Isolation and Characterization of a Novel Chicken Egg White Protein with Scavenger Receptor Cysteine-rich Domains

Whayoung Yoo¹, Tomohiro Araki², Junna Saito¹, Yamato Kurata¹, Kazuhiko Tokita¹, Kohtaro Kato³ and Misao Matsushita¹

¹ Department of Applied Biochemistry, School of Engineering, Tokai University, Hiratsuka 259–1292, Japan
² Department of Bioscience, School of Agriculture, Tokai University, Aso 869–1404, Japan
³ Department of Cellular Physiological Chemistry, Graduate School, Tokyo Medical and Dental University, Tokyo 113–0034, Japan

There are more than 70 proteins in chicken egg white, most of which have a role in host defense. We isolated a novel protein from chicken egg white which we termed EW135 by means of polyethylene glycol precipitation and ion-exchange chromatography, and an amino acid sequence analysis of its tryptic peptides showed it to be a member of the group B scavenger receptor cysteine-rich domain superfamily. EW135 was speculated to be complexed with a substance (s) in egg white in a Ca²⁺-dependent manner. From a structural point of view, EW135 may play a role in host defense.

Key words: Ca²⁺-dependent complex, chicken, egg white protein, scavenger receptor cysteine-rich domain


Introduction

Egg white consists of about 75% water, 12% proteins, 12% lipids and small amounts of other substances such as minerals and vitamins (Kovacs-Nolan et al., 2005). Egg white proteins include ovoalbumin, lysozyme, ovomucin, ovomucoid, ovotransferrin and others. The major function of egg white proteins is to defend the egg yolk against invasion by pathogens (Mine, 2007). Lysozyme is a glycosidase that hydrolyzes the bond between N-acetyl-muramic acid and N-acetyl-glucosamine in peptidoglycan of bacterial cell walls, thus exerting bactericidal activity (Vocadlo et al., 2001). Ovomucin is a heavily glycosylated protein that acts as a hemagglutination inhibitor by defending against certain kinds of viruses including influenza virus (Tsuge et al., 1996). Ovomucoid, cystatin, ovomacroglobulin and ovoinhiber are protease inhibitors (Tomimatsu et al., 1966; Anastasi et al., 1983; Kato et al., 1987; Molla et al., 1987). Ovotransferrin prevents bacterial use of iron by acting as an iron scavenger (Valenti et al., 1983; Bou Abdallah and el Hage Chahine, 1998). In addition to these known proteins, many new proteins have been identified in chicken egg white as a result of recent advances in proteomic and genomic analyses. Mann (2007) reported 78 chicken egg white proteins including 54 which were newly identified. Guérin-Dubiard et al. (2006) identified two new proteins in chicken egg white, Tenp and VMO-1.

In the present study, we isolated a novel protein in chicken egg white which we termed EW135. EW135 was speculated to be complexed with a substance (s) in egg white in a Ca²⁺-dependent manner. Partial amino acid sequences have revealed that EW135 is composed of scavenger receptor cysteine-rich (SRCR) domains. Proteins belonging to the SRCR domain superfamily are known to have a role in innate immunity. From a structural point of view, EW135 in egg white may play a role in host defense.

Materials and Methods

Materials

Eggs of Shaver White chickens were purchased from a local dealer. Q Sepharose and Sephadex G-50 were purchased from GE Healthcare (Uppsala, Sweden). Polyvinylidene difluoride membranes (Immobilon) were purchased from Millipore (Billerica, MA, USA). Block Ace was purchased from AbD serotec (Oxford, UK). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Except where indicated, all other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).
Purification of EW135 from Chicken Egg White

EW135 purification was performed in 4 steps.

1) Chicken egg white was diluted with a threefold volume of water, and the diluted solution was stirred for 30 minutes and centrifuged (4°C, 15,000×g, 20 min). 2) The collected supernatant was precipitated with 10% polyethylene glycol (PEG) 4,000 and centrifuged (4°C, 15,000×g, 20 min). The precipitates were then dissolved in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂, pH 7.8 (TBS-Ca). After leaving at 4°C overnight, the mixture was centrifuged (4°C, 15,000×g, 20 min) and the precipitates were washed with TBS-Ca. 3) Buffer containing 50 mM Tris-HCl, 200 mM NaCl, 10 mM EDTA, pH 7.8 (TBS-EDTA) was then added to the precipitates and the mixture was left at 4°C for 30 min. After centrifugation (4°C, 1,400×g, 20 min), the supernatant was collected and adjusted to pH 5 with HCl. Following further centrifugation (4°C, 1,400×g, 20 min), it was dialyzed against 20 mM Tris-HCl, 50 mM NaCl (pH 8.0). 4) The dialyzed solution was then chromatographed on a Q Sepharose column that had been equilibrated with the above buffer. Elution was performed by applying a linear NaCl gradient to 0.6 M. EW135 eluted at a concentration between 0.35 and 0.45 M NaCl. Fractions containing EW135 were collected and concentrated using an Amicon Ultra (Millipore, Billerica, MA, USA).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the Laemmli (1970) method. 2-mercaptoethanol was used as a reducing reagent.

Solubilization of EW135 at High Salt Concentrations

The procedure for the preparation of the precipitates containing EW135 was the same as that of Step 1 and Step 2 described above. A 50 mM Tris-HCl buffer (pH 7.8) with varying concentrations of NaCl (0.3 M to 1 M) was added to the precipitates and left at 4°C overnight. After centrifugation (4°C, 1,400×g, 20 min), the supernatant was subjected to SDS-PAGE under reducing conditions. Proteins were then transferred from the gels to polyvinylidene difluoride membranes and the blots were probed with rabbit polyclonal antibody against EW135. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was used as a secondary antibody and the blots were visualized with TMB.

Assay for Ca²⁺-dependent Binding of EW135 to a Substrate (s) in Chicken Egg White

Microplate wells (IWAKI, Tokyo, Japan) were coated with the solution of EW135 solubilized with 50 mM Tris-HCl buffer containing 0.5 M NaCl as described above. After leaving at 4°C overnight, the wells were blocked with Block Ace, and then washed with 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂, 0.1% Tween 20, pH 7.8 (TBS-Ca-T) or 50 mM Tris-HCl, 200 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Tween 20, pH 7.8 (TBS-EDTA-T). TBS-Ca-T or TBS-EDTA-T was added to the wells, followed by incubation at 37°C for 30 min. After washing the wells with either of these buffers, rabbit polyclonal anti-EW135 antibody diluted with the respective buffer was added, and the wells were incubated at 37°C for 1 hr. The wells were then washed, HRP-anti-rabbit IgG was added, and incubation was continued for an additional hour. TMB was added after incubation to allow for visualization. After termination of the HCl treatment, the optical density was measured at 450 nm.

Preparation of Antibodies Against EW135

Polyclonal antibodies against EW135 were produced by immunizing rabbits with 150 μg of purified EW135, followed by five booster injections of the EW135 antigen at 2-week intervals. Antisera were collected 2 weeks after the last booster injection. IgG of antibodies against EW135 was purified by ammonium sulfate precipitation followed by affinity chromatography using Protein A-agarose (GE Healthcare).

Carboxymethylation of EW135

EW135 (0.2 mg) was reduced and carboxymethylated for structural analysis, according to the method of Crestfield et al. (1963). First, 0.2 mg of EW135 was dissolved in 100 ml of 1.4 M Tris-HCl buffer, pH 8.6. Then 0.12 g of urea, 10 ml of 5% EDTA, and 3.3 ml of 2-mercaptoethanol were added. The solution was left for 60 min at 37°C under N₂ gas. After the reduction, 17.8 mg of monooiodoacetic acid in 60 ml of 1.0 M NaOH were added and the mixture was left for 60 min at room temperature in the dark. The reaction mixture was desalted in 0.2 M NH₄OH on a Sephadex G-50 column (1.7 × 46 cm) and the protein fraction was lyophilized (Cm protein).

Tryptic Digestion and Peptide Separation of EW135

Cm protein (0.1 mg) was suspended in 50 ml of 100 mM Tris-HCl buffer, pH 8.0, and then digested with trypsin (1/50, w/w, TR-TPCK, Cooper Biomedical Co.) at 37°C for 4 h. The digested EW135 peptides were purified on a reversed-phase HPLC column (YMC ODS 120A S-5; 4.6 × 250 mm, Yamamura Chemical Co., Japan) using a JASCO 800 series HPLC apparatus (Japan Spectroscopic Co., Japan). The peptides were developed using a gradient elution system of 0.1% trifluoroacetic acid (solvent A), and 60% acetonitrile in solvent A (solvent B). A gradient of 0–60% of solvent B was achieved in 300 min. The peptide was measured at 220 nm (Thammasirirak et al., 2002). The amino acids of tryptic peptides were sequenced by a protein sequencer (Model PPSQ21A, Shimadzu Co., Kyoto, Japan).

Results and Discussion

Purification of EW135 from Chicken Egg White

Egg white was first diluted with water. After centrifugation, the supernatant was precipitated with PEG 4,000 and the resulting precipitates were dissolved and incubated. The newly generated precipitates contained a protein which had not been observed in the starting material (Fig. 1A, lane 5). This protein had a molecular weight of 135 kDa under reducing conditions. By incubation of the precipitates with TBS-EDTA buffer, the 135 kDa protein was released into the buffer (Fig. 1A, lane 6). This protein tentatively named EW135 was purified to homogeneity by ion-exchange chromatography using Q Sepharose (Fig. 1A, lane 7). The molecular weight of EW135 under non-reducing conditions was
Partial Amino Acid Sequences of EW135 proteins.

was undetectable on an SDS-PAGE of the egg white protein judging from the fact that the band corresponding to EW135 in chicken egg white seems to be relatively low approximately 100 kDa (Fig. 1B). The concentration of EW135 in chicken egg white seems to be relatively low judging from the fact that the band corresponding to EW135 was undetectable on an SDS-PAGE of the egg white protein.

Partial Amino Acid Sequences of EW135

The EW135 protein was first digested with trypsin. The peptides generated were then separated and sequenced. In a homology search, the amino acid sequences of the tryptic peptides of EW135 were found to have features common to those of the group B scavenger receptor cysteine-rich (SRCR) domain. Mann (2007) identified many novel egg white proteins by means of proteomic analysis. After egg white proteins were separated by SDS-PAGE, protein bands were excised from gels and subjected to tryptic digestion followed by MS analysis. One of the novel egg white proteins identified had a molecular weight around 116 kDa and the amino acid sequences of its peptides analyzed were found in the protein database under accession numbers UPI0000611E45 (formerly IPI00595253) and UPI000044AB0D (formerly IPI00584163) (Fig. 2). Mann (2007) speculated that these two entries overlap and belong to one single protein with eight SRCR domains. The amino acid sequences of the tryptic peptides of EW135 were also found in these two entries and were distributed in distinct SRCR domains (Fig. 2).

The SRCR superfamily, whose members consist of 100–110 amino acid residues, is divided into two groups (A and B) according to the respective type of the SRCR domain (Sarrias et al., 2004). The SRCR domain of group A has 6 cysteine residues, whereas that of group B has 6 to 8 cysteine residues. Most of the proteins belonging to the SRCR superfamily consist of tandem repeats of the SRCR domain. Some of the proteins containing group A SRCR domains include SR-AI/II, SR-AIII and MARCO, whereas some of those with group B domains include DMBT1/gp-340 and 18-B. Various protein sequences of the SRCR superfamily have been deposited in the protein database. To date, 18-B is the only one of these reported to be isolated from chicken (Iwasaki et al., 2001). 18-B, which is present in chicken serum, is composed of four SRCR domains and has a size of 66 kDa under reducing conditions. It is speculated that it regulates cell function by inhibiting the overproduction of reactive oxygen species.

Solubilization of EW135 at High Salt Concentrations

As described above, EW135 was included in the precipitates obtained by incubation of the dissolved PEG precipitates. To examine whether EW135 could be solubilized at high salt concentrations, the latter precipitates were incubated with Tris buffer containing NaCl at concentrations ranging from 0.2 M to 1 M. After centrifugation, the supernatant was examined for the presence of EW135 by immunoblotting using anti-EW135. As shown in Fig. 3, EW135 was not detected in the supernatant when the precipitates were treated with 0.2 M NaCl-containing buffer. However, it was detected at NaCl concentrations above 0.3 M. The intensity of the EW135 band increased with increasing NaCl concentrations. This result indicates that EW135 was solubilized at high NaCl concentrations in a dose-dependent manner. It is possible that not only EW135 but other proteins were solubilized.

Ca²⁺-dependent Binding of EW135 to a Substance (s) in Chicken Egg White

EW135 was released by EDTA treatment of the precipitates that had been generated by incubation of the dissolved PEG precipitates. This suggested that EW135 was complexed with a substance (s) in the precipitates in a Ca²⁺-dependent manner and was dissociated by treatment with EDTA. To determine whether this was the case, we carried out an ELISA using a solubilized EW135 preparation obtained by treatment of the precipitates with Tris-HCl buffer containing 0.5 M NaCl. The preparation was coated on the microplate wells and incubated with Tris-HCl buffer containing either Ca²⁺ or EDTA. After incubation, the EW135 level on the wells was measured using anti-EW135. As shown in Fig. 4, the level on wells treated with Tris-Ca was approximately 1.8 times higher than that on wells treated with TBS-EDTA. This result strongly suggests that EW135
forms a Ca\(^{2+}\)-dependent complex with an as yet unknown substance (s) and that the complex was released from the precipitates by means of Tris buffer containing 0.5 M NaCl.

In conclusion, we isolated for the first time a chicken egg white protein with group B SRCR domains. This protein tentatively named EW135 could form a complex with an as yet unknown substance (s) in a Ca\(^{2+}\)-dependent manner. It is most likely that EW135 is identical to the protein Mann (2007) analyzed using proteomic methods. Proteins of the SRCR superfamily mainly have a role in host defense. For instance, DMBT1/gp-340 binds and agglutinates certain bacteria (Carlén et al., 1998). The functions of EW135 remain unknown. From a structural point of view, however, it is possible that EW135 has a role in host defense by protecting against invading microbes in egg white. It is of particular interest that EW135 could be complexed with an...
Fig. 3. Solubilization of EW135 from the precipitates using high concentrations of NaCl. The precipitates generated from the solubilized PEG precipitates were incubated with Tris buffer containing NaCl at concentrations ranging from 0.2 M to 1 M. After centrifugation, the supernatant was examined for the presence of EW135 by immunoblotting using anti-EW135 as a probe. The arrow indicates EW135.

Fig. 4. Ca\textsuperscript{2+}-dependent binding of EW135 to a substance(s) in chicken egg white. The EW135 preparation obtained by treatment of the precipitates with 0.5 M NaCl-containing buffer was coated on the microplate wells and incubated with buffer containing either Ca\textsuperscript{2+} or EDTA. After incubation, EW135 levels in the wells were measured using anti-EW135. **P<0.01 (n=3).

egg white substance(s). Although the identification and functions of the EW135 -binding substance(s) remain to be elucidated, EW135 may exert its function in concert with them in the complex form.

Acknowledgments

We are grateful to Mr. Hideo Tsukamoto, the Education and Research Support Center, Tokai University, for generating anti-EW135 antibody. This work was supported in part by the Kieikai Research Foundation (to M. M).

References


