V. RESULTS

1. Lactic acid bacteria

1.1 Selection of antimicrobial producing lactic acid bacteria

From the previous study, ten isolates of lactic acid bacteria demonstrated the strong antimicrobial activities. Three of these, B85/4, B282 and B63/8 which exhibited the potent activity against *P. gingivalis* W50 were recruited for performing in the present study.

1.2 Identification of potent antimicrobial producing lactic acid bacteria

The species identifications by using API® database were interpreted as *Lactobacillus paracasei* subsp. *paracasei* with the 99.7 %, 99.6 % and 99.7 % identity, respectively (Table 10). The identifications by using BBL™ database were interpreted as *L. casei* with the confidence value 0.911, 0.977 and 0.935, respectively (Table 11).

Table 10 Identification of potent antimicrobial producing lactic acid bacteria by API® software database

Strains or isolates	Interpretations	% Identity
L. casei TISTR 390	*L. paracasei* subsp. *paracasei*	99.9
L. fermentum TISTR 055	*L. fermentum*	96.9
L. plantarum TISTR 541	*L. plantarum*	91.1
L. rhamnosus TISTR 108	*L. rhamnosus*	99.5
B85/4	*L. paracasei* subsp. *paracasei*	99.7
B282	*L. paracasei* subsp. *paracasei*	99.6
B63/8	*L. paracasei* subsp. *paracasei*	99.7

The species identification was acceptable at the levels of 80% identity.

Table 11 Identification of potent antimicrobial producing lactic acid bacteria by BBL™ software database

Strains or isolates	Interpretations	Confidence value
L. casei TISTR 390	*L. casei*	0.923
L. fermentum TISTR 055	*L. fermentum*	0.676
L. rhamnosus TISTR 108	*L. rhamnosus*	0.855
B85/4	*L. casei*	0.911
B282	*L. casei*	0.977
B63/8	*L. casei*	0.935

1.3 Determination of casein utilization

The casein utilization activity of these lactobacilli was detected by compared with *L. casei* TISTR 390 and *L. plantarum* TISTR 541 as the positive and negative controls. Three isolates showed no activity of the casein utilization as shown in Figure 4. It was concluded that these lactobacilli belonged to *L. paracasei* subsp. *paracasei*.

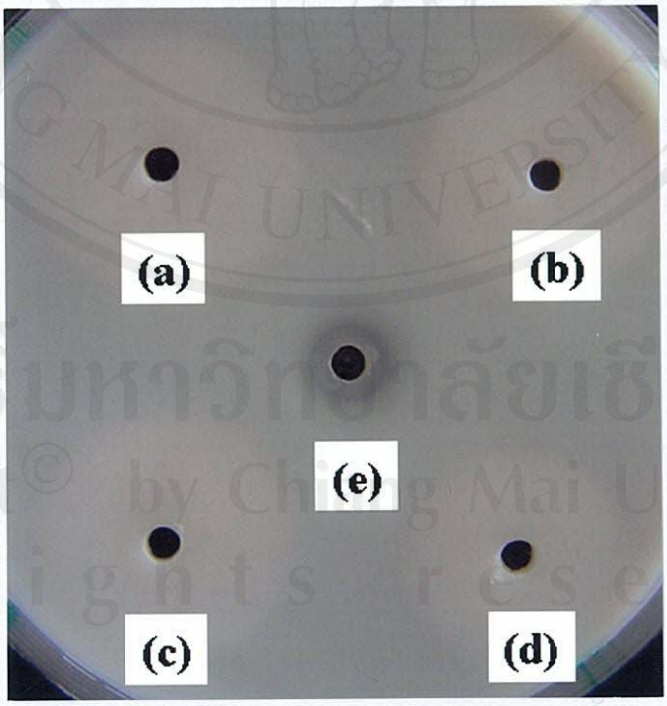


Figure 4 The casein utilizations of (a) B85/4, (b) B282, (c) B63/8, (d) *L. plantarum* TISTR 541 and (e) *L. casei* TISTR 390. The test was done in 15-cm petridish.

1.4 Determination of antimicrobial susceptibility

The results of antimicrobial susceptibility of the selected lactobacilli were demonstrated in Table 12. All tested isolates were susceptible to erythromycin. But their susceptibility patterns to clindamycin were differently demonstrated. The MICs of erythromycin and clindamycin for B85/4, B282 and B63/8 by using E-test were 0.032, 0.032 and 0.032 μg/ml; and 0.047, 0.064 and 0.016 μg/ml, respectively. It was interpreted that all isolates were susceptible to erythromycin and clindamycin. The example of MIC according to E-test was shown in Figure 5.

Table 12 Antibiogram patterns of the selected lactobacilli by using agar-disc diffusion technique

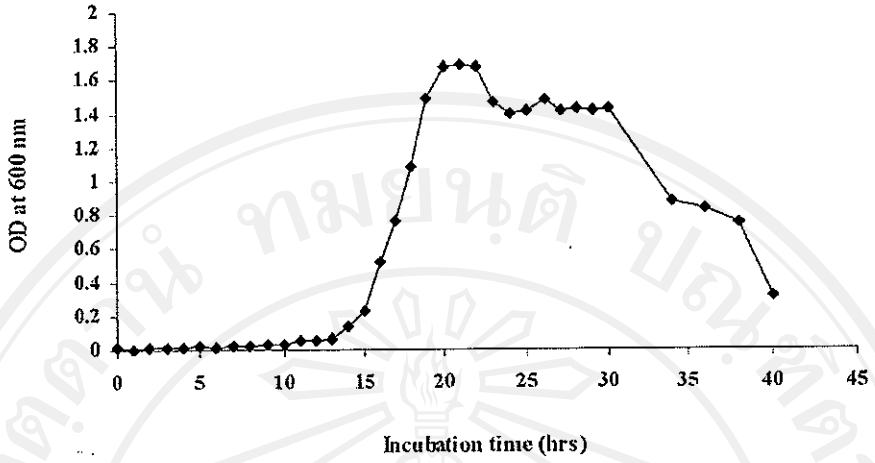
Antibiotics	**Isolates**		
	B85/4	**B282**	**B63/8**
Penicillin G	R	R	R
Ampicillin	R	R	R
Cephalothin	R	R	R
Ceptazidime	R	R	R
Gentamicin	R	R	R
Erythromycin	S	S	S
Norfloxacin	I	I	I
Chloramphenicol	I	I	S
Clindamycin	R	I	S

S, susceptible; I, intermediate; R, resistant
Interpreted according to the table of antibiotic susceptibility provided by Oxoid (Oxoid®; Basingstoke, Hampshire, England)

Figure 5 The MIC of clindamycin after tested against B282 isolate. The result showed MIC = 0.064 μg/ml

1.5 Analysis of growth curve and generation time

The growth curves of the potent antimicrobial producing lactobacilli were plotted from the absorbance at 600 nm of each collecting time. Lag, log, stationary and declined phases of all isolates were interpreted from their curves. The growth curves of B85/4 and B282 were demonstrated in the same manner of lag, log, stationary and declined phases such as hrs 0^{th}-13^{th}, 14^{th}-20^{th}, 21^{st}-32^{nd} and 33^{rd}-40^{th}, respectively. While the growth manners of B63/8 were 0^{th}-13^{th}, 14^{th}-22^{nd}, 23^{rd}-31^{st} and 32^{nd}-40^{th}, respectively. These growth curves showed in Figure 6. The generation time of all isolates was calculated from hrs 14^{th} for early-log phase, and hrs 18^{th} for mid-log phase. The generation time of B282, B85/4 and B63/8 isolate were 18.2, 21.5 and 23.6 min, respectively as shown in Table 13.



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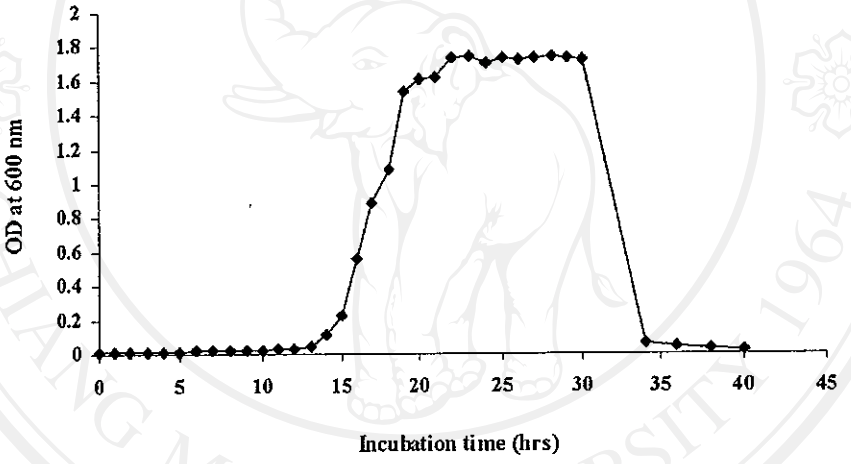

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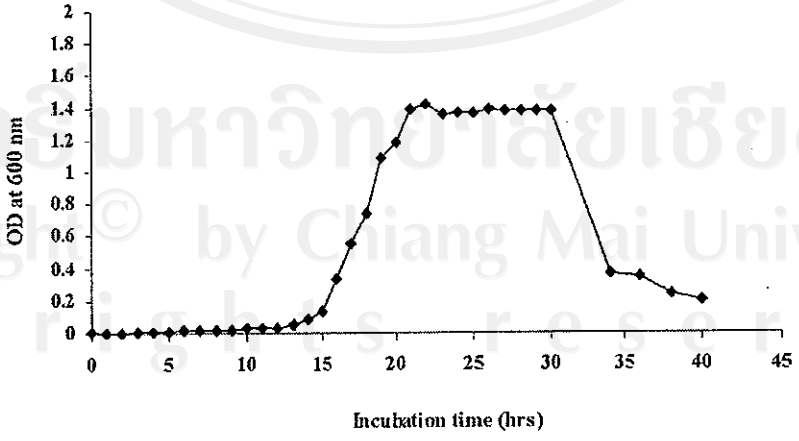


(c)

Figure 6 The growth curves of the potent antimicrobial producing lactobacilli, (a) B85/4, (b) B282 and (c) B63/8

Table 13 The generation time of the potent antimicrobial producing lactobacilli

Isolates	Hour of incubation		Time (hr)	Bacterial number (CFU/ml)		Generation time (hr or min)
	B_i	B_f		B_i	B_f	
B85/4	14	18	4	1.475×10^6	3.31×10^9	0.36 or 21.5
B282	14	18	4	1.725×10^6	1.58×10^{10}	0.30 or 18.2
B63/8	14	18	4	2.930×10^5	3.320×10^8	0.40 or 23.6

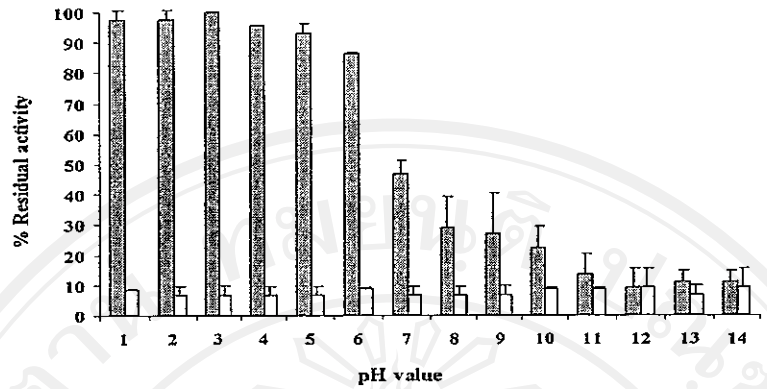
2. Characterization of cell-free supernatants

2.1 Determination of pH sensitivity

The effects of pH, high temperature and proteolytic enzyme towards the cell-free supernatant of all potent antimicrobial producing lactobacilli were analysed as percentage of residual antimicrobial activity against *P. gingivalis* W50 compared with the original supernatant (at pH value of 4.3) as 100% activity and the activity of MRS broth (original pH = 6.2) was measured in parallel. For the effects of pH, the residual activities of the tested lactobacilli, B85/4, B282 and B63/8, were still exhibited after adjusted the pH below 6. The residual activities of these treatments were gradually reduced after pH 6 (Figure 7).

2.2 Determination of heat sensitivity

For the effect of heat, the residual activity of the tested lactobacilli (B85/4, B282 and B63/8) was partially demonstrated at 121°C. The residual activities of these treatments were increased at lower temperature, 80°C and 60°C (Figure 8).



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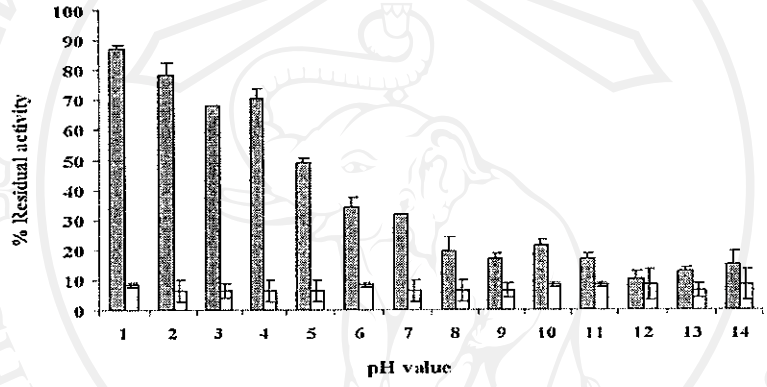

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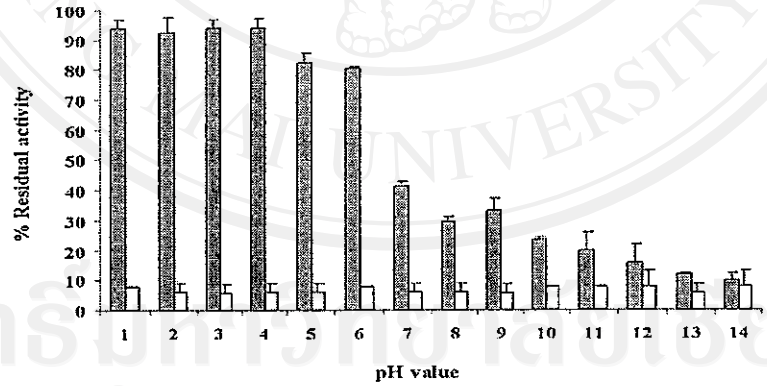
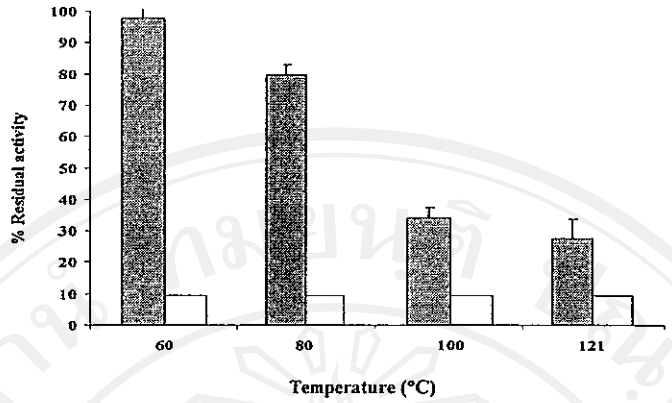


(c)

Figure 7 The effect of pH toward the antimicrobial activity of the tested lactobacilli, (a) B85/4, (b) B282 and (c) B63/8. The results demonstrated as the percentage of residual activity at various pH levels. Gray and white bars represented the activity of pH adjusted supernatant; and pH-adjusted MRS broth, respectively. The percentage of residual activity of the freshly prepared cell-free supernatant was 100%. The original MRS broth (pH = 6.2) showed 8% residual activity.



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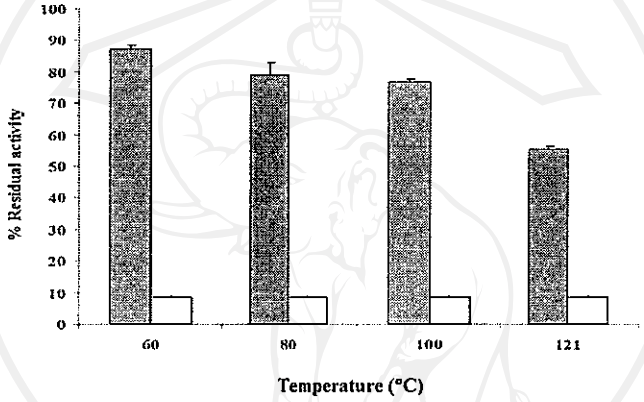

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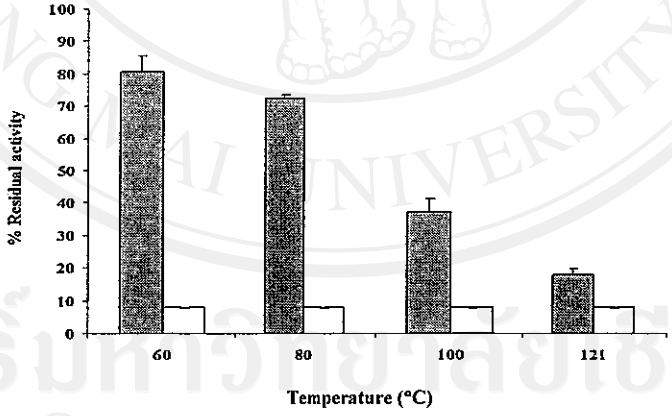


(c)

Figure 8 The effect of heat toward the antimicrobial activity of the tested lactobacilli, (a) B85/4, (b) B282 and (c) B63/8. The results demonstrated as the percentage of residual activity at various temperatures. Gray and white bars represented the activity of heated supernatant and heated MRS broth, respectively. The percentage of residual activity of the freshly prepared cell-free supernatant was 100%. The original MRS broth showed 8% residual activity.

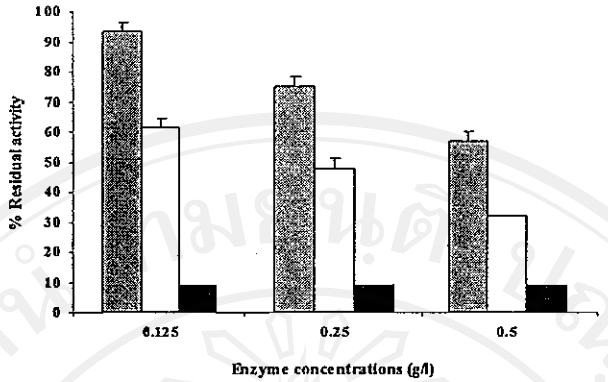
2.3 Determination of enzyme sensitivity

The percentage of residual activity compared with the original supernatant was significantly decreased after digesting with 0.125 g/l of both trypsin and pepsin and it was dose dependent (Figure 9). Pepsin showed more effect to the residual activity than trypsin. The higher levels of antimicrobial activity against *P. gingivalis* W50 could be found in acidic condition of supernatant.

After treatments with pH, high temperature and proteolytic enzymes, the affected proteins in each treatment could not be detected by using SDS-PAGE as shown in Figure 10-11.

2.4 Extraction of crude bacteriocins by ammonium sulphate precipitation

The crude bacteriocins of all isolates were precipitated with various concentration of ammonium sulphate; 20%, 40%, 60% and 80% (Figure 12-15). The optimum protein precipitation of all isolates was 40-80% ammonium sulphate. The 40% saturated ammonium sulphate was selected to use as the protocol for crude bacteriocins precipitation.



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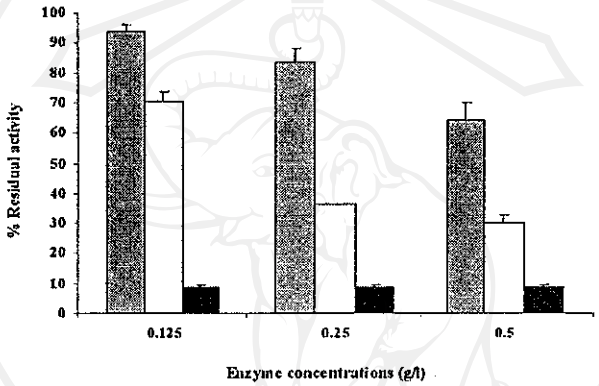

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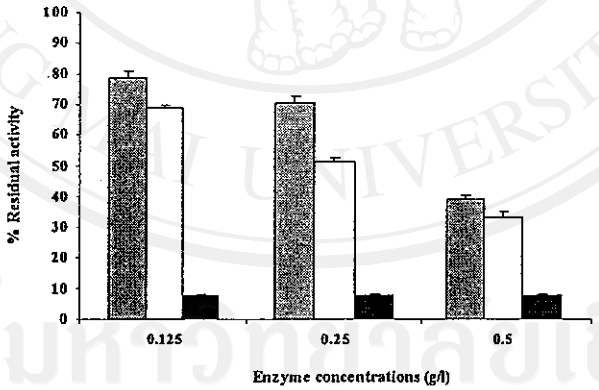
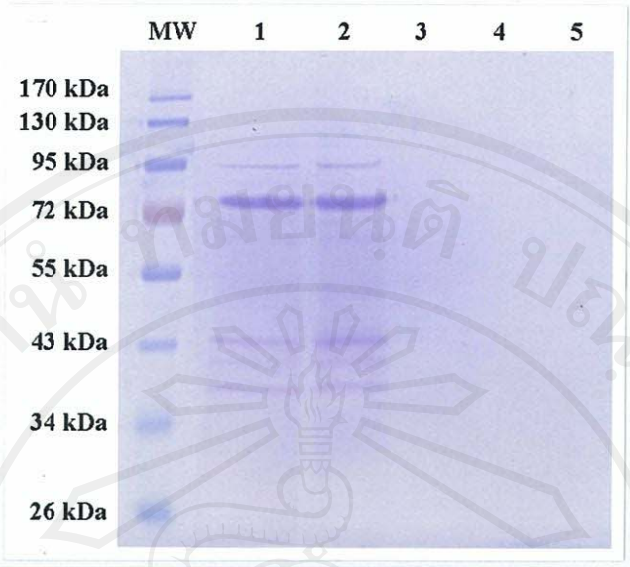


(c)

Figure 9 The effect of enzyme toward the antimicrobial activity of the tested lactobacilli, (a) B85/4, (b) B282 and (c) B63/8. The results demonstrated as the percentage of residual activity at various concentrations of enzyme. Gray, white and dark bars represented the activity of trypsin-digested supernatant, pepsin -digested supernatant and trypsin- or pepsin-digested MRS broth with the same condition. The percentage of residual activity of the freshly prepared cell-free supernatant was 100%. The origin MRS broth showed 8% residual activity.



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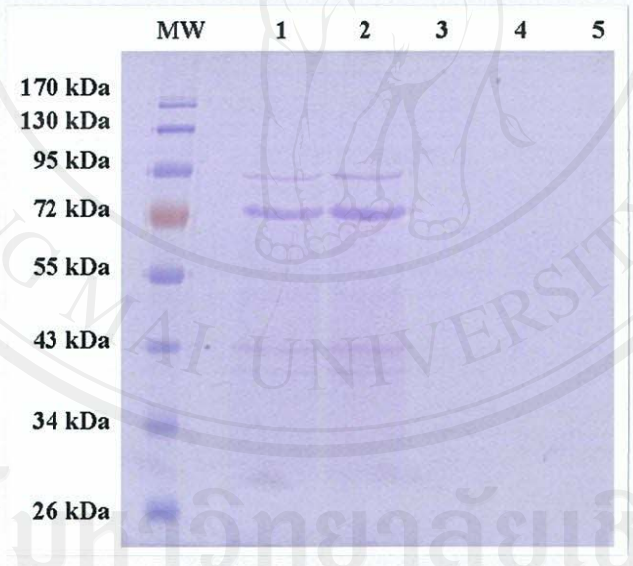
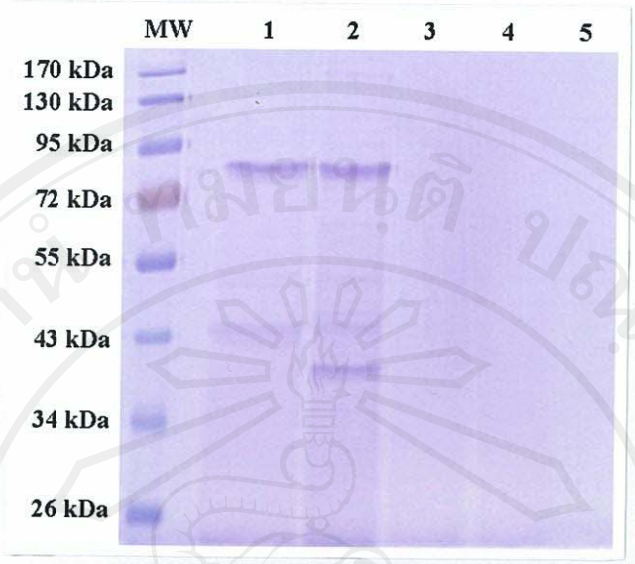


(b)

Figure 10 The affected proteins of (a) B85/4 and (b) B282 after characterized; lane MW, molecular weight marker (Page Ruler™ Prestained Protein Ladder; Fermentas INC, Burlington, ON, Canada); lane 1, crude bacteriocins; lane 2, pH 5-adjusted proteins; lane 3, heated-proteins; lane 4, trypsin-digested proteins and lane 5, pepsin-digested proteins



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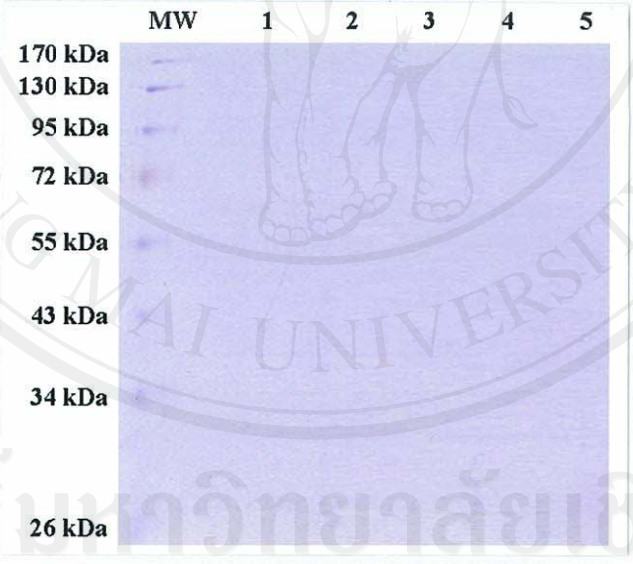
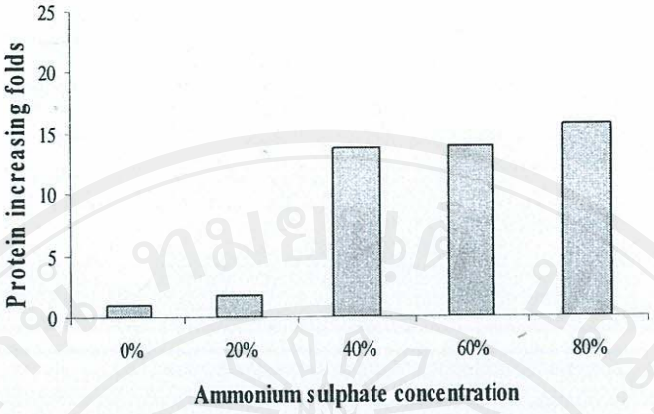


(b)

Figure 11 The affected proteins of (a) 63/8 and (b) MRS broth after characterized; lane MW, molecular weight marker (Page Ruler™ Prestained Protein Ladder; Fermentas INC, Burlington, ON, Canada); lane 1, crude bacteriocins; lane 2, pH 5-adjusted proteins; lane 3, heated-proteins; lane 4, trypsin-digested proteins and lane 5, pepsin-digested proteins



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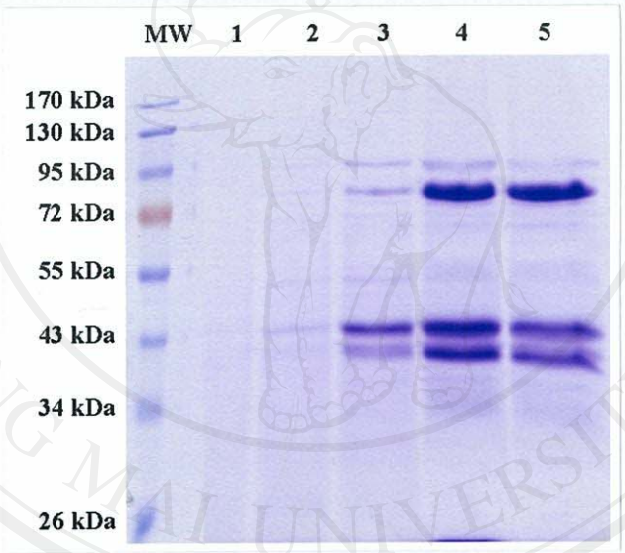
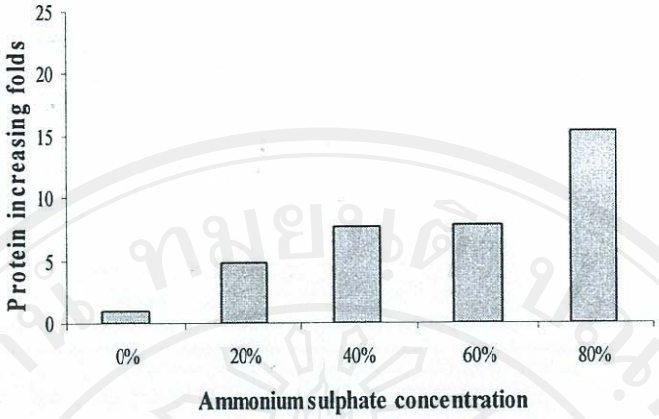


(b)

Figure 12 Crude bacteriocins of B85/4 extracted by ammonium sulfate precipitation, (a) protein increasing folds, (b) crude bacteriocins. Lane MW, molecular weight marker (Page Ruler™; Fermentas INC); lane 1, freshly prepared cell-free supernatant; lane 2, crude bacteriocins precipitated by 20% ammonium sulphate; lane 3, crude bacteriocins precipitated by 40%; ammonium sulphate; lane 4, crude bacteriocins precipitated by 60% ammonium sulphate and lane 5, crude bacteriocins precipitated by 80% ammonium sulphate.



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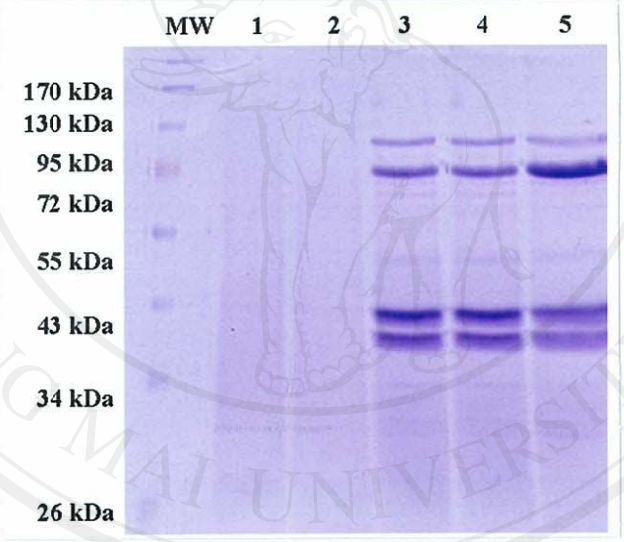
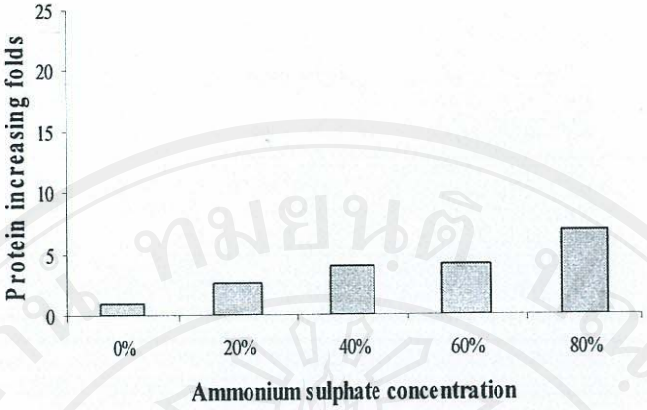


(b)

Figure 13 Crude bacteriocins of B282 extracted by ammonium sulfate precipitation, (a) protein increasing folds, (b) crude bacteriocins. Lane MW, molecular weight marker (Page Ruler™; Fermentas INC); lane 1, freshly prepared cell-free supernatant; lane 2, crude bacteriocins precipitated by 20% ammonium sulphate; lane 3, crude bacteriocins precipitated by 40%; ammonium sulphate; lane 4, crude bacteriocins precipitated by 60% ammonium sulphate and lane 5, crude bacteriocins precipitated by 80% ammonium sulphate.



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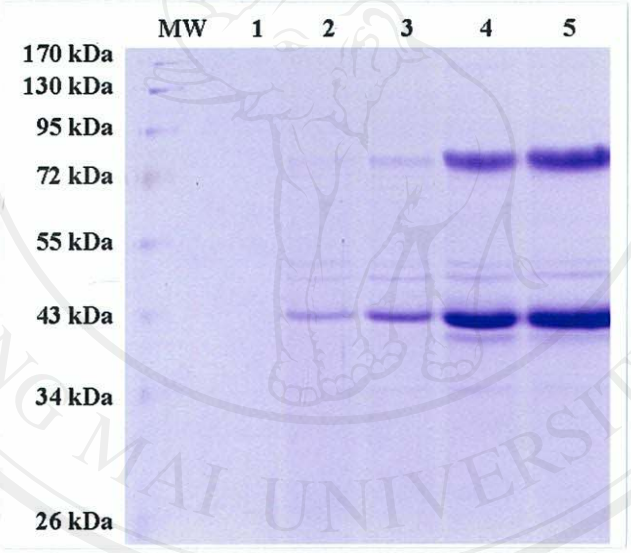
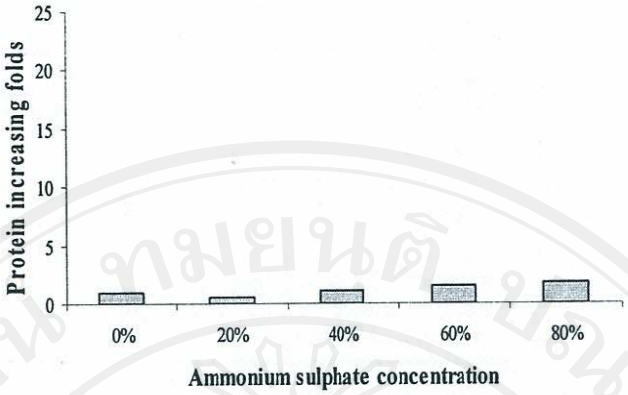


(b)

Figure 14 Crude bacteriocins of B63/8 extracted by ammonium sulfate precipitation, (a) protein increasing folds, (b) crude bacteriocins. Lane MW, molecular weight marker (Page Ruler™; Fermentas INC); lane 1, freshly prepared cell-free supernatant; lane 2, crude bacteriocins precipitated by 20% ammonium sulphate; lane 3, crude bacteriocins precipitated by 40%; ammonium sulphate; lane 4, crude bacteriocins precipitated by 60% ammonium sulphate and lane 5, crude bacteriocins precipitated by 80% ammonium sulphate.



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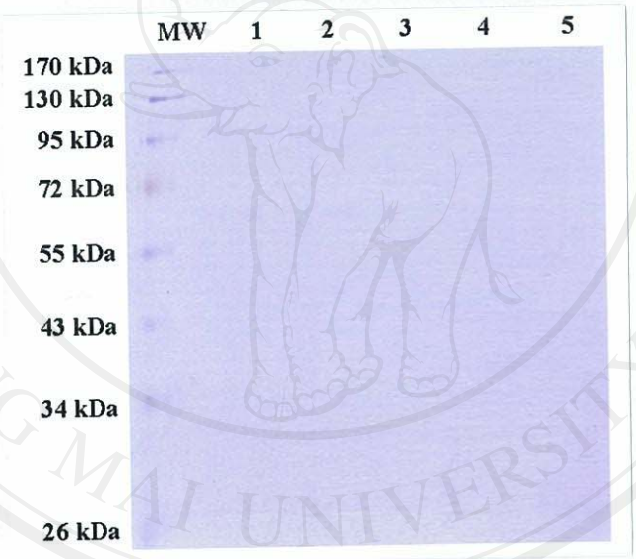


(b)

Figure 15 Crude protein of MRS broth extracted by ammonium sulfate precipitation, (a) protein increasing folds, (b) crude proteins. Lane MW, molecular weight marker (Page Ruler™; Fermentas INC); lane 1, MRS broth; lane 2, crude proteins by 20% ammonium sulphate; lane 3, crude proteins precipitated by 40%; ammonium sulphate; lane 4, crude proteins precipitated by 60% ammonium sulphate and lane 5, crude proteins precipitated by 80% ammonium sulphate.

2.5 Total protein assay of crude bacteriocins

The total protein concentrations of crude bacteriocins extracted from these isolates and crude proteins extracted from MRS broth were determined by BCA protein assay kit. The total protein concentrations of B85/4, B282, B63/8's crude bacteriocins and MRS's crude proteins were 35.0, 26.9, 28.6 and 6.4 mg/ml, respectively.

2.6 One-dimensional polyacrylamide gel electrophoresis

The protein patterns in SDS-PAGE of three potent bacteriocins were shown in Figure 16. There was no protein band of MRS broth precipitant (lane 1). The major proteins bands of B282, B85/4 and B63/8 were observed at 95, 87, 46 and 42 kDa (lane 2); 93, 83, 46, 42 and 38 kDa (lane 3); 83 and 45 kDa (lane 4), respectively.

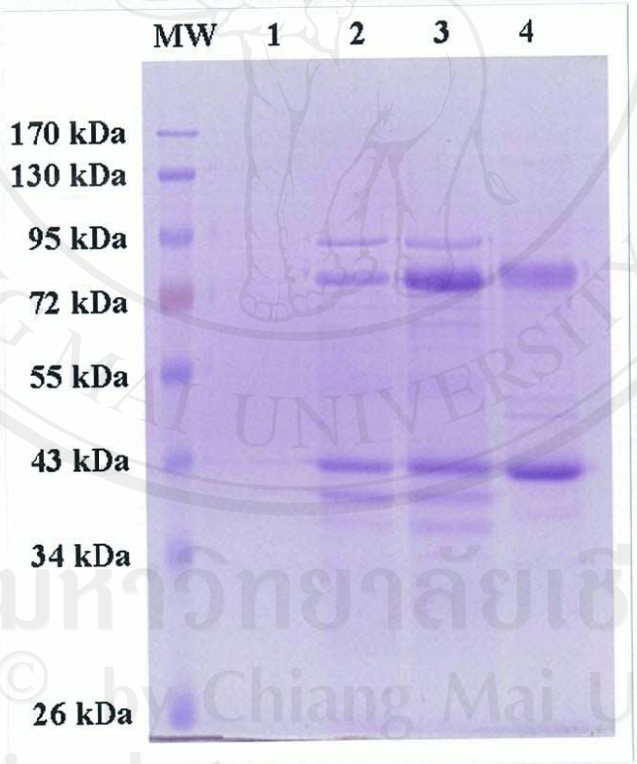


Figure 16 The protein patterns of each crude bacteriocins from ammonium sulphate precipitation; lane MW, molecular weight marker (Page Ruler™; Fermentas INC); lane 1, crude proteins extracted from MRS broth; lane 2, crude bacteriocins extracted from B282 isolate; lane 3, B85/4 and lane 4, B63/8

2.7 Two-dimensional polyacrylamide gel electrophoresis

The protein patterns in 2D-PAGE of three crude bacteriocins were shown in Figure 17-19. Their molecular weights were demonstrated in a range of between 35-90 kDa and their charges were located in both cationic and anionic zones. In the anionic zone, pI of major cluster of proteins was 4 - 6 and minor cluster was 3 - 4. In the cationic zone, the slight protein spots were found at a range of pI 8 - 9.

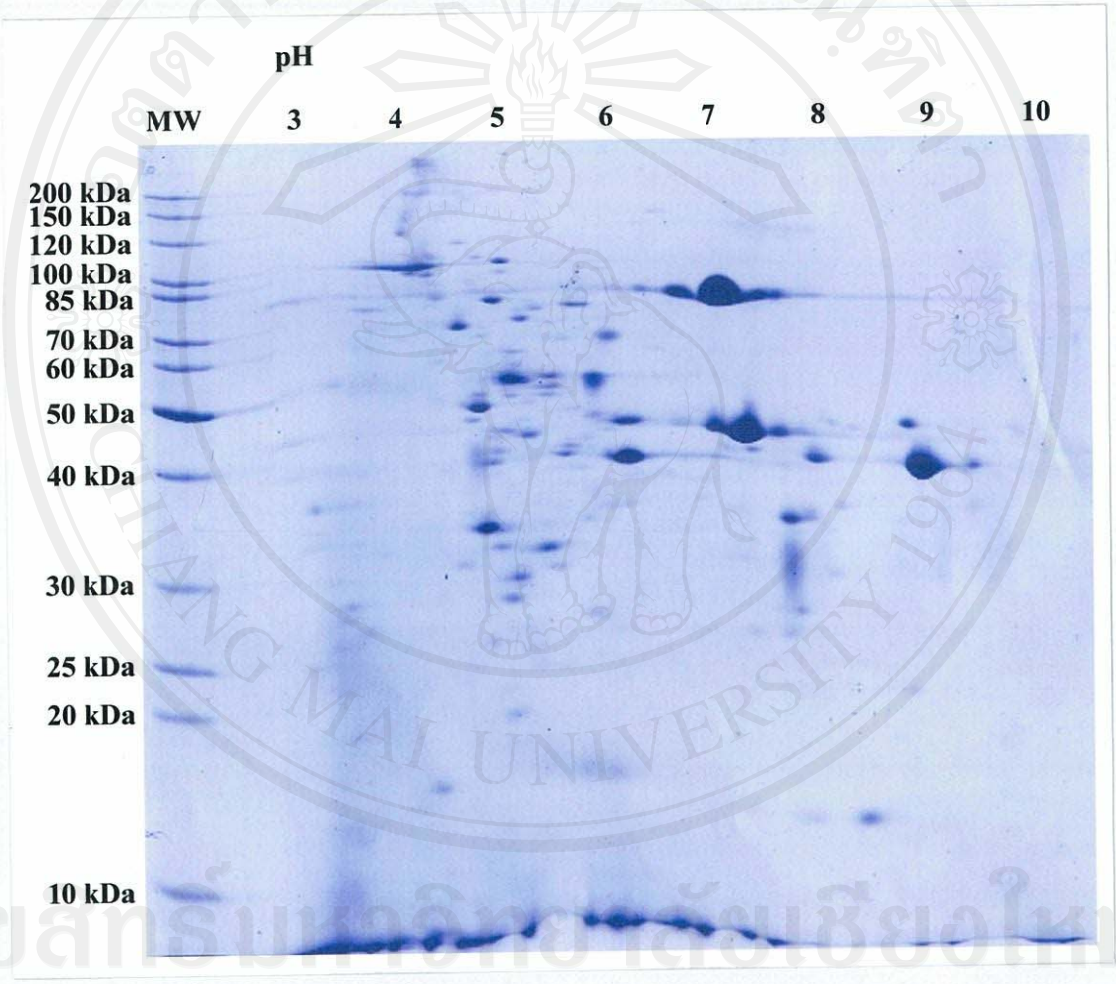


Figure 17 The proteins pattern of B85/4's crude bacteriocins observing by 2D-PAGE. These crude bacteriocins were extracted from the cell-free supernatant of B85/4.

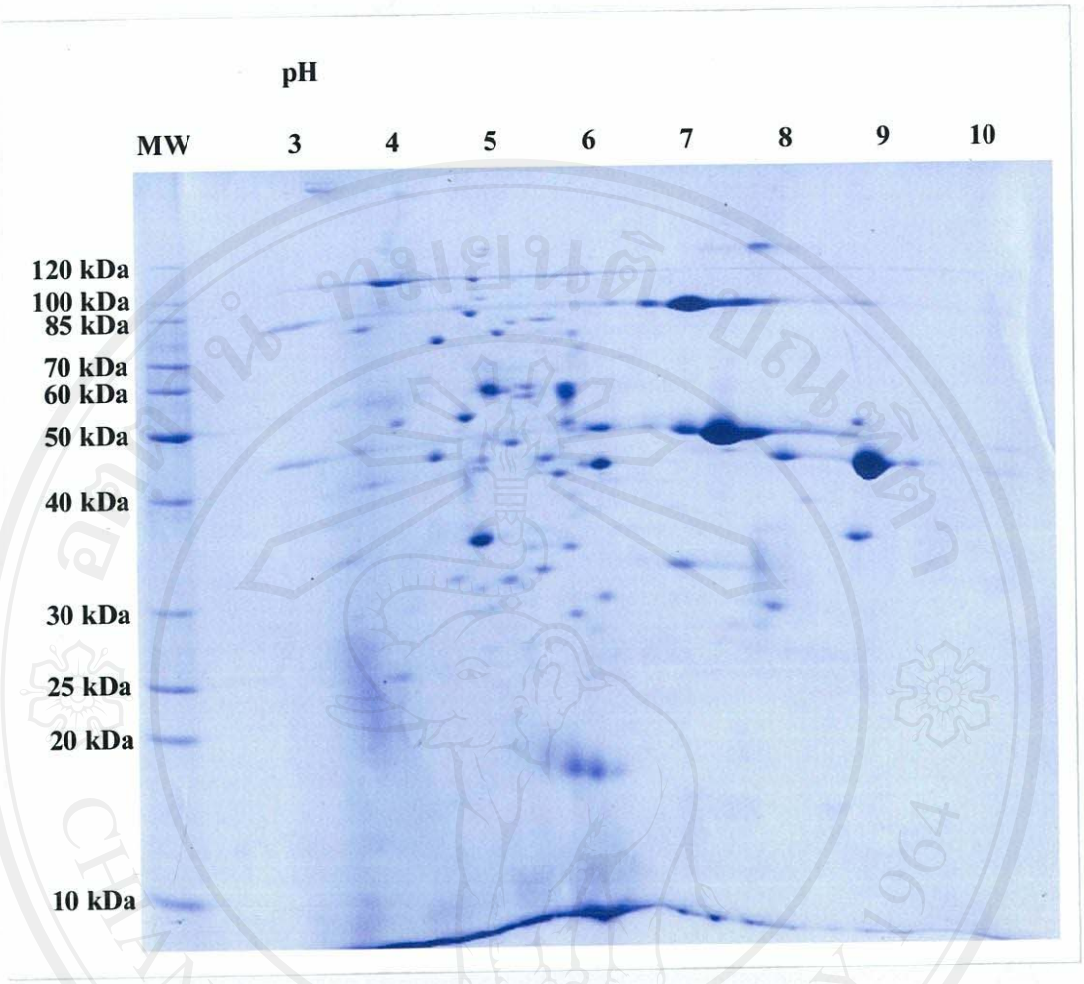


Figure 18 The proteins pattern of B282 crude's bacteriocins observing by 2D-PAGE. These crude bacteriocins were extracted from the cell-free supernatant of B282.

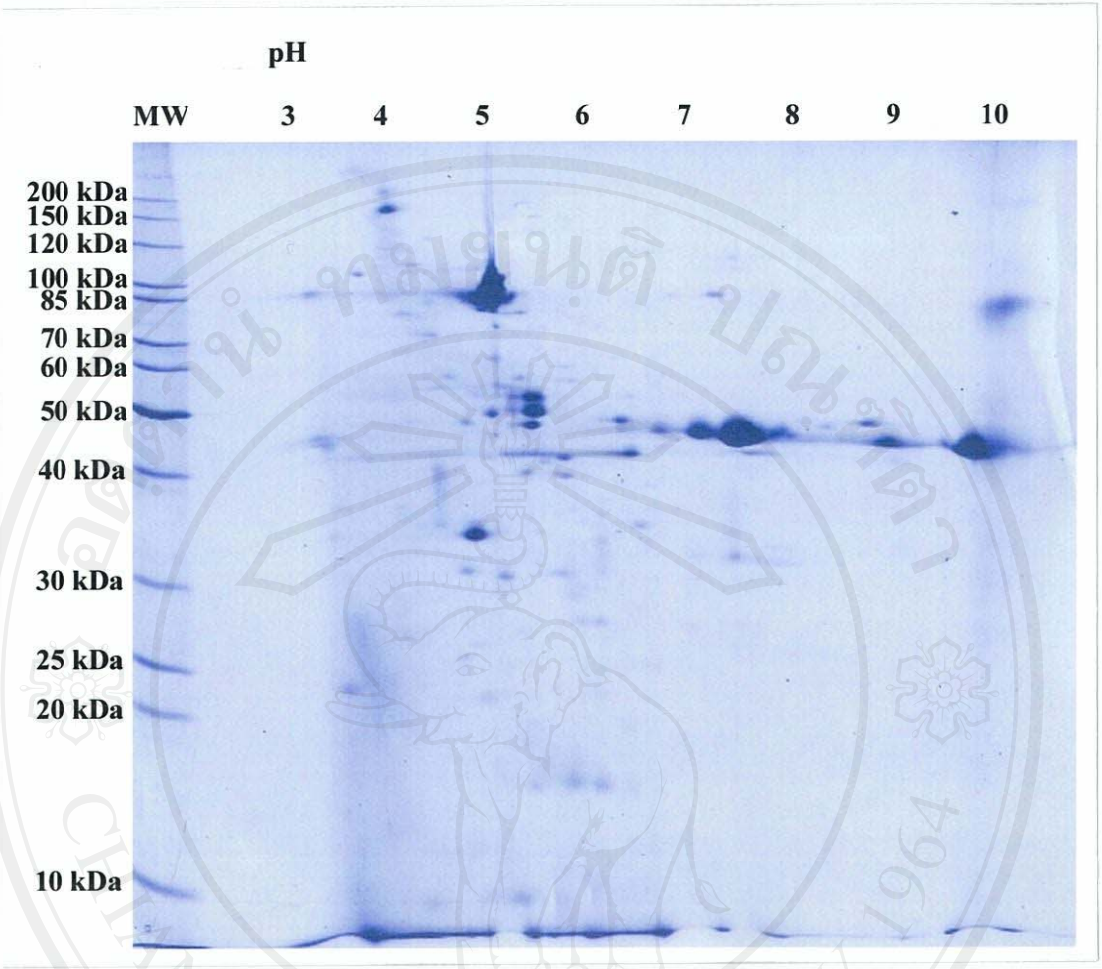


Figure 19 The proteins pattern of B63/8's crude bacteriocins observing by 2D-PAGE. These crude bacteriocins were extracted from the cell-free supernatant of B63/8.

3. Partial purification of crude bacteriocins

3.1 Anion exchange column chromatograms

According to an anion exchange column chromatography, the eluted fractions were demonstrated in the purification chromatograms (Figure 20-23). The unbound fractions, including cationic proteins and other impurities were eluted out from the column at the fractions 3-5 (A2-A4). The first anionic peak of each isolate was demonstrated in the second segmented gradient elution (55% NaCl of buffer B) with the detectable level of 3,300, 5,000 and 3,300 mAU for B85/4, B282 and B63/8, respectively. These anionic peaks were collected in the manners of the pooled fraction 23-27 (C1-C5) or called as front peak and the pooled fraction 28-32 (C6-C10) or called as back peak. The second anionic peak of each isolate was detected in the third segmented gradient elution (100% NaCl of buffer B) with the detectable level of 250, 400 and 500 mAu for B85/4, B282 and B63/8, respectively. These anionic peaks were collected as the pooled fraction 47-52 (E2-E7). All pooled fractions were carried out to concentrate, run in SDS-PAGE, determine the antimicrobial activity, assayed the protein quantity before determining the bacteriocin units. Total protein concentration of each pooled fraction was shown in the Table 14. Their protein patterns were shown in SDS-PAGE gel (Figure 24-25).

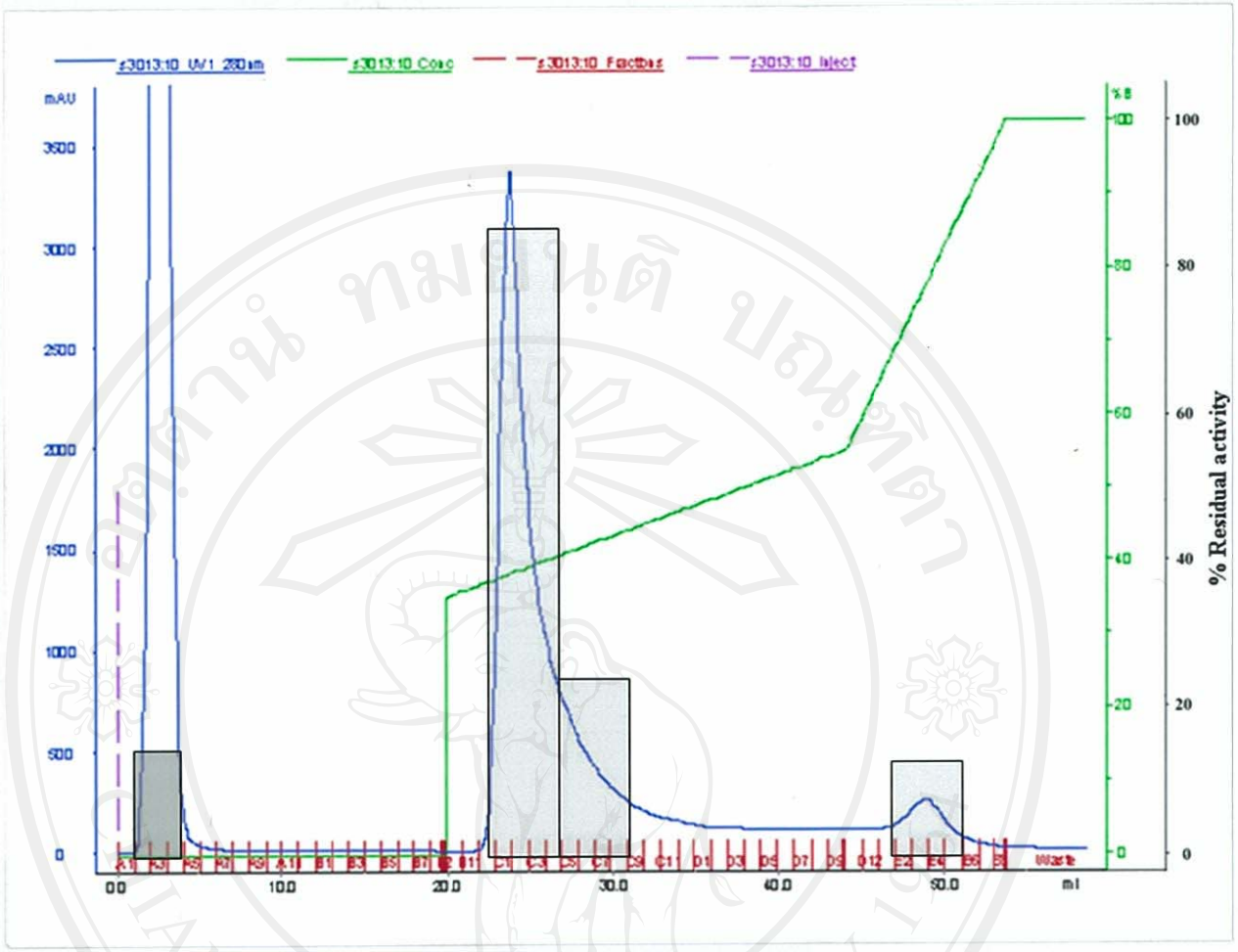


Figure 20 The purification chromatograms of the B85/4's bacteriocins according to an anion exchange column chromatography. Blue line represents the absorbance value in mAU at 280 nm related to the left Y axis; green line represents the percentage of NaCl in eluting buffer B related to the green Y axis; gray bars represent the percentage of residual antimicrobial activity related to the black Y-axis and red X-axis represents each eluted fraction.

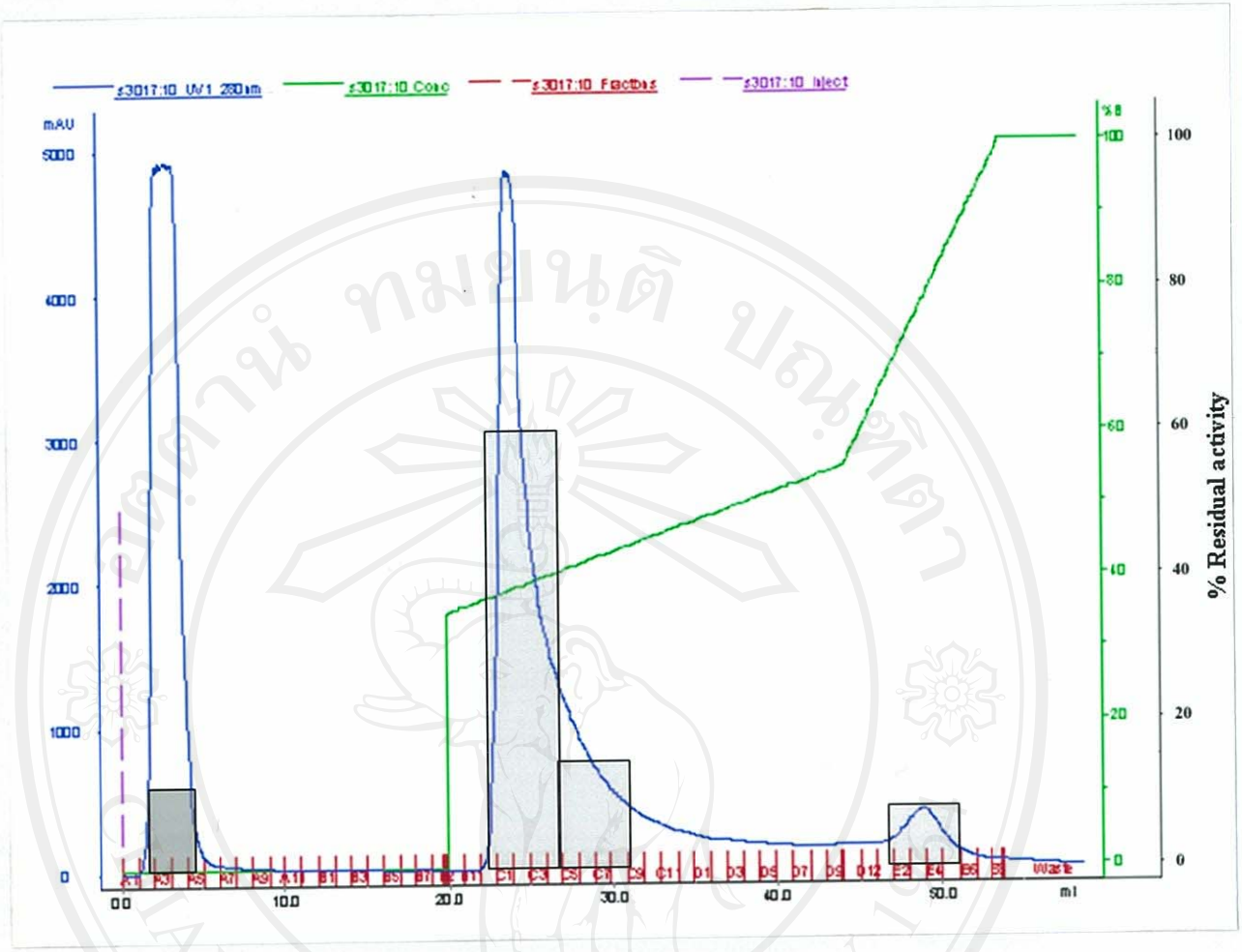


Figure 21 The purification chromatograms of the B282's bacteriocins according to an anion exchange column chromatography. Blue line represents the absorbance value in mAU at 280 nm related to the left Y axis; green line represents the percentage of NaCl in eluting buffer B related to the green Y axis; gray bars represent the percentage of residual antimicrobial activity related to the black Y-axis and red X-axis represents each eluted fraction.

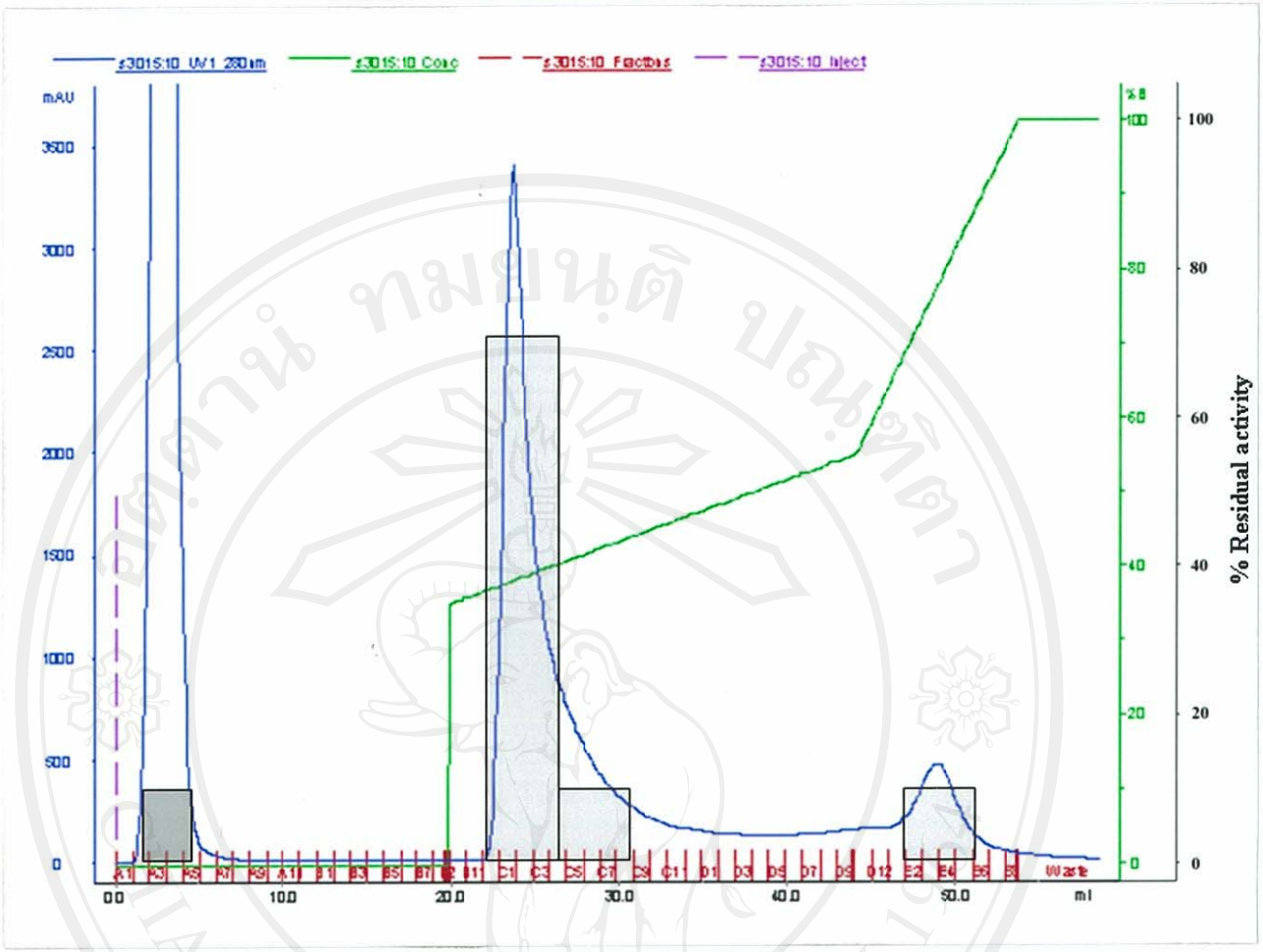


Figure 22 The purification chromatograms of the B63/8's bacteriocins according to an anion exchange column chromatography. Blue line represents the absorbance value in mAU at 280 nm related to the left Y axis; green line represents the percentage of NaCl in eluting buffer B related to the green Y axis; gray bars represent the percentage of residual antimicrobial activity related to the black Y-axis and red X-axis represents each eluted fraction.

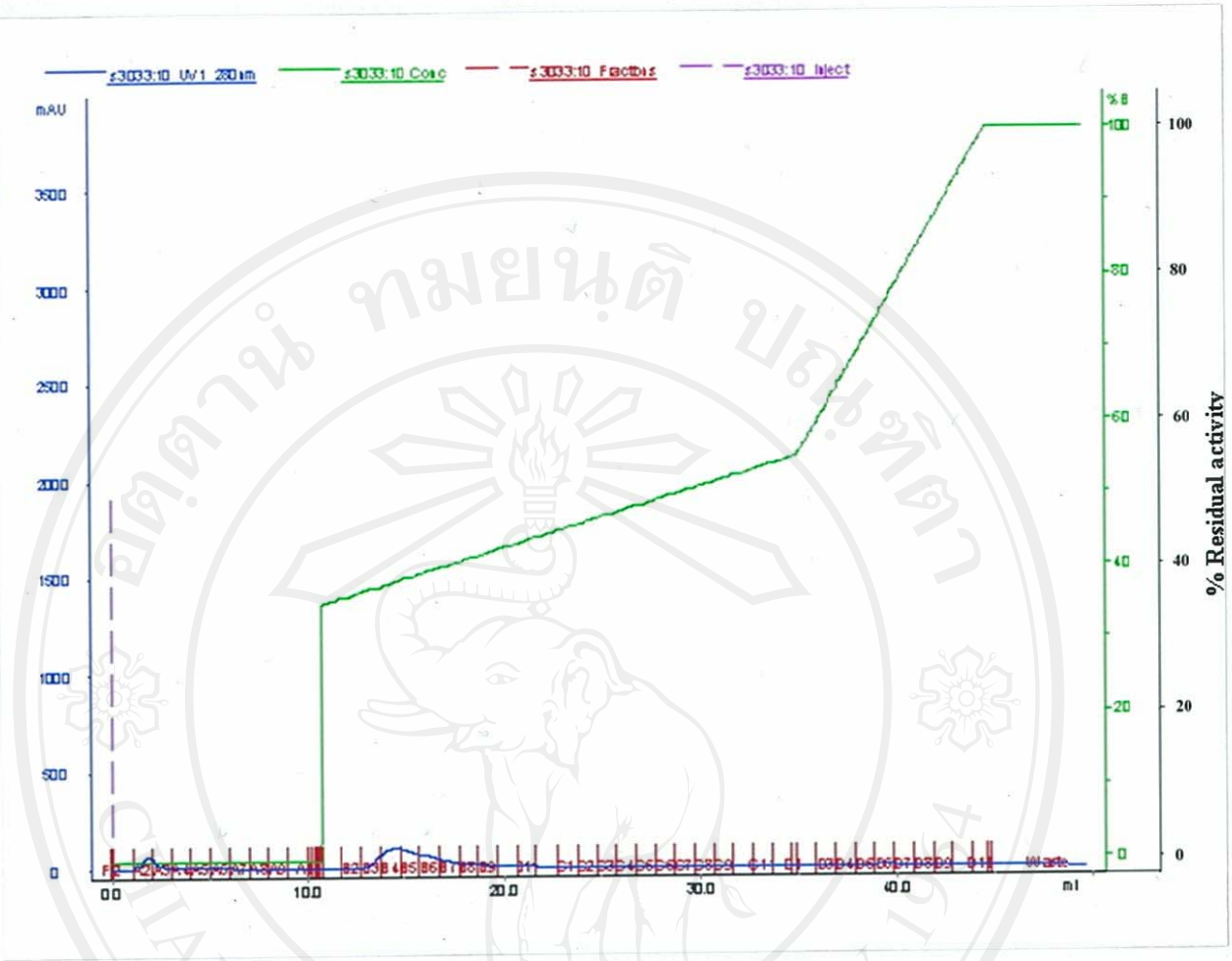
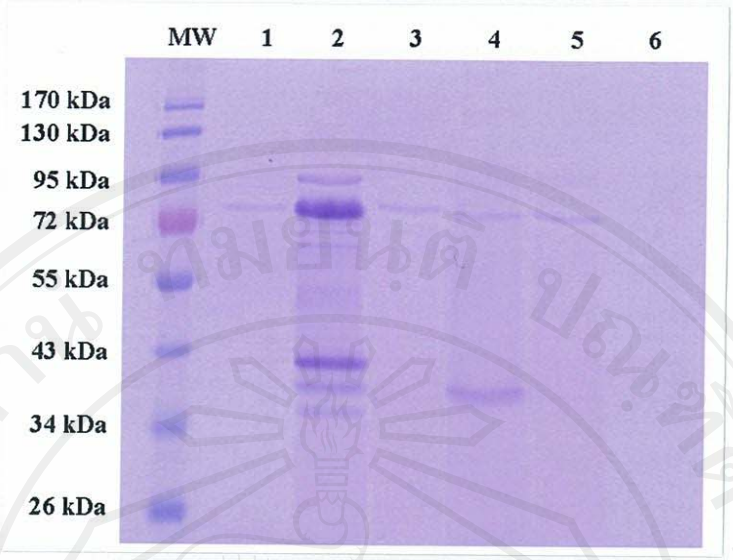


Figure 23 The purification chromatograms of the MRS's crude proteins according to an anion exchange column chromatography. Blue line represents the absorbance value in mAU at 280 nm related to the left Y axis; green line represents the percentage of NaCl in eluting buffer B related to the green Y axis; black Y-axis represent the percentage of residual antimicrobial activity and red X-axis represents each eluted fraction.

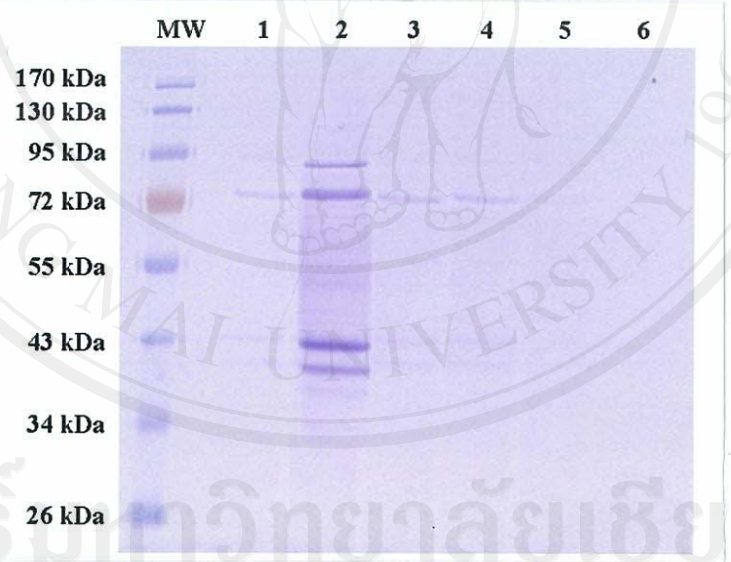
Table 14 Total protein concentration of each pooled fraction of the partially purified bacteriocin by using BCA protein assay kit

	Bacteriocin fractions	Total protein concentration (mg/ml)
B85/4	cell-free supernatant	3.5
	crude bacteriocins	35.0
	unbound fraction	15.8
	pooled fraction 23-27	16.9
	pooled fraction 28-32	0.6
	pooled fraction 47-52	ND
B282	cell-free supernatant	2.9
	crude bacteriocins	26.9
	unbound fraction	16.4
	pooled fraction 23-27	12.8
	pooled fraction 28-32	3.5
	pooled fraction 47-52	0
B63/8	cell-free supernatant	6.4
	crude bacteriocins	28.6
	unbound fraction	16.4
	pooled fraction 23-27	12.8
	pooled fraction 28-32	ND
	pooled fraction 47-52	ND
MRS broth	cell-free supernatant	4.7
	crude proteins	6.4
	unbound fraction	ND
	pooled fraction 16-21	ND

ND; not detectable (The result gave a minus value in the analysis.)

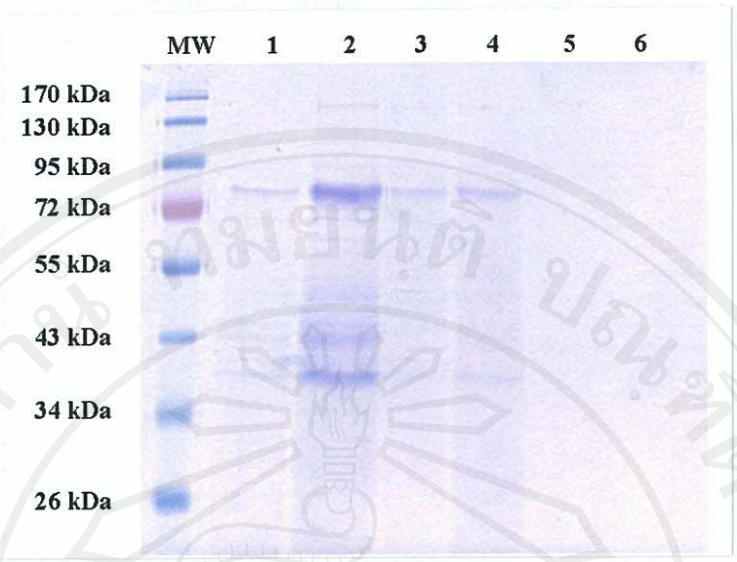


(a)

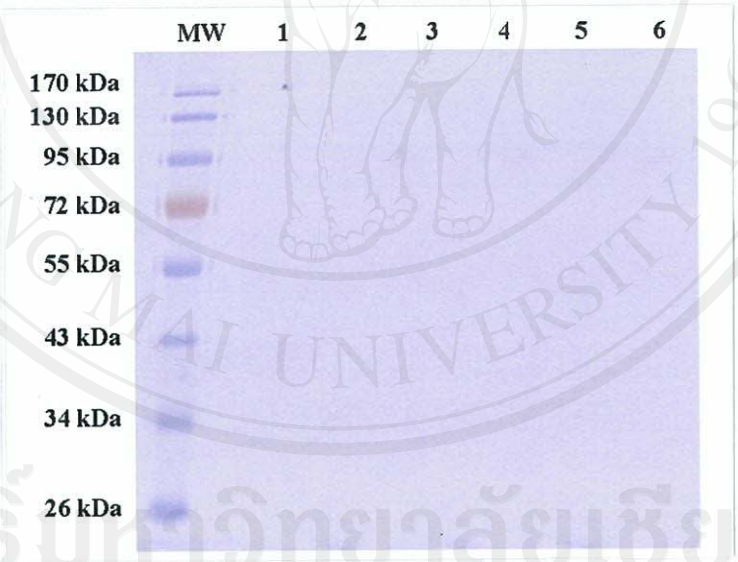


(b)

Figure 24 The protein patterns of the partially purified bacteriocins extracted from (a) B85/4 and (b) B282. Lane MW, Molecular weight marker (Page Ruler™; Fermentas INC); lane 1, cell-free supernatant; lane 2, crude precipitated; lane 3, unbound fractions; lane 4, the pooled fraction 23-27; lane 5, the pooled fraction 28-32; lane 6, the pooled fraction 47-52



(a)



(b)

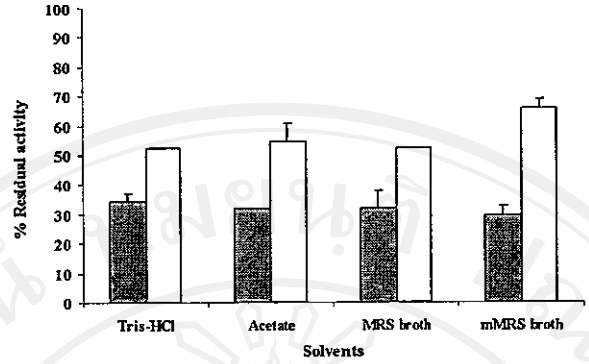
Figure 25 The protein patterns of the partially purified bacteriocins extracted from (a) B63/8 and (b) MRS. Lane MW, Molecular weight marker (Page Ruler™; Fermentas INC); lane 1, cell-free supernatant; lane 2, crude precipitated; lane 3, unbound fractions; lane 4, the pooled fraction 23-27; lane 5, the pooled fraction 28-32; lane 6, the pooled fraction 47-52

3.2 Determination of the antimicrobial activity

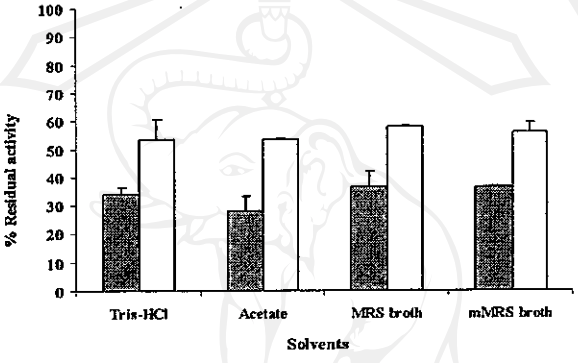
The percentage of residual antimicrobial activity of each pooled fraction was determined by using agar-cup diffusion method. The highest percentage was demonstrated in the pooled fraction 23-27 (C1-C5) after compared with the freshly prepared cell-free supernatant of each lactobacillus isolate which exhibited 100% residual antimicrobial activity. They were shown in Figure 20-22. The pooled fractions 23-27 (C1-C5) of all isolates were carried to the further study.

3.3 Effect of the solvent towards antimicrobial activity

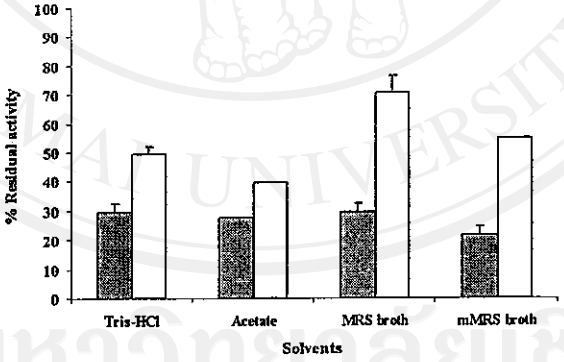
Among three isolates, the stronger antimicrobial activities of the partially purified bacteriocins were detected in the anionic pooled fraction 23-27 in various solutions (Figure 26). Among various solvents, B282 and B63/8's bacteriocins which dissolved in MRS broth and B85/4's bacteriocins which dissolved in mMRS broth were exhibited the strongest residual antimicrobial activity after compared with the freshly prepared cell-free supernatant (100% residual activity) and the unbound fraction in each solvent. These bacteriocins in the appropriate solvent as described above were carried out to determine their bacteriocin unit against *P. gingivalis* W50.



(a)



(b)



(c)

Figure 26 The percentage of residual activity of unbound fraction (gray bars) and pooled fractions 23-27 (white bars) of (a) B85/4, (b) B282, (c) B63/8 in each solvent after compared with the freshly prepared cell-free supernatant of each isolate. The percentage of activity of the freshly prepared cell-free supernatant was 100%.

3.4 Determination of bacteriocin unit

The supernatant, crude bacteriocins and pooled fractions 23-27 of each isolate were determined the bacteriocin unit in the appropriate diluent. Results of the bacteriocin purification steps, bacteriocin unit and activity recovered of these bacteriocins were demonstrated in Table 15. Bacteriocin titers in cell-free supernatant, crude bacteriocins and pooled fractions 23-27 of B85/4 that could inhibit 50% growth of *P. gingivalis* were 158, 55 and 485. Likely to B85/4, bacteriocin titers of B282 and B63/8 were 255, 3 and 363 and 258, 14 and 363, respectively. These titers were performed to calculate the bacteriocin activity according to the bacteriocin equation (see Appendix D). Among these isolates, high bacteriocin activity was found in the pooled fractions 23-27. The bacteriocin activities, total activities, specific activities, purification folds and activity recovered of B85/4, B282 and B63/8's pooled fractions were shown in Table 15. From these results, B85/4's pooled fractions 23-27 demonstrated the strongest bacteriocin activity in this study. It was selected to study the time killing assay towards *P. gingivalis* W50.

Table 18 Bacteriocin purifications of *L. paracasei* subsp. *paracasei* strain B85/4, B282 and B63/8

Isolate	Sample	Volume (ml)	Bacteriocin activity ($\times 10^2$ BU/ml)	Total activity ($\times 10^4$BU)	Protein concentration (mg/ml)	Total protein ($\times 10^1$mg)	Specific activity ($\times 10^1$ BU/mg)	Purification folds ($\times 10^0$)	Activity (%)***
B85/4*	Cell-free supernatant	475.00	6.65	31.60	18.00	855.00	36.96	1.00	100.00
	Crude bacteriocins	12.00	91.57	11.00	35.00	42.00	26.16	7.08	34.78
	Pooled fraction 25-29	5.40	1,794.96	96.93	16.90	9.13	1,062.11	287.37	306.73
B282**	Cell-free supernatant	475.00	6.65	31.60	14.50	688.75	4.58	1.00	100.00
	Crude bacteriocins	12.00	2.20	2.64	26.90	32.28	8.18	1.78	8.35
	Pooled fraction 25-29	5.40	194.00	10.48	12.80	6.91	151.56	33.03	33.15
B63/8**	Cell-free supernatant	475.00	8.42	40.00	18.30	869.25	4.60	1.00	100.00
	Crude bacteriocins	12.00	82.33	9.88	28.60	34.32	28.79	6.26	24.70
	Pooled fraction 25-29	5.40	1,686.59	91.08	12.80	6.91	1,317.65	286.34	227.69

*mMRS broth were performed as the appropriate diluent

** MRS broth were performed as the appropriate diluent

*** activity recovered

3.5 Determination of minimum inhibitory concentration (MIC)

MIC values of B85/4's supernatant, partially purified bacteriocin and ampicillin against *P. gingivalis* W50 were 9.0, 4.1 mg/ml and 1.5 μg/ml, respectively.

3.6 Time-killing assay

The time-killing assay was performed to assess the minimal exposure time between the active compound and the tested bacteria in a period of 12 hrs. MIC of cell-free supernatant, partially purified bacteriocin, and 2-folds MIC of partially purified bacteriocin were carried out to determine the minimal exposure time. MIC of ampicillin was performed in parallel. The killing time was determined at the 99.9% growth inhibition compared with viable count of *P. gingivalis* W50 in each time. The results of killing time were shown in Table 16. The killing time of ampicillin, cell-free supernatant, MIC of the partially purified bacteriocin and 2-folds MIC of the partially purified bacteriocin were 3, 12, 6 and 6 hrs.

Table 16 The killing times of ampicillin, B85/4's cell-free supernatant, MIC of the partially purified bacteriocin 2-folds MIC of the and partially purified bacteriocin

Time	% Growth inhibition			
	Ampicillin (1.5 μg/ml)	Cell-free supernatant (9 mg/ml)	Partially purified bacteriocin (4.1 mg/ml)	Partially purified bacteriocin (8 mg/ml)
0 min	0	0	0	0
30 mins	93.68	31.58	31.58	49.12
1 hr	76.36	69.09	66.36	57.27
2 hrs	93.00	62.50	83.75	52.50
3 hrs	99.90	98.75	99.78	99.88
6 hrs	100	99.57	99.97	99.98
12 hrs	100	99.90	99.99	99.99