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A Bacteriocin Produced by *Lactobacillus lactis* subsp. *lactis* Isolated from Thai Fermented Foods

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ABSTRACT Lactic acid bacteria isolated from local fermented foods were screened for bacteriocin activity under conditions that eliminated the effects of hydrogen peroxide, and organic acids. The antibacterial substance produced by one bacterial isolate identified as *Lactobacillus lactis* subsp. *lactis* was shown to have characteristics of bacteriocins which are being sensitive to proteolytic enzymes, being resistant to heat and having antibacterial activity against specific bacteria. The bacteriocin produced by *L. lactis* subsp. *lactis* was shown to have bacteriocidal mode of action against *Leuconostoc mesenteroides* (TISTR 473) without breaking its cells. Compared to the growth of *L. lactis* subsp. *lactis*, the activity of the bacteriocin was detected for the first time in the log phase and its maximal activity was detected in the stationary phase.

KEYWORDS: bacteriocin, antibacterial substance, lactic acid bacteria.

INTRODUCTION

Lactic acid bacteria (LAB) are gram-positive, non-sporulating microaerophilic bacteria whose main fermentation product from carbohydrates is lactate. They comprise both cocci (*Lactococcus, Vagoccocus, Leuconostoc, Pediococcus, Aerococcus, Tetragenococus, Streptococcus, Enterococcus*) and rods (*Lactobacillus, Carnobacterium, Bifidobacterium*).[1]

In fermented food industry, lactic acid bacteria have been used as starter cultures in fermentation process.[2-3] Substances produced by these bacteria do not only contribute to flavor and aroma development but also have inhibitory activity against spoilage bacteria and food-borne pathogens.[1,4-7] The antibacterial activity of lactic acid bacteria is due to the production of organic acids, hydrogen peroxide, and bacteriocins.[1,8-13]

Bacteriocins are antibacterial substances which are produced by many different bacterial species.[4,14-17] They are proteinaceous in nature and are bacteriocidal against other, mostly closely related bacteria. Collectively, bacteriocins form a heterogeneous group with regard to producing bacteria, antibacterial spectrum, mode of action and chemical properties.[1] Because of its specificity to sensitive bacteria, many research groups have been interested in screening for bacteriocin producing bacteria that can be used in food industry to inhibit the growth of spoilage bacteria and/or food-borne pathogens without any effect on useful normal flora residing in human body.

Bacteriocin-producing bacteria have been isolated from many food products such as meat,[5,15] fermented sausage,[18-19] dairy products[6-7], and vacuum-package foods.[20] In this study, a number of lactic acid bacteria isolated from fermented foods available in local market was screened for the production of bacteriocins and some characteristics of the substance were also determined.

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

Lactic acid bacterial strains isolated from fermented foods were propagated and maintained in 0.2% glucose MRS medium; indicator organisms were propagated and maintained in NB (Nutrient Broth) medium, unless stated otherwise. Indicator organisms used in this study were *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922) *Leuconostoc mesenteroides* (TISTR 473), *Proteus vulgaris* (ATCC 13315), and *Staphylococcus aureus* (ATCC 25923)

2. Screening of isolated lactic acid bacteria for production of bacteriocins

For the screening of bacteriocin-producing bacteria isolated from fermented foods, 0.2% glucose MRS medium was used to eliminate the effect of organic acids. Thai fermented foods used in this study included fermented vegetable, fermented beef, fermented pork and fermented fish available in local

market. 10 g of each fermented food was mixed with 90 ml of sterile phosphate buffer. The liquid part of the mixture was diluted in phosphate buffer and spread on 0.2% glucose MRS plates. The plates were incubated anaerobically overnight at 37°C. Anaerobic condition was used to minimize the formation of hydrogen peroxide. Only the plates that provided separated bacterial colonies were used for detection of antibacterial activity of isolated bacteria. 10 ml of soft 0.2% glucose MRS agar (0.7% agar) containing approximately 1 x 10^6 cells of *Leuconostoc mesentroides* was poured onto the surface of the plates containing separated bacterial isolates. After anaerobically incubated at 37°C for 24 hr, the plates were checked for inhibition zone.

3. Detection of antibacterial activity

For the detection of antibacterial activity, the swab-paper disc technique[21] was used. Approximately 1 x 10^6 cells of the indicator strains grown in appropriate media were used for testing the antibacterial activity. Each indicator organism was spread with a swab on appropriate agar plates. Sterile filter paper discs (Schleicher & Schuell) of 6 mm in diameter were placed on the surface of the agar plates containing the indicator strain. 25 ml of culture supernatants were dropped on the paper discs. After overnight incubation at 37°C, the plates were checked for inhibition zones around the paper discs.

4. Preparation of culture supernatant

Each bacterial isolate was grown in 0.2% glucose MRS broth for 24 hr at 37°C. Cell free culture supernatant was obtained by centrifuging the culture at 10,000 xg for 10 min, followed by filtration of the supernatant through a 0.2 mm pore-size filter.

5. Sensitivity to heat and proteolytic enzyme

Cell free culture supernatant of *Lactobacillus lactis* subsp. *lactis* was heated at 100°C for 10, 20, and 30 min and the remaining activity was assayed by the swab-paper disc technique. To test the sensitivity of the bacteriocin to proteases, the culture supernatant was treated with proteinase K and pepsin, each at a final concentration of 0.5 mg/ml. Samples were incubated at 37°C for 12h before tested for antibacterial activity.

6. Mode of action

To study the mode of action of the bacteriocin on sensitive cells, 1 ml of the supernatant prepared from the culture of *L. lactis* subsp. *lactis* was added to a 15 ml of an 8 hour culture of *Leuconostoc mesentroides* (TISTR 473). At every 30 min for 2 hours, the optical density of the *L. mesentroides* culture was determined at the wavelength of 660 nm and the number of viable *L. mesentroides* cells was also determined.

7. The relationship between the growth of *L. lactis* subsp. *lactis* and its bacteriocin activity

To study the relationship between the growth of *L. lactis* subsp. *lactis* and its bacteriocin activity, 2 ml of the overnight bacterial culture were inoculated into the 100 ml of fresh 0.2% glucose MRS medium. At every hour for 12 hr, samples were taken from bacterial culture for determining the optical density at the wavelength of 660 nm and bacteriocin activity. For determining bacteriocin activity, serial dilution of the supernatant prepared from bacterial culture was performed. Each dilution was tested for antibacterial activity against *Leuconostoc mesenteroides*. The bacteriocin activity reported, as arbitary units per milliliter (AU/ml), was calculated from the reciprocal of the highest dilution of a bacteriocin which had antibacterial activity against the indicator strain.

8. Identification of lactic acid bacteria

The bacteriocin-producing LAB was identified by the Thailand Institute of Scientific and Technological Research, Chatuchak, Bangkok, Thailand. The method used to identify the bacteriocin-producing LAB was the rapid method for identification of lactic acid bacteria (Api).

Results

1. Screening of isolated lactic acid bacteria for production of bacteriocins

Lactic acid bacteria isolated from fermented foods were screened for the production of bacteriocin using *Leuconostoc mesenteroides* (TISTR 473) as the indicator organism. The experiment was performed by using low glucose MRS medium (0.2% glucose) and in anaerobic condition to minimize the extent of organic acid and hydrogen peroxide production, respectively. Eleven isolates of lactic acid bacteria, designated as FS1 to FS11, were shown to be able to inhibit the growth of the indicator strain. The antibacterial activity of the eleven isolates against *Leuconostoc mesenteroides* was confirmed by using the swab-paper disc technique. Six out of the eleven LAB isolates were shown to have antibacterial activity against *Leuconostoc mesenteroides*. The bacterial isolate FS1 isolated from fermented green onion was

found to produce the largest inhibition zone against the indicator organism (Table 1). Therefore, we selected this bacterial isolate for further study. The bacterial isolate FS1 was identified as *Lactobacillus lactis* subsp. *lactis*.

2. Protease and heat sensitivity of the antibacterial substance

The activity of the antibacterial substance produced by *L. lactis* subsp. *lactis* was abolished by protease treatments (proteinase K and pepsin) but was resistant to heat (Table 2). There was no reduction of the antibacterial activity after heating the supernatant prepared from the culture of *L. lactis* subsp. *lactis* for 30 min at 100°C. These results demonstrate that the antibacterial substance produced by *L. lactis* subsp. *lactis* is heat stable protein.

3. Inhibitory spectrum

The antibacterial activity of the supernatant prepared from the culture of *L. lactis* subsp. *lactis* on various gram-positive and gram-negative pure indicator culture bacteria was tested using the swab-paper disc technique. The indicator organisms used in this experiment included *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922) *Leuconostoc mesenteroides* (TISTR 473), *Proteus vulgaris* (ATCC 13315), and *Staphylococcus aureus* (ATCC 25923). The culture supernatant was active against *Leuconostoc mesenteroides*. The growth of other gram-positive and gram-negative indicator organisms tested was not inhibited.

Table 1. Sizes of inhibition zones produced by supernatant of isolated lactic acid bacteria against *Leuconostoc mesenteroides* (TISTR 473)

Bacterial isolates	Fermented foods	Sizes of inhibition zone (mm)
FS1	Fermented green onion	15
FS2	Fermented green onion	0
FS3	Fermented bamboo shoot	0
FS4	Fermented cucumber	12
FS5	Fermented cucumber	10
FS6	Fermented pork	13
FS7	Fermented pork	12
FS8	Fermented pork	0
FS9	Fermented fish	0
FS10	Fermented beef	0
FS11	Fermented beef	11

Table 2. Effects of heat and proteolytic enzymes on the antibacterial substance produced by *L. lactis* subsp. *lactis*

Supernatant prepared from the culture of *L. lactis* subsp. *lactis*	Sizes of inhibition zones (mm)
No treatment	15
Heated at 100°C for 10 min	15
Heated at 100°C for 20 min	15
Heated at 100°C for 30 min	15
Treated with Proteinase K	0
Treated with Pepsin	0

4. Mode of action

The addition of the supernatant prepared from culture of *L. lactis* subsp. *lactis* to a 8 hr culture of *Leuconostoc mesenteroides* resulted in a significant inhibition of its growth. In the plate count experiment, the number of viable cells per ml declined from 4.91×10^8 to 3.26×10^4 after 120 min of incubation. In contrast, the control indicator culture that was not added with the *L. lactis* subsp. *lactis* culture supernatant showed constant number of cells in every plate count (Figure 1). However, the optical density at the wavelength of 660 nm of the indicator culture treated with the supernatant prepared from culture of *L. lactis* subsp. *lactis* and that of the control was stable throughout the experiment (Fig 1).

5. The relationship between the growth of *L. lactis* subsp. *lactis* and its bacteriocin activity

When *L. lactis* subsp. *lactis* was grown in 0.2% glucose MRS broth at 37°C, the growth of the bacterial culture and its bacteriocin production was found as shown in Fig 2. The bacterial culture was in the lag phase for 3 hours before entering the log phase and stayed in log phase for 3 hours before entering stationary phase. Compared to the growth of *L. lactis* subsp. *lactis*, the bacteriocin activity was detected for the first time in the log phase and the maximal activity of the bacteriocin was detected in the stationary phase.

Discussion

Bacteriocin-producing lactic acid bacteria originally isolated from Thai fermented foods are probably one of the best candidates for improving the microbiological safety of these foods, because they are adapted to the traditional conditions better than those isolated from other sources. Initially we screened for the ability to produce bacteriocins of lactic acid bacteria isolated from fermented foods

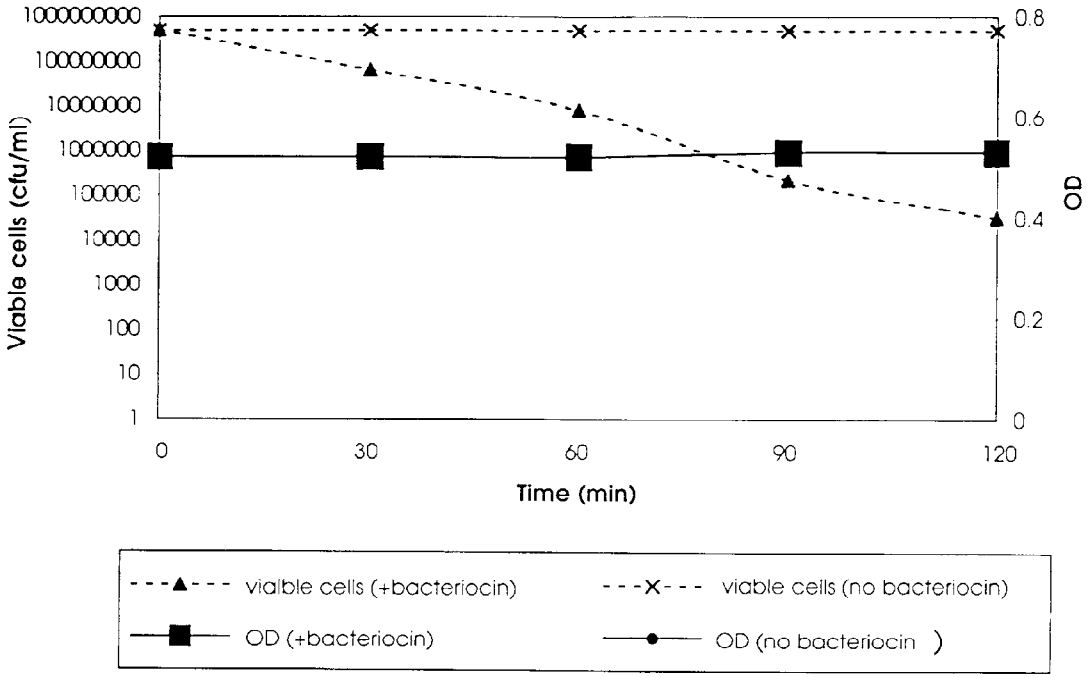

Fig 1. Effects of the bacteriocin produced by *L. lactis* subsp. *lactis* on *Leuconostoc mesenteroides*

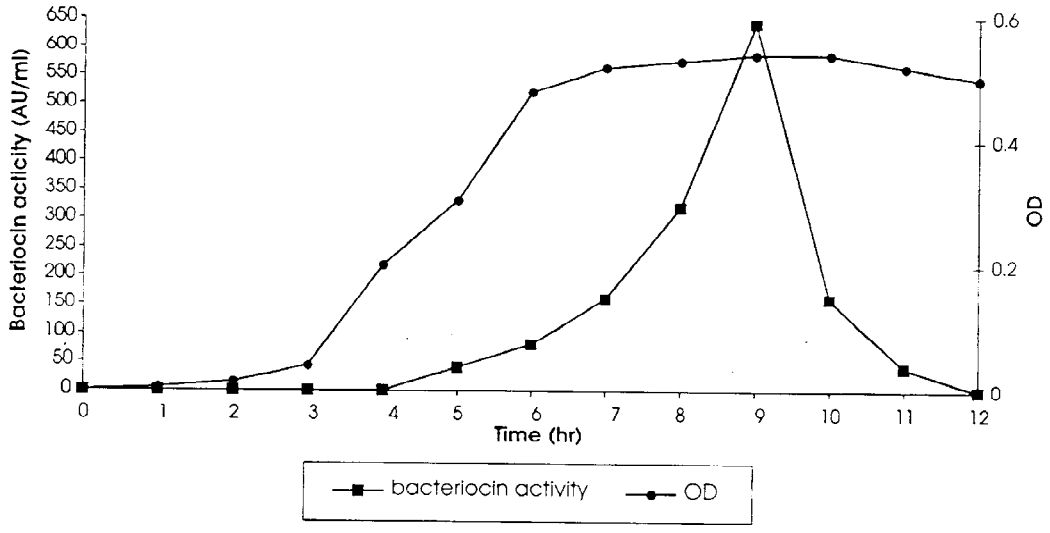

Fig 2. Growth of *L. lactis* subsp. *lactis* and its bacteriocin activity

available in local markets. From the results, six out of eleven lactic acid bacterial isolates that produce bacteriocins were showed to have antibacterial activity against *Leuconostoc mesenteroides* on the swab-papar disc technique. In liquid culture, proteolytic enzymes might be released from the producer strain into the medium where the bacteriocin might be digested by the enzymes.

The reasons that we used *L. mesenteroides* as an indicator in the screening experiment are the followings. Since bacteriocins normally have antibacterial activity against bacteria that are closely related to bacteriocin-producing bacteria, it would be more practical to use *L. mesenteroides* which belongs to the group of lactic acid bacteria as an indicator in the screening experiment. Additionally, since some strains of *L. mesenteroides* are spoilage bacteria, the bacteriocin found in this study might be useful in food industry for inhibiting strains of *L. mesenteroides* that are spoilage bacteria.

In this study, the antibacterial activity of *L. lactis* subsp. *lactis* could not be attributed to the production of organic acids and hydrogen peroxide. By using low glucose MRS medium (0.2%), the carbon source was reduced so that the lactic acid bacteria cannot produce organic acids to the level that affects the growth of other bacteria.[5-7, 15] In the screening experiment that was conducted under anaerobic condition, the lactic acid bacteria cannot produce hydrogen peroxide to inhibit the growth of the indicator organisms.

Treatments of the antibacterial substance produced by *L. lactis* subsp. *lactis* with proteases and heat showed that its activity was abolished by protease treatments (proteinase K and pepsin) but resistant to heat. These results coincide with those from previous studies of bacteriocins produced by lactic acid bacteria includin *Lactobacillus acidophilus*,[22-23] *Lactobacillus helveticus*,[24-25] *Lactobacillus plantarum*,[26-27] and *lactobacillus sake*.[15]

Mode of action of the bacteriocin produced by *L. lactis* subsp. *lactis* was studied. The number of viable cells of *Leuconostoc mesenteroides* treated with the bacetriocin was dramatically reduced. The bacteriocin comply with the definition of bacteriocins being protein-containing macromolecules which exert a bacteriocidal mode of action.[14] Interestingly, the OD at the wavelength of 660 nm of *Leuconostoc mesenteroides* culture treated with the culture supernatant prepared from *L. lactis* subsp. *lactis* was stable throughout the experiment. These results suggest that the total number of *L. mesenteroides* cells was stable, eventhough the viable cells of *L. mesenteroides* was reduced. This indicates that the bacteriocin may have a bacteriocidal effect on *L. mesenteroides* without lysing the cells. The mode of action of the bacteriocin is similar to that of the gassericin A which is a bacteriocin produced by *Lactobacillus gasseri* LA39.[28]

When the relationship between the growth and the bacteriocin activity of *L. lactis* subsp. *lactis* isolated from Thai fermented food was studied, it was found that the activity of bacteriocin produced by *L. lactis* subsp. *lactis* were detected at the log phase. This result suggests that the bacteriocin may not be essential for the growth of *L. lactis* subsp. *lactis*. Therefore, it is likely that the bacteriocin of interest is a secondary metabolite. Many bacteriocins produced by lactic acid bacteria have been found to be secondary metabolite such as Leucocin S and Mesenteroicin 5 which are produced by *Leuconostoc paramesenteroides* strain OX [5] and *Leuconostoc mesenteroides* strain UL5.[29]

Many of the bacteriocin-like agents produced by gram positive bacteria are known to kill species other than those that are likely to have the same ecological niche. We have demonstrated the killing of a bacteriocin-sensitive strain of *Leuconostoc mesenteroides* in MRS broth/agar by an isolated strain of lactic acid bacteria. Many known food-borne pathogens may be bacteriocin sensitive, and *Lactobacillus lactis* subsp. *lactis* isolated in this study may be useful for the inactivation of related pathogens.

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