator bacteria.

Bactericidal and lytic effect

Indicator strains such as *Bacillus subtilis* IFO 3025 (*B. subtilis*), *Staphylococcus aureus* IAM 1011 (*Staph. aureus*), *Listeria monocytogenes* VTU 206 (*L. monocytogenes*), *Escherichia coli* RB (*E. coli*), and *Ps. fragi*, were incubated at their optimal growth temperatures in Mueller Hinton broth, the cells were harvested by centrifugation at the early- to mid-logarithmic phase of growth. Pelleted cells were washed twice with sterile 0.1 M NaCl solution (pH 4.5), resuspended in the same solution at an optical density (660 nm) of 0.3, and used as the cell suspensions. After addition of antibacterial substance (20 units/ml) into the cell suspensions, the samples were incubated during 6 h at their optimal growth temperatures. Control used sterile 0.1 M NaCl solution (pH 4.5) without addition of antibacterial substance. At the intervals of 2 h, viable cells were counted on SPC agar (Nissui) plates, and the optical density (660 nm) of the samples were monitored by a spectrophotometer (Hitachi U-1100).

Effect of Enzymes

The crude antibacterial substance (20 units/ml) was treated with enzymes (Sigma) such as, catalase, pepsin, pronase E, proteinase K, chymotrypsin, trypsin, aminopeptidase and carboxypeptidase. This crude substance was adjusted to pH 4.5 and 5.5, and treated for 2 h at 35℃ with each filter sterilized enzymes at a final concentration of 1 mg/ml in 10 mM potassium phosphate buffer (pH 4.5 and pH 5.5, respectively). Enzyme activity was stopped by heating at 90℃ for 5 min. Antibacterial activity remaining after enzyme treatment was assayed with *Ps. fragi* as indicator organism. Controls consisted of enzyme plus buffer and crude substance plus buffer.

RESULTS

Production of antibacterial substance

L. amylovorus IMC-1 was incubated in the skim milk medium during 120 h at 37℃ for the production of antibacterial substance (Fig. 2). The strain produced a detectable amount of the substance in the culture supernatant after 24 h incubation. Its maximum production was achieved after 72 h incubation at 37℃, and further incubation resulted in the same activity.

Inhibitory activity of crude antibacterial substance

The inhibitory activity of crude antibacterial substance was determined against foodborne spoilage bacteria such as *B. subtilis*, *Staph. aureus*, *L. monocytogenes*, *E. coli*, and *Ps. fragi* (Table 1). The substance inhibited all strains used at the concentration of 20 units/ml. However, at the concentration of 10 units/ml, *L. monocytogenes* was not sensitive. Surprisingly, the substance was active against two Gram-negative foodborne spoilage bacteria tested.

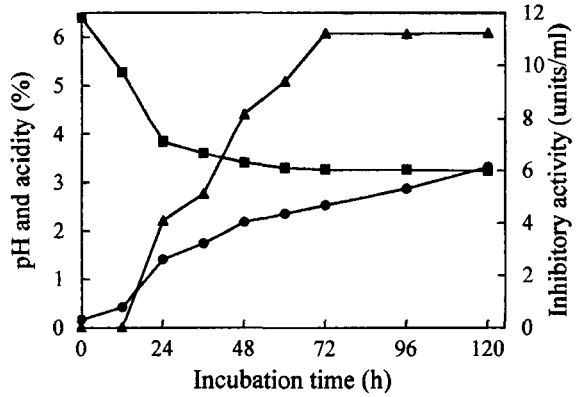

Fig. 2. Changes in pH values (■), acidities (●) and inhibitory activities (▲) during cultivation with *Lactobacillus amylovorus* IMC-1 in skim milk medium at 37℃.

Table 1. Inhibitory activity of antibacterial substance produced by *Lactobacillus amylovorus* IMC-1

Test organisms	Source or reference	Inhibitory zone (mm)* Antibacterial substance (units/ml)		
		30	20	10
Bacillus subtilis IFO 3025	IFO	ND	17.3	15.2
Staphylococcus aureus IAM 1011	IAM	ND	17.5	15.3
Listeria monocytogenes VTU 206	VTU	16.9	15.0	–
Escherichia coli RB	Milk	ND	17.8	15.6
Pseudomonas fragi IFO 3458	IFO	ND	19.5	17.7

*Inhibitory zone was estimated by paper disc assay (ϕ 13 mm) using the gel-filtrated antibacterial substance, the values were expressed as the mean of three experiments. ND, not determined. IFO, Institute for Fermentation, Osaka, Japan; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; VTU, Department of Biomedical Science, Faculty of Agriculture, University of Tokyo, Tokyo, Japan.

Bactericidal and lytic effect of crude antibacterial substance

Effects of antibacterial substance on the viabilities of indicator strains, such as *B. subtilis*, *Staph. aureus*, *L. monocytogenes*, *E. coli*, and *Ps. fragi*, were determined in Table 2. The addition of the inhibitor resulted in above 99% reduction of the viable cell counts of *B. subtilis*, *E. coli*, and *Ps. fragi* compared with control after 6 h incubation. In addition, the optical density of the same strains gradually decreased. These results showed that their death was due to cellular lysis. However, the presence of antibacterial substance did not showed any change in the viable cell counts and the optical density of both *Staph. aureus* and *L. monocytogenes* compared with control during 6 h incubation.

Therefore, the substance was found to show bactericidal action against *B. subtilis*, *E. coli*, and *Ps. fragi*, and bacteriostatic activity against both *Staph. aureus* and *L. monocytogenes*.

Table 2. Bactericidal and lytic activity of antibacterial substance produced by *Lactobacillus amylovorus* IMC-1 against several indicator bacteria

Indicator strains	Bactericidal action	Cellular lysis
Bacillus subtilis IFO 3025	+[1)]	+[2)]
Staphylococcus aureus IAM 1011	–	–
Listeria monocytogenes VTU 206	–	–
Escherichia coli RB	+	+
Pseudomonas fragi IFO 3458	+	+

20 units/ml of antibacterial substance was added to the cell suspension (O.D. = 0.3) in 0.85% NaCl (pH 4.5), followed by incubation during 6 h at the optimal growth temperature of indicator strain. The optical density was measured at 660 nm for cellular lysis.

[1)]Positive of bactericidal action was expressed as reduction of above 10^2/ml of the viable cell counts compared with control after 6 h incubation.

[2)]Positive of cellular lysis was expressed as reduction of above 0.1 of optical density compared with control after 6 h incubation.

Effect of Enzymes

Treatments of the crude antibacterial substance with enzymes such as, catalase, pepsin, pronase E, proteinase K, chymotrypsin, trypsin, aminopeptidase and carboxypeptidase, did not affect activity. Therefore, these data indicate that the substance is not hydrogen peroxide and proteinaceous compound.

DISCUSSION

L. amylovorus IMC-1 from Inner Mongolian cheese, belonged to the *L. acidophilus* group strains, produces antibacterial substance in the skim milk medium. The amount of antibacterial substance in the culture supernatant reached the maximum after 72 h incubation at 37°C.

The substance produced by *L. amylovorus* IMC-1 had a wide spectrum of inhibitory activity against lactic acid bacteria.[12)] This substance showed inhibitory activity against five foodborne spoilage strains used in this study.

Table 3. Effect of enzymes on the inhibitory activity of the antibacterial substance produced by *Lactobacillus amylovorus* IMC-1

Enzymes	Residual antibacterial activity (%)*	
	pH 4.5	pH 5.5
Catalase	100	100
Pepsin	100	100
Pronase E	100	100
Proteinase K	100	100
Trypsin	100	100
Chymotrypsin	100	100
Aminopeptidase	100	100
Carboxypeptidase	100	100

Sample used was 20 units/ml of crude antibacterial substance after gel filtration, and *Pseudomonas fragi* IFO 3458 was used as the indicator strain.

*Residual antibacterial activity was expressed as follows;

$$\frac{\text{Inhibitory units after enzyme treatment}}{\text{Inhibitory units before enzyme treatment}} \times 100$$

There are very few antibacterial substances produced by lactic acid bacteria that inhibit growth of Gram-negative bacteria.[2,4,5,11,15] Interestingly, this substance was active against Gram-negative foodborne spoilage strains such as *Ps. fragi* and *E. coli*. However, these data differ from lactobin A produced by *Lactobacillus amylovorus* LMG P-13139, which was not active against Gram-negative strains.[1]

The substance showed bacteriostatic activity against *Staph. aureus* and *L. monocytogenes* of Gram-positive bacteria. Despite Gram-positive strain, the death of *B. subtilis* was caused by its addition. *B. subtilis* is Gram-positive strain to form spore under adverse environmental conditions as the NaCl solution. When the cell structure of the strain is weak by some change during forming spore in the solution, the substance attacks and kills rapidly the strain. However, the substance failed to kill all cells at concentration of 20 units/ml after 12 h incubations, thus, it indicates that the spore-formed cells could be surviving in the solution containing antimicrobial. The inhibitor showed bactericidal activity against both *Ps. fragi* and *E. coli*, and their death was due to cell lysis.

This substance had higher activity at relatively low pH value, and retained its activity at pH 7.0.[12] All treatments of the substance with catalase, protease, and peptidase did not affect activity at pH 4.5 and 5.5. However, at pH 4.5, proteolytic enzymes (pronase, proteinase K, trypsin, chymotrypsin and pepsin; 1 mg/ml) totally inhibited the activity of mesentericin Y105, produced by *Leuconostoc mesenteroides*, after 2 h treatment.[5] Therefore, these data indicate that the substance is not organic acid, hydrogen peroxide and compound of proteinaceous nature such as bacteriocin.[8-9]

L. acidophilus group strains are an important natural inhabitant of the intestinal tracts. It is of great interest that this substance shows inhibitory activity against foodborne spoilage bacteria. Therefore, it would be especially important to use *L. amylovorus* IMC-1 as a starter culture for controlling undesirable bacteria in lactic fermented foods and intestinal tracts. The antibacterial substance produced by *L. amylovorus* IMC-1 could be used as a food biopreservative.

국문요약

본 연구에서는 발효 식품의 제조에 유용한 항균성 물질을 생산하는 유산균 starter의 개발의 일환으로 장내 상존균으로 알려진 *Lactobacillus acidophilus* group 유산균인 *Lactobacillus amylovorus* IMC-1 균주가 생산한 항균성 물질의 식품오염세균에 대한 항균 활성을 검토하였다. 내몽골 원산 치즈에서 분리된 *L. amylovorus* IMC-1 균주는 탈지유배지에서 37°C에서 72시간 배양하였을 때 최대로 항균성 물질을 생산하였으며, 더 이상의 배양은 항균성 물질의 생산에 영향을 미치지 않았다. 겔 여과후의 항균성 물질은 식품오염세균인 *Bacillus subtilis* IFO 3025, *Staphylococcus aureus* IAM 1011, *Listeria monocytogenes* VTU 206, *Escherichia coli* RB, 및 *Pseudomonas fragi* IFO 3458 등과 같은 모든 피검균에 대하여 20 units/ml 첨가로 항균활성을 나타내었다. 그리고 이 물질은 *B. subtilis*, *E. coli* 및 *Ps. fragi*에 대해서는 살균 작용을 나타내었으며, *Staphy. aureus*와 *L. monocytogenes*에 대해서는 정균 작용을 보였다. 그 살균 작용은 용균 작용에 기인한 것임이 밝혀졌다. 또한 이 물질은 유기산, 과산화수소 및 단백질성 물질이 아님이 밝혀졌다.

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