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Primary Structure of Potential Allergenic Proteins in Emu (*Dromaius novaehollandiae*) Egg White

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The emu (*Dromaius novaehollandiae*) egg is considered promising as an alternative egg product. To obtain basic biochemical information on emu egg white, the major protein compositions in emu and chicken egg whites and the primary structures of potential allergenic proteins were compared. The dominant protein in emu egg white was ovotransferrin (OVT), followed by ovalbumin (OVA) and TENP protein. The OVA and ovomucoid (OVM) levels in emu egg white were estimated as significantly lower than those in chicken egg white by Western blotting and enzyme-linked immunosorbent assays using anti-chicken OVA or OVM antibodies. Lysozyme and its enzymatic activity were not detected in emu egg white. OVT, OVA, and OVM genes were also cloned, and their nucleotide and amino acid sequences were determined. The protein sequences of OVT, OVA, and OVM from emu showed lower similarities to those of chicken than other avian species, such as quail and turkey. These results emphasize the low allergenicity of emu egg white and its potential as an alternative to chicken egg white.

KEYWORDS: Egg white; emu; ovalbumin; ovomucoid; ovotransferrin; TENP

INTRODUCTION

The emu (*Dromaius novaehollandiae*) is native to Australia and is the second largest member of the ratite family, which includes the ostrich, rhea, and cassowary. Emus have come to be considered as an alternative form of livestock, and their production has increased in Japan. These birds produce eggs that are 10 times larger than chicken eggs and, thus, are of interest as an alternative egg product. However, before emu eggs can be used in foods, their allergenicity must be assessed, because chicken egg white is one of the most common allergenic foods and causes serious immediate hypersensitivity reactions or anaphylaxis, which are mediated by immunoglobulin (IgE), in infants and young children (*1*, *2*). The emu egg has traditionally been believed by native Australians to have low allergenicity, although there is no scientific evidence to support this. Significantly fewer studies have been conducted on emu egg white compared with chicken egg white, which has been extensively characterized with respect to its biochemical, physicochemical, and immunological properties. The characterization of emu egg white proteins is essential for further industrial use of emu eggs. Therefore, we have identified the major proteins present in emu egg white and predicted the allergenicity of emu egg white compared to chicken egg white on the basis of the composition of potential allergenic proteins and their primary sequences.

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MATERIALS AND METHODS

Materials. Eggs and tissue samples from egg-laying emus were obtained from Tokyo Nodai Bio-Industries Co., Ltd. (Abashiri, Japan). White Leghorn eggs were obtained from a local market. The egg whites were homogenized in a blender for 10 s at room temperature. A piece of tissue was excised from the emu oviduct and immediately stored in RNA*later* (Ambion, Inc., Austin, TX) until use.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli (*3*). The homogenized egg whites were diluted 10 times with water and mixed with an equal volume of loading buffer prior to heat treatment. The resulting egg white samples of emu and chicken eggs were run on an SDS-PAGE. The molecular weight of the proteins was estimated by SDS-PAGE under reducing conditions. Low molecular weight markers (GE Healthcare Bio-Sciences Corp.) included phosphorylase *b* for 97 kDa, albumin for 66 kDa, ovalbumin (OVA) for 45 kDa, carbonic anhydrase for 30 kDa, trypsin inhibitor for 20 kDa, α-lactalbumin for 14 kDa, and a BenchMark Protein ladder (Invitrogen Co., Carlsbad, CA). These were used to obtain a calibration curve to determine molecular weights of < 45 kDa with 15% gel and > 50 kDa with 7.5% gel. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (CBB). To determine the proportion of different proteins present in the total protein, the CBB-stained bands separated on SDS-PAGE were analyzed by

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Table 1. Primers Used for Cloning of cDNAs for Emu Egg White Proteins

primer name	sequence (5′→3′)	designed on the basis of[a]
OT-3F	GGGGACAGTCTGTGACCAA	oOVT (AJ786651)
OT-2R	CATCTGGAGGAGATCTGATGG	oOVT (AJ786651)
OT-6F	CCCATGGGCTTGATTCACAACA	oOVT (AJ786651)
OA-10F	GTTGATGCCATTTACTTCAAAGG	eOVA−V8 fragment[b]
OA-15F	GATGTATTCAAGGAGCTCAAAGTC	cOVA (NM_205152)
OA-12R	GCCTGATACATCATCTGCACAG	OA-10/AP-2 fragment
OA-21F	GGTCAAACTTCTGAAGGGAACC	cOVA (J00895)
OA-19R	CTTACTGGCAAGGCTGAGCG	OA-15/-12 fragment
OA-23F	CACAGCTGGAAAGCTGTATTGC	OA-20/-19 fragment
OA-13R	CTGATTGTAGTCTCAAGCTGCTC	OA-10/AP-2 fragment
OM-1F	TTGCTACGGTGGACTGCAG	third domain of eOVM[b]
OM-2R	GCATTTTCCAAAGTGCCC	third domain of eOVM[b]
OM-5F	CCTCTGTGTGGCTCTGAC	OM-1/-2 fragment
OM-9F	AGCCGGGCAGTACCTCACC	cOVM (J00902)
OM-8R	TGCTGTCAGAGCCACACAGAGG	OM-1/-2 fragment
OM-15F	GTGCTGCTCTCTCTTGTGCTTTG	cOM-9/-8 fragment
OM-16R	GCATTCGTTGCTGTAAGTGACTC	cOM-9/-8 fragment
OM-21F	CAAGAGATGAAAACAAGCCAGAGG	OM-15/-16 fragment
OM-23R	CCTCTGCAGCATCTTGAGAG	OM-15/-16 fragment
OM-24F	TAGCAGCCTGCAGACTCGAG	OM-23/-14 fragment
OM-14R	GCAAGATGAACTGAGGTGAGATC	OM-5/AP-2 fragment

[a] o, ostrich; c, chicken; e, emu. Accession numbers are indicated in parentheses. [b] Codons for these protein sequences were selected on the basis of the cOVM nucleotide sequence.

densitometric quantification using a Gel-Pro analyzer (Media Cybernetics, Inc., Bethesda, MD).

Western Blotting. After separation of egg white proteins by SDS-PAGE, bands were electroblotted on a polyvinylidene fluoride (PVDF) membrane. A Morinaga FASPEK egg protein Western blot kit (ovalbumin) (Morinaga Institute of Biological Science, Yokohama, Japan) and a Morinaga FASPEK egg protein Westen blot kit (ovomucoid) were used for detection of OVA and ovomucoid (OVM), respectively, according to the manufacturer's instruction. Chicken and emu egg white samples were diluted 12000 and 60000 times, and then 10 μL of these samples was loaded and separated on an SDS-PAGE. The total protein contents of the 12000 and 60000 times diluted samples were 10 and 2 μg/mL in chicken egg whites and 9.2 and 1.8 μg/mL in emu egg whites. Protein concentration was determined according to the bincinchoninic acid (BCA) method using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL), following the manufacturer's instructions. Primary antibodies to chicken OVA (anti-OVA antibodies) and to chicken OVM (anti-OVM antibodies) obtained from immunized rabbits included in the kit were used for emu and chicken egg whites. A Vectastain ABC-AP Rabbit IgG kit (Vector Laboratories, Ltd., Burlingame, CA) was used as a secondary antibody, and an alkaline phosphatase substrate kit IV BCIP/NBT (Vector Laboratories, Ltd.) was used to detect the secondary antibody.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was performed to estimate antigenicity to chicken OVA using a Morinaga Egg Protein ELISA kit (Morinaga Institute of Biological Science), which was originally intended for the determination of egg protein content in food samples based on the reactivity to the anti-OVA antibody (*4*). The procedure followed the manufacturer's instructions. Emu and chicken egg white samples were serially diluted from 2.8×10^5 to 8.8×10^6 times and from 4.8×10^6 to 1.5×10^8 times, respectively, with distilled water to give protein concentrations of 6.25−400 and 0.78−25 ng/mL, respectively. Protein concentration was determined by using the BCA method.

Protein Sequencing. To determine the partial amino acid sequences of sample proteins, in-gel digestion was performed following the method of Cleveland et al. (*5*) using *Staphylococcus aureus* V8 protease (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described in our previous paper (*6*). After electroblotting on a PVDF membrane (Bio-Rad, Hercules, CA), each separated band of a proteolytic fragment was excised from the membrane and subjected to a protein sequencer PPSQ-21 (Shimazdu, Kyoto, Japan) for amino (N)-terminal analysis. The resultant protein sequence data were analyzed using the NCBI protein BLAST program.

Measurement of Lysozyme (LYZ) Enzyme Activity. The bacteriolytic activity of LYZs toward *Micrococcus lysodeikticus* cells (Sigma-Aldrich Japan, Osaka, Japan) was measured turbidimetrically in a cell suspension of 0.2 mg/mL using a homogenized egg white sample. According to Pooart et al. (*7*), 0.1 M Britton−Robinson buffer at pH 5−11 was used to measure. The decrease in absorbance due to bacteriolysis was then monitored at 450 nm with a Beckman DU640 spectrophotometer (Beckman Coulter, Inc.).

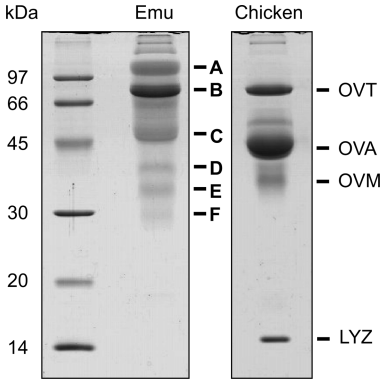

Figure 1. SDS-PAGE patterns of emu and chicken egg whites. Homogenized egg whites from emu and chickens were diluted 1:10 and loaded on a 15% gel. The gels were stained with CBB.

Cloning of cDNAs for Emu Egg Proteins. Emu oviduct cDNA was prepared as described previously (*6*). Briefly, total RNA was extracted from an emu oviduct using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). Single-stranded cDNA synthesis was performed using a SuperScript III First-Strand Synthesis SuperMix kit and an oligo-dT(20) primer (Invitrogen Co.). The amplified cDNA was used for subsequent PCR amplifications with Ex*Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan). The primers used for amplification of OTF, (OVM), and OVA are listed in **Table 1**. The amplicons were cloned into pCR2.1-TOPO (Invitrogen Co.) and sequenced by Macrogen Japan Corp. (Tokyo, Japan).

3′ Rapid Amplification of cDNA end (3′ RACE). To isolate the downstream region of the 3′ end of the cDNA, we performed 3′ RACE. Single-stranded cDNA synthesis was performed using an oligo-dT primer with the adaptor sequence 5′-AACTGGAAGAATTCGCGGCCGCAG-GAATTTTTTTTTTT TTTTTTT-3′ and RNA prepared from the emu oviducts. This amplicon was used as a template for subsequent PCR using emu cDNA primers for OVA or OVM and an adaptor primer AP-2 (5′-AACTGGAAGAATTCGCGGCCGCAGGAA-3′). The amplicons were cloned into pCR2.1-TOPO (Invitrogen Co.) and sequenced by Macrogen Japan Corp. The resultant nucleotide sequence data were analyzed with the NCBI nucleotide BLAST program.

Inverse PCR. To determine the nucleotide sequence of the 5′ end of the OVM gene, we performed inverse PCR (*8*). Emu genomic DNA was extracted from the emu ovary using a QIAGEN Blood & Cell Culture DNA Mini Kit (QIAGEN K.K., Tokyo, Japan), according to the manufacturer's instructions. First, we decided to use a restriction enzyme such as *Kpn*I from the sequence of the amplified fragment from emu genomic DNA with the primers OM-15F and OM-16R. Then the genomic DNA was digested with *Kpn*I, and the digest was self-ligated using a FastLink DNA Ligation Kit (Epicenter Biotechnologies, Madison, WI). PCR amplification was then performed using the primers OM-23R and OM-21F to obtain a fragment that included the 5′ untranslated region of the emu OVM gene.

RESULTS AND DISCUSSION

Identification of Major Proteins in Emu Egg White. Total protein content in emu egg white has been reported as 8.9%, which was less than the 10.5% of standard chicken egg (*9*). We diluted emu and chicken egg white samples 10 times and compared their SDS-PAGE patterns. As shown in **Figure 1**, chicken egg white showed four major protein bands: ovotransferrin (OVT), OVA, OVM, and LYZ with proportions of 19, 54, 7, and 5%, respectively (data not shown). This composition of

Table 2. Identification of the Major Proteins in Emu Egg White

band	partial amino acid sequence	identified as	MW (kDa)	approximate proportion[a] (%)
A	MVLVDAIYFKGTCEKACKDE[b]	ovalbumin	102	15
B	AAPKATVRWCTISS[c]	ovotransferrin	78	33
C	TKSPDCGGILSPDGLSYFA[c]	TENP protein	52	16
D	VEVDCSKYPNTTNEDGKEVL[c]	ovomucoid	39	7[d]
E	VEVDCSKYPNTTNEDGKEVL[c]	ovomucoid	34	
F	VEVDCSKYPNTTNEDGKEVL[c]	ovomucoid	30	
others				29
				total 100

[a] The proportion of each protein was densitometrically estimated from the CBB-stained bands on SDS-PAGE shown in **Figure 1**. [b] Internal sequence. [c] N-Terminal sequence. [d] Sum of three ovomucoids of D, E, and F. These amino acid sequences are listed in UniProt Knowledgebase under accession numbers P86380, ovalbumin; P85895, ovotransferrin; P86384, TENP protein; and P05560, ovomucoid.

major proteins found in chicken egg white was nearly consistent with data obtained previously by others (*10*). In contrast, emu egg white exhibited three major protein bands, A, B, and C, with molecular masses of 102, 78, and 52 kDa, respectively. The 78 kDa band was identical in size with OVT in the chicken egg protein; however, the other bands in the emu egg white differed in size from those in the chicken egg white. Notably, no similar band was observed in emu egg white at 45 kDa, which is the most abundant protein, OVA, in chicken egg white.

To identify the major proteins found in emu egg white by SDS-PAGE, the bands were electroblotted onto a PVDF membrane and subjected to the protein sequencer. As a result of N-terminal amino acid sequencing, seven bands, A−F, found in SDS-PAGE for the emu egg white were identified (**Table 2**). The most abundant protein in emu egg white band B comprised 33% of the total emu egg white proteins and was identified as OVT because of its high N-terminal sequence similarity to that of chicken egg white. Emu OVT has the same molecular mass as chicken OVT. The protein with the highest molecular mass among the major proteins in emu egg white, band A, was not identified directly by N-terminal sequencing, indicating that its N-terminus was blocked by modified amino acid residues. Therefore, in-gel digestion was conducted using the band A protein excised from the SDS-PAGE gel. Amino acid sequencing of a fragment from the band A after cleavage with V8 protease revealed the partial internal amino acid sequence of the band A protein. As this was very similar to the chicken OVA sequence based on the BLAST protein search, band A was identified as OVA despite its molecular mass being double that of chicken OVA. Thus, it was found that OVA was contained in emu egg white and its molecular mass was significantly different from those in other avians such as chicken, quail, and turkey (*11*), although it has been unidentified in emu egg white because a 45 kDa band corresponding to chicken OVA is not seen in emu egg white on SDS-PAGE (*9*, *11*). The 19 N-terminal amino acids of band C were sequenced, and band C was found to have 73.7% similarity with chicken TENP protein. TENP protein has been reported to be transiently expressed in the chicken retina and brain (*12*), but its biological function has yet to be clarified. Recently, it was reported that chicken TENP protein has strong homology with a bacterial permeability-increasing protein family (*13*). Bands D, E, and F were found to have the same N-terminal sequences and were identified as OVM. Chicken OVM is a 28 kDa protein with trypsin inhibitor activity containing 20−25% carbohydrate. It has been reported previously that chicken OVM consists of several variants with different carbohydrate contents (*14*), although one major band was seen in chicken egg white. In contrast, three bands of OVM were evenly seen in emu egg white. The estimate of the proportion of OVM in emu egg white based on SDS-PAGE (**Figure 1**) suggests that the OVM content in emu egg white is less than that in chicken egg white (**Table 2**). Surprisingly, the LYZ band was not found in emu egg white despite its being a major protein in chicken and also several other avian egg whites, because it plays an important role in protecting the egg against bacterial infections.

Detection of LYZ in Emu Egg White. LYZ, a bacteriolytic enzyme that catalyzes the hydrolysis of β-1,4 glycosidic bonds in the peptidoglycan of bacterial cell walls, is classified into several types including two major types of chicken (c-LYZ) and goose types (g-LYZ). Several ratite family members, such as the ostrich (*15*), cassowary (*16*), and rhea (*7*), have g-LYZ in their egg whites, whereas chicken, quail, and turkey have c-LYZ in their egg whites. However, neither c-LZY (MW of approximately 14 kDa) nor g-LYZ (MW of approximately 20 kDa) was detected in emu egg white by SDS-PAGE with CBB (**Figure 1**) or silver staining (data not shown). Furthermore, no LYZ bacteriolytic activity was detected in emu egg white (data not shown), although Feeney et al. reported a LYZ content in emu egg white of 0.05% based on its bacteriolytic activity (*17*). Other ratite LYZs have been reported to show optimum lytic activity at lower pH than chicken LYZ (*18*). Therefore, we measured the lytic activity in emu egg white at a broad range of pH levels. However, no lytic activity was detected at pH 5−11 (data not shown). In several cases, a g-type LYZ exhibits higher lytic activity, for example, 6 and 10 times higher in goose and ostrich g-LYZs, respectively (*15*) than in chicken c-LYZ. As we did not detect any lytic activity in emu egg white, c-LYZ, which is a major allergen, must not exist in substantial amounts in emu egg white. To our knowledge, this is the first report of an avian egg white in which LYZ was not detected, although Feeney et al. reported just a small amount (< 0.02%) of LYZ in *Agapornis personata* egg white (*17*). Further detailed study of the expression of the emu LYZ gene is needed.

Molecular Cloning and Primary Structure of Emu OVT. We cloned genes for OVT, OVA, and OVM from emu oviduct cDNA preparations by PCR to elucidate their complete amino acid sequences because these are known to be the major allergenic proteins in chicken egg white (*19*).

For cloning of emu OVT cDNA, a 2 kbp fragment from the 5′ untranslated region of OVT cDNA was first obtained by PCR amplification using the primer OT-3F and OT-2R. The 3′ end of OVT was obtained by 3′ RACE with the primers OT-6F. As a result of PCR amplification, cDNA with 2052 bp nucleotides coding 19 amino acids of signal peptide, 684 amino acids of mature emu OVT protein, and about 200 bp of the 3′ untranslated region, including a polyadenylation signal was obtained, and the sequence data were deposited in DDBJ under accession no. AB455549. The predicted signal cleavage site and deduced amino acid sequence of the N-terminus of mature OVT were consistent with the N-terminal sequence experimentally determined for egg protein. The amino acid sequences of emu and chicken OVTs were aligned, as shown in **Figure 2A**. Osuga and Feeney reported isoelectric pHs (p*I* values) of cassowary and kiwi OVTs of 9.0 and 8.0, respectively, whereas those of chicken and turkey were 6.0 and 5.8, respectively (*18*). The theoretical p*I* of emu OVT calculated from its amino acid sequence is 8.6, which is in agreement with that of other ratites. Chicken OVT has a glycosylation site at Asn473; in the emu OVT sequence an extra potential glycosylation site was found at Asn52. However, because emu OVT and chicken OVT were the same size on SDS-PAGE (**Figure 1**), it is predicted that this position may not participate in glycosylation. The iron ligand residues in chicken OVT (**Figure 2A**) were completely conserved in emu OVT. It is

(A)

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emu OVT      AAPKATVRWCTISSPEQKKCNDLKERMQQESFGLSCVQKT  40
chicken OVT  APPKSVIRWCTISSPEEKKCNNLRDLTQQERISLTCVQKA  40

emu OVT      TYMDCIMAIARNETDAISLDGGQIFEAGLGPYKLKPIAAE  80
chicken OVT  TYLDCIKAIANNEADAISLDGGQVFEAGLAPYKLKPIAAE  80

emu OVT      VYEGTQGSTTYYYAVAVVKKGTDFTINDLKGKTSCHTGLG  120
chicken OVT  IYEHTEGSTTSYYAVAVVKKGTEFTVNDLQGKNSCHTGLG  120

emu OTF      RSAGWNIPIGTLIRRGDIPWDGIGSGSLEQEVAKFFSASC  160
chicken OVT  RSAGWNIPIGTLLHWGAIEWGIESGGSVEQAVAKFFSASC  160

emu OVT      VPGAN-EKKLCRQCKGDAKTKCAHGGPYYGYSGAFRCLKD  199
chicken OVT  VPGATIEQKLCRQCKGDPKTKCARNAPYSGYSGAFHCLKD  200

emu OVT      GNGEVAFVKHTTVEENAAKEKDQYELLCLDGTRQPVDNYK  239
chicken OVT  GKGDVAFVKHTTVNENAPDLNDEYELLCLDGSRQPVDNYK  240

emu OVT      ACHWARVPGHAVVTRDDGKAEEIWTFLSKAQEKFGVGTTS  279
chicken OVT  ACHWARVAAHAVVARADSKAEEIWSFLSKAQEKFGVGKSS  280

emu OVT      DFHLFGTPDKKDPHLKDLLFKDSAIKLKKIPQLMDSGLYL  319
chicken OVT  DFHLFGTPDKKDLHLKDLLFKDSAIMLKRVPSLMDSQLYL  320

emu OVT      GFEYYSAIQNLRKDVMTSTPRKNKVQWCAVGKEEKSKCDW  359
chicken OVT  GFEYYSAIQSMRKDQLTPSPRENRIQWCAVGKDEKSKCDR  360

emu OVT      RSVVSNGEVECAVGDNTKDCIIKIMKGEADAISLDGGFVY  399
chicken OVT  WSVVSNGDVECTVVDETKDCIIKIMKGEADAVALDGGLVY  400

emu OVT      TAGVCGLVPVMGENYEDETSCSKVGQ-PASYFAVAVVKKS  438
chicken OVT  TAGVCGLVPVMAERYDDESQCSKTDERPASYFAVAVARKD  440

emu OVT      DRDITWDNLQGKKSCHTAVGRTAGWNIPMGLIHNRTGSCN  478
chicken OVT  S-NVNWNNLKGKKSCHTAVGRTAGWVIPMGLIHNRTGTCN  479

emu OVT      FDEYFREGCAPGSPSGSRLCKLCQGSGRIPSEKCVANSHE  518
chicken OVT  FDEYFSEGCAPGSPPNSRLCQLCQGSGGIPPEKCVASSHE  519

emu OVT      RYYGYTGALRCLVEQGDVAFIKHSIVEENTNGKNKEEWAK  558
chicken OVT  KYFGYTGALRCLVEKGDVAFIQHSTVEENTGGKNKADWAK  559

emu OVT      NLKMDQFELLCTNGRRANIMDYSSCHLAKVPAHAVISRPE  598
chicken OVT  NLQMDDFELLCTDGRRANVMDYRECNLAEVPTHAVVVRPE  599

emu OVT      KTSKIRELLERQEKLFGEKGTDKERFTMFKSHTKDLLFKD  638
chicken OVT  KANKIRDLLERQEKRFGVNGSEKSKFMMFESQNKDLLFKD  639

emu OVT      LTKCLVKLREGITYKEFLGDQYYASVASLNTCNPSDLLQM  678
chicken OVT  LTKCLFKVREGTTYKEFLGDKFYTVISNLKTCNPSDILQM  679

emu OVT      CTFLEG-   684
chicken OVT  CSFLEGK   686
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(B)

	emu	ostrich	duck	turkey
ostrich (CAH10347)	84.0%			
duck (P56410)	77.1%	74.1%		
turkey (CAI84849)	76.0%	73.3%	81.5%	
chicken (NM_205304)	75.3%	71.9%	80.6%	90.5%

Figure 2. (**A**) Comparison of amino acid sequences between emu and chicken OVTs. Potential N-glycosylation sites are highlighted by the black background. The amino acids determined by direct sequencing are underlined. Potential hinge regions are boxed. Triangles indicate potential iron-coordinating ligands. (**B**) Percentage of protein sequence similarity in OVT. Accession numbers are indicated in parentheses.

known that chicken OVT consists of two similarly sized homologous N- and C-lobes that are further divided into two similarly sized domains. The four hinge regions for connecting the four domains were well conserved in the emu and chicken OVTs, although one amino acid deletion (corresponding to Arg427 in chicken OVT) was found in one of the hinge regions in emu OVT. Because this was also seen in ostrich OVT (*20*), it is presumed that the deletion may not influence the molecular structure of emu OVT.

Molecular Cloning and Primary Structure of Emu OVA. For cloning of emu OVA cDNA, a 1.3 kbp fragment from the 3′ end of OVA cDNA was first obtained by 3′ RACE with the primer OA-10F. Subsequently, a 550 bp fragment near the 5′ end of OVA was obtained by PCR amplification with OA-15F and OV-12R primers. The 5′ untranslated region of emu OVA was amplified from genomic DNA using OA-23F primer that was designed on the basis of OA-21F and OA-19R. This was then amplified from

(A)

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emu OVA     GSIGAASTEFCFDMFKELKVHHVNENIIYSPLSIISILSM   40
chicken OVA GSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAM   40

emu OVA     VFLGARENTKTQMEKVIHFDKITGFGESLESQCGTSVSVH   80
chicken OVA VYLGAKDSTRTQINKVVRFDKLPGFGDSIEAQCGTSVNVH   80
            E1
emu OVA     ASLKDILSEITKPSDNYSLSLASKLYAEETYPVLPEYLQC  120
chicken OVA SSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQC  120
                           E2
emu OVA     IKELYKGSLETVSLQTAADQARELINSWVETQTNGVIKNF  160
chicken OVA VKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNV  160

emu OVA     LQPGSVDPQTEMVLVDAIYFKGTWEKAFKDEDTQEVPFRI  200
chicken OVA LQPSSVDSQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRV  200
                                           E3
emu OVA     TEQESKPVQMMYQAGSFKVATVAAEKMKILELPYASGELS  240
chicken OVA TEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMS  240

emu OVA     MFVLLPDDISGLEQLETTISIEKLSEWTSSNMMEDRKMKV  280
chicken OVA MLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKV  280
            E4      E5
emu OVA     YLPHMKIEEKYNLTSVLVALGMTDLFSPSANLSGISTAQT  320
chicken OVA YLPRMKMEEKYNLTSVLMAMGITDVFSSSANLSGISSAES  320

emu OVA     LKMSEAIHGAYVEIYEAGSEMATSTGVLVEAASVSEEFRV  360
chicken OVA LKISQAVHAAHAEINEAGREVVGSAEAGVDAASVSEEFRA  360

emu OVA     DHPFLFLIKHNPSNSILFFGRCIFP  385
chicken OVA DHPFLFCIKHIATNAVLFFGRCVSP  385
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(B)

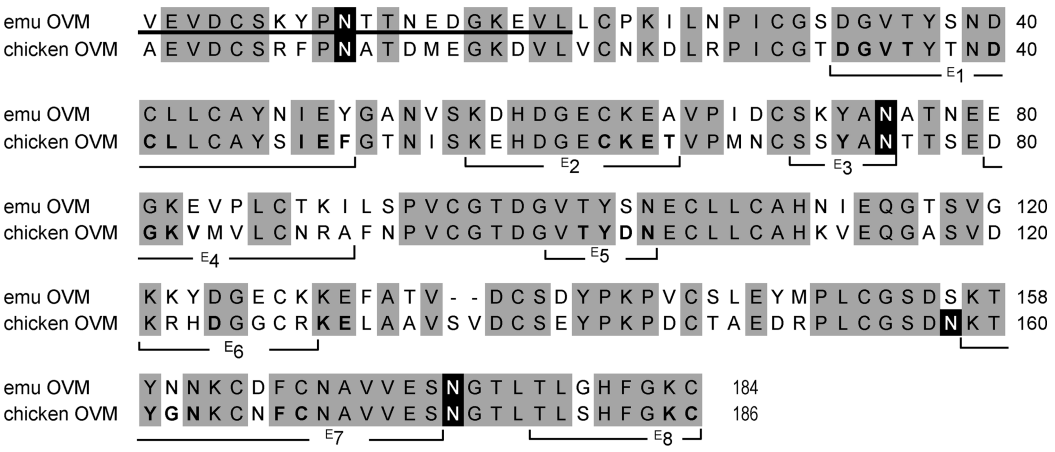
	emu	turkey	chicken
turkey (AAC16664)	73.4%		
chicken (AY354922)	71.3%	90.6%	
quail (AAQ22769)	69.0%	85.8%	86.8%

Figure 3. (**A**) Comparison of amino acid sequences between emu and chicken OVAs. The amino acids determined by direct sequencing after in-gel digestion are underlined. Potential N-glycosylation sites are highlighted by a black background. Potential phosphorylation sites are boxed. $^{E}1-^{E}5$ regions indicate IgE epitopes as determined by Mine and Rupa (*18*). Bold letters represent the amino acids critical for IgE binding. (**B**) Percentage of protein sequence similarity in OVAs. Accession numbers are indicated in parentheses.

cDNA by primers OA-23F and OA-13R. As a result of combined sequence data from three fragments of emu OVA amplified by PCR, the complete nucleotide sequence of emu OVA from the 5′ untranslated region to the 3′ polyadenylation signal was determined and deposited in DDBJ under accession no. AB525084. This cDNA encodes 385 amino acids including the initiation methionine at the N-terminus. Despite this protein being double the size of chicken OVA, the amino acid number deduced from the nucleotide sequence of emu OVA was the same as that of chicken OVA, and the amino acid sequences of emu and chicken OVAs were aligned, as shown in **Figure 3A**. The N-terminus of mature OVA was predicted to be acetylglycine, as is the case for chicken OVA, because direct protein sequencing of the N-terminus was not possible. A potential N-glycosylation site at position 292 of chicken OVA was conserved in emu OVA; however, other glycosylation sites that could account for the larger molecular mass were not found in the emu OVA sequence. Recently, Guerin-Dubiard et al. (*13*) identified several OVA variants with higher molecular masses in chicken egg white by two-dimensional electrophoresis and mass spectrometry; however, those have not been characterized. Further study should be made of dissecting the structure of emu OVA with significantly larger molecular mass than 43 kDa calculated from its amino acid sequence. Whereas chicken OVA has two phosphorylation sites at Ser78 and Ser318, emu OVA lacked a potential phosphorylation site at position 317. Allergenic determinants that induce hypersensitivity to chicken OVA have been well studied. Therefore, the sequences of epitopes on chicken OVA recognized by IgE antibodies ($^{E}1-^{E}5$) reported by Mine and Rupa (*21*) were compared with the corresponding sequences in emu OVA, as shown in **Figure 3A**. Of the five epitopes, E3 and E4 were relatively conserved in their amino acid sequences. However, differences in the critical amino acids for binding to IgE were seen in all of the epitopes. The protein sequence similarity of emu OVA with chicken OVA was 71.3%, which was relatively low compared to that between chicken OVA and three other avian OVAs (**Figure 3B**).

Molecular Cloning and Primary Structure of Emu OVM. For cloning emu OVM cDNA, a 120 bp fragment was first amplified using the primers OM-1F and OM-2R, which were designed on the basis of the third domain of emu OVM reported by Laskowski et al. (*22*). Then, based on the sequence, the OM-8R primer was designed and approximately a 500 bp fragment of emu OVM was amplified with OM-9F. Primer OM-24F was designed on the basis of the 5′ untranslated region of the OVM gene in emu genomic DNA sequenced by inverse PCR, and approximately 700 bp of emu OVM was amplified with primer OM-14R. A 280 bp fragment including the 3′ end of emu OVM was obtained by 3′ RACE with OM-5. As a result of PCR amplification, emu OVM precursor cDNA containing a 621 bp open-reading frame sequence and encoding 207 predicted amino acid residue was obtained. The sequence data were deposited in DDBJ under accession no. AB462349. The amino acid sequence of the mature

(A)



(B)

	emu	turkey	quail
turkey (P68390)	73.4%		
quail (P01003)	68.5%	82.2%	
chicken (NM_001112662)	68.5%	82.2%	75.8%

Figure 4. (**A**) Comparison of amino acid sequences between emu and chicken OVMs. The amino acids determined by direct sequencing are underlined. Potential N-glycosylation sites are highlighted by a black background. $^{E}1-^{E}8$ regions indicate IgE epitopes as determined by Mine and Zhang (*26*). Bold letters represent amino acids critical for IgE binding. (**B**) Percentage of protein sequence similarity in OVMs. Accession numbers are indicated in parentheses.

protein deduced from the nucleotide sequence consisted of 184 amino acids and was aligned with the chicken OVM sequence, as shown in **Figure 4A**. Chicken OVM is a unique egg protein consisting of three tandem domains, each of which possesses some anti-trypsin activity (*23*). However, the major trypsin reactive site is in domain II in chicken OVM (*24*). Zhang and Mine have reported that there are significantly more human IgG and IgE binding activities to domain III than to domain I or II of chicken OVM in sera derived from egg-allergic patients (*25*). The potential IgE binding regions in chicken OVM reported by Mine and Zhang (*26*) are indicated by $^{E}1-^{E}8$ in **Figure 5A**. A two amino acid deletion site in emu OVM domain III was found when this was compared with chicken OVM; however, this was not in the region of the potential epitopes for IgE binding. Three N-glycosylation sites have been reported in chicken OVM. Emu OVM lacked a N-glycosylation site at position 156, which is adjacent to epitope $^{E}7$ in domain III. The role of the carbohydrate moiety in relation to immunogenic activity remains controversial because Matsuda et al. found that the carbohydrate chain played an important role in antigenic determinants against human IgE antibody (*27*) and Cooke et al. have reported a decrease in the binding of OVM-specific IgE antibody to deglycosylated OVM in some patients (*28*). On the other hand, Zhang and Mine reported that the carbohydrate moieties in OVM had an inhibiting effect on IgE binding activity (*25*). The percentage of protein sequence similarity in four avian OVMs revealed that the similarity to chicken was lowest in the emu (**Figure 4B**).

Immune Cross-Reactivities of Emu OVA with Chicken OVA. As shown in **Figure 5A**, when anti-chicken−OVA antibody was employed, a large amount of OVA was detected in both dilutions of chicken egg white samples. In contrast, very few bands were detected at approximately 100 kDa, which is where OVA should be detected. Instead, a faint band was observed at around 55 kDa. This 55 kDa band was likely to be another OVA rather than 102 kDa OVA, although it was not found on SDS-PAGE with CBB staining in **Figure 1**. However, because of the low content, it was not confirmed as OVA by the amino acid sequencing. Because the 102 kDa OVA was detected as one of major proteins as shown in **Figure 1** and **Table 2**, this result would indicate that the 102 kDa OVA hardly had a cross-immune reactivity against antichicken OVA antibody. This considerably fewer cross-immune reactivity of emu 102-kDa OVA with chicken OVM should be further investigated in the future. On the other hand, when anti-chicken OVM antibody was employed, very faint bands of three variously sized OVMs at the same position as seen in **Figure 1** were detected in emu egg white sample, whereas a large amount of OVM was detected in chicken egg white sample (**Figure 5A**). This result indicated a considerably lower level of OVM in emu egg white on the basis of its antigenecity against anti-chicken OVM antibody.

To determine immune cross-reactivity between emu and chicken OVAs, an ELISA was performed using an Egg Protein ELISA Kit with a specific anti-chicken OVA antibody for the inspection of egg protein levels in processed foods (*29*). **Figure 5B** shows significant binding of chicken egg white to anti-chicken OVA antibody but considerably lower binding of emu egg white to the same antibody. The binding activity of emu egg white was only 0.64% of that of chicken egg white. Therefore, the level of OVA in emu egg white was estimated to be 0.35% on the basis of its antigenicity against anti-chicken OVA antibody, compared with 54% in chicken egg white.

We found that emu egg white has a different aspect in its composition of major proteins compared with chicken egg white; OVA appears to be present in lower amounts, and LYZ was substantially absent in emu egg white. We also found that amino acid sequence similarities for OVA and OVM, which are known to be major allergenic proteins in chicken egg, were lower between emu and chicken than between other ratites and chicken. In addition, low reactivity on immunoblot analysis and ELISA using antibodies against chicken OVA indicated that emu egg white possessed low levels of OVA and OVM on the basis of their antigenetic reactivity and, therefore, will be expected to have a lower allergenicity in

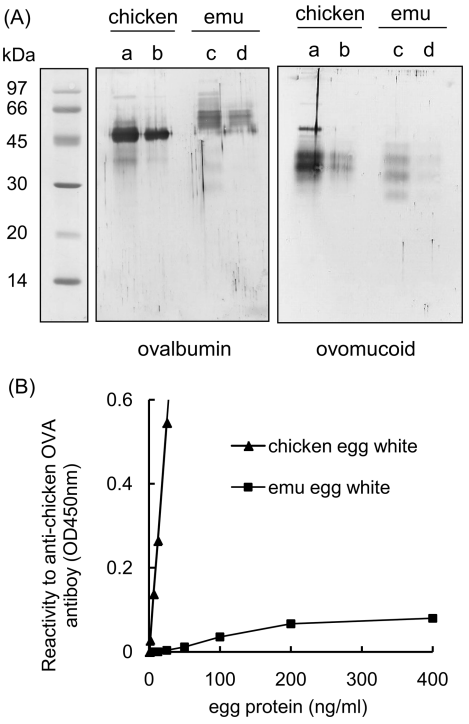


Figure 5. Western blot analysis for OVA and OVM (**A**) and ELISA for OVA (**B**) in emu and chicken egg whites using anti-chicken OVA and anti-chicken OVM antibodies. Homogenized chicken and emu egg white samples were diluted with water at 1:12000 (lanes a and c, respectively) or at 1:60000 (lanes b and d, respectively) for Western blot analysis.

egg-allergy patients than chicken egg white. However, further clinical analysis, such as immunoblots using sera from egg-allergy patients and diagnostic tests, are essential for determining the actual allergenicity of emu egg white. The sequence information relating to the emu egg white proteins presented in this study will aid not only immunological but also physicochemical research on emu egg white, thereby accelerating further research and development of industrial products using emu egg white.

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