

Degradation of wheat allergen in Japanese soy sauce

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Abstract. Soy sauce is a traditional fermented seasoning of East Asian countries and is available throughout the world. Wheat and soybeans are the 2 main raw materials of soy sauce, and soy sauce also contains a high concentration of salt. Since wheat allergy is considered a serious problem globally, it is significant to examine the allergenicity of soy sauce. In this study, by immunoblotting, inhibition ELISA and direct ELISA using sera from 5 children with wheat allergy, it was clearly demonstrated that wheat allergens were degraded into amino acids and peptides losing the IgE-binding ability in both salt-soluble and salt-insoluble fractions of soy sauce during fermentation. Furthermore, no wheat allergen was detected in 10 items of commercial soy sauce in Japan, by inhibition ELISA or direct ELISA using the sera of patients. In the brewing process of soy sauce, first salt-insoluble wheat allergen was solubilized to salt water during the *koji* stage (mold cultivation and enzyme production), and second both the resultant salt-solubilized and initially salt-soluble wheat allergens were completely degraded during the *moromi* stage (fermentation) by microbial proteolytic enzymes. Therefore, it was concluded that no wheat allergen is contained in soy sauce.

Introduction

IgE-mediated hypersensitive responses to wheat have long been emerging as an important public health problem (1). α -amylase inhibitor from the salt-soluble fraction of wheat flour and other proteins in the salt-insoluble fraction were identified as allergens (2-7), and their IgE-binding epitope was also determined (8-10). Generally, wheat proteins are comprised of salt-soluble and salt-insoluble fractions, both of which are likely responsible for the IgE-mediated allergy to wheat (6,11).

Soy sauce has been traditionally used in Japan and several oriental countries as a liquid seasoning and is currently used

in cooking worldwide (12,13). The daily consumption of soy sauce in Japan is estimated at about 30 ml per person according to data from the Japan Soy Sauce Brewers Association (1988). Recent studies suggest that soy sauce contains certain bioactive components in addition to taste and aroma compounds, and various biological activities of soy sauce have been reported, including anticarcinogenic (14,15), antimicrobial (16), anti-oxidative (13,17,18), antiplatelet (19) and inhibition of angiotensin I-converting enzyme activities (20).

In Japanese soy sauce, soybeans and wheat are the 2 main raw materials present in almost the same quantity, and soy sauce might contain a salt-soluble fraction because of a high salt concentration (12,21,22). However, soy sauce is well known to possess microbial proteolytic activity during fermentation which degrades soybean proteins, including a major soybean allergen (Gly m Bd 30K), into peptides and amino acids after fermentation (23,24). Although it has been clarified that the allergenicity of soybeans in soy sauce is reduced, another major material of soy sauce, wheat, has not been investigated for IgE-mediated allergy.

In this study, by immunoblotting, inhibition ELISA and direct ELISA using sera from 5 children with wheat allergy, we showed the degradation of wheat allergens in both salt-soluble and salt-insoluble fractions. For this purpose, each brewing process between raw materials and the final soy sauce was examined. In addition, we confirmed by both inhibition ELISA and direct ELISA using the sera of patients, that no wheat allergen was detected in 10 items of commercial soy sauce in Japan. In this report, we explain the reduction of wheat allergen in soy sauce from 2 aspects: solubilization of salt-insoluble protein and degradation of salt-soluble proteins during fermentation.

Materials and methods

Soy sauce. Soy sauce was prepared by Higashimaru Shoyu Co. Ltd. (Tatsuno, Hyogo, Japan) as shown in Fig. 1 (12,21,22,25). To examine wheat allergen in the brewing of soy sauce, both salt-soluble and salt-insoluble fractions of soy sauce were obtained from each brewing process between raw materials and the final soy sauce. The quantity (weight or volume) of the fractions was equalized (Fig. 1): 22.4 g of raw materials was equal to 20.0 g of the *koji*, and then 20 g of the *koji* was mixed with 50 ml of 23% NaCl. This mixture was called *moromi* mash. The initial volume of the *moromi* was about 80 ml, and gradually decreased during fermentation and aging

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to a final volume of about 70 ml after 3 months. Finally, the aged *moromi* was pressed to obtain liquid as raw soy sauce, and then the raw soy sauce was pasteurized at over 85°C for 30 sec to produce the final soy sauce after filtration. The solid waste part of the by-product was termed 'soy cake'.

Freeze-dried solid samples such as 22.4 g of raw materials, 22.4 g of treated materials, and 20.0 g of the *koji* were prepared as follows. Each solid sample was extracted with 50 ml of 23% NaCl for 30 min, and then centrifuged at 1300 x g for 10 min to obtain a supernatant as the salt-soluble fraction. The precipitate obtained by centrifugation was washed twice with 23% NaCl (50 ml each), extracted with 50 ml of 0.1 M Tris-HCl buffer (pH 8.6) containing 4 M urea (26,27) for 30 min, and centrifuged at 1300 x g for 10 min to obtain a supernatant as the salt-insoluble fraction. In contrast, each *moromi* sample was centrifuged at 1300 x g for 10 min to obtain a supernatant as the salt-soluble fraction. The precipitate was washed twice with 23% NaCl (50 ml each), extracted with 50 ml of 0.1 M Tris-HCl buffer (pH 8.6) containing 4 M urea for 30 min, and centrifuged at 1300 x g for 10 min to obtain a supernatant as the salt-insoluble fraction.

In addition, 10 items of commercial soy sauce in Japan (4 typical kinds of soy sauce as recognized by the JAS (Japanese agricultural standards) 'Koikuchi' as dark soy sauce, 'Usukuchi' as light soy sauce, 'Shiro' as extra light soy sauce and 'Saishikomi' as refermented soy sauce) were also used in the assay. 'Koikuchi' is dark in color and made from approximately equal weights of soybeans and wheat. 'Usukuchi' is also made from a mixture containing approximately equal weights of soybeans and wheat. Sometimes, rice and gluten are added to these materials in 'Usukuchi'. The principles of the 'Usukuchi' preparation are almost the same as those of 'Koikuchi', except that all of the procedures are directed towards producing a lighter color in the final product. 'Shiro' is very light in color and is made mostly from wheat with very little soybean. 'Saishikomi' is made from the *moromi* mixed with both the *koji* and raw soy sauce instead of the more commonly used salt water.

Pooled sera. Sera were obtained from 5 patients with wheat allergy, 2 girls (patients A and B) and 3 boys (patients C, D and E) (mean age $\pm$ SD, 3.7 $\pm$ 2.2), at the Department of Pediatrics, Kansai Medical University (Moriguchi, Osaka, Japan). These children had an episode of wheat-induced anaphylaxis within 6 months. Informed consent was obtained from the parents of all the donors. The wheat-specific IgE titers of the patients (A-E) were >100, >100, >100, >100 and 82.87 U/ml, respectively, as estimated with RAST (CAP-System-FEIA, Pharmacia Diagnostic, Uppsala, Sweden). The RAST-score was 5-6 for wheat allergy according to the manufacturer's instructions. Sera were pooled and used for ELISA after appropriate dilution.

Electrophoresis and immunoblotting. The salt-soluble and salt-insoluble fractions were diluted 18-fold with distilled water and 0.1 M Tris-HCl buffer (pH 8.6) containing 4 M urea, respectively, and aliquots (5 μ l each) were electrophoresed by the procedure of Laemmli (26-28). SDS 10-20% gradient polyacrylamide gel was obtained from Atto Co. (e-PAGEL, Tokyo, Japan). A mixture of rabbit muscle phospholylase b

(MW 97 kDa), bovine serum albumin (MW 66 kDa), rabbit muscle aldolase (MW 42 kDa), bovine erythrocyte carbonic anhydrase (MW 30 kDa), soybean trypsin inhibitor (MW 20 kDa) and egg white lysozyme (MW 14 kDa) was used as the molecular-weight markers (Daiichi III, Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). The gel was divided into 2 parts. Proteins separated on part of the gel were stained with Coomassie brilliant blue R-250 (Quick-CBB for electrophoresis, Wako Pure Chemical Industries Ltd., Osaka, Japan), and those separated on the other part of the gel were transferred electrophoretically onto a polyvinylidene difluoride membrane (AE-6666, Clear blot membrane-P, Atto) for 1 h at a fixed current of 2 mA/cm² (AE-6675-P/N, Atto). The membrane was blocked overnight with 1% bovine serum albumin (FIA/RIA grade, essentially globulin free, Nacalai Tesque Inc., Kyoto, Japan) in TBS Tween-20 (0.1%, Sigma Chemical Co., St. Louis, MO, USA) at 4°C. The proteins on the membrane were probed with the pooled sera of patients at 25°C for 30 min. After washing 3 times with 1% bovine serum albumin in TBS Tween-20 (0.1%), the membrane was incubated with biotinylated anti-human IgE (BA-3040, Vector Laboratories Inc., Burlingame, CA, USA) at 25°C for 30 min. After washing 3 times with TBS Tween-20 (0.1%), this was detected by an avidin-biotinylated enzyme complex-peroxidase kit using 3, 3'-diaminobenzidine tetrahydrochloride (Vectastain ABC-PO kit, Vector Laboratories Inc.).

Inhibition ELISA and direct ELISA. Inhibition ELISA and direct ELISA were performed as previously described (9,10, 29,30). Commercially available hard flour (1.0 mg, Nippon Flour Mills Co. Ltd., Tokyo, Japan) was extracted with 0.1 M Tris-HCl buffer (pH 8.6, 1.0 ml) containing 4 M urea, and the extract (100 μ l) was coated as antigen on a microplate (Nunc-Immuno plate, F96 Maxisorp, Nunc A/S, Roskilde, Denmark) overnight at 4°C. Unoccupied sites in the wells were saturated by incubation with 200 μ l of bovine serum albumin (2%) at 37°C for 2 h. Pooled sera of patients were used at a 10-fold dilution with PBS Tween-20 (0.05%). Either salt-soluble fractions or soy sauce samples were diluted 18-fold with distilled water to adjust the physiological saline concentration because soy sauce contains 16-18% NaCl. As a positive allergen, a pancreatic hydrolysate of wheat flour was used as described previously (9,10). The test solution (60 μ l) was preincubated with the pooled sera (60 μ l) at 25°C for 1 h. Aliquots (100 μ l each) of the treated sera were used as the antibody for inhibition ELISA, and untreated sera were used as a control. A 2 h incubation at 25°C was followed by the addition of biotinylated anti-human IgE (BA-3040, Vector Laboratories Inc.), streptavidin-peroxidase conjugate (500U, Roche Diagnostics GmbH, Mannheim, Germany), and o-phenylenediamine (OPD Tablet, ICN Biomedicals Inc., Aurora, OH, USA). The wells were washed 3 times between each addition with PBS Tween-20 (0.05%). After the reaction, the OD value was read by a plate reader (FLUOstar Galaxy, BMG Labtechnologies GmbH, Offenburg, Germany), using a 490 nm filter. A test sample was judged to be allergenic if it inhibited antigen-antibody binding by >5% (29).

Direct ELISA was performed as follows (26,27): the salt-soluble and salt-insoluble fractions that were prepared from each brewing process, between raw materials and the final soy

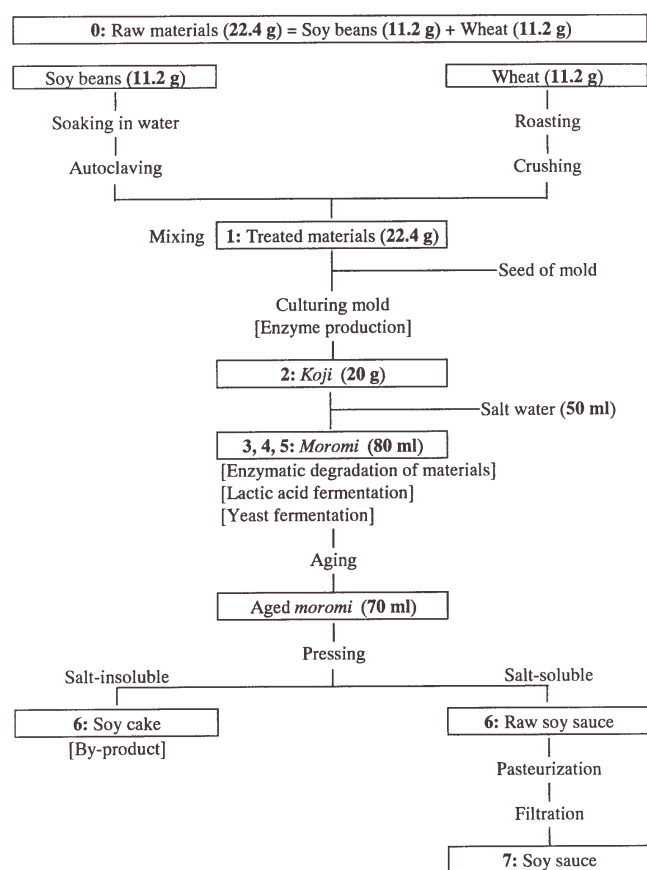
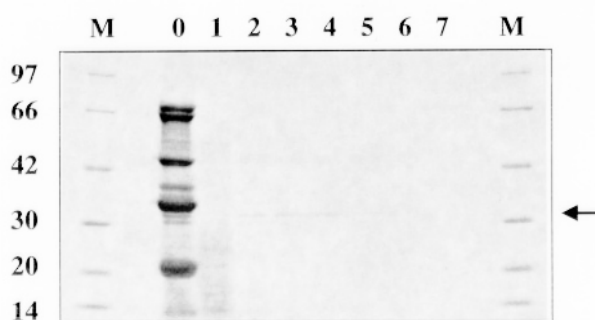


Figure 1. Brewing process of soy sauce from the raw materials to the final product. The quantity (weight and volume) in this experiment was calculated on the basis of industrial mass balance in Higashimaru Shoyu Co. Ltd. (Tatsuno, Hyogo, Japan). The nos. for each process (0-7) correspond to lanes (0-7) in Figs. 2, 4 and 5.

sauce, were diluted 18-fold with distilled water and 0.1 M Tris-HCl buffer (pH 8.6) containing 4 M urea, respectively. Soy sauce samples were also diluted 18-fold with distilled water. The test solution (100 µl each) was directly coated on a microplate (Nunc-Immuno plate, F96 Maxisorp, Nunc A/S) overnight at 4°C. For the positive allergen, commercially available hard flour (1.0 mg, Nippon Flour Mills Co. Ltd.) was extracted with 0.1 M Tris-HCl buffer (pH 8.6, 10 ml) containing 4 M urea. The extract was diluted with the same buffer, and a series of extracts (1, 10, and 100 µg/ml) were coated on the microplate as standards for antigen. Unoccupied sites in the wells were saturated by incubation with 200 µl of bovine serum albumin (2%) at 37°C for 2 h. For the assay, the pooled sera from 5 children with wheat allergy were used at a 10-fold dilution with PBS Tween-20 (0.05%). The pooled sera were added to the wells and incubated for 2 h at 25°C. This was followed by the same reactions as described above, and then the OD value at 490 nm was read by a plate reader. Concentrations of wheat allergen in Fig. 5 were estimated from a standard curve ($r=0.99$) using the series of positive extracts.

From preliminary experiments, we confirmed that components of soy sauce, for example, salt (less than 1% at a final concentration) and pigments had no effects on the inhibition ELISA and direct ELISA.

A



B

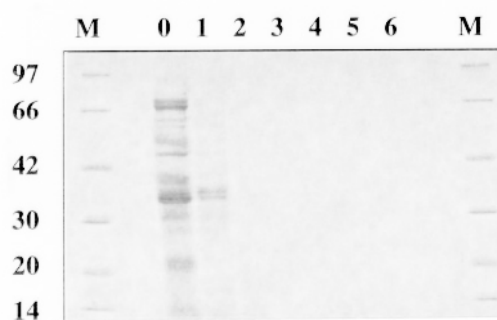


Figure 2. SDS-PAGE of the salt-soluble (A), and salt-insoluble (B), fractions obtained from each brewing process between raw materials and the final soy sauce. For details of the fractionation procedure, see Materials and methods. The salt-soluble (A), and salt-insoluble (B), fractions were diluted 18-fold with distilled water and 0.1 M Tris-HCl buffer (pH 8.6) containing 4 M urea, respectively, and the aliquots were subjected to electrophoresis. The gel sheets were stained with Coomassie brilliant blue. Lanes of the salt-soluble fractions (A): 0, raw materials; 1, treated materials; 2, *koji*; 3, *moromi* at 10 days; 4, *moromi* at 48 days; 5, *moromi* at 67 days; 6, raw soy sauce after pressing; 7, final soy sauce after pasteurization; M, marker proteins (kDa). Lanes of the salt-insoluble fractions (B): 0, raw materials; 1, treated materials; 2, *koji*; 3, *moromi* at 10 days; 4, *moromi* at 48 days; 5, *moromi* at 67 days; 6, soy cake after pressing; M, marker proteins (kDa). The arrow in (A) indicates the protein, which appeared between the *koji* and *moromi* stages (lanes 2-5).

Results

Protein degradation in soy sauce brewing. Tested soy sauce was prepared by the brewing process shown in Fig. 1. To examine the degradation of proteins in both salt-soluble and salt-insoluble fractions obtained between raw materials and the final soy sauce, the diluted fractions were electrophoresed in order of the brewing process (Fig. 2). Salt-soluble proteins (Fig. 2A) were detected in raw materials (lane 0), and were gradually degraded during fermentation (lanes 1-7). Interestingly, the protein indicated by the arrow, disappeared after the treatment of materials (lane 1), but again appeared between the *koji* and *moromi* stages (lanes 2-5). The MW of the protein was estimated as 31 kDa. After that, no salt-soluble protein was detected in either raw soy sauce or the final soy sauce (lanes 6 and 7). In contrast, salt-insoluble proteins (Fig. 2B) were detected in raw materials (lane 0), and disappeared after the *koji* stage (lane 2).

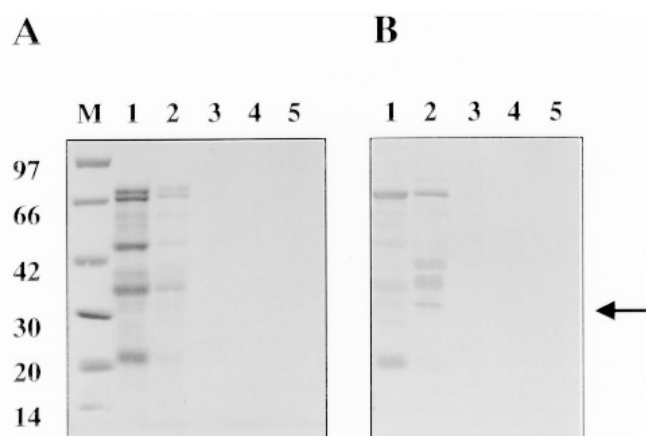


Figure 3. SDS-PAGE and immunoblotting analyses of the salt-soluble and salt-insoluble fractions obtained from each brewing process such as raw materials, pressing and pasteurization. For details of the fractionation procedure, see Materials and methods. The salt-soluble and salt-insoluble fractions were diluted 18-fold with distilled water and 0.1 M Tris-HCl buffer (pH 8.6) containing 4 M urea, respectively, and the aliquots were subjected to electrophoresis. The gel sheet was stained with Coomassie brilliant blue (A), or blotted onto a membrane and immunostained with the pooled sera from 5 children with wheat allergy (B). Lanes of fractions: 1, salt-soluble raw materials; 2, salt-insoluble raw materials; 3, raw soy sauce (salt-soluble) after pressing; 4, soy cake (salt-insoluble) after pressing; 5, final soy sauce (salt-soluble) after pasteurization; M, marker proteins (kDa). The arrow in (B) indicates the salt-insoluble protein, which solubilized between the *koji* and *moromi* stages.

Although proteins of raw materials were almost degraded into peptides and amino acids in soy sauce during fermentation, it was necessary to exclude the possibility that IgE-binding wheat proteins remain in soy sauce after fermentation, using immunological methods. For this purpose, immunoblotting analyses were performed using the pooled sera from 5 children with wheat allergy. Although most soy sauce is of the pasteurized-type, a small amount of raw soy sauce is also produced in Japan. Therefore, either 'salt-soluble fractions' such as raw materials, raw soy sauce after pressing and the final soy sauce after pasteurization, or 'salt-insoluble fractions' such as raw materials and soy cake after pressing were immunostained with the pooled sera (Fig. 3). As shown in Fig. 3B, several immunostained proteins in raw materials were detected as allergens in both fractions (lanes 1 and 2). In contrast and as expected, no allergen was detected in raw soy sauce, soy cake or the final soy sauce (lanes 3-5). Furthermore, the protein indicated in Fig. 3B was detected in the salt-insoluble fraction (lane 2) of raw materials, and corresponded to that indicated (31 kDa) in Fig. 2A which appeared after the *koji* stage.

Allergen degradation in soy sauce brewing. To examine the degradation of wheat allergens during the brewing of soy sauce, it was further investigated whether each salt-soluble fraction of soy sauce retained its IgE-binding ability by inhibition ELISA using the pooled sera from 5 children with wheat allergy (Fig. 4). When 0.1 mg/ml of a pancreatic hydrolysate of wheat flour was used as a positive wheat allergen (9,10), the ELISA inhibition reached about 60-70% (data not shown), and thus the pooled sera were judged to be appropriate for antibody in this assay. As shown in Fig. 4, the ELISA

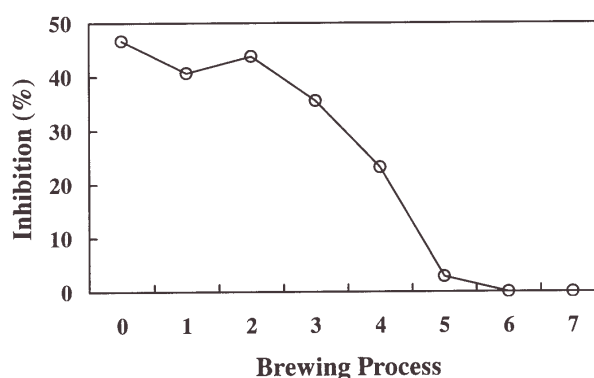


Figure 4. Inhibition ELISA of the salt-soluble fractions obtained from each brewing process. Wheat flour was used as the antigen in the assay. Pooled sera from 5 children with wheat allergy were preincubated at 25°C for 1 h with the salt-soluble fractions diluted 18-fold with water, and the aliquots were used as antibody in the assay. Inhibition value (%) was calculated by using intact sera as a control. For details of the ELISA procedure, see Materials and methods. Lanes of the salt-soluble fractions: 0, raw materials; 1, treated materials; 2, *koji*; 3, *moromi* at 10 days; 4, *moromi* at 48 days; 5, *moromi* at 67 days; 6, raw soy sauce after pressing; 7, final soy sauce after pasteurization.

inhibition generally decreased from the raw material to the *moromi* stage. However, the inhibition slightly increased from treated materials to the *koji* stage, and then extensively decreased during the *moromi* stage. Finally, the inhibition reached 0 in raw soy sauce and remained as 0 up to the final soy sauce. Furthermore, 10 items of commercial soy sauce in Japan (4 typical kinds of soy sauce as recognized by the JAS: 'Koikuchi', 'Usukuchi', 'Shiro', and 'Saishikomi') were used in this assay. As shown in Table I, ELISA inhibition was not seen in all soy sauce tested.

Although degradation of wheat allergens in salt-soluble fractions was confirmed by the inhibition ELISA, the salt-insoluble fraction was not subjected to this assay because the fraction contained a high concentration of urea. To further examine the degradation of wheat allergens in both fractions, the IgE-binding ability of each fraction was detected by direct ELISA using the pooled sera from 5 children with wheat allergy, after each fraction was directly coated as antigen in a microplate (Fig. 5). As shown in Fig. 5, concentrations of wheat allergen in both fractions gradually decreased during the brewing.

Interestingly, wheat allergen in the salt-insoluble fraction greatly decreased from treated materials to the *koji* stage, and the concentrations of the salt-insoluble fraction were lower than those of the salt-soluble fraction in both the *koji* and the *moromi* at 10 days (lanes 2 and 3 in Fig. 5). These results indicated that salt-insoluble proteins in treated materials were solubilized to salt water during the *koji* stage by enzymatic degradation. This corresponded to the pattern indicated by the arrow in SDS-PAGE (Fig. 2A). Next, in the *moromi* stage, both the resultant salt-solubilized and initial salt-soluble wheat allergens were degraded extensively, and also corresponded to the results of the inhibition ELISA (Fig. 4). Similarly, the decrease in the salt-insoluble fraction continued in the *moromi* stage. After the *moromi* pressing, the residual wheat allergen in both fractions of soy sauce (raw soy sauce and soy cake) was evaluated as 0, and the final soy sauce contained no wheat allergen after pasteurization.

Table I. Inhibition ELISA and direct ELISA of commercial soy sauce samples in Japan^a

Sample	Kind of soy sauce ^b	Company	Cereal materials ^c	ELISA inhibition (%) ^d	Wheat allergen (mg/ml) ^e
1	Koikuchi	A	Soybean, wheat	0	0
2	Koikuchi	B	Soybean, wheat	0	0
3	Raw-Koikuchi	C	Soybean, wheat	0	0
4	Usukuchi	A	Soybean, wheat, rice	0	0
5	Usukuchi	A	Soybean, wheat, barley, rice	0	0
6	Usukuchi	A	Soybean, wheat, rice	0	0
7	Usukuchi	B	Soybean, wheat, rice	0	0
8	Raw-Usukuchi	A	Soybean, wheat, rice	0	0
9	Raw-Saishikomi	A	Soybean, wheat	0	0
10	Shiro	D	Wheat, soybean	0	0

^aFor details of the ELISA procedure, see the legends to Figs. 4 and 5. ^bJAS rule name. 'Raw' means ultrafiltrated-raw soy sauce (no pasteurization). ^cMain materials are in the order of their content in the product except no cereal such as salt, water, etc. ^dThe percentage inhibition value was calculated by using intact sera as a control. ^eConcentrations of wheat allergen were estimated from the OD values of direct ELISA based on a standard curve ($r=0.99$) using the extracts of wheat allergen.

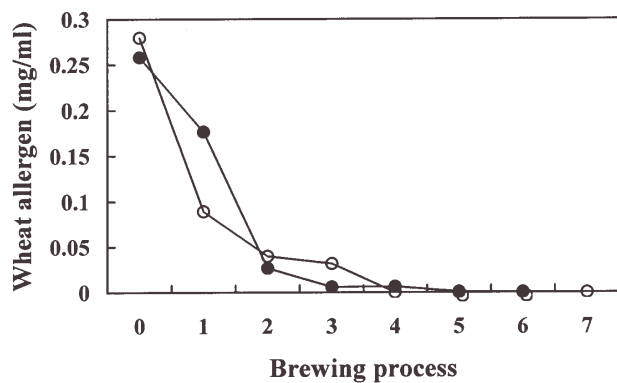


Figure 5. Direct ELISA of the salt-soluble and salt-insoluble fractions obtained from each brewing process. The salt-soluble and salt-insoluble fractions were diluted 18-fold with distilled water and 0.1 M Tris-HCl buffer (pH 8.6) containing 4 M urea, respectively, and the aliquots were used as antigen samples in the assay. For details of the ELISA procedure, see Materials and methods. Symbols (concentration of wheat allergen): salt-soluble (open) and salt-insoluble (closed) fractions. Lanes of the fractions: 0, raw materials; 1, treated materials; 2, *koji*; 3, *moromi* at 10 days; 4, *moromi* at 48 days; 5, *moromi* at 67 days; 6, raw soy sauce (salt-soluble) and soy cake (salt-insoluble) after pressing; 7, final soy sauce (salt-soluble) after pasteurization.

Furthermore, 10 items of commercial soy sauce in Japan were also subjected to this assay. As shown in Table I, the concentrations of wheat allergen were confirmed as 0 in all soy sauce tested.

Discussion

Wheat is the most widely cultivated cereal and an important source of food and dietary protein worldwide. The ingestion of wheat products and inhalation of wheat flour are the 2 major routes of sensitization. Wheat allergy is considered to be a serious problem globally, since wheat is the most consumed crop in the world (1). Wheat proteins are classified into 2 major fractions on the basis of water/salt solubility (1,6,11): a water/salt-soluble fraction (albumin and globulin) and a water/salt-

insoluble fraction (glutenin and gliadin). The components of the water/salt-soluble fraction were reported to be the causative allergens in cases of baker's asthma, based on a typical IgE-mediated allergy to inhaled wheat flour (1). In the water/salt-soluble fraction, several 14-17 kDa proteins from the α -amylase/trypsin inhibitor family (3,31), a 27 kDa protein similar to acyl-coenzyme A oxidase (31), a 35 kDa protein similar to fructose-bisphosphate-aldolase (32) and a 36 kDa glycoprotein with peroxidase activity (33,34) were identified as allergens for asthma. Furthermore, several proteins in the water/salt-insoluble fraction have been identified as inhalant allergens (1).

Compared with baker's asthma, the number of studies on allergy to ingested wheat is surprisingly small. In wheat-allergic children the IgE response appears heterogeneous, and some immunoblotting studies have detected IgE binding to a number of water/salt-soluble proteins with molecular weights ranging from 15 to 69 kDa (5,35-37). James *et al* (5) showed IgE binding to a 15 kDa α -amylase inhibitor in the sera of 5 children with ingested wheat allergy. In addition, IgE binding to water/salt-insoluble proteins and a polysaccharide have also been reported (1,11,29). Tanabe *et al* (9) found that most patients with wheat allergy were sensitive to salt-insoluble gluten (glutenin and gliadin) and identified the allergen to be a low molecular weight glutenin subunit containing a pentapeptide repeat motif, Gln-Gln-Gln-Pro-Pro, which was identified to be an IgE-binding epitope. Takizawa *et al* (7) detected IgE binding to water/salt-soluble proteins (16, 35-67 and 94 kDa) and water/salt-insoluble proteins (16, 38 and 70 kDa) in the sera of 7 patients with wheat-induced anaphylaxis. Therefore, wheat proteins in both water/salt-soluble and insoluble fractions would be responsible for IgE-mediated allergy to ingested wheat.

Soy sauce is one of the most traditional seasonings in Japan and several oriental countries, and is currently used in cooking worldwide (12). Many varieties of soy sauce are produced in Japan and other oriental countries (13). Of all the types of soy sauce consumed in Japan, the total of 2 major types, 'Koikuchi' and 'Usukuchi', accounts for about 95% (12). Although

'Koikuchi' has a strong aroma and dark color during cooking, 'Usukuchi' is used mainly for cooking in order to preserve the original color and flavor of food. Food allergens are sometimes characterized by their high stability against heating and digestive enzymes, with their epitope structures remaining unchanged. These characteristics often cause allergy, even if cooked foods are eaten. Tanabe *et al* (27) succeeded in the preparation of hypoallergenic wheat flour using microbial enzymes other than gut digestive enzymes. Since soy sauce also possesses potent microbial proteolytic enzymes such as protease and peptidase during fermentation (12), all allergens are expected to be degraded into peptides and amino acids after fermentation.

In this study, using immunoblotting, inhibition ELISA and direct ELISA, with the pooled sera from 5 children with wheat allergy, it was clearly demonstrated that no allergen remained in soy sauce. In fact, the concentrations of wheat allergen were confirmed to be 0 in all commercial soy sauce tested (Table I). By inhibition ELISA (Fig. 4), the salt-soluble allergen of wheat was slightly increased after the *koji* stage, and then completely decreased during the *moromi* stage. By direct ELISA (Fig. 5), degradation of salt-insoluble allergen was faster than that of the salt-soluble allergen between the *koji* and the *moromi* stages. These results indicated that wheat allergens of raw materials were degraded as follows. At either the *koji* or the *moromi* stage, salt-soluble allergens of raw materials were degraded well, and most of the salt-insoluble allergens were hydrolyzed to become salt-soluble by the action of microbial enzymes and thereafter were extensively degraded at these stages. Especially, the protein (31 kDa) as shown in Figs. 2A and 3B which was immunostained by the sera from 5 children with wheat allergy, disappeared after the treatment of materials, but again appeared between the *koji* and *moromi* stages, and finally disappeared in both raw soy sauce and the final soy sauce. The 31 kDa protein was significant to reduce wheat allergen in the brewing of soy sauce, and further study would be necessary to clarify the protein as an allergen. However, several immunoblotted proteins in raw materials (containing both wheat and soybeans) did not completely coincide with wheat allergens identified previously (1), and the IgE binding ability of the pooled sera used in this study might crossreact with soybeans. As for soy sauce, it has been reported that the allergenicity of soybeans was greatly reduced in soy sauce after fermentation (23,24). Therefore, both allergens, wheat and soybeans, in raw materials of soy sauce are judged to be degraded completely in the *moromi* stage, and soy sauce would be useful as a seasoning for both wheat- and soybean-associated allergic patients.

In conclusion, we firstly clarified in this study that wheat allergens were completely degraded into amino acids and peptides losing the IgE-binding ability during the fermentation of soy sauce, and that no allergen was thus contained in soy sauce. There is a great degradation of wheat allergen during the brewing, especially during the 2 processes *koji* (mold cultivation, enzyme production and solubilization from salt-insoluble to salt-soluble proteins) and then *moromi* (enzymatic degradation of both salt-soluble and salt-insoluble proteins). These results are quite informative for wheat-allergic patients as well as pediatricians, physicians and dermatologists.

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