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Multiple Biological Functions of Novel Basic Proteins Isolated from Duck Egg White: Duck Basic Protein Small 1 ($dBPS_1$) and 2 ($dBPS_2$)

Supaporn Naknukool, Shigeru Hayakawa,* and Masahiro Ogawa

Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Ikenobe, Miki, Kagawa 761-0795, Japan

ABSTRACT: Biological functions of duck basic protein small 1 ($dBPS_1$) and 2 ($dBPS_2$) were investigated by in vitro experiments. Results of agarose gel retardation assay indicated that $dBPS_1$ and $dBPS_2$ associate with RNA. Addition of NaCl or urea induced partial dissociation of $dBPS_1$/$dBPS_2$-RNA complex, implying that electrostatic interaction, hydrophobic interaction, and hydrogen bonds are involved in the association of $dBPS_1$/$dBPS_2$ to RNA. $dBPS_1$ and $dBPS_2$ inhibited pancreatic lipase activity with the fifty percent inhibitory concentration (IC_{50}) of 250 and 100 μg/mL, respectively. Peptic hydrolysates of $dBPS_1$ and those of $dBPS_2$ showed a potent angiotensin I-converting enzyme (ACE) inhibition with an IC_{50} of 22.5 and 49.6 mg/L. The most potent ACE-inhibitory peptide was a nanopeptide (EKKGFCAGY) from $dBPS_1$ and an octapeptide (KYCPKVGY) from $dBPS_2$. These multiple biological functions of $dBPS_1$ and $dBPS_2$ may contribute to reducing the risk of lifestyle diseases.

KEYWORDS: egg white protein, basic protein, RNA-binding protein, lipase inhibition, angiotensin I-converting enzyme inhibitor

INTRODUCTION

Egg white (EW) is an excellent source of protein. Many EW proteins have been investigated for their structures and functionalities since 1920s. Well-characterized EW proteins include ovalbumin, ovotransferrin, ovomucoid, ovomucin, lysozyme, ovoflavoprotein, avidin, cystatin, ovoinhibitor, and ovomacroglobulin. EW protein possesses various functional properties such as gelling, foaming, emulsifying, crystallization control, proteolytic enzyme inhibition, and antimicrobial effect, so that it is used as an essential ingredient in food.[1,2] Recently, several new biological functions have been discovered for some EW proteins and EW protein derived peptides: antiadhesive and antimicrobial properties and immunomodulatory, anticancer, antihypertensive, and biospecific ligand-binding activities.[3,4] Thus, EW is now recognized as a source of biologically active substances, with significant therapeutic potential.

Despite extensive research to identify and characterize all the proteins in EW, especially from chicken egg, the inventory of EW proteins is far from being complete. Duck egg, besides chicken egg, is commonly used in the Chinese and Southeast Asian kitchens. Several studies suggested that some of proteins of duck EW possess diverse structure and function from the counterparts of chicken EW.[5−7] Duck ovalbumin possesses a larger number of disulfide bonds than chicken counterpart does, leading to clear differences in the translucency and viscoelasticity of heat-induced EW gel between duck and chicken.[5] Duck ovostatin is capable of inhibiting serine proteinase as well as metalloproteinase, while chicken ovostatin inhibits only metalloproteinase.[6] We also reported that duck lysozyme has higher antimicrobial activity than chicken lysozyme.[7] Thus, more detailed and extended investigations of duck EW proteins may provide crucial annotations for duck egg.

Recently, we isolated two novel basic proteins from duck EW and named them duck basic protein small 1 ($dBPS_1$) and 2 ($dBPS_2$).[8] $dBPS_1$ and $dBPS_2$ both consist of 39 amino acid residues with a molecular mass of 4.4 kDa. The proteins have relatively high lysine, cysteine, and glycine contents. $dBPS_2$ is also rich in serine. The isoelectric points of $dBPS_1$ and $dBPS_2$ are 9.31 and 9.35. The amino acid sequences of the two proteins show 45% homology and are similar to the sequences of low molecular weight EW proteins from other avian species: cygnin from black swan, meleagrin from turkey, and similar to meleagrin from chicken. $dBPS_1$ and $dBPS_2$ are localized to the oviduct and gallbladder of duck as well as to EW. Despite these structural features, the biological functions of $dBPS_1$ and $dBPS_2$ remain unknown.

Several basic proteins with a high number of positive charges, such as protamine, avidin, and lysozyme, were reported to possess nucleic acid binding activity and lipase inhibition activity.[9−11] Thus, the object of this study was to investigate whether $dBPS_1$ and $dBPS_2$ have such biological functions. We also prospectively evaluated angiotensin I-converting enzyme (ACE) inhibitory activity of peptide derived from peptic hydrolysis of $dBPS_1$ and $dBPS_2$ as several ACE inhibitory peptides have been identified from hydrolysate of chicken egg white.[12]

MATERIALS AND METHODS

Chemicals and Materials. Duck eggs were obtained from the Agriculture, Food, and Environmental Sciences Research Center of Osaka Prefecture (Osaka, Japan). Sephacryl™ S-300 HR and SP-Sepharose™ Fast Flow were obtained from Amersham Biosciences Inc. (Uppsala, Sweden). TSKgel ODS-80 (4.6 × 250 mm) column was from TOSOH Co., Ltd. (Tokyo, Japan). Shodex SB402.5−4E (4.6 × 250 mm) was from Showa Denko K. K. (Tokyo, Japan). Baker's yeast RNA, protamine sulfate salt from salmon, pepsin A from porcine gastric mucosa, ACE from rabbit lung, hippuryl-L-histidyl-L-leucine (HHL), triolein, lipase from porcine pancreas, colipase from porcine pancreas, and *N*-tris (hydroxymethyl)-2-aminoethanesulfonic acid (TES) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Phosphatidylcholine from egg yolk, taurocholic acid, and bathocuproine were obtained form

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Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Iodoacetamide (IAM), dithiothreitol (DTT), and 3-*tert*-buty-4-hydroxylanisol were supplied by Nacalai Tesque Inc. (Kyoto, Japan). Unless specified otherwise, all other chemicals used were guaranteed reagent grade.

Protein Purification. $dBPS_1$ and $dBPS_2$ were purified by the method of Naknukool et al.[8] All the purification procedures were conducted at 4 °C. Duck EW (120 mL) was mixed with 3-fold volumes of Milli-Q water. Then, the sample was adjusted to pH 6.0 with 1 N HCl. The resultant precipitate was removed by centrifugation at 8800*g* for 20 min. The supernatant was applied to a column of SP-sepharose (100 mL), equilibrated with 10 mM sodium acetate buffer, pH 6.0, beforehand, and then washed out with 150 mL of 10 mM sodium acetate buffer, pH 6.0. After addition of 150 mL of sodium phosphate buffer (ionic strength, 0.05 mol/L, pH 7.5) (PB) in the column, $dBPS_1$ and $dBPS_2$ were eluted by a 300 mL linear gradient of 0−0.5 M NaCl in PB and 150 mL of 0.5 M NaCl in PB. Fractions containing $dBPS_1$ and $dBPS_2$, confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[12] were collected and dialyzed against PB using Spectra/Por 3,500 MWCO molecular porous membrane tubing (Spectrum Laboratories, INC., Ranch Dominguez, CA), overnight. Then, the sample was applied to a column of Sephacryl S-300 (150 mL) equilibrated with PB. $dBPS_1$ and $dBPS_2$ were eluted from the column with PB. The purity of the proteins was determined by SDS-PAGE.[12] Finally, purified $dBPS_1$ and $dBPS_2$ were dialyzed against Milli-Q water using the same membrane tubing, overnight, lyophilized, and stored at −20 °C, until used. Protein concentration of the purified was determined by Lowry method.[13]

Determination of RNA-Binding Property. RNA-binding capacity of $dBPS_1$ and $dBPS_2$ was determined by a gel retardation assay. Baker's yeast RNA was treated with various concentrations of $dBPS_1$ or $dBPS_2$ in 10 mM Tris-HCl buffer, pH 7.6 (TB). RNA solution (25 μL, 3 mg/mL) was mixed with an equal volume of protein solution (3, 6, 12, 18, 24, and 30 mg/mL). These mixtures were incubated at 4 °C for 1 h and then centrifuged at 8000*g* for 5 min. The supernatant (10 μL) was subjected to agarose gel electrophoresis on 1% agarose gel containing 1% ethidium bromide. RNA was visualized at 302 nm using an UV transilluminator.

Effect of NaCl and Urea on dBPS-RNA Complexes. dBPS-RNA mixtures were prepared in TB containing different concentrations of NaCl and urea. dBPS 75 μg and RNA 7.5 μg were mixed in 50 μL of TB containing NaCl (0.1, 0.3, or 0.5 M) or urea (2, 4, or 6 M). After being incubated at 4 °C for 1 h, the mixture was centrifuged at 8000*g* for 5 min. The resultant supernatant was subjected to the agarose gel electrophoresis as described above.

Inhibition of Pancreatic Lipase Activity. Lipase activity was determined by measuring the rate of release of oleic acid from triolein according to the method of Tsujita et al.[11] The substrate solution was prepared by mixing 90 μmol triolein, 12.6 μmol phosphatidylcholine, and 9.45 μmol taucholic acid in 9 mL of 0.1 M TES buffer, pH 7.0, containing 0.1 M NaCl (TES-S). The substrate solution was then sonicated for 5 min. Thereafter, 100 μL of the sonicated substrate solution was taken and added into mixed solution containing 25 μL of 6 U/mL pancreatic lipase, 25 μL of 10 μg/mL colipase, and 50 μL of a given concentration of $dBPS_1$ or $dBPS_2$ solution. After incubation at 37 °C for 30 min, the amount of oleic acid produced in the reaction solution was determined by the method of Zapf et al.[14] with slight modifications.[15] The reaction solution was added to 3 mL of the 1:1 (v/v) mixed solution of chloroform and 2% (v/v) methanol-containing heptanes and then shaken horizontally for 10 min in a shaker. The mixture was centrifuged at 2000*g* for 10 min. Then, the upper aqueous phase was removed by suction. The lower organic phase was mixed with 1 mL of copper solution containing 0.45 M triethanolamine, 0.05 M acetic acid, 3.4% (w/v) copper sulfate pentahydrate, and 20% (w/v) NaCl. After being shaken for 10 min, the mixture was centrifuged at 2000*g* for 10 min. One milliliter of upper phase was added to 1 mL of 0.1% (w/v) bathocuproine containing 0.05% (w/v) 3-*tert*-butyl-4-hydroxyanisol. The absorbance of the mixture was measured at 480 nm. Triplicate tests were performed for each sample. Lipase activity in the presence of dBPS was calculated from the equation $((As-Ab)/(Ac-Ab)) \times 100$, where As is the absorbance of the reaction solution with both lipase and dBPS. Ac is the absorbance of solution without dBPS, and Ab is the absorbance of solution without lipase.

Peptide Preparation. dBPS 150 μg was dissolved in 100 μL of 50 mM Tris-HCl buffer, pH 8.0 containing 5 mM EDTA, 5 mM DTT, and 8 M urea, and the protein solution was incubated at 30 °C for 4 h under a nitrogen atmosphere. The reduced protein solution was added to an equal volume of 50 mM Tris-HCl buffer, pH 8.0, containing 15 mM IAM and 8 M urea, and then incubated at 30 °C for 4 h in the dark. The excess IAM in the resultant carboxymethylated sample was removed by dialyzation against Milli-Q water for 1 day. The pH of the dialyzed protein solution was adjusted to 2.0 using HCl. Fifteen microgram of pepsin was added to 100 μL of the acidic protein solution. After incubation at 37 °C for 24 h, the peptic hydrolyzation was stopped by neutralizing the reaction solution by adding 10 μL of 1 M NaOH. The hydrolysates were lyophilized and stored at −20 °C until analyzed.

Peptide Purification. The peptic hydrolysates of dBPSs were separated by reverse-phase high-performance liquid chromatography (HPLC). Reverse-phase HPLC was carried out with a TSKgel ODS-80 column (4.6 × 250 mm) (TOSOH, Tokyo, Japan) connected to a JASCO LC system (Tokyo, Japan). Mobile phases for gradient elution were 0.1% (v/v) trifluoroacetic acid (TFA) aqueous solution (solvent A) and 80% (v/v) acetonitrile solution containing 0.1% (v/v) TFA (solvent B). Peptic hydrolysates of dBPSs (0.5 mg/mL, 100 μL) were applied to the column, and dBPS-derived peptides were eluted with a linear gradient from 2.5% of solvent B to 60% of solvent B over a period of 65 min at flow rate of 0.8 mL/min. The eluate was collected when absorbance at 220 nm increased. Fifteen microliters of each collected fraction was subjected to ACE-inhibitory activity assay. Fractions possessing high ACE-inhibitory activity were further purified by gel filtration chromatography using a Shodex SB402.5−4E column (4.6 × 250 mm) (Showa Denko K. K., Tokyo, Japan) connected to the JASCO LC system. Peptides were eluted with 0.1% (v/v) TFA in 10% (v/v) acetontrile at flow rate of 0.3 mL/min. The eluate was collected when absorbance at 220 nm increased. The samples collected were subjected to ACE-inhibitory activity assay.

Determination of ACE-Inhibitory Activity. ACE-inhibitory activity of the peptides was measured in vitro by the spectrophotometric assay described by Cushman and Cheung[16] with some modifications. Fifteen microliter of peptide solution was mixed with 110 μL of 13.63 mM HHL in 0.1 M borate buffer with 0.3 M NaCl, pH 8.3, followed by the addition of 25 μL of 156 mU/mL ACE. The mixture was incubated at 37 °C for 80 min. The reaction was stopped by addition of 110 μL of 1 N HCl. After mixing with 1 mL of ethyl acetate, the solution was centrifuged at 3000*g* for 10 min. A 750-μL amount of the organic layer was taken and dried out at 95 °C for 10 min. The residue was redissolved in 1 mL of Milli-Q water, and the absorbance of the solution was measured at 228 nm. Triplicate tests were preformed for each sample. The percent of ACE-inhibitory activity (% IACE) of peptides was calculated from the equation $((Ec-Es)/(Ec-Eb)) \times 100$, where Es is the absorbance of the reaction solution with peptides, Ec the absorbance of reaction solution without peptides, and Eb the absorbance of solution to which the stop solution was added beforehand addition of enzyme.

Peptide Identification. N-terminal amino acid sequences of peptides were determined by Edman degradation using an Applied Biosystems 491 Procise Protein Sequencer (Foster City, CA). Molecular mass of peptides was determined with a Bruker Autoflex matrix-assisted laser desorption ionization time-of-fight (MALDI-TOF) mass spectrometer (Bremen, Germany) using α-cyano-4-hydroxycinnamic acid (HCCA) matrix. Mass spectra were collected in the reflex and positive ion mode.

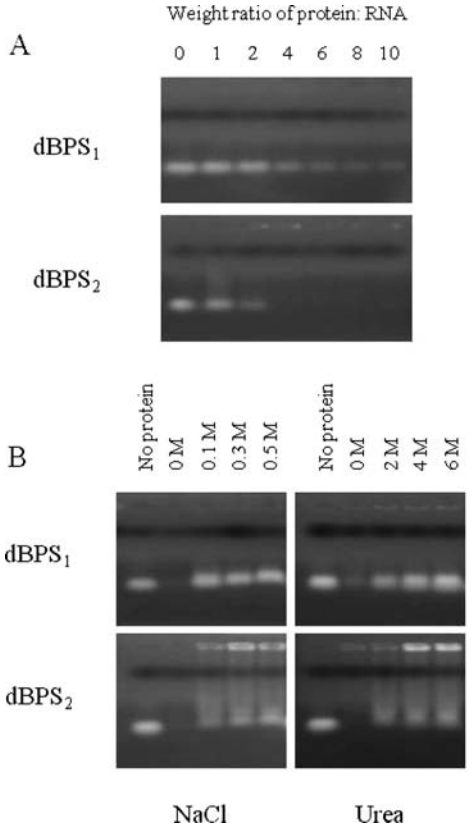

Figure 1. Agarose gel retardation pattern of baker's yeast RNA mixed with either $dBPS_1$ or $dBPS_2$. (A) The mixture was prepared at different weight ratios of protein: RNA in 10 mM Tris-HCl buffer, pH 7.6. Final concentration of RNA was 1.5 mg/mL in all samples. (B) Protein-RNA mixtures were prepared at protein:RNA ratio of 10 in 10 mM Tris-HCl buffer, pH 7.6 with NaCl (0.1−0.5 M) or urea (2−6 M). Final concentration of RNA was 1.5 mg/mL in all samples.

RESULTS AND DISCUSSION

RNA-Binding Property. Interactions between proteins and nucleic acids play an essential role in controlling many paramount cellular processes, such as transcription, replication, and recombination. In this study, interaction of dBPSs with nucleic acids was investigated by agarose gel migration assay using yeast RNA. A band of RNA treated with $dBPS_1$ gradually faded as the amount of the protein added increased (Figure 1A). The RNA band was almost invisible at the $dBPS_1$:RNA weight ratio of 10. A band of RNA treated with $dBPS_2$ completely faded away from the agarose gel when the $dBPS_2$:RNA ratio was >2. The losses in RNA band intensity might be explained by the neutralization of negative charges of RNA molecule by positive-charged $dBPS_1$ and $dBPS_2$. The RNA molecules thus neutralized are incapable of migrating from anode to cathode in an electrical field. In short, the fade away of RNA band implies that $dBPS_1$ and $dBPS_2$ bind to RNA molecule. The presence of $dBPS_2$ at $dBPS_2$:RNA ratio higher than 2 caused most of the RNA molecules to precipitate; whereas in the case of $dBPS_1$, a slight amount of RNA molecules migrated even when the $dBPS_1$:RNA ration was 10. Thus, it is suggested that $dBPS_2$ possesses higher RNA-binding affinity than $dBPS_1$ does.

Effect of NaCl and Urea on the RNA-Binding Property. Effects of NaCl and urea on the protein-RNA binding were investigated to comprehend interaction forces between dBPSs and RNA. The RNA band which disappeared by the presence of $dBPS_1$ at the $dBPS_1$:RNA ratio of 10 regained upon the addition of ≥0.1 M NaCl or ≥2.0 M urea (Figure. 1B), suggesting that complexes formed between $dBPS_1$ and RNA are dissociated upon the addition of NaCl and urea. The $dBPS_2$−RNA complex showed different behavior from the $dBPS_1$−RNA complex. Upon the addition of ≥0.3 M NaCl and ≥4 M urea, a clear and intense RNA band appeared at the position of the application well. The appearance of the bands at the position of wells reveals no migration of RNA.

A large amount of salt which breaks electrostatic interaction disrupts the association of the basic groups of protein with the phosphate anions of nucleic acid.[17] Urea attenuates hydrophobic interaction and hydrogen bonds, which are closely involved in protein−substrate binding.[18,19] Thus, it is presumed that three noncovalent interactions, i.e., electrostatic interaction, hydrophobic interaction, and hydrogen bonding, are engaged in the dBPS−RNA association. In addition, it seems that $dBPS_2$ binds to RNA with stronger force than $dBPS_1$ does, because higher concentrations of NaCl and urea are required to disrupt the association of $dBPS_2$ with RNA. The dissimilarity of the two proteins in RNA binding may be due to the difference in the number of basic amino acid residues. $dBPS_1$ has six Lys residues, while $dBPS_2$ has the same number of Lys residues and one Arg residue. Standke et al.[20] reported that increasing the number of basic amino acid residues in a peptide chain elevates the binding affinity of the peptide with nucleic acid. Furthermore, Arg has stronger attractive force to nucleic acid than Lys.[21,22] At least a 10-fold higher concentration of Lys-containing peptide is required to achieve a nucleic acid-binding rate similar to Arg-containing peptide.[23] Thus, the stronger RNA-binding affinity of $dBPS_2$ can be explained by the presence of an Arg residue which occurs only in $dBPS_2$. The presence of 0.3 M NaCl, the higher salt concentration than physiological saline, did not induce the dissociation of $dBPS_2$−RNA complex. This suggests the possibility of in vivo interaction of $dBPS_2$ with nucleic acids. On the contrary, the $dBPS_1$−RNA complex is easily dissociated even at the NaCl concentration of 0.1 M lower than physiological saline, suggesting no existence of physiological interaction of $dBPS_1$ with RNA.

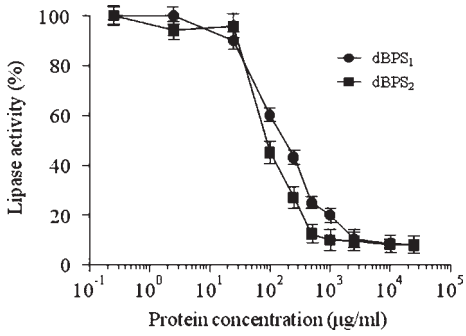

Figure 2. Effect of $dBPS_1$ and $dBPS_2$ on pancreatic lipase activity. Each point represents the mean ± standard deviation of three separate experiments.

Pancreatic Lipase Inhibition by dBPSs. Figure 2 shows inhibition effects of dBPSs on lipase activity using triolein emulsified with phosphatidylcholine as substrate. $dBPS_1$ and $dBPS_2$ inhibited lipase activity in a dose-dependent manner. Fifty percent inhibitory concentration (IC_{50}) was 250 μg/mL for $dBPS_1$ and 100 μg/mL for $dBPS_2$. Complete lipase inhibition

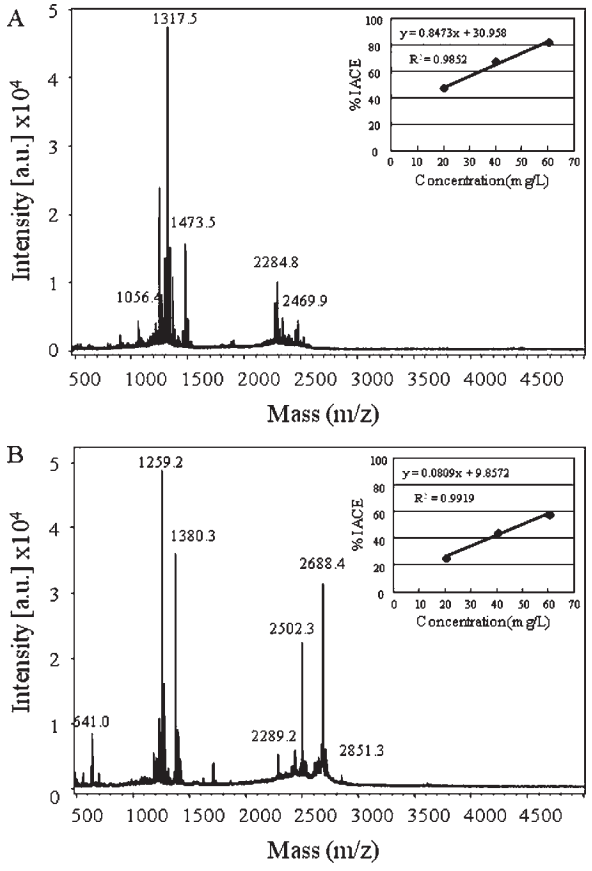

Figure 3. MALDI-TOF mass spectra and % IACE of peptic digests of $dBPS_1$ (A) and $dBPS_2$ (B).

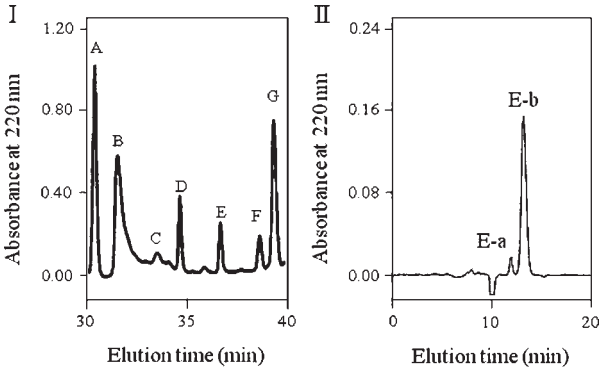

Figure 4. Chromatographic separation of ACE-inhibitory peptides derived from $dBPS_1$. (I) Reverse-phase HPLC chromatogram (TSKgel ODS-80) of peptic digests of $dBPS_1$ is shown in the retention time range of 30−40 min. Capital letters of A−G in the chromatogram indicates the fractions collected. (II) Gel filtration chromatogram (Shodex SB402.5−4E) of fraction E from $dBPS_1$ is shown. E-a and E-b indicates the fractions collected.

was brought about by the presence of 2.5 mg/mL $dBPS_1$ and $dBPS_2$. This suggests that $dBPS_1$ and $dBPS_2$ are the pancreatic lipase inhibitors that are more potent than an amphiphilic protein, BSA, which inhibits less than 10% of lipase activity at the concentration of 2.5 mg/mL.[11] Basic polymers such as protamine and ξ-polylysine also inhibit the hydrolysis of triolein emulsified with phosphatidylcholine.[24,25] It was mentioned that ξ-polylysine interacts with taurocholate, a bile acid, through electrostatic interaction and forms a surface-active complex. This complex occupies the interface between lipid micelles and water, thereby blocking access of lipase to trioleins on micelle surface.[25] Since similar in basicity to ξ-polylysine, $dBPS_1$ and $dBPS_2$ are assumed to form a surface-active complex with taurochlate, thereby retarding or blocking access of lipase to trioleins. Thus, the inhibition of lipid hydrolysis by dBPSs may be indicative of physiological function of dBPSs in gallbladder tissue where the proteins are located.[8]

ACE-Inhibitory Activity of Peptides Derived from dBPSs. Enzymatic hydrolysis of food proteins results in the release of oligopeptides, which are capable of exerting diverse biological activities. Some peptides are reported to exhibit a high ACE-inhibitory activity.[26−29] The peptic digests of $dBPS_1$ and $dBPS_2$ showed ACE-inhibitory activity with IC_{50} of 22.5 mg/L for $dBPS_1$ and 49.6 mg/L for $dBPS_2$ (Figure 3); whereas intact $dBPS_1$ and $dBPS_2$ did not exhibit any ACE-inhibitory activity at the concentration of ≤4 g/L. Peptides produced by pepsin digestion had the molecular masses below 3000 Da (Figure 3). The IC_{50} values of the dBPSs digests were lower than those reported for the digests of EW protein (40.2 mg/L)[28] and ovalbumin (45.3 mg/L).[26] The protein hydrolysates with the IC_{50} value of 0.5 g/L or less are generally recognized as significant in vitro and potential in vivo ACE-inhibitors.[29] When $dBPS_1$ and $dBPS_2$ are taken orally, their digests may work as in vivo ACE inhibitors.

An ACE-inhibitory peptide was purified from the $dBPS_1$ digests by reverse-phase HPLC and gel filtration chromatography. In preliminary RP-HPLC analysis, the highest ACE-inhibitory activity was observed at the retention time of 30−40 min. The column eluate from 30 to 40 min was collected, and its concentrate was subjected to rechromatography. The peptides were fractionated into seven fractions (A−G) (Figure 4I). The fraction E exhibited a potent ACE-inhibitory activity with 84.5% IACE (data not shown). The fraction was further purified using gel filtration chromatography. Two major peaks, E-a and E-b, were observed in the chromatogram (Figure 4II). Compared to the E-a peak with 5.6% IACE, the E-b peak had a higher ACE-inhibitory activity with 87.4% IACE. MALDI-TOF mass spectra proved that the peak E-b contains a homogeneous peptide with the molecular mass of 1041 Da (Figure 5). The amino acid sequence of the 1041 Da-peptide determined with a protein sequencer was EKKGFCAGY, which corresponds to Glu_1−Tyr_9 of $dBPS_1$. The theoretical molecular mass calculated from the sequence agreed with the molecular mass of 1041 Da determined with the MALDI-TOF mass spectrometer. Consequently, the potent ACE-inhibitory peptide purified from the peptic digests of $dBPS_1$ turn out to be the N-terminal nanopeptide of $dBPS_1$ (Figure 6).

The same analytical approach was applied to $dBPS_2$ peptic digests. The experimental results indicate that the strong ACE-inhibitory peptide was an octapeptide with the amino acid sequence of KYCPKVGY, corresponding to Lys_4−Tyr_{11} of $dBPS_2$ that has the molecular mass of 1014 Da (Figure 6).

It is reported that the presence of an aromatic amino acid in the COOH-terminal three residues of an oligopeptide is deeply involved in the activity level of ACE-inhibitory peptide.[27] In the $dBPS_1$ nanopeptide and $dBPS_2$ octapeptide, there exists a Tyr residue located at each of their COOH terminuses. The COOH terminal Tyr residue may play an important role in the potent ACE-inhibitory activity of oligopeptides. The ACE-inhibitory

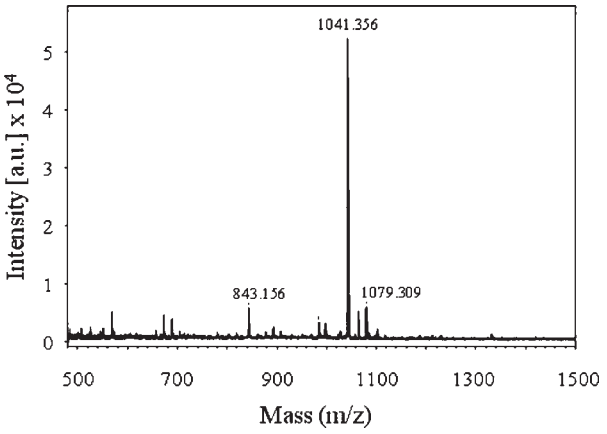

Figure 5. MALDI-TOF mass spectra of fraction E-b purified from the peptic digest of $dBPS_1$.

A.

$dBPS_1$ EKKGFCAGYCSYSCAKTDEWTFHQTCGKMYCCIPPPKKG
1 10 20 30 39

B.

$dBPS_2$ EVRKYCPKVGYCSSKCSKADVWSLSSDCKFYCCLPPGWK
1 10 20 30 39

Figure 6. Amino acid sequences of ACE-inhibitory peptides derived from $dBPS_1$ (A) and $dBPS_2$ (B). The underlines show the amino acid sequences of ACE-inhibitory peptides.

peptides derived from $dBPS_1$ and $dBPS_2$, however, will be processed to smaller peptides, as further digestion may take place during gastrointestinal transition. Peptides generated by pancreatic enzymes trypsin and α-chymotrypsin are presumed on the basis of their preferential cleavage sites to be EKK, GF, and CAGY for the $dBPS_1$-nanopeptide and CPK, VGY, and EVR for the $dBPS_2$-octapeptide. The tetrapeptide CAGY and tripeptide VGY, which contain aromatic amino acid at COOH-terminus, might maintain ACE-inhibitory activity. Since ACE plays a key role in the regulation of blood pressure, oral administration of $dBPS_1$ and $dBPS_2$ might prevent hypertensive disease and disorder.

In egg, there exist some EW proteins that help protect against microbial infection. Lysozyme exhibits bacteriolytic activity and ovotransferrin exerts bacteriostatic action. Ovomucoid seems to function as a protective or antinutrient agent against parasites through proteolytic inhibition. The secondary structures of $dBPS_1$ and $dBPS_2$ are similar to β-defensins, β-sheet-rich basic proteins containing 3 disulfide bonds.[8] β-defensins are well-known as powerful antimicrobial agents against microorganisms, including bacteria and fungi, whereas $dBPS_1$ and $dBPS_2$ did not show any antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella enteritidis*, *Escherichia coli*, and *Candida albican* at the concentration of 0.25 mg/mL (data not shown). In addition, $dBPS_1$ and $dBPS_2$ at the concentration of 1 mg/mL did not show any protease inhibitory activity against papain, trypsin, chymotrypsin, pepsin, and protease V8, indicating that the duck basic proteins do no act as a protease inhibitor (data not shown).

In conclusion, this paper is the first report showing the biological functions of $dBPS_1$ and $dBPS_2$, the novel basic proteins from duck EW in vitro. The present finding concerning the ACE and lipase inhibitory actions of these proteins suggest that they might be use in prevention of hypertriglyceridemia and hypertension. Nevertheless, further exploration of the in vivo activity of these proteins should be performed. The finding may enhance the industrial utilization of egg proteins and their value for health enhancement.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81-87-891-3099. Fax: +81-87-891-3021. E-mail: hayakawa@ag.kagawa-u.ac.jp.

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