Antimicrobial Peptides Derived From Lysozymes

การศึกษาเพปไทด์ที่มีฤทธิ์ต้านเชื้อแบคทีเรียจากไลโซไซม์

Yuwatida Pukcothanung (ยุวธิดา ปักโคทานัง)[1]
Somruedee Booncharoen (สมฤดี บูรณ์เจริญ)[1]
Sompong Thammasirirak (สมปอง ธรรมศิริรักษ์)[2]*

Abstract

This work aimed at the purification and characterization of antimicrobial peptides from C-type lysozyme (soft shelled turtle lysozyme; SSTL) and G-type lysozyme (goose egg white lysozyme; GEWL) which were purified and then hydrolyzed by pepsin and trypsin. The hydrolysated peptides were purified by HPLC techniques. The tryptic peptides derived from SSTL showed broad spectrum activity to both Gram-positive bacteria (*S. aureus and S. epidermidis*) and Gram-negative bacteria (*E. coli, K. pneumoniae and V. cholerae*). In the same manner, tryptic peptides from GEWL demonstrated effective activity against *S. epidermidis, S. typhi* and *V. cholerae*. The amino acid sequence of tryptic peptides from SSTL were deduced by MALDI-TOF mass spectrometry. One peptide had the sequence I-V-S-D-G-D-G-M-N-A-W in the residues 98-108. Another peptide was determined to have the sequence H-G-L-D-N-Y-R corresponding to the amino acid residues 15-21 of lysozyme. Further, the primary structure of tryptic peptides from GEWL is currently under evaluation. The results obtained in this work indicate that C-type and G-type lysozymes possess antimicrobial peptides in their primary structure and they can be released by protease hydrolysis.

บทคัดย่อ

งานวิจัยนี้ได้ศึกษาเพปไทด์ที่มีฤทธิ์ต้านเชื้อแบคทีเรียจากไลโซไซม์ 2 ชนิดคือ ไลโซไซม์ชนิด C จากไข่ขาวของตะพาบน้ำและไลโซไซม์ชนิด G จากไข่ขาวของห่าน พบว่าเพปไทด์จากไลโซไซม์ทั้ง 2 ชนิด สามารถยับยั้งหรือทำลายแบคทีเรียได้ดีทั้งชนิดแกรมบวกและแกรมลบ โดยการนำไลโซไซม์ทั้ง 2 ชนิดมาตัดด้วยเอนไซม์เพปซิน และเอนไซม์ทริปซิน จากนั้นแยกเพปไทด์ที่ได้ด้วยเทคนิค HPLC และนำเพปไทด์ที่แยกได้มาทดสอบผลการยับยั้งการเจริญเติบโตของแบคทีเรีย ซึ่งพบว่าเพปไทด์ที่ได้จากไลโซไซม์ทั้ง 2 ชนิด สามารถยับยั้งการเจริญเติบโตของแบคทีเรียได้ทั้งแกรมบวกและแกรมลบ จากนั้นนำเพปไทด์นี้มาหาลำดับกรดอะมิโนโดยแมสสเปคโตรมิตรี พบว่าเพปไทด์ที่ได้จากไลโซไซม์ชนิด C มีลำดับกรดอะมิโนเป็น I-V-S-D-G-D-G-M-N-A-W และอีกหนึ่งเพปไทด์มีลำดับกรดอะมิโนเป็น H-G-L-D-N-Y-R ส่วนลำดับกรดอะมิโนของเพปไทด์ที่ได้จากไลโซไซม์ชนิด G กำลังอยู่ในระหว่างการศึกษา

Keywords: Soft shelled turtle lysozyme, Goose egg white lysozyme, antimicrobial peptide
คำสำคัญ: ไลโซไซม์จากไข่ขาวของตะพาบน้ำ ไลโซไซม์จากไข่ขาวของห่าน เพปไทด์ที่มีฤทธิ์ต้านเชื้อแบคทีเรีย

[1]*Graduate student, Department of Biochemistry, Faculty of Science, Khon Kaen University, Thailand*
[2]*Assistant professor, Department of Biochemistry, Faculty of Science, Khon Kaen University, Thailand*
**corresponding author, e-mail: somkly@kku.ac.th*

Introduction

Over many years, the continuous use of conventional antibiotics has resulted in multi-resistant bacterial strains all over the world. This phenomenon has accelerated the development of alternative drugs and/or natural materials that can control pathogen-caused diseases. The materials should be as natural as possible, with broad-spectrum antibiotics and a wide rage of action over numerous pathogens (Marshall et al., 2003 and Dubin et al., 2005). Several studies have emphasized antimicrobial peptides as extremely interesting; to date clinical Phase I and Phase II trials have shown a limited resistance for the bacterial strains tested (Zasloff, 2002).

The antimicrobial peptides are a large group of short peptides which naturally exhibit antimicrobial activity. They are an abundant and diverse group of molecules that are generated from many sources in a variety of invertebrate, plant and animal species (Brogden, 2005). Moreover, antimicrobial peptides also involve the discovery of antimicrobial peptides released upon the digestion or breakdown of protein, such as from ovalbumin (Pellegrini et al., 2004), ovotransferrin (Ibrahim et al., 2000), lactoferricin (Farnaud et al., 2004), and various lysozyme peptides released from the proteolytic digestion of lysozyme (Düring et al., 1999; Mine et al., 2004; Ibrahim et al., 2005).

Lysozyme (N-acetylmuramide glyconohydrolase, (EC 3.2.1.17)) is one of the best characterized hydrolases. It cleaves β-1,4 linkages of GlcNAc homopolymer and $(GlcNAc-MurNAc)_n$ heteropolymer, which causes lysis of the bacteria containing these polymers in the cell wall. Lysozyme is considered to be a self-defense enzyme which is produced in serum, mucus and many organs of vertebrates. Lysozymes have been classified into three distinct forms by their amino acid sequences and tertiary structures, namely, C-type (Canfield, 1963; Jollès et al., 1963; Blake et al., 1965), T_4-type (Inoue et al., 1970; Matthews and Remington, 1974) and G-type (Grütter et al., 1979; Simpson et al., 1980; Weaver et al., 1985). This enzyme group is effective against Gram-positive bacteria but it is largely ineffective against Gram-negative bacteria. Thus, to change the bacteriocidal activity of lysozyme to Gram-negative bacteria, the antimicrobial peptides derived by protease digestion of this enzyme need to be studied.

Recently, there have been reports about antimicrobial peptides from several types of lysozymes that are effective against Gram-negative bacteria, without lytic activity (Ibrahim et al., 2001). An internal peptide (residues 98-112) has been found in hen egg white lysozyme (C-type), obtained after digestion with clostripain, which possesses broad spectrum antimicrobial action in vitro. This internal peptide is part of a helix-loop-helix domain (87-114 sequence of hen lysozyme) located at the upper lip of the active site cleft of lysozyme. Furthermore, Mine et al. (2004) reported two derived antimicrobial peptides from hen egg white lysozyme hydrolysate, obtained by peptic digestion and subsequent tryptic digestion. These peptides are located in a part of the helix. Therefore, in this study, we aim to purify C-type lysozyme from soft shelled turtle egg white and G-type lysozyme from goose egg white by cation exchange column chromatography and search for new antimicrobial peptides derived from protease digestion of two lysozymes.

Material and Methods

Material. Freshly laid soft shelled turtle (*Trionyx sinensis Chinese*) eggs were purchased from a local breeding farm at Chonburi province. Goose eggs were purchased from a local farm at Samutprakan province. The enzymes pepsin (EC 3.4.23.1, activity 2,500-3,500 unit/mg) extracted from porcine stomach mucosa (Sigma Chemical Co.) and trypsin (TPCK treated, EC 3.4.21.4, activity 7490 u/mg) extracted from bovine pancreas (Fluka Biochemica) were purchased from Sigma.

Microorganisms. Bacterial strains used in this study were *Staphylococcus aureus*, *Salmonella typhi*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Klepsiella pneumoniae*, *Vibrio cholerae* and *Escherichia coli* (clinical isolates). All bacteria strains were maintained in nutrient agar at 4 °C.

Purification of soft shelled turtle lysozyme (SSTL). Egg white was diluted with two volumes of 0.03 M phosphate buffer, pH 7.0 and stirred at 4 °C for 30 min. The homogenate was centrifuged at 12,000 x g for 15 min. The supernatant was used as crude extract, and subjected to isoelectric precipitation at pH 4.0 and pH 7.0. At each step of pH treatment, the solution was adjusted to the desired pH using 1.0 M HCl or 1.0 M NaOH and incubated at 4 °C for 1 h and then centrifuged at 12,000 x g for 30 min. The final clarified supernatant was applied to a CM-Toyopearl 650 M cation exchange column (size 1.3 x 90 cm), equilibrated with 0.03 M phosphate buffer, pH 7.0. The column was washed with the same buffer and the adsorbed protein was eluted with a linear gradient of NaCl concentration from 0.0 to 0.25 M in the same buffer, at a flow rate of 15 ml/h. Then the active fractions were pooled and dialyzed against distilled water. The enzyme was lyophilized for use as the purified enzyme.

Purification of goose egg white lysozyme (GEWL). Goose egg white was diluted three times with 0.05 M phosphate buffer, pH 7.0 and stirred at 4 °C for 30 min. The homogenate was centrifuged at 10,000 x g for 15 min, 4 °C. The resulting supernatant was used as crude extract for protein purification. The crude extract was precipitated at pH 4.0 and pH 7.0, with adjustment to pH 4.0 and pH 7.0 effected using 1.0 M HCl followed by 1.0 M NaOH and mixing for 1 h at 4 °C. The solution was then centrifuged at 10,000 x g for 30 min. Then the supernatant was applied to a CM-Toyopearl 650 M cation exchange column chromatography (size 2.5 x 85 cm), previously equilibrated with 0.05 M sodium phosphate buffer pH 7.0. After washing the column with the same buffer, it was eluted stepwise by changing NaCl concentration to 0.1, 0.2, 0.3 and 0.5 M in 0.05 M sodium phosphate buffer pH 7.0 at a flow rate of 30 ml/h. Fractions exhibiting lysozyme activity at 540 nm were measured, collected and then dialyzed against distilled water several times and concentrated by lyophilization. The enzyme was re-chromatographed on the same cation exchange column chromatography (size 2.2 x 105 cm.). The column was washed with the same buffer and eluted with a linear gradient of NaCl concentration from 0 to 0.4 M in the same buffer at a flow rate of 15 ml/h. The fractions exhibiting lysozyme activity were pooled and dialyzed against distilled water several times. The enzyme was lyophilized to use as the purified enzyme.

Muramidase activity assay. Muramidase activity was assayed using the lyophilized cell of *Micrococcus lysodeikticus* (ATCC 4618, Sigma) as a substrate. The substrate was suspended in 0.1 M sodium phosphate buffer pH 7.0, adjusted to O.D. 0.800 –

1.000 at 540 nm. 100 ml of enzyme solutions were added to 3 ml of the substrate solution. The activity was evaluated as the decrease of absorbance at 540 nm after 3 min. One enzyme unit was defined as the amount causing a decrease of 0.1 absorbance units at 540 nm in the reaction for 1 min at 25 °C.

Molecular mass measurement. To examine the purity of the lysozyme preparation and to obtain a molecular mass, sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970), using 4% (w/v) stacking and 12.5% (w/v) resolving gel. The protein bands were stained with Coomassie brilliant blue R-250 (CBB). The low molecular weight calibration kit for SDS electrophoresis (Amersham Biosciences, U.S.A) was used as standard molecular weight marker.

Enzymatic hydrolysis of lysozymes. Two lysozymes were digested with pepsin followed by trypsin. The native SSTL was initially dissolved in 0.03 M HCl pH 1.0 and digested with 1:20 (w/w) of pepsin at 37 °C for 4 h. After that, the enzymatic reaction was stopped by adjusting the pH to 8.0 with 0.5 M Na_2CO_3 solution. Then the peptic digest was further digested with 1:250 (w/w) of trypsin at 37 °C overnight and then heated at 90 °C for 10 min. For the GEWL, it was heat-denatured at 80 °C for 10 min. Then the digestion was performed by pepsin at pH 2.0, ratio 1:25 (w/w). After that, the reaction was treated in the same manner with SSTL. The SSTL hydrolysate was centrifuged at 5,000 x g for 10 min. The precipitate and supernatant were collected and each part was tested for antimicrobial activity using the growth inhibition assay. On the other hand, the insoluble solids of GEWL were removed by centrifugation at 5,000 x g for 30 min and the resulting supernatants were aliquots for testing antimicrobial activity and were freeze-dried for further purification by HPLC.

Tris-tricine SDS-PAGE. Tris-tricine ready gel, 16.5% acrylamide (Amersham Biosciences) was used to resolve the peptides and small proteins produced during proteolytic digestion of lysozymes. Sample were mixed with a sample buffer composed of 10% (v/v) 1.0 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.2% (w/v) Bromophenol Blue and 2% (v/v) 2-mercaptoethanol (ratio sample-to-sample buffer of 1:1 (v/v)). Each sample (20 µl) was loaded into a well. Electrophoresis was carried out at 120 volt at room temperature, in running buffer; cathode buffer containing 0.1 M Tris, 0.1 m tricine and 0.1% SDS, anode buffer, pH 8.9 containing 0.2 M Tris. The protein band was stained by a silver stain kit (Amersham Biosciences, U.S.A.).

Growth inhibition assay. Antimicrobial activity assay was performed in growth inhibitor in microtiter cell-culture plates. Mid-logarithmic phase cells grown in Mueller Hinton Broth (MHB) were diluted in the same medium to OD 0.001 at absorbance 600 nm (2X concentrate). The bacterial suspension (100µl) was mixed with an equal volume of peptide (dissolved in distilled water). Then the reaction was incubated for 18-24 h at 37 °C. After incubation, the absorbance at 550 nm was determined using a microtiter plate reader with 3 h time interval until 24 h.

Purification of antimicrobial peptides. Lyophilyzed lysozyme hydrolysates of supernatant from protease digestion were isolated using C18 reversed-phase high performance liquid chromatography (RP-HPLC). Both enzyme hydrolysates were dissolved in deionized-water and applied to a C18

reverse phase column (Apollo C18 5u; size 4.6 x 250 mm). After 10 min at 100% Solvent A (0.1% TFA), elution was performed using a linear gradient from 0% to 20% solvent B (60% (v/v) acetonitrile in 0.1% TFA) over 10 min and increased to 100% solvent B in 40 min at a flow rate of 1.0 ml/min. The fractions of each peak were pooled and then dried by speed vacuum concentrator. Each peak was assayed for antimicrobial activity. The GEWL hydrolysate fractions active against bacteria were dried and re-chromatographed in the same column. The elution gradient was performed from 0% to 50% solvent B (60% acetonitrile in 5mM potassium phosphate buffer, pH 6.0) in 40 min and increased to 100% solvent B in 50 min at a flow rate of 1.0 ml/min. Each fraction was pooled, dried and frozen at 20 ^{o}C to test antimicrobial activity.

MALDI-TOF MS. The molecular mass of antimicrobial peptide was analyzed by MALDI-TOF MS at The Bioservice Unit (Bangkok, Thailand).

Results and Discussion

Purification of lysozymes

The SSTL was simply purified from *Trionyx sinensis taiwanese* egg white. From the purification profile, two active peaks of SSTL were obtained, designated as peak A (SSTL A) and peak B (SSTL B) (Figure 1A). On the other hand, GEWL showed only one homogeneous lysozyme after re-chromatography on CM-Toyopearl 650M (Figure 1B).

The SDS-PAGE patterns of purified lysozymes are shown in Figure 2. The final product of lysozyme purification showed a single protein band by Coomassie staining. The molecular masses of SSTLs were estimated to be 14.8 kDa by SDS-PAGE, which corresponds to hen egg white lysozyme (HEWL) (Canfield et al., 1963). The final step of purification of GEWL showed a single protein band and the molecular mass was estimated to be 21 kDa. This molecular mass is corresponding to the reported G-type lysozyme (Canfield and McMurray, 1967). From this result, two lysozymes from soft shelled turtle egg white were obtained (SSTL A and SSTL B). However, from the SDS-PAGE result and the reported multiple lysozymes (Araki et al., 1998; Thammasirirak et al., 2006) only 1-2 amino acid residues are different. The amino acid sequence of these two lysozymes were analyzed and found to be very similar and differing from each other by only two amino acids (data not shown). Thus, we propose that there is no significant difference between the primary structures of these enzymes. Thus, in further steps, the pool of these enzymes was used.

Enzymatic Hydrolysis of Lysozyme

The native SSTL was digested with pepsin followed by trypsin. Examination of the digestion of SSTL by Tris-tricine SDS-PAGE showed the combination of peptic digest and tryptic digest were able to hydrolyze lysozyme to smaller peptides of less than 4 kDa and had no enzymatic activity (Figure 3A). It has been reported that the susceptibility of lysozyme was induced to digestion by protease with heat at high temperature in a short time (Ibrahim et al., 2001). In the present work, the digestion by protease of native GEWL forms and heat denatured forms were studied (Figure 3B). Heat denatured GEWL gave smaller fragments than native GEWL and the muramidase activity was completely abolished when lysozymes were digested with pepsin followed by trypsin (data not shown). In these results, we use the native SSTL and denatured form of GEWL to produce novel antimicrobial peptides.

Purification of antimicrobial peptides

The lysozymes hydrolysates were clarified by centrifugation, and in the resulting precipitate the insoluble peptides did not demonstrate significant antimicrobial activity but the water soluble part of SSTL hydrolysate absolutely inhibited growth of Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli, Ps. aeruginosa and S. typhi*) (Figure 4). The present study clearly demonstrates that soft shelled turtle lysozyme contains peptide that can induce noncatalytic bacterial inhibition, which differs from enzymatic lysis of cell wall. The supernatant of digested-SSTL was separated by gel filtration (Superdex 30 prep grad) FPLC (data not shown), and further purified by HPLC (Figure 5). The peptide was collected in fourteen fractions (1-14).

For the characterizations of antimicrobial peptides from the digestion of GEWL, hydrolyzed GEWL was clarified by centrifugation. Then the supernatant showing antimicrobial activity was separated by C18 reversed-phase HPLC as shown in Figure 6. Twenty fractions of peptides were obtained (1-20).

After isolating peptides by HPLC, the antimicrobial activities of each peptide from the two lysozymes were investigated against several bacterial strains. Aliquots of the fractions were tested for their ability to inhibit growth of the Gram-positive bacteria (*S. aureus and S. epidermidis*) and the Gram-negative bacteria (*E. coli, K. pneumoniae, Ps. aeruginosa, S. typhi* and *V. cholerae*). For the SSTL hydrolysate, the pooled fractions 2-5, 7 and 8 were active against *S. aureus, S. epidermidis, K. pneumoniae and V. cholerae.* For GEWL hydrolysate we found that pooled fractions 5 and 7 were strongly active against *S. epidermidis, S. typhi and V. cholerae* (Table 1). The antimicrobial peptides derived from SSTL hydrolysates (5, 7 and 8) were then subjected to amino acid sequence analysis and MALDI-TOF mass spectrometry. Peptide mass analyses were searched for and compared with amino acid sequences of C-type lysozyme in the database. The results indicate that the peptides may be located in the helix structure domain of lysozyme. The sequences of these parts are residues 15-21 (HGLDNYR) and residues 98-108 (IVSDGNGMNAW). These results coincide with the report of Mine et al. (2004). However, the amino acid sequence of tryptic peptides from GEWL hydrolysate is currently under evaluation.

The peptide HGLDNYR located in the N-terminal helix and another part of this peptide is located in the loop. The other, the peptide IVSDGNGMNAW is located in the middle part of the helix-loop-helix motif of lysozyme. This helix structure might be helpful to penetrate into the lipid bilayer membrane of bacteria which has been reported by Ibrahim et al. (2001) and Mine et al. (2004). As we mention in the introduction, GEWL has an overall three dimensional structure the same as C-type lysozyme. The antimicrobial peptide activity from the non-catalytic enzyme is also obvious. Taking this data into account, we propose that the helix structure contained in the structure might be important for the antimicrobial activity.

In conclusion, in this study we have purified soft shelled turtle lysozyme and goose egg white lysozyme. These two enzymes were used to study antimicrobial peptides by protease hydrolysis. The protease digestion generated two potent antimicrobial peptides from SSTL hydrolysate, which killed *S. aureus, S. epidermidis, K. pneumoniae and*

V. cholerae. The digested-GEWL shows antimicrobial activity against *S. epidermidis*, *S. typhi and V. cholerae*. These results show that the N-terminal part with helix-loop-helix structure derived from SSTL is important for antimicrobial activity. These studies reveal that peptides exhibiting bacteriocidal activity can be released by proteolytic digestion of lysozyme.

Acknowledgements

This research was financially supported by TRF Master Research Grants, Thailand Research Fund (MRG475S041 and MRG485S026).

References

Araki, T., Yamamoto, T. and Torikata, T. 1998. Reptile Lysozyme: The complete amino acid sequence of soft-shelled turtle lysozyme and its activity. **Biosci Biotechnol Biochem** 62(2): 316-324.

Blake, C.C.F., Koenig, D.F., Mair, G.A., North, A.C.T., Phillip, D.C. and Serma, V.R. 1965. Structure of hen egg white lysozyme. A three-dimensional fourier synthesis at 2 Å resolution. **Nature** 206: 757-761.

Brogden, K.A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?: Review. **Nature** 3: 238-248.

Canfield, R.E. 1963. The amino acid sequence of egg white lysozyme. **J Biol Chem** 238: 2698-2707.

Canfield, R.E. and McMurray, S. 1967. Purification and characterization of lysozyme from goose egg white. **Biochim Biophys Res Commun** 26: 38-42.

Dubin, A., Mak, P., Dubin, G., Rzychon, M., Stec-Niemczyk, J., Wladyka, B., Maziarka, K. and Chmiel, D. 2005. New generation of peptide antibiotics. **Acta Biochim Pol** 52(3): 633-638.

Düring, K., Porsch, P., Mahn, A., Brinkmann, O. And Gieffers, W. 1999. The non-enzymatic microbicidal activity of lysozymes. **FEBS Letters** 449: 93-100.

Farnaud, S., Patel, A., Odell, E.W. and Evans, R.W. 2004. Variation in antimicrobial activity of lactoferricin-derived peptides explained by structure modeling. **FEMS Microbiol Lett** 238: 221-226.

Grütter, M.G., Rine, K.L. and Matthews, B.W. 1979. Crystallographic data for lysozyme from the egg white of the embden goose. **J Biol Chem** 135: 1029-1032.

Ibrahim, H.R., Sugimoto, Y. and Aoki, T. 2000. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. **Biochim Biophys Acta** 1523: 196-205.

Ibrahim, H.R., Thomas, U. and Pellegrini, A. 2001. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. **J Biol chem** 276: 43767-43774.

Ibrahim, R.I., Inazaki, D., Abdou, A., Aoki, T. and Kim, M. 2005. Processing of lysozyme at distinct loops by pepsin: A novel action for generating multiple antimicrobial peptide motifs in the newborn stomach. **Biochim Biophys Acta** 1726: 102-114.

Inoue, M., Imada, M. and Tsugita, A. 1970. The amino acid sequence of T4 phage lysozyme. IV. Diluted acid hydrolysis and order of tryptic peptides. **J Biol Chem** 245: 3479-3484.

Jollès, J., Jauregui-Adell, J., Bernier, I., and Jollès, P. 1963. La structure chimique du lysozyme de blanc d'oeuf de poule: etude detailee. **Biochim Biophys Acta** 78: 668-689.

Laemmli, U.K. 1970. Cleavage of structure protein during the assembly of the head of bacteriophage T4. **Nature** 277: 680-685.

Marshall, H.S. and Arenas, G. 2003. Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology. **Electron J Biotechn** 6(2): 271-282.

Matthews, B.W. and Remington, S.J. 1974. The three dimensional structure of the lysozyme from bacteriophage T4. **Proc Natl Acad Sci U.S.A.** 71: 4178-4182.

Mine, Y., Ma, F. and Lauriau, S. 2004. Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. **J Agric Food Chem** 52: 1088-1094.

Pellegrini, A., Hülsmeier, A., Hunziker, P. and Thomas, U. 2004. Proteolytic fragments of oval-bumin display antimicrobial activity. **Biochim Biophys Acta** 1672: 76-85.

Simpson, R.J., Begg, G.S., Dorow, D.S. and Morgan, F.J. 1980. Complete amino acid sequence of goose type lysozyme from egg white of black swan. **Biochemistry** 19: 1814-1819.

Thammasirirak, S., Ponkham, P., Preecharram, S., Khanchanuan, R., Phonyothee, P., Daduang, S., Srisomsap, C., Araki, T. and Svasti, J. 2006. Purification, characterization and comparison of reptile lysozyme. **Comp Biochem Phys Part C** 143: 209-217.

Weaver, L.H., Grütter, M.G., Remington, S.J., Gray, T.M., Isaace, N.W. and Matthews, B.W. 1985. Comparison of goose type, chicken type, and phage type lysozyme illustrates the changes that occur in both amino acid sequence and three-dimensional structure during evolution. **J Mol Evol** 21: 97-111.

Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. **Nature** 415: 389-395.

Table 1. The antimicrobial activity of peptide fraction from GEWL and SSTL against *S. aureus*, *S. epidermidis*, *S. typhi*, *Ps. aeruginosa*, *K. pneumoniae*, *V. cholerae*, *E. coli*.

Bacterial strain	Peptide fraction	
	GEWL	SSTL
S. aureus	-	P4, P8
S. epidermidis	P5, P7	P8
S. typhi	P5, P7	-
Ps. aeruginosa	-	-
K. pneumoniae	-	P2, 4, 5, 7, 8
V. cholerae	P5, P7	P3, 4, 5, 7, 8
E. coli	-	P8

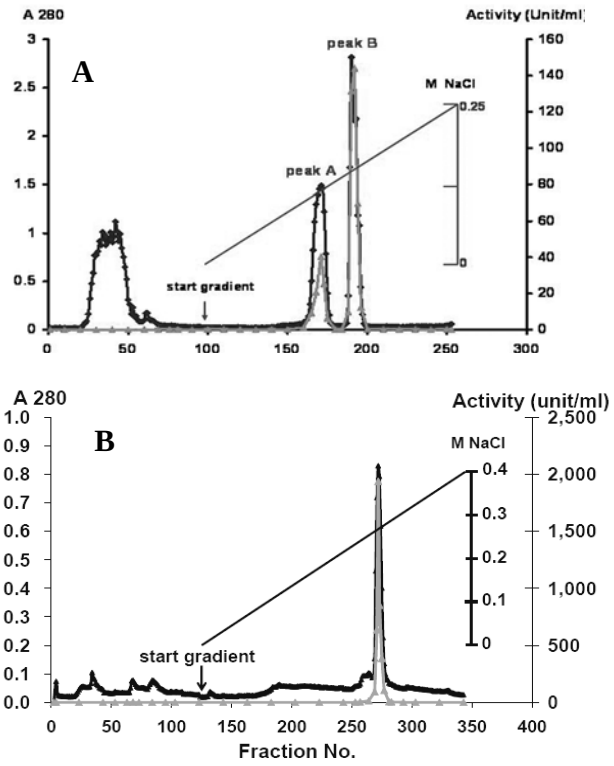

Figure 1. Purification of SSTL (A) and GEWL (B) on CM-toyopearl 650M cation exchange column chromatography.

A: Chromatography of purified SSTL cation exchange column. The column was eluted with a linear gradient of NaCl (0 - 0.25 M) in 0.05 M phosphate buffer, pH 7.0 at a flow rate of 15 ml/h (3 ml/ fraction). B: Re-chromatogram of purified GEWL by cation exchange column. The column was eluted with a linear gradient of NaCl (0 - 0.4 M) in 0.05 M phosphate buffer, pH 7.0 at a flow rate of 15 ml/h (3 ml/ fraction). The arrow indicates the start gradient, (-●-) protein at 280 nm and (-△-) enzyme activity (unit/ml) at 540 nm.

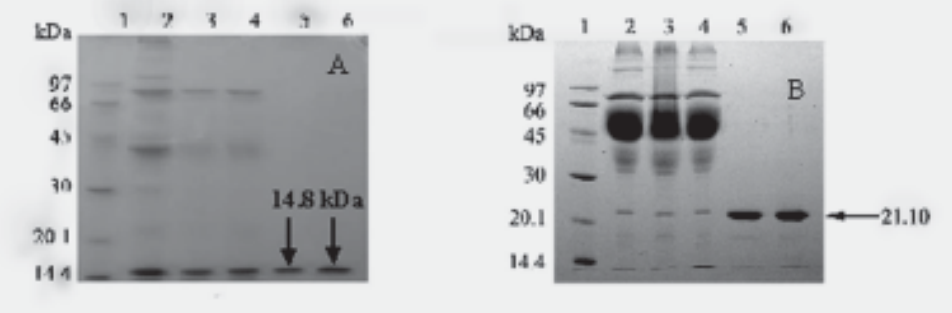

Figure 2. SDS-PAGE of the purification steps of SSTLs (A) and GEWL (B).

Samples from each step of the purifications were analyzed. A; Lane1: standard molecular weight marker proteins, Lane 2: crude extract, Lane 3: pH 4.0 treatment, Lane 4: pH 7.0 treatment, Lane 5: pooled fraction from peak A and Lane 6: pooled fraction from peak B. B; Lane1: standard molecular weight marker proteins, Lane 2: crude extract, Lane 3: pH 4.0 treatment, Lane 4: pH 7.0 treatment, Lane 5: pooled fraction from CM-stepwise, Lane 6: pooled fraction from CM-gradient.

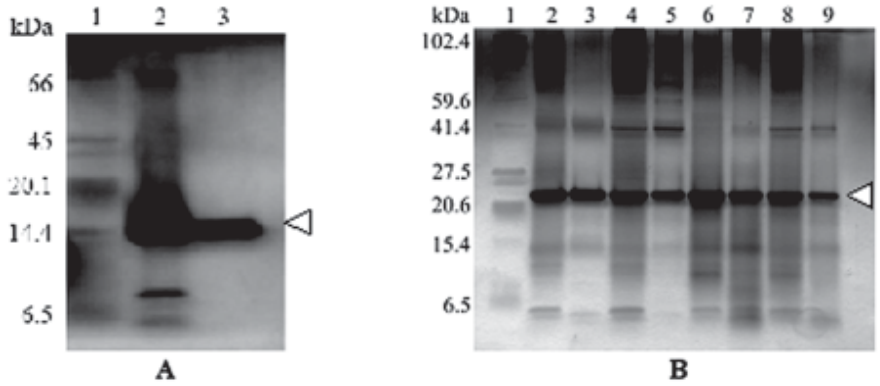

Figure 3. Tris-tricine SDS-PAGE of digested-SSTL (A) and digested-GEWL (B).

A; Lane 1: marker proteins, Lane 2: SSTL hydrolysate and Lane 3: intact SSTL. B; Lane 1: marker proteins, Lanes 2-5: native GEWL, Lanes 6-9: heat denatured GEWL, Lanes 2 and 6: intact GEWL (non heat denatured), Lanes 3 and 7: heat-denatured GEWL, Lanes 4 and 8: peptic digestion, Lanes 5 and 9: peptic followed by tryptic digestion.

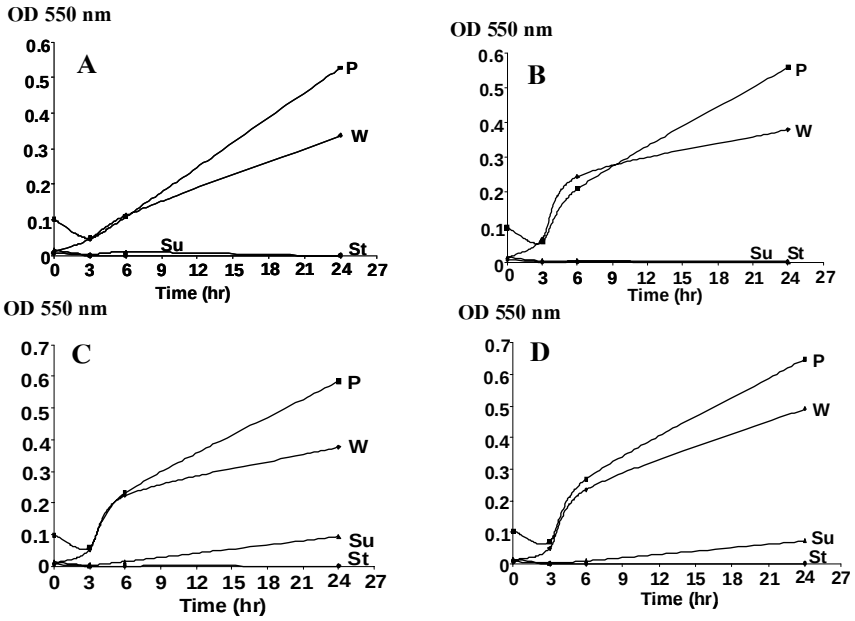

Figure 4. Antimicrobial activity against Gram-negative bacteria (*S. typhi*:A, *E. coli*:B and *Ps. aeruginasa*:C) and Gram-positive bacteria (*S.aureus*:D). Each bacterial strain was incubated with a pellet from peptic and tryptic digestion (P), supernatant from peptic and tryptic digestion (Su), sterile water (W: positive control), and steptomycin (St: negative control).

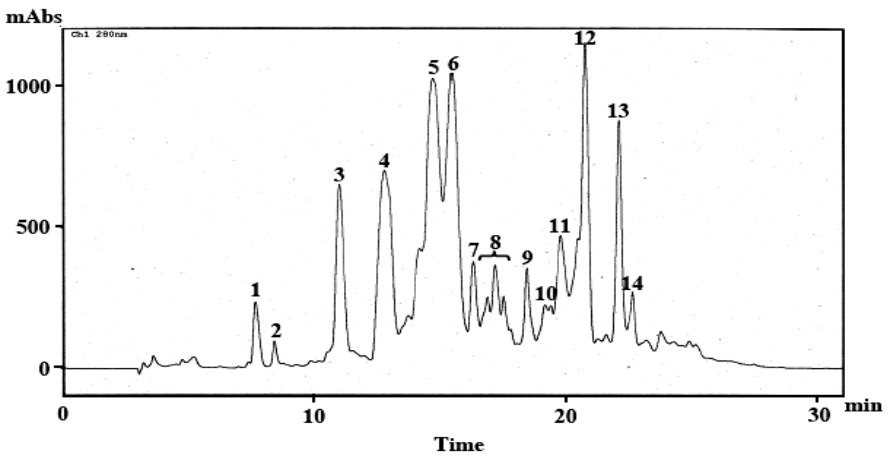

Figure 5. C18 reversed-phase HPLC Chromatography of SSTL digested with pepsin followed by trypsin.

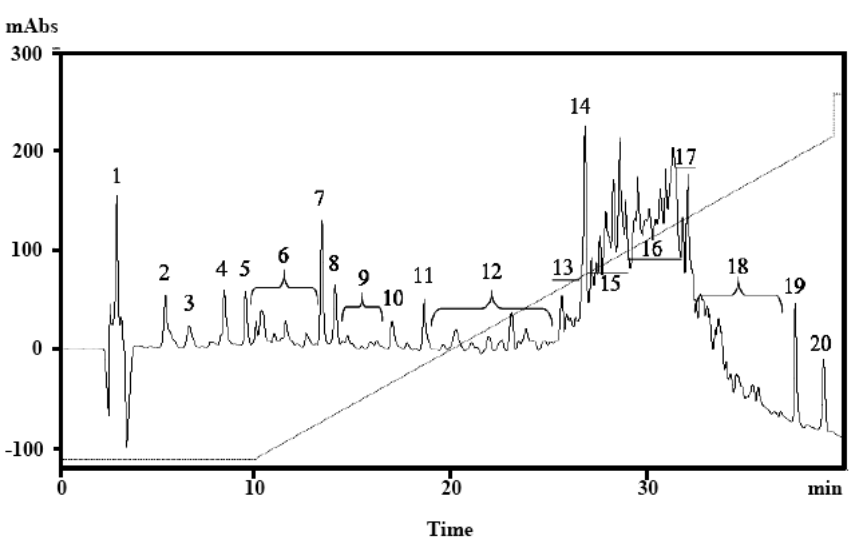

Figure 6. C18 reversed-phase HPLC Chromatography of GEWL digested with pepsin and subsequently by trypsin. The buffer gradient is shown by a dotted line.