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

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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.

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 RNA*later* (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.

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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′-AGAACGGGTTGCTACGG-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′-TCACAGCCGGCAGCCTCTGATCCA -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′-CCACATCGTTGGCATAGTC-3′ and 5′-ATGGAAGTTCTGACTACGGG-3′ and 5′-GTTGCGCCATGCGACCCA-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′-GTGACCTGACAGACTACCT-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′-AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTT-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′-AGGGACCTACGAAGTATGG-3′) or the forward primer designed on the basis of a sequence of emu LZC cDNA fragment (5′-GTTGGTGGTGCAATGATGGCA-3′) and an adaptor primer (5′-AACTGGAAGAATTCGCGGCCGCAGGAA-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′-CAAGACATCCTACAGGAAGCAG-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 μg 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 *Sal*I 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