Novel basic proteins, duck basic protein small 1 (dBPS\textsubscript{1}) and 2 (dBPS\textsubscript{2}), were isolated from duck egg white by cation-exchange and gel filtration chromatography. Protein sequence analyses indicated that they possessed 39 amino acid residues with three disulfide bonds. The amino acid sequence of dBPS\textsubscript{1} showed 45% identity with dBPS\textsubscript{2}. The amino acid sequence of dBPS\textsubscript{2} was the same as cygnin, a small protein from black swan, and strongly homologous with meleagrin from turkey and chicken. Phylogenic relationships implied that dBPS\textsubscript{1} and dBPS\textsubscript{2} share a common ancestry with cygnin and meleagrin. Based on MALDI-TOF mass spectra, the molecular masses of dBPS\textsubscript{1} and dBPS\textsubscript{2} were 4,373, and the 4,486 Da. pI of dBPS\textsubscript{1} showed 101.2 and 98.3. Both dBPS\textsubscript{1} and dBPS\textsubscript{2} have been sequenced and are well characterized. The molecular masses of lysozyme and avidin are 14 and 68 kDa respectively. But little is known about basic proteins with molecular masses lower than 10 kDa in EW components. In 1983, a low molecular weight basic protein (MW 4,454), called cygnin, was found in black swan (Cygnus atratus) EW.\textsuperscript{11} After that, meleagrin (MW 4,552), another basic protein, was isolated during the preparation of ovomucoid from turkey (Meleagris gallopavo) EW.\textsuperscript{12} Both cygnin and meleagrin showed strong homology in their amino acid sequences and tightly folded structures with three disulfide bonds. In chicken EW, the meleagrin homologous proteins, having 63 amino acid residues, called similar to meleagrin (accession nos. XP429907 and XP429908 in GenBank), were derived from an annotated genomic sequence in 2006. The presence of these small basic proteins in a related avian species suggests the importance of these small basic proteins.

Investigation of the amino acid sequences of small basic proteins in many species might provide valuable information in the relation of structures and functions for those proteins, as in the case of lysozyme,\textsuperscript{13–15} cystatin\textsuperscript{16} and ovostatin.\textsuperscript{17} It has been found that duck EW lysozyme shows a difference in amino acid sequence from chicken lysozyme. Duck lysozyme contains three isoforms with higher enzymatic activity than that of chicken lysozyme, which has only one isoform.\textsuperscript{13–15} Cystatin from duck EW presents glycosylated and phosphorylated properties, while chicken cystatin is lacking in both properties.\textsuperscript{16} Differences
between duck and chicken ovostatin have also been mentioned, as duck ovostatin possesses the thio-ester group and serine proteinase inhibitory activity while chicken ovostatin does not. These results led us to investigate the small basic proteins in duck EW.

During lysozyme purification from duck EW, unknown basic proteins were discovered. In this study, we isolated two novel basic small proteins in duck EW, and investigated their structures, physicochemical properties, phylogenetic relations, and the distribution of these proteins in duck tissues.

Materials and Methods

Materials. Duck eggs were obtained from the Agriculture, Food, and Environmental Sciences Research Center of Osaka Prefecture (Osaka, Japan). Duck internal organ tissues were supplied by the Kagawa Prefecture Livestock Experiment Station (Kagawa, Japan). Sephacryl™ S-300 HR and SP-Sepharose™ Fast Flow, used in protein purification and tissue distribution experiments, were obtained from Amersham Biosciences (Uppsala, Sweden). Pyroglobutamate aminopeptidase (EC3.4.19.3) from Pyrococcus furiosus and chymotrypsin (type I, EC3.4.21.1) from bovine pancreas were from Sigma Chemical (St. Louis, MO). V8 Protease (EC3.4.21.19) from Staphylococcus aureus and lysyl endopeptidase (EC3.4.21.50) from Achomobacter lyticus were from Wako Pure Chemical Industries (Tokyo). Iodoacetamide (IAM) and dithiothreitol (DTT) were from Nacalai Tesque. (Kyoto, Japan). An immobilized linear pH gradient polyacrylamide gel strip with a pI range of 3–10 and calibration kit for broad range pI were from Amersham Biosciences (Uppsala, Sweden). Pyroglobutamate aminopeptidase (EC3.4.19.3) from Pyrococcus furiosus and chymotrypsin, while the dBPS counterpart was hydrolyzed with pyroglutamate aminopeptidase, lysyl endopeptidase, V8 protease, and chymotrypsin. Hydrolyzation was done under the following conditions: Pyroglobutamate aminopeptidase in 50 mM sodium phosphate buffer, pH 7.0, with 10 mM DTT and 1 mM EDTA, substrate/enzyme ratio = 1 nmol/5 mU; lysyl endopeptidase in 10 mM Tris–HCl buffer, pH 9.0, substrate/enzyme = 20:1 in weight ratio; V8 protease in 50 mM ammonium hydrogen carbonate buffer, pH 8.0, substrate/enzyme = 50:1 in molar ratio; chymotrypsin in 10 mM Tris–HCl buffer, pH 8.0, substrate/enzyme = 50:1 in molar ratio. All the proteolyses were conducted at 30°C for 16 h except for pyroglobutamate aminopeptidase hydrolyzation which was conducted at 4°C overnight. The sample was applied to a column of Sephacryl S-300 (150 ml) equilibrated with PB. The purity of the purified proteins was determined by SDS–PAGE with a 15% gel.

Polyacrylamide gel electrophoresis. The samples were electrophoresed on a 15% polyacrylamide gel in the presence of SDS and mercaptoethanol, by the method of Laemmli. The gel was stained with Comassie Brilliant Blue R-250. The molecular weight of the samples was determined by comparing electrophoretic mobility with molecular weight standard proteins purchased from New England BioLabs (Boston, MA).

Identification by mass spectrometry. Mass spectra of the samples were determined with a Bruker Autoflex matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bremen, Germany) equipped with a nitrogen laser and a delayed extraction ion source. A sample solution (1 μl) at a final concentration of 2–3 pmol/μl was thoroughly mixed with a saturated matrix solution, α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) (1:4, v/v) before crystallization on polished steel TF. Mass spectra were collected in the reflex and positive ion mode. MALDI-TOF mass spectra were acquired by an average of 300 laser shots.

Reduction of disulfide bonds and carboxymethylation. Samples of 150 μg were incubated in 100 μl of 5 mM EDTA, 5 mM dithiothreitol (DTT), 8 mM urea and 50 mM Tris–HCl buffer, pH 8.0, and incubated at 30°C for 4 h under a nitrogen atmosphere. The reduced samples were added with an equal volume of 50 mM Tris–HCl buffer, pH 8.0, containing 15 mM iodoacetamide (IAM) and 8 mM urea, and then incubated at 30°C for 4 h in the dark. The excess IAM was removed by dialyzation against Milli-Q water for 1 d. The sample was lyophilized and stored at –20°C until use.

Determination of amino acid sequences. Carboxymethylated dBPS1 was hydrolyzed with pyroglobutamate aminopeptidase, lysyl endopeptidase, V8 protease, and chymotrypsin. The samples were electrophoresed on a 15% polyacrylamide gel in the presence of SDS and mercaptoethanol, by the method of Laemmli. The gel was stained with Comassie Brilliant Blue R-250. The molecular weight of the samples was determined by comparing electrophoretic mobility with molecular weight standard proteins purchased from New England BioLabs (Boston, MA).
conducted at 50 °C for 10 h. Each reaction was ended by the addition of 1% TFA solution, giving a final pH of 3.0. The resulting peptides were separated by RP-HPLC. The peptides of dBPS\textsubscript{1} and dBPS\textsubscript{2} were determined as to their N-terminus amino acid sequences by Edman degradation using an Applied Biosystem 491 Procise Protein Sequencer (Foster City, CA). The peptide sequences obtained were compared by BLAST and UniProt search of the proteins at the National Center for Biotechnology Information (NCBI) of United States and the European Bioinformatics Institute (EBI) public database respectively. Sequence similarity between proteins was determined by WU-Blast.\textsuperscript{19}

**Phylogenetic determination.** The amino acid sequence of cygnin from black swan (access no. P02785), meleagrin from turkey (P21376), “similar to meleagrin” from chicken (XP 429908 and XP 429907), ovalbumin from chicken (NP 990483), ovotransferrin from duck (P56410) and chicken (NP 990635), and lysozyme from duck (8082160A) and chicken (NP 990612) EW was obtained from GenBank. Sequence alignment of studied proteins was done with the Clustal W multiple sequence alignment program\textsuperscript{20} at the DNA Data Bank of Japan (DDBJ) website (http://www.ddbj.nig.ac.jp/). A molecular phylogenetic tree was constructed from p-distance estimation by the neighbor-joining method\textsuperscript{21} with 1,000 bootstrapping analyses, using the PHYLIP 3.582 software package.\textsuperscript{22} A phylogenetic tree was drawn using Tree View program version 1.6.6 (Division of Environmental and Evolution Biology, Institute of Biomedical and Life Science, University of Glasgow).

**Fourier transform infrared spectroscopy (FT-IR).** Samples at 5% (w/v) were placed between two CaF\textsubscript{2} cuvettes before applying them to a Jasco FT-IR 670 plus spectrometer (Tokyo). FT-IR spectra were recorded at 4 cm\textsuperscript{-1} resolution from 400 to 4,000 cm\textsuperscript{-1} after 32 scans. Spectra of Milli-Q water were collected under the same conditions. The transmittance of Milli-Q water was subtracted from the spectra of the sample solution to obtain the FT-IR spectra of the protein. The PCR program from Jasco (Tokyo) was used to translate the spectra.

**Isoelectric focusing.** Samples were dissolved with 100 μl of rehydration buffer (7 M urea, 2 M thiourea, 1 M DTT, 0.001% bromophenol blue (BBP), 3% Triton X-100, 0.1% IPG buffer, and 0.1% TBP). Then the solution was applied to an 18-cm immobilized linear pH gradient polyacrylamide gel strip with a pI range of 3–10. Isoelectric focusing was carried out on Amersham Biosciences Ettan IPGphor 3 IEF system (Amersham) at 20 °C under the following conditions: dehydration for 12 h, focusing at 500 V for 1 h, 1,000 V for 1 h, 3,500 V for 4 h, 5,500 V for 4 h, and 8,000 V for 1 h. Protein bands were stained with Coomassie Brilliant Blue R-250 after fixation with 20% trichloroacetic acid (TCA) for 45 min. Decolorization was done using 5% methanol and 7% acetic acid. A calibration curve was constructed using relative electrophoretic mobility (Rf) from cathode to anode of the Calibration Kit for broad range pI (3–10) determination.

**Denaturation temperature.** Differential scanning calorimetry was performed in Setaram Micro DSC VIII-Commissioning/Utilizations (Caluire, France). Samples of 0.5 mg/ml in Milli-Q water (0.5 ml) were heated in the calorimeter at a scan rate of 0.5 °C/min over a range of 20–120 °C. Milli-Q water was used as a reference. The calorimetric data was analyzed by SETSOFT 2000, thermal analysis software from Setaram.

**Distribution of dBPS\textsubscript{1}/dBPS\textsubscript{2}-related protein in duck internal tissues.** After washing with saline water, duck internal organs tissues, oviduct, gallbladder, kidney, ovary, intestine, heart, spleen, gizzard, pancreas, bone, leg muscle, serum, breast muscle, liver, and skin, were homogenized with 3 times volumes of saline water. Following centrifugation at 5,000 × g, at 4 °C for 20 min, 5 ml of supernatant was applied to the column of SP-Sepharose (5 ml) and eluted stepwise with PB (10 ml) and PB containing 0.5 M NaCl (10 ml). Protein solutions from the fractions that were eluted with PB containing 0.5 M NaCl were collected and determined by indirect ELISA.

Each well of a 96-well flat-bottom polystyrene microplate was coated with 100 μl per well of tissue extracted protein solution in 0.1 M carbonate buffer, pH 9.6, and its serial dilution in 8 steps (about 3\textsuperscript{-7}). After overnight incubation at 4 °C, each well of the plate was washed 3 times with 200 μl phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T). The unbound site was blocked by incubation with 200 μl per well of blocking reagent (0.2%, w/v, BSA in PBS-T) at 37 °C for 2 h. After incubation, the plate was washed 3 times with PBS-T solution, as mentioned above. Then 100 μl of rabbit anti-dBPS\textsubscript{1} polyclonal antibody, diluted 1:10,000 (v/v) with blocking reagent, was added to each well and this was incubated at 37 °C for 1 h. After washing 3 times with PBS-T, 100 μl of goat anti-rabbit IgG peroxidase-conjugate, diluted 1:10,000 (v/v) with blocking reagent, was added to each well and this was incubated at 37 °C for 1 h. After washing 3 times with PBS-T, the calorimetric data was analyzed by SETSOFT 2000, thermal analysis software from Setaram.

**Results**

**Purification of small duck basic proteins**

Proteins in ovomucin-free solution, supernatant of duck EW solution adjusted to pH 6.0, were separated by a cation exchange chromatography using an SP-Sephar-
ose column. The elution pattern of the proteins is shown in Fig. 1A. Ovalbumin and ovotransferrin were eluted in regions (a) and (b) respectively. Proposed basic proteins, possessing net positive charges at pH 7.5, were eluted in region (c), after applying a gradient of 0–0.5 M NaCl in PB. The major EW basic protein lysozyme was eluted in region (d), after the addition of 0.5 M NaCl in PB. Fractions of two major peaks (fraction numbers 97–102 and 109–114) eluted in region (c) mainly contained small-sized basic proteins with molecular masses of less than 9.4 kDa, corresponding to the SDS–PAGE pattern (Fig. 1B). Hereafter, a small sized protein eluted in the first peak is designated ‘duck basic protein small 1 (dBPS1),’ while a similar molecular weight protein in the second peak is designated ‘duck basic protein small 2 (dBPS2).’ To get rid of other proteins, fractions containing dBPS1 and dBPS2 were applied to gel filtration chromatography with a column of Sephacryl/S-300 (Fig. 2A and B). This resulted in generating homogeneous proteins, as found in the SDS–PAGE pattern. Recovery from total duck EW proteins was 0.1% (w/w) for dBPS1 and 0.16% (w/w) for dBPS2.

**Molecular weight and isoelectric point**

dBPS1 and dBPS2 molecular masses determined by MALDI-TOF Mass spectrometer are shown in Fig. 3. The molecular mass of dBPS1 was 4,374.9 Da, and the dBPS2, 4,486.9 Da. The isoelectric focusing results showed that dBPS1 and dBPS2 were basic proteins with different pI values, 9.35 and 9.44 respectively.

**Amino acid sequences and disulfide bonds**

When the protein sequence of dBPS1 and dBPS2 were determined by automated Edman degradation, no PTH amino acid was eluted, but treatment of both dBPS1 and dBPS2 with pyroglutamate aminopeptidase before application to the protein sequencer enabled analysis of the amino acid sequence. Sequence of KKGFCAG and VRKYCPKVGYC were found for treated dBPS1 and dBPS2 respectively. This implies that the first N-terminus amino acid in both proteins is glutamine, which spontaneously cyclizes to become pyroglutamate. Internal sequences were determined with peptides derived from the proteins. The sequence of dBPS1 was aligned according to the sequence of five peptides of PG (hydrolyzed with pyroglutamate aminopeptidase), CT-3 and CT-11 (hydrolyzed with chymotrypsin), LE-3 (hydrolyzed with lysyl endopeptidase), and V8-4 (hydrolyzed with V8 protease). In the case of dBPS2, the sequence was deduced from the sequence of four peptides of PG, CT-10, and LE-3, and LE-4 (hydrolyzed with lysyl endopeptidase). (Fig. 4A) The results indicate that both proteins contained 39 amino acid residues,
with some differences in their sequences. These proteins possessed high contents of cysteine (six residues), lysine (six residues), and glycine (six residues). dBPS$_2$ was also rich in serine (six residues).

Six sulfhydryl groups in dBPS$_1$ and dBPS$_2$, according to the number of cysteine in amino acid sequences, were consistent with the results calculated from MALDI-TOF Mass (data not shown). It was found that molecular masses of dBPS$_1$ and dBPS$_2$ increased by 343 Da after carboxymethylation with iodoacetamide, corresponding to a modification of six sulfhydryl groups in proteins molecules. All of these sulfhydryl groups are formed three disulfide bonds in native dBPS$_1$ and dBPS$_2$, since native dBPS$_1$ and dBPS$_2$ did not react with the DTNB solution$^{23}$ (data not shown), indicate that there is no free sulfhydryl group in either protein.

Fig. 2. Gel Filtration Chromatogram and SDS–PAGE Pattern of dBPS$_1$ (A) and dBPS$_2$ (B) Eluted from Sephacryl$^\text{TM}$ S-300 Column with Phosphate Buffer (ionic strength, 0.05 mol/l), pH 7.5.

Fig. 3. MALDI-TOF Mass Spectra of dBPS$_1$ (above) and dBPS$_2$ (below). HCCA was used as matrix, performed in reflex and positive modes.
Results of chymotrypsin and lysyl endopeptidase digestion of insufficient carboxymethylated protein derived peptides, CT-3 from dBPS1 and LE-3 and LE-4 from dBPS2, that consisted of subpeptides, as more than one amino acid residues were detected in each cycle, in sequencing analysis of these peptides by the Edman degradation method. The results showed that CT-3 contained two subpeptides of Cys6-Tyr9 and Cys31-Lys38. For the peptide from dBPS2, LE-3 possessed three subpeptides of Tyr5-Lys8, Cys16-Lys18, and Phe30-Lys39, while LE-4 possessed two subpeptides of Val9-Lys15 and Ala19-Lys29. The linkage between the subpeptides was attributable to a disulfide bond, as each subpeptide contains cysteine. The results of the subpeptide sequences suggest a connection between the subpeptides and indicate two possibilities in the position of two disulfide bonds in the dBPS1 and dBPS2 structure, 1stCys5-Cys and 3rdCys6-Cys or 1stCys5-Cys and 3rdCys6-Cys. In both patterns of disulfide formation, however, there was no difference in topological structure. Since there is no free sulfhydryl group in dBPS1 and dBPS2, the linkage between 2ndCys and 4thCys is identified as another disulfide bond in these proteins. Hence the disulfide bonds of both proteins were assumed to be as shown in Fig. 4B.

Phylogeny of small duck basic proteins

The amino acid sequence of dBPS1 did not match any proteins in the protein databases. It showed 45% identity with dBPS2, as determined. The amino acid sequence of dBPS2 was totally homologous with cygin and strongly homologous with meleagrin from turkey EW, and with “similar to meleagrin” from chicken. Multisequence alignment of dBPS1, dBPS2, with previously reported small basic proteins, cygin, meleagrin, and part of “similar to meleagrin” (Val24-Lys63), showed that there were 14 identical, seven strongly similar, and three weak similar residues among these proteins (Fig. 4B). A phylogenetic tree of these proteins and other EW proteins, ovalbumin, ovotransferrin, lysozyme, and Basic Small Proteins from Various Avians. The neighbor-joining method with p-distance estimation was used. Bootstrap values (based on 1,000 replications) are shown on interior branches. The scale bar indicates a distance of 0.1.

Fig. 4. Primary Structures of dBPS1 and dBPS2.

A, the complete amino acid sequences of dBPS1 and dBPS2. Numbers denote the positions of amino acid residues in its structure. PG, peptide derived by pyroglutamate aminopeptidase; LE, peptide derived from lysyl endopeptidase; V8, peptide derived from protease V8; CT, peptide derived from chymotrypsin. The amino acid sequence data reported are available in the EMBL databases under accession nos. P85123 and P85124. B, Sequence alignment of dBPS1, dBPS2, and small basic proteins from other species. The alignment was determined by CLUSTAL W. Identical residues (*), strong similar residues (:), and weakly similar residues (.). Sulfhydryls are shown in blocks. Disulfide bonds are displayed as (–S–S–).
meleagrin” or dBPS1. Moreover, this group of small basic proteins did not share a common ancestor with ovalbumin, ovotransferrin, or lysozyme.

**Secondary structure**

The secondary structure contents of dBPS1 and dBPS2, measured with a FT-IR spectrometer, are shown in Table 1. Both proteins were β-sheet rich proteins, as 60% of dBPS1 and 62% of dBPS2 were β-sheet and β-turn. Moreover, these proteins possessed comparable secondary structures, with 2% differences in the α-helix and β-sheet contents.

**Denaturation temperature of small duck basic proteins**

A DSC thermogram of dBPS1 and dBPS2 is shown in Fig. 6. The denaturation temperature (Td) and the enthalpy of denaturation (ΔH) values were 101.2°C and 22.43 J/g for dBPS1, and 98.3°C and 20.42 J/g for dBPS2.

**The distribution of dBPS1/dBPS2-related proteins in duck tissues**

The immunological cross-reactivity of dBPS1 with dBPS2 polyclonal antibody was assessed by indirect ELISA (Fig. 7). The polyclonal antibody showed a strong reaction with dBPS2. Consequently, dBPS2 polyclonal antibody was used to investigate the distribution of dBPS1 and of dBPS2-related (dBPS1/dBPS2-related) proteins in duck internal tissues.

Basic proteins were extracted from duck internal tissues. Cross-reaction of dBPS2 polyclonal antibody was observed only for extracts from the oviduct and gallbladder (Fig. 7), while no immunoreactivity of dBPS2 antibody with other tissue extracts was observed. This implies that dBPS1/dBPS2-related proteins were distributed in the oviduct and gallbladder.

**Discussion**

Among avian EW proteins, the basic proteins include only lysozyme and avidin. In the current study, two novel basic proteins with small molecular weights in

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**Table 1. Secondary Structures of dBPS1 and dBPS2**

<table>
<thead>
<tr>
<th>Protein</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>β-turn</th>
<th>Random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>dBPS1</td>
<td>14</td>
<td>36</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>dBPS2</td>
<td>12</td>
<td>38</td>
<td>24</td>
<td>26</td>
</tr>
</tbody>
</table>

Determined by FT-IR. Proteins were determined at a concentration of 5% (w/v). Spectra were translated by PCR.

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**Fig. 6. DSC Thermogram of dBPS1 (above) and dBPS2 (below) Solutions.**

The experiment was performed over a range 20–120°C, using Milli Q water as a reference.

**Fig. 7. Distribution of dBPS1/dBPS2-Related Proteins in Duck Tissues.**

The investigation was done by indirect ELISA at an initial protein concentration of 10 μg/ml, and then aliquots were serially diluted through eight steps (about 3^−1) using rabbit anti-dBPS2 as first antibody. Mean value of three replications. ○, dBPS1; ■, dBPS2; ●, oviduct; ●, gallbladder; ×, kidney; ★, ovary; ○, intestine; □, heart; Δ, spleen; ○, gizzard; ○, pancreas; ※, bone; +, leg muscle; †, serum; $$$, breast muscle; Δ, liver; @, skin.
duck EW were isolated by ion-exchange and gel filtration chromatography. Their characteristics as basic and small proteins roused our curiosity about duck EW proteins. They were named dBPS$_1$ and dBPS$_2$, according to their physical characteristics and order of elution by ion-exchange chromatography. The elution order accounts for the different pI's of dBPS$_1$ (pI = 9.35) and dBPS$_2$ (pI = 9.44). The dissimilarity in basicity of dBPS$_1$ and dBPS$_2$ may be due to the difference in the number of positively charged amino acids in dBPS$_1$ (six lysine) and dBPS$_2$ (six lysine and one arginine). The amounts of dBPS$_1$ (0.1%) and dBPS$_2$ (0.16%) from total EW proteins that were obtained from purification suggest that these proteins were minor proteins of EW, since the major proteins are recognized to be ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovoglobulin G2 and G3 (8%), lysozyme (3.4%), and ovomucin (1.5%).$^{1,2}$ Even though the amounts of both proteins in EW were sufficient to be detected, there is no report about the presence of dBPS$_1$ and dBPS$_2$.$^{8-10}$ This may be due to the extremely low molecular masses of dBPS$_1$ and dBPS$_2$.

This group of small basic proteins, consisting of dBPS$_1$, dBPS$_2$, cygnin, meleagrin, and “similar to meleagrin,” showed close phylogenetic relationships to each other. The phylogenetic tree implied that these proteins evolved from a common ancestor. Besides dBPS$_1$, which was the most distantly related protein within this group, the definite homology of cygnin and dBPS$_2$ was clearly explained by the common family of Anatidae, order Anseriformes that swan and duck belong to.$^{24}$ Meleagrin and “similar to meleagrin” also showed a close relationship to each other. This result corresponds to the close evolutionary relationship within Galliformes according to the absent of Galx1-4Gal in all tested glycoproteins of Galliformes EW.$^{25}$ In addition, the relationship of meleagrin with “similar to meleagrin,” from the same order (Galliformes), is closer than with cygnin or dBPS$_2$, from the different order (Anseriformes). A close relationship within Galliformes order than with other from Anseriformes order was also suggested in ovotransferrin.$^{26}$ Mature turkey ovotransferrin showed higher sequence identity to chicken ovotransferrin (89%), than to duck ovotransferrin (78%). In this study, dBPS$_2$ and cygnin from the Anseriformes order showed closer relationships to meleagrin from turkey (family Meleagrididae, order Galliformes) than to that from the same order but a different family, such as “similar to meleagrin,” from chicken (family Phasianidae, order Galliformes). Sun$^{27}$ has reported the differences in numbers of the SH group from the ovalbumin of hen, turkey, guinea fowl, Peking duck, and Mallard duck. She mentioned that even though there was only one disulfide bond in all the studied ovalbumins, there were four cysteinyl residues (Cys11, Cys30, Cys367, and Cys382) for hen, three cysteinyl residues (Cys11, Cys367, and Cys382) for turkey and guinea fowl, and two cysteinyl residues (Cys11 and Cys331) for Peking duck, Mallard duck, and Edmen goose ovalbumin. Therefore, the closer phylogenetic relationship of dBPS$_2$ and cygnin to meleagrin than to “similar to meleagrin” might be due to evolution within the Galliformes and Anseriformes. dBPS$_1$, the most distantly related protein in the small basic protein group, can also be found in other species of avian. The relationship of meleagrin and cygnin to ovotransferrin was mentioned in a previous report.$^{12}$ In contrast, the results of the phylogenetic tree indicate that a group of small basic protein did not evolve from the same common ancestor as ovotransferrin like lysozyme, one of the basic proteins, and ovalbumin, the most abundant protein, in EW.

The secondary structure of dBPS$_1$ and dBPS$_2$ was similar in the ratio of structure contents. The alpha-helix content, of 12–14%, implies that only one alpha-helix turn is theoretically formed in dBPS$_1$ and dBPS$_2$, since one helical turn requires 3.4 amino acids to participate in formation. However, the alpha-helix naturally prefers to form long chains more than small chains. This implies that there is perhaps no alpha-helix structure in dBPS$_1$ and dBPS$_2$. Consequently, both proteins are classified as beta-proteins.

Heating is a popular process in the food industry. During this process, denaturation of proteins occurs. Denaturation is usually followed by changes in physical or functional properties, e.g., solubility. Denaturation of globular proteins can lead to a great variety of properties, such as gelling, clotting, emulsifying, and the water binding property, each of which can affect food products.$^{26}$ EW protein begins to denature when heated to 60°C, according to CD spectra of native and denatured EW protein.$^{20}$ In spite of that, individual proteins of chicken EW have different denaturation temperatures: ovotransferrin (61°C), lysozyme (75°C), ovalbumin (84°C), ovomucoid (79°C), G2 globulin (92.5°C), and avidin (85°C).$^{2,30}$ The DSC thermogram of EW at pH 7.0 showed only three main peaks, corresponding to ovotransferrin, lysozyme, and ovalbumin.$^{31}$ Even though there have been many reports on the denaturation of EW proteins, no other protein was assigned as high a heat stability as dBPS$_1$ (Td = 101.2°C, DH = 22.43 J/g) and dBPS$_2$ (Td = 98.3°C, DH = 20.42 J/g). The high thermostability of dBPS$_1$ and dBPS$_2$ may be due to their structures which contain three disulfide bonds. High disulfide bonds containing compact-shaped protein requires fairly high thermal energy to disrupt the sulfhydryl linkages and unfold the molecule.$^{28}$ Moreover, the high content of proline (three residues) in both proteins might be attributable to heat stability, as it was mentioned to stabilize protein conformation in previous studies.$^{12,33}$ A report of Zscherp$^{33}$ revealed a pronounced loss of thermal stability by tendamist, a small beta-sheet protein containing three prolines after it was mutated into proline-free tendamist. According to those results, dBPS$_1$ and dBPS$_2$ are the most heat stable proteins in EW, to our knowledge.
The clear result of a high immunological reaction between dBPS_1 and rabbit anti-dBPS_2 antibody confirmed a high homology in primary and secondary structures of dBPS_1 and dBPS_2. Using anti-dBPS_2 antibody, the presence of dBPS_1/dBPS_2-related protein was detected. According to these results, dBPS_1/dBPS_2-related protein was expressed in the oviduct and gallbladder of duck. There was no surprise in the presence of dBPS_1/dBPS_2-related protein in the oviduct, where most EW proteins are synthesized. The gallbladder is a place where bile salt, produced in the liver, is stored before it is transported to the duodenum. The availability of these proteins in the gallbladder but not in the liver, according to the result in this experiment, implies that they are not connected with bile salt and may possess other specific functions in the gallbladder. Further studies on how dBPS_1 and dBPS_2 function in the gallbladder are required.

Even though the function of dBPS_1 and dBPS_2 was not identified in this study, the presence of these proteins, including cygnin, meleagrin, and "similar to meleagrin" in various avians, duck, chicken, turkey, and swan, suggests the importance of these proteins in avian species. Moreover, the discovery of these unique small basic proteins in EW and their physicochemical properties might provide valuable information for egg utilization in the food industry and in the pharmacology.

References

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