

Ovalbumin-Related Gene Y Protein Bears Carbohydrate Chains of the Ovomuroid Type

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We have recently established the monoclonal antibodies (mAbs) specific to the major food allergen, ovomucoid, as mAb 7D, recognizing the carbohydrate moiety of ovomucoid, and mAb 6H, the peptide moiety (*Biosci. Biotechnol. Biochem.*, 68, 2490–2497, (2004)). Using these mAbs, we found commercially available ovalbumin preparations contaminated with a considerable amount of ovomucoid together with other glycoproteins. To examine the contaminants, egg white was subjected to cation-exchange chromatography. An unidentified protein was found in egg white that reacted with mAb 7D but not with mAb 6H, having a molecular size of about 52 kDa and a blocked N-terminus. Two internal amino acid sequences of the fragments obtained after a lysyl endopeptidase and a hydroxylamine treatment revealed the protein to be ovalbumin Y (ovalbumin-related gene Y protein). We conclude that ovalbumin Y is a unique chimeric glycoprotein having an amino acid sequence similar to that of ovalbumin, but having a carbohydrate moiety similar to that of ovomucoid.

Key words: egg-white protein; food allergen; monoclonal antibody; ovalbumin Y; ovomucoid

Hen's eggs are the most frequent causes of food allergy in Japan, approximately two-thirds of the children diagnosed with food allergy being sensitive to egg white.^{1,2)} Egg white contains many different and well-characterized proteins.^{3,4)} Ovalbumin which constitutes 54% of the egg-white protein, is a glycoprotein with an acetylated N-terminal residue. It is easily denatured and coagulated by heating. Ovomuroid constituting 11% is also a glycoprotein with 22% carbohydrate, but is incoagulable by heating. Ovomuroid has four glycosylated asparaginyl residues to which complex oligosaccharide chains are attached, while ovalbumin contains only a single glycosylated residue with either a

high-mannose or hybrid oligosaccharide.⁵⁾ Although both proteins are generally considered to be the most allergenic egg proteins, ovalbumin has been almost exclusively used as a model allergen for egg white in basic research and clinical diagnosis.^{6–9)} However, the immunodominance of ovomucoid among egg-white proteins has been gradually noticed,^{10,11)} stemming from the observation that the use of commercially purified ovalbumin fractions that are contaminated with ovomucoid has led to an overestimation of the dominance of ovalbumin as the major egg allergen.¹²⁾ Urisu *et al.* have clinically indicated the predominance of ovomucoid over other proteins in egg white and pointed out the important role of heat-treated ovomucoid in the pathogenesis of allergic reactions to egg white by a double-blind, placebo-controlled food challenge.¹³⁾ A method to prepare a low-ovomuroid egg-white fraction for allergic patients has been developed, while maintaining the functional properties of the egg white.¹⁴⁾

However, the situation involved in the degree of contamination observed in commercial ovalbumin preparations appears to be more complicated. In addition to ovomucoid, commercial samples of chicken ovalbumin have been reported to contain other unidentified glycoproteins with carbohydrate chains of the ovomucoid type.⁵⁾ We have also noticed the presence of a protein in egg white that was recognized by the monoclonal antibody (mAb) specific to the carbohydrate moiety of ovomucoid.¹⁵⁾ Characterization of these minor, although potentially allergenic, unidentified proteins in egg white should be carried out to understand the precise mechanism for egg allergy, as well as their nutritional and food-chemical functions in the egg industry.

The ovalbumin gene family in the chicken is composed of three genes, the X, Y, and ovalbumin genes, located within a 40-kb region on chromosome 2.^{16–18)} These genes are transcribed in oviduct under a

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM phosphate-buffered saline at pH 7.4

similar hormonal control and code for the respective proteins, gene X protein, gene Y protein and ovalbumin, although gene X protein has not been identified in eggs.^{19,20} Very recently, gene Y protein has been isolated from egg white as a glycoprotein with a molecular mass of about 53 kDa.²¹ In the present study, we have identified ovalbumin-related gene Y protein, hereafter referred to ovalbumin Y, in commercial ovalbumin preparations as well as in egg white and show that it has carbohydrate chains of ovomucoid type by using mAbs specific to ovomucoid.¹⁵

Materials and Methods

Materials. The preparation of mAbs 6H and 7D and their details have been described in the previous paper.¹⁵ The following materials were obtained from the sources indicated in parentheses: ovalbumin, reagent grade (Tokyo-Kasei, Tokyo, Japan); ovalbumin grades II, III, V, VI and VII (Sigma, St. Louis, MO, U.S.A.); anti-ovalbumin IgG (Polysciences, Warrington, PA, U.S.A.); affinity purified anti-ovalbumin IgG with an antigen-immobilized column (Cosmo Bio, Tokyo, Japan); lysyl endopeptidase from *Achromobacter lyticus* (Wako, Osaka, Japan); TSKgel CM-5PW for cation-exchange chromatography (Tosoh, Tokyo, Japan); Maxisorp microtiter plate for an enzyme-linked immunosorbent assay (Nunc, Roskilde, Denmark); alkaline phosphatases conjugated to goat anti-mouse IgG (ICN, Aurora, Ohio, U.S.A.), anti-rabbit IgG (Cappel, Durham, NC, U.S.A.), and streptavidin (Oncogene, Boston, MA, U.S.A.). All other chemicals used were of the highest purity available.

Preparation of proteins from egg white. To freshly prepared hen's egg white was added an equal volume of 0.1 M acetate buffer at pH 3.8, and the mixture was dialyzed against the same buffer overnight. After centrifugation, the resulting supernatant was applied to a cation-exchange column (TSKgel CM-5PW, 21.5 mm × 15 mm). The column was washed with a 20 mM acetate buffer at pH 4.6, and the proteins were successively eluted with a 0–0.2 M NaCl gradient, 1 M NaCl in the same buffer, and 2 M of NaCl in a 0.2 M phosphate buffer at pH 7.0. The flow rate was 6 ml/min. The ovalbumin fractions were also purified by the ammonium sulfate procedure according to the method of Sørensen and Höyrup.²²

Preparation of ovomucoid-free ovalbumin and the antibody against it. MAb 7D (10 mg) was coupled to 1 ml of Affi-Gel 10 (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's protocol. Two mg of commercial ovalbumin (Sigma Grade VI) in PBS was applied to the column. Ovomucoid-free ovalbumin purified in the through fractions did not show any bands by a western analysis with mAb 7D.

Three rats were intraperitoneally immunized with 200 µg each of ovomucoid-free ovalbumin in Freund's

complete adjuvant, and the immunization was boosted three times with 100 µg each of the antigen in Freund's incomplete adjuvant at 2-week intervals. Antisera were withdrawn from them, and the IgG fractions were purified in a Protein G-Sepharose column (Amersham Biosciences Corp. NJ, U.S.A.). A portion of the purified IgG was biotinylated with EZ-Link sulfo-NHS-LC-Biotin (Pierce, IL, U.S.A.). These animals were given a standard laboratory diet and water *ad libitum*. The experiments were done under the control of the Guidelines for Animal Experiments in the Law (No. 105) and Notification (No. 6) of the government.

Protein sequencing. Purified protein was reductively carboxymethylated according to Mine and Zhang.²³ To 70 µl of carboxymethylated protein (0.5 mg/ml), 10 µl of lysyl endopeptidase (1.3 mg/ml) was added, and the mixture was incubated at room temperature. The reaction was stopped by adding 20 µl of 2-mercaptoethanol. Hydroxylamine cleavage was done essentially according to Bornstein and Balian.²⁴ Carboxymethylated protein (0.7 mg/ml) was completely dialyzed against 6 M guanidine-HCl, then against 2 M hydroxylamine at pH 9.0 45 °C for 4 h and finally against a 0.1 M Tris-HCl buffer at pH 8.1. The cleaved samples were subjected to SDS-PAGE and blotting.²⁵ The membrane was briefly stained with Coomassie Brilliant Blue and washed well with a 10 mM CAPS buffer at pH 11 containing 10% methanol. The bands of interest were cut off and directly subjected to an amino acid sequence analysis with a 476A gas-phase protein sequencer (Applied Biosystems).

Other methods. SDS-PAGE was performed under reducing conditions with Mini Protean II apparatus (Bio-Rad). The proteins in a gel for the western analysis were transferred to a polyvinylidene difluoride membrane with a Mini Trans Blot apparatus (Bio-Rad)²⁶ and visualized with alkaline phosphatase-conjugated goat anti-mouse IgG via mAbs 6H and 7D. The protein concentration was determined with Bio-Rad DC Protein Assay kit.

Results and Discussion

Ovomucoid in the ovalbumin preparations

Commercially available ovalbumin preparations, usually without confirmation of purity and any further purification, have been widely used in basic and clinical research as a model antigen, even though they have been shown to contain non-negligible amounts of contaminated ovomucoid. We therefore examined the purity of six different commercial ovalbumin fractions by a western analysis, using the mAbs specific to ovomucoid that we have recently established.¹⁵ MAb 6H specifically recognizes a peptide region of ovomucoid exposed outside by heating. MAb 7D reacted with chicken ovomucoid, but neither with deglycosylated chicken

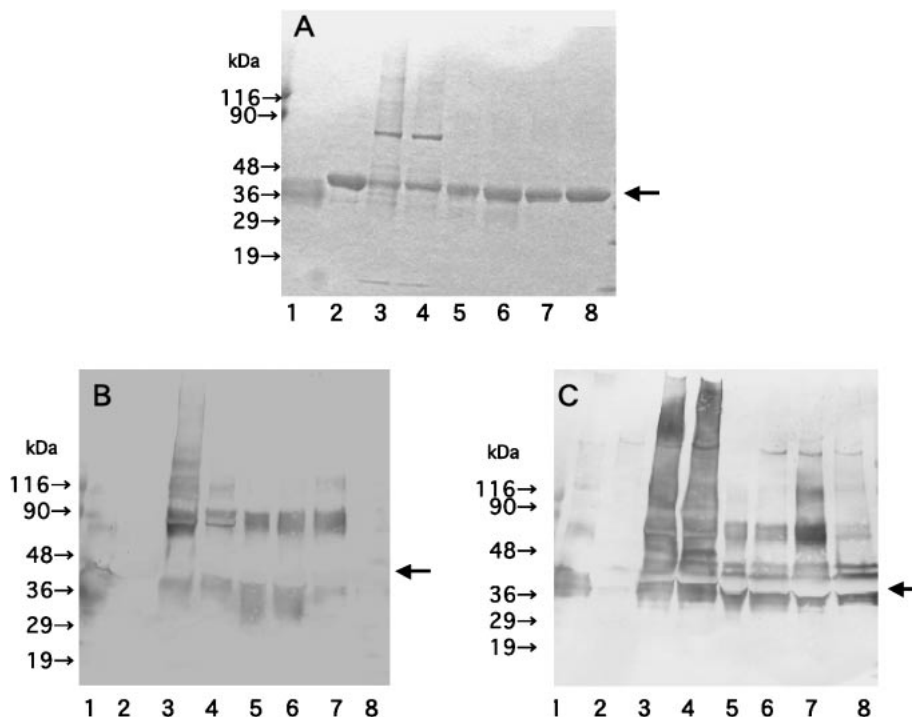


Fig. 1. Contamination by Ovomuroid of Ovalbumin Preparations.

In each panel, purified ovomucoid (lane 1), ovalbumin purified by five-times crystallization (lane 2), and commercially available ovalbumin preparations of reagent grade from Tokyo-Kasei, and grades II, III, V, VI and VII from Sigma, respectively (lanes 3–8) were loaded for SDS-PAGE. Three micrograms of each protein was applied to a gel and stained directly with Coomassie Brilliant Blue (*panel A*). Two microgram of purified ovomucoid and 10 μ g of each ovalbumin were applied to the gels, transblotted on polyvinylidene difluoride membranes and immunologically stained with anti-ovomucoid mAb 6H (*panel B*) and mAb 7D (*panel C*). Linear gradient gels (5–20%) were used. An arrow in each panel indicates the migration position corresponding to ovalbumin (45 kDa).

ovomucoid nor with other proteins with different types of carbohydrate moieties such as ovalbumin and ovomucoid from Japanese quail and duck. Thus, mAb 7D was specific to the carbohydrate moiety of chicken ovomucoid and therefore equally recognized both native and heat-denatured ovomucoid. Five-times crystallization of egg white with ammonium sulfate gave an ovalbumin fraction of good purity without any obviously stained bands with the mAbs (lane 2 in Figs. 1A, B and C). On the other hand, the crude commercial ovalbumins (lanes 3 and 4 in Fig. 1A) contained a considerable amount of contaminating protein with a molecular size of about 80 kDa, which was probably an undissociable dimer of ovomucoid whose monomeric form showed an apparent molecular size of 40 kDa (lanes 1 and 3–8 in Figs. 1B and C). The apparent molecular size of many glycoproteins often appears larger by SDS-PAGE than that calculated from its molecular constituents, as is the case of ovomucoid whose molecular mass is 28 kDa. Although the commercially purified ovalbumin fractions give clear protein bands by SDS-PAGE (lanes 5–8 in Fig. 1A), the mAb staining in the western analysis reveals that they contained significant amounts of both monomeric and dimeric ovomucoid (lanes 3–8 in Figs. 1B and C), together with other minor contaminants, in consistent with the previous observations.^{5,12,15)}

To make matters worse, two commercially available

anti-ovalbumin antibodies purchased from different sources reacted almost equally against ovalbumin and ovomucoid in an enzyme-linked immunosorbent assay (Figs. 2A and B). They must have been prepared from antisera raised against commercial ovalbumin fractions contaminated with ovomucoid. The contaminated ovomucoid in these ovalbumin fractions could be easily eliminated by using a mAb 7D-immobilized column (see the Materials and Methods section). As shown in Fig. 2C, the polyclonal antibody obtained from antisera raised against the ovomucoid-free ovalbumin did not react at all with ovomucoid. It is strongly recommended for researchers using commercial ovalbumin samples as a model allergen to keep these findings in mind and to pay particular attention to the purity of the antigen and the specificity of the antibody to guarantee the validity of the data obtained with them.

Separation of the proteins stained with mAb 7D

In Fig. 1, it appears that at least two bands at around 50 kDa, slightly above ovalbumin, were specifically stained with mAb 7D but not with mAb 6H (lanes 3–8 in Figs. 1B and C). It is possible that they represent glycoproteins having identical carbohydrate chains to ovomucoid but different amino acid sequences. In order to identify these proteins in egg white, cation-exchange chromatography was carried out.

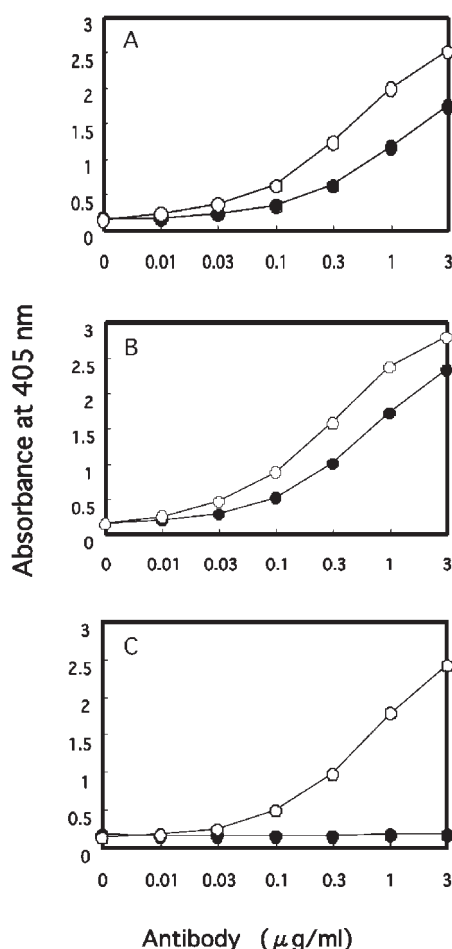


Fig. 2. Reactivity of the Anti-Ovalbumin Antibodies against Ovomucoid in an Enzyme-Linked Immunosorbent Assay.

The wells of a microtiter plate were coated with 2 μg/ml of ovomucoid-free ovalbumin (unfilled circle) or ovomucoid (filled circle) in PBS. After blocking the wells with 1% BSA in PBS, were added varying amounts of an anti-ovalbumin IgG fraction from Polysciences (panel A), an affinity-purified fraction with an antigen-immobilized column from Cosmo Bio (panel B), and biotinylated IgG prepared in the present study against ovomucoid-free ovalbumin (panel C). The antibodies bound to the solid-phase antigens were detected either with alkaline phosphatase-conjugated anti-rabbit IgG or alkaline phosphatase-conjugated streptavidin.

Figure 3 shows the protein elution pattern of egg white by carboxymethyl Toyopearl chromatography. Judging from both the amounts and order of elution and the molecular sizes estimated by SDS-PAGE (data not shown), we conclude that the three major peaks, 4, 5 and 6, correspond to ovalbumin, lysozyme, and ovo-transferrin, respectively. The proteins in peak 2, which is composed of subfractions 2a and 2b, were stained with both mAbs 6H and 7D in the western analysis, indicating that peak 2 corresponds to ovomucoid (Fig. 4). The heterogeneity detected here may have been due to that of the carbohydrate moiety of ovomucoid which has at least three different types of carbohydrate chain containing sialic acid residues.²⁷⁾ A protein found in the fractions of peak 1 which were yellow in color gave a molecular size of about 40 kDa

and was stained only with mAb 7D (Fig. 4). This protein is assumed to be the riboflavin binding-protein that has been reported to carry the carbohydrate moiety of the ovomucoid type.^{5,28)}

The protein detected in peak 3 was stained only with mAb 7D and with an estimated molecular size of about 52 kDa by SDS-PAGE (Fig. 4). Since this protein was eluted immediately before ovalbumin (peak 4), it would have had a slightly lower pI value than that of ovalbumin, pI = 4.5. Among the well-known egg-white proteins, only ovoinhibitor and ovoglobulins G2 and G3 exhibit a similar molecular size (49 kDa). However, the pI values of these proteins, 5.1, 5.5 and 5.8, respectively, are much higher than that expected, making their candidacy unlikely.

Identification of the 52-kDa protein

To identify the 52-kDa protein of peak 3, it was subjected to N-terminal amino acid sequencing. The analysis, however, was unsuccessful, probably due to N-terminus blocking. The protein was then reduced, carboxymethylated and partially digested by lysyl endopeptidase. Three large fragments (b–d in Fig. 5) obtained after SDS-PAGE and blotting were analyzed for their N-terminal amino acid sequences. Two fragments gave an identical sequence for the first eight residues, KVLHFDSI, and the rest gave no terminal residue. A BLAST analysis (Search for short nearly exact matches) [<http://www.ncbi.nlm.nih.gov/BLAST/>] was carried out on this sequence using the chicken (*Gallus gallus*) database. Ovalbumin Y (ovalbumin related-gene Y protein; accession number P01014 in the Swiss-Prot databank)¹⁸⁾ was the only protein found having a consistent sequence.

Ovalbumin Y is a protein highly homologous to ovalbumin, having 388 amino acid residues, including the initiator methionine residue (Fig. 6). For further confirmation, we performed a hydroxylamine treatment which specifically cleaves the peptide bond between Asn-Gly residues. Ovalbumin Y has only a single cleavable site between Asn 155 and Gly 156, and the sequence determination of the successive residues following Gly 156 should give an unambiguous identification of the 52-kDa protein if it is ovalbumin Y. As expected, two fragments obtained by the treatment show apparent molecular sizes of 31 and 21 kDa, which may correspond to the C- and N-terminal fragments, respectively (Fig. 7). The sequence of GQIKDLLVS was found for the first nine residues of the larger C-terminal fragment, proving that the 52-kDa protein is indeed ovalbumin Y. The N-terminal determination for the N-terminal fragment was unsuccessful, as was the case for the whole protein.

Both the fragments obtained by the hydroxylamine treatment were stained with mAb 7D to indicate their glycosylation (Fig. 7). Among the four N-glycosylation sites predicted in ovalbumin Y, therefore, the first site and at least one of other three sites should be

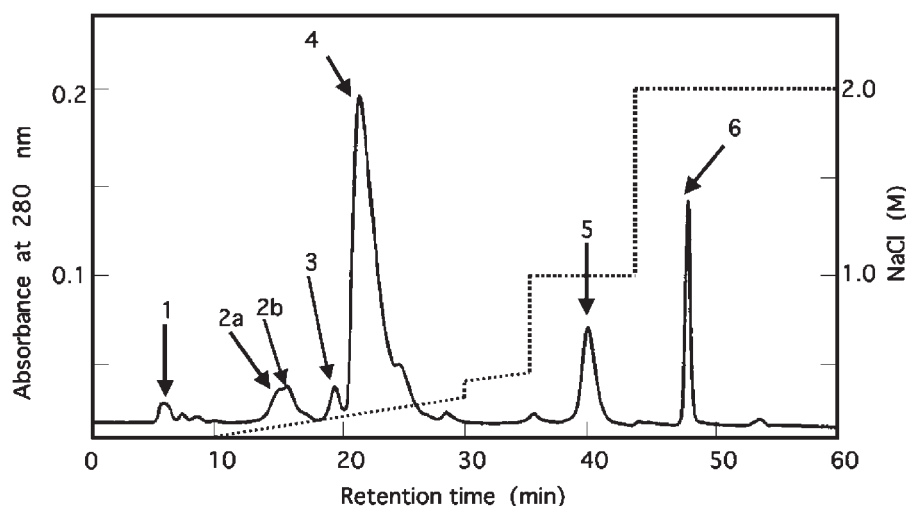


Fig. 3. Separation of the Egg-White Proteins by Cation-Exchange Chromatography.

A solution of egg-white proteins (0.12 g) was applied to a column of Toyopearl CM-650 cation-exchange resins (21.5 mm $\times$ 150 mm). After washing the column with a 20 mM acetate buffer at pH 4.6, the proteins were successively eluted with a 0–0.2 M NaCl linear gradient, 1 M NaCl in the same buffer, and then with 2 M of NaCl in 0.2 M phosphate buffer at pH 7.0. The phosphate buffer was required for the efficient elution of ovotransferrin. The flow rate was 6 ml/min. The fractions of peaks 1–6 indicated by the arrows were analyzed to identify the proteins included. Peak 2 was further fractionated into subfractions 2a and 2b. Solid line: absorbance at 280 nm; broken line: NaCl concentration (M).

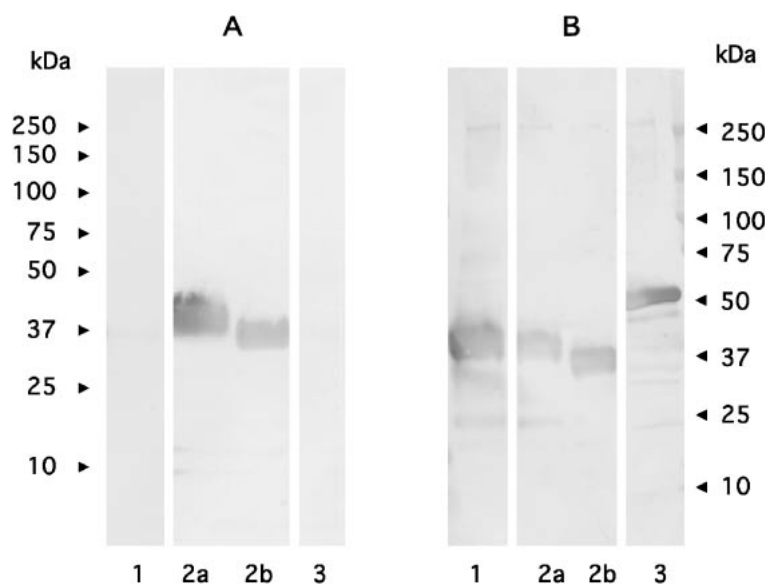


Fig. 4. Western Analysis of Egg-White Proteins with Anti-Ovomucoid mAbs 6H and 7D.

The proteins (2 μ g each) in peaks 1, 2a, 2b and 3 were applied to SDS-PAGE and immunologically stained with anti-ovomucoid mAbs 6H (panel A) and 7D (panel B) after blotting on polyvinylidene difluoride membranes (see the Materials and Methods section). Linear gradient gels (5–20%) were used for PAGE.

glycosylated (Fig. 6). In contrast to ovalbumin, multiple glycosylation of ovalbumin Y may occur in view of its apparent molecular size being larger than that (43 kDa) expected for a simple protein.

Chemical deglycosylation of the 52-kDa protein with trifluoromethanesulfonic acid resulted in loss of staining with mAb 7D in the western analysis (data not shown), similar to the case with ovomucoid.¹⁵⁾ It should be mentioned that an unidentified protein with carbohydrate chain(s) of the ovomucoid type has been identified in

commercial samples of ovalbumin by a mass spectro-metric analysis for the carbohydrate moiety.⁵⁾

We conclude from these results that ovalbumin Y is a glycoprotein having at least two carbohydrate chains of ovomucoid type with a molecular size of about 52 kDa that readily contaminates in commercial albumin preparations. The ovalbumin Y content in egg white is estimated to be around 5% from its recovery by chromatography (Fig. 2). Since egg white contains about 54% ovalbumin, the ratio of ovalbumin to

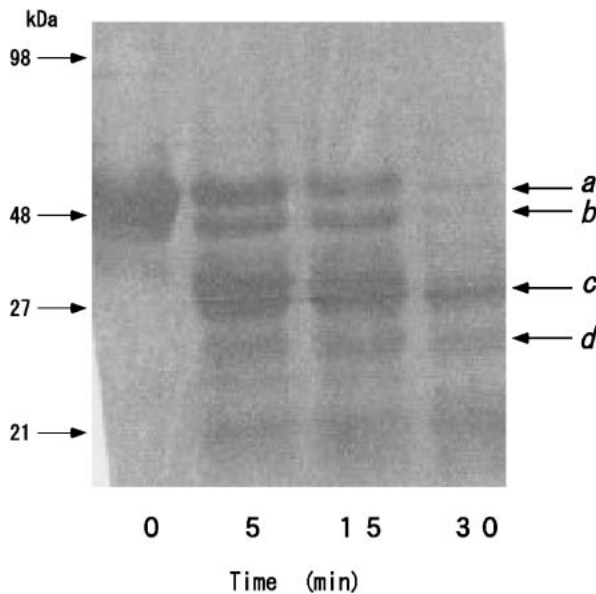


Fig. 5. Fragments Obtained by a Limited Lysyl Endopeptidase Treatment of the 52-kDa Protein.

The reduced-carboxymethylated 52-kDa protein was digested with lysyl endopeptidase at room temperature for the indicated times (see the Materials and Methods section). Samples (3 µg) before digestion and those (10 µg) after digestion were subjected to SDS-PAGE with a linear gradient gel (5–20%) and blotted for staining with Coomassie Brilliant Blue. The bands indicated by arrows *a–d* were cut out for N-terminal analyses. Both bands *b* and *d* gave the identical N-terminal sequence of KVLHFDSI. The analyses for bands *a* and *c* were unsuccessful.

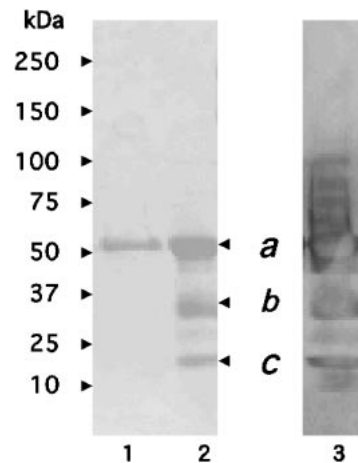


Fig. 7. Fragments Derived from the 52-kDa Protein by Hydroxylamine Treatment.

The reduced-carboxymethylated 52-kDa protein (3 µg, lane 1) and the hydroxylamine-treated protein (10 µg each, lanes 2 and 3) were subjected to SDS-PAGE and transblotted on to a membrane which was then stained with Coomassie Brilliant Blue (lanes 1, 2). The transblotted sample was also analyzed by staining with mAb 7D (lane 3). Linear gradient gels (5–20%) were used for PAGE. The bands indicated by arrows *a–c* were cut out for N-terminal analyses. Band *b* gave the N-terminal sequence of GQIKDLLVS. The analyses for bands *a* and *c* were unsuccessful.

	10	20	30	40	50	60	70	80	90	100
Ovalbumin Y	MDSISVTNAKFCFDVFNEMKVHHVNIENILYCLPLSILALAMVYLARGNTESQMKVLEFDSITGAGSTTDSQCSSEYVHNLFKELLSEITRPMATYSL									
Ovalbumin	M SI + +FCFDVF E+KVHH NENI YCP++I++ALAMVYLGA+ +T +Q+ KV+ FD + G G + ++QCG+S VH+ +++L++IT+PN YS									
	110	120	130	140	150	160	170	180	190	200
Ovalbumin Y	EIADKLYVDKTFVSLPEYLSKARFYTGVEEVNFTAAEERQLINSWVEKETNGQIKDLLVSSSIDFGTTMVFINTIYFGIWKIATNTEDTREMPPS									
Ovalbumin	+A +LY ++ + +LPEYL C ++ Y GG+E +NF+TAA++AR+LINSWE +TNG I++L SS+D T MV +N I FRG++ AF EDT+ MPF									
	210	220	230	240	250	260	270	280	290	300
Ovalbumin Y	MTKEESKPVQMMCHNSFNATLPAEKMKILELPYASGDLMLVLLPDEVSGLERIEKTINFQKLEWTSTNAMAKSKMKVYLPKMKIEEYNTLSILMA									
Ovalbumin	+T++ESKPVQMM F VA++ +EKMKILELP+ASG +SHLVLLPDEVSGLE++E INF+KL EWTST+N M ++ +EVYLPKMK+EEKYNLTS+LMA									
	310	320	330	340	350	360	370	380		
Ovalbumin Y	LGMTDLFSSANLGTISSVDNLMISDAVGVFMEVNEEGTEATGSTGAIGNIKHSELEEFADHPFLFFIRYNPTNAILFFGRYNSP 388									
Ovalbumin	+G+TD+FS SANL+GISS ++L IS AVH E+NE G E GS A + + EEFADHPFLF I++ TNA+LFFGR SP									
	310	320	330	340	350	360	370	380		
Ovalbumin Y	MGITDVFSSANLSSAESSKISQAVHAHAHAEINEAGREVVGSAEA--GVDAASVSEEFADHPFLFCIKHIATNAVLFFGRVSP 386									
Ovalbumin										

Fig. 6. The Pairwise Alignment between Ovalbumin Y and Ovalbumin.

The sequence alignment was obtained by performing a PSI-BLAST analysis. The identities are echoed about the central lines of the alignment, and homologous substitutions are indicated by '+' symbols. Underlined residues of ovalbumin Y indicate those determined in the present study. The locations of putative N-linked glycosylation sites are shown in italics.

ovalbumin Y becomes 11, this value being consistent with that estimated from the level of their transcripts.²⁰⁾ All characteristics of ovalbumin Y revealed here are in agreement with the recent results reported by Nau *et al.*²¹⁾ Nonetheless, it is important for a precise understanding of ovalbumin Y to determine the structure of its carbohydrate moiety by a direct analysis such as that by mass spectrometry.

Serpins (serine protease inhibitors) are a large,

structurally heterogeneous, and functionally diverse family of proteins found in organisms ranging from viruses to vertebrates.²⁹⁾ Ovalbumin as well as ovalbumin Y are members of this family and form the ovalbumin-type of serpin subfamily which is characterized by the absence of a cleavable N-terminal signal peptide, conserved phosphorylation sites, and a serine rather than asparagine residue in the penultimate position.³⁰⁾ A comparison between ovalbumin and

ovalbumin Y appears to provide some indication for their functions. Considering the similarity in the primary structure between ovalbumin Y and ovalbumin, ovalbumin Y may exhibit similar antigenicity to that of ovalbumin. In addition, the fact that ovalbumin Y has at least two carbohydrate chains of ovomucoid type instead of the single chain in ovalbumin may endow the protein with additional potential for antigenicity. Therefore, significant care should be taken to evaluate the possible contribution of glycans attached to contaminated ovalbumin Y, despite of controversial reports on the role of the carbohydrate moiety of ovomucoid in its antigenicity and allergenicity.^{11,31)} Furthermore, the chimeric nature of ovalbumin Y found in this study gives rise to interesting issues in terms of the biosynthesis of carbohydrate chains, as well as of developing foods with novel functional properties.

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