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Isolation and characterization of antimicrobial proteins and peptide from chicken liver

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Abstract: Endogenous antimicrobial peptides and proteins are crucial components of the innate immune system and play an essential role in the defense against infection. Antimicrobial activity was detected in the acid extract of livers harvested from healthy adult White Leghorn hens, *Gallus gallus*. Two antimicrobial proteins and one antimicrobial polypeptide were isolated from the liver extract by cation-exchange and gel filtration chromatography, followed by two-step reverse-phase high-performance liquid chromatography (RP-HPLC). These antimicrobial components were identified as histones H2A and H2B.V, and histone H2B *C*-terminal fragment using peptide mass fingerprinting and partial sequencing by tandem nanoelectrospray mass spectrometry. The proteins and the peptide identified in the present study, which exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria, were thermostable and showed salt-resistant activity. The antimicrobial properties of histones and histone fragment in chicken provide further evidence that histones, in addition to their role in nucleosome formation, may play an important role in innate host defense against intracellular or extracellular microbe invasion in a wide range of animal species.
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Keywords: antimicrobial protein/peptide; histones; liver; chicken; mass spectrometry

INTRODUCTION

Antimicrobial peptides and proteins are widely distributed throughout the plant and animal kingdoms [1]. These molecules, which are either constitutive or inducible, serve as crucial components in the innate host defenses and represent a potential source of useful natural antibiotics for pharmaceutical applications [2,3]. Antimicrobial peptides and proteins show broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and some enveloped viruses [4]. Unlike many natural antimicrobials synthesized via specialized metabolic pathways, these peptides/proteins are gene-encoded and are often synthesized as precursor molecules, which are then proteolytically cleaved to generate active molecules [1]. Antimicrobial peptides are generally thought to act by disrupting the membrane integrity. It has recently been reported that, in addition to their antimicrobial activity, some antimicrobial peptides and proteins possess multifunctional properties, such as antitumor [5,6], anti-inflammatory [7,8] and immunomodulatory activities [9–11].

Currently, over 880 antimicrobial peptides and proteins have been described and are listed on the comprehensive, periodically updated Antimicrobial Sequences Database (http://www.bbcm.univ.trieste.it/~tossi/amsdb.html), in which the majority of those reported are from insects, plants, amphibians and mammals. Over the past decade, a few antimicrobial peptides and proteins from chicken have been reported. Cationic antimicrobial peptides homologous to β-defensins, known as gallinacins (Gal-1, -1α and -2) have been isolated and characterized from chicken heterophils [12,13]. Recently, Lynn *et al.* [14] and Xiao *et al.* [15] have reported independently the discovery of seven novel chicken β-defensins using a bioinformatics approach and demonstrated their differential gene expression across a panel of chicken tissues. The deduced prepropeptide sequences of these β-defensins vary from 64 to 104 amino acid residues in length. Lynn *et al.* also identified a novel cathelicidin, which is expressed across a wide variety of tissues [14], and a novel, unique, liver-expressed antimicrobial peptide 2 (LEAP-2), which is also expressed in the intestine, gall bladder and kidney [14,16]. LEAP-2 is upregulated in the small intestine and liver after oral *Salmonella enterica* infection [17]. More recently, Nile *et al.* [18] purified and characterized a 3.5 kDa antimicrobial peptide from the mucosa and epithelial cells of chicken intestine, which was identified as the carboxy-terminal fragment of a novel 767 amino acid avian protein containing multiple domains with homology to protease inhibitory modules.

Although many antimicrobial peptides and proteins have been discovered in chicken using bioinformatic

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algorithms and molecular biological approaches at the nucleic acid level [14–17,19–21], little information is available regarding the isolation and structural characterization of antimicrobial peptides/proteins at the amino acid level and their antimicrobial profiles from chicken tissues. Moreover, post-translational modifications of antimicrobial proteins and peptides, such as proteolytic processing, glycosylation, carboxy-terminal amidation, phosphorylation and halogenation, have been described [1,4]. The vertebrate liver plays a central role in the innate immune response to infection. The 'acute phase' response to infection or inflammation is accompanied by a pattern of increased hepatic synthesis of many secreted proteins involved in host defense and the selective suppression of synthesis of other secreted proteins [22]. To date, no antimicrobial peptides or proteins from chicken liver have been isolated and characterized at the amino acid level. In this study, we report the purification and structural characterization of antimicrobial proteins and peptide from chicken liver.

MATERIALS AND METHODS

Preparation of Liver Crude Extract

Twelve healthy adult White Leghorn laying hens (*G. gallus*) were obtained from the Arkell Poultry Research Station, University of Guelph, Canada. The animals were humanely sacrificed by cervical dislocation. The livers were immediately removed and frozen in liquid nitrogen. The tissues were ground into a powder under liquid nitrogen using a mortar and pestle (Fisher Scientific Company, Ontario, Canada). The tissue powder was suspended in ice-cold 1% acetic acid (1 : 2, w/v) supplemented with 3 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma–Aldrich Canada, Ontario, Canada). Following extraction by stirring for 1 h on ice, the homogenate was boiled for 5 min. The homogenate was then centrifuged at 10 000 *g* for 30 min at 4 °C. The supernatant was collected and stored at −80 °C until analysis.

Radial Diffusion Antimicrobial Assay

The antimicrobial activity of the crude extracts and purified proteins during each purification step was examined by the radial diffusion assay (RDA) as previously described with some modifications [23]. Briefly, *Bacillus subtilis* (kindly provided by Dr Poppe, C.C., Health of Animal Lab., Health Canada, Guelph, ON, Canada) and *E. coli* D31 (CGSC E. coli genetic stock center, Yale University, MA, USA) were grown overnight in Brain Heart Infusion (BHI, BD Biosciences, Ontario, Canada) broth and trypticase soy broth (TSB, BD Biosciences, Ontario, Canada), respectively. The bacteria were centrifuged at 1500 *g* for 15 min at 4 °C, washed twice using cold 20 mM phosphate buffered saline (PBS), pH 7.4 and resuspended in cold PBS, pH 7.4. The bacterial concentration was adjusted to 1×10^6 CFU/ml using a Vitek colorimeter (Hach Company, CO, USA). The bacterial suspension was then added to the previously autoclaved agar broth composed of 1.5% low-EEO agar (Sigma–Aldrich), 0.5% yeast extract, 1% tryptone (BD Biosciences) and 100 mM phosphate buffer (PB, pH 6.8) to a final bacterial concentration of 1×10^5 CFU/ml. The agar suspension was poured into sterile Petri dishes (Fisher Scientific) to form a uniform layer of approximately 1mm thickness. After the agar solidified, wells were made by using a puncher with 3 mm diameter. The sample (3–5 µl) was added to wells and the dishes were incubated at 37 °C for 18 h. The antimicrobial activities were assayed by observing the suppression of bacterial growth around the wells. Clear zones indicating no growth of bacteria were seen when the samples contained antimicrobial factors.

Bacterial Gel Overlay Antimicrobial Assay

The bacterial gel overlay assay (GOA) was performed as previously described with modifications [23]. Specifically, the tissue crude extracts were analyzed using acid-urea–polyacrylamide gel electrophoresis (AU–PAGE). After electrophoresis, the gel was washed by rinsing twice for 10 min in 0.01 M phosphate buffer, pH 7.2. The gel was placed on a bacterial plate prepared exactly as described earlier for RDA, and incubated at 37 °C to allow proteins in the gel to diffuse into the agarose. After 3 h, or overnight incubation if necessary, the gel was removed from the agarose and stained with Coomassie Blue. Clear zones were seen where the growth of bacteria was suppressed by protein bands with antimicrobial activity.

Purification of Antimicrobial Proteins

Crude extracts were loaded onto a carboxymethyl cellulose (CM, Sigma–Aldrich) cation-exchange column (3 × 15 cm) that was previously equilibrated with 20 mM ammonium acetate (pH 5.0). The proteins bound to the CM were eluted using a gradient from 20 to 1500 mM ammonium acetate at a flow rate of 1 ml/min and 30 min fractions were collected. Fractions were lyophilized and then reconstituted in 0.01% acetic acid for determination of antimicrobial activity by RAD or GOA. Pooled active fractions were loaded onto a Superdex 75 10/30 gel filtration column (1 × 30 cm, Amersham Pharmacia Biotech, Sweden) equilibrated with 0.2 M ammonium acetate buffer solution (pH 5.0) and eluted with the same buffer solution at a flow rate of 0.5 ml/min. The elution was monitored at 280 nm. The fractions were lyophilized and redissolved in 0.01% acetic acid for antimicrobial activity assay. Fractions with antimicrobial activity were subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a Sephasil Peptide C8 5 µm ST 4.6/250 column (4.6 × 250 mm, Amersham Pharmacia Biotech, Sweden). The separation was performed with a linear gradient from 100% solvent A (0.1% TFA in Mili Q water) to 60% of solvent B (100% acetonitrile containing 0.085% TFA) over 60 min and then another linear gradient from 60% of solvent B to 100% of solvent B for 5 min, at a flow rate of 1 ml/min. The elution was monitored at 214 nm. Active fractions of interest were further chromatographed by RP-HPLC on µRPC C2/C18 5 µm ST 4.6/100 column (4.6 × 100 mm, Amersham Pharmacia Biotech, Sweden) under a shallower gradient from 30 to 60% or from 25 to 55% acetonitrile in 0.1% TFA over 60 min, at a flow rate of 0.5 ml/min. After each step, the protein profiles of the active fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% separating gel and 4% stacking gel. After electrophoresis the gel was stained with silver stain

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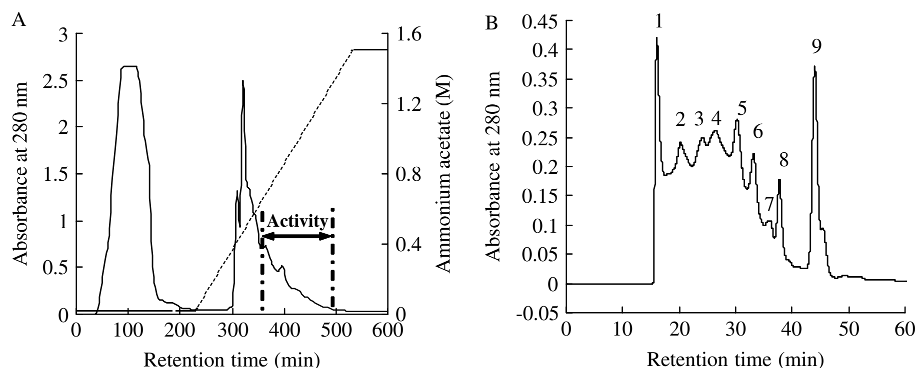

Figure 1 Isolation of antimicrobial proteins from chicken liver. (A) Cation-exchange chromatography of liver acetic acid extract on a CM-cellulose cation-exchange column (3 × 15 cm). The proteins bound to the CM were eluted using a gradient from 20 to 1500 mM ammonium acetate at a flow rate of 1 ml/min and 30 min fractions were collected. The fractions eluting between 0.6 and 1.2 M ammonium acetate demonstrated antimicrobial activity against both *E. coli* D31 and *B. subtilis*. (B) Gel filtration chromatography of pooled active fractions from the cation-exchange column on a Superdex 75 10/30 column (1 × 30 cm). Separation was performed at a flow rate of 0.5 ml/min with 0.2 M ammonium acetate buffer solution (pH 5.0). The elution profile was monitored at 280 nm.

using a commercial kit (Sigma) according to the manufacturer's manual. The purities of the purified antimicrobial proteins were further assayed by SDS-PAGE and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Mass Spectrometry and Sequence Analysis

Protein identification by mass spectrometry analysis was performed at the Mass Spectrometry Facility, Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, Canada. All MALDI-TOF mass spectra of intact proteins were obtained on a QSTAR XL MALDI Qq TOF instrument (Applied Biosystems MDS-SCIEX, Ontario, Canada). All nanoelectrospray mass spectrometry (nES-MS) experiments for peptide mapping of tryptic digests of proteins were conducted on a Q-TOF hybrid quadrupole/time-of-flight instrument (Micromass, UK), for high-resolution and on-line liquid-chromatography-tandem mass spectrometry (LC-MS/MS) experiments. MS/MS experiments on trypsin-digested proteins identified in the survey scan were performed using a nanoelectrospray source. Precursor ions were selected by the first quadrupole, while a pusher electrode was pulsed to transfer fragment ions formed in hexapole cell to the TOF analyzer. Collision activation was performed using argon collision. The spectral data were analyzed using the MassLynx program from Micromass. To identify potential peptide matches, the MS/MS data were used to conduct database searches with the Mascot peptide search program (http://www.matrixscience.com). The list of peptide masses were searched against the nonredundant protein sequence database (NCBInr database) provided by the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov).

RESULTS

Antimicrobial Activity of Liver Extracts and Purification of Antimicrobial Proteins

Crude liver extract with or without heat-treatment showed weak antimicrobial activity against both *E. coli* D31 and *B. subtilis* in the RDA owing to the low concentration of antimicrobial factors contained in the supernatant of the liver extract (data not shown). However, the antimicrobial agents were thermostable, retaining their activity even after boiling for 5 min. The liver crude extract was loaded onto the cation-exchange column and eluted with a linear gradient of ammonium acetate buffer from 20 to 1500 mM. The fractions eluting between 0.6 and 1.2 M ammonium acetate demonstrated antimicrobial activity against both *E. coli* D31 and *B. subtilis* (Figures 1(A) and 2(A)). The active fractions were pooled and subjected to AU-PAGE on a 12.5% homogeneous acid-urea polyacrylamide gel for bacterial gel overlay antimicrobial assay. AU-PAGE is a native-polyacrylamide gel electrophoresis that separates proteins on the basis of their charge density. Three distinct inhibitory zones were observed on the bacterial agarose plate after blotting with the electrophoresed pooled active fractions from the CM-cellulose column (Figure 2(B)), indicating that at least three types of antimicrobial proteins or peptides with different ionic charge strength were present in the liver extract. As can also be seen from Figure 2(B), the antimicrobial proteins that have more positive charges and therefore correspondingly migrate far through the gel under the acid electrophoresis condition had stronger antimicrobial activity. In particular, the protein band at the lower position that is hardly visualized on the gel had very strong activity.

Pooled active fractions from the CM-cellulose column were applied to a Superdex 75 10/30 gel filtration column and nine fractions were eluted in this chromatography (Figure 1(B)). Antimicrobial activities against both Gram-positive and Gram-negative bacteria were observed in fractions 1–4, whereas fractions 5 and 6 were active against only *B. sutilis* (Figure 2(C)). Antimicrobial protein present in fraction

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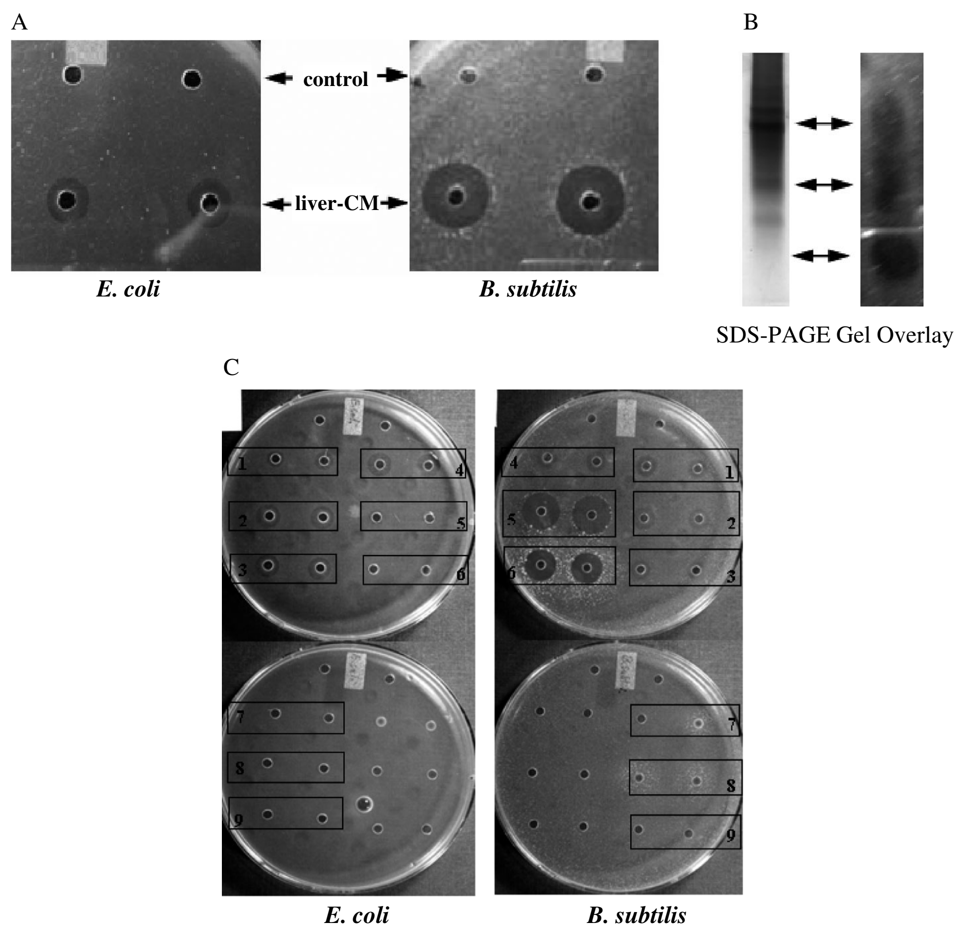

Figure 2 Antimicrobial activity of proteins from chicken liver. (A) Radial diffusion assay of pooled active fractions from cation-exchange chromatography against *E. coli* D31 and *B. subtilis*. Antimicrobial activity was tested by placing 5-μl samples in each well. Control, 0.01% acetic acid; liver-CM, pooled active fractions from CM cation-exchange column. (B) AU-PAGE gel overlay antimicrobial assay against *E. coli* D31 (results for *B. subtilis* not shown). Antimicrobial activity was visualized as clear zones shown in the stained agarose bacterial plate after blotting with the electrophoresed gel. (C) Radial diffusion assay of fractions from gel filtration chromatography against *E. coli* D31 and *B. subtilis*. Antimicrobial activity was tested by placing 3-μl samples in each well.

5 was identified as lysozyme (data not shown). Because of the limited amounts of fractions 1, 4 and 6, we focused on the re-purification of fractions 2 and 3. Fractions 2 and 3 were further purified by C8 RP-HPLC. Chromatography by C8 RP-HPLC of fraction 2 resulted in five active fractions, designated as P2-A, P2-B, P2-C, P2-D and P2-E (Figure 3(A)). P2-D and P2-E were purified to homogeneity by a second RP-HPLC on C2/C18 column using a shallower water/acetonitrile gradient (Figure 3(B) and (C)). The same procedure was used to purify the antimicrobial activity contained in fraction 3 from gel filtration chromatography. A single peak designated P3-A with strong activity against both *E. coli* D31 and *B. subtilis* was obtained after the second RP-HPLC (Figure 4(A) and (B)). SDS-PAGE confirmed the single protein purity of P2-D, P2-E and P3-A (Figure 5). The purified proteins were then subjected to mass spectrometry and protein identification.

Structural Characterization

Mass spectrometry analysis of the purified proteins revealed molecular masses of 13 823.3 and 13 851.2 (data not shown) for P2-D and P2-E, respectively. The molecular masses of these antimicrobial factors determined by MALDI-TOF were lower than the relative masses indicated by mobility on SDS-PAGE (Figure 5). The MALDI-TOF-MS spectra of the proteins following digestion with trypsin are shown in Figure 6. For the automated MS/MS protein identification, the most abundant peptide precursor ions at any given moment were automatically selected for collision-induced dissociation (CID), and the sequence information of the corresponding peptides was obtained as shown in Table 1. The sequence information was used to conduct database searches with the Mascot peptide search program. Taken together with the molecular masses obtained by MALDI-TOF of the intact proteins, MS/MS sequencing results and peptide mass fingerprinting, we

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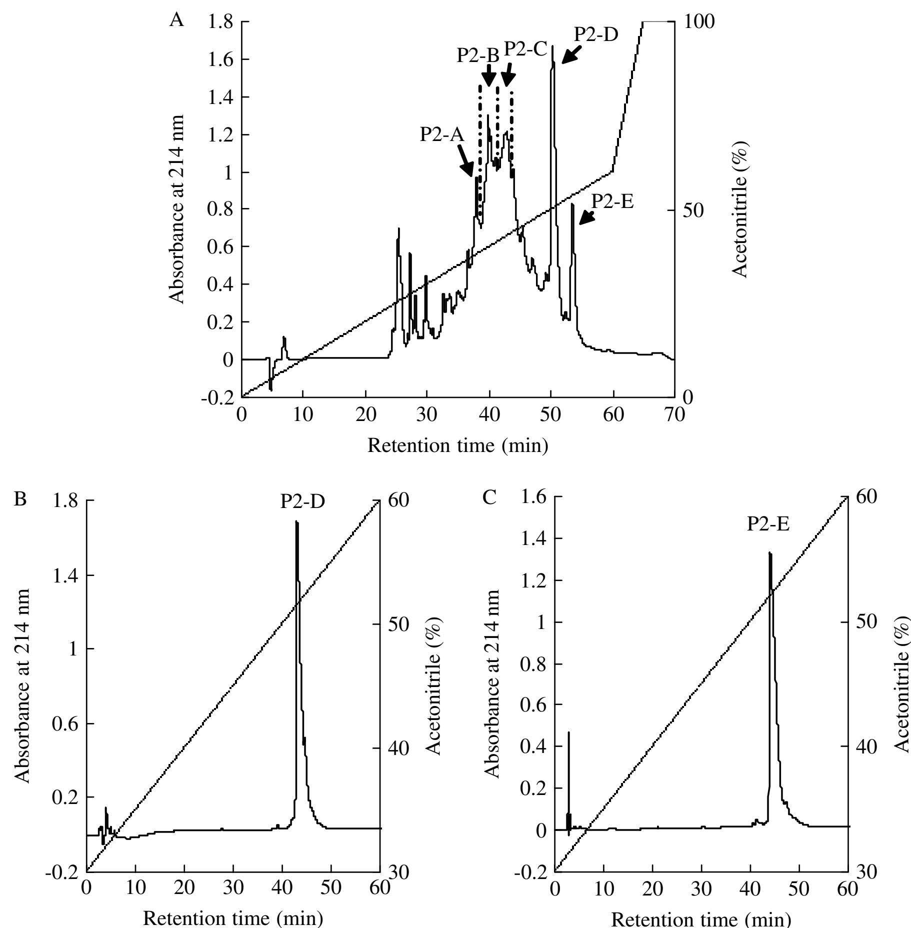

Figure 3 (A) Purification of fraction 2 from gel filtration chromatography by RP-HPLC on a C8 column. Fractions designated as P2-A, P2-B, P2-C, P2-D, P2-E and P3-A showed antimicrobial activity against both *E. coli* D31 and *B. subtilis*. (B) (C) Re-chromatography of 2-D, P2-E on a C2/C18 RP-HPLC column using a shallower acetonitrile gradient. The dashed line indicates the acetonitrile gradient. The elution profile was monitored at 214 nm.

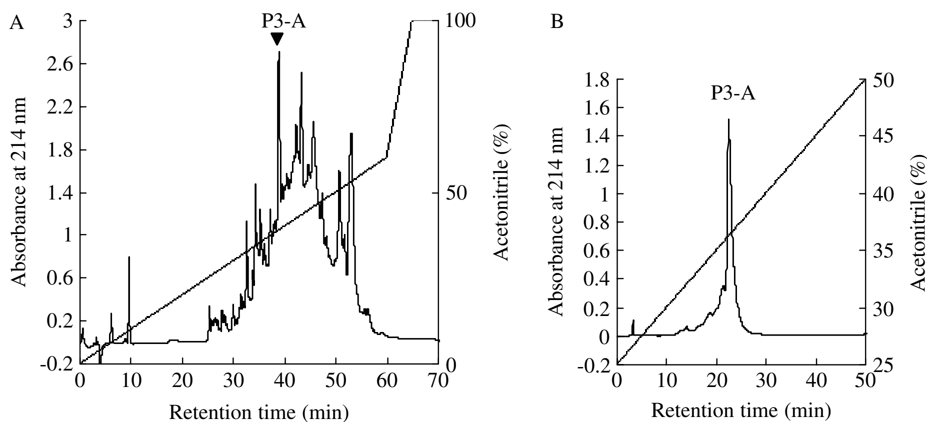

Figure 4 (A) Purification of fraction 3 from gel filtration chromatography by RP-HPLC on a C8 column. Fractions designated as P3-A showed antimicrobial activity against both *E. coli* D31 and *B. subtilis*. (B) Re-chromatography of P3-A on a C2/C18 RP-HPLC column using a shallower acetonitrile gradient. The dashed line indicates the acetonitrile gradient. The elution profile was monitored at 214 nm.

identify P2-D and P2-E as the histones H2B.V and H2A, respectively. However, our attempt to identify P3-A using MALDI-MS/MS was unsuccessful owing to the weak signal. P3-A was then subjected to LC-MS/MS, and the resulting peptide tandem spectra were analyzed using the software Mascot database to match

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chicken histone H2B. Only one matching peptide fragment derived from the histone H2B *C*-terminus was detected in the tryptic digests of P3-A (Figure 6(C)). By comparing the location and relative molecular mass of P3-A with those of P2-D (Figure 5), we therefore concluded that P3-A was the *C*-terminal fragment of histone H2B.

DISCUSSION

In this study, we describe the isolation and identification of antimicrobial proteins from chicken liver. These antimicrobials were identified as histones H2A, H2B and the *C*-terminal fragment of histone H2B, all of which are active against both Gram-positive and Gram-negative bacteria. To the best of our knowledge, this is the first report to demonstrate that histone H2A- and histone H2B-derived *C*-terminal fragment in avian species have antimicrobial activity against both Gram-positive and Gram-negative bacteria.

Several research groups have demonstrated that a number of antimicrobial peptide genes, including β-defensins and cathelicidin, are constitutively expressed in chicken liver [14–17,19–21]. However, in our assay method, the major antimicrobials from liver were found to be histones and a histone-derived fragment. Some antimicrobial peptides or proteins from animals are salt-sensitive and their activity can be altered depending on the salt concentration [24–27]. Taking into account the high salt (up to 140 mM) in RAD and GOA, it is unlikely that salt-sensitive antimicrobial peptides such as defensins and some cathelicidins could be active in such a milieu despite their constitutive expression in the liver of chicken, which at the same time suggests that histones H2A, 2B and its fragment identified from chicken liver in the present study are salt-tolerant. Like the insect fat body (functional equivalent of the mammalian liver) where abundant antimicrobial peptides are produced, transcriptionally induced and released into the hemolymph in response to infection or injury [28,29], the vertebrate liver is centrally involved in the innate immune response to infection. It is also possible that the healthy chickens used in this study express low levels of other antimicrobial peptides or proteins that were not detected in our assay method. Additionally, in some cases, an antimicrobial factor exerts an antimicrobial activity only when it synergizes with other antimicrobial components [30]. The synergistic effects would obviously be lost during the purification procedure in which interacting factors are separated. It is noteworthy that developmentally expressed antimicrobial peptides or proteins would no longer be present in the adult laying hens used in this study. As can be seen from Figures 1(B), 2(B) and 3(A), in fact, there are numerous unidentified antimicrobial components in the acid liver extract, especially relatively low molecular weight antimicrobial peptides with high positive charge as indicated in Figure 2(B), which are possibly salt-resistant. In the present study, the fractions used for isolation of antimicrobial proteins/peptides were selected according to both the intensity of their antimicrobial activity as well as their protein composition and amounts. Further purification and characterization of other antimicrobial proteins and peptides from chicken liver will be an obvious extension of this work.

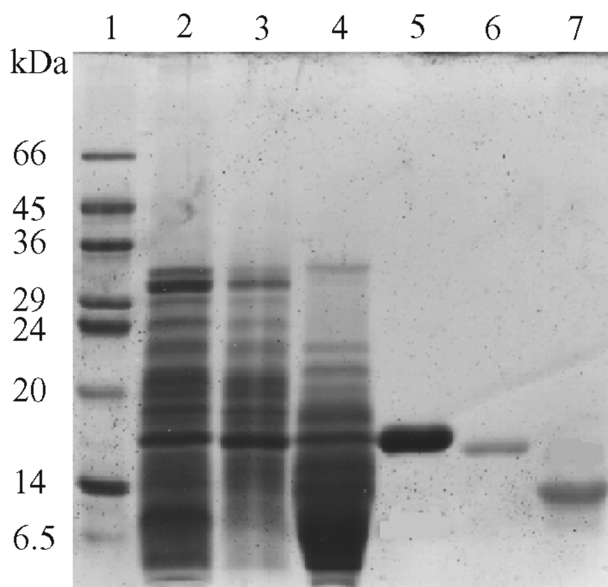

Figure 5 SDS-PAGE analysis of the active fractions obtained at each purification step. The gel was stained with silver. Lane 1, mass standards; lane 2, pooled active fractions from CM cation-exchange chromatography; lane 3, fraction 2 from gel filtration chromatography; lane 4, fraction 3 from gel filtration chromatography; lane 5, peak P2-D from the second RP-HPLC; lane 6, peak P2-E from the second RP-HPLC; lane 7, peak P3-A from the second RP-HPLC.

Interestingly, the molecular masses of histones H2A and 2B determined by MALDI-MS are far lower than the apparent relative masses determined by SDS-PAGE (Figure 5). Similar results have also been reported by other investigators [31,32] and in our recent study [33]. The strong positive charge of histones H2A and 2B.V with the theoretical isoelectric points (pI) of 10.90 and 10.32 calculated by Compute pI/Mw tool (http://www.expasy.org/tools/), respectively, slows their mobility in SDS-PAGE, giving an exaggerated apparent mass. This fact would explain the discrepancy between the apparent molecular mass determined by SDS-PAGE and the absolute mass measured by MS. On the basis of the amino acid sequence deduced from cDNA of histone H2B.V, the predicted molecular mass of histone H2B.V is 13 950.2 Da. If methionine 1, which is a putative initiation amino acid [34], is cleaved, the predicted molecular mass becomes 13 819.0 Da. The observed molecular mass for histone H2B.V by MALDI-MS is 13 823.3 Da, which

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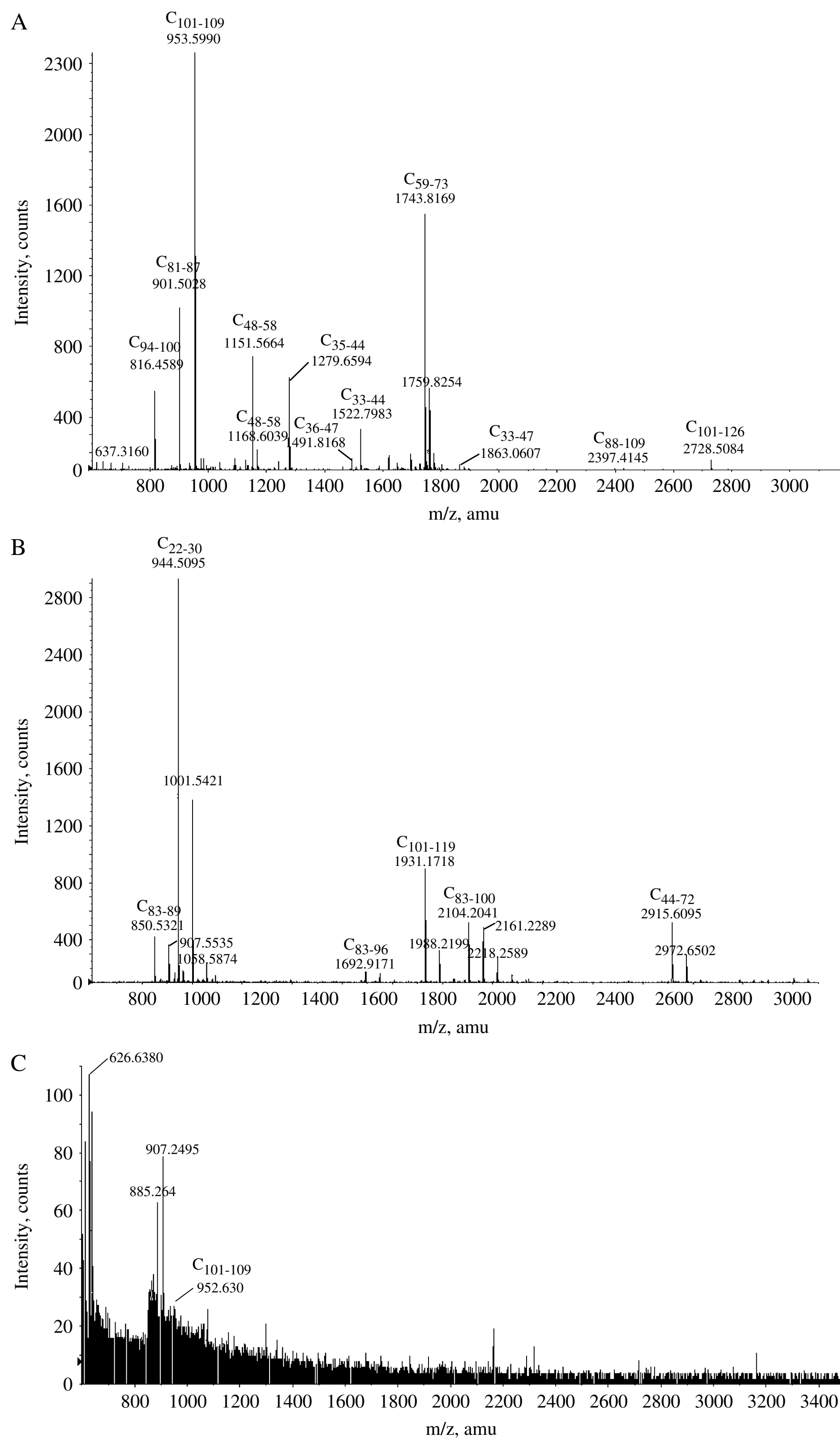

Figure 6 MALDI-TOF-MS spectra of tryptic digests from P2-D (A), P2-E (B) and P3-A (C). The masses of the identified monovalent peptide ions as well as their positions within the protein are indicated.

is within the experimental error of the instrument. On the other hand, the post-translational modifications of histones, mainly acetylation and methylation [35], can also explain the molecular mass differences between that theoretically calculated and that observed by MS. For histone H2A, the observed molecular mass

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Table 1 Peptide mass fingerprint of the antimicrobial proteins and peptide from chicken liver

Antimicrobial proteins[a]	m/z (Measured)	m/z (Calculated)	Protein fragment[b]	Peptide sequence (post-translational modification)
P2-D	816.459	816.458	H2B.V f94–100	EIQTAVR
P2-D	901.503	901.501	H2B.V f81–87	LAHYNKR
P2-D	953.599	953.604	H2B.V f101–109	LLLPGELAK
P2-D	1151.566	1151.570	H2B.V f48–58	QVHPDTGISSK Pyro-Glu (*N*-terminal Q)
P2-D	1168.604	1168.596	H2B.V f48–58	QVHPDTGISSK
P2-D	1279.659	1279.657	H2B.V f35–44	KESYSIYVYK
P2-D	1491.817	1491.810	H2B.V f36–47	ESYSIYVYKVLK
P2-D	1522.798	1522.791	H2B.V f33–44	SRKESYSIYVYK
P2-D	1743.817	1743.820	H2B.V f59–73	AMGIMNSFVNDIFER
P2-D	1863.061	1863.038	H2B.V f33–47	SRKESYSIYVYKVLK
P2-D	2396.498	2396.388	H2B.V f88–109	STITSREIQTAVRLLLPGELAK
P2-D	2728.508	2728.525	H2B.V f101–126	LLLPGELAKHAVSEGTKAVTKYTSSK
P2-E	850.532	850.526	H2A f83–89	HLQLAIR
P2-E	944.509	944.532	H2A f22–30	AGLQFPVGR
P2-E	1692.917	1692.903	H2A f83–96	HLQLAIRNDEELNK
P2-E	1931.172	1931.169	H2A f101–119	VTIAQGGVLPNIQAVLLPK
P2-E	2104.204	2104.188	H2A f83–100	HLQLAIRNDEELNKLLGK
P2-E	2915.610	2915.588	H2A f44–72	VGAGAPVYLAAVLEYLTAEILELAGNAAR
P3-A	952.630	952.600	H2B f101–109	LLLPGELAK

[a] P2-D, P2-E and P3-A corresponded to chicken histones H2B.V (NCBI Accession No. JH0362; ExPASy Accession No. P0C1H4), H2A (NCBI Accession No. NP_001025924), and H2B-derived *C*-terminal fragment, respectively.
[b] Amino acid residues are numbered according to the sequences of the chicken histones H2A and H2B (Figure 7), which showed the highest matched score with peptides from P2-D, P2-E and P3-A.

(13 851.2 Da.) by MALDI-MS is in a good agreement with the calculated monoisotopic molar mass (13 851.0 Da) when the putative methionine 1 is truncated, which also suggests that the antimicrobial histone H2A isolated from chicken liver in the present study is neither acetylated nor methylated. The observed molecular mass of histone H2A in our study is consistent with that reported by Zhang *et al.* [36], although one- and two-site acetylated isoforms of histone H2A from chicken erythrocyte was also detected in that study. Kim *et al.* demonstrated by immunocytochemistry that buforin I is unacetylated and that it is derived from a cytoplasmic unacetylated histone H2A protein [37]. However, in the rainbow trout, the histone H2A active protein is acetylated at its *N*-terminus [38].

Accumulating evidence has demonstrated that endogenous antimicrobial peptides and/or proteins play a key role in the host innate immune system [1–3]. Among the numerously reported antimicrobial peptides/proteins, one class of these antimicrobial molecules is the group of known proteins with other well-known functions or of fragments thereof, such as phospholipase A2 [39], ribosomal proteins [27,40] and histones [27,31,32]. The histone proteins, which are rich in lysine and/or arginine residues, play a critical role in the compaction of DNA into nucleosomes as well as in the overall organization of eukaryotic chromosomes. The four core histones H2A, H2B, H3 and H4 form a tripartite, octameric assembly. Recently many reports have shown that histones from frog [41], shrimp [32], fish [27,31,38,42,43] and mammals [44–48] possess broad-spectrum antimicrobial activity and are linked to the innate immune system in addition to their classical function in chromatin conformation. In our more recent study, we isolated and identified histones H1 and H2B as two antimicrobial proteins present in the adult female reproductive system of *Gallus gallus* [33]. Investigations have indicated that histones are not only localized in the nucleus but also in the cytoplasm [44,45,48,49], suggesting that histones in the cytoplasm may help protect against either intracellular pathogens or extracellular microorganisms through release into mucosal surfaces or tissue fluids after infection-induced cell lysis or apoptosis. Histones can also be secreted; for instance, histone H2A in both the toad and catfish is secreted prior to enzymatic cleavage to active peptides [50,51]. Moreover, histones H2A and H2B isolated from human placenta showed dose-dependent inhibition of the endotoxin activity of lipopolysaccharide (LPS) by binding it. These histones are released by epithelial cells to contribute to bactericidal activity against bacteria in the amniotic fluid [45]. Histone H1 was also shown to possess LPS-binding property and to modulate the production of cytokines [47]. These facts imply that histone functions as an effector molecule of the immune

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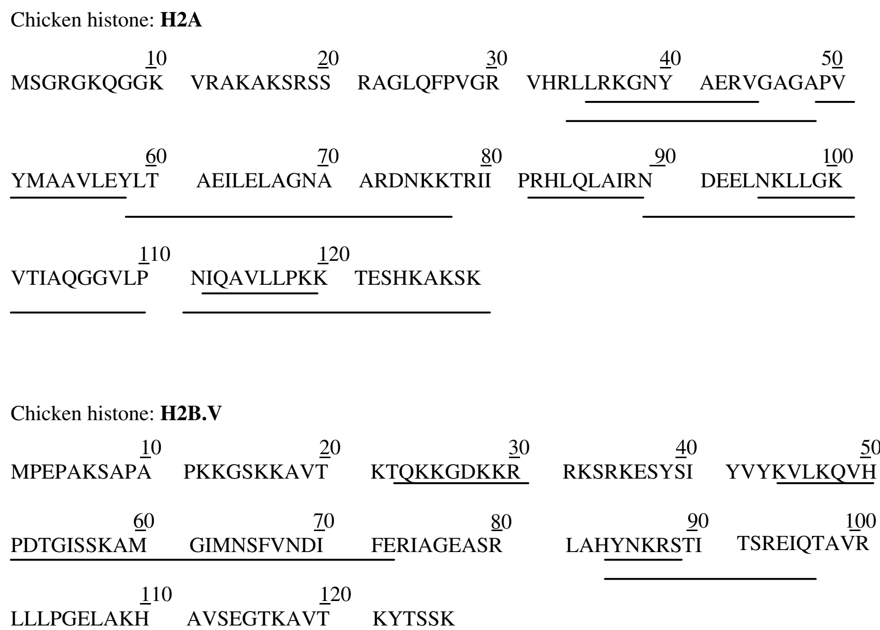

Figure 7 Primary amino acid sequence of chicken histones H2A and H2B.V. The underline covered identified in this study by MALDI-TOF-MS fingerprinting.

system in a fashion similar to other antimicrobial peptides.

In addition to intact histones, histone-derived fragments with antimicrobial activities have been well documented. Park *et al.* [50] isolated a potent 39-amino acid antibacterial peptide (buforin I) from the stomach tissue of the Asian toad. Buforin I is derived from *N*-terminal domain of unacetylated histone H2A and produced by pepsin after the unacetylated histone H2A is secreted into the stomach [50]. A similar mechanism was demonstrated for the antimicrobial peptide parasin I, a histone H2A-derived 19-residue *N*-terminal fragment that is produced by cathepsin D in the skin mucus of catfish and was shown to be induced in the epidermal mucus upon stimulation [51]. Another histone H2A-derived antimicrobial peptide is hipposin, a 51-residue *N*-terminal fragment from the skin mucus of the Atlantic halibut [52]. Histone H1 and H2B *N*-terminal peptide fragments with broad-spectrum antimicrobial properties have been identified from the skin mucus of the Atlantic salmon [53] and human wound fluid [49], respectively. In addition to the active *N*-terminal fragments from histones, a few reports have described the antimicrobial properties of histone *C*-terminal fragments. Wang and colleagues [46] isolated two *C*-terminal fragments of histone H1A and three *C*-terminal fragments of histone H1D from human peripheral granulocytes of a healthy donor who had been treated with granulocyte-colony-stimulating factor and cortisol. All these fragments exhibited activity against the Gram-positive bacterium *Bacillus megaterium*. More recently, a potent antimicrobial peptide, named oncorhycin II, was isolated from the rainbow trout skin secretions. Oncorhycin II is a 69-residue *C*-terminal fragment of histone H1 and has minimal inhibitory concentrations in the submicromolar range against Gram-positive as well as Gram-negative bacteria [54]. In the present study, we isolated an antimicrobial polypeptide from chicken liver with relatively low molecular mass compared to histones H2A and H2B, as indicated in Figure 5. Peptide mass fingerprint and tandem mass spectrometry data as well as homology database searches jointly indicate that this peptide is likely to be the *C*-terminal fragment of histone H2B. However, the exact amino acid sequence of this fragment and the mechanism underlying the generation of this fragment, i.e. whether it is produced intracellularly by enzymatic hydrolysis due to the subcellular distribution of histone-degrading enzymes in various tissues [55] or produced extracellularly after histone H2B is secreted, remain to be elucidated. Taken together, antimicrobial properties of histones and fragments thereof from different animal species reinforce the hypothesis that multifunctional histone proteins may be a relatively ubiquitous component of the host defenses.

In conclusion, the present study demonstrates that histones H2A, H2B.V and H2B-derived *C*-terminal fragment isolated from chicken liver exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria. The antimicrobial properties of histones and histone fragment in chicken provide further evidence that histones may play an important role in innate host defense against intracellular or extracellular microbe invasion in a wide range of animal species in addition to their role in nucleosome formation. Moreover,

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further purification and identification of other antimicrobial peptides or proteins in the chicken liver will provide insights into the role of the liver in the innate immune response of chicken.

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REFERENCES

1. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; **415**: 389–396.
2. Koczulla A, Bals R. Antimicrobial peptides: current status and therapeutic potential. *Drugs* 2003; **63**: 389–406.
3. Otvos L Jr. Antibacterial peptides and proteins with multiple cellular targets. *J. Pept. Sci.* 2005; **11**: 697–706.
4. Andreu D, Rivas L. Animal antimicrobial peptides: an overview. *Biopolymers* 1998; **47**: 415–433.
5. Rozek T, Wegener KL, Bowie JH, Olver IN, Carver JA, Wallace JC, Tyler MJ. The antibiotic and anticancer active aurein peptides from the Australian bell frog *Litoria aurea* and *Litoria raniformis*. The solution structure of aurein1.2. *Eur. J. Biochem.* 2000; **267**: 5330–5341.
6. Papo N, Shai Y. Host defense peptides as new weapons in cancer treatment. *Cell. Mol. Life Sci.* 2005; **62**: 784–790.
7. Tomee JF, Koeter GH, Hiemstra PS, Kauffman HF. Secretory leukoprotease inhibitor: a native antimicrobial protein presenting a new therapeutic option? *Thorax* 1998; **53**: 114–116.
8. Scott MG, Hancock REW. Cationic antimicrobial peptides and their multifunctional role in the immune system. *Crit. Rev. Immunol.* 2000; **20**: 407–431.
9. Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schroder JM, Wang JM, Howard OM, Oppenheim JJ. β-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* 1999; **286**: 525–528.
10. Yang D, Biragyn A, Kwak LW, Oppenhein JJ. Mammalian defensins in immunity: more than microbicidal. *Trends Immunol.* 2002; **23**: 291–296.
11. Gifford JL, Hunter HN, Vogel HJ. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell. Mol. Life Sci.* 2005; **62**: 2588–2598.
12. Evans EW, Beach GG, Wunderlich J, Harmon BG. Isolation of antimicrobial peptides from avian heterophils. *J. Leukoc. Biol.* 1994; **56**: 661–665.
13. Harwig SSL, Swiderek KM, Kokryakov VN, Tan L, Lee TD, Panyutich EA, Aleshina GM, Shamova OV, Lehrer RI. Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes. *FEBS Lett.* 1994; **342**: 281–285.
14. Lynn DJ, Higgs R, Gaines S, Tierney J, Janmes T, Lloyd AT, Fares MA, Mulcahy G, O'Farrelly C. Bioinformatic discovery and initial characterization of nine novel antimicrobial peptides genes in the chicken. *Immunogenetics* 2004; **56**: 170–177.
15. Xiao Y, Hughes AL, Ando J, Matsuda Y, Cheng JF, Skinner-Noble D, Zhang G. A genome-wide screen identifies a single β-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. *BMC Genomics* 2004; **5**: 56.
16. Lynn DJ, Lloyd AT, O'Farrelly C. In silico identification of components of the Toll-like receptor (TLR) signaling pathway in clustered chicken expressed sequence tags (ESTs). *Vet. Immunol. Immunopathol.* 2003; **93**: 177–184.
17. Townes CL, Michailidis G, Nile CJ, Hall J. Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to *Salmonella enterica* infection. *Infect. Immun.* 2004; **72**: 6987–6993.
18. Nile CJ, Townes CL, Hirst BH, Hall J. The novel avian protein, AWAK, contains multiple domains with homology to protease inhibitory modules. *Mol. Immunol.* 2006; **43**: 388–394.
19. Brockus CW, Jackwood MW, Harmon BG. Characterization of beta-defensin prepropeptide mRNA from chicken and turkey bone marrow. *Anim. Genet.* 1998; **29**: 283–289.
20. Zhao C, Nguyen T, Liu L, Sacco RE, Brogden KA, Lehrer RI. Gallinacin-3, an inducible epithelial β-defensin in the chicken. *Infect. Immun.* 2001; **69**: 2684–2691.
21. Dijk Avan, Veldhuizen EJ, van Asten AJ, Haagsman HP. CMAP27, a novel chicken cathelicidin-like antimicrobial protein. *Vet. Immunol. Immunopathol.* 2005; **106**: 321–327.
22. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J. Biol. Chem.* 2001; **276**: 7806–7810.
23. Lehrer RI, Rosenman M, Harwig SS, Jackson R, Eisenhauer P. Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods* 1991; **137**: 167–173.
24. Bals R, Goldman MJ, Wilson JM. Mouse β-defensin 1 is a salt-sensitive antimicrobial peptide present in epithelia of the lung and urogenital tract. *Infect. Immun.* 1998; **66**: 1225–1232.
25. Shamova O, Brogden KA, Zhao C, Nguyen T, Kokryakov VN, Lehrer RI. Purification and properties of praline-rich antimicrobial peptides from sheep and goat leukocytes. *Infect. Immun.* 1999; **67**: 4106–4111.
26. Relf JM, Chisholm JRS, Kemp GD, Smith VJ. Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from the granular haemocytes of the shore crab, *Carcinus maenas*. *Eur. J. Biochem.* 1999; **264**: 350–357.
27. Bergsson G, Agerberth B, Jornvall H, Gudmundsson GH. Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*). *FEBS J.* 2005; **272**: 4960–4969.
28. Lehrer R, Ganz T. Antimicrobial peptides in mammalian and insect host defence. *Curr. Opin. Immunol.* 1999; **11**: 23–27.
29. Laszlo Otvos JR. Antibacterial peptides isolated from insects. *J. Pept. Sci.* 2000; **6**: 497–511.
30. Patrzykat A, Zhang L, Mendoza V, Iwama GK, Hancock REW. Synergy of histone-derived peptides of coho salmon with lysozyme and flounder pleurocidin. *Antimicrob. Agents Chemother.* 2000; **45**: 1337–1342.
31. Richards RC, O'Neil DB, Thibault P, Ewart V. Histone H1: an antimicrobial protein of atlantic salmon (*Salmo salar*). *Biochem. Biophys. Res. Commun.* 2001; **284**: 549–555.
32. Patat SA, Carnegie RB, Kingsbury C, Gross PS, Chapman R, Schey KL. Antimicrobial activity of histones from hemocytes of the pacific white shrimp. *Eur. J. Biochem.* 2004; **271**: 4825–4833.
33. Silphaduang U, Hincke MT, Nys Y, Mine Y. Antimicrobial proteins in chicken reproductive system. *Biochem. Biophys. Res. Commun.* 2006; **340**: 648–655.
34. Takami Y, Higashio M, Fukuoka T, Takechi S, Nakayama T. Organization of the chicken histome genes in a major gene cluster and generation of an almost complete set of the core histone protein sequences. *DNA Res.* 1996; **3**: 95–99.
35. Spencer VA, Davie JR. Role of covalent modifications of histones in regulating gene expression. *Gene* 1999; **240**: 1–12.
36. Zhang K, Tang H. Analysis of core histones by liquid chromatography-mass spectrometry and peptide mapping. *J. Chromatogr. B* 2003; **783**: 173–179.
37. Kim HS, Yoon H, Minn I, Park CB, Lee WT, Zasloff M, Kim SC. Pepsin-mediated processing of the cytoplasmic histone H2A to strong antimicrobial peptide buforin I. *J. Immunol.* 2000; **165**: 3268–3274.

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38. Fernandes JM, Kemp GD, Molle MG, Smith VJ. Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Biochem. J.* 2002; **368**: 611–620.
39. Harwig SS, Tan LL, Qu X, Cho Y, Eisenhauer PB, Lehrer RI. Bactericidal properties of murine intestinal phospholipase A2. *J. Clin. Invest.* 1995; **95**: 603–610.
40. Tollin M, Bergman P, Svenberg T, Jornvall H, Gudmundsson GH, Agernberth B. Antimicrobial peptides in the first line defence of human colon mucosa. *Peptides* 2003; **24**: 523–530.
41. Kawasaki H, Isaacson T, Iwamuro S, Conlin JM. A protein with antimicrobial activity in the skin of Schlegel's green tree frog *Rhacophorus schlegelii* (Rhacophoridae) identified as histone H2B. *Biochem. Biophys. Res. Commun.* 2003; **312**: 1082–1086.
42. Robinette D, Wada S, Arroll T, Levy MG, Miller WL, Noga EJ. Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. *Cell. Mol. Life Sci.* 1998; **54**: 467–475.
43. Noga EJ, Fan Z, Sihphaduang U. Histone-like proteins from fish are lethal to the parasitic dinoflagellate *Amyloodinium ocellatum*. *Parasitology* 2001; **123**: 57–65.
44. Rose FR, Bailey K, Keyte JW, Chan WC, Greenwood D, Mahida YR. Potential role of epithelial cell-derived histone H1 proteins in innate antimicrobial defense in the human gastrointestinal tract. *Infect. Immun.* 1998; **66**: 3255–3263.
45. Kim HS, Cho JH, Park HW, Yoon H, Kim MS, Kim SC. Endotoxin-neutralizing antimicrobial proteins of the human placenta. *J. Immunol.* 2002; **168**: 2356–2364.
46. Wang Y, Griffiths WJ, Jornvall H, Agerberth B, Johansson J. Antibacterial peptides in stimulated human granulocytes: characterization of ubiquitinated histone H1A. *Eur. J. Biochem.* 2002; **269**: 512–518.
47. Jacobsen F, Baraniskin A, Mertens J, Mittler D, Mohammadi-Tabrisi A, Schubert S, Soltau M, Lehnhardt M, Behnke B, Gatermann S, Steinau HU, Steinstraesser L. Activity of histone H1.2 in infected burn wounds. *J. Antimicrob. Chemother.* 2005; **55**: 735–741.
48. Hiemstra PS, Eisenhauer PB, Harwig SS, van den Barselaar MT, van Furth R, Lehrer RI. Antimicrobial proteins of murine macrophages. *Infect. Immun.* 1993; **61**: 3038–3046.
49. Frohm M, Gunne H, Bergman C, Agerberth B, Bergman T, Boman A, Liden S, Jornvall H, Boman HG. Biochemical and antibacterial analysis of human wound and blister fluid. *Eur. J. Biochem.* 1996; **237**: 86–92.
50. Park CB, Kim MS, Kim SC. A novel antimicrobial peptide from *Bufo bufo gargarizans*. *Biochem. Biophys. Res. Commun.* 1996; **218**: 408–413.
51. Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC. Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. *FASEB J.* 2002; **16**: 429–431.
52. Birkemo GA, Luders T, Amdersen O, Nes IF, Nissen-Meyer J. Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Biochim. Biophys. Acta* 2003; **1646**: 207–215.
53. Luders T, Birkemo GA, Nissen-Meyer J, Amdersen O, Nes IF. Proline conformation-dependent antimicrobial activity of a proline-rich histone H1 N-terminal peptide fragment isolated from the skin mucus of Atlantic salmon. *Antimicrob. Agents Chemother.* 2005; **49**: 2399–2406.
54. Fernandes JMO, Molle G, Kemp GD, Smith VJ. Isolation and characterization of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secrections of rainbow trout, *Oncorhynchus mykiss*. *Dev. Comp. Immunol.* 2004; **28**: 127–138.
55. Haas R, Weiss E, Jusic M, Heinrich PC. Limited proteolysis of the very-lysine-rich histone H1 by a membrane-bound proteinase from rat liver. *FEBS Lett.* 1976; **63**: 117–119.

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