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DETECTION AND PARTIAL CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *LEUCONOSTOC* ISOLATED FROM THAI FERMENTED FOOD

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ABSTRACT

Lactic acid bacteria isolated from various Thai fermented foods were tested for bacteriocin activity. One isolate of the lactic acid bacteria, designated as *Leuconostoc mesenteroides* TFF5, was shown to produce an antimicrobial substance that had characteristics of a bacteriocin. The bacteriocin produced by *L. mesenteroides* TFF5 was heat stable, retained activity after heating at 100°C for 30 min and at 121°C for 15 min and stable at pH values ranging from 2 to 10. The bacteriocin was sensitive to all proteolytic enzymes used in this study, but resistant to non-proteolytic enzymes. Inhibitory activity of the bacteriocin was limited to the gram-positive indicator bacteria tested. It exhibited bacteriocidal effect without cell lysis against sensitive cells. Bacteriocin activity was detected initially in log phase of culture growth and its maximal activity coincided with the onset of stationary phase. *L. mesenteroides* TFF5 was shown to contain no plasmids suggesting that genetic determinant for bacteriocin production was likely to be chromosomal encoded.

Keywords : Bacteriocin, *Leuconostoc*, fermented food.

INTRODUCTION

Bacteriocins are antimicrobial substances produced by varieties of bacteria. They are proteinaceous in nature and are bacteriocidal against other, mostly closely related bacteria. Many strains of lactic acid bacteria have been shown to be able to produce bacteriocins against specific species of spoilage bacteria and food-borne pathogens (Daeschel, 1989; Lewus et al., 1991).

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These findings have attracted many researchers to screen for bacteriocin-producing lactic acid bacteria that can be used in food industry to inhibit the growth of specific undesired bacteria without any effect on normal flora residing in human body.

Lactic acid bacteria are considered to be important in fermented food production because many substances that they produce such as organic acids and diacetyl contribute to taste and texture of the food. In addition, some substances produced by lactic acid bacteria including organic acids, hydrogen peroxide and bacteriocins have been reported to be able to inhibit the growth of spoilage bacteria and food-borne pathogens (Daeschel, 1989; Dahiya and Speck, 1968; Lewus et al., 1991). In the production of fermented food, fermentation can depend on lactic acid bacteria contaminated from the environment or lactic acid bacteria intentionally added into the food by manufacturers. It is difficult to control the quality of fermented food in term of taste, texture, amount and type of microorganisms presented in the food when the production of fermented food relies on lactic acid bacteria contaminated from the environment. Manufacturers of most Thai fermented food have faced this difficulty. To alleviate this difficulty, the use of lactic acid bacteria that can produce bacteriocins as protective cultures in Thai fermented food has been considered.

Lactic acid bacteria that are suitable for use as protective cultures in Thai fermented food should be isolated from Thai fermented food and produce bacteriocins against microorganisms that cause public health problem in Thailand such as *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. However, almost all of the reported bacteriocin-producing lactic acid bacteria were isolated from non-Thai fermented food such as Moroccan fermented food (Benkerroum et al., 2000), Korean fermented vegetable (Choi et al., 2000; Hur et al., 2000), Nigerian fermented food (Olasupo et al., 1999) and Argentinean regional fermented sausages (Piari et al., 1998). Those bacteriocins are unlikely to inhibit the growth of microorganisms causing public health problem in Thailand. Therefore, we are interested in screening of lactic acid bacteria isolated from Thai fermented food for the production of bacteriocins against microorganisms that cause public health problem in Thailand and determining some characteristics of the peptides.

MATERIALS AND METHODS

Bacterial cultures, media and culture conditions

Indicator organisms used in this study were *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 27736), *Proteus mirabilis* (ATCC 21100), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella paratyphi* A (DMS 5785), *Salmonella typhi* (DMS 5784), *Shigella dysenteriae* (DMS 2137), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (P.785), and *Streptococcus pyogenes* (DMS 3393). *S. pneumoniae* and *S. pyogenes* were propagated in M17 broth at 37°C. The other indicator organisms were propagated in nutrient broth (NB) medium at 37°C. Lactic acid bacteria isolated from Thai fermented foods were grown in MRS broth at 37°C. Cultures of indicator organisms and bacteriocin-producing strain were maintained as frozen stock cultures at -20°C in broth supplemented 20% (v/v) glycerol.

Screening of isolated lactic acid bacteria for bacteriocin production

Thai fermented food used in this study included fermented meat and fermented vegetable. Ten 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 MRS agar plates. The plates were

incubated overnight at 37°C. Only the plates that provided separated bacterial colonies were used for detection of antimicrobial activity of isolated bacteria. Ten ml of soft nutrient agar (NA) medium (0.7% agar) containing approximately 10^6 cells of *S. aureus* was poured onto the surface of the plates containing the separated bacterial colonies. After incubated at 37°C for 24 h, the plates were checked for inhibition zones around the bacterial colonies.

Preparation of cell-free supernatant containing bacteriocin

Cell-free culture supernatant (CFSB) of *L. mesenteroides* TFF5 was prepared by centrifuging overnight culture of *L. mesenteroides* TFF5 at 10,000 x g for 10 min. CSFB was adjusted to pH 6.8 and treated with catalase (Sigma) at final concentration of 1 mg/ml and passed through a 0.22 μm sterile filter (Sigma).

Spectrum of antimicrobial activity

To determine the spectrum of antimicrobial activity of the bacteriocin produced by *L. mesenteroides* TFF5, a wide range of bacteria were used as indicator organisms in swab-paper disc test (Rattanachaikunsopon and Phumkhachorn, 1998). Approximately 10^6 cells of each indicator organism were spread with a swab on M17 agar plates or nutrient agar (NA) plates. M17 agar plates were used for *S. pyogenes* and *S. pneumoniae* while NA plates were used for the other indicator organisms. Sterile filter paper discs of 6 mm in diameter were placed on the surface of the agar plates containing the indicator strain. Twenty-five μl of CFSB was dropped on the paper discs. After overnight incubation at 37°C, the plates were examined for the presence of inhibition zones around the paper discs.

Bacteriocin assay

For determining bacteriocin activity, serial two-fold dilution of CFSB was performed. Each dilution was tested for antimicrobial activity against *S. aureus* by using swab-paper disc test. The bacteriocin activity reported, as arbitrary units per milliliter (AU/ml), was calculated from the reciprocal of the highest dilution of the bacteriocin, which had antimicrobial activity against the indicator organism.

Effect of temperature, enzymes and pH on bacteriocin activity

The effect of temperature on bacteriocin activity was studied by heating CFSB at 100°C for 10, 20, or 30 min or autoclaving it at 121°C for 15 min. Each of the heat treated CFSB was then cooled down to room temperature and assayed for bacteriocin activity. To examine the effect of enzymes on bacteriocin activity, a collection of enzymes including papain, pepsin, pronase E, proteinase K, trypsin, α-amylase, lipase A, and lysozyme (all from Sigma) were used. Each enzyme was dissolved in sterile 4 m mol/l phosphate buffer (pH7) before adding into CFSB at final concentration of 1 mg/ml. After each of the enzyme treated CFSB was incubated at 37°C for 1 h, it was tested for the remaining bacteriocin activity. To determine the effect of pH on bacteriocin activity, CFSB was adjusted with sterile 5 m mol/l NaOH or 5 m mol/l HCl to different pH values between 1 and 14. Each of the treated CFSB was incubated at 25°C for 12 h, adjusted to pH 7 with sterile 5 m mol/l NaOH or 5 m mol/l phosphoric acid and then assayed for bacteriocin activity.

Mode of action

To study the mode of action of the bacteriocin on sensitive cells, cells of log phase or stationary

phase culture of *S. aureus* were harvested, washed and suspended in sterile 50 m mol/l of phosphate buffer (pH 6.8) to a final concentration of 10^8 CFU/ml. Fifteen ml of the sensitive cell suspension were mixed with 1 ml of CFSB and then incubated at 37°C. Samples were taken from the bacteriocin treated cell suspension at 0, 30, 60, 90, and 120 min. The optical density of each sample was determined at the wavelength of 660 nm and the number of viable *S. aureus* cells of each sample was also determined by plating them onto NA plates. Sterile MRS broth (pH 6.8), instead of CFSB, was used as a control.

Bacteriocin production

To study bacteriocin production, 2 ml of overnight culture of *L. mesenteroides* TFF5 were used to inoculate 100 ml of fresh MRS broth. The culture was incubated at 37°C without shaking. At every hour for 12 h, samples were taken from bacterial culture for determining the optical density at the wavelength of 660 nm and bacteriocin activity.

Identification of lactic acid bacteria

The bacteriocin-producing lactic acid bacteria was identified by the rapid method for identification of lactic acid bacteria (Api) (the API 50 CHL kit) with some additional tests including Gram stain, catalase test, arginine hydrolysis test, and test for growth at 4, 10 and 45°C.

Plasmid DNA isolation

L. mesenteroides TFF5 was subjected to plasmid DNA isolation using the method described by Anderson and McKay (1983). DNA was visualized following electrophoresis in 0.8% (w/v) agarose gel in Tris-acetate buffer at 60 V and staining with ethidium bromide solution (0.5 μg/ml, Sigma).

RESULTS

Screening of isolated lactic acid bacteria for bacteriocin production

Lactic acid bacteria isolated from various Thai fermented food were tested for bacteriocin production using *S. aureus* as the indicator organism. From the initial screening tests, 15 isolates of lactic acid bacteria were found to be able to inhibit the indicator organism. The antagonistic activity against *S. aureus* of the bacterial isolates was confirmed by swab-paper disc test. Twelve out of fifteen tested bacterial isolates were shown to inhibit the indicator organism with different degree of inhibitory ability (Table 1). One of the bacterial isolates giving positive results in swab-paper disc tests, designated as TFF5, was selected to be used for further study because it produced the largest inhibition zone against *S. aureus* (Table 1). Since this bacterial isolate was identified as *Leuconostoc mesenteroides*, it was named as *L. mesenteroides* TFF5.

Table 1. Antimicrobial activity of lactic acid bacteria isolated from Thai fermented food against *S. aureus* in nutrient broth.

Bacterial isolates	Food sources	Sizes of inhibition zones from swab-paper disc test (mm)
TFF1	Fermented fish	10
TFF2	Fermented fish	0
TFF3	Fermented fish	10
TFF4	Fermented fish	12
TFF5	Fermented pork	15
TFF6	Fermented pork	12
TFF7	Fermented pork	10
TFF8	Fermented beef	10
TFF9	Fermented beff	10
TFF10	Fermented vegetable	12
TFF11	Fermented vegetable	12
TFF12	Fermented vegetable	0
TFF13	Fermented vegetable	10
TFF14	Fermented vegetable	0
TFF15	Fermented vegetable	10

Effect of temperature, enzymes and pH on bacteriocin activity of *L. mesenteroides* TFF5

Boiling of the CFSB prepared from culture of *L. mesenteroides* TFF5 for 10, 20 and 30 min was found to have no effect on its antagonistic activity against *S. aureus*. The same result was found with the autoclaved CFSB. When the CFSB of *L. mesenteroides* TFF5 was treated with various enzymes, it was found that some enzymes including papain, pepsin, pronase E, proteinase K, and trypsin abolished the antimicrobial activity of the bacteriocin. The other enzymes including α-amylase, lipase A, and lysozyme did not have any effect on the inhibitory ability of the CFSB. No change in the antimicrobial activity was detected from the CFSB exposed to pH 2 to 10. However, exposing the CFSB to pH 1 and pH 11 to 14 resulted in no detectable antimicrobial activity.

Spectrum of antimicrobial activity of CFSB of *L. mesenteroides* TFF5

The CFSB of *L. mesenteroides* TFF5 was tested for antimicrobial activity against varieties of gram positive and gram negative bacteria. It inhibited *B. cereus*, *S. aureus*,*S. pneumoniae* and *S. pyogenes* (Table 2). The degree of inhibition of the CFSB against the tested organisms varied from strain to strain. However, the CFSB did not inhibit any of the eight gram-negative bacteria tested including *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*, *S. paratyphi* A, *S. typhi* and *S. dysenteriae* (Table 2).

Table 2. Antimicrobial activity of CFSB of *L. mesenteroides* TFF5 against various indicator organisms.

Indicator organisms	Sizes of inhibition zones (mm)
Bacillus cereus (ATCC 11778)	13
Escherichia coli (ATCC 25922)	0
Klebsiella pneumoniae (ATCC 27736)	0
Proteus mirabilis (ATCC 21100)	0
Proteus vulgaris (ATCC 13315)	0
Pseudomonas aeruginosa (ATCC 27853)	0
Salmonella paratyphi A (DMS 5785)	0
Salmonella typhi (DMS 5784)	0
Shigella dysenteriae (DMS 2137)	0
Staphylococcus aureus (ATCC 25923)	10
Streptococcus pneumoniae (P. 785)	15
Streptococcus pyogenes (DMS 3393)	15

Mode of action

The effect of the CFSB of *L. mesenteroides* TFF5 on the number of viable cells and on the optical density of the suspension of log phase and stationary phase cells of *S. aureus* were very similar. The addition of CFSB of *L. mesenteroides* TFF5 to the suspension of *S. aureus* cells resulted in significant decline of the number of viable cells of *S. aureus* from 10^8 CFU/ml to about 3 x 10^3 CFU/ml within 2 h (for log phase cells of *S. aureus*) and from 10^8 CFU/ml to about 7 x 10^5 CFU/ml within 2 h (for stationary phase cells of S. *aureus*) (Fig. 1). However, the optical density at the wavelength of 660 nm of the suspension of log phase and stationary phase cells of *S. aureus* was stable throughout the experiments. No change in the number of viable cells of *S. aureus* and the optical density of the suspension of log phase and stationary phase cells of *S. aureus* was detected in control experiment.

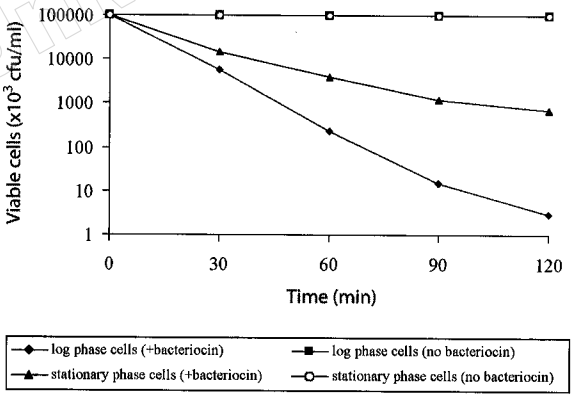

Figure 1. Effects of the bacteriocin produced by *L. mesenteroides* TFF5 on log phase and stationary phase cells of *S. aureus*.

Bacteriocin production

When *L. mesenteroides* TFF5 was grown in MRS broth at 37°C, the growth of the bacterial culture and its bacteriocin activity was found as shown in Fig. 2. Bacteriocin activity was not detectable when the *L. mesenteroides* TFF5 culture was in the lag phase and early log phase of growth. The bacteriocin activity was detectable for the first time when the *L. mesenteroides* TFF5 culture was in the mid log phase of growth. Then, the bacteriocin activity increased rapidly to the maximal level (640 AU/ml) when the *L. mesenteroides* TFF5 culture was in the stationary phase of growth. Once the bacteriocin activity reached maximal level, it did not decline throughout the stationary phase of growth.

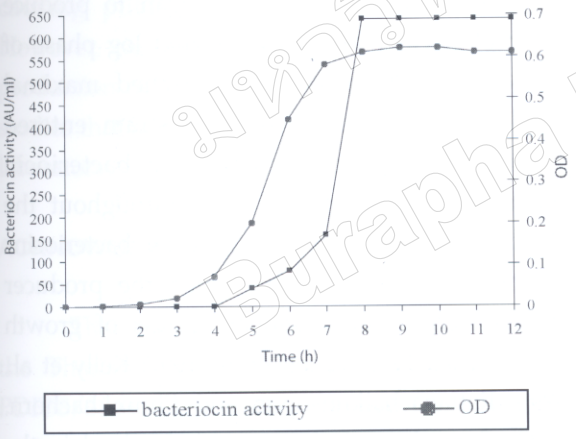

Figure 2. Growth of *L. mesenteroides* TFF5 and its bacteriocin activity.

Plasmid DNA isolation

From plasmid DNA isolation experiment, agarose gel electrophoresis revealed that *L. mesenteroides* TFF5 contained no plasmid.

DISCUSSION

In this study, lactic acid bacteria isolated from Thai fermented food were screened for bacteriocin activity against pathogenic bacteria causing health problems in Thailand. In the initial screening test, *S. aureus* was used as an indicator organism for a couple of reasons. Firstly, *S. aureus* is a major food borne pathogen that can be found in Thai fermented food. Secondly, *S. aureus* would be more sensitive to bacteriocin produced by lactic acid bacteria than gram negative bacteria because *S. aureus* is gram positive bacteria which are more closely related to lactic acid bacteria than gram negative bacteria are. Although 15 isolates of lactic acid bacteria were found to have antagonistic activity against *S. aureus* in the initial screening test, only 12 out of the 15 bacterial isolates were confirmed to have such activity in swab-paper disc test. The other 3 bacterial isolates may produce bacteriocin only on the agar medium but not in liquid medium. Some bacterial strains have been reported to produce bacteriocins only on the agar medium (Barefoot and Klaenhammer, 1983; Fricourt et al., 1994). Based on the size of inhibition zone, one isolate of lactic acid bacteria giving positive result in swab-paper disc test, designated as *L. mesenteroides* TFF5, was selected for further studies. Several strains of lactic acid bacteria in the genus *Leuconostoc* have been reported to be able to produce bacteriocin, for example, *L. mesenteroides* BC2 (Janes et al., 1999), *L. mesenteroides* subsp. *mesenteroides* (Kyung et al., 1996), *L. carnosum* La54a (Hastings et al., 1994), *L. carnosum* Ta11a (Hastings et al., 1994), *L. gelidum* UAL187 (Hastings et al., 1994), *L. paramesenteroides* La7a (Hastings et al., 1994), L. *paramesenteroides* XO (Lewus et al., 1991; Lewus et al., 1992) and L. *mesenteroides* UL5 (Daba et al., 1991)

Lactic acid bacteria can produce several substances such as organic acids, hydrogen peroxide and bacteriocins to inhibit growth of other bacteria (Daeschel, 1989). Since this study is interested in bacteriocin activity of *L. mesenteroides* TFF5 against indicator organisms, CFSB of *L. mesenteroides* TFF5 used in this study was neutralized and treated with catalase to minimize the effect of organic acids and hydrogen peroxide on the growth of indicator organisms. Like several other bacteriocins, antibacterial activity of the bacteriocin produced by *L. mesenteroides* TFF5 was sensitive to all proteolytic enzymes used in this study but resistant to α-amylase, lipase A and heat. These results indicated that the bacteriocin was heat stable protein or peptide and its antibacterial activity was not dependent on the presence of either carbohydrate moiety or lipid moiety. Almost all of the bacteriocins produced by *Leuconostoc* are resistant to amylase, except leucocin S (Lewus et al., 1992). Furthermore, it was found that the bacteriocin activity of *L. mesenteroides* TFF5 was stable over a wide pH range (pH 2 to 10) which agrees with results for bacteriocins produced by *Leuconostoc* (Janes et al., 1999) and other lactic acid bacteria (Franz et al., 1998; Kelly et al., 1996).

The bacteriocin produced by *L. mesenteroides* TFF5 inhibited only gram positive bacteria used in this study. This characteristic of the bacteriocin is similar to that of most bacteriocins, which inhibit gram positive bacteria better than gram negative bacteria. Compared to the bacteriocin produced by *L. mesenteroides* BC2 which inhibits B. *cereus* ATCC 1178 but not *S. aureus* ATCC 25923 (Janes et al., 1999), the bacteriocin produced by L. *mesenteroides* TFF5 was shown to inhibit both of the bacterial strains.

The bacteriocin of *L. mesenteroides* TFF5 caused the decline of both log phase and stationary phase cells of *S. aureus*. However, the bacteriocin had no effect on turbidity of the both *S. aureus* cultures. These results suggested that the bacteriocin produced by *L. mesenteroides* TFF5 had bacteriocidal effect without cell lysis on *S. aureus* cells. Among known bacteriocins, some bacteriocins were shown to have bacteriocidal effect without cell lysis on sensitive cells (Green et al., 1997; Kelly et al., 1996), while other bacteriocins were shown to have bacteriolytic effect on sensitive cells (Gonzalez et al., 1994; Yildirim and Johnson, 1998). However, there were bacteriocins found to have both bacteriocidal effect without cell lysis and bacteriolytic effects on sensitive cells. For example, a bacteriocin produced by *Lactobacillus curvatus* IFPL 105 was proved to have different effects on log phase and stationary phase cells of *Lactococcus lactis* subsp. *lactis* IFPL 186. It had bacteriolytic effect on the log phase sensitive cells but had bacteriocidal effect without cell lysis on the stationary phase sensitive cells (Casla et al., 1996).

L. mesenteroides TFF5 began to produce the bacteriocin when it was in mid log phase of growth and the bacteriocin reached maximal level as soon as the producer strain entered stationary phase of growth. The bacteriocin remained in the maximal level throughout the stationary phase of growth. Several bacteriocins reached the maximal level when the producer strains were in the stationary phase of growth (Casla et al., 1996; Green et al., 1997; Kelly et al., 1996; Rattanachaikunsopon and Phumkhachorn, 2000). Some bacteriocins were maintained in the maximal level throughout the stationary phase of growth (Green et al., 1997; Kelly et al., 1996) but other bacteriocins remained in the maximal level for a short period of time (Casla et al., 1996; Rattanachaikunsopon and Phumkhachorn, 2000).

Although genetic determinants for bacteriocin production of bacteriocins can be either chromosomally encoded (Franz et al., 1998; Kelly et al., 1996) or plasmid encoded (Hyronimus et al., 1998), those of *Leuconostoc* bacteriocins which have been genetically characterized appear to be plasmid

encoded (Hastings et al., 1994).To determine the situation for *L. mesenteroides* TFF5, plasmid isolation was performed and found that *L. mesenteroides* TFF5 contained no plasmid. This result suggested that genetic determinant for bacteriocin production of *L. mesenteroides* TFF5 was likely to be chromosomally encoded.

One of the disadvantages of the production of Thai fermented food is the difficulty in controlling type and number of contaminated microorganisms present in the food. This disadvantage sometimes leads to the presence of the pathogens in Thai fermented food. The use of bacteriocin-producing lactic acid bacteria as a protective culture may improve the safety of the food. *L. mesenteroides* TFF5 obtained from this study is interesting as a potential protective culture because of its natural occurrence in Thai fermented food and its antimicrobial activity against some pathogens causing public health problem in Thailand. However, the use of the bacterial strain in the production of Thai fermented food requires more studies that have been currently in progress in our laboratory.

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