



Molecular characterization of goose- and chicken-type lysozymes in emu (*Dromaius novaehollandiae*): Evidence for extremely low lysozyme levels in emu egg white

Kenji Maehashi ^{a,*}, Mami Matano ^a, Tomohiro Irisawa ^b, Masataka Uchino ^b,
Yutaka Kashiwagi ^a, Toshihiro Watanabe ^c

^a Department of Fermentation Science, Faculty of Applied Bio-Science, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

^b Department of Applied Biology and Chemistry, Faculty of Applied Bio-Science, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

^c Department of Food and Cosmetic Science, Faculty of Bio-Industry, Tokyo University of Agriculture, 196 Yasaka, Abashiri, Hokkaido 099-2493, Japan

ARTICLE INFO

Article history:

Accepted 1 October 2011

Available online 25 October 2011

Received by A.J. van Wijnen

Keywords:

Lysozyme

Emu

Egg white

Ostrich

Lytic activity

ABSTRACT

Lysozyme (LZ), a bacteriolytic enzyme, is found in the egg white of many avian eggs and plays an important role in host defense; however, LZ activity in emu (*Dromaius novaehollandiae*) egg white is exceptionally undetectable. We cloned and characterized emu goose-type LZ (LZG) and chicken-type LZ (LZC) genes. RT-PCR analysis revealed very low LZG gene expression levels and absence of LZC gene expression in the emu oviduct. Sequencing of full-length LZG and LZC cDNAs indicated that their amino acid sequences show high similarities to ostrich LZG and LZC, respectively, with conserved catalytic residues for enzymatic activities. Whereas recombinant emu LZG prepared using *Pichia pastoris* exhibited similar enzyme activity as ostrich LZG, recombinant emu LZC exhibited significantly higher lytic activity than chicken LZC. We concluded that emus have functional genes for both LZG and LZC like many other avians, and the LZG gene is expressed in oviduct probably as in other ratite, however, its expression levels in egg white were low to be detected.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Emu (*Dromaius novaehollandiae*) is the second largest member of the ratite family, which also includes the ostrich, rhea, and cassowary, and is native to Australia. Recently, emus have begun to be considered as an alternative form of livestock, causing an increase in their breeding in Japan. Emu eggs are 10 times larger than chicken eggs, and their size makes them a potentially attractive alternative to traditional egg products. Chicken egg white has been extensively studied and its biochemical properties have been well characterized, however, significantly few studies have been performed on emu egg white. We have previously reported that considerable differences exist in the compositions of the major proteins between emu and chicken egg white, including the fact that ovotransferrin is the dominant protein in emu egg white (Maehashi et al., 2010), whereas ovalbumin is the dominant protein followed by ovotransferrin, ovomucoid, and lysozyme (LZ) in chicken egg white (Mine and Yang, 2008). A significant feature of emu egg white is the lack of LZ activity.

LZ (EC3.2.1.17) is a bacteriolytic enzyme that catalyzes the hydrolysis of the β -1,4 glycosidic bonds of the peptidoglycan in bacterial cell walls. Thus its main biological function is protecting the host

from bacterial infections as an innate immune system. LZs are classified according to the similarity among amino acid sequences into three major types: chicken-type (LZC), goose-type (LZG), and invertebrate-type. Most avians, including chickens, have LZC in their egg white, but some avians, such as geese and ostriches, have LZG in their egg white. Because no studies have reported avian egg white without LZ and because it has unique immune functions and possibly lower allergenicity in individuals with egg allergy, emu egg white is of particular interest.

In this study, we identified the emu genes for LZC and LZG and characterized them using recombinant proteins prepared in *Pichia pastoris*.

2. Materials and methods

2.1. Materials

Eggs and tissues of egg-laying female emus (*D. novaehollandiae*) were obtained from a local emu farm at Abashiri-shi, Hokkaido, Japan. The pieces of tissue were excised from the oviduct and stored in RNAlater (Ambion, Inc., Austin, TX) until use. White Leghorn eggs and Japanese quail eggs were obtained from a local market and ostrich eggs from a local farm. Emu and ostrich genomic DNAs were prepared from the meat using Blood & Cell Culture DNA Mini Kit (QIAGEN KK, Tokyo, Japan).

Abbreviations: LZC, chicken-type lysozyme; LZG, goose-type lysozyme.

* Corresponding author. Tel./fax: +81 3 5477 2748.

E-mail address: maehashi@nodai.ac.jp (K. Maehashi).

2.2. RT-PCR amplification of LZC and LZG cDNAs

Total RNA was extracted from emu tissues using the TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). First-strand cDNA synthesis was performed using SuperScript III First-Strand Synthesis SuperMix and oligo-dT (20) primer (Invitrogen Co., Carlsbad, CA). The amplified cDNA was used for subsequent PCR amplifications with Ex Taq DNA polymerase (Takara Bio Inc. Shiga, Japan). For cloning of emu LZG cDNA, the primers 5'-AGAACGGGTGCTACGG-3' and 5'-TAGTATCCATGCTGCTT-3' were designed on the basis of a nucleotide sequence of ostrich LZG predicted from its amino acid sequence (GenBank ID: P00719). For cloning of emu LZC cDNA, the primers 5'-AAAGTCTTTGGACGATGTGAGCTAGC-3' and 5'-TCACAG-CCGGCAGCCTCTGATCCA-3' were designed from chicken LZC mRNA (GenBank ID: V00428).

For tissue expression profiling of LZG and LZC mRNAs, primer sets of 5'-GATCCAGCTGTGATTGCTGG-3' and 5'-CCACATCGTTGGCATA-GTC-3' and 5'-ATGGAAGTTCTGACTACGGG-3' and 5'-GTTGCGCCAT-GCGACCA-3', respectively, were used for RT-PCR amplification. PCR was performed at 94 °C for 30 s followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final cycle of 72 °C for 1 min. A set of β -actin primers (5'-GTGACCTGACAGACTCT-3' and 5'-CAGGAAGGATGGCTGGAAGAG-3') served as a control for amount and quality of cDNA.

2.3. 3'-Rapid amplification of cDNA end (RACE)

To isolate the downstream regions of the 3' end of LZG and LZC cDNAs, we performed 3'-RACE. Single-strand cDNA synthesis was performed using an oligo (dT) primer with the adapter sequence (5'-AACTGGAAGAATTCGCGGCCGAGGAATTTTTTTTTTTTTTTT-3') and RNA prepared from the emu oviduct. This amplicon was used as a template for subsequent PCR using the forward primer designed on the basis of a sequence of emu LZG cDNA fragment (5'-AGGGACCTAC-GAAGTATGG-3') or the forward primer designed on the basis of a sequence of emu LZC cDNA fragment (5'-GTTGGTGGTGCAATGAT-GGCA-3') and an adaptor primer (5'-AACTGGAAGAATTCGCGGCCGAG-GAA-3'). The amplicons were cloned into pCR2.1-TOPO (Invitrogen Co.) and DNAs were sequenced.

2.4. Inverse PCR

To determine the nucleotide sequence of the 5' regions of LZG and LZC genes, we performed inverse PCR (Collins and Weissman, 1984). The genomic DNA was digested with *Bgl*I for LZG gene or *Hind*III for LZC gene, and the digests were self-ligated using a Fast link DNA ligation kit (Epicentre Biotechnologies, Madison, WI, USA). PCR amplification was performed using the primer sets of 5'-CAAGA-CATCCTACAGGAAGCAG-3' / 5'-GTGAGCTGAATCCAGGACTAC-3' and 5'-GCACAGACTGCAGGTATTGTC-3' / 5'-TCTTCATAGCTGCTGCCAGCT-3' to obtain fragments those included the 5' untranslated regions of emu LZG and LZC genes, respectively.

2.5. DNA sequencing

Nucleotide sequences of cloned DNAs in pCR2.1-TOPO were analyzed by MacroGen Japan Co. (Tokyo, Japan). T7 promoter primer and M13 reverse primer (Invitrogen Co.) were used as sequencing primers. The resultant nucleotide sequence data was assembled using the ATGC program (Genetyx Corp., Tokyo, Japan) and analyzed by NCBI nucleotide BLAST program.

2.6. Phylogenetic tree analysis

The amino acid sequences of LZG and LZC were obtained from the GenBank and SWISS-PROT databases. The ClustalW 1.81 multiple

sequence alignment program was used for multiple alignments of protein sequences of various LZGs and LZCs, determination of amino acid substitution, and construction of the neighbor-joining phylogenetic tree. Bootstrap analysis was replicated 1000 times to evaluate the phylogenetic tree topology (Felsenstein, 1985). The accession numbers of LZGs used in this analysis are as follows: human g1, Q8N1E2; mouse g1, Q9D7Q0; Western clawed frog g2, NM_001015739; African clawed frog g2, NM_001094684; chicken g2, P27042; cassowary, Q7LZR3; ostrich g, P00719; rhea, P84504; black swan, P00717; and goose, P00718. The accession numbers of LZC used in this study are as follows: sheep C1, P17607; bovine stomach, Q06285; rainbow trout, P11941; Japanese flounder, Q90VZ3; mouse M, P08905; bovine milk, Q6B411; human c, P61626; Andrew's toad, P85045; African clawed frog, AAH72985; green sea turtle, P84492; Chinese softshell turtle, Q7LZQ1; guinea fowl, P00704; turkey, XM_003202070; Japanese quail, P00701; chicken c, P00698; horse milk, P11376; dog milk, P81708, pigeon, P00708; and hoatzin stomach, L36032.

2.7. Southern blot analysis

For Southern blot analysis, isolated emu genomic DNA was digested with two restriction endonucleases *Hind*III and *Pst*I. The digested DNA samples (10 μ g each) were separated on a 0.8% agarose gel and blotted on a Hybond + Nylon membrane (GE Healthcare Bio-Sciences Corp., NJ, USA). The membrane was hybridized with the digoxigenin (DIG)-labeled emu LZG or LZC DNA probes prepared using the DIG DNA labeling and detection kit (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's instructions.

2.8. Construction of *P. pastoris* expression vector

The entire gene encoding open reading frames of mature emu LZC and LZG were amplified by PCR with Platinum *Pfx* DNA polymerase (Invitrogen Co.). Because the emu LZG gene had a *Xho*I recognition site, a silent mutation was introduced into the gene in advance to modify the *Xho*I site by overlap extension PCR. Next, amplicons of emu LZG and LZC were digested with *Xho*I and *Not*I. These insert DNAs from emu LZC and LZG were ligated with pPIC9 (Invitrogen Co.) using Fast Link DNA ligase and subjected to transformation with *Escherichia coli* TOP10 to obtain constructs of pPIC9-eLZC and pPIC9-eLZG, respectively.

2.9. Expression of emu LZ in *P. pastoris*

The constructed expression vectors pPIC9-eLZC and pPIC9-eLZG were linearized by *Sall* and transformed into cells of *P. pastoris* GS115 (Invitrogen Co.) by electroporation using MicroPulser (Bio-Rad, CA, USA) with a pre-set program Pic. The cells were spread on MD plates [2% glucose, 1.34% yeast nitrogen base (YNB), 4×10^{-5} % biotin and 2% agar] for the selection of positive clones. The positive clones were further selected for productivity of recombinant LZ according to lytic activity and SDS-PAGE analysis after culturing in 3 mL of BMM (0.5% methanol, 1.34% YNB, 4×10^{-5} % biotin, 100 mM potassium phosphate, pH 6.0) at 30 °C for 5 days.

For preparation of recombinant LZG and LZC on a large scale, *P. pastoris* transformant cells were grown in 3 L of BMG [1% glycerol, 1.34% YNB, 4×10^{-5} % biotin, 100 mM potassium phosphate, pH 6.0] using a baffled flask for 2 days at 30 °C with shaking. The cells were collected by centrifugation and resuspended in 3 L of BMM and cultivated in a jar fermenter (MDL-4CR, B. E. Marubishi Co., LTD., Japan) at 28 °C for 120 h with an aeration rate of 6 L/min and stirring at 500 rpm. Methanol was added at a concentration of 0.5% every 12 h. A silicon antifoam agent (Shin-Etsu Chemical Co. Ltd, Tokyo, Japan) was dissolved in methanol. LZs secreted in the media after cultivation were purified by cation-exchange chromatography. In brief, the culture supernatant was diluted 5-fold with water, and the secreted

LZs were separated on a CM-Sepharose Fast-Flow column (GE Healthcare Bio-Sciences Corp.), which was eluted with a gradient of 20 mM phosphate buffer and the same buffer containing 0.5 M NaCl at pH 7. The eluted LZ fractions were further separated on the same column but eluted with a gradient from 0.2 to 0.45 M NaCl in the same buffer. The eluted LZ fractions were desalted by ultrafiltration and lyophilized. The lyophilized powder of purified mutant LZs was dissolved in distilled water.

2.10. Measurement of enzyme activity

Enzyme activity was assayed using the lyophilized cell wall of *Micrococcus luteus* (Sigma-Aldrich Japan, Osaka, Japan) as a substrate by the method of Pooart et al. (2005). A cell suspension of 0.2 mg/mL prepared using 0.1 M Britton-Robinson buffer (pH 2–11) gave an absorbance between 0.7 and 0.8 at 450 nm. To 3 mL of the cell suspension, we added 50 μ L of the LZ solution, and the decrease in turbidity was monitored by absorbance at 450 nm using the Beckman DU640 spectrophotometer (Beckman Coulter, Inc., CA, USA) at 30 °C. Lytic activity was represented as the decrease in OD₄₅₀ value in 1 min by 1 μ g of protein. The protein concentration of the LZ solution was determined by the bicinchoninic acid (BCA) method using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Chitinase activity of LZ was determined according to the method of Imoto and Yagishita (1971) using ethylene glycol chitin (Wako Pure Chemical Industries Co., Osaka, Japan) as a substrate.

2.11. Lysoplate assay

The lysoplate assay was performed according to Minagawa et al. (2001) using *M. luteus* NRIC1094 as a substrate. A gel plate containing 1% agarose in 50 mM phosphate buffer (pH 6.2) and *M. luteus* (OD₆₆₀ = 2.0) was prepared. To each 2-mm diameter well on the plate 5 mL of sample was added. The plate was incubated at 30 °C for 48 h, and diameter of each cleared zone (mm) was measured.

3. Results and discussion

3.1. Detection of LZ in emu egg white

In a previous study, we demonstrated that lytic activity toward *M. luteus* cell suspension was not detected in emu egg white by turbidimetric analysis, and that no protein bands indicating LZ were detected by SDS-PAGE analysis with Coomassie Brilliant Blue staining (Maehashi et al., 2010). Here we further investigated the lytic activity of emu egg white against *Micrococcus* cells by the lysoplate assay. As shown in Fig. 1(A), whereas chicken egg white caused a large lysed circle with 22 mm diameter, emu egg white caused a slight lysed circle with 4.4 mm diameter. This indicated that emu egg white contains lower LZ and lytic substance levels than chicken egg white. Next, we performed SDS-PAGE in which 2.5 μ L of 10 times diluted emu, ostrich, and chicken egg white samples was run and subjected to silver staining. As shown in Fig. 1B, bands were not seen for emu egg white at the positions corresponding to the 20-kDa band of ostrich LZG and the 14-kDa band of chicken LZC. Instead, unknown bands of less than 14 and 20 kDa, respectively, were seen for emu egg white. Because the unknown bands for emu egg white were too faint to identify, emu egg white was concentrated by chromatography, separated by SDS-PAGE, and then subjected to protein sequencing. As a result, the band that was less than 14 kDa for emu egg white was identified as β -defensin 11 with a sequence of LPRHTLHCVAYH-GYCFHSKV (UniProtKB ID: P86385). This band had a sequence similarity of 76% with chicken β -defensin 11, one of the widely studied family of cysteine-rich antimicrobial peptides (Herve-Gre'pinet et al., 2010). The band that was less than 20 kDa for emu egg white

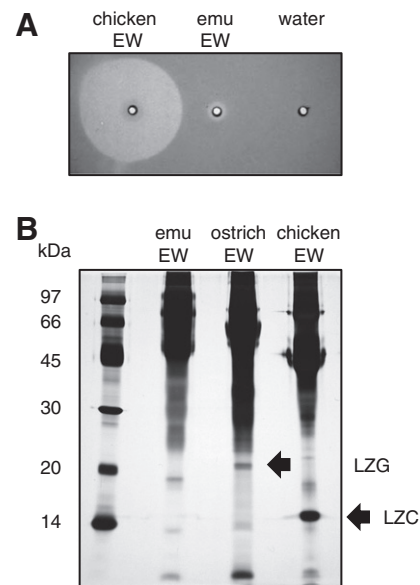


Fig. 1. Detection of LZs in emu egg white. (A) Lysoplate assay for chicken and emu egg white samples. Homogenized egg white samples were added into the wells of the agarose plate containing *M. luteus* cells and incubated for 48 h at 30 °C. (B) SDS-PAGE of homogenized emu, ostrich, and chicken egg white samples with silver staining.

was not identified because of a possible N-terminal blocking (data not shown). When emu egg white was separated on a CM-Sepharose column under the same conditions as those employed for chicken or ostrich egg white separation, fractions with lytic activity were absent. Furthermore, the band that was less than 20 kDa was detected in the fraction by separation using the DEAE-Sepharose column, but it did not have lytic activity (data not shown). These results suggested the absence or an extremely low LZ levels in emu egg white.

3.2. Primary structure of emu LZG and LZC

To investigate whether LZG and LZC genes are expressed in the oviduct, we performed PCR amplification using the primer set designed for emu LZC and LZG genes on the basis of chicken LZC and ostrich LZG, respectively, from an emu oviduct cDNA preparation. Thus, LZC and LZG cDNA fragments were obtained from the oviduct. We performed 3'-RACE and obtained the complete sequences of LZC and LZG gene coding regions. The determined emu LZG cDNA consisted of an open reading frame of 618 bp, which coded for 206 deduced amino acid residues (DDBJ ID: AB451700). Based on the similarity among the amino acid sequences of emu LZG and other ratite LZGs, the signal sequence of emu LZG was predicted as N-terminal 19 amino acids. The determined emu LZC cDNA consisted of an open reading frame of 441 bp, which coded for 147 amino acid residues (DDBJ ID: AB513854). From the similarity of the amino acid sequences to other ratite LZCs, the signal sequence of emu LZC was predicted as N-terminal 18 amino acids. N-terminal amino acid of emu LZG was found to have a glutamine residue that was identical to that in cassowary LZG. Since the N terminal in cassowary LZG is blocked by a pyroglutamic acid residue (Thammasirirak et al., 2001), emu LZG may also have a blocked N-terminal blocked in egg white. These are the first reports regarding the gene sequences of ratite-origin LZG and LZC. Furthermore, we cloned LZG and LZC genes from ostrich genomic DNA and determined their complete nucleotide sequences by similarities to emu LZG and LZC cDNAs. The sequence data of ostrich LZG and LZC genes were deposited to the DDBJ database under the accession numbers AB469329 and AB627846, respectively. Multiple alignments of the amino acid sequences of LZG and LZC (Fig. 2) illustrated that both LZG and LZC in emu had conserved catalytic residues for enzyme activity (Glu73,

LZG

Emu	- - - - - MHLMLVLLGLAALLGTSQSQTGCGYGVVNRIDTT	14
Cassowary	- - - - - QTGCGYGVVNRIDTT	14
Ostrich	- - - - - MHLMLVLLGLAALLGTSQSRTGCGYGDVNRVDTT	14
Goose	- - - - - RTDCYGNVNRIDTT	14
Chicken	MLGKNDPMCLVLVLLGLTALLGICQGGTGCYGSVSRIDTT	14
Emu	GASCETAKPEKLNLCYGVAAASRMATAERDLRSMDRHKTLIKK	54
Cassowary	GASCETAKPEKLNLCYGVAAASRKIAEGDLQSMdryKTLIKK	54
Ostrich	GASCKSAKPEKLNLCYGVAAASRKIAERDLQSMdryKALIKK	54
Goose	GASCKTAKPEGLSYCGVSASKKIAERDLQAMdryKTLIKK	54
Chicken	GASCRtAKPEGLSYCGVRSRTIAERDLGSMNkyKVLIKR	54
Emu	VGQKLCVDPAVIAGIISRESHAGKALKNGWGDRNGNGFGLM	94
Cassowary	VGQKLCVDPAVIAGIISRESHAGKALKDNGWGDRNGNGFGLM	94
Ostrich	VGQKLCVDPAVIAGIISRESHAGKALRNGWGDRNGNGFGLM	94
Goose	VGEKLCVDPAVIAGIISRESHAGKVLKNGWGDRNGNGFGLM	94
Chicken	VGEALCIEPAVIAGIISRESHAGKILKNGWGDRNGNGFGLM	94
Emu	QVDKRSHTPVGEWNGERHLTQGTIELISMICKIQKKFPRW	134
Cassowary	QVDKRSHTPVGKWNGERHITQGTIELISMICKIQKKFPRW	134
Ostrich	QVDRRSHPKPVGEWNGERHLMQGTIELISMICKIQKKFPRW	134
Goose	QVDKRSHPKQGTWNGEVHITQGTIELINFIKTIQKKFPSW	134
Chicken	QVDKRYHKIEGTWNGEAHIRQGTIRILIDMVKKIQKKFPRW	134
Emu	TKEQQLKGGISAYNAGSGNVRSYERMDIGTTHNDYANDVV	164
Cassowary	TKEQQLKGGISAYNAGSGNVRTYERMDIGTTHNDYANDVV	164
Ostrich	TKEQQLKGGISAYNAGPGNVRSYERMDIGTTHDDYANDVV	164
Goose	TKDQQLKGGISAYNAGAGNVRSYARMDIGTTHDDYANDVV	164
Chicken	TRDQQLKGGISAYNAGVGNVRSYERMDIGTLHDDYSNDVV	164
Emu	ARAQYYKQHGYY	185
Cassowary	ARAQYYKQHGYY	185
Ostrich	ARAQYYKQHGYY	185
Goose	ARAQYYKQHGYY	185
Chicken	ARAQYFKQHGYY	185

LZC

Emu	MKFFLILGFCLLPLIAQGGKVFGRCELAAAMKKHGLSNYRG	22
Ostrich	MKSLLILGFCLLPLVAQGGKIYERCELAAAMKRLGLDNFRG	22
Chicken	MRSLLILVLCFLPLAALGKVFGRCELAAAMKRHGLDNYRG	22
Turkey	MRSLLILVLCFLPLAALGKVFGRCELAAAMKRLGLDNYRG	22
Quail	MRSLLVLCFLPLAALGKVFGRCELAAAMKRHGLDNYRG	22
Emu	YSLGHWVCAAKYESNFNTAATNRNRDGSSTDYGILQINSRW	62
Ostrich	YSLGHWVCAAKFESGFDTRAIRNRNRDGSSTDYGILQINSRW	62
Chicken	YSLGNWVCAAKFESNFNTQATNRNTDGSSTDYGILQINSRW	62
Turkey	YSLGNWVCAAKFESNFNTAATNRNRDGSSTDYGILQINSRW	62
Quail	YSLGNWVCAAKFESNFNSQATNRNTDGSSTDYGVLLQINSRW	62
Emu	WCNDGRTSGAKNLCKISCSALLSSDITASVNCARVVSDDK	102
Ostrich	WCNDGRTSRAKNACNISCSALLSSDITASVNCARVVSDDK	102
Chicken	WCNDGRTPGSRNLNIPCSALLSSDITASVNCARVVSDDK	102
Turkey	WCNDGRTPGSKNLNIPCSALLSSDITASVNCARVVSDDK	102
Quail	WCNDGRTPGSRNLNIPCSALLSSDITATVNCARVVSDDK	102
Emu	NGMNAWVAWRNHCKGRDVSQWIRGCRV	129
Ostrich	NGMNAWVAWKHCKGRDVSQWIRGCRV	129
Chicken	NGMNAWVAWRNRCKGTDVQAWIRGCRV	129
Turkey	DGMNAWVAWRNRCKGTDVHAWIRGCRV	129
Quail	NGMNAWVAWRNRCKGTDVHAWIRGCRV	129

Fig. 2. Multiple alignments of LZG and LZC amino acid sequences. Emu and ostrich sequences are deduced from their nucleotide sequences. Triangles indicate the predicted signal cleavage sites. The sequences are numbered after signal cleavage sites. Emu sequences and identical amino sequences are marked with a gray background. Conserved catalytic sites are marked with a black background.

Asp86 and Asp97 for LZG and Glu35 and Asp52 for LZC) (Weaver et al., 1985), with other LZG and LZC, respectively. Therefore, it was expected that emus have functional genes for LZG and LZC and elicit enzyme activity as egg white LZ.

3.3. Sequencing of emu LZG and LZC genes

We amplified emu LZG and LZC genes from the emu genomic DNA preparation using gene-specific primers based on two exons crossing

over the intron predicted from the corresponding chicken LZG and LZC genes. As the result, 2148 bp of a genomic DNA fragment containing 5 exons and 4 introns of the LZG gene (DDBJ ID: AB462632) and 4065 bp of a genomic DNA fragment containing 4 exons and 3 introns of the LZC gene (DDBJ ID: AB513676) were obtained. Sequencing of these fragments confirmed the sequences of the cDNA clones of LZG and LZC genes and the boundaries between exons and introns. Because there have been no reports on the genes of ratite-origin LZG and LZC, we cloned ostrich LZG and LZC genes from the ostrich

genomic DNA preparation and sequenced them to find the structural characteristics of both ratite LZ genes compared with those of chicken. As illustrated in Fig. 3A, emu and ostrich LZG and LZC genes were similar in their intron and exon sizes, whereas chicken LZG and LZC genes were shorter. Emu and ostrich LZC genes had longer introns between exon 1 and exon 2 and between exon 3 and exon 4, and shorter introns between exon 3 and exon 4 compared with chicken LZC gene. Many inserted and deleted regions were seen in the introns of the emu LZG gene that those in the chicken LZG gene, whereas a relatively higher similarity was seen between emu and ostrich sequences (data not shown).

Chickens, humans, and mice have two LZG genes (Irwin and Gong, 2003; Nakano and Graf, 1991; Nile et al., 2004). Therefore, Southern blot analysis was performed to examine the number of emu LZG and LZC genes. The emu genomic DNA digested by restriction enzymes *Pst*I and *Hind*III showed a simple hybridization band pattern with the probes for LZG and LZC as shown in Fig. 3B. These probes were prepared by PCR using the primers corresponding to the regions within exon 4 of LZG and exon 1 of LZC, respectively. The results demonstrated the presence of a single copy of LZG and LZC genes in the emu genome.

Fig. 4 shows the phylogenetic tree of LZG and LZC based on their amino acid sequences. Emu LZG formed a ratite cluster with cassowary, ostrich, and rhea within the bird group apart from the mammalian LZGs. Mammals and chickens have two types of LZGs: one is secretory (g1 or g) and the other is non-secretary (g2) (Irwin and Gong, 2003; Nile et al., 2004). The emu LZG sequence was closest to the cassowary LZG sequence with 97.22% similarity and predicted to be a secretory type, the same as other ratite LZGs. LZC includes the digestive type such as bovine stomach LZC, calcium-binding type such as horse milk LZC, and digestive calcium-binding type such as hoatzin stomach LZC (Kornegay et al., 1994) other than conventional type represented by chicken LZC. It was found that emu LZC was a conventional non-calcium-binding and non-digestive

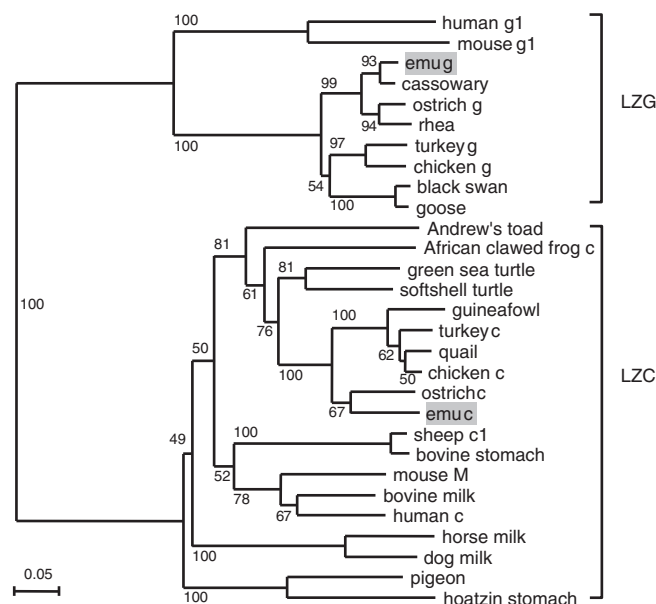


Fig. 4. Phylogenetic tree of LZGs and LZCs based on their amino acid sequences. Numbers at the nodes represent bootstrap proportions (%) from 1000 replicates.

type, similar to chicken LZC, but formed a ratite cluster with ostrich apart from other avians including chicken. We did not examine the genes for digestive and/or calcium-binding type lysozymes those may exist in emu.

3.4. RT-PCR analysis of LZC and LZG expression in emu tissues

To investigate the tissue distribution of emu LZG and LZC mRNA expression, RT-PCR analysis of RNA prepared from various tissues of female egg-laying emus was performed. A 340-bp band of LZG was well amplified from the bone marrow and lung, weakly from the liver and faintly from the spleen and oviduct, as shown in Fig. 5. Although there is no report regarding ratite LZG gene expression, it has been reported that the chicken LZG gene is expressed in the bone marrow and lung but not in the liver, spleen, and oviduct (Nakano and Graf, 1991). The LZG expression patterns in emu and chicken suggest that LZGs in the bone marrow and lung are common, however, emu LZGs in the liver, spleen, and oviduct have other characteristic functions. Although other ratites have sufficient LZG in their egg white to prevent bacterial infections, it is uncertain if the low LZG levels in the emu oviduct would be sufficient for the same function. A 200-bp band of emu LZC was weakly amplified in the ovary and liver but was undetectable in the other tissues examined compared to that of chicken LZC, which is abundantly expressed in the oviduct (Nakano and Graf, 1991). There is a report that LZC was detected as the major protein in chicken vitelline membrane (De Boeck and Stockx, 1986), and therefore, emu LZC expressed in the ovary may exist in the vitelline membrane as well as in chicken egg white. It was confirmed for

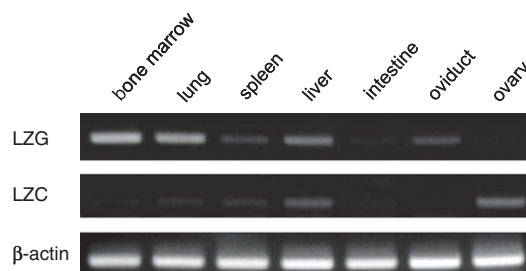


Fig. 5. RT-PCR analysis of LZG and LZC gene expression in different emu tissues.

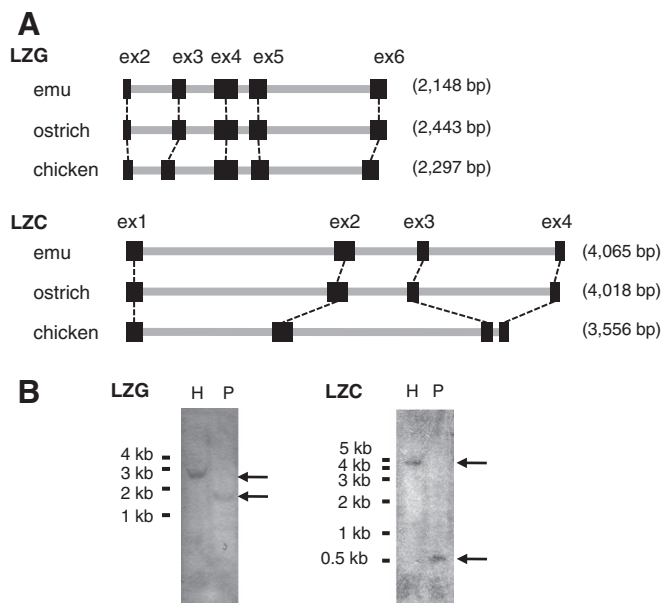


Fig. 3. (A) Diagram of the genomic structures of LZG and LZC genes from emu, ostrich and chicken. Solid boxes represent the region coding for the structural sequences of the protein. Lines indicate the sequences of introns. Gene regions from the start to the stop codons are illustrated and their nucleotide lengths are indicated in parentheses. Exons and introns are represented by boxes and solid lines, respectively. (B) Southern blot analysis of emu genomic DNA digested with *Hind*III and *Pst*I. Membranes were hybridized with DIG-labeled emu LZG or LZC probes. Lane H, *Hind*III digests of emu genome; lane P, *Pst*I digests of emu genome. Sizes indicated on the left are the positions of molecular mass markers. Arrows indicate the positions of positive bands.

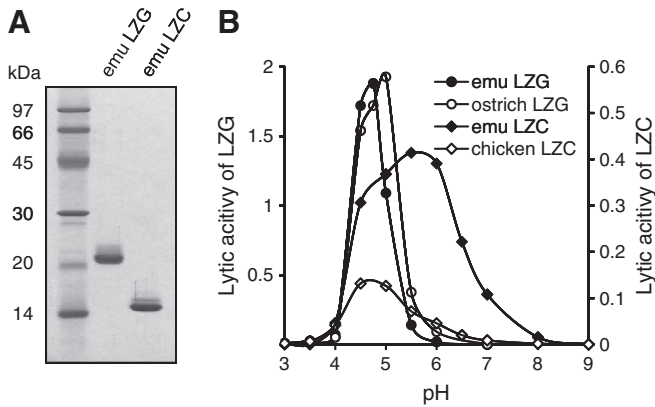


Fig. 6. (A) SDS-PAGE analysis of recombinant emu LZG and LZC purified from culture supernatants of *P. pastoris*. (B) Lytic activities of emu and ostrich LZGs and emu and chicken LZCs at various pHs. Lytic activities are represented as the decrease in OD₄₅₀ value in 1 min by 1 µg of protein.

the first time that the LZC gene is not expressed in the ratite oviduct as evidence that LZC protein is undetectable in ratite egg white because there is no data on the expression pattern of LZC in other ratites of species to closest emu, such as ostrich, rhea, and cassowary. It was considered that the successful cloning of LZC cDNA from emu oviduct was due to the concentrated template cDNA, therefore, LZC gene was substantially not expressed in emu oviduct.

3.5. Enzyme activities of recombinant emu LZC and LZG

Neither LZG nor LZC protein could be detected in emu egg white, but the genes were expressed at very low levels in the oviduct. Therefore, to investigate the enzymatic activities of these products, we prepared recombinant emu LZG and LZC using their cDNAs cloned from the emu oviduct in *P. pastoris* as described in the [Materials and methods](#) section. Expression vectors were constructed for emu mature LZG and LZC and their expression in *P. pastoris* allowed the detection of recombinant emu LZG and LZC in culture supernatants by monitoring their lytic activity. Purified recombinant emu LZG and LZC were obtained from each culture supernatant by ion exchange chromatography as a single protein ([Fig. 6A](#)), and their N-terminal amino acid sequences were confirmed to be processed correctly from secretion signal sequence. LZG purified from ostrich egg white has been characterized previously ([Jolles et al., 1977; Schoentgen et al., 1982](#)), but its DNA has not been reported. We prepared recombinant ostrich LZG from an assembled DNA with 5 exons of LZG gene amplified from the ostrich genome by PCR. We preliminarily confirmed that recombinant chicken LZC and ostrich LZG had the same enzyme activities as each native one prepared from egg white (data not shown).

To compare the enzyme activities of emu LZG and LZC with those of ostrich LZG and chicken LZC, respectively, their lytic activity against *M. luteus* was examined at various pHs. [Fig. 6B](#) shows that emu and ostrich LZG had relatively similar lytic activity within a narrow and acidic pH of 4.5–5. This was similar to that of cassowary LZG reported by [Pooart et al. \(2005\)](#). Emu LZG exhibited about 15 times more activity than chicken LZC and same as ostrich LZG, which has been reported to have 10 times more lytic activity than chicken LZC. But at pH 5, the lytic activity of emu LZG was about half that of ostrich LZG. Emu LZC had a broad and higher pH optimum at a range of 5–6 in contrast to chicken LZC, which had an optimum pH of 4.5–5. The most significant difference between chicken and emu LZCs was that emu LZC exhibited 3 times more lytic activity than chicken LZC at their optimum pHs. Furthermore, in comparison at pH 6.5, emu LZC activity was about 10 times higher than chicken LZC. It is known that human LZC has 3–4 times more activity than chicken

LZC ([Imoto, 1996](#)) and many other mammalian LZCs also have more activity than chicken LZC. However, to our knowledge, no avian LZCs have such high activity as emu LZC.

Goose and ostrich LZGs are known to have very low hydrolytic activity for ethylene glycol chitin (chitinase activity) compared with chicken LZC ([Matsumura and Kirsch, 1996](#)). In our experiment, neither emu nor ostrich LZG showed chitinase activity, while emu LZC showed 44% of chitinase activity against chicken LZC (data not shown). This indicates that chitinase activity of LZG has not been well studied.

4. Conclusions

Despite the presence of lytic activity in chickens and many avians, as defense systems from bacterial infections, it was hardly detected in emu egg white. However, LZG mRNA expression was detected in the emu oviduct, indicating the existence of LZG in egg white. Cloning of LZG cDNA and characterization of recombinant LZG revealed that emu LZG has a very similar structure to that of ostrich LZG, including two conserved catalytic residues, and predicted that emu egg white contained functional LZG like chicken egg white. Probably, in general, ratite members have relatively low LZG levels in egg white, but in emu egg white, LZG level is extremely low. However, for confirmation of this hypothesis, western blot analysis using emu LZG antibody needs to be performed. Further study is also necessary to clarify the control mechanism of LZ synthesis and the reason why the emu does not require LZ in the egg white, unlike many other avians.

References

- Collins, F.S., Weissman, S.M., 1984. Directional cloning of DNA fragments at a large distance from an initial probe: a circularization method. *Proc. Natl. Acad. Sci. U. S. A.* 81, 6812–6816.
- De Boeck, S., Stockx, J., 1986. Egg white lysozyme is the major protein of the hen's egg vitelline membrane. *Int. J. Biochem.* 18, 617–622.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Herve-Gre'pinet, V., et al., 2010. Purification and characterization of avian β -defensin 11, an antimicrobial peptide of the hen egg. *Antimicrob. Agents Chemother.* 54, 4401–4408.
- Imoto, T., 1996. Engineering of lysozyme. In: Jolles, P. (Ed.), *Lysozyme: Model Enzyme in Biochemistry and Biology*. Birkhauser Verlag, Basel/Switzerland, pp. 163–181.
- Imoto, T., Yagishita, K., 1971. A simple activity measurement of lysozyme. *Agric. Biol. Chem.* 35, 1154–1156.
- Irwin, D.M., Gong, Z., 2003. Molecular evolution of vertebrate goose-type lysozyme genes. *J. Mol. Evol.* 56, 234–242.
- Jolles, J., Perin, J.-P., Jolles, P., 1977. The ostrich (*Struthio camelus*) egg-white lysozyme. *Mol. Cell. Biochem.* 17, 39–44.
- Kornegay, J.R., Schilling, J.W., Wilson, A.C., 1994. Molecular adaptation of a leaf-eating bird: stomach lysozyme of the hoatzin. *Mol. Biol. Evol.* 11, 921–928.
- Maehashi, K., et al., 2010. Primary structure of potential allergenic proteins in emu (*Dromaius novaehollandiae*) egg white. *J. Agric. Food Chem.* 58, 12530–12536.
- Matsumura, I., Kirsch, J.F., 1996. Is aspartame 52 essential for catalysis by chicken egg white lysozyme? The role of natural substrate-assisted hydrolysis. *Biochemistry* 35, 1881–1889.
- Minagawa, S., Hikima, J., Hirono, I., Aoki, T., 2001. Expression of Japanese flounder cDNA in insect cells. *Dev. Comp. Immunol.* 25, 439–445.
- Mine, Y., Yang, M., 2008. Recent advances in the understanding of egg allergens: basic, industrial, and clinical perspectives. *J. Agric. Food Chem.* 56, 4874–4900.
- Nakano, T., Graf, T., 1991. Goose-type lysozyme gene of the chicken: sequence, genomic organization and expression reveals major differences to chicken-type lysozyme gene. *Biochim. Biophys. Acta* 1090, 273–276.
- Nile, C.J., Townes, C.L., Michailidis, G., Hirst, B.H., Hall, J., 2004. Identification of chicken lysozyme g2 and its expression in the intestine. *Cell. Mol. Life Sci.* 61, 2760–2766.
- Pooart, J., Torikata, T., Araki, T., 2005. Enzymatic properties of rhea lysozyme. *Biosci. Biotechnol. Biochem.* 69, 103–112.
- Schoentgen, F., Jolles, J., Jolles, P., 1982. Complete amino acid sequence of ostrich (*Struthio camelus*) egg-white lysozyme, a goose type lysozyme. *Eur. J. Biochem.* 123, 489–497.
- Thammasirirak, S., Torikata, T., Takami, K., Murata, K., Araki, T., 2001. Purification and characterization of goose type lysozyme from cassowary (*Casuaris casuaris*) egg white. *Biosci. Biotechnol. Biochem.* 65, 584–592.
- Weaver, L.H., Grutter, M.G., Remington, S.J., Gray, T.M., Isaacs, N.W., Matthews, B.W., 1985. Comparison of goose-type, chicken-type, and phage-type lysozymes illustrates the changes that occur in both amino acid sequence and three-dimensional structure during evolution. *J. Mol. Evol.* 21, 97–111.