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# Properties of emu (*Dromaius novaehollandiae*) albumen proteins

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ABSTRACT

The emu, a flightless bird native to Australia, lays eggs that have different properties than hen eggs: emu eggs begin to coagulate at a lower temperature than do hen eggs, and hard-boiled emu albumen has less gel strength than hen albumen. To investigate the mechanisms behind the differences, three major albumen proteins, ovotransferrin, ovalbumin, and tenp, were analyzed calorimetrically and physicochemically. In the calorimetrical analysis, emu ovotransferrin and ovalbumin exhibited similar thermostability to their hen counterparts. Tenp, a major component of emu but not hen albumen, showed high thermostability. Ovotransferrin, the major protein in emu albumen, produced a softer gel than ovalbumin when denatured in boiling water. Tenp did not coagulate completely in boiling water. The above results suggest the usefulness of emu eggs for processed food production. Changes in the antigenicity of emu ovalbumin during digestion were also examined in vitro.


## 1. Introduction

The emu (*Dromaius novaehollandiae*) is a flightless bird from Australia that produces eggs only during the winter. Emu eggs weigh 10 times more than hen eggs and are known to possess properties amenable to gelation, whipping, and foaming (Takeuchi & Nagashima, 2010). For example, emu eggs begin to coagulate at a lower temperature than do hen eggs. Hard-boiled emu albumen has 1/3 the gel strength of hen albumen. Foam stability is also superior in the emu egg, which means, for example, that a cake made with emu eggs is softer than one made with hen egg products. Local confectioneries are utilizing emu eggs because these unique properties are advantageous for various production processes.

Emu albumen is composed of components similar to those of hen albumen but at a different ratio (Maehashi et al., 2010). The difference in albumen quality could be ascribed to this ratio. However, the properties of each composing protein have not yet been analyzed precisely. Thus, this study investigated the processing properties of the major constituent proteins. Emu albumen contains the following major proteins: ovotransferrin, ovalbumin, and tenp protein (abbreviated as tenp). The ovotransferrin in emu albumen is 75.3% identical to the hen counterpart (Maehashi et al., 2010). It is the most abundant protein component of an emu egg but the second most abundant protein component of a hen egg. Therefore, it is conceivable that ovotransferrin strongly influences the properties of emu eggs. Hen ovotransferrin is known to coagulate at the low temperature of 65 °C (Donovan, Mapes, Davis, & Garibaldi, 1997). It is an ion-binding protein that is known to stabilize by ion loading at the binding sites (Kurokawa, Mikami, & Hirose, 1995; Lin, Mason, Woodworth, & Brandts, 1991; Mine, 1995). However, little is known about the properties of emu ovotransferrin.

Emu ovalbumin behaves like a 102-kDa high-molecular-weight protein on SDS-PAGE analysis, although its primary structure is 71.3% identical to 44.5-kDa hen ovalbumin. Interestingly, it has been reported that emu ovalbumin is not recognized by anti-hen ovalbumin polyclonal antibody (Maehashi et al., 2010). However, consumers may hesitate to use emu egg for allergic patients, because of the high level of similarity between the primary structures of emu and hen ovalbumin. To confirm the safety of emu eggs, it must be proven that no antigenic fragments emerge from emu ovalbumin during digestion. About the properties of emu ovalbumin, the mobility pattern and difference in antigen recognition suggest ovalbumin polymer formation occurs in addition to protein modifications such as phosphorylation and glycosylation. Therefore, it may be possible that there are some differences in the properties of hen and emu ovalbumin.

Tenp has been isolated as a minor component of infertile hen albumen (Guérin-Dubiard et al., 2006). It is also known to be a putative membrane protein from which mRNA is transiently expressed during embryonic development (Yan & Wang, 1998). In the emu egg, tenp constitutes 16% of the total albumen protein; thus, its characteristics may play a role in the unique properties of the emu egg.

In this study, we separately purified ovotransferrin, ovalbumin, and tenp and then performed a calorimetric analysis and heat denaturing

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gel strength test. Ovalbumin was also subjected to immunoblotting using anti-hen ovalbumin antibody to examine antigenicity during in vitro digestion.

## 2. Materials and methods

### 2.1. Materials

Commercially available fertile emu eggs were kindly provided by Tokyo Nodai Bio-industry Co., LTD. (Hokkaido, Japan). Fertile hen eggs (White leghorn) were provided by Dr. Gaku Shimoi of Tokyo University of Agriculture. All experimental reagents were purchased from Kanto Chemical Co., INC. (Tokyo, Japan) unless otherwise noted.

### 2.2. Purification of emu albumen components

Fresh emu albumen was removed from the yolk and homogenized in a whirling blender (AM-7, Nihonseiki, Tokyo, Japan). The pH of the homogenized albumen was adjusted to 6.0 by adding HCl, and the albumen was stirred overnight at 4 °C to produce ovomucin precipitate (Guerin-Dubiard et al., 2005). The precipitated ovomucin was removed by centrifugation for 10 min at 17,000 *g* (Avanti HP-30I, Beckman Coulter, CA, USA). The supernatant was dialyzed against 50 mM Tris–HCl (pH 8.0) and 50 mM NaCl and then stored at −20 °C until use. This mucin-free albumen was loaded onto an anion-exchanging column (DEAE 650M, Tosoh, Tokyo, Japan) that had been equilibrated with 50 mM Tris–HCl (pH 8.0) and 50 mM NaCl. Protein elution was performed by gradually increasing the NaCl concentration to 500 mM. Ovotransferrin (78 kDa) was collected in an unbound fraction. Ovalbumin (102 kDa) and tenp (47 kDa) were jointly eluted out as the NaCl concentration increased. The eluate containing ovalbumin and tenp was dialyzed and then loaded onto a hydroxyapatite Biogel HTP column (BioRad, Hercules, CA, USA) that had been equilibrated with 10 mM potassium phosphate (pH 6.7) and 50 mM NaCl. Ovalbumin and tenp were separately eluted out as the phosphate concentration increased. The fractions that contained both ovalbumin and tenp were reloaded onto the hydroxyapatite Biogel HTP to clearly fractionate the two proteins. Isolated 102-kDa and 47-kDa proteins were analyzed by an amino acid sequencer and identified as ovalbumin and tenp, respectively (data not shown). Purified protein was dialyzed against PBS (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 140 mM NaCl, and 2.7 mM KCl, pH 6.8), and then concentrated using a membrane filter (10-kDa pore; Millipore, Billerica, MA, USA).

The crude mixture without ovotransferrin that was to be used for the calorimetric experiment was prepared by anion exchange chromatography of the mucin-free emu albumen as described above. Elution was performed isocratically with 0.5 M NaCl. The crude mixture without tenp was prepared by overloading mucin-free emu albumen into the DEAE 650M column and collecting the flow-through fraction.

The recombinant ovalbumin, the hexahistidine (6His)-tagged version at the carboxy terminus, was purified through Ni-NTA superflow resin (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The protein was eluted by increasing the imidazole concentration, collected, and subjected to further purification by hydroxyapatite Biogel HTP, as described above.

### 2.3. Calorimetric analysis

The purified protein in PBS was concentrated using a membrane filter to obtain a protein concentration of 5~10 mg/ml. Protein solution (60 μl) was transferred into an aluminum canister (AL-70 capsule, Epolead Service Inc., Chiba, Japan) that had been oxidized, and then sealed by an automatic sealer (Epolead Service, Inc.). The canister was set into a differential scanning calorimeter (DSC-6100, Seiko Instruments Inc., Chiba, Japan) along with a control canister that contained PBS. Calorimetric analysis was performed by raising the temperature from 30 °C to 150 °C at a rate of 1 °C/min.

### 2.4. Gel strength analysis

Two hundred microliters of the protein solution was transferred to a 2-ml plastic tube and boiled for 10 min. The tube was immediately cooled under running water. After cooling, the tube was fixed on a stand and then analyzed using a Tensipresser (TTP-50BXII-2006, Taketomo Electric, Tokyo, Japan), which was equipped with a cylindrical plunger whose diameter was 3 mm. The test was run in one-bite mode with a bite speed of 1 mm/sec. Experiments were performed in triplicates. Resulting mean values were statistically evaluated by Welch's *t*-test.

### 2.5. In vitro digestion and immunoblotting

The purified emu ovalbumin was digested with simulated gastric fluid (SGF) as previously reported (Astwood, Leach, & Fuchs, 1996). The SGF contained 0.02%(w/v) pepsin (from porcine gastric mucosa, Sigma-Aldrich, St Louis, MO, USA) and 30 mM of NaCl. The SGF was adjusted to a pH of 1.2 using HCl. The reaction with SGF was performed as follows: an 80-μl aliquot of SGF and 70 μl of 0.5 mg/ml protein solution were separately preheated at 37 °C. The SGF was then added to the protein solution and continuously incubated. At every time point, 40 μl of 160 mM $Na_2CO_3$ was added to the reaction mixture to quench the reaction. A 10-μl reaction mixture was mixed with 2× Laemmli SDS-PAGE buffer, boiled and subjected to SDS-PAGE. Hen ovalbumin (Nacalai Tesque, Kyoto, Japan) was used as a control. For the denaturing condition, the ovalbumin protein solution was boiled for 10 min prior to digestion in SGF.

The gels were electroblotted onto PVDF membranes (GE Healthcare UK Ltd., Little Chalfont, England). The membranes were then blocked by incubation in 5% non-fat dried milk in TBST (25 mM Tris–HCl (pH 7.5) and 15 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. A rabbit anti-hen ovalbumin polyclonal antibody (Millipore) was diluted to 1:8000 in TBST and used as a primary antibody. A secondary antibody (HRP conjugated goat anti-rabbit IgG (GE Healthcare)) was diluted to 1:10,000 in TBST. Detection was performed according to the manufacturer's instruction, and the illuminant was captured by a light capture II AE-6981 image scanner (ATTO Corp., Tokyo, Japan).

## 3. Results and discussion

### 3.1. Denaturing temperature of emu albumen proteins

Because emu egg behaves differently than hen egg during heat denaturation, it was hypothesized that the albumen proteins play important roles. First, the thermostability of crude albumen during a temperature shift was examined to confirm the previously reported results (Nakamura et al., 2011). As shown in Fig. 1A, three peaks were observed in the emu albumen. The exact peak temperatures are summarized in Table 1. Two obvious peaks were detected in a lower temperature range in the emu albumen and corresponded to the hen albumen. The peak observed above 90 °C was small but unique in the emu albumen. To identify which component was responsible for which peak, we then fractionated the emu albumen. The prepared fraction was analyzed by SDS-PAGE prior to the calorimetric analysis. The tenp-removed fraction is shown in Fig. 2A. This fraction did not show a band at 45 kDa, and apparently lost the peak above 90 °C (Fig. 1B) even though it had unidentified peaks in the lower temperature range. The ovotransferrin-removed fraction was smeared in an SDS-PAGE analysis (Fig. 2B) but showed only two obvious peaks at 74.2 °C and 91.9 °C (Fig. 1B). Lastly, we separately purified and then examined the three major components of emu albumen: ovotransferrin, ovalbumin, and tenp. The results of the SDS-PAGE analysis of the purified proteins are shown in Fig. 2C.