

Recognition of Native and/or Thermally Induced Denatured Forms of the Major Food Allergen, Ovomucoid, by Human IgE and Mouse Monoclonal IgG Antibodies

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Human sera obtained from children with egg allergy reacted well with both native and heated ovomucoid (OM). Ovalbumin is present in egg white in a 5 times greater quantity than OM; however, it easily aggregates and becomes difficult to extract by heating. For accurate food allergen labeling of processed food, therefore, OM should be evaluated with the determination of egg white protein in consideration of heat denaturation. Three kinds of monoclonal antibodies and sandwich ELISA tests were established which are able to recognize the native and/or heat-denatured forms of OM. The usefulness of these characteristic mAbs and ELISA tests are discussed in relation to allergen labeling, monitoring food processing, and movement or change of dietary protein *in vivo*.

Key words: allergen labeling; food allergy; hen's egg protein; monoclonal antibody; ovomucoid

It is estimated that about 2% of adults and 5–8% of infants are afflicted with IgE-mediated food allergy.^{1,2)} The prevalence of food allergy in infants is one of the most urgent problems to be solved, because it has a bad influence on the normal growth of infants and the quality of life of their families and, in the worst case, frequently develops into atopic dermatitis or asthma by the so-called allergy march mechanism.³⁾ The primary way to prevent food allergy is making patients take as few food allergens as possible. This can be accomplished by hypoallergenization of foods and correct labeling of allergens. Heating is thought to be the simplest and most general method for hypoallergenization. However, food allergy occurs while we usually eat heat-treated foods. This is attributed to the presence of some unchanged or newly formed epitopes by heating, in addition to incomplete heating. For example, the allergenicity of

peanuts *Ara h I* is not weakened by heating,⁴⁾ and the IgE-binding capacity of soy 2S-globulin is strengthened by heating.⁵⁾ Thus, heating is not a complete method for reducing the allergenicity of foods, so that more attention needs to be paid to the determination of heat-denatured food proteins. There is worldwide demand for adequate labeling of foods containing allergenic substances. Since April 2002, mandatory labeling of five foods containing allergic substances (eggs, milk, wheat, buckwheat, and peanuts) has been operating in Japan. In this case, heat-denatured allergen must be properly evaluated as already mentioned. The production of monoclonal antibodies (mAbs) is an appropriate and well-used strategy for stably and precisely assessing the denaturation state of a protein. MAbs specific to some thermally denatured states of β -lactoglobulin⁶⁾ or ovalbumin⁷⁾ have therefore been produced.

Hen's egg is the most frequent cause of food allergy, and approximately two-thirds of children diagnosed with food allergy are reactive to egg white.^{8,9)} Ovomucoid (OM), a glycoprotein with a molecular weight of 28 kDa, is thought to be the major allergen in egg white,¹⁰⁾ despite the existence of a controversial report.¹¹⁾ Since its high allergenicity is associated with its thermal stability, relationship between them has been immunologically discussed.^{12–15)} Uris *et al.* have clinically indicated the predominance of OM as an allergen in egg white and the importance of the allergenicity of heat-treated OM by a double-blind, placebo-controlled food challenge.¹⁶⁾ MAbs specific to OM have been reported, although the specificity concerning the thermal denaturation of OM has not been presented so far.^{17–20)}

We introduce here mAbs that can recognize native and/or thermally induced denatured forms of OM and some examples of their application. They have recently been used to reveal the occurrence of OM as an immune

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Abbreviations: mAb, monoclonal antibody; OM, ovomucoid; N-OM, native OM; H-OM, heated OM; PBS, 10 mM phosphate-buffered saline at pH 7.4; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis

complex with its specific IgA in breast milk.²¹⁾ The establishment of the assay system for total OM, irrespective of the degree of its heat denaturation, described here would provide a new concept for the relevant labeling of foods containing allergens.

Materials and Methods

Materials. The following materials were obtained from the sources indicated: Protein G Sepharose, monoclonal antibody isotyping kit and ECL glycoprotein detection system (Amersham Biosciences Corp., NJ, USA); Maxisorp microtiter plate for ELISA (Nunc, Roskilde, Denmark); and alkaline phosphatase-conjugated goat anti-mouse IgG (ICN, Aurora, Ohio, USA). All other chemicals used were of the highest purity available. Human sera were obtained from allergic children (1–6 years old) with informed consent of their parents. Their RAST values (0.9–57.7 UA/ml) against egg white proteins were determined for the clinical diagnosis.

Preparation of OM and ovalbumin. Native OM (N-OM) and ovalbumin were chromatographically purified. To freshly prepared hen's egg white was added an equal volume of a 0.1 M acetate buffer at pH 3.8, and the mixture was dialyzed overnight against the same buffer. After centrifugation, the resulting supernatant was applied to a column of carboxymethyl-Toyopearl 650 M (Tosoh, Tokyo, Japan). OM and ovalbumin were independently eluted by a linear gradient of 0–0.2 M of NaCl in a 20 mM acetate buffer at pH 4.6. OM was identified by determining the partial amino acid sequence. To prepare heated OM (H-OM), 1 mg/ml of N-OM in 10 mM phosphate-buffered saline (PBS) at pH 7.4 was heated at 100 °C for 30 min. Reductively carboxymethylated OM was prepared according to the method of Mine and Zhang²²⁾ and was chemically deglycosylated by using a commercial kit (Glyco, Novato, CA, USA).

Generation of antibodies. Hybridomas producing monoclonal antibodies were established by fusing splenocytes of BALB/c mice that had been immunized with N- or H-OM and myeloma cells (NSI/1-Ag4-1) essentially as described previously.^{21,23)} Those of interest were cloned twice, and mAbs named after them were prepared from their ascitic fluids and the purified in Protein G-Sepharose columns. Portions of the purified mAbs were biotinylated with EZ-Link sulfo-NHS-LC-Biotin (Pierce, IL, USA).

The rabbits and rats were immunized in the same way as the mice, and antisera were withdrawn 2 weeks after the last boost. 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 (Law No. 105 and Notification No. 6) of the government.

Enzyme-linked immunosorbent assay (ELISA). Three types of ELISA were done essentially as described previously.^{24,25)} For standard ELISA, the wells of a microtiter plate were coated with 2 µg/ml of N- or H-OM in PBS. After blocking the wells with 1% BSA in PBS, the antibody (the culture supernatant of a hybridoma or serum) was added. The antibodies bound to OM were detected with alkaline phosphatase-conjugated second antibodies (anti mouse, rat, or rabbit-IgG or anti human IgE) and *p*-nitrophenylphosphate. Competitive ELISA was carried out in the same manner as standard ELISA, except that the antibody was added to the antigen-coated wells together with different amounts of a competitor. For sandwich ELISA, the wells in a plate were first coated with 2 µg/ml of purified mAb 7D. After blocking, standard OM or a sample was added to the wells. The amount of OM bound to a well was indirectly assayed by using alkaline phosphatase-conjugated streptavidin (Oncogene, Boston, MA, USA) *via* biotinylated mAb 10D, 6H, or 7D. Sandwich ELISA for ovalbumin was constructed with purified rat IgG against native ovalbumin and its biotinylated derivative, like that for OM.

Other methods. SDS-PAGE was performed under reducing conditions with Mini protean II apparatus (Bio-Rad, Hercules, CA, USA). For the western analysis, the proteins in a gel were transferred to a polyvinylidene difluoride membrane with Mini Trans Blot apparatus (Bio-Rad). The protein concentration was determined with Bio-Rad DC Protein Assay kit.

Results

Polyclonal human IgE antibodies against OM

The reactivity of IgE in sera obtained from 18 patients with egg allergy was tested against N-OM, H-OM, and ovalbumin by the standard ELISA method. All the sera showed weak reactivity against ovalbumin (data not shown). On the other hand, the sera with high reactivity to OM (Nos. 1–8) had shown high RAST values against egg-white proteins (Fig. 1), suggesting the importance of OM as the major allergen in egg white. Among the high responders, Nos. 3, 4, 6, and 7 were almost specific to N-OM, while the rest also reacted with H-OM. In particular, Nos. 1 and 8 reacted with H-OM more strongly than with N-OM. This result provides strong evidence to indicate that IgE could be produced against the epitope(s) which is (are) hidden inside of N-OM and appear(s) outside of H-OM by heating at 100 °C for 30 min. Therefore, the heat-denatured forms of allergens must be properly evaluated at the labeling stage of allergens. The antisera obtained from N-OM-immunized rats and rabbits reacted with N-OM more strongly than with H-OM, and those from H-OM-immunized rats and rabbits reacted with H-OM more strongly than with N-OM (data not shown). The extent of N- or H-preference was different for individual sera, and an antiserum specific to N- or H-OM could not be obtained.

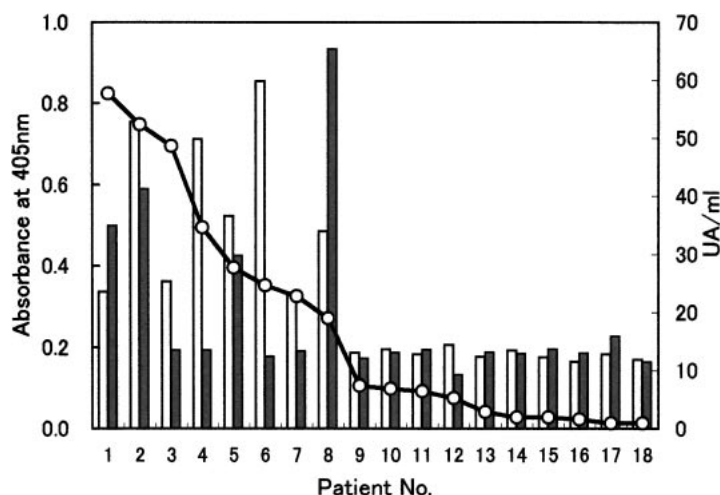


Fig. 1. Reactivity of IgE in Sera Obtained from Patients with Egg Allergy.

The experiments were performed according to the standard ELISA method (see the Materials and Methods section) by using N-OM (open bars) or H-OM (closed bars) as a solid-phase antigen. Human IgE bound to the solid phase was determined with alkaline phosphatase-conjugated anti human IgE. The absorbance at 405 nm was measured for the enzyme reaction. The RAST value of each serum is shown as UA/ml (unfilled circles).

Monoclonal antibodies against N- and/or H-OM and their properties

The failure to get polyclonal antibodies capable of distinguishing between N- and H-OM prompted us to produce mAbs. We finally established 6 hybridoma clones designated 1H, 2H, 5C, 7D, 10D, and 6H from 12 BALB/c mice. The former five were derived from mice immunized with N-OM, while 6H was from one of 6 mice immunized with H-OM. They produced respective mAbs with characteristic reactivity against OM fixed to the assay plate by standard ELISA. MAbs 1H, 5C and 10D reacted with N-OM more strongly than with H-OM, mAbs 2H and 7D reacted equally with both N- and H-OM, and 6H only reacted with H-OM (data not shown). The subclass of all mAbs was IgG1, and the light chain was κ . These mAbs did not cross-react with proteins from such other sources as cow's milk, wheat flour, buckwheat flour, soy bean, peanut, yeast extract and beef, pork or chicken meat in the standard ELISA test (data not shown).

Their reactivity against free OM was further analyzed by the competitive ELISA test, in which the inhibitory effect of the unfixed free antigen on the binding of mAbs to the fixed antigen could be tested (Fig. 2). The preference against free N- and H-OMs observed for all mAbs except mAb 2H was similar to that against fixed OM as already mentioned. However, no competition was apparent against either OM in the case of mAb 2H. Fixation of an antigen to a plastic plate often causes a conformational change by hydrophobic denaturation of the antigen.⁷⁾ It is therefore possible that mAb 2H could recognize a structure appearing only on the surface of fixed OM, but not on the surface of free N- and H-OMs.

OM has a unique structure with 9 disulfide bonds and 4 or 5 carbohydrate chains corresponding to about 25% of its molecular size. Their contribution to the binding of

mAbs was then analyzed by using reductively carboxymethylated OM and deglycosylated OM in a western analysis. As shown in the SDS-PAGE data (Fig. 3-A), reductively carboxymethylated OM gave a slightly higher molecular size than OM due to irreversible cleavage of the disulfide bonds which made OM adopt a tightly folded conformation. A similar result has been reported with OM of Japanese quail.¹⁹⁾ Due to the reactivity against reductively carboxymethylated OM, it seems unlikely that mAbs 7D and 6H could recognize conformation of N-OM (Figs. 3-C and -D). Deglycosylation made OM 5–7 kDa smaller, this being confirmed by glycostaining (Figs. 3-A and -B). Gu *et al.* have reported that 75 mol% of the carbohydrate moiety was removed from OM by the same method.¹⁸⁾ The epitope of mAb 7D is clearly shown in Fig. 3-C to be a carbohydrate moiety of OM by the lack of reactivity only against deglycosylated OM. This result is compatible with the impartial binding capacity of mAb 7D to both N- and H-OMs, since the carbohydrate moiety with high hydrophilicity must be exposed outside of OM, irrespective of heat treatment. The positive reactivity of mAb 6H only to H-OM by ELISA and to all OM used in the experiment of Fig. 3 indicates that mAb 6H recognized a sequential epitope that was hidden inside of N-OM and appears outside of H-OM by heating. MAbs 1H, 2H and 10D seem to react with conformational epitopes of OM because of their weak or negative reactivity against all OM used in the western analysis (data not shown).

MAb 6H only reacted with OM among the hen's egg white proteins by the western analysis, although mAb 7D reacted with two other minor proteins, suggesting the presence of proteins with a similar carbohydrate moiety to that of OM but a different amino acid sequence (data not shown). One is probably a riboflavin-binding protein

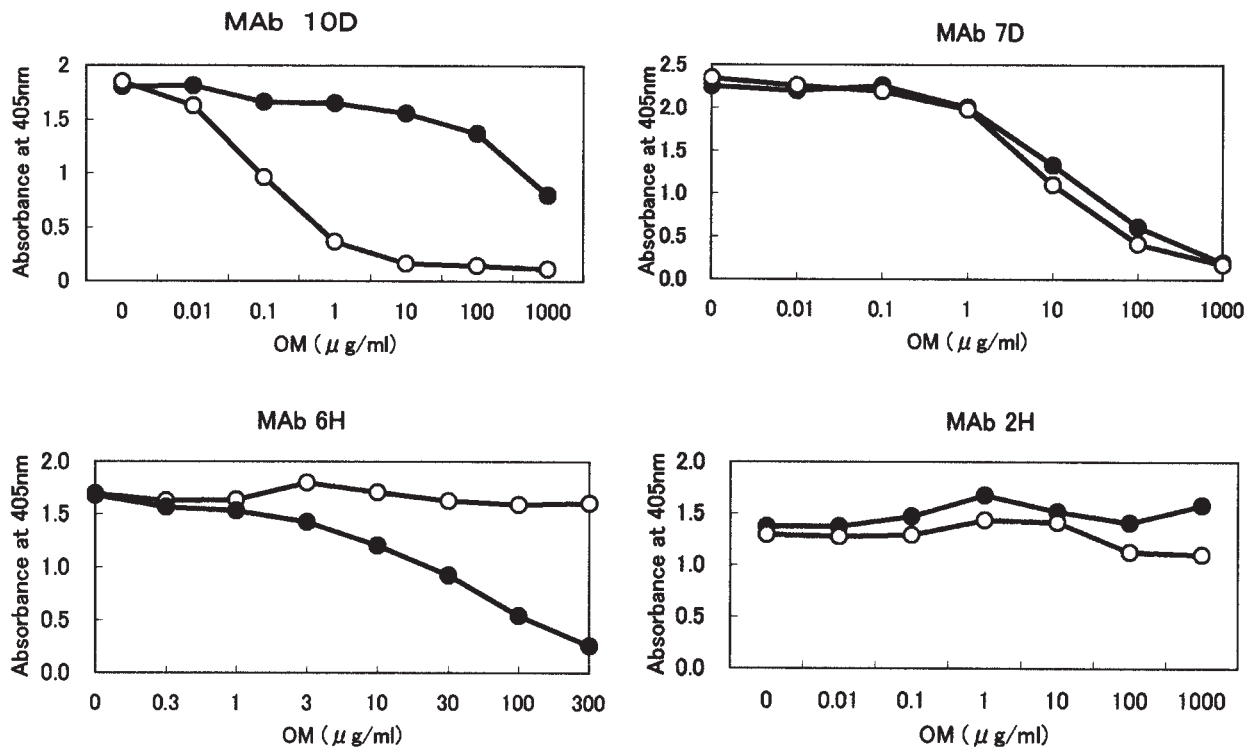


Fig. 2. Characterization of mAbs against OM by Competitive ELISA.

The experiments were performed according to the competitive ELISA method (see the Materials and Methods section) in the presence of various amounts of N-OM (unfilled circles) or H-OM (filled circles) as a competitor. The culture medium of each clone was used as an antibody. N-OM was used as a solid-phase antigen in the experiments with mAbs 1H, 2H, 5C, 10D, and 7D, while H-OM was used in that with mAb 6H. Since similar results were obtained with mAbs 1H, 5C, and 10D, the result with mAb 10D was shown as a representative.

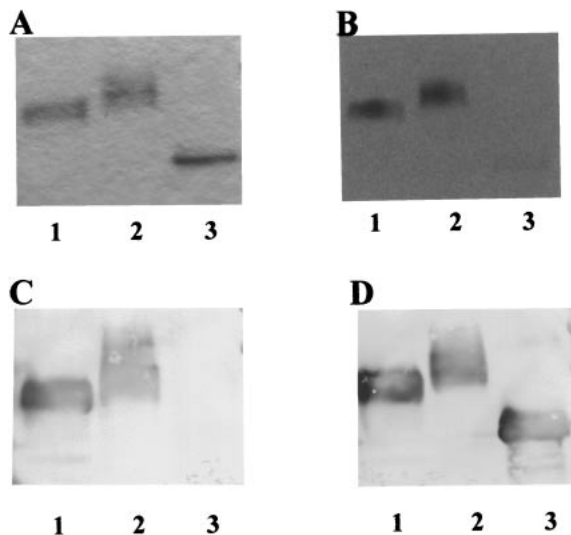


Fig. 3. Epitope Analysis of mAbs against OM.

One μg each of N-OM (lane 1), reduced and carboxymethylated OM (lane 2), and chemically deglycosylated OM (lane 3) was subjected to SDS-PAGE under reducing conditions. OM was stained directly with Coomassie Brilliant Blue in panel A. After blotting on a polyvinylidene difluoride membrane, OM was stained with a glycoprotein detection kit (panel B) or immunologically stained with mAbs 7D (panel C) and 6H (panel D).

because of its yellow color,²⁶⁾ while the other is an unknown protein with a molecular weight of about 50 kDa (under investigation). In contrast, mAb 6H also

reacted with duck and quail OM, while mAb 7D did not. This is thought to have been caused by the heterogeneity of the carbohydrate moiety of each OM.²⁷⁾

Application of mAbs

Egg white proteins are frequently added to processed foods to improve their functional properties, which often triggers an allergic reaction. MABs 6H and 7D did not cross-react with proteins from other sources, as just described, nor by the western analysis (data not shown). They are therefore suitable for checking the contamination or addition of egg proteins into processed foods. As shown in Fig. 4 (lanes 3–8) for example, OM was clearly detected only in commercial ham with labeling of egg usage by the western analysis with mAb 6H. The appearance of protein bands of higher molecular weights implies the aggregation of OM itself or with other proteins during processing. It is notable that the aggregate was stable, even after boiling for 5 min in the presence of 5% β-mercaptoethanol and 2% SDS. Although Kato *et al.* have reported reductive allergenicity for the aggregates of OM with wheat proteins,²⁸⁾ the allergenicity of those described here is unknown. If mAb 7D is used instead of mAb 6H, the carbohydrate moiety-specific or hen's OM-specific profile could be obtained.

A western analysis is useful for a confirmative analysis as the secondary screening for allergen detec-

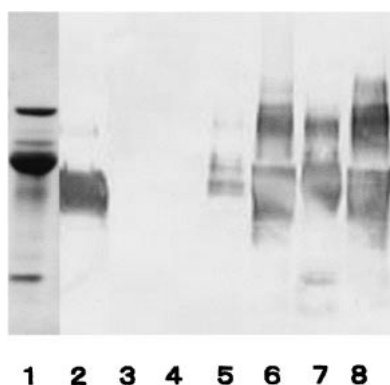


Fig. 4. Detection of OM in Commercial Ham by a Western Analysis with mAb 6H.

Commercial ham (0.5 g) was mashed in one ml of PBS with a mortar and a pestle. After centrifuging at 13,000g for 10 min, the supernatant was collected, and 5 mg of protein was subjected to a western blotting analysis (lanes 3–8) with mAb 6H. Lane 1, Coomassie Brilliant Blue staining of egg-white proteins (15 µg); lane 2, purified OM (1 µg); lanes 3 and 4, ham samples without labeling of egg usage; lanes 5–8; ham samples with labeling of egg usage.

tion. On the other hand, sandwich ELISA is suitable for a quantitative analysis with high sensitivity or as the first screening which is necessary to handle many samples at once. We therefore developed sandwich ELISA systems capable of distinguishing N-OM and/or H-OM. MAb 7D was selected as the first antibody because of its binding capacity to both N- and H-OMs. Each biotinylated mAb was used as the second antibody, and OM that bound to the plate *via* mAb 7D was assayed by alkaline phosphatase-conjugated streptavidin. The property of each sandwich ELISA was dependent on that of the biotinylated second antibody (Fig. 5). In the case of biotinylated mAb 10D, the 10D/7D system was about 30 times more sensitive to N-OM than to H-OM. In the case of biotinylated mAb 6H, however, the 6H/7D system was almost specific to H-OM. Several ng/ml of N- or H-OM could be selectively detected by each system. Although it is not usually possible to set up sandwich ELISA with a single mAb, it was successful in the case of mAb 7D because of the 4 or 5 carbohydrate chains per molecule of OM. The 7D/7D system showed equal reactivity against N- and H-OMs, indicating the possibility of determining the total OM content irrespective of the degree of heat denaturation of OM. The detectable limit for the 7D/7D system was about 100 ng/ml.

The time-course characteristics of the heat denaturation of OM at 100 °C were then precisely monitored by using these three sandwich ELISA systems (Fig. 6). Total OM measured by the 7D/7D system was unchanged as expected. The increase in H-OM (appearance of the epitope for mAb 6H) proceeded somewhat faster than the decrease in N-OM (disappearance of the epitope for mAb 10D). This discrepancy might have been caused by the independence of the epitopes of

mAbs 10D and 6H.

The 7D/7D system was applied for the determination of OM in cooked and processed foods (Table 1). Ovalbumin was also determined in a comparative study. Raw egg white generally contains about 1% of OM and 5% of ovalbumin, and this ratio was also shown by our methods. Providing that the weight ratio of egg white and yolk is 2:1 and that the recovery is 100%, the OM and ovalbumin contents in egg soup prepared according to the legend to Table 1 can be expected to be 1,300 and 6,500 µg/g, respectively. The value determined for OM (1,280 µg/g) in Table 1 is quite reasonable, but that for ovalbumin (4 µg/g) is incredibly low. This was caused by the fact that ovalbumin had been removed by filtration as an aggregate, but OM is non-aggregable, and that although our 7D/7D system can determine the total OM content irrespective of the degree of heat denaturation of OM, the antibody used for ovalbumin determination was prepared against the native antigen. The aggregation of egg white is used for removing harshness or for clarification of a liquid-type of food. Therefore, OM can sometimes be detected in a superior grade of soup or wine. Furthermore, egg white is widely used as an additive to processed foods for improving their texture, taste and nutritional value. We can monitor egg white usage in these foods by the 7D/7D system with a lower risk of extraction efficiency and heat denaturation. Table 1 summarizes the data obtained from commercial foods with labeling of egg usage. OM and ovalbumin in commercial foods without labeling of egg usage were undetectable (data not shown).

Discussion

OM is thought to be one of the most potent allergenic food proteins because it is indigestible and thermally stable. However, the reasons are sometimes misunderstood. First, quail OM inhibits human trypsin, but hen's OM does not.²⁹⁾ OM is difficult to be digested because of the 4 or 5 carbohydrate chains and 9 disulfide bonds in its polypeptide chain. Second, OM does not aggregate by heating and H-OM still expresses allergenicity.¹⁶⁾ The reversible conformational change of OM during heat treatment has been analyzed,^{30,31)} however, this does not mean that OM is native even after heating. As we have shown in this paper, the establishment of mAbs specific to N- or H-OM provides strong evidence for the presence of an irreversibly heat-denatured form of OM. The selective IgE binding to H-OM seen in Fig. 1 reflects the production of IgE specific to the epitope(s) newly appearing on the surface of OM by heating. There thus exist at least two immunologically distinct forms of OM, and both are allergenic in heat-treated foods containing egg white. Therefore, they need to be suitably evaluated.

In order to prevent the occurrence of health hazards caused by foods containing allergens, the Joint FAO/WHO Codex Alimentarius Commission Session agreed

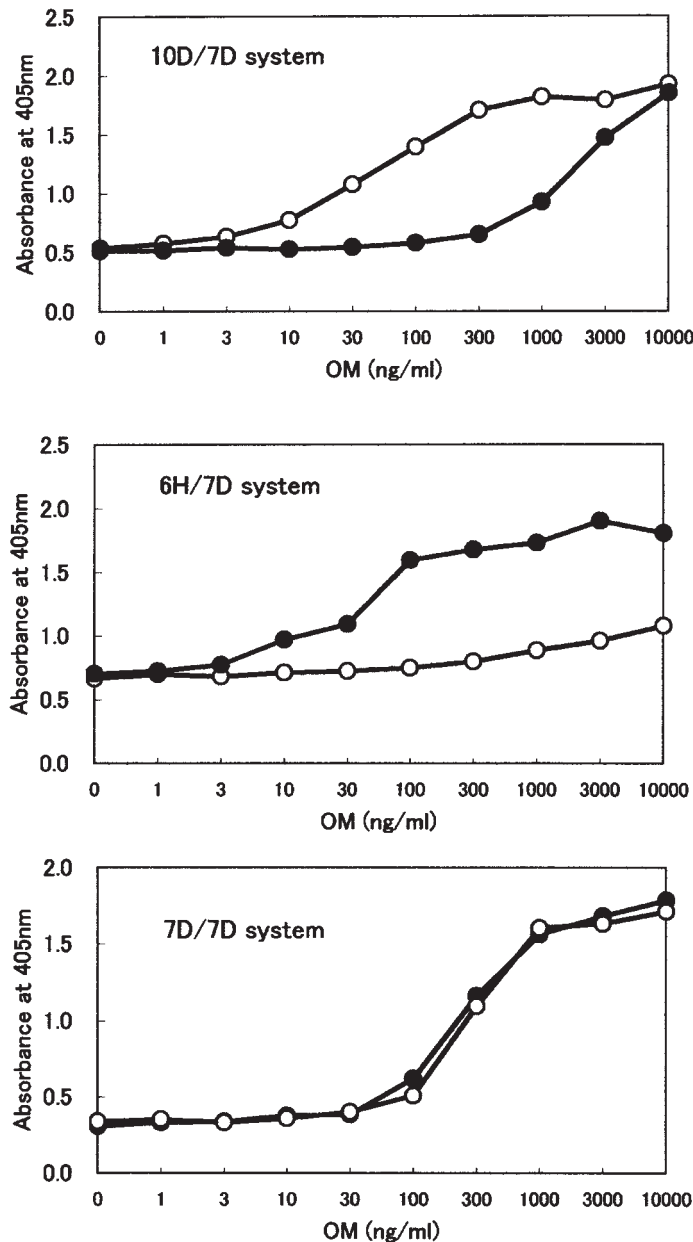


Fig. 5. Sandwich ELISAs for the Quantitative Analysis of N- and H-OMs.

Three sandwich ELISAs were constructed with mAb 7D as a common solid-phase antibody and biotinylated mAb 10D, 6H or 7D as a second antibody in the 10D/7D, 6H/7D, or 7D/7D system, respectively. N-OM (unfilled circles) and H-OM (filled circles) were used as standards, and the amount of OM bound to a well was assayed by alkaline phosphatase-conjugated streptavidin.

in 1999 to include eight kinds of food in allergen labeling. The mandatory labeling of five specified ingredients started from 2002 in Japan. Two types of sandwich ELISA using polyclonal antibodies have been notified by Ministry of Health, Labour and Welfare of Japan to comply with the requirements of this labeling. However, further improvements of these methods have been requested in connection with scientific progress. The 7D/7D system described in this paper has characteristic and superior benefits for monitoring egg protein in foods. First, OM is more suitable as the target than ovalbumin because of its strong allergenicity (Fig. 1) and good extraction efficiency (Table 1). Second, the

7D/7D system enables us to determine the total OM content, irrespective of the degree of heat denaturation of OM (Fig. 6). Third, the method is very stable in specificity and quantity due to the nature of mAb. These properties of the 7D/7D system will provide important information for establishing a common international method for allergen determination.

Besides antigen determination, many analyses such as following the heating history of foods by the three sandwich ELISA methods, secondary confirmation analysis of allergen detection by western blotting, and discrimination of birds' eggs have become possible by using these mAbs. We have previously revealed the

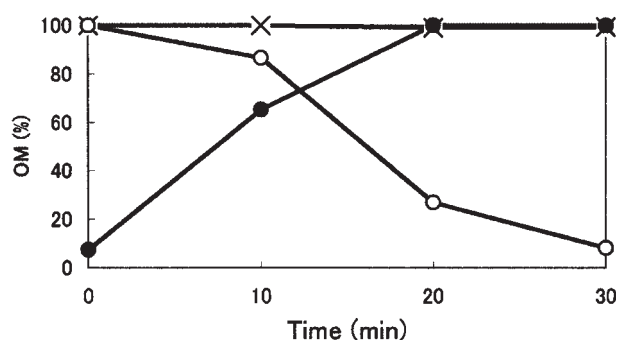


Fig. 6. Monitoring the Heat Denaturation of OM by Using Three Sandwich ELISAs.

N-OM (1 mg/ml) in PBS was heated at 100°C. Aliquots (0.1 ml each) were removed at the times indicated and poured into 0.9 ml of PBS in an ice bath. OM was determined by the three sandwich ELISAs described in Fig. 5. N-OM in the 10D/7D (unfilled circles) and the 7D/7D (×) systems or H-OM in the 6H/7D system (filled circles) was used as a standard. Values are expressed as percentage of 1 mg/ml.

Table 1. Determination of OM and Ovalbumin in Food Samples

Mixed whole egg (60 g) was quickly dispersed into 300 ml of boiling water. After boiling for 1 min, the aggregate was removed by filtration, and the filtrate was used as egg soup. One gram each of commercial Chinese noodles, ham, kamaboko (semicylindrically molded fish paste) or chikuwa (bamboo-like molded fish paste) was mashed in 2 ml of PBS with a mortar and a pestle. After centrifuging at 13,000g for 10 min, the supernatant was used as an extract. OM was determined with the 7D/7D system. Ovalbumin was determined by sandwich ELISA constructed with purified rat IgG against native ovalbumin and its biotinylated derivative, like that for the 7D/7D system. Each value is expressed as µg/g.

Food sample	Ovomucoid	Ovalbumin
Raw egg white	10,300	50,000
Egg soup	1,280	4
Canned soup	140	ND
Chinese noodle	184	486
Ham	952	814
Kamaboko	130	3
Chikuwa	226	50

ND: not detected.

occurrence of OM in human breast milk as an immune complex with specific IgA by using mAbs 10D and 7D.²¹⁾ We can now suggest, after learning about their epitopes, that OM is absorbed, transported, and then secreted into breast milk as it holds a rather native structure and carbohydrate moieties. Furthermore, a new protein with the OM type of carbohydrate chain have been discovered in hen's egg white by using mAb 7D, details of which will be reported in the near future. MAbs 7D, 6H and 10D each have characteristic specificity, and an individual polyclonal antibody is a mixture of such mAbs in different ratios. Demand for the establishment of a series of mAbs to an antigen with definite epitopes or specificity will greatly expand hereafter.

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