Decrease in Antigenic and Allergenic Potentials of Ovomucoid by Heating in the Presence of Wheat Flour: Dependence on Wheat Variety and Intermolecular Disulfide Bridges

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The antigenic and allergenic activities of ovomucoid (OM) remaining in soluble fractions of pasta-like model samples of wheat flour mixed with egg white were investigated by ELISA competitive inhibition and immunoblotting analyses using a rabbit anti-OM IgG and the serum IgE specific for OM in patients allergic to egg white. The mixture of egg white and wheat flour of soft, hard, and durum varieties was kneaded for 10−50 min and benched for 1 h at RT, and then small pieces of the dough were heated in boiling 1% NaCl solution for 15 min. Even before heating, only after the kneading for 30 min or more, but not after kneading for only 20 min, followed by the benching, the antigenic activity of OM which remained in the phosphate-buffered saline extract from the dough markedly decreased. Almost no antigenic activity of OM was detected in the extracts of heated samples. Furthermore, in the extracts of heated durum samples, only a trace of or almost no IgE-reactive OM was detected against the five patients' sera. These reductive effects of wheat on the OM antigenicity and allergenicity were more remarkable in the durum variety than in the others. No detectable proteins were extracted with 1% SDS from the heated samples, whereas OM was extracted with 1% SDS containing 10% 2-mercaptoethanol, suggesting heat-induced polymerization through intermolecular disulfide bonds among OM and wheat.

Keywords: *Egg white; ovomucoid; wheat flour variety; pasta; reductive allergenicity; reductive antigenicity, intermolecular disulfide bridges*

INTRODUCTION

Egg white is frequently used for various processed foods as an ingredient to improve the nutritional value and functional properties. A typical example is the case of pasta, in which egg white is added to wheat flour mainly to improve the rheological properties of cooked pasta products. Although egg white protein exhibits excellent functional properties as a food ingredient, it also has a negative property against a certain number of the population, especially babies and infants, as a major causative component in food allergy.

Antigenic and allergenic properties of egg white proteins have been intensively investigated from the viewpoints of food science, immunology, and clinical allergology (*1*−*4*). Among the major egg white proteins, ovomucoid (OM) has been identified as a main causative antigen in egg allergy and this strong allegenicity is believed to depend on the stability of OM against denaturation, aggregation, and proteolytic degradation (*5*−*9*).

A recent clinical study by Urisu et al. (*10*) using the double-blind, placebo-controlled, food challenge with heated and ovomucoid-depleted egg white indicated that sufficient heat treatment markedly reduced the ability to induce immediate type hypersensitive reaction to the egg white and that the subsequent washing treatment to remove residual soluble proteins, mostly ovomucoid, almost completely eliminated the clinically relevant allergenicity of the heated egg white. These clinical data suggest that the aggregated insoluble proteins in the heated egg white cause allergic symptoms much less frequently than soluble ones on the oral challenge; in other words, the soluble proteins remaining in the heated egg white were the major causative component responsible for the immediate type reaction.

The denaturation and aggregation of egg white proteins by heating has been investigated for isolated proteins, egg white, and whole egg. Many data have shown that isolated OM is not aggregated and precipitated by heating. Furthermore, OM was reported to remain soluble and retain the reactivity to patients' IgE in heat-coagulated egg white even after boiling for 1 h (*11*). Thus, heat-induced changes in antigenic and allergenic activities of OM in egg white are well characterized. However, little is known concerning changes in such immunological properties of OM heated in the presence of the other food proteins as in the cases of various processed foods.

In the present study, we investigated the heat-induced insolubilization of OM, as measured by its antigenic and allergenic activities, in the egg white mixed with wheat flour as a model of pasta. By heating the model pasta OM was effectively insolubilized, and almost no antigenic activity of OM was detected for not only rabbit IgG but also human IgE antibodies probably

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due to aggregation through intermolecular disulfide bonds with wheat proteins. The effect of kneading time on the OM insolubilization upon heating was also demonstrated. Such effective insolubilization of egg white OM by heating with wheat flour may lead to a decrease in the allergenic potential of egg white contained in pasta.

MATERIALS AND METHODS

Preparation of Pasta. The wheat flours used were commercially milled durum semolina, hard wheat flour, and soft flour provided by Nisshin Mills Co., Ltd.

The pasta ingredients (65 g of flour, 30 g of raw egg, 4 g of olive oil, and 1 g of NaCl) were kneaded for 10 to 50 min on a board. The mixed pasta ingredients were benched for 1 h at room temperature (RT), resulting in dough formation. The dough was extended and cut into noodle form of 0.1 × 0.2 × 15 cm, and boiled in 40 mL of 1% NaCl solution for 15 min.

Extraction of Soluble Proteins from Pasta Samples. Samples of dough and pasta (500 mg) were suspended in 1 mL each solution of phosphate-buffered saline (PBS, pH 7.4), 4% sodium dodecyl sulfate (SDS), and 4% SDS plus 10% 2-mercaptoethanol (2-ME). The solution and sample were homogenized with a vortex mixer (Vortex Genie Scientific Industries, Inc., Bohemia, NY) for 1 min at the maximum speed, and subsequently ultrasonicated for 15 min (100 w, 39 kHz) on ice. The suspended samples were kept overnight at 4 °C and centrifuged at 16000*g* for 20 min to remove insoluble materials. The SDS or SDS/2-ME extract mixed with an equal volume of SDS−PAGE sample buffer was heated at 95 °C for 5 min and directly used for the SDS−PAGE analysis. The PBS-soluble fraction and NaCl solution in which the pasta had been boiled was used as soluble protein fractions for the analyses of ELISA competitive inhibition as described below.

Patient Sera. The sera with IgE against egg white (RAST-score 2 to 5) were selected from sera of patients with known clinical histories of egg allergy, and the five sera with high titer of IgE antibody to ovomucoid were further selected by ELISA using purified OM as antigen. Informed consent was obtained from the patients and/or their parents.

Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting. First, the proteins were separated by SDS-gel electrophoresis using 12.5% acrylamide gel according to the method of Laemmli (*12*). One sheet of the gel was stained with Coomassie Brilliant Blue (CBB) R-250, and another gel was used for immunoblotting. The proteins separated by the electrophoresis were transferred electrophoretically onto a nitrocellulose sheet (0.45-μm, Advantec Toyo, Tokyo) by the method of Towbin et al. (*13*). The nitrocellulose sheet was incubated overnight at 4 °C in 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS, pH 7.5) containing 0.02% Tween-20 (TBST). After being washed with TBST, the sheet was incubated at 37 °C for 2 h in 5 mL of 1% BSA containing 5 μL of a rabbit antiserum specific for OM. The antiserum for OM was prepared as described previously (*14*). After being washed with TBST, the sheet was incubated at 37 °C for 1 h in peroxidase-coupled goat anti-rabbit immunoglobulin G (Cappel Laboratories, Aurora, OH) appropriately diluted with 1% BSA/TBST. After washing with TBST, the protein bands with reactivity to the specific antiserum were visualized by activity staining for peroxidase using 4-chloro-1-naphthol (Bio-Rad, Hercules, CA).

ELISA Competitive Inhibition. The antigenic activity of OM in the PBS extract from each pasta sample was measured by the ELISA competitive inhibition analyses (*15*) using the rabbit antiserum to OM. Flat-bottomed ELISA plates were coated with OM solution (1 μg/mL in PBS). As competitors, 75 μL of the PBS extract, diluted serially with PBS, was mixed with the same volume of the antiserum diluted (1000 ×)with PBS, incubated overnight at 4 °C, and added to the wells of the ELISA plates. The rabbit anti-OM serum, which reacted with the plate-bound OM, was determined using peroxidase-coupled goat anti-rabbit immunoglobulin G (Cappel Laboratories, Aurora, OH), as described previously (*16*).

Extraction of PBS-, EtOH-, and Alkaline-Soluble Proteins from Wheat Flour. First, salt-soluble proteins were extracted with 0.5 mL of PBS from 0.1 g of each wheat flour sample (durum semolina, hard flour, and soft flour). The wheat flour was suspended in PBS, kept overnight at 4 °C, and centrifuged at 16000*g* for 20 min. The supernatant was separated and the precipitate was re-suspended with 250 μL of PBS. The soluble proteins were extracted from the precipitate in the manner described above. The extraction with PBS from precipitate was repeated once more. These three supernatants containing the salt-soluble proteins were pooled and kept at −20 °C before use. Next, alcohol-soluble proteins were extracted with 70% ethanol 3 times in the same manner as above, and the supernatants were pooled and kept at −20 °C before use. Finally, from the resultant precipitate, alkaline-soluble proteins were extracted 3 times with 2% Na_2CO_3/0.1 mol/L NaOH in the same manner as above. The protein concentration in each extract was determined by the method of Lowry et al. (*17*).

RESULT AND DISCUSSION

Change in OM Antigenicity During the Process of Pasta-Making. The egg white/wheat flour mixture as a model of pasta was prepared with hand kneading for 50 min on a board and benched for 1 h at RT. Antigenicity of OM in the PBS extract from the dough samples kneaded for 0−50 min and from the benched

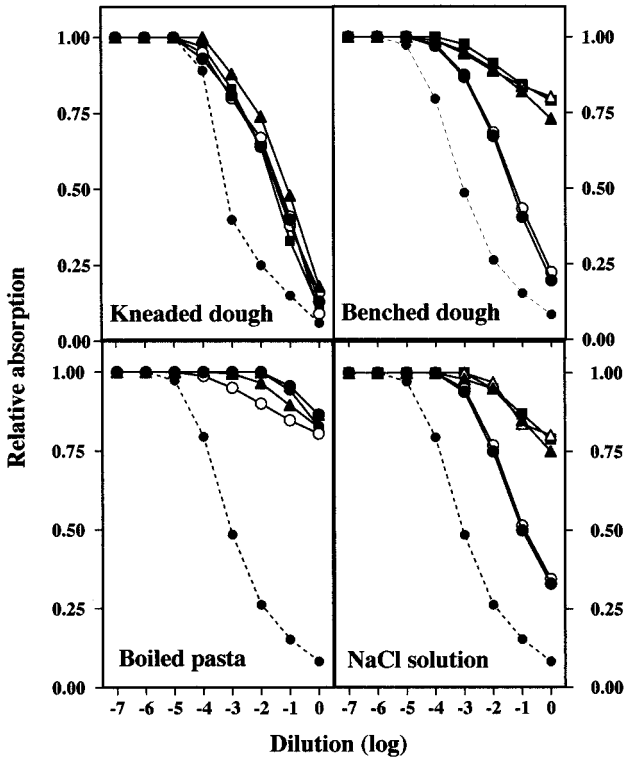

Figure 1. ELISA competitive inhibition analysis for OM antigenic activity change in kneaded dough, kneaded/benched dough, boiled pasta samples, and NaCl solution using rabbit anti-OM serum. The pasta ingredients containing egg white were kneaded for 10(●), 20(○), 30(▲), 40(△), or 50(■) min, and these kneaded dough samples were each benched for 1 h at RT. Each benched dough sample was extended and cut into noodle form and heated in 1% NaCl solution. Applied samples were extracted with PBS (1 mL) from 0.5 g of each dough and pasta sample and the NaCl solution. EW solution (dotted line) was prepared by replacing other ingredients in the dough with the same amount of PBS. The relative absorbance value is given as 1.0 against an absorbance value of 0.917 at 492 nm.

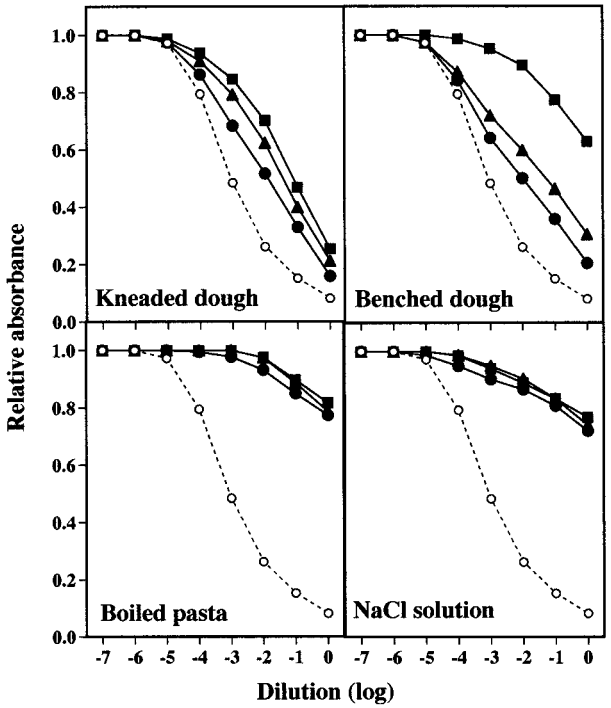

Figure 2. Comparison of OM antigenic activities in the processes of pasta-making with three kinds of wheat varieties using rabbit anti-OM serum. Pasta containing egg white was prepared with three kinds of flour: soft (●), hard (▲), and durum (■). The dough was kneaded for 30 min and benched for 1 h at RT. Applied samples were extracted with PBS (1 mL) from 0.5 g of each dough and pasta sample and the NaCl solution. EW solution (dotted line) was prepared by replacing other ingredients in the dough with the same amount of PBS. The relative absorbance value is given as 1.0 against an absorbance value of 0.917 at 492 nm.

dough was examined by ELISA competitive inhibition using the rabbit anti-OM serum (Figure 1). The concentration of antigenic and PBS-soluble OM was estimated based on a standard curve of pure OM obtained under the same ELISA condition. OM extracted with PBS from the pasta sample before kneading was estimated to be 75 μg/mL. The OM concentration in the PBS extract from the kneaded dough was slightly decreased with kneading time and after 50 min of kneading decreased to about 3%. Benching the dough for 1 h after the kneading remarkably insolubilized OM in the dough samples, that were kneaded for 30 min or more; though benched dough samples kneaded for 20 min or less markedly inhibited the OM and the antibody binding. These results clearly indicate that some structural and/or physicochemical changes were indicated for OM mixed with wheat flour by kneading for the longer time followed by benching.

The benched dough samples were cut into small pieces (0.1 × 0.2 × 15 cm) and 20 g of this pasta was boiled in 40 mL of 1% NaCl solution for 15 min. The boiled samples were separated from the NaCl solution, and the remaining NaCl solution was completely removed from the boiled pasta by being absorbed with pieces of filter paper. Soluble proteins were then extracted from the boiled pasta (0.5 g) with PBS (1 mL). The NaCl solution in which the pasta had been boiled was diluted three times and used for analysis. OM concentrations in these salt solutions were determined as described above (Figure 1). In ELISA inhibition, the boiled pasta samples, which had been kneaded for 10 to 50 min, showed no influence on the binding reaction between anti-OM

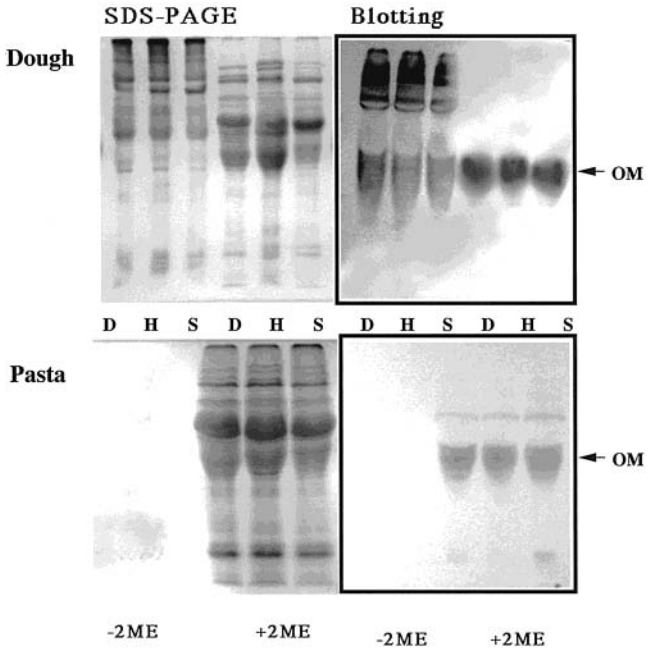

Figure 3. SDS−PAGE and immunoblotting patterns of SDS and SDS + 2ME soluble proteins extracted from benched dough and boiled pasta. Samples were prepared from durum (D), hard (H), and soft (S) flours. Applied samples were extracted with 4% SDS (−2ME) or 4% SDS plus 10% 2-ME (+2ME) from dough or heated pasta samples.

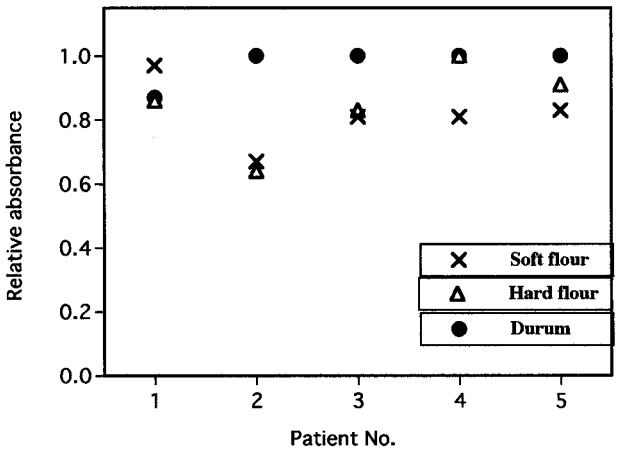

Figure 4. ELISA competitive inhibition analysis for OM allergenicity in boiled pasta samples using anti-human IgE. The pastas were prepared from soft, hard, and durum flours, and applied samples were extracted with PBS (1 mL) from 0.5 g of each boiled sample. Relative absorbance value is given as 1.0 against an absorbance value of each patient's specific IgE for OM at 492 nm.

antibody and OM, and the kneading time of dough little affected the binding activities. The OM concentration in the NaCl solution used for boiling the pasta was also estimated by the ELISA inhibition analysis. The solution used for boiling the pasta kneaded for 20 min or less contained a trace amount of OM (about 1% of the OM of the added to the pasta). On the other hand, OM was scarcely detected in the NaCl solution in which the pasta kneaded for 30 min or more had been boiled.

OM is highly heat-stable and can be extracted with salt solution even from hard-boiled shell eggs (*11*). It is reasonable to presume that OM was insolubilized in the pasta kneaded for longer than 30 min because OM was not detected in either the boiled pasta or the NaCl solution used for boiling.

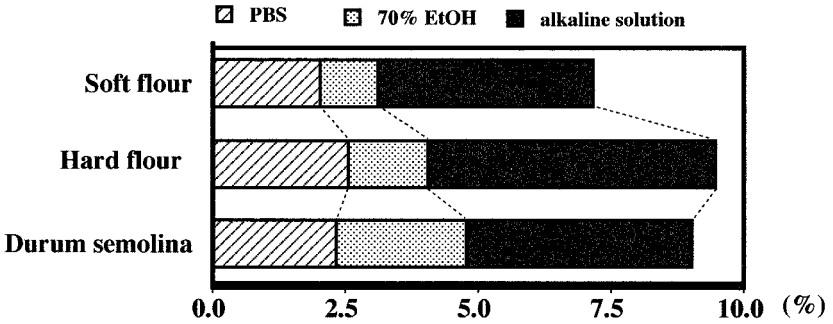

Figure 5. Comparison of protein contents in PBS, 70% EtOH, and alkaline solutions extracted from soft, hard, and durum flours.

Comparison of OM Antigenicity in Pasta Samples Prepared from Wheat Flour Varieties. To estimate whether the varieties of wheat flour affect the heat-induced changes in OM antigenicity and solubility, soft and hard flour samples were also examined. The other ingredients for pasta and the experimental conditions were the same as those for the durum pasta making, i.e., the pasta was made by the process of 30-min kneading, 1-hr benching at RT, and 15-min boiling in 1% NaCl solution. OM antigenicities in dough samples after kneading and after kneading/benching were compared among the three kinds of wheat varieties (Figure 2). The antigenicities of OM in the PBS extracts from soft, hard, and durum flour dough samples were estimated to be about 1/15, 1/30, and 1/100 of that of the egg white without wheat flour as the control solution, respectively. The effect of benching time on the antigenicity of OM was also examined. The OM antigenicity was hardly detectable in the PBS extract of the dough prepared from durum flour after benching, whereas the antigenicity in those of the benched doughs prepared from soft and hard flours were not changed during benching. The OM antigenicity was detected in none of the boiled pasta samples or the NaCl solutions used for pasta boiling. The boiled pasta prepared from soft and hard flours also made OM insoluble similarly to that of the pasta prepared from durum, although soluble OM was detectable in the benched doughs prepared from soft and hard flours.

Because PBS-extractable OM antigenicity of the wheat flour mixed with egg white was markedly decreased by heating compared to that of egg white alone, homologous or heterogeneous intermolecular interaction was expected to occur during the processes including kneading and boiling. To examine the intermolecular interaction through hydrophobic and covalent bonds, proteins were extracted with 4% SDS and 4% SDS containing 10% 2-ME from the benched dough and boiled pasta samples and analyzed by SDS−PAGE and immunoblotting using the rabbit anti-OM serum (Figure 3). A large amount of polymerized proteins was detected by CBB staining in the SDS extracts from the dough samples. In the dough samples, OM was polymerized with wheat proteins and/or OM and they reduced with 2-ME as shown in the immunoblotting. There were no detectable proteins in SDS extracts from boiled pasta samples, whereas many proteins were stained by CBB in the SDS+2ME extracts from them. Furthermore, one of the major protein bands defined as OM was detected with the specific antibody in the SDS+2ME extracts. Thus, reducing agents in addition to a detergent are required for the solubilization of OM in boiled pasta samples. It is suggested that OM which interacted with wheat proteins was polymerized in the kneading/benching process and was insoluble with a disulfide exchange reaction in the process of heating.

The inhibition activities of PBS-soluble proteins from boiled pasta samples prepared from the varieties of flours were compared by competitive ELISA analysis using the sera from 5 patients with egg allergy (Figure 4). Their inhibition activities were estimated against the binding between OM and five patient-specific IgE to OM. The inhibition activities of PBS extracts from the three boiled-pasta samples were different from each other. The allergenicity of OM in the boiled pastas prepared from soft and hard flours remained from 35 to 20%, respectively. The PBS extract from boiled durum pasta had no effect on the binding of IgE to OM without one patient IgE.

The different OM antigenic or allergenic activities in three kinds of boiled pasta were possibly dependent on protein concentrations of, and/or different protein components in, their flour varieties. The protein concentrations in the PBS, 70% EtOH, and alkaline solutions extracted from the three kinds of flour were determined (Figure 5). The sum protein content of three extracts in hard flour was the highest, but the protein content in the 70%-EtOH extract from durum flour was higher than the protein contents in the extracts from the other types of flours. The protein contents (% of total protein) of the 70% EtOH extract were 26.5, 15.8, and 14.9% for durum, hard, and soft flours, respectively. Therefore, such a higher protein content, as well as the amount of the 70%-EtOH extract (gliadin) might be ascribed to the effective inactivation of OM allergenicity in the pasta. The gliadin content (26.5%) estimated in the present study was a little lower that the values (34−37%) determined by the Kjeldahl method in an earlier report (*18*). The lower value is due to the analytical method used in the present study.

The disulfide exchange reaction takes place during dough formation after kneading of the wheat flour/water mixture, and a small amount of thiol group in wheat protein is a dominant factor for the reaction. It is suggested that a disulfide exchange reaction may occur between ovomucoid and gliadin and/or glutenin. Whereas the role of gliadin is not clear in the exchange interaction, durum flour contains more gliadin than the other types of flour. We had reported that baked bread containing egg white induced irreversible denaturation of OM, with a disulfide exchange reaction leading to the loss of its antigenic activity (*19*). The reaction occurs in a mixture of gluten and egg white, but not in a mixture of casein and egg white (*20*). Some processed foods using wheat flour mixed with egg white make possible hypoallergenic foods for patients allergic to egg, if they

reproduce no antigenicity after enzyme digestion. Studies on the in vitro digestion of bread and pasta containing egg white are now in progress.

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