Antimicrobial peptides derived from goose egg white lysozyme

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Peptide fragments possessing antimicrobial activity were obtained by protease digestion of goose egg white lysozyme. Digested peptide purified from RP-HPLC which showed no lysozyme activity exhibited bactericidal activity toward Gram-negative and Gram-positive bacteria. LC/MS–MS and automated Edman degradation revealed the amino acid sequence to be Thr-Ala-Lys-Pro-Glu-Gly-Leu-Ser-Tyr. This sequence corresponds to amino acid positions 20–28, located at the N-terminal outer part of goose lysozyme. The peptide acted on bacterial membrane as shown by scanning electron microscopy. The mechanism of action could be explained from a helical structure that may be formed by the centered Pro residue and the terminal Lys residue after the peptide attaches to a cell membrane. This is the first study to report that a peptide derived from the protease digests of G-type lysozyme possesses antimicrobial activity with broad spectrum activity. Our result is comparative to the previous reports of Chicken lysozyme and T4 phage lysozyme, which showed antimicrobial activity after digestion with protease. These results might contribute to the usage of antimicrobial peptides engineered by genetic or chemical synthesis.

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1. Introduction

Antimicrobial peptides represent an ancient host defense mechanism of a diverse range of organisms including mammals, birds, amphibians, crustaceans, fish, insects, plants and microbes (Bachère, 2003; Papagianni, 2003; Thomma et al., 2003). Some antimicrobial peptides are produced constitutively while others are synthesized in response to microbial attack (Gallo et al., 2002). The rapid availability of antimicrobial peptides is important for innate immune systems, making it a highly effective first line of defense in organisms (Ganz, 2003). Antimicrobial peptides are capable of killing a range of cells and microbes including bacteria, fungi, protozoa, enveloped viruses, malignant cells and parasites (Vizioli and Salzet, 2002a,b).

Over the past decade, research on antimicrobial agents has been widely performed, giving the discovery of numerous naturally occurring antimicrobial peptides. Recently, attention has mainly focused on the antimicrobial peptides generated from edible proteins. Antimicrobial peptides could be generated during the digestion of food proteins in the gastrointestinal tract. One novel branch of this work involves the discovery of antimicrobial peptides released upon the digestion or breakdown of proteins such as lactoferrin (Farnaud and Robert, 2003), ovotransferrin (Ibrahim et al., 2000), ovalbumin (Pellegrini et al., 2004), and various peptides released from the protease digestion of lysozyme (Mine et al., 2004; Ibrahim et al., 2005). Lysozyme (Muramidase, EC 3.2.1.17) is found in various biological fluids and tissue, including avian eggs, animal secretions, tears, saliva, and respiratory and cervical secretions and is secreted by polymorphonuclear leukocytes. It is one of the best characterized hydrolases. It catalyzes the hydrolysis of bacterial cell wall by cleavage of β-1, 4 linkages between N-acetylmuramic acid (NAM) and N-acetylgalactosamine (NAG) of peptidoglycan in the bacterial cell wall and acts as a nonspecific innate immunity molecule against the invasion of bacterial pathogens (Jollès and Jollès, 1984). Lysozyme is well known as an antibacterial protein. The in vitro antimicrobial activity of lysozyme is directed against certain Gram-positive bacteria such as Staphylococcus aureus, Micrococcus luteus, Bacillus steaerotherophilus, Clostridium tyrobutyricum (Cunningham et al., 1991), and to a lesser degree against Gram-negative bacteria (Banks et al., 1986; Brooks et al., 1991; Ibrahim et al., 2002). Lysozyme has many other functions, including antiviral (Hasselberger, 1978; Lee-Huang et al., 1999), immune modulatory (Kokoshis et al., 1978), anti-inflammatory (Jollès and Jollès, 1984) and antitumor activities (Sava et al., 1998). Antimicrobial peptides are classified into six types: chicken type lysozyme (C-type) which includes stomach lysozyme and calcium-binding lysozyme, goose-type lysozyme (G-type), plant lysozyme, bacterial lysozyme, T4 phage lysozyme (phage-type), and invertebrate lysozyme (i-type) (Beintema and Terwisscha van Scheltinga, 1996). Four types of lysozyme, C-type lysozyme, T4 lysozyme and G-type lysozyme (Grutter...
et al., 1983; Weaver et al., 1995) as well as i-type lysozyme (Goto et al., 2007), share the similarity of their three-dimensional structures.

So far, the C-type of lysozyme has been extensively studied, and several lysozyme proteins have already been purified and cloned. The antimicrobial action of C-type lysozyme appears to depend not on its enzymatic activity but rather on a structural phase transition (Ibrahim et al., 1996; Ibrahim, 1998). Partially unfolded C-type lysozyme showed enhanced bactericidal action against Gram-negative bacteria and still retains microbial activity to Gram-positive bacteria (Ibrahim et al., 1996; Ibrahim and Funkhouser, 1997). Recently, Ibrahim et al. (2001) have reported that hen egg white lysozyme exhibits a broad spectrum of antimicrobial activity after digestion with cestropin. This peptide is active against both Gram-positive and Gram-negative bacteria and the fungus Candida albicans. In addition, in 2004, Ibrahim et al. found novel antimicrobial peptides from hen egg white lysozyme hydrolysate, obtained by peptic digestion and subsequent tryptic digestion.

The G-type lysozyme has a similar three-dimensional structure to C-type and T4 lysozyme. Although much information on structural properties and enzymatic mechanism including antimicrobial peptides of C-type and T4-type has been explored, there is only limited information on primary structure and catalytic mechanisms for G-type lysozyme (Simpson and Morgan, 1983; Hikima et al., 2001; Thammasirirak et al., 2002; Zhao et al., 2007). In particular, no antimicrobial peptide derived from this lysozyme has been reported. In the present study, therefore, we purified a G-type lysozyme from goose egg white, which was then enzymatically digested to obtain peptides possessing antibacterial activity. The amino acid sequence of this peptide was identified and the membrane disrupting activity of the peptide was shown by SEM using bacterial cells.

2. Materials and methods

2.1. Materials

Goose (Anser Cygnoides) eggs were purchased from a local farm. The microbial substrate of lysozyme (M. luteus cell; ATCC 4618) was purchased from Sigma. The microorganisms Salmonella typhi (ATCC 11778), Escherichia coli (0157:H7), S. aureus (ATCC 25923), Staphylococcus epidermidis (clinical isolates), Klebsiella pneumoniae (ATCC 27736), Pseudomonas aeruginosa (clinical isolates) and Vibrio cholerae (clinical isolates) were used in this study. All bacterial strains were maintained in nutrient agar slant at 4 °C.

2.2. Purification of lysozyme from goose egg white

The method for purification was modified from Thammasirirak et al. (2001). Namely, goose egg white (50 mL) 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 ×g for 15 min, 4 °C. The resulting supernatant was used as a crude extract for protein purification. The crude extract was precipitated at pH 4.0 and pH 7.0. At each step of pH treatment, the solution was adjusted to pH 4.0 and pH 7.0 desired using 1.0 M HCl followed by 1.0 M NaOH and mixed around 1 h at 4 °C. Then, the solution was centrifuged at 10,000 ×g for 30 min. The supernatant was applied onto a CM-Toyopearl 650 M cation exchange column (size 2.5 × 85 cm) which was equilibrated with 0.05 M sodium phosphate buffer, pH 7.0. After the sample solution was applied, the column (size 2.5×85 cm) which was equilibrated with 0.05 M sodium phosphate buffer, pH 7.0 and stirred at 4 °C for 30 min. The column was washed with the same buffer and eluted with a linear gradient of NaCl from 0 to 0.4 M in the same buffer at a flow rate of 15 mL/h. The active fractions were pooled and dialyzed against distilled water several times. The enzyme was lyophilized to use as the purified enzyme for characterization.

2.3. Lysozyme activity assay

The lysozyme activity was assayed during purification based on the method of Parry et al. (1965), using lyophilized cells of M. luteus ATCC 4618 (Sigma) as a substrate. Substrate suspension in 0.1 M sodium phosphate buffer, pH 7.0, was diluted and adjusted to 0.8–1.0 of optical density (OD) at 540 nm. Enzyme solution (100 µL) was added to 3 mL of the substrate suspension. The activity unit was evaluated from the decrease of absorbance at 540 nm for 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. The assay was performed in triplicate per sample.

2.4. Measurement of protein concentration

Protein concentration was measured by the method of Lowry et al. (1951) with commercial chicken egg white lysozyme as standard.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was conducted according to the method of Laemmli (1970), using 13% separating gel and 4% stacking gel. Samples were prepared by mixing with 2× solubilizing buffer (0.5 M Tris–HCl buffer, pH 6.8, 0.5% (w/v) bromophenol blue, 10% (v/v) glycerol, 2% (w/v) SDS, and 10% (v/v) β-mercaptoethanol) ratio of 1:1 (v/v) and then boiled for 3 min. The protein bands were stained with Coomassie brilliant blue R250. Low molecular weight calibration kit (Amersham Bioscience, Sweden) was used as the standard proteins marker.

2.6. Zymogram or refolding gel electrophoresis

M. luteus whole cells were labeled with the vinyl-sulfone reactive dye RBB according to the method for synthesis of RBB-labeled starch (Rinderknecht et al., 1967) and the refolding gel analysis was performed by the method of Hardt et al. (2003). Polyacrylamide gel electrophoresis (PAGE) was performed using a 4% (w/v) stacking gel and a 12.5% (w/v) resolving gel containing 0.1% (w/v) blue M. luteus cells. Polyacrylamide gels and buffers contained 0.1% (w/v) SDS. Samples were boiled for 2 min in 2× sample buffer without reducing agent (62.5 mM Tris–HCl buffer, pH 6.8, 0.006% (w/v) bromophenol blue as tracking dye, 20% (v/v) glycerol, 2% (w/v) SDS). Samples of 10 µL were used for loading. Electrophoresis was performed using Mighty Small SE 245 (Hoefer Scientific Instruments, San Francisco) at a constant of 120 V for 1 h until the dye front approached the lower gel margin. The gel was washed twice with distilled water for 30 min to remove SDS, incubated in a covered tray with 300 mL of refolding buffer (0.05 M sodium phosphate buffer, pH 7.0 containing 1% (v/v) Triton X100), and gently shaken at 37 °C. The image of the gel was taken by a digital camera (Nikon Coolpix4500, Japan) with a white background. The activity of enzymes was measured by the clear zone in the otherwise opaque bluish gel.

2.7. Enzymatic hydrolysis of lysozyme

Enzymatic hydrolysis of lysozyme was carried out through the two steps. Initially, lysozyme was heated at 80 °C for 20 min and then digested with pepsin at pH 2.0. The lysozyme was dissolved in water, heated at 80 °C for 20 min and then the solution was adjusted to pH 2.0 with 1.0 M HCl (final concentration was 2 mg/mL). Pepsin A (2500–3500 unit/mg, Sigma) in 0.001 M HCl was added to (1:50 W/W) a reaction mixture, which was then incubated at 37 °C for 2 h. Then the
enzymatic reaction was stopped by adjusting the pH to 8.0 with 0.5 M sodium carbonate (NaHCO₃) solution. Further, the TPCK trypsin (7490 unit/mg, Fluka) solution (5 mg/mL in 0.1 M Tris-HCl containing 0.03 M NaCl, pH 8.0) was added to peptic digests at a ratio of enzyme-to-substrate of 1:250 (w/w). The reaction mixture was incubated at 37 °C for 18 h and then the enzymatic reaction was terminated by heating at 90 °C for 10 min. As a control experiment, the lysozyme solution (2 mg/mL) was subjected to the process described above without the addition of pepsin A and trypsin. Insoluble materials were removed by centrifugation at 5000 × g for 30 min and the resulting supernatant was analyzed for lysozyme activity and lyophilized for succeeding experiments.

2.8. Enzymatic activity of peptide

The enzymatic activities of lysozyme and lysozyme hydrolysate were determined using lyophilized M. luteus cells which were resuspended in 0.1 M sodium phosphate buffer, pH 7.0 and adjusted to 0.8–1.0 of OD at 540 nm, as a substrate (Gorin et al., 1971). Ninety microliter aliquots of the samples were added to 900 µL of M. luteus cell suspension. The activity is expressed as the rate of decrease in absorbance per min of the initial velocity of reaction (OD₅₄₀ nm/min), by monitoring OD at 540 nm over 60 min. The muramidase activity was expressed as a percentage relative to that of untreated lysozyme. The assay was performed in triplicate per sample.

2.9. Purification of antimicrobial peptide by HPLC

Lyophilized lysozyme hydrolysate obtained from protease digestion was further purified by C18 reversed-phase high performance liquid chromatography (RP-HPLC). Samples were dissolved in deionized water and applied to a C18 reverse phase column (Apollo C18 5 μm, size 4.6 × 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 (solvent B was 60% (v/v) acetonitrile in 0.1% TFA) in 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 fractions containing antimicrobial activity were dried and rechromatographed on C18 reversed-phase chromatography. After 10 min elution of 100% solvent A (5 mM potassium phosphate buffer, pH 6.0) the peptide was eluted by a linear gradient from 0% to 50% solvent B (solvent B was 60% (v/v) acetonitrile in 5 mM potassium phosphate buffer, pH 6.0) in 40 min and increased to 100% solvent B within 50 min at a flow rate of 1.0 mL/min. Each fraction was pooled, dried and frozen at −20 °C until the antimicrobial activity was tested.

2.10. Measurement of peptide concentration

The peptides were quantified by UV absorbance at 215 and 225 nm using the formula concentration (mg/mL) = (A₂₁₅ − A₂₂₅) × 0.144 following the method of Waddell (1956).

2.11. Antimicrobial activity by growth inhibition assay

In this study, the bacteriocidal assay was performed by 5 mL portions of nutrient broth (NB), which were inoculated with a single colony of bacteria and incubated overnight at 37 °C. The bacterial suspension was diluted in the same medium to OD 0.5 at 600 nm and aliquot 1% to mix...
with 5 mL NB. Bacteria were grown at 37 °C, 160 rpm in 5 mL nutrient broth until the logarithmic phase and then diluted with the same medium to obtain OD 0.001–0.01. Antimicrobial activity of the peptide fractions from HPLC was monitored using 96-well microtiter cell-culture plates. Each well containing 100 µL of the bacterial suspension was mixed with an equal volume of peptide fractions (300 µg/mL was dissolved in the same medium), incubated for 24 h at 37 °C. After incubation, OD at 550 nm was measured using a microtiter plate reader with 3 h time interval until 24 h. All antimicrobial assays were performed in triplicate.

2.12. Amino acid sequence analysis

Each peptide solution at a final concentration of 100–500 pmol/µL dried by speed vacuum concentrator was used to analyze the amino acid sequence by LC/MS–MS at The Bioservice Unit (Bangkok, Thailand). Protein identification was performed using the Turbo SEQUEST algorithm in the Bioworks™ 3.1SR1 software package (Thermo Electron) and Fasta database. Furthermore, we confirmed amino acid sequence by automated amino acid sequencer (Applied Biosystem 477, USA).

2.13. Scanning electron microscopy (SEM)

Antibacterial activity was determined by scanning electron microscopy. After bacterial strains were grown to mid-logarithmic phase in Muller Hinton Broth (MHB), the cells were obtained by centrifugation at 1500 × g for 10 min, and then washed with 0.15 M NaCl and followed by 20 mM sodium phosphate buffer pH 7.0. The resultant cells were resuspended with 150 mM NaCl in 20 mM sodium phosphate buffer pH 7.0 to obtain the OD 550 nm of 0.001–0.005, and incubated with peptides (5–10 µg) at 37 °C for 2 and 4 h. After incubation, the cells were fixed with 5% glutaraldehyde–sodium phosphate buffer, pH 7.0 for 2 h, and spotted onto the polycarbonate membrane and then dehydrated in graded ethanol concentrations (25%×10 min, 50%×10 min, 75%×10 min, two times with 95%×10 min and two times with 100%×10 min, respectively). The dehydrated materials in the absolute ethanol were dried in a critical point drier (CPD7510; Thermo VG Scientific, England) with carbon dioxide as the drying agent. Dry materials were coated by sputter Coater (SC7620; Polaron, England) with gold palladium and examined by scanning electron microscopy (LEO1435VP; LEO Electron Microscopy Ltd, England) operating at 12–20 kV. The negative control was performed in a similar manner except that the bacterial cells were incubated with PBS buffer instead of the antibacterial compound.
3. Results

3.1. Purification of lysozyme from goose egg white

G-type lysozyme was purified from goose egg white by pH precipitation (pH 4.0 and pH 7.0) and cation exchange column chromatography using CM-Toyopearl 650M. In the first column, protein peak eluted at 0.2 M NaCl contained high muramidase activity, indicating the presence of lysozyme (Fig. 1A). The corresponding fractions were pooled, dialyzed and concentrated for second purification step using cation exchange column. After rechromatography with a linear gradient of NaCl, a symmetric single protein peak with high lytic activity was obtained (Fig. 1B). Analysis of each purification step by SDS-PAGE is shown in Fig. 2A. The final step of purification showed a single protein band with the molecular mass around 21 kDa. We designated this band as goose egg white lysozyme (GEWL). To confirm the enzymatic activity of GEWL after purification, an on-gel refolding experiment was performed. The result showed clear zones at approximately 21 kDa at all purification steps; i.e. crude extract, GEWL from stepwise gradient, and GEWL from linear gradient (Fig. 2B).

3.2. Study of antimicrobial peptide from GEWL

3.2.1. Enzymatic hydrolysis of GEWL

Purified lysozyme was used as the starting source to generate antimicrobial peptides. To facilitate protease digestion, GEWL was denatured by heating at 80 °C for 20 min prior to digestion. As shown in Fig. 3, however, folded GEWL was found to be slightly denatured by the heat treatment. Tris–tricine SDS-PAGE of the products digested by pepsin A showed small fragments of less than 20 kDa with no intact GEWL. Pepsin A digestion followed by trypsin treatment resulted in no detectable protein components. Therefore, the proteolytic digestion of GEWL appeared to be successfully achieved by pepsin A followed by trypsin treatments. The enzyme activity was investigated comparing between native and hydrolyzed lysozyme. Heat-treated lysozyme retained 74.5% of its native activity. The peptic digests of heat-treated lysozyme possessed 2.3% of activity, while the activity was completely abolished after heat-denatured lysozyme underwent peptic and tryptic digestion (Fig. 3).

3.2.2. Purification of antimicrobial peptides

After protease digestion, fragmented lysozyme was purified by RP-HPLC using the C18 column. As shown in Fig. 4A, 10 fractions were collected from HPLC and only fraction number 9 demonstrated antibacterial activities against V. cholerae, P. aeruginosa and S. epidermidis. Active peptides from fraction number 9 were further purified by rechromatography on the same column. Six peptide peaks were resolved (Fig. 4B). Peptides IV and VI showed activity against Gram-positive bacteria (S. epidermidis) and stronger activity toward Gram-negative bacteria (V. cholerae) (Fig. 5). The yield of purified peptide, determined by the method of Waddell (1956) was approximately 10 ng.

3.2.3. Scanning electron microscopy

To visualize the effect of peptide IV on susceptible bacteria, SEM was used to observe disruption of bacterial membrane by antimicrobial peptide. After treatment with the peptide fraction, Gram-negative V. cholerae and Gram-positive S. epidermidis were visualized by scanning electron micrographs (Fig. 6). With both strains, control cells (in the absence of peptides) exhibited a regular, smooth surface (Fig. 6A and F), whereas cells treated with peptide IV (Fig. 6B–E) and peptide VI (Fig. 6C–J) revealed membrane damage due to disturbance of membrane integrity. Further, incubation time was varied to observe the degree of killing. From the results, when S. epidermidis cells were incubated with 500 µg/mL of peptide IV for 2 h (Fig. 6B), the cell membrane revealed small globular surface protrusions, i.e. blebs. The numbers of blebs increased and their size also expanded when incubated for 4 h (Fig. 6C). It can be seen that, when incubating peptide VI with bacterial cells, cell deformation and blebs increased with time (Fig. 6D–E). In the same manner with V. cholerae, the cells had a large numbers of small blebs when treated with peptide (Fig. 6G, H). When reducing peptide VI concentration to 250 µg/mL, bacterial cells still displayed membranes with blebs that increased with time (Fig. 6D–E, I–J). These results indicate that the killing mechanism of these peptides is time dependent.

3.2.4. Amino acid sequence analysis

The amino acid sequences of peptides IV and VI were analyzed by LC/MS–MS and automated Edman degradation. The amino acid sequence of peptide IV was determined to be TAKPEGLSY, corresponding to amino acid residues 20–28 of GEWL (Fig. 7). However, the amino acid sequence of peptide VI could not be obtained by both Edman and Mass spectrometry. This result may be due to the peptide VI not being obtained in sufficient purity resulting in a complicated mass spectrum.

4. Discussion

Lysozyme is a hydrolase that cleaves the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine heteropolymer of the peptidoglycan, the components of the bacterial cell walls (Salton, 1957; Chipman and Sharon, 1969; Mine et al., 2004). These enzymes are strongly active against Gram-positive bacteria but inactive against Gram-negative bacteria (Banks et al., 1986; Brooks et al., 1991; Ibrahim et al., 2002). However, Ibrahim et al. (1996) have reported that the denaturation of hen egg white lysozyme increases the antimicrobial activity against Gram-negative bacteria, besides having inherent action against Gram-positive bacteria. Further, During et al. (1999) have reported the antimicrobial peptide of T4 lysozyme to be independent of the enzymatic activity. Recently, hen egg white lysozyme has been reported to possess nonenzymatic bacteriostatic regions in its primary sequences after digesting with pepsin and subsequently trypsin (Mine et al., 2004). This result uncovered the capability of digested lysozyme fragments to display antimicrobial activity against both Gram-positive and Gram-negative bacteria. Moreover, antimicrobial activity was also reported on protease-digested peptides derived from other proteins such as clastripain (Ibrahim et al., 2001), subtilisin (Liepke et al., 2001) and pepsin (Ibrahim et al., 2005). Mine et al. (2004) have demonstrated that, after digestion by pepsin and trypsin, sequentially, hen egg white lysozyme generates small peptides of less than 5 kDa with antimicrobial activity.

Fig. 6. Effect of antimicrobial peptides against susceptible bacteria. Scanning electron micrographs of Staphylococcus epidermidis (A–E) and Vibrio cholerae (F–J) incubated without (A, F) and with (B–E, G–J) antimicrobial peptides derived from GEWL. Peptide IV was used at 500 µg/mL and incubated at 37 °C for 2 h (B, G) and 4 h (C, H). Peptide VI was used at 250 µg/mL and incubated at 37 °C for 2 h (D, I) and 4 h (E, J).
There is growing evidence that peptides derived from lysozyme family exhibited antimicrobial activity that acts against both Gram-positive and Gram-negative bacteria (Mine et al., 2004; Ibrahim et al., 2005). However, no information on antimicrobial peptides derived from G-type lysozyme has been available, even though they share the same core three-dimensional structures. In this study, we purified GEWL, confirmed lysozyme activity on gel by the refolding method and we found the antibacterial activity of peptic- and trypsin-digested peptides derived from this protein. Regarding the G-Type lysozyme, to current knowledge, our study is the first study reporting antimicrobial activity of G-Type lysozyme after digestion with proteases. One active peptide obtained from this study, peptide IV, exhibited antimicrobial activity against V. cholerae and S. epidermidis. From SEM analysis, we observed a significant effect of the peptide on the bacterial surface membrane. The blebs were found in bilayer structure on the bacterial surface, extruding from the cytoplasmic membrane and releasing to the surroundings of the bacteria (Ulvatne et al., 2001). This result coincides with electron micrographs of E. coli treated with 30 µg/mL of Lactoferricin B, the antibacterial peptide from digested lactoferrin. This phenomenon is also similar to the action of SMAP-29 against Gram-negative E. coli and Gram-positive S. aureus (Skerlavaj et al., 1999). This result suggests that the bacterial membrane is a target for this peptide. Applying a similar mechanism, we suggest that peptides IV and VI are able to interact with the outer membrane surface of bacterial cells and then insert into the lipid bilayer of the cytoplasmic membrane, inducing the globular surface protrusions (blebs) depending on time and concentration.

The amino acid sequence of peptide IV, analyzed by automated Edman degradation and LC/MS–MS, was Thr-Ala-Lys-Pro-Glu-Gly-Leu-Ser-Tyr and corresponds to amino acid residues at positions 20–28 of GEWL. Peptide IV is located at the N-terminal outer surface of the active site part of the GEWL molecule. This part does not contribute to the activity and substrate binding of this enzyme (Weaver et al., 1995). Blast similarity search indicates that this peptide does not show any resemblance with other antimicrobial peptides or the one derived from C-type lysozyme. The three-dimensional structure shows that this part carries a helix–loop–helix structure. Even though there is no similarity on the primary sequence, surprisingly this position is located similarly to that of antimicrobial peptide derived from C-type lysozyme at positions 15 to 21 which showed the same helix–loop–helix structure (Mine et al., 2004). This result implies that the antimicrobial activity of each peptide does not directly depend on its amino acid sequence. Comparing the data from all reports of antimicrobial peptides released by protease digestion from lysozyme family, it is clearly seen that the helix structure is important to perturbing the permeabilization of the bacterial membrane as well as being found in chicken, human and T4 phage lysozyme (During et al., 1999). Thus, it may be proposed that the helical structure is important for antimicrobial activity for peptides derived from lysozyme family.

From the sequence of peptide IV, it might adopt a helix structure in aqueous solution (Fig. 8) which was predicted by http://expasy.org/cgi-bin/pi_tool, and it is assumed to keep the helix structure when penetrating a bacterial membrane (Christensen et al., 1988). In addition, it is notable that the proline residue in the structure may be important for antimicrobial action. The report by Suh et al. (1999) showed that the antimicrobial peptide gaegurin displayed α-helical structure and had a central proline residue. The proline residue contributes to stability of the region on the concave side of the curved helix, and the internal proline hinge in buforin II is important for peptide penetration (Park et al., 2000). Regarding the amino acid sequence of peptide IV, the proline residue may facilitate a formation of helix structure when the peptide interacts with the outer membrane of bacteria. Further, we propose that Lys residue may play an important role in interacting with the negative charge(s) in lipid A of lipopolysacharide (LPS) for Gram-negative bacteria and negative charge(s) of teichoic acids on the outer surface of the peptidoglycan (PDG) (Dennison et al., 2005).

In conclusion, peptide IV derived from protease digestion of GEWL is an antimicrobial peptide which this activity is different from the hydrodase one. This peptide may be executed by the penetration to the bacterial membrane with the specific peptide structure carrying center Pro residue and terminal Lys residue as well as the property of this peptide to form a helix structure. This hypothesis is in accordance with other reported antimicrobial peptides. However, further investigations aim to evaluate the killing mechanism of this peptide, for application use as antimicrobial peptides engineered by genetic or chemical synthesis.

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