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Antimicrobial Peptides Released by Enzymatic Hydrolysis of Hen Egg White Lysozyme

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This work was aimed at the isolation, purification, and characterization of novel antimicrobial peptides from chicken egg white lysozyme hydrolysate, obtained by peptic digestion and subsequent tryptic digestion. The hydrolysate was composed of over 20 small peptides of less than 1000 Da, and had no enzymatic activity. The water-soluble peptide mixture showed bacteriostatic activity against Gram-positive bacteria (*Staphylococcus aureus* 23-394) and Gram-negative bacteria (*Escherichia coli* K-12). Two bacteriostatic peptides were purified and sequenced. One peptide, with the sequence Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp, inhibited Gram-negative bacteria *E. coli* K-12 and corresponded to amino acid residues 98−108, which are located in the middle part of the helix−loop−helix. Another novel antimicrobial peptide inhibited *S. aureus* 23-394 and was determined to have the sequence His-Gly-Leu-Asp-Asn-Tyr-Arg, corresponding to amino acid residues 15−21 of lysozyme. These peptides broadened the antimicrobial activity of lysozyme to include Gram-negative bacteria. The results obtained in this study indicate that lysozyme possesses nonenzymatic bacteriostatic domains in its primary sequence and they are released by proteolytic hydrolysis.

KEYWORDS: Egg white lysozyme; lytic activity; bacteriostatic activity; antimicrobial peptides; pepsin; trypsin; SEM; reversed-phase HPLC

INTRODUCTION

Hen egg white lysozyme is an enzyme that makes up about 3.4% of egg white proteins. The molecular mass of egg white lysozyme, computed from the amino acid sequence of 129 residues, is 14307 Da. These residues are cross-linked by four disulfide bridges, and the isoelectric point is 10.7 (*1*). The FAO/WHO and many countries such as Austria, Australia, Belgium, Denmark, Finland, France, Germany, Italy, Japan, Spain, and the United Kingdom have acknowledged the nontoxicity of lysozyme and have approved its use in some foods and for pharmacological and therapeutic applications (*2*). It has been estimated that more than 100 tons of lysozyme is used annually for this purpose (*3*). Fresh vegetables, fish, meat, fruit, shrimp, and other seafood have been preserved by coating the surface of the food with lysozyme (*4*). Lysozyme also can be used to preserve soya bean curd by adding it to soya milk during processing. Other products such as kimuchi pickles, sushi, Chinese noodles, and creamed custard also can be preserved by adding lysozyme to them (*5*). Several patents claim the effectiveness of lysozyme at low concentrations to prevent the development of undesirable microorganisms in butter and cheese for more than 24 months (*6*). In combination with EDTA (ethylenediaminetetraacetic acid), lysozyme can effectively control bacterial contamination in some meat products such as sausage, salami, pork, beef, or turkey frankfurters (*7*, *8*). As a partial replacement of SO_2, lysozyme inhibits malolactic fermentation of red wine, promotes microbial stabilization, and prevents an increase in the content of acetic acid and biogenic amines. Also, it offers continuing protection to the wine (*9*). In addition to the direct bacteriolytic action, many other biological functions have been reported for lysozyme, such as antiviral action to inactivate certain viruses (*10*), potential antibiotic effects (*11*), anti-inflammatory and antihistaminic activities (*12*), direct activation of immune cells (*13*), antitumor action (*14*), fusogenic activity to phospholipid (*15*), and agglutinating and antiheparinic activity (*16*).

Lysozyme belongs to a class of enzymes that lyse the cell wall of certain Gram-positive bacteria by splitting $\beta(1-4)$ linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan, the components making up bacterial cell walls. It is the most effective against some specific Gram-positive bacteria such as *Staphylococcus aureus*, *Micrococcus lysodeikkticus*, *Bacillus cereus*, *Bacillus stearothermophilus*, *Clostridium thermosaccarolyticum*, and *Clostridium tyrobutyricum* (*2*), but it is largely ineffective against Gram-negative bacteria. The antimicrobial spectrum of lysozyme can be broadened by pretreating the target microbial cells in a number of ways. Pretreatment with some chelating agents such as EDTA will sensitize some Gram-negative bacteria to the action of lysozyme (*17*). Conjunction of lysozyme with dextran has been shown to increase its activity against Gram-positive and Gram-negative bacteria, particularly if the temperature is raised during treatment (*18*). Lysozyme and the bacteriocin nisin

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act synergistically under certain conditions to inhibit the growth of and to inactivate cells of *Listeria monocytogenes* (*19*). Freeze−thaw treatments sensitize *Escherichia coli* to lysozyme. Gram-negative bacteria, including *Salmonella*, become lysozyme-sensitive following an osmotic downshift (*20*). To broaden the bactericidal action of lysozyme to Gram-negative bacteria and food-borne pathogens, some research has been carried out, such as heating of lysozyme at increasing temperatures to get partially denatured or denatured lysozyme (*21*, *22*), lipophilization of lysozyme by different length fatty acid chains (*23*−*25*), and inserting hydrophobic peptides into the C-terminal of lysozyme by genetic modification (*26*, *27*). These attempts promoted the antimicrobial effects of lysozyme against Gram-negative bacteria while retaining whole or lower activity against Gram-positive bacteria. Moreover, the synergistic effect of other preservatives with lysozyme has resulted in significant improvement in lysozyme activity against a wide range of bacteria. In most cases, lysozyme in combination with some natural preservatives such as nisin, lactoferrin, glycine, organic acids, trypsin, aprotinin, and gelatin, as well as ultrahigh pressure and electroporation, has performed better against a wide range of bacteria than lysozyme alone (*28*, *29*).

Recently, it has been reported that chicken lysozyme contains peptide sequences which can induce noncatalytic bacterial death differing from enzymatic lysis of cell membranes. The clostripain-digested lysozyme yielded a pentadecapeptide (amino acids 98−112) with antimicrobial activity without muramidase activity (*30*, *31*). During et al. (*32*) identified amphipathic peptide stretches in T4 and hen egg white lysozyme. Two synthetic peptides, A23 (amino acids 126−141) and A4 (amino acids 143−155), were analyzed. Peptide A4 displayed a strong bactericidal and fungistatic activity, whereas peptide A23 was only active toward fungi. No enzymatic activity could be detected for those peptides (*32*). Some proteolytic enzymes such as trypsin, chymotrypsin, and papain do not hydrolyze lysozyme, unlike pepsin. However, these enzymes do hydrolyze denatured lysozyme (*33*). Increasing knowledge of lysozyme brings us a new insight that the antimicrobial action of hen egg white lysozyme depends not only on its enzymatic activity but rather on a structural phase transition, and a specific antibacterial domain which may be involved in the antimicrobial action of lysozyme (*31*). Proteolytic action on lysozyme has not been well documented in the past since it has been recognized that lysozyme is resistant to proteolytic action. Therefore, this study aimed to digest lysozyme and search for peptides exhibiting novel antibacterial activity in vitro. In this paper, we report on the peptic and tryptic digestion of lysozyme and the isolation of two antimicrobial peptides.

MATERIALS AND METHODS

Materials. Egg white lysozyme was kindly supplied by Canadian Inovatech Inc. (Abbotsford, BC, Canada). The enzymes pepsin (Pepsin A, EC 3.4.23.1, activity 600−1130 units/mg) extracted from porcine stomach mucosa and trypsin (TPCK treated, EC 3.4.21.4, activity 13600 units/mg) were obtained from Sigma Chemical Co. (Saint Louis, MO). Some chemicals and organic solvents were from Fisher Scientific (Fair Lawn, NJ). *E. coli* K-12 and *S. aureus* 23-394 were used for antimicrobial assay. Both strains were kindly provided by Dr. Poppe, C.C. (Health of Animal Laboratory, Health Canada, Guelph, ON, Canada). Peptone water, agar, and tryptic soy broth were purchased from Difco (Detroit, MI). All other reagents and chemicals were of analytical grade. Buffers used for the peptide purification were of high-performance liquid chromatography grade and were purchased from Fisher.

Enzymatic Hydrolysis of Lysozyme. Enzymatic hydrolysis of lysozyme was carried out in a two-stage process, using digestion with pepsin followed by trypsin. Lysozyme (2.5 g) was initially dissolved in 98 mL of solution A (0.03 M HCl solution, pH 1.0) and mixed with 2.0 mL of pepsin solution (50 mg/mL in solution A) for an enzyme-to-substrate ratio of 1:25. This mixture was incubated at 37 °C for 1 h. Then the enzymatic reaction was stopped by adjusting the pH to 8.0 with 0.5 M sodium carbonate solution. For the second stage, a 1.0 mL trypsin solution (5.0 mg/mL in 0.1 M Tris−HCl containing 0.03 M sodium chloride, pH 8.0) was added to the peptic digests at a ratio of enzyme to substrate of 1:500. It was incubated at 37 °C overnight and then heated at 90 °C for 10 min. Heat treatment of intact lysozyme was carried out by first heating lysozyme solution (2.5 g/100 mL) at 95 °C for 20 min and then subjecting it to the proteolytic digestions described above.

Measurement of Lysozyme Enzymatic Activity. The enzymatic activity of lysozyme and its digests was measured with lyophilized *M. lysodeikticus* (ATCC 4698) cells (Sigma) resuspended at 0.5 mg/mL in 10 mM potassium phosphate buffer (pH 7.0) as a substrate, using a method adapted from Gorin (*34*). Thirty microliter aliquots of different dilutions of the sample were added to 300 μL of *M. lysodeikticus* cell suspension, and the lysis of cells was measured as a decrease in turbidity, by taking readings at 600 nm over 40 min at 20 °C. The enzymatic activity was expressed as a percentage relative to that of untreated lysozyme.

Tricine Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. Tris−tricine ready gels, 16.5% acrylamide (Bio-Rad Laboratories, Hercules, CA), based on the formulation by Schagger and von Jagow (*35*), were used to resolve the peptides and small proteins produced during proteolytic digestion of lysozyme. Samples were diluted to 2.0 mg/mL in sample buffer containing 20% (v/v) 1.0 M Tris−HCl, pH 6.8, 40% (v/v) glycerol, 2% (v/v) sodium dodecyl sulfate (SDS), 0.04% (v/v) Coomassie Blue R-250 (Sigma), and 2% (v/v) 2-mercaptoethanol (Fisher). Ten microliters (20 μg) of each sample was loaded onto each well. Electrophoresis was carried out at 100 V, for approximately 110 min, at 4 °C, in running buffer composed of 0.1 M Tris−HCl, 0.1 M tricine, and 0.1% (w/v) SDS. Gels were fixed in a 40% (v/v) methanol, 10% (v/v) acetic acid solution, followed by staining with 0.025% (w/v) Coomassie Blue R-250 in 10% acetic acid, and destaining in 10% (v/v) acetic acid.

Protein Assay. The Bio-Rad DC assay kit (Bio-Rad Laboratories) was used to determine protein concentration in the experiment. The assay was a modified version of the Lowry method, and bovine serum albumin (Fisher) was used as a protein standard.

Purification and Isolation of Antimicrobial Peptides. The lysozyme hydrolysate was dialyzed against Milli-Q water with a 100 Da molecular mass cutoff membrane for 2 days. During this process, some peptides formed coagulum and were precipitated in the dialysis tubing. The precipitate and supernatant were separated by centrifugation at 5000*g* for 10 min and then lyophilized separately. Both precipitate and supernatant were subjected to antimicrobial activity assay. The mixture of peptides from the supernatant was dissolved in 0.05 M sodium acetate buffer, pH 4.2, and applied to a Bio-Rad S5 cation exchange column (Bio-Rad Laboratories). The column was washed with the same buffer, and peptides were eluted with a linear gradient of 0−0.5 M sodium chloride in the same buffer at a flow rate of 1.0 mL/min. Fractions were collected, dialyzed against Milli-Q water using a 100 Da cutoff membrane, lyophilized, and assayed for antimicrobial activity. Fractions that showed antimicrobial activity were further purified using reversed-phase high-performance liquid chromatography (RP-HPLC). Each of these fractions was dissolved in Milli-Q water containing 0.1% (v/v) TFA (solvent A) and applied to a C18 reversed-phase column (250 mm × 4.0 mm) (Vydac, Hesperia, CA). Solvent B was 0.085% (v/v) TFA in acetonitrile. After 2 min at 100% solvent A, elution was performed using a linear gradient from 0% to 50% B in 40 min at a flow rate of 1.0 mL/min. Several subgroup fractions of all peaks were collected, dialyzed, lyophilized, and assayed for antimicrobial activity. The fractions that had antimicrobial activity were resubjected to further purification. Reversed-phased chromatography by using a Sephasil protein C4 5 μm ST 4.6/250 column (Amersham Pharmacia Biotech, Piscataway, NJ) was performed to separate the active peptides with the same buffer conditions as C18 RP-HPLC.

Peptide Sequence Analysis and MALDI. The peptide sequence analysis was carried out at the Nucleic Acid Protein Services (NAPS) Unit, Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada. Sequencing services at the NAPS Unit were performed on the Perkin-Elmer ABI 476A automated sequencer (Perkin-Elmer Instruments, Norwalk, CT) using the Edman degradation procedure. In this standard method for sequencing peptides, the amino-terminal residue is reacted with phenyl isothiocyanate and removed as phenylthiohydantoin (PTH) derivatives (*36*). Matrix-assisted laser desorption ionization (MALDI) using Applied Biosystems Voyager System 4377 was performed to determine the molecular mass of isolated peptides.

Antimicrobial Activity Assay. In this study, we refer to bacteriostatic activity as an antimicrobial activity. To determine antimicrobial activity, 10 mL portions of trypticase soy broth (TSB) were first inoculated with a colony of bacteria (*E. coli* or *S. aureus*) and incubated overnight at 37 °C. One milliliter of the bacterial suspension was then diluted (1:50) in TSB. Bacteria were grown at 37 °C until the logarithmic phase was reached as determined by the absorbance at 660 nm. Bacteria were harvested by centrifugation at 5000*g* at 4 °C for 10 min, then washed with a 10 mM sodium phosphate and 137 mM sodium chloride buffer, pH 7.4, and resuspended (3×10^5 cfu/mL) in the same media. One milliliter of the bacterial suspension was mixed with an equal volume of various concentrations of lysozyme digests or their purified fractions in the same medium. The mixtures of peptide and bacteria were incubated at 37 °C for 15 h, and the absorbance at 660 nm was measured using a Novospec 2 Pharmacia spectrophometer (Amersham Pharmacia Biotech). A 100 μL aliquot was placed onto nutrient agar plates. Colony-forming units were obtained after incubation of plates at 37 °C for 24 h. Assays were performed in triplicate.

Scanning Electron Microscopy (SEM). Suspensions of *S. aureus* and *E. coli* at midlog phase (3×10^5 cells/mL) grown in TSB were incubated with 400 μg/mL isolated peptide at 37 °C for 15 h. The bacteria were washed with 0.85% NaCl. After fixing by the addition of glutaraldehyde (Sigma) (final concentration 2.5% w/v), cell pellets obtained by centrifugation were washed in 20 mM phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde. The fixed cells were carefully spotted onto the slide and then dehydrated in ethanol gradients. Dried cells were coated with gold and examined by scanning electron microscopy (Hitachi model S-570, Hitachi, Tokyo).

Statistical Analysis. Data were analyzed by ANOVA (SPSS version 8.0 for Windows; SPSS, Chicago, IL), and the means were separated by Duncan's multiple ranges test. The significance was defined at $P < 0.05$.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis of Lysozyme. It has been reported that heat treatment of lysozyme induces its susceptibility to proteolytic digestion. Generally, lysozyme is resistant to trypsin, but is digested by pepsin (*32*). **Figure 1** shows a Tris−tricine SDS−PAGE of peptic and tryptic digests of native and heat-denatured lysozyme. Heat-denatured lysozyme formed a small amount of dimer. Peptic digests of both intact and heat-induced denatured lysozyme showed no siginificant difference in SDS−PASGE pattern and produced smaller peptides of less than 5 kDa. Trypsin digested both lysozyme forms and exhibited two large fragments as well as a nondigested one. This is in conflict with a previous report of During (*32*). Probably, this is because of different enzyme conditions and analytical techniques for picking the smaller fragment up. We used Tris−tricine SDS−PAGE, which is suitable for peptide analysis. The peptic digest and the following tryptic digest were more efficient to hydrolyze lysozyme to smaller peptides. Therefore, we used a combination of both enzymes in this study for searching antimicrobial peptides.

The enzyme activity of the hydrolysate of lysozyme was investigated (**Figure 2**). With heating at 90 °C for 10 min, the lysozyme solution still retained 26% of its enzyme activity.

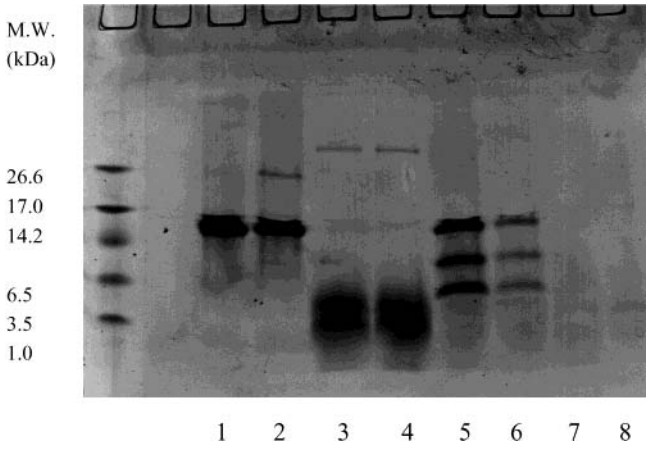

Figure 1. Tris–tricine SDS–PAGE of peptic and tryptic digestion of native and heat-denatured lysozyme. Bands 1, 3, 5, and 7 are native and bands 2, 4, 6, and 8 are heat-denatured lysozyme. Bands 1 and 2: nondigested lysozyme. Bands 3 and 4: peptic digestion. Bands 5 and 6: tryptic digestion. Bands 7 and 8: peptic digestion followed by tryptic digestion.

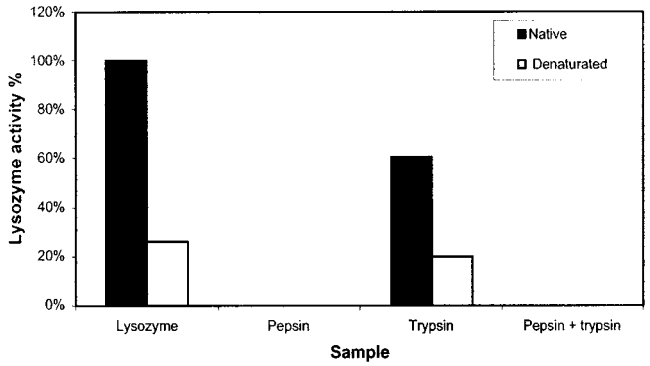

Figure 2. Enzyme activity of lysozyme hydrolysate on *M. lysodeikticus*.

Tryptic digests of native and heat-denatured lysozyme possessed 60.5% and 20% enzyme activity, respectively. This is because the tryptic digest still contained intact protein in the mixture. However, enzyme activity was completely abolished by treatment with pepsin or a combination of pepsin and trypsin (**Figure 2**). On the basis of these results, we focused on the hydrolysate derived by peptic digestion and followed by tryptic digestion of native lysozyme to search for novel nonenzymatic antimicrobial peptides which were used for the following experiments.

Purification of Antimicrobial Peptides. In the preliminary experiment as a function of peptide mixture concentration, the minimum concentration exhibiting bacteriostatic activity was determined to be 400 μg/mL (data not shown). The lysozyme hydrolysate was clarified by centrifugation, and the resulting precipitate contained the non-water-soluble (insoluble) peptides, while the supernatant contained water-soluble (soluble) peptides. The insoluble peptides did not demonstrate significant antimicrobial activity against either *E. coli* or *S. aureus* (data not shown). Thus, we focused on the water-soluble fraction for screening for antimicrobial peptides at the peptide concentration of 400 μg/mL. The soluble peptide mixture obtained from the lysozyme hydrolysate digested by pepsin and trypsin was separated on a Bio-Rad S5 cation exchange column as shown in **Figure 3**. The peptide mixture was collected in four fractions (1−4), and each was tested using the bacteriostatic activity assay. As shown in **Figure 4**, F1 was the most active against *E. coli* K-12, which inhibited up to 70% of bacterial growth. The F3 fraction was found to be the most active against *S. aureus* 23-394, and inhibited up to 35% of bacterial growth.

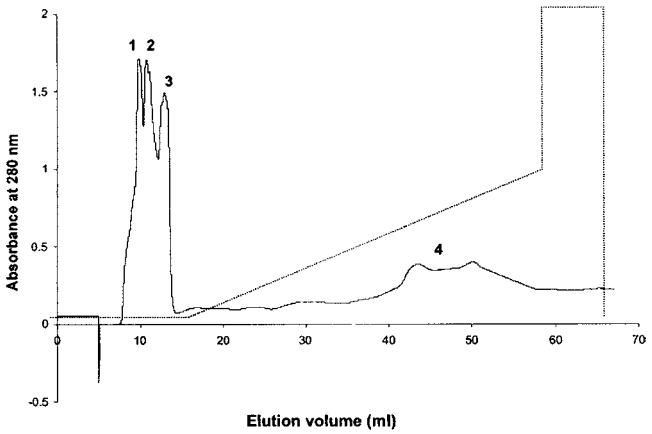

Figure 3. Bio-Rad S5 ion-exchange column chromatography of soluble peptides obtained by lysozyme digestion by peptic and subsequent tryptic digestion. The salt gradient is shown by a dotted line.

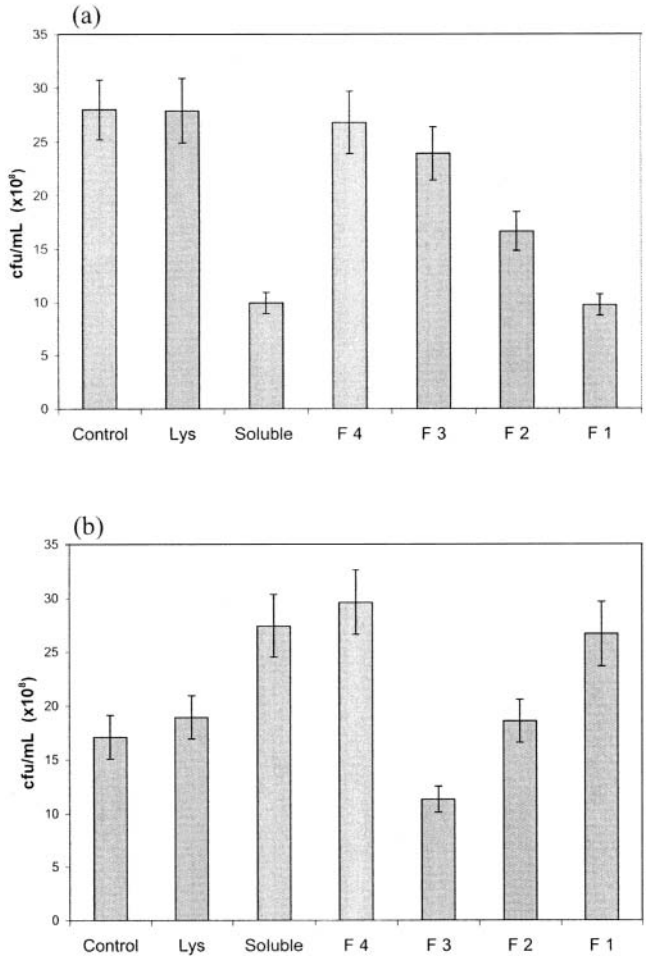

Figure 4. Antimicrobial activity against *E. coli* K-12 (a) and *S. aureus* 23-394 (b) of peptide fractions obtained by Bio-Rad S5 column chromatography: control, bacteria only; Lys, native lysozyme; fractions 1–4, obtained on a Bio-Rad S5 column. The concentration of peptides was 400 μg/mL. The standard error was expressed by triplicate measurements.

Fractions F3 and F4 had no significant antimicrobial activity against *E. coli* K-12, while F1 and F4 actually increased bacterial growth of *S. aureus*. Therefore, F1 and F3 were further purified using a C18 reversed-phase HPLC column. The profile of C18 reversed-phase chromatography of F1 is shown in **Figure 5a**. Four groups (F1a−F1d) were collected, and were subjected to the bacteriostatic assay. The results are shown in **Figure 6a**.

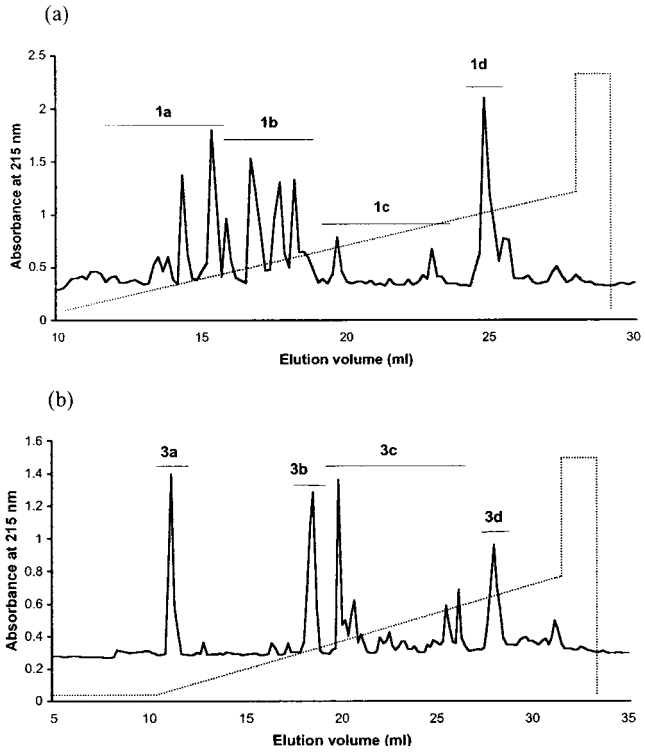

Figure 5. C18 reversed-phase HPLC profiles of peptide fraction 1 (a) and fraction 3 (b). The buffer gradient is shown by a dotted line.

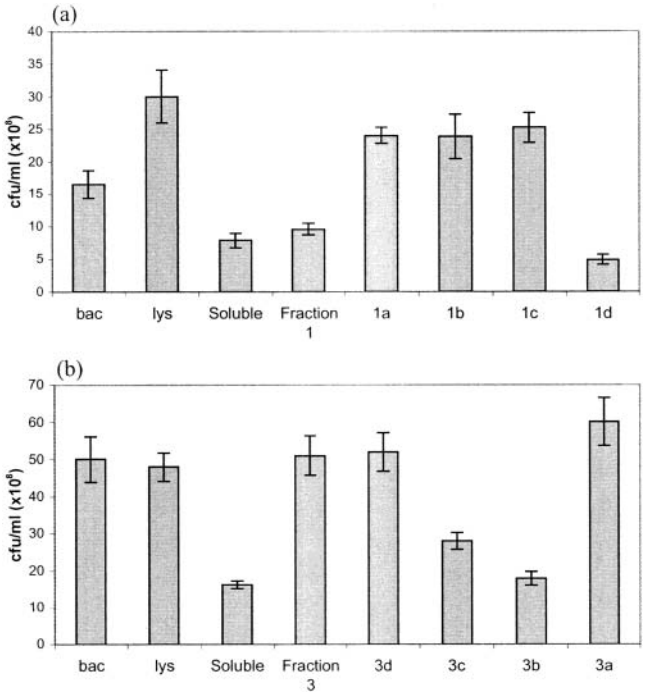

Figure 6. Antimicrobial activity against *E. coli* K-12 (a) and *S. aureus* 23-394 (b) of peptide fractions obtained by C18 reversed-phase column chromatography: control, bacteria only; Lys, native lysozyme; each fraction was obtained on a C18 reversed-phase column. The concentration of peptides was 400 μg/mL. The standard error was expressed by triplicate measurements.

The C18 reversed-phase profile of F3 is shown in **Figure 5b**. Four peaks (F3a−F3d) were also collected, and antimicrobial assay results are shown in **Figure 6b**. As shown in **Figure 6a**, F1d was the most active fraction against *E. coli* K-12, inhibiting up to 75% of bacterial growth at a concentration of 400 μg/

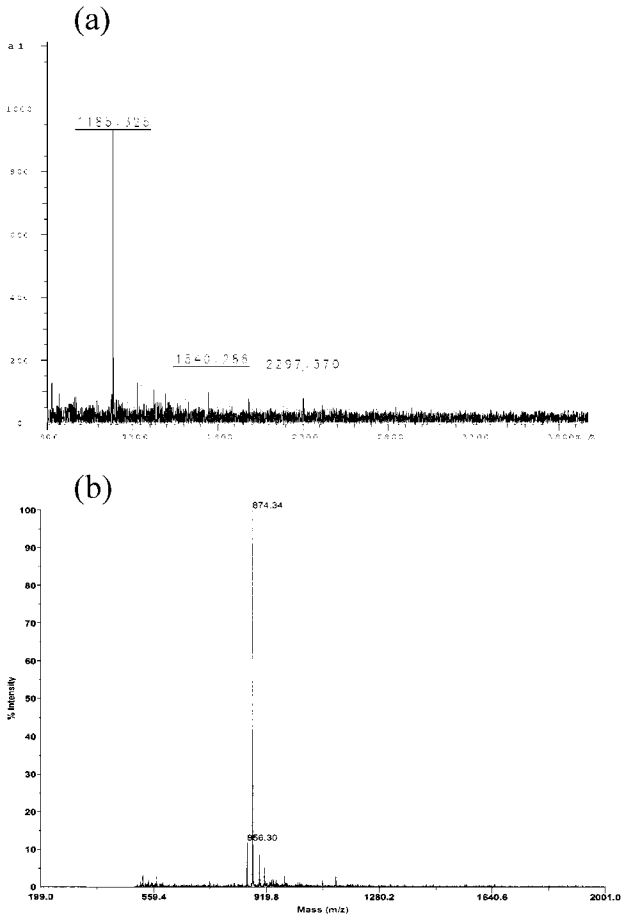

Figure 7. MALDI-MS of isolated peptides F1d (a) and F3b (b).

mL. These results suggested that one of the antimicrobial peptides was in this fraction. Moreover, there was just one major peak in this mixture, as shown by the HPLC profile of reversed-phase chromatography, and this peptide had an elution volume at 25 mL. This major peak was therefore assumed to be an antimicrobial peptide against *E. coli* K-12, and was further purified by C4 RP-HPLC as described before. As shown in **Figure 6b**, F3b was the most active fraction against *S. aureus* 23-394, inhibiting up to 70% of bacterial growth. Therefore, the antimicrobial peptide against Gram-positive bacteria appeared to be in this fraction. There was just one peak, and this peptide peak had an elution volume at 19 mL. It was further purified on a C4 RP-HPLC column. These two isolated peptides were then subjected to amino acid sequence analysis and MALDI. The amino acid sequence of F1d was determined to be Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp (residues 98−108 of lysozyme) and that of F3b to be His-Gly-Leu-Asp-Asn-Tyr-Arg (residues 15−21 of lysozyme) (data not shown). The molecular masses of F1d and F3b as determined by MALDI were 1185 and 874 Da, respectively (**Figure 7**).

The lysozyme molecule consists of two domains or lobes, the α- and β- domains, linked by a long α-helix between which lies the active site. Helix A (4−15), helix B (24−36), helix C (88−99), helix D (108−115), and a 3_{10} helix (120−125) form the α-domain. A triple-stranded β-sheet (41−60), central 3_{10} helix (79−84), and large loop (61−78) form the β-domain. The active site of hen egg white lysozyme consists of six subsites which are sufficient to bind six sugar residues, A, B, C, D, E, and F. These six subsites are along the active cleft position of the catalytic groups Glu35 and Asp52 (*37*). The peptide inhibiting *E. coli* K-12, isolated from F1d, contained 11 amino

Figure 8. Schematic ribbon representation of hen egg white lysozyme. The two bacteriostatic peptide sequences 98–108 and 15–21 are shown. The structure was drawn using Dep View/Swiss-Pdb Viewer version 3.7 from Glaxo Smithkline (Geneva, Switzerland).

acids and was identified to be residues 98−108 of lysozyme. This peptide was located in the middle part of the helix−loop−helix motif of lysozyme (**Figure 8**). Amino acid residues 98−108 are in the N-terminal helix. The loop is composed of residues 101−106. Residues 107−108 are in the C-terminal helix (*31*). This peptide has two helices to exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria. A very similar peptide, amino acid residues 98−112 (Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg), which was obtained by clostripain digestion of lysozyme, was shown to possess a broad spectrum of antimicrobial action against Gram-negative and Gram-positive bacteria but without muramidase activity (*30*). The sequence 107−110 (Trp-Val-Ala), as part of the active center of lysozyme, was not substantially involved in the noncatalytic mechanism of bacterial inhibition (*30*). The sequence of the isolated peptide in this study was composed of residues 98−108. The present study provides the potential of releasing antimicrobial peptide to prevent bacterial infection in the GI since it was released by peptic digestion and the following trypric digestion, which mimic the digestive system. Another antimicrobial peptide, that inhibiting *S. aureus* 23-394, isolated from F3b contained seven amino acids (residues 15−21 of lysozyme). This peptide is a novel antimicrobial peptide in chicken egg white lysozyme being reported for the first time here. Residues 15 and 16 are in the N-terminal helix (helix Gly4−Gly16). Another part of this peptide is located in the loop. This loop is composed of residues 17−25 (**Figure 8**).

The inhibition of *E. coli* K-12 and *S. aureus* 23-394 by peptides 98−108 and 15−21 was demonstrated by SEM observation of the morphological changes associated with the promoted susceptibility of Gram-negative *E. coli* and Gram-positive *S. aureus* (**Figure 9**). Upon incubation with a 400 μg/mL concentration of each isolated peptide, the bacterial membrane had direct damage after 15 h. These results indicated both peptides directly interact with bacterial surface and damage membrane integrity.

The present study clearly demonstrates that egg white lysozyme contains peptide sequences that can induce noncatalytic bacterial inhibition, which differs from enzymatic lysis of cell membranes. Structural and functional characterization of natural antimicrobial peptides is of growing interest because of their possible therapeutic applications and food industry applications. The α-helix and net positive charge play an important role in bacteriolytic activity. A major group of these antimicrobial peptides share a common structural motif, namely, a

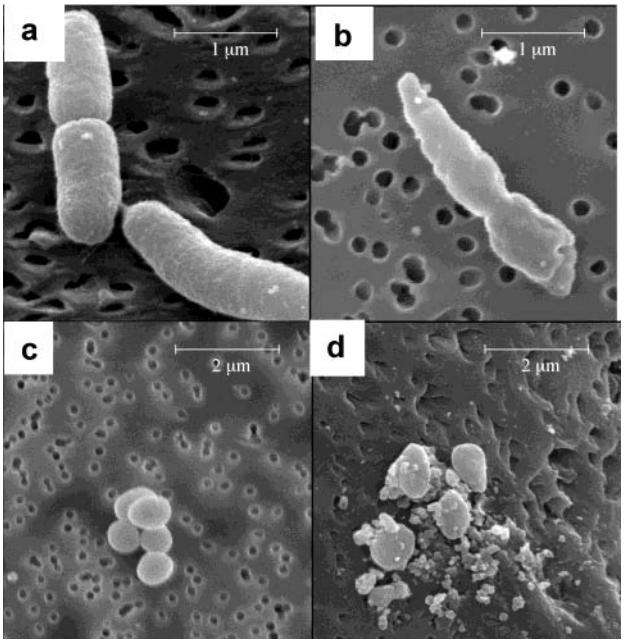

Figure 9. Scanning electron micrographs of *E. coli* K-12 (a, b) and *S. aureus* 23-394 (c, d) incubated without (a, c) and with (b, d) antimicrobial peptides derived from lysozyme. (b) Peptides 98–108 and 15–21 were used at 400 μg/mL and incubated for 15 h at 37 °C for the test, respectively.

helix−loop−helix or a α-helical hairpin (*31*), and are composed of short sequences (≤40 residues) that vary considerably in chain length, hydrophobicity, and overall charge.

The active site of lysozyme divides the molecule into two domains, the α-domain and the β-domain, whereas the helix−loop−helix motif is uniquely located at the upper lip of the active site of the enzyme. Ibrahim et al. (*31*) have reported that the N-terminal helix was sufficient to exert antimicrobial action against Gram-positive bacteria, whereas the α-helical hairpin structure or the C-terminal helix was needed to extend the activity toward Gram-negative bacteria (*31*). Most of the α-helical hairpin antimicrobial peptides, such as ceropins (*38*, *39*) and those found in the C-terminal region of colicins (*40*, *41*), are active against both Gram-positive and Gram-negative bacteria by disrupting bacterial membrane function. The cellular target for most α-helical peptides is the cytoplasmic membrane. To achieve this, the α-helical peptides must penetrate the outer membrane of Gram-negative bacteria. They may induce disruption of the inner membrane of bacteria as well. However, the inner membrane disintegration could result in a lethal event (*31*). The peptide Ile98−Trp108 was assumed to be able to permeate the outer membrane of *E. coli*. The C-terminal helix was the portion to partition into the lipid bilayer of the outer membrane. This peptide permeated the bacterial membrane by spanning the membrane via the C-terminal helix, probably forming a spiral-like channel, as an oligomer or stacks in the lipid bilayer of the bacterial cell wall. However, another peptide, His15−Arg21, obviously had no C-terminal helix, just an N-terminal helix and loop; therefore, it could not permeate the outer membrane of *E. coli* to reach and disrupt the inner membrane.

The present study shows that hen egg white lysozyme possesses nonenzymatic bacteriostatic domains in its primary sequence and these peptides exhibiting bacteriostatic activity could be released by proteolytic digestion of native lysozyme.

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