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Antibacterial properties of *Lactobacillus plantarum* isolated from fermented mustards against *Streptococcus mutans*

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Antibacterial properties of *Lactobacillus* strains isolated from traditional fermented mustard were investigated. Among the strains tested, strain B0105 was found to produce bacteriocin-like compounds against *Streptococcus mutans* BCRC 10793. Strain B0105 was identified as *Lactobacillus plantarum* by phenotypical and physiological tests as well as16S rDNA identification. The molecular weight of bacteriocin-like compounds was 3.5 and 4.7 kDa by Tris-Tricine SDS-PAGE. In addition, this bacteriocin-like exhibited a strong antibacterial activity, heat stability (15 min at 121°C) and pH stability (pH 2.0–4.0) against *S. mutans* BCRC 10793, however, it was sensitive to proteolytic enzyme. Overall, the results obtained, demonstrate that strain B0105 is able to produce bacteriocin-like compounds inhibiting *S. mutans* BCRC 10793, making it potential candidate for antibacterial agents.

Key words: *Lactobacillus plantaru*m, bacteriocin, *Streptococcus mutans*.

INTRODUCTION

Dental caries is influenced by diverse bacterial, dietary, environmental, socioeconomic and physiological risk factors and is one of the most prevalent diseases in humans (Sánchez-Pérez et al., 2004; Marthaler, 2004). One of the properties required for a caries-preventive sweetener is that it decreases fermentation of sugar to acid by oral-microorganisms, including *Streptococcus mutans* which is a primary causative agent of human dental caries (Padilla et al., 2006). *S. mutans* present good adherent capacity owing to the production of glycocalyx and are responsible for initiating the cariogenic process (Cvitkovitch et al., 2003). To control dental caries formation, antibacterial agents that reduce the number of *S. mutans* are commonly used, for exam-ple xylitol, chlorhexidine and triclosan (Fejerskov, 2004). Using such antibacterials may induce resistance in micro-organisms (Badet et al., 2004; Diekema et al., 2004). Therefore, it is important to search for new antibacterials that may control the effect of *S. mutans* (Padilla et al., 2006).

Bacteriocins are defined as antibacterial peptide or proteins generally active against closely related species (Cleveland et al., 2001). Bacteriocins from lactic acid bacteria (LAB) have attracted more interest than those from other resources (Gao et al., 2010). Antagonistic effects produced by LAB towards other organisms may play an important role in maintaining a proper microbial

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balance in intestine tract and preserving certain foods (Millette, et al., 2007; Udhayashree et al., 2012). LAB are widely used as a starter culture and play an important role in food preservation, microbial stability and aroma compounds (Ravi et al., 2011, Abriouel et al., 2012). Many novel bacteriocin-producing LAB have been isolated from various foods and characteristics of these bacteriocins have been investigated (Gao et al., 2010). The properties of bacteriocins, that is, sensitivity to proteolytic enzymes, a narrower spectrum of antibacterial activity, and the antagonism phenomena, could make bacteriocins promising chemotherapeutic agents for the treatment of dental caries. During the last few years, a large number of new LAB bacteriocins have been identified and characterized (Cizeikiene et al., 2013). However, few studies have described bacteriocins activities against *S. mutans*.

Suan-tsai is a traditional fermented mustard which is widely consumed in Taiwan. It is made from green mustard and its production is a spontaneous fermentation process by a mixed microbial population mainly composed of LAB. Some LAB from fermented foods have been described to produced bacteriocins, e.g. *Lactobacillus pentosus* B96 (Delgado et al., 2005), *Lactobacillus plantarum* BS (Elegado et al., 2004), Lactobacillus strains (Omar et al., 2008), *L. acidophilus* La-5 (Tabasco et al., 2009), *L. plantarum* strains (Sánchez-Pérez et al., 2004) and *Lactobacillus sake* C2 (Gao et al., 2010). The information related to antibacterial activity of LAB against *S. mutans* is limited. Thus, the aims of this study were to investigate the antibacterial activity of LAB against *S. mutans* isolated from traditional fermented mustard and the characteristics of bacteriocin-like compounds were also determined.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The samples of fermented mustard were collected from southern areas of Taiwan. The fermented liquor samples (0.1 ml with serial dilutions) were spread on the surface of MRS agar and then incubated at 30°C for 1-2 days. Colonies were randomly selected and purified by re-plating on MRS agar plates. The purified colonies were primarily identified by milk agglutination. The acid producing bacteria were then identified by Gram staining and catalase tests. Only both Gram positive and catalase negative strains were selected and stored in MRS broth with 20% glycerol (v/v) at -80°C. The *Lactococcus lactis* subsp. *lactis* (BCRC 10791), *L. plantarum* (BCRC 10069) and *S. mutans* (BCRC 10793), used in determining of antibacterial activity were obtained from Bioresource Collection and Research Center (BCRC), Hsin Chu, Taiwan.

Screening for LAB producing bacteriocin from traditional fermented mustard samples

For screening of antibacterial activity of LAB isolates, 1% (v/v) of these cultures were inoculated into 50 ml MRS broth individually and incubated at 35°C for 24 h without agitation. Bacterial cell adjusted to pH 2.0 to release bacteriocins from the cell (Yang et al., 1992) were removed by centrifugation (5000 g, 20 min, 4°C) and filtration with 0.22 µm filter, then, the supernatants were examined with the diameters of inhibition zones using the agar diffusion assay method (Cizeikiene et al., 2013). Overnight test culture of *S. mutans* BCRC 10793, was diluted in saline and was inoculated in TSA agar medium to final concentration of 10^8 CFU/mL. Briefly, 200 µl of cell free supernatant were placed into wells (10.0 mm in diameter) on TSA agar plates seeded with the above test strains. After incubation at 35°C for 24 h, the diameter of inhibitory zones was determined. The pH of MRS broth was also adjusted to the same value as control. *L. lactis* subsp. *lactis* (BCRC 10791) overnight culture (10^8 CFU/mL) adjusted pH and filtrated, was used as the positive control. As a blank control, aliquots of MRS broth, treated as filtered supernatants, were used.

The pH value of the fermented supernatants was adjusted to 4.0 (Yang et al., 1992) with NaOH to eliminate the effect of low pH value and the pH of MRS broth was also adjusted to the same value as control, respectively. After adding catalase (50 U ml^{-1}, sigma), the cell free supernatants were incubated at 37°C for 3 h to eliminate the effect of hydrogen peroxide and the same supernatants without catalase were used as controls (Gao et al., 2010). Diameters of inhibition zones were recorded using *S. mutans* as indicator. *L. lactis* subsp. *lactis* (BCRC 10791) was used as the positive control. As a blank control, aliquots of MRS broth, treated as filtered supernatants, were used.

To determine the possible protein nature of the detected antibacterial substances, after eliminating hydrogen peroxide and low pH effects, the bacterial cell-free supernatants were incubated 37°C overnight with pepsin (Sigma, St. Louis, Missouri) at a final concentration of 2 mg ml^{-1} and those without enzyme treatment were used as controls (Ennahar et al., 2000). Diameters of inhibition zones were determined. *L. lactis* subsp. *lactis* (BCRC 10791) was used as the positive control. As a blank control, aliquots of MRS broth, treated as filtered supernatants, were used.

Strain identification

A PCR assay was performed using genomic DNA from strains that showed antibacterial activity against *S. mutans*. Amplification of 16S rDNA sequences by PCR was performed using the primers 27F AGAGTTTGATCMTGGCTCAG and 1492R GGYTACCTTGTTACGACTT described by Tanner et al. (2000). The reference strain was *L. lactis* subsp. *lactis* (BCRC 10791). For the PCR identification, genomic DNA was extracted using the Genomic isolation kit (GeneMark, Georgin, USA) according to the manufacture's instructions. Genomic DNA concentration was determined spectrophotometrically (Hitachi, U-2800A, Tokyo, Japan). PCR primers were used to amplify a 1484 bp DNA fragment. The reaction mixture contained 10 µl genomic DNA, 2.5 units of *Taq* polymerase (Promega, Madision, WI), 2 µl each of 10 mM dATP, dTTP, dCTP and dGTP, 5 µl of 10 X reaction buffer (10 mM Tris-HCl (pH 8.3 at 25°C) containing 50 mM KCl, 0.01% Triton X-100, 0.01% gelatin, 6.0 mM $MgCl_2$), and 50 pmol of each primers in a final volume of 50 µl. The DNA was denatured at 94°C for 2 min and amplified for 35 cycles at 94°C for 40 s, 45°C for 50 s and 72°C for 50 s. A final extension incubation of 2 min at 72°C was included. Amplification reactions were performed on a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400, Foster city, CA). The PCR products were purified with Gel/PCR DNA fragments extraction kit (Geneaid, Taipei, Taiwan) and sequenced by automated sequencing core laboratory, National Cheng Kung University (Tainan, Taiwan).

Estimation of bacteriocin activity

Overnight culture broth was heated at 70°C for 30 min to kill the cells and adjusted to pH 6, and stirred at 4°C for 4 h. Cells were collected by 20 min of centrifugation at 17000 x*g* , 4°C and washed

Table 1. Zones of growth inhibition of *S. mutans* by lactic acid bacteria.

Strains	Supernatant adjusted to pH 2.0	Supernatant adjusted to pH 4.0 and treated with catalase	Supernatant adjusted to pH 4.0 and treated with pepsin
	Inhibition zone (mm)		
MRS	15.0±0.2[a]	10.0±0.0[a]	10.0±0.1[a]
BCRC10791	17.5±0.2[b]	12.0±0.4[b]	10.5±0.1[b]
B0032	20.5±0.3[d]	14.0±0.6[c]	12.0±0.2[d]
B0105	20.5±0.2[d]	14.8±0.2[d]	10.5±0.1[b]
B0106	20.8±0.3[de]	14.0±0.5[c]	11.0±0.2[c]
B0115	20.8±0.3[de]	14.0±0.4[c]	11.0±0.2[c]
B0117	21.0±0.2[e]	14.3±0.3[cd]	12.0±0.2[d]
B0125	20.0±0.1[c]	14.5±0.4[cd]	11.0±0.1[c]
B0157	20.5±0.2[d]	14.0±0.2[c]	11.0±0.3[c]
B0158	20.5±0.2[d]	14.8±0.3[d]	11.0±0.2[c]

The data values are expressed as the mean ± SD (n = 3). Values in a row with different superscript letters are significantly different (P<0.05).

twice with 0.1 volume of 5 mM sodium phosphate buffer (pH 6.0). The cells were resuspended in a 0.25 volume of 100 mM NaCl (pH 2.0) and stirred at 4°C for 4 h. The resultant samples were centrifuged at 17000 x*g*, 4°C for 20 min. After the pH of the supernatants had been adjusted to 4.0, the resultant samples were filtered through a 0.22 µm filter (Yang et al., 1992; Wu et al., 2004). The protein concentration (Lowry et al., 1951), and bacteriocin activities were determined.

Bacteriocinogenic activity of bacteriocin was quantified using microtiter plate assay (Rojo-Bezares et al., 2007). Each well of the microtiter plate contained: 90 µl of twofold-concentrated MRS broth, 100 µl of cell-free supernatants at serial double dilutions and 10 µl of the indicator *S. mutans* (10^5 c.f.u. ml^{-1}, as final concentration), respectively. Microtiter plate cultures were incubated for 12–24 h at 35°C after which growth inhibition of the indicator strain was measured by optical density at 595 nm (OD595) in a Bio-Rad microtiter reader (model 450, Bio-Rad Laboratories, Hercules, California). Half maximal inhibitory concentration (IC50) was defined as the concentration of a bacteriocin that is required for 50% inhibition *in vitro* (50% of the OD595 of the positive growth control).

SDS-PAGE analysis and identification of the activity band

The molecular weight of pH purified bacteriocin preparations were determined by Tris-Tricine SDS-PAGE with 12% acrylamide resolving gel and 10% acrylamide spacer gel (Schagger and Von Jagow, 1987). The pH purified bacteriocin preparations along with low molecular weight markers (Sigma) were electrophoresed together in one gel at 20 mA for the first 2 h and then at 30 mA for another 12 h. Half of the gel was stained with Coomassie brilliant blue R-250 for molecular weight determination, meanwhile the another half of the gel was used for antibacterial activity assay by washing in sterile water, and overlaying with TSB agar plate seeded with 1% *S. mutans* in a Petri plate. The plate was incubated at 35°C for 24 h (Gao et al., 2010).

Characterization of bacteriocin

(A) Stability after different temperature and extreme pH treatments: pH purified bacteriocin preparations were incubated, respectively, in either thermostatic water bath at 80 and 100°C for 20, 40 and 60 min or in autoclave at 121°C for 15 min. By adjusting the pH value in a range of 2.0 to 11.0 and maintaining the bacteriocins for 2 h, the effect of pH was tested. For all the experiments described here, *S. mutans* was used as indicator and controls were maintained without any treatment (Gao et al., 2010).

(B) Sensitivity to proteolytic enzymes: The following enzymes were assayed (Rojo-Bezares et al., 2007): pepsin, protease, pronase, α-chymotrysin, bromelain and ficin (5 mg ml^{-1}) (all of them were purchased from Sigma, St. Louis, Missouri). Under conditions recommended by the manufacturer, antibacterial activity was assayed. For all the experiments, *S. mutans* was used as indicator and controls were maintained without any treatment. The percentage of inhibition activity was determined as: [(diameter of inhibition zone)^2 - (10 mm)^2]/[(diameter of maximum inhibition zone)^2 – (10 mm)^2] x 100%.

RESULTS

The antibacterial activity of LAB isolates

In this work, presumptive strains were first determined by phenotypical and physiological tests including Gram staining, catalase test and acid production. Acid-producing bacteria isolated from fermented mustard were cultivated in MRS broth and the activities of spent cell supernatants (SCS) against *S. mutans* using agar well diffusion test method were obtained (Table 1). The effect of low pH and hydrogen peroxide were eliminated by adjusting pH and catalase hydrolysis, respectively. These isolates with antibacterial activity against *S. mutans* were selected for the further experiments. After treatment by pepsin, the antibacterial activity of SCS of test strains, especially for B0105 and BCRC10791, almost disappeared, indicates that the substance with antibacterial activity was sensitive to pepsin.

Strain identification

Amplification of 16S rDNA sequence by PCR was performed using the primers described by Tanner et al. (2000). For 16S rDNA molecular identification of strain B0105, 1496 bp fragment was amplified from its genomic

DNA and the nucleotide sequence was determined. It revealed that the 16S rDNA nucleotide sequence of strain B0105 was 100% identical with that of *L. plantarum* strain WCFS1. Thus, strain B0105 was identified as *L. plantarum* and the GenBank access number is AL935258 was assigned.

Extraction of adsorbed bacteriocin and determination of its molecular weight

Bacteriocin-like compounds were adsorbed at pH 6.0 to B0105 and the cells were then centrifuged and resuspended in 100 mM NaCl at pH 2.0 for 4 h to release the bacteriocin-like compounds from the cell of B0105. The protein concentrations of released bacteriocin-like and activity was determined. The protein concentration of released bacteriocin-like compound was 867.9 μg ml^{-1} and was used to inhibit the indicator strain. According to the results from Table 2, pH purified bacteriocin-like compound significantly inhibited the growth of *S. mutans*. The IC_{50} ranged from 7.81-15.62 μg ml^{-1}.

According to the Tris-tricine-SDS–PAGE analysis, bacteriocins-like B0105 are small polypeptides with a molecular weight in the range of 3.5 and 4.7 kDa (Figure 1A). Interestingly, after Tris-tricine-SDS–PAGE, the bands of bacteriocin B0105 were still active against the indicator strain *S. mutans* (Figure 1B).

Characterization of bacteriocin

The bacteriocin B0105 treated with pH and its antibacterial activity was determined with agar-well diffusion test against *S. mutans* (Table 2). The antibacterial activity of bacteriocin B0105 against *S. mutans* decreased with increased pH values. No antibacterial activity was obtained when above 6.0. Table 3 also shows that the characteristics of the bacteriocin-like compound were stable after it was treated at 100°C for 1 h with 84.5% of antibacterial activity remaining. As far as proteases were concerned, the antibacterial activity of the bacteriocin-like compound was decreased when it was treated with different proteases, including trypsin, a-chymotrypsin, pronase, protease, bromelain and ficin.

DISCUSSION

It was observed that fermented vegetables or fruits are good sources of LAB. Many bacteriocin-producing LAB such as Lactobacillus sake C2, *L. plantarum* C19, *Lactobacillus brevis* P-319 and *Lactobacillus sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7, *Pediococcus pentosaceus* KTU05-8, KTU05-9 and KTU05-10 strains, showing activity towards various indicator strains, were successively isolated from fermented vegetables (Gao et al., 2010; Delgado et al., 2005; Li et al., 2008; Cizeikiene et al., 2013). Similiarity, strain B0105 was isolated from fermented vegetables and identified as *L. plantarum*. In this study, the antibacterial activity of B0105 and B0158 isolates against *S. mutans* was maintained after adjustment of pH and removal of catalase, indicating that the antibacterial agents of B0105 and B0158 could be bacteriocin-like compounds. After treatment with pepsin, no antibacterial activity of B0105 was obtained, indicating that bacteriocin-like compound is peptide (Rojo-Bezares et al., 2007). In another report, the bactericidal effect might be from the production of organic acids and/or in combination with the production of bacteriocin (Lin et al., 2008).

Adsorption of the bacteriocins onto cells was strongly influenced by the pH of the suspending environment. Maximum adsorption of nisin to both producer and indicator bacteria occurred at pH 6.5, showed complete loss of adsorption at pH 3.0 and below (Yang et al., 1992). Yildirimi et al. (2002) also noted that the adsorption of buchnericin LB was maximal between pH5.0 and 8.0 (100%) but below or above these values, the adsorption was decreased to 50%. The effects of pH value on isolation of bacteriocin-like B0105 was adsorbed at pH 6.0 to B0105, and then the cells were resuspended in pH 2.0 to release the bacteriocin from the cell of B0105. Extraction at alkaline pH was not used because like nisin, pediocin AcH, and some other bacteriocins of lactic acid bacteria are inactivated at alkaline pH (Yang et al., 1992). It has been reported that *L. acidophilus* produced a acidocin B, it retained 50% activity at pH 5.0 (Han et al., 2007). Lin et al. (2008) also indicated that cell cultures of LAP5 strain were neturalized to pH 7.0, the antagonistic effects of LAP5 against the *Salmonella* growth showed no inhibitory activity. Therefore, the pH ranges of antibacterial activities of B0105 was pH 2.0 to 6.0, the inhibition activity to *S. mutans* became negligible at pH 7.0 or above. Rojo-Bezares et al. (2007) reported that the MIC50 value of nisin against *Oenococcus oeni* was 0.024 and 12.5 μg ml^{-1} for other wine LAB species. Our results also revealed bacteriocin B0105 as an efficient antibac-terial agent again *S. mutans* with IC_{50} from 7.81-15.62 μg m^{-1}.

According to the Tris-tricine-SDS–PAGE analysis, bacteriocin-like B0105 are small polypeptides with a molecular weight in the range of 3.5 and 4.7 kDa. However, the range of bacteriocins for *L. plantarum*, *plantaricin* ST31 (2.76Da) from *L. plantarum* ST31 (Todorov et al., 1999), bacST202Ch and bacST216Ch (3.5 and 10.0 kDa) from *L. plantarum* (Todorov et al., 2010), bacteriocins ST28MS and ST26MS (5.5 and 2.8 kDa) from *L. plantarum* (Todorov and Dicks, 2005) are also reported. The results of measured molecular weight of bacteriocin B0105 suggested that its molecular weight is almost the same as the reports. The bacteriocins of LAB against LAB, Gram positive or other pathogenic bacteria were studied (Elegado et al., 2004; Yildirim et al., 2002; Lin et al., 2008; Gao et al., 2010), but *S. mutans* was not included in their study.

It is well known that heat stability of bacteriocins are important if they are to be used as food preservative agent, because many procedures of food preparation involve

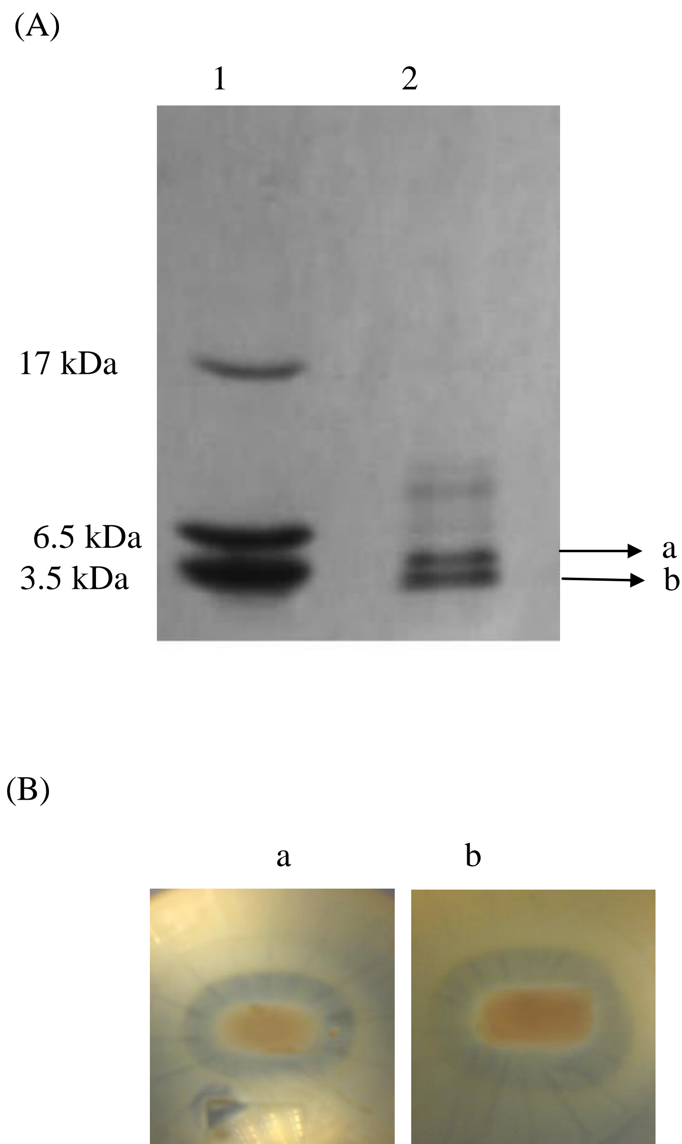

Figure 1. (A) A bacteriocin profile of SDS-PAGE of *L. plantarum* B0105. Lane 1: Ultra-low range molecular weight marker; Lane 2: Bacteriocin of *L. plantarum* B0105; (B) The gel band a, b overlaid with Tryptic soy soft agar surface seeded with 1% *S. mutans*.

Table 2. Antibacterial activity of extracted bacteriocin against *S. mutans* at different protein concentration.

Protein concentration (μg/ml)	Bacterial growth (OD_{595})		
	12 h	18 h	24 h
Control[a]	0.667±0.010[a]	0.669±0.012[a]	0.678±0.014[a]
250	0.054±0.011[b]	0.074±0.008[b]	0.085±0.009[b]
62.25	0.134±0.008[c]	0.139±0.018[c]	0.141±0.015[b]
15.62	0.351±0.020[d]	0.335±0.016[d]	0.328±0.018[ab]
7.81	0.348±0.028[d]	0.358±0.037[d]	0.348±0.037[ab]

Control[a]: containing *S. mutans* but no bacteriocins; The data values are expressed as the mean ± SD (n = 3). Values in a row with different superscript letters are significantly different ($P<0.05$).

Table 3. Effect of pH, heat and proteolytic enzyme on the antibacterial activity of bacteriocins from *L. plantarum* against *S. mutans*.

Treatment	Inhibition zone (mm)/Inhibition activity (%)[a]
pH	
2.0	20.0 ±0.0[a]/(100)
3.0	17.8±0.1[b]/(72.3)
4.0	16.0±0.1[c]/(52.0)
5.0	13.3±0.1[d]/(25.6)
6.0	11.5±0.1[e]/10.8)
7.0	10.0±0.0[f]/(0)
8.0	10.0±0.0[f]/(0)
9.0	10.0±0.0[f]/(0)
10.0	10.0±0.0[f]/(0)
11.0	10.0±0.0[f]/(0)
Heat	
80°C,20 min	20.0±0.0[a]/(100)
80°C,40 min	20.0±0.0[a]/(100)
80°C,60 min	20.0±0.0[a]/(100)
100°C,20 min	20.0±0.0[a]/(100)
100°C,40 min	20.0±0.0[a]/(100)
100°C,60 min	20.00.0[a]/±(100)
121°C,15 min	18.8±0.1[b]/(84.5)
Proteolytic enzyme	
control	20.0±0.0[a]/(100)
pepsin	14.0 ±0.1[b]/(34.4)
protease	15.5±0.1[c]/(50.0)
pronase	14.5±0.1[d](39.3)
α-chymotrysin	14.5±0.1[d]/(39.3)
bromelain	14.0±0.1[b](44.6)
ficin	15.0±0.1[f]/(29.8%)

[a]Inhibition activity (%) = [(diameter of inhibition zone) ^{2} – (10 mm) ^{2}/(diameter of maximum inhibition zone) ^{2} – (10 mm) ^{2}] × 100%. *The data values are expressed as the mean ± SD (n = 3). Values in a row with different superscript letters are significantly different (P<0.05).

heat treatment. Major classes of bacteriocins produced by LAB include lantibiotics (class I) and large heat-labile protein (class III) and complex proteins (class IV) whose activity requires the association of carbohydrate or lipid moieties (Llaenhammer, 1993). In this study, the antibacterial activity of bacteriocin-like B0105 treated at 121°C for 15 min was 84.5% against *S. mutans*. Consequently, bacteriocin-like B0105 has a stable behavior similar to other bacteriocin reported (Rojo-Bezares et al., 2007; Han et al., 2007; Yildirim et al., 2002). In other words, bacteriocin-like B0105 has considerable heat and pH tolerance which could make it useful additive in foods.

Conclusions

Traditional fermented mustard is a rich source of probiotic LAB. From isolated LAB strains, strain B0105 which produced a bacteriocin-like compound which strongly inhibited *S. mutans* was isolated from traditional fermented mustard. This strain was identified as *L. plantarum* by phenotypical, physiological tests and 16S rDNA sequence. This may be the first bacteriocin-producing strain of *L. plantarum* against *S. mutans*. After extraction by cell adsorption-desorption, the molecular weight of bacteriocin B0105 was 3.5 and 4.7 kDa in Tris-tricine SDS-PAGE. After Tris-tricine-SDS-PAGE, the bands of bacteriocin were still active against the indicator strain *S. mutans*. Bacteriocin-like compounds produced from B0105 had the strongest antibacterial activity and exhibited heat stability, pH stability and sensitivity to proteolytic enzyme. The present study recommended that bacateriocins produced from strain B0105 may be a potential candidate for development as an antibacterial agent. Further work would be done to determine the sequence of bacteriocin gene and ascertain the mode of action of bacteriocin and investigate the effects of bacteriocin B0105 on food

quality.

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