tients can eat different kinds of fish roe safely. The cross-reactivity among fish roe was investigated using a CAP-RAST system[4] and inhibition immunoblot,[5] but there have as yet been no articles demonstrating this. Moreover, the cross-reactivity between SR and CE is controversial.

In this study, we attempted to demonstrate the cross-reactivity between SR and other kinds of fish roe (herring roe : HR, Pollock roe : PR), salmon, or CE.

## METHODS

### EXTRACTS

Extracts of salmon ( *Oncorhynchus kisutch* ) and fish roe from salmon ( *Oncorhynchus keta* ), pollock (*Theragra chalcogramma* ) and herring ( *Clupea pallasii* ) were obtained from Toyo Suisan Kaisha, Ltd. Raw or frozen salmon and fish roe were cleared of parasites and minced with a speed cutter (Matsushita Industry Company, Tokyo, Japan). Five grams each of salmon and fish roe were vortexed in 15 ml of 1M KCl-PBS in sterile centrifuge tubes, and placed overnight in a cold room (4℃). After the addition of a further 5 ml 1 M KCl-PBS, the samples were centrifuged at 13,000 rpm (20000 xg). The supernatants were dialyzed against distilled water with a dialysis tube (cut off 6,000−8,000 of molecular weight) for 1−2 days in a cold room. The concentrates were lyophilized and stored at -20℃.

Chicken egg extracts (egg white and egg yolk) were obtained in the same way as previously described.[6]

The protein concentrations of these samples were determined by a BCA protein assay (Pierce, Rockford, IL, USA).

### MEASUREMENT OF SPECIFIC IGE ANTIBODIES TO EXTRACTS FROM FISH, ROE AND CHICKEN EGG WHITE (CEW) USING THE RADIOALLERGOSORBENT TEST (RAST)

The freeze-dried samples were dissolved in 25 ml coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl) and centrifuged at 13,000 rpm (20000 xg). Cyanogen bromide-activated paper disks were soaked in each fish extract solution and incubated with rotation at 4℃. After the supernatant was aspirated, 25 ml of blocking buffer (0.2 M glycine buffer) was added to the disks and incubated with rotation at room temperature (RT) for 5 hours. The disks were alternately washed with 0.1 M acetate buffer and blocking buffer 5 times. The disks were washed once with PBS-Tween and suspended in 40 ml of PBS-Tween. The disks in PBS-Tween were stored in a cold room (4℃).

These extract-conjugated paper disks were incubated for 5 hours with 25 μl of patient serum and 25 μl of PBS-Tween. After washing with PBS-Tween, 25 μl of¹²⁵I-labeledanti-IgE (IgE-RIABEAD, DAINABOT

CO., LTD, Tokyo, Japan), approximately 2,200 Bq, and 25 μl of PBS-Tween were added and incubated overnight. After the free radioisotope was removed by rinsing with PBS-Tween, the bound radioisotope was measured in a gamma counter. Results were expressed as the percent binding of the total radioactivity added.

### ELISA ( ENZYME-LINKED IMMNUNOSORBENT ASSAY)

The freeze-dried samples were dissolved (100 μg/ml) with PBS buffer and placed (100 μl/well) in each hole on Nunc-Immuno Plate I (Nunc A/S, Roskilde, Denmark) for 1.5 hr at RT. Samples were discarded and SuperBlock Blocking Buffer in PBS (150 μl/well, Pierce, Rockford, IL, USA) was added and stored overnight at 4℃. Each well was washed with 200 μl/well of PBS-Tween and 100 μl/well of the serum diluted by SuperBlock Blocking Buffer (1 : 5) was added and stored overnight at RT. After being washed with PBS-Tween, Mouse Anti-Human IgE-BIOT (1 : 1,000, 100 μl/well, Southern Biotechnology Associates, Inc. Birmingham, AL, USA) was added for 1 hr at RT. This was washed well, then streptavidine-HRP (1 : 5,000, 100 μl/well, Southern Biotechnology Associates, Inc. Birmingham, AL, USA) was added for 1 hr at RT. This was washed well and followed by incubation with 100 μl/well of TMB (ICN Biomedicals, Inc. Aurora, OH, USA) for 30 minutes under a light shield. The reaction was stopped with 100 μl/well of 1N HCl and measured with LS-PLATE manager 2001 (Wako, Osaka, Japan).

### ELISA INHIBITION

Before addition to the ELISA plate that was pre-coated with extract of SR, salmon or chicken egg yolk (CEY), serum samples were pre-incubated with solutions containing extracts (SR, HR, PR, salmon, CEW, CEY and anisakis) of 5 different concentrations (0, 1, 10, 100, 1,000 and 10,000 μg/ml ) as inhibitors at RT. The subsequent procedure was the same as that for ELISA described above.

### TRANSFER AND IMMUNOBLOTTING

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 4−20% Tris-glycine precast gel (Tefco Corporation, Machida, Japan) according to Laemmli under reducing conditions. Each sample was separated at 120 V for 2 h. Immunoblot of the proteins and detection of bound serum IgE were performed as previously reported.[7]

### N-TERMINAL AMINO ACID SEQUENCE

After blotting, Immobilon™-P membranes were stained with 0.1% amido black (Sigma Diagnostics, St. Louis, MO, USA) in 50% methanol and 10% acetic acid, destained with 40% methanol and 10% acetic acid and air-dried. Protein bands with IgE-binding activity
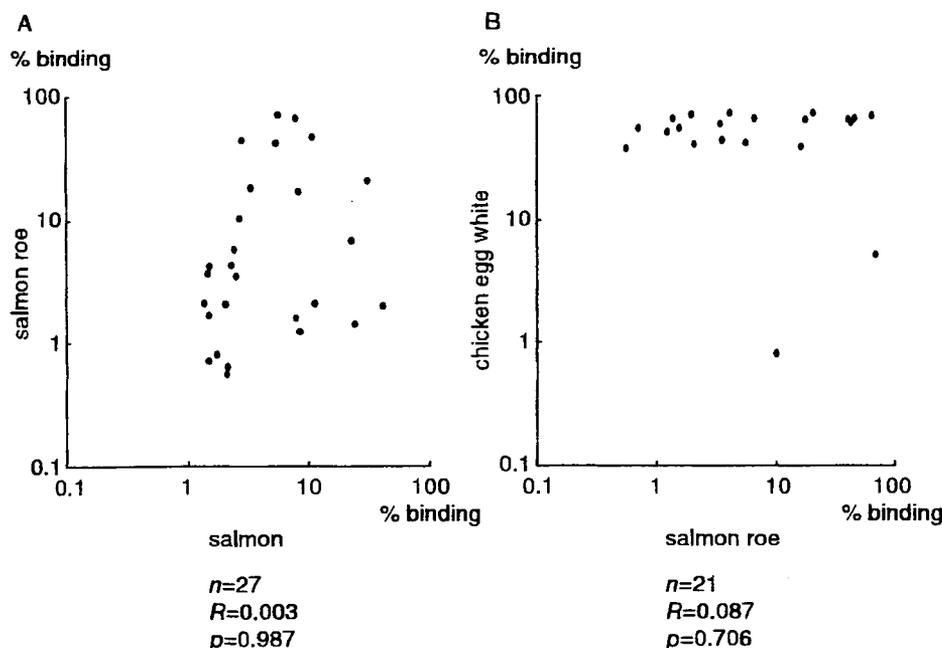
Fig. 1 Relationship of the specific IgE value between salmon and salmon roe (A) and between salmon roe and chicken egg white (B). IgE values of each extract were measured by radioallergosorbent test (RAST) in serum from 27 fish allergy patients and 26 non-fish allergy patients (control subjects). When the IgE values of both extracts were less than the mean + 2SD of the control subjects, the sample was excluded from this study. There were no significant relationships of IgE values between salmon and salmon roe (R=0.003), and between salmon roe and chicken egg white (R=0.087).

were excised and subjected to N-terminal amino acid sequencing via automated Edman degradation using an Applied Biosystems model 470 A protein sequencer.[8] The resulting phenylthiohydantion (PTH) amino acid derivatives were identified using a model 120A one-line PTH analyzer and the standard Applied Biosystems program. The N-terminal amino acid sequence of each protein was determined at least twice.

## HUMAN SERA

Twenty-seven patients (male : female = 20 : 7, range of age ; 6 months (m) to 11 years (y) ; mean ± SD = 4y4 m ± 3 y 6 m,) allergic to fish were enrolled in the RAST study. Fish allergy was diagnosed based on at least one convincing report of a hypersensitive reaction to fish ingestion and positive results (more than class 2) to at least one item (salmon) of the fish-specific IgE using the CAP system (Pharmacia Diagnostics, Uppsala, Sweden). They had episodes of clinical allergic reactivity to one or more fish. Twenty-six children (male : female = 18 : 8, range of age ; 3 m to 11 y 9 m; mean ± SD = 4 y 8 m ± 3 y 6 m ) were enrolled as control subjects for the RAST study. They had no histories of allergic reactions to fish ingestion and had negative IgE results (class 0) to at least five

items from fish in a commercial CAP-system.

As samples for the ELISA inhibition and immunoblot study, we choose sera from 3 patients. Two patients had obvious hypersensitive episodes (anaphylaxis) to SR and another patient had no episodes of hypersensitive reaction because of their avoidance of SR due to its high-IgE values (class 2) to salmon, SR and CEW. All samples were stored at -20°C until use.

## RESULTS

### THE RELATIONSHIP OF THE IgE VALUES BETWEEN SALMON AND SR (Fig. 1A), AND BETWEEN SR AND CEW IN THE RAST STUDY (Fig. 1B)

We measured the IgE value to each extract in the serum of fish allergy patients and control subjects. When the IgE values to both extracts were less than the mean + 2SD of the control subjects, the sample was excluded from this study. Finally, 27 patients were enrolled, as shown in Figure 1A, and 21 samples were used, as shown in Figure 1B. There were no relationships in the IgE values between salmon and SR ( R = 0.003) or between SR and CEW ( R = 0.087).
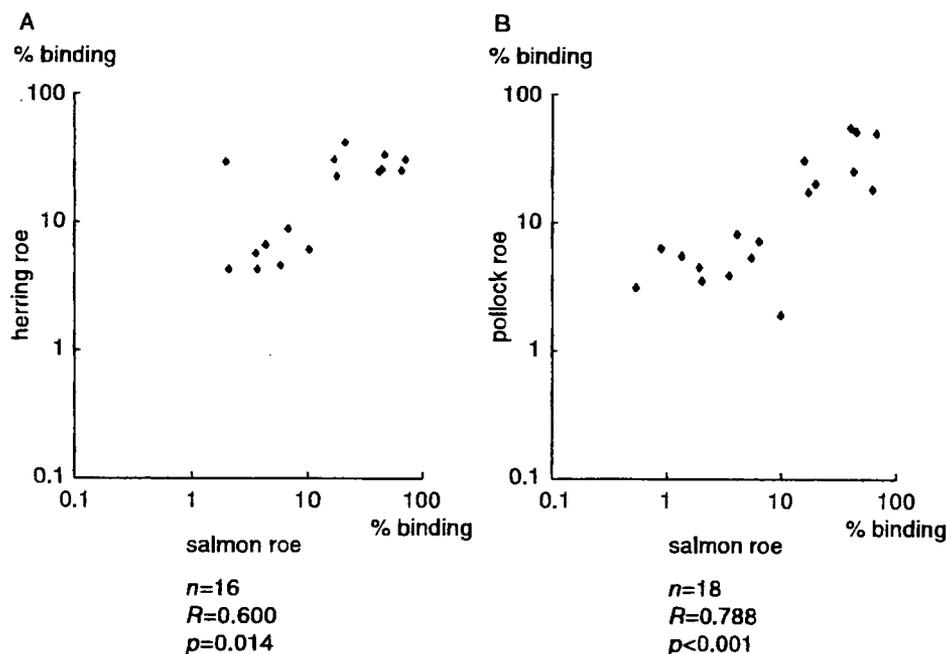
**Fig. 2** Relationship of the IgE value between salmon roe and herring roe (**A**) or pollock roe (**B**). Each sample was measured in the same way as described in Figure 1. There were positive correlations between salmon roe and herring roe ($R$=0.600) and between salmon roe and pollock roe ($R$=0.788).

## RELATIONSHIP OF IgE VALUES BETWEEN SR AND HR OR PR (Fig. 2)

The samples were measured in the same way as described above and the results are shown in Figure 2. Sixteen (A) and eighteen (B) samples were matched in each study. There were positive correlations between SR and HR ($R$ = 0.600), and between SR and PR ($R$ = 0.788).

## ELISA INHIBITION

As shown in Figures 3A, B, the binding of IgE from two anaphylaxis patients to SR was inhibited more than 50% by pre-incubation of the serum with HR and almost 50% by those with PR in a dose dependent manner ; however, there was no efficient inhibition with CEY and anisakis. CEW also did not efficiently inhibit the binding of IgE to SR (data not shown). Because the binding of IgE to salmon and CEY was quite low, the assessment of inhibition of these two allergens was not possible. Inhibition of the IgE binding to SR was achieved more than 50% by salmon in patient A. In another patient (B), inhibition of the IgE to SR was achieved nearly 50% by salmon inhibitor at one level below the maximum concentration. Because the solution of salmon inhibitor was very sticky, testing the maximum concentration (10,000 µg/ml ) was not possible.

As shown in Figure 3C, the binding of IgE to each extract in patients who avoided SR because of the high IgE values to salmon, SR and CEW, was not in-

hibited by any heterogeneity inhibitor.

## IMMUNOBLOTTING OF SR WITH SERA FROM 3 PATIENTS (Fig. 4)

The IgE binding patterns to SR on the membrane were different between patients with anaphylaxis to SR and without any anaphylactic episodes to SR. The two anaphylactic patients reacted to the protein with relatively low molecular weight bands (15 and 17 kDa), while the patient with no episodes of hypersensitivity to SR reacted to that with a relatively high molecular weight protein band (21 kDa ). Partial protein sequences of these bands were determined and screened for homology with sequences in the Swiss Prot Data Base. These protein bands were suspected as being fragments of vitellogenin, because of an almost complete identity with the amino-acid sequence of vitellogenin precursor (JC 4956) from rainbow trout.[9]

## DISCUSSION

SR allergy is well known to cause severe anaphylaxis, and it was thus recommended to be labeled as allergenic. Despite its nature, there were no articles about its allergenicity, including cross-reactivity. In clinical work we were at a loss to account for whether a patient with egg allergy can eat SR safely or a fish allergic patient can eat SR.

Regarding the cross-reactivity between SR and CEW, Ito et al. recently described that there was no
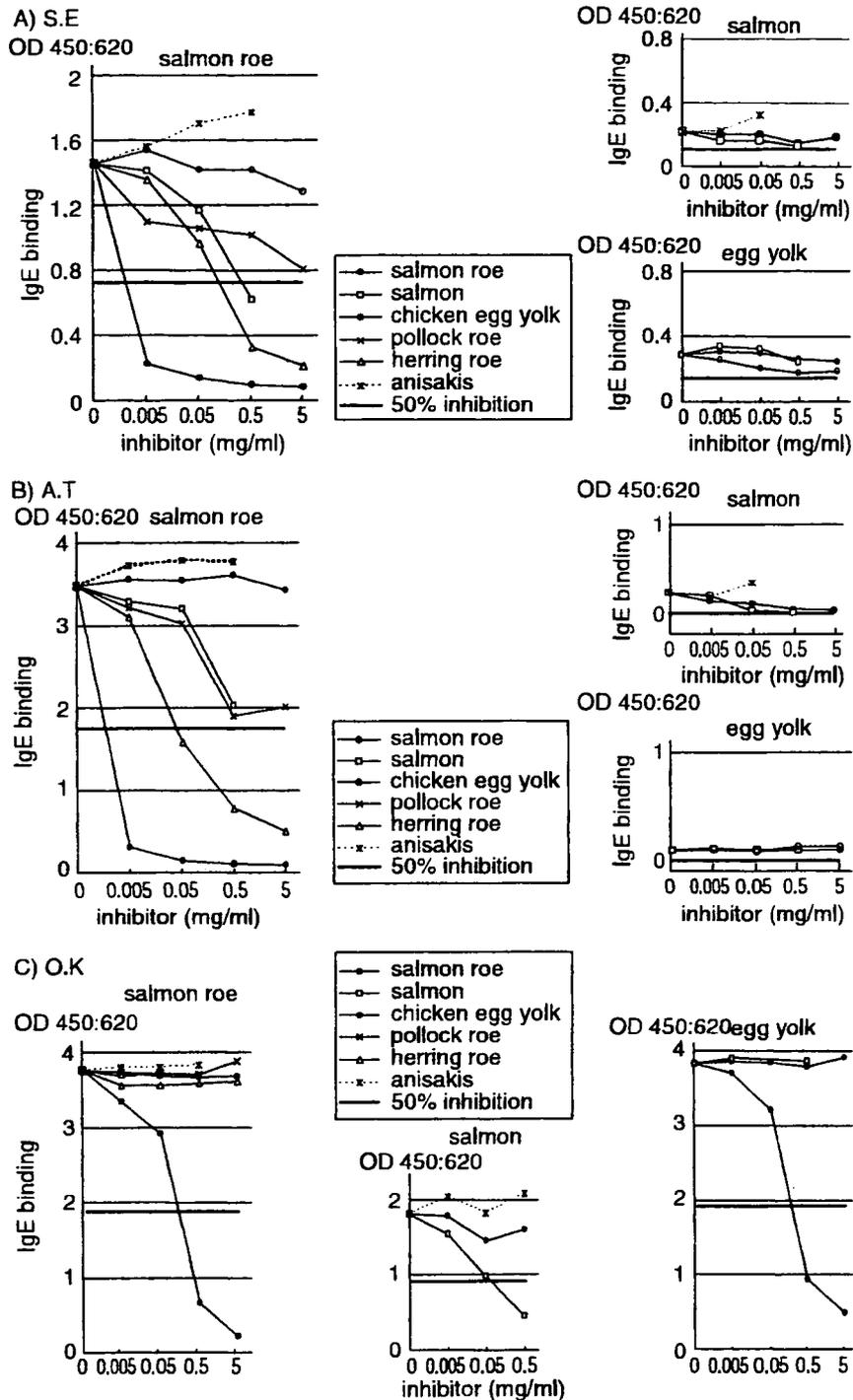
Fig. 3　ELISA inhibition between salmon roe (SR) and various antigen (salmon, herring roe: HR, pollock roe: PR or chicken egg yolk: CEY) using sera from two patients who had anaphylactic reactions to salmon egg (A and B), and sera from a patient with fish allergy who had no allergic reactions to SR but avoided SR because the IgE value of salmon, SR, chicken egg white were high (C). In the two sera from patients with anaphylaxis to SR (A, B), the binding of IgE to SR was inhibited more than 50% by pre-incubation of the serum with HR and almost 50% by those with PR in a dose dependent manner; however, no efficient inhibition of IgE to SR was seen by pre-incubation with CEY or anisakis. Salmon inhibited the IgE binding to SR more than 50% in patient A. On the other hand, in the sera from patient C the IgE binding to each extract was not inhibited by pre-incubation of the serum with any heterogeneity extract.
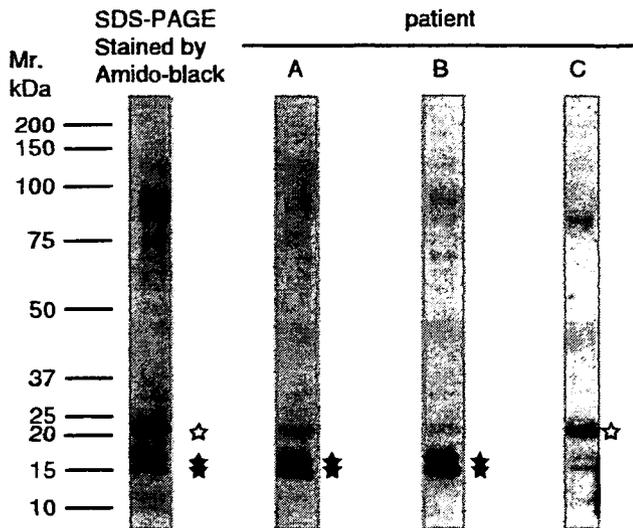
**Fig. 4** Immunoblot of salmon roe with sera from the same patients described in Figure 3.

The IgE binding patterns were different between the patients with anaphylaxis to salmon roe (A and B) and fish allergy without anaphylactic episodes to salmon roe (C). Both IgE from anaphylaxis patients bound to relatively low molecular weight protein bands, while IgE from the fish allergy patient reacted with the relatively high molecular weight protein band. Outlined and solid stars indicate the protein bands of salmon roe that were strongly bound by the patient's IgE.

correlation between them based on measuring the specific IgE with the CAP-RAST system.[10] The same results were seen in our RAST study. Moreover, Tanaka et al. reported in the minutes of the Japanese Society of Allergology (2001) that there were no cross-reactivities between SR and CEW or CEY in their inhibition immunoblot study.[5] These results corresponded with those of our ELISA inhibition study. These data suggested that there were no cross-reactivities between SR and CEW or CEY ; however, our examinations were performed only in a small number of samples and further investigations are needed to clear this important problem.

With regard to the cross-reactivity between SR and salmon, a recent study has described that there was no significant relationship between them.[10] That was also observed in our RAST data. Additionally, there was no cross-reactivity in the inhibition immunoblot study.[5] However, we think that it is undeniable that there is cross-reactivity between SR and salmon. Shibata et al. described in their discussion the need for careful attention when feeding fish roe to patients with fish allergy, because the patients with anaphylaxis to fish roe often have fish allergy.[11] Actually, in our ELISA inhibition study using the serum from two patients having anaphylaxis to SR without allergy to

salmon, salmon extract inhibited more than 50% of IgE binding to SR in one patient, while in another patient salmon was inhibited by almost 50%. Neglecting the effect of anisakis on ELISA inhibition between salmon and SR, we used anisakis extract as an inhibitor and confirmed there was no inhibition of the IgE binding to salmon and SR. We also confirmed there was no contamination of SR to salmon using a patient who had no history of hypersensitive reactions to SR but high IgE values to salmon, SR and CEW, as shown in Figure 3C. Our data were different from previous data.[5] This difference may account for the reason why the analysis of the inhibition with the immunoblot was qualitative, while the ELISA inhibition was quantitative. Our data suggested that there is cross-reactivity between SR and salmon in some cases.

Cross-reactivity between SR and different kinds of fish roe was suspected by Watanabe et al. using the CAP-RAST system only in abstract form.[4] In our RAST examination, there were positive correlations between SR and HR or PR. In another report, using an inhibition immunoblot study, HR and PR inhibited the IgE binding to SR.[5] In our ELISA inhibition, using two sera from patients who had anaphylaxis to SR, the binding of IgE to SR was inhibited by both kinds of roe (HR and PR) to different degrees. This means that HR inhibited the IgE binding to SR more than 50%, while PR inhibited it to almost, but less than 50%. Those results suggested that there is a significant cross-reactivity between SR and HR. On the other hand, we suspected a partial cross-reactivity between SR and PR, because PR inhibited the IgE binding to SR in a dose-dependent manner, but less than 50%. We have been unable to come to a conclusion regarding the cross-reactivity between SR and PR, because of the small number of samples in our study.

Between two anaphylactic patients and a patient without anaphylactic episodes to SR there were differences in the IgE binding pattern to SR. Two anaphylaxis patients' IgE binding patterns were very similar, with reactions to protein bands of 15 and 17 kDa, while the pattern of IgE binding from another patient who had no experiences of anaphylaxis to SR reacted mainly to the relative higher molecular weight protein band (21 kDa) in Figure 4. The N-terminal amino acid sequences of these proteins were almost identical to the vitellogenin precursor of rainbow trout ( Oncorhynchus mykiss ). Two protein bands of 15 and 17 kDa had the same amino acid sequence and they were thought of as fragments from vitellogenin. The difference in molecular weight between them might have been due to a modification by carbohydrates or a different breaking point at the C-terminal. The 21 kDa protein had a different sequence to those of 15 and 17 kDa and was thought of as another fragment of vitellogenin. The smaller fragment was suspected to be the beta'-component and the large fragment was

speculated to be lipovitellin, due to the sequence similarity and molecular weight, respectively. These results were similar to Kubo's report[3] that the beta'-component has a relationship with the symptoms.

Moreover, recent research on molecular analysis has verified the presence of multiple vitellogenins in at least some fish species.[12] From these data we can say that vitellogenin exists in many kinds of fish roe, fish liver and even in chicken yolk with various mutations, and those mutations increase with species change. Symptoms of anaphylaxis to SR may develop in patients whose IgE is bound on a certain epitope of vitellogenin, especially on the amino acid sequence of the beta'-component ; however, we can not state conclusively that the cross-reactivity was caused by vitellogenin because of insufficient data to demonstrate this.

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# Cross-reactive Carbohydrate Determinant Contributes to the False Positive IgE Antibody to Peanut

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## ABSTRACT

**Background:** The importance of peanut allergy has not been well recognized in Japanese society. IgE antibody to peanut can be, however, detected in patients without clinical peanut allergy.

**Methods:** Clinical characteristics of 14 patients (aged 1–8 years) with peanut allergy were evaluated. IgE antibodies to peanut from patients with and without clinical peanut allergy were compared with those to soybean and other nuts. To examine the role of cross-reactive carbohydrate determinant (CCD) on the clinically false positive detection of peanut IgE, horseradish peroxidase (HRP) and bromelain specific IgE were measured by Uni CAP IgE kit. Inhibition of peanut IgE by HRP was also examined.

**Results:** The patients repeatedly experienced potentially life-threatening symptoms, including anaphylaxis. Sera from patients with peanut allergy had negative or relatively low IgE antibodies to other nuts. However, clinically false positive peanut IgE showed significant correlation-coefficients with soybean, almond, chestnut, pistachio, macadamia and cashew ($r$ = 0.61–1.00). Anti-HRP and anti-bromelain IgE antibodies were detected in the clinically false positive sera, but not in the sera from patients with peanut allergy. Two out of four clinically false positive peanut IgE antibodies were significantly inhibited by HRP.

**Conclusions:** Social education about the features of peanut allergy is needed in Japan. Anti-CCD IgE antibody was suggested to be one of the mechanisms contributing to the false positive detection of peanut IgE. Detection of anti-HRP or anti-bromelain IgE can be a useful tool to recognize the presence of anti-CCD antibodies.

## INTRODUCTION

Allergies to peanut account for the majority of fatal and near-fatal anaphylactic reactions to foods.[1] In the United States, 3 million people are allergic to peanut or tree nuts, and peanut-induced anaphylaxis causes 50 to 100 deaths per year.[2] In Japan, peanut contributes to 2.4% of immediate type allergic reactions to food.[3] According to the results from this nation-wide study, and because of the severity of allergic symptoms to peanut reported overseas, the Japanese National Ministry of Health, Labor and Welfare designated peanut as one of the five major food allergens required to be specified on the label of food products.

However, peanut allergy is not well understood in the community, partially because of the lack of precise information about the prevalence or clinical importance of peanut allergy among the Japanese population.

The first aim of this paper is to report the clinical importance of peanut allergy in Japanese children, and allergic cross-reactivity of peanut to soybean and other nuts.

Measurement of IgE antibody to peanut is a screening test for diagnosis of peanut allergy. However, positive IgE antibody does not always indicate a definitive diagnosis of peanut allergy.[4] The presence of IgE antibody to the carbohydrate (oligosaccharide) moieties in the plant antigen is one of the well-known

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**Table 1** Clinical features of peanut allergy

| No | Peanut IgE (UA/ml) | Age (Years) | First episode (Years) | Symptoms | Other food allergies | Total IgE (IU/ml) |
|---|---|---|---|---|---|---|
| 1 | 100 | 6.7 | 3 | Anaphylaxis x2, asthma * | milk, soybean (itch) | 689 |
| 2 | 49.6 | 4.2 | 2 | Anaphylaxis, urticaria | milk | 812 |
| 3 | 45.6 | 3.6 | | Anaphylaxis x2 | milk, walnut (urticaria) | 741 |
| 4 | 10.3 | 6.1 | | Anaphylaxis | — | 1180 |
| 5 | 2.7 | 4.8 | 1 | Anaphylaxis | milk, eggs | 608 |
| 6 | 100 | 5.6 | | Urticaria | — | 1918 |
| 7 | 85.6 | 5.0 | 2 | Urticaria, vomiting, diarrhea | — | 8316 |
| 8 | 16.1 | 2.8 | 3 | Urticaria | — | 307 |
| 9 | 8.17 | 2.1 | 1 | Urticaria | eggs, wheat, soybean (urticaria) | 215 |
| 10 | 1 | 2.8 | | Urticaria, abdominal pain | — | 26 |
| 11 | 15.4 | 1.3 | 1 | Erythema | eggs | 583 |
| 12 | 2.01 | 3.0 | 2 | Erythema, itchy skin | — | 217 |
| 13 | 1.08 | 8.2 | 3 | Erythema | eggs, gelatin, soybean (eczema) | 203 |
| 14 | 100 | 2.3 | | Eczema after 2 days | milk, eggs, shrimp, chocolate (eczema) | 11270 |

\* Induced by prick-to-prick test with peanut butter

**Table 2** Clinical features of nut allergy.

| No | Age (Years) | Nuts | Symptoms | Walnut IgE (UA/ml) | Peanut IgE (UA/ml) | Peanut allergy |
|---|---|---|---|---|---|---|
| 1 | 3 | Walnut | Urticaria | 0.35 | 45.6 | + |
| 2 | 5 | Walnut, pine nut | Anaphylaxis | 5.52 | 0.8 | - |
| 3 | 2 | Walnut | Cough | 0.65 | 2.2 | - |
| 4 | 3 | Walnut | Anaphylaxis | 3.36 | 0.5 | - |
| 5 | 6 | Walnut | Urticaria | 4.17 | 14.1 | - |
| 6 | 5 | Mixed nuts † | Erythema | 4.21 | 3.4 | - |

† Almond, cashew, walnut

mechanisms contributing to the clinically false positive detection of IgE antibodies.[5] The common structures of N-linked glycan in plants (fruits, vegetables and pollens) have been well characterized and designated as cross-reactive carbohydrate determinant (CCD).[6] Natural Ara h 1, the major peanut allergen, has a single N-glycosylation site bearing five glycan species in a one to one ratio.[7]

The second aim of this paper is to investigate the role of anti-CCD IgE antibodies in the clinically false positive IgE antibodies to peanut, soybean and other nuts.

## METHODS

Fourteen patients (aged 1−8 years, mean ± SD : 4.17 ± 2.0 years) with apparent history of immediate type peanut allergy were recruited to reveal the clinical characteristics of peanut allergy. Six patients with tree nut allergy (aged 2−6 years, 4.63 ± 1.69 years) were also analyzed. Oral challenge tests were not always performed to confirm the diagnosis, because severe anaphylaxis might occur in those with peanut

and tree nut allergy.[1]

Sera from the 14 patients with peanut allergy and 8 patients without clinical peanut allergy despite the detection of IgE to peanut (designated as clinically false positive sera) were served for measurement of IgE antibodies to other nuts by UniCAP specific IgE kit (Pharmacia Diagnostics AB, Sweden). The nuts examined were soybean, walnut, almond, cashew nut, chestnut, pistachio, macadamia and pine nut, although some data were lacking due to the limitations of the sera obtained.

Four representative sera from patients with peanut allergy and four clinically false positive sera were examined for IgE antibodies to horseradish peroxidase (HRP)[8] and bromelain (from pineapple stem)[9] by UniCAP specific IgE kit. The UniCAP inhibition test was also performed using peanut ImmunoCAP and HRP (Sigma P6782, St. Louis, MO, USA) as an inhibitor. HRP contains 6 N-linked glycans and bromelain carries only one IgE-binding glycan.[10]

Informed consent was obtained from parents of the subjects to donate their sera for this study.
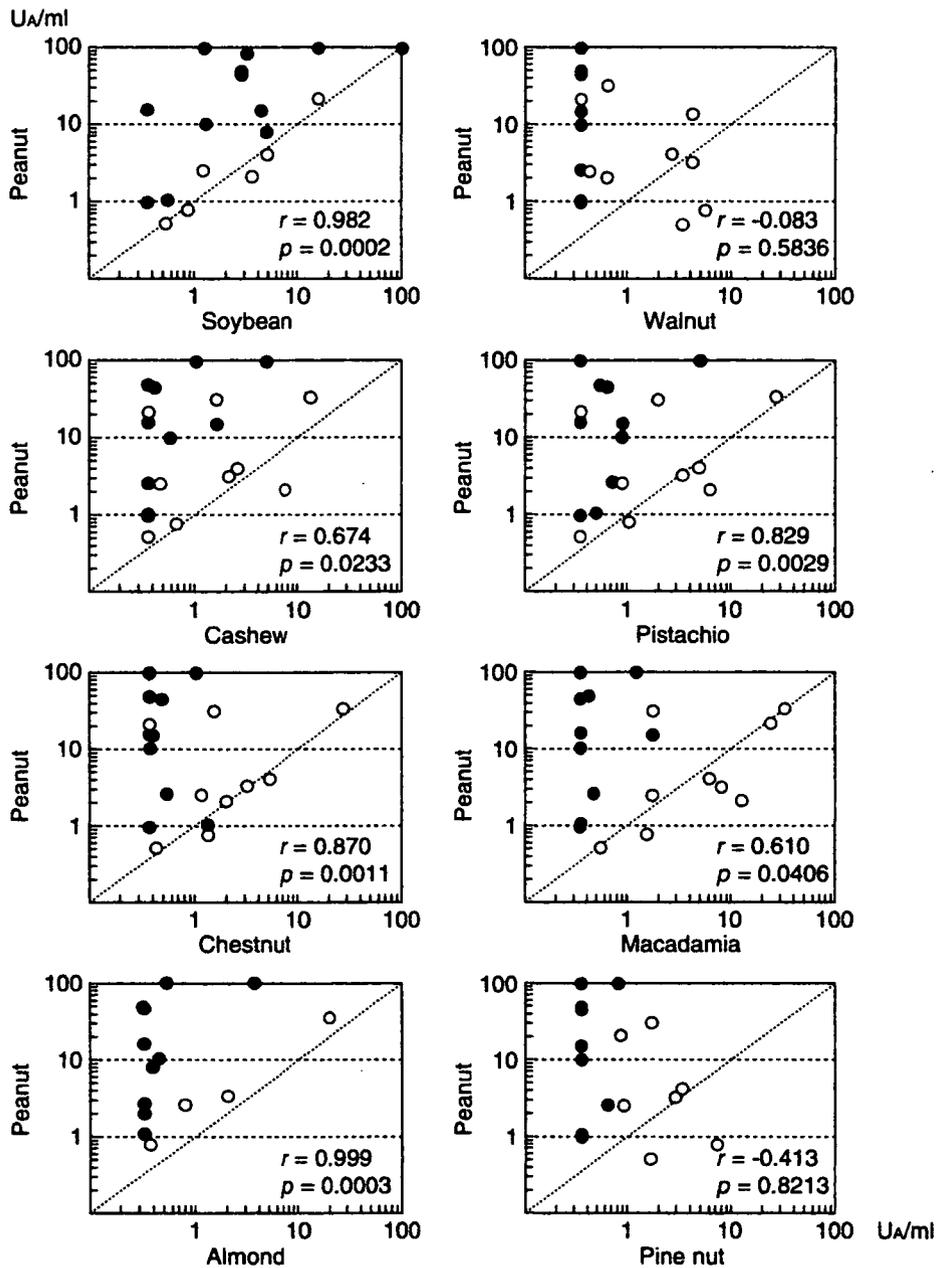
**Fig. 1** Correlation of IgE antibodies to peanut and other nuts. Closed circles indicate sera from patients with peanut allergy, and open circles indicate sera from patients without peanut allergy. Pearson's correlation-coefficient was calculated only with the clinically false positive sera.

## RESULTS

The clinical symptoms of 14 patients with peanut allergy were mostly severe, including anaphylaxis ( $n$ = 5), systemic urticaria ( $n$ = 5), erythema ( $n$ = 3), asthma and gastrointestinal symptoms (Table 1). The severity of symptoms was compatible to that reported in the United States or European countries.[1] Most of the patients had their first episode at 1-3 years of age. Although the parents were careful to avoid any

food contaminated with peanut, some patients repeated the symptoms by eating processed foods such as curry, sandwiches and crackers (contamination from the manufacturing line), or by contact to nutshells.

Three patients were also allergic to soybean, but the symptoms were mild and consisted of atypical immediate reactions (eczema, urticaria). One patient had an allergy to walnut (urticaria), but IgE antibody to walnut was negative.

**Fig. 2** IgE antibodies to HRP and bromelain in the sera from patients with and without peanut allergy.

Six patients had allergies to other nuts, including walnut ($n$ = 5), pine nut ($n$ = 1) and mixed nuts (almond, cashew and walnut, $n$ = 1), which caused anaphylaxis ($n$ = 2), urticaria ($n$ = 2) and cough ($n$ = 1). Only one patient was cross-reactive to peanut (Table 2).Although most of the patients avoided peanut and tree nuts, clinical cross-reactivity between peanut and other tree nuts was not common.

Correlations of IgE antibodies between peanut and other nuts are shown in Figure 1. Most of the sera from patients with peanut allergy had negative IgE to walnut, chestnut, macadamia, almond and pine nut. Although IgE antibody to soybean was positive in most of the patients with peanut allergy, IgE antibody to peanut was higher than that to soybean. These data suggested that IgE antibody from patients with peanut allergy tend to bind to the peanut-specific epitope which did not cross-react to other nuts.

On the other hand, clinically false positive sera showed significant correlation-coefficients between IgE antibodies to peanut and soybean ($r$ = 0.98, $p$ = 0.0024), almond ($r$ = 1.00, $p$ = 0.0003), chestnut ($r$ = 0.87, $p$ = 0.0011), pistachio ($r$ = 0.83, $p$ = 0.0029), macadamia ($r$ = 0.61, $p$ = 0.0406) and cashew ($r$ = 0.67, $p$ = 0.0233). These data suggested that false positive peanut IgE recognized the common structure between peanut and these nuts. No significant correlation was observed between clinically false positive IgE to peanut and walnut or pine nut (Fig. 1).

Anti-CCD IgE antibodies were measured to reveal one of the mechanisms of the clinically false positive IgE to peanut. Anti-HRP and anti-bromelain IgE were detected in 4 representative sera from patients without peanut allergy. However, the 4 representative sera from patients with peanut allergy showed negative IgE to HRP and bromelain (Fig. 2).

IgE inhibition tests showed that pre-incubation of the sera with HRP significantly abrogated IgE binding to peanut in 2 of the 4 sera with false positive peanut IgE in a dose-dependent manner. Another serum

showed partial (40%) inhibition, and the other was not inhibited. These findings suggested that CCD might contribute to the clinically false positive peanut IgE in some patients. On the other hand, no inhibition was observed in the sera from any of the 4 patients with peanut allergy (Fig. 3).

## DISCUSSION

In the United States and Europe, many authors have emphasized the importance of peanut allergy because of the life-threatening symptoms and increasing number of affected patients.[1,2] The prevalence of peanut allergy in the Japanese population is not clear. Here we report on 14 children with peanut allergy in our clinic. This number was compatible to that of buckwheat, fish or shrimp allergy in our clinic (data not shown). The severity of allergic symptoms of each patient was similar to that reported previously, and the patients repeatedly experienced potentially life-threatening symptoms.

It is known that almost 30% of patients with peanut allergy also respond to other tree nuts.[11] Our findings also suggested that 28.6% (4 out of 14) patients with peanut allergy reacted to soybean or walnut. However, the reactions to soybean observed in the 3 cases were not typical immediate-type responses, such as worsening of eczema.

Ara h 1, the major peanut allergen, belongs to the cupin superfamily, named vicilin.[12] Although homologous proteins exist in the other legume families,[12] patients with peanut allergy had no or relatively lower IgE antibodies to other tree nuts or soybean. These findings suggested the presence of peanut-specific epitopes that are preferentially recognized by IgE antibodies of patients with peanut allergy.

Shin et al. reported that three Ara h 1 molecules assemble to form a highly stable trimeric complex,[13] and IgE-binding epitopes are clustered near the regions of monomer-monomer contact.[14] According to this trimeric structure, Ara h 1 may possess resistance to protease digestion, and the increased number of IgE-binding epitopes in one molecule may induce a strong release of chemical mediators from mast cells. Cross-reactivity of this trimeric Ara h 1 between homologous proteins of other tree nuts may be of interest.

On the other hand, IgE antibody to peanut can be detected from atopic patients without clinical symptoms to peanut. The presence of IgE antibody to the carbohydrate moiety (CCD) is a well-known mechanism to explain the detection of clinically false positive IgE to plant allergens.[6] Figure 4 shows the structures of N-linked glycans of HRP, bromelain and Ara h 1. Common structures of glycans have been characterized from a variety of plant allergens.[15] The presence of $\beta(1, 2)$-Xylose and $\alpha(1, 3)$-Fucose residues linked to the core $Mann GlcNAc_2$ backbone is known to contribute to the IgE binding.[16] Anti-CCD IgE anti-
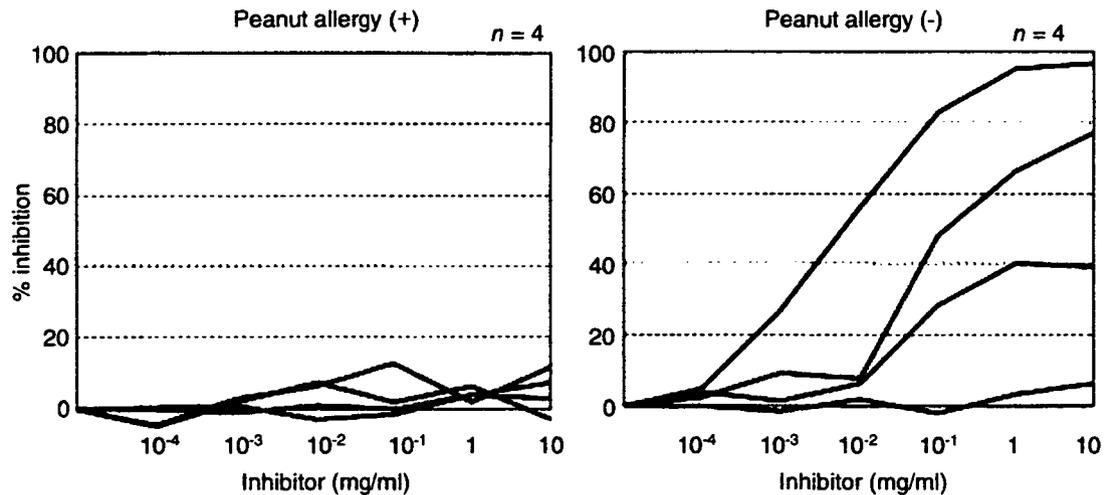
**Fig. 3** Peanut IgE inhibition tests with HRP, a representative CCD antigen. Individual sera from 4 patients with and without peanut allergy were pre-incubated with the indicated concentrations of HRP, and peanut IgE antibodies were detected by UniCAP. Percent inhibition of peanut IgE titers are shown.
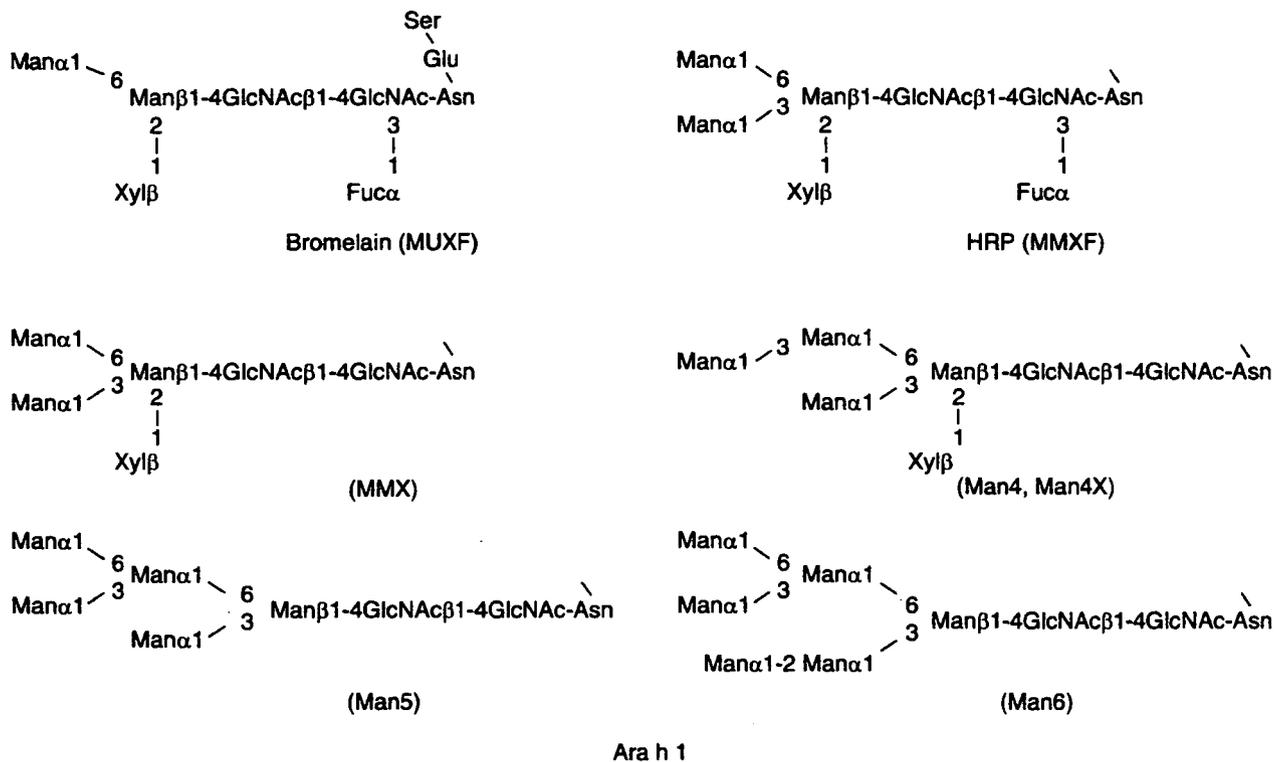


Ara h 1

**Fig. 4** Structures of the N-linked glycans known as cross-reactive carbohydrate determinant (CCD). Bromelain from pineapple stem has one xylose-containing N-linked glycan (MUXF). Horseradish peroxidase (HRP) has 6 xylose and fucose-containing glycans (MMXF). Ara h 1 has one glycosylation site bearing either one of the 5 oligosaccharides (MMX, Man4X, Man4, Man5, Man6).

bodies have poor biologic activity in terms of weak basophil histamine release and negative skin prick tests,[4] because CCD on a monoglycosylated allergen can not cross-link high-affinity IgE receptors on mast

cells and basophils. Van der Veen et al. have reported that positive IgE antibodies to peanut without clinical symptoms were the consequence of anti-CCD IgE that cross-reacted to peanut and grass-pollen aller-

gens.[17]

Our findings also support the idea that anti-CCD IgE antibodies contribute to the clinically false positive IgE antibodies to peanut and tree nuts in some patients. In general, anti-CCD IgE antibody binds to multiple plant allergens including pollens, vegetables, fruits and nuts. Care should be taken to consider the presence of anti-CCD IgE in the sera with positive IgE antibodies to multiple allergens, especially in the Japanese clinical practice where detection of IgE antibodies is more commonly used than skin prick tests or oral food challenge for the screening of food allergy.

Detection of anti-HRP or anti-bromelain IgE by UniCAP is a practical tool for the detection of anti-CCD IgE. Furthermore, the RAST inhibition test may be required to confirm the presence of CCD in the allergen. In the present study, one false positive serum showed negative inhibition of peanut IgE with HRP, suggesting that CCD is not always the feature of clinically false positive IgE.

In conclusion, peanut allergy causes severe, sometimes life-threatening reactions in Japanese children. Social education about the importance of peanut allergy is needed. However, detection of IgE antibody to peanut does not always indicate a definitive diagnosis of peanut allergy, and the presence of anti-CCD IgE antibody should be considered in a case of multiple positive IgE antibodies to plant allergens, including peanut.

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Research paper

# Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing agent

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## Abstract

Enzyme-linked immunosorbent assay (ELISA) has been considered extremely useful for the detection of markers of allergenic substances in food, because it is simple, offers a suitable sensitivity, and is useful in providing quantitative results. Allergenic protein present in processed food can be denatured or altered, hindering therefore their possibility to be extracted and detected. This paper reports the development of an ELISA method that can be used for the determination of allergenic proteins in buffer solutions containing SDS, a surfactant, and 2-mercaptoethanol, a reducing agent. Measurement by ELISA in solutions containing 1% SDS and 7% 2-mercaptoethanol has been made possible by using an antibody prepared through immunization with an antigen denatured with SDS and 2-mercaptoethanol. This ELISA technique can be used to measure proteins in food that have been denatured by various manufacturing processes. An example is egg white albumin, which is susceptible to heat denaturation and has been difficult to recover from food in the past. Its recovery was improved 10- to 100-fold by the new ELISA method as compared with previous methods. This means that allergenic substances in food can now be detected quantitatively. This method can be very useful in allergy prevention and control strategies.
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*Keywords:* ELISA; SDS; 2-Mercaptoethanol; Food allergy

## 1. Introduction

The immune system, which is the self-defense system of the body, sometimes responds in a way that is harmful to the body. Some of these responses are referred to as allergic reactions environment of food. Allergies caused in part by the surrounding are increasing in incidence and have recently become a societal problem. The number of children suffering from allergic reactions is also reported to have increased. Allergic

symptoms are diverse and vary according to each individual, some of whom show a severe symptom called 'anaphylactic shock', which is life-threatening. In order to prevent the inducement of allergic symptoms, it is important to avoid consuming foods with unknown ingredients some of which can be known allergens. In order to keep the diets of allergy patients safe, research is under way to determine the threshold intake doses of allergenic foods (Taylor et al., 2002). Subsequently regulations have made necessary to detect and quantify allergenic substances in food as accurately as possible.

Detection methods for allergenic substances in food include enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlman, 1972), immunochromatography and Western blotting, which target proteins, and polymerase chain reaction (PCR), which targets genes. Of these methods, ELISA is considered extremely useful for the detection of allergenic substances in food, because it is simple, high in detection sensitivity, and excellent in terms of quantification.

Food products can be subjected to processing treatments such as heating, pressurization and sterilization, during which proteins contained in food are often denatured in various manners. When an antibody generated against a native protein is used to detect such denatured proteins, its reactivity is often decreased as compared with its reactivity towards the native protein, because the denatured protein has undergone a change in 3D structure. In addition, it is necessary to extract the protein from the food commodity before enabling its detection check on the presence of allergenic substances by ELISA. Denatured or altered proteins are often less water-soluble than native proteins, which sometimes renders their extraction from food difficult. Surfactants and denaturants can be used to solubilize and extract insoluble proteins. However, measurement by ELISA with an extraction solution containing a denaturant cannot be made accurately because the antibody is affected by the denaturant. Furthermore, dilution of the extract to the extent that the denaturant does not influence the antibody sometimes makes detection of the extracted protein difficult.

In order to avoid this problem, investigations were carried out where a plate is coated with a protein treated with a surfactant and then an antigen–antibody reaction is enabled in a buffer system without the surfactant (Lechtzier et al., 2002). However, to our knowledge, no study has referred a method for quantification by an antigen–

antibody reaction in a buffer system containing surfactants or reducing agents. This paper describes a new ELISA that has been developed with the aim of solving the above-mentioned problems. This study focuses on the detection of egg protein that can be difficult to detect by ordinary ELISA after processing treatments such as heating. With this new approach, it is possible to extract these proteins more efficiently from food, carry out an antigen–antibody reaction, and detect the egg protein with a high recovery rate in a buffer system containing a surfactant and a reducing agent. This method is applicable to proteins in other food products.

## 2. Materials and methods

### 2.1. Materials

2-Mercaptoethanol (2-ME), sodium dodecyl sulfate (SDS), polyoxyethylene-sorbitan monolaurate (Tween 20), and methanol special grade were supplied by Nakarai Co., Ltd. 10× Tris/glycine buffer (pH, 8.3) and 10× Tris/glycine/SDS buffer (pH, 8.3) were provided by Bio-Rad Laboratories, Inc. Bovine serum albumin RIA grade (BSA), horseradish peroxidase (POD), 3,3'5,5'-tetramethylbenzidine (TMB), and Hybond-P (PVDF membrane) were from Wako Pure Chemical Industries Co., Ltd., Toyobo Co. Ltd., Moss, Inc., and Amersham Biosciences Co., Ltd., respectively. Freeze-dried whole egg powder was prepared from fresh egg of white leghorn.

In addition, the following buffers were prepared for the experiment: 120 mM Tris/HCl (pH 7.4) containing 0.1% BSA and 0.05% Tween 20 (Buffer A), and 20 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl and 0.05% Tween 20 (Buffer B).

### 2.2. Methods

#### 2.2.1. Preparation and purification of antibodies for specificity

##### 2.2.1.1. Preparation of a specific anti-OVA antibody (anti-native-OVA antibody). For immunization, an adjuvant containing about 0.2 mg of OVA (egg albumin, 5 × cryst., supplied by Seikagaku Corp.) per injection was hypodermically injected as an antigen about five times (~1 mg per rabbit) at appropriate

intervals (1 week) (Coligan et al., 1995). Whole blood was collected and the serum was separated for purification. An affinity column prepared by the fixation of OVA to a HiTrap NHS-activated HP column (Pharmacia Biosciences Co., Ltd.) was used to purify the OVA-specific antibody. The serum was applied to the column equilibrated with 20 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl, and the antibody that eluted with 0.1 M glycine/HCl buffer (pH 2.3) was taken to be a specific anti-OVA antibody (anti-native-OVA antibody).

### 2.2.2. Preparation of a specific antibody against OVA denatured with SDS and 2-ME (anti-SDS-OVA antibody)

For immunization, 5 mg/mL of OVA in PBS was added 1% SDS and undiluted 2-ME at final concentrations of 1% and 0.07%, respectively, for over 12 h. Then 1% SDS and 0.07% 2-ME exposed antigen was added adjuvant to inject rabbits (~1 mg per rabbit) in several applications as described above. The serum obtained from whole blood was purified for the denatured-OVA-specific antibody with a HiTrap column to which denatured OVA was fixed. The antibody obtained through this purification was taken to be a specific anti-SDS-OVA antibody.

### 2.3. Standard and sample extraction

#### 2.3.1. Homogenization of samples

A Millser IFN-700G homogenizer (Iwatani International Corp.) was used to homogenize samples. Samples were treated with the Millser a few times for 30 s for homogeneity.

#### 2.3.2. System without SDS and 2-ME

Thirty-eight milliliters of Buffer A (see Materials) was added to 2 g of a homogenized sample, which was then treated with the Millser three times for 30 s for extraction. After confirming the pH was around 6.0–8.0 with pH paper, the sample was centrifuged at 3000×g for 20 min and the supernatant was filtered through 5A filter paper (Advantec Tokyo Kaisha, Ltd.) to obtain the extract.

#### 2.3.3. System containing SDS and 2-ME

Nineteen milliliters of Buffer A containing 1% SDS and 7% 2-ME was added to 1 g of a homoge-

nized sample. The sample was then shaken back and forth at 90–110 rpm (moving distance, 3 cm) overnight (over 12 h at room temperature) for extraction. The subsequent centrifugation and filtration steps to obtain the extract were the same as those described for the system without SDS and 2-ME.

### 2.4. Protein assay

The extracts obtained were analyzed with a 2D Quant Protein Assay Kit (Pharmacia Biosciences Co., Ltd.).

### 2.5. ELISA

#### 2.5.1. ELISA in the system without SDS and 2-ME

A solution of egg extract was used as a standard and adjusted to make up the following concentration gradient by means of a protein assay: 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0 ng/mL as a blank (Buffer A only). These standards, plus the sample, which was diluted stepwise with Buffer A, were applied to an ELISA plate coated with the anti-native-OVA antibody at 100 µL/well and allowed to stand at 25 °C for 1 h for the primary reaction. Buffer B, 300 µL/well, was used to wash the plates, and the washing was repeated six times. An anti-native-OVA antibody labeled with POD was added to the ELISA plate at 100 µL/well and allowed to stand for 30 min for the secondary reaction. After a second washing, TMB, 100 µL/well, was added and the enzyme reaction was allowed to run at 25 °C for exactly 10 min. The reaction was stopped by the addition of 100 µL/well of 1 N $H_2SO_4$. Absorbance was measured at 450 nm, the dominant wavelength, and at 630 nm, the subdominant wavelength. The amount of egg protein was calculated from the absorbance of the sample by using the standard curve obtained.

#### 2.5.2. ELISA in the system containing SDS and 2-ME

A solution of egg extract denatured with SDS and 2-ME was used as a standard and adjusted to a concentration of 1 µg/mL by means of a protein assay. It was diluted 20-fold to 50 ng/mL with Buffer A and then serially diluted 2-fold to make up a concentration gradient of 25, 12.5, 6.25, 3.125, 1.56, 0.78, and a blank (0 ng/ml). The concentrations of SDS and 2-ME were set at 0.05% SDS and 0.35% 2-ME, respectively, for the

extraction. In addition, the sample extract was diluted 20-fold with Buffer A so that its SDS and 2-ME concentrations were made the same as those in the standards. The standards and sample extracts, 100 μL/well each, were applied to the ELISA plate, coated with the anti-SDS-OVA antibody to enable the primary reaction to occur. An anti-SDS-OVA antibody labeled with the POD was used as the enzyme-labeled antibody (Yoshitake et al., 1982) and diluted with Buffer B containing 1% BSA. The conditions and the reaction times for the washing and secondary reaction steps were the same as those described above for the ELISA in a system without SDS and 2-ME.

### 2.5.3. ELISA to SDS and 2-ME untreated OVA and treated OVA

The reaction of anti-OVA antibody to SDS and 2-ME treated OVA was performed using the freeze-dried egg powder dissolved in a solution containing 1% SDS and 7% 2-ME. Then it was left for 0, 1, 2, 4, and 6 h and then exposed to first reaction buffer containing SDS and 2-ME. The solution was subsequently subjected to ELISA as described above. Two antibodies were used: an anti-native-OVA antibody, specific for native OVA; and an anti-SDS-OVA antibody, specific for SDS-treated OVA.

### 2.6. Western blotting

The egg extract obtained from the assay where SDS and 2-ME were used and an OVA sample denatured with SDS and 2-ME, as described in the above methods, were mixed with a loading buffer (a mixture of Laemmli Sample Buffer [Bio-Rad Laboratories, Inc.] and 2-ME at 19:1) at a ratio of 1:2. After boiling in a boiling water bath for 5 min, the samples were cooled on ice and used for electrophoresis. SDS-PAGE (Laemmli, 1970) was performed at a constant electric current of 30 mA/gel by applying 20 μL/lane of the egg extract sample and the OVA extract sample to a 15% separation gel (TEFCO, Ltd.). A kaleidoscope standard (Bio-Rad Laboratories, Inc.) was used as a molecular weight marker. The proteins were blotted onto PVDF membranes in a transblot SD cell (Bio-Rad Laboratories, Inc.) at a constant electric current of 120 mA (2 mA/cm) for 1 h. The membranes were shaken in a blocking buffer (Buffer B containing a final concentration of 0.1% BSA) for 1 h for blocking treatment.

For the primary immunostaining reaction, the anti-SDS-OVA antibody at a concentration of 0.5 μg/mL was shaken with the membranes for 1 h. Buffer B (see Materials) was used for washing by shaking (three times for 5 min). For the secondary reaction, the membranes were shaken in the presence of a biotin-conjugated anti-rabbit-IgG antibody for 30 min using a VECTASTAIN ABC-AP Rabbit IgG kit (Vector Laboratories, Ltd.). After a second washing step, the membranes were shaken for a tertiary reaction with an avidin–biotin complex (an ABC solution) labeled with alkaline phosphatase (AP), which was included in the VECTASTAIN ABC IgG kit (Vector Laboratories, Ltd.). After additional washing step, the membranes were shaken with 100 mM Tris/HCl (pH 9.5) for 15 min. An Alkaline Phosphatase Substrate Kit IV ⟨BCIP/BNT⟩ (Vector Laboratories, Ltd.) was used for detection. Color was developed by shaking for about 5 min.

### 2.7. Preparation of model processed foods

Freeze-dried whole egg powder was mixed with raw foods to prepare processed food items containing the egg protein at two different concentrations: 10 μg/g and 50 μg/g. The following were prepared as model processed foods: orange juice (main raw ingredients: orange juice concentrate, sugar, and citric acid), biscuit (main raw ingredients: wheat flour, shortening, sugar, and salt), and strawberry jam (main raw ingredients: strawberry, sugars, and pectin).

## 3. Results

### 3.1. Improvement in protein extraction with SDS and 2-ME

Surfactants and reducing agents are useful for the solubilization of insoluble proteins. We extracted proteins from food with buffer solutions containing various concentrations of SDS and 2-ME and examined the relationship between the amount of protein extracted and the concentrations of SDS and 2-ME (Fig. 1). A raw egg (Fig. 1A), a boiled egg (Fig. 1B), and egg-fried noodles (Fig. 1C) were used to determine the amount of protein that could be extracted from food samples, which had been processed in various manners. All

**A.** Raw Egg



SDS concentration (%)

**B.** Boiled Egg



SDS concentration (%)

**C.** Fried Noodle
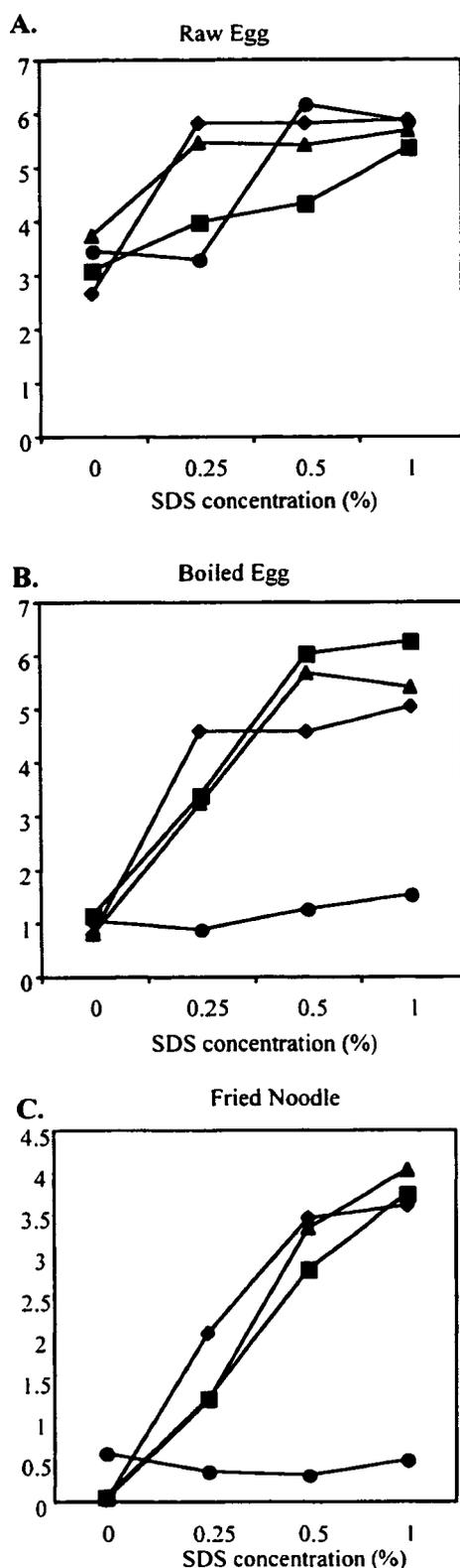


SDS concentration (%)

three samples showed an increase in the amount of protein extracted as the concentrations of SDS and 2-ME increased. Furthermore, the amount of protein almost reached a plateau in extract solutions containing 0.5% SDS and 2% 2-ME.

In addition, other food types were also tested for differences between the amount of protein extracted with a buffer (Buffer A) without SDS and 2-ME and the amount of protein extracted with a buffer containing 1% SDS and 7% 2-ME (Table 1). It was evident that the amount of protein extracted from all types of food increased markedly when the solution contained SDS and 2-ME. From these results, it was found that the amount of protein extracted increased by 10- to 100-fold when the extracting solution contained sufficient amounts of SDS and 2-ME.

### 3.2. Development of ELISA in solutions containing SDS and 2-ME

An antigen–antibody reaction must take place in the presence of SDS and 2-ME in order to perform ELISA for proteins extracted with solutions containing SDS and 2-ME. We have attempted such development for the determination of egg albumin (OVA) by ELISA in presence of SDS and 2-ME. The occurrence of binding between of the anti-OVA antibody and the OVA in the presence of SDS and 2-ME was assessed through the experimental procedure. SDS and 2-ME treated OVA was dispensed to the wells coated anti-native-OVA antibody or anti-SDS-OVA antibody. The anti-native-OVA antibody showed a reaction with native OVA that still remained at time 0, but did not react with denatured or altered OVA that had been exposed to SDS and 2-ME for 1 h (Fig. 2). By contrast, it was shown that the anti-SDS-OVA antibody was able to detect the denatured and altered OVA sample even in the presence of high concentrations of SDS and 2-ME, and the measured values were not affected even after 6 h of exposure to SDS and 2-

Fig. 1. Improvement in protein extraction with SDS and 2-ME. A raw egg (A), a boiled egg (B), and egg-fried noodles (C) were extracted with buffer solutions containing SDS at the concentrations indicated in the figure and 2-ME at concentrations of 0 (solid circle), 1% (solid square), 2% (solid triangle), and 7% (solid diamond). The extraction method is described in Materials and methods. Duplicate measurements were taken and the graph shows the average values.

Table 1
Improvement of protein extraction with SDS and 2-ME

| Food sample | SDS 0%, 2-ME 0% | SDS 1%, 2-ME 7% |
|---|---|---|
| | Protein concentration (mg/ml) | |
| Whole barley | 0.1 | 10 |
| Pepper | 0.02 | 3.4 |
| Corn | 0.09 | 11 |
| Soybean | 2.6 | 27 |
| Rice | 0.06 | 5 |

Samples were extracted with a solution without SDS and 2-ME and a solution containing them. Extracted proteins were measured by a protein assay, as described in Materials and methods. Results are representative of three independent experiments.

ME. We also confirmed that anti-SDS-OVA antibody recognizes untreated OVA.

With treated a cross reactivity state reaching 35% (considering OVA as a reference). Such cross reactivity could be attributed to the polyclonal native of the antibody, which is able to recognize various epitopes including those of non-denatured egg protein. In addition, OVA was subjected to Western blotting using the anti-SDS-OVA antibody in order to confirm whether or not the anti-SDS-OVA antibody recognized OVA (Fig. 3). Fig. 3 confirmed such specific recognition. The above results corroborate the specific reaction of
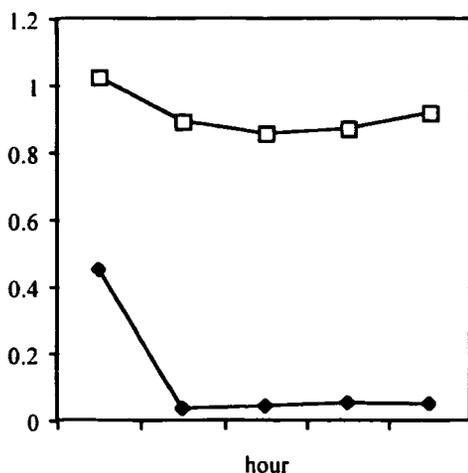


Fig. 2. Antigen–antibody reactions in the presence of SDS and 2-ME. Egg protein, 1.28 µg/mL, was dissolved in a solution containing 1% SDS and 7% 2-ME and the solution was allowed to stand for the durations indicated in the figure. The treated egg protein was detected by ELISA using an anti-native-OVA antibody (solid dimond) and an anti-SDS-OVA antibody (open square). The ELISA procedure is described in Materials and methods. Triplicate measurements were taken and the graph shows the average values.
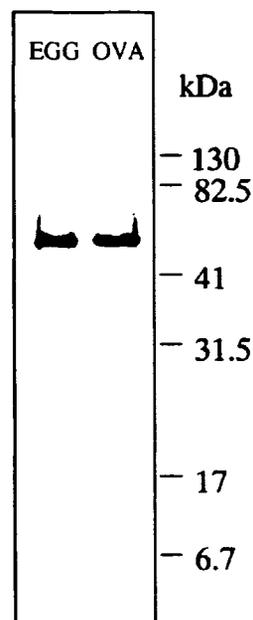


Fig. 3. Immunoblots of egg protein with an antibody against OVA treated with SDS. Egg and egg white albumin were treated with a solution containing SDS and 2-ME and then prepared for SDS-PAGE as described in Materials and methods. After electrophoresis, the anti-SDS-OVA antibody was used for immunoblot analysis. Immunostaining was done as described in Materials and methods. The immunoblots shown are representatives of two independent experiments with similar results.

the anti-SDS-OVA antibody with both denatured and altered OVA in the presence of SDS and 2-ME.

Next, a study was undertaken to examine how high the concentrations of SDS and 2-ME could be used without inhibiting the reactions involved in a sandwich ELISA using the anti-SDS-OVA antibody. The ELISA was performed in buffer solutions (Buffer A) containing SDS and 2-ME at various concentrations and standard curves were prepared (Fig. 4). The result showed that standard curves could be drawn up to an SDS concentration of 1% and a 2-ME concentration of 7%, making it possible to perform ELISA in solutions containing high concentrations of SDS and 2-ME.

3.3. Rates of protein recovery from model processed foods and measurements of proteins extracted from commercially available foods by ELISA

Model processed foods, to which known amounts of egg protein was added, were prepared in order to examine how accurately the ELISA could detect the
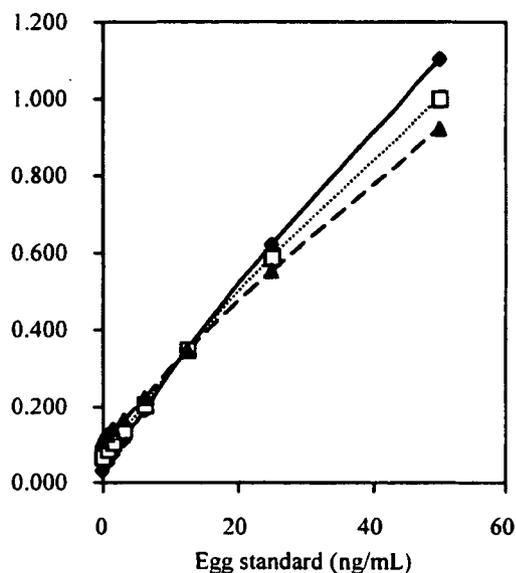
Fig. 4. Standard curves generated in the presence of various concentrations of SDS and 2-ME. Egg protein, 50 ng/mL, was used as a standard and the following 2-fold dilution series was prepared using Buffer A containing SDS and 2-ME: blank, 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 ng/mL. ELISA was performed with a system in which an anti-native-OVA antibody was used in a buffer without SDS and 2-ME (solid diamond), a system in which an anti-SDS-OVA antibody was used in a buffer containing 0.25% SDS and 1% 2-ME (open square), and a system in which the anti-SDS-OVA antibody was used in a buffer containing 1% SDS and 7% 2-ME (solid triangle). The ELISA procedure was done as described in Materials and methods. The graph shows the average values of triplicate measurements.

target protein in food that had undergone processing such as heating and pressurization. Proteins were extracted with Buffer A without SDS and 2-ME and Buffer A containing 1% SDS and 7% 2-ME for ELISA. The anti-native-OVA antibody was used for the former extract and the anti-SDS-OVA antibody was used for the latter extract. The extracts were diluted so that the measured values fell within ranges quantification of the standard curves. Recovery rates were determined by comparing the values obtained by ELISA measurement with the added amount of protein. The model-processed foods were prepared by adding egg protein at 10 μg/g and 50 μg/g to orange juice, biscuit, and jam. It was confirmed that none of the raw food items was contaminated with egg protein. The recovery rate of protein from the extracts containing SDS and 2-ME ranged from 75% to 93% for orange juice, from 36% to 47% for biscuit, and

Table 2
Improvement of protein recovery rate measured by ELISA in the presence of SDS and 2-ME

| Processed food | Recovery | | | |
|---|---|---|---|---|
| | SDS, 2-ME negative | | SDS, 2-ME positive | |
| | 10 ppm | 50 ppm | 10 ppm | 50 ppm |
| Orange juice | 1.23 | 1.22 | 92.8 | 75.8 |
| Biscuit | 1.48 | 0.82 | 47.2 | 36.2 |
| Jam | 0.65 | 0.52 | 46.9 | 38.4 |

Processed food samples, to which 10 μg/g or 50 μg/g of egg protein was added, were prepared. The samples were extracted with a solution without SDS and 2-ME, and the extracts were subjected to ELISA using an anti-native-OVA antibody (SDS, 2-ME negative). In addition, the samples were extracted with a solution containing SDS and 2-ME for ELISA using an anti-SDS-OVA antibody (SDS, 2-ME positive). The extraction and ELISA procedures are described in Materials and methods. Results are representative of three independent experiments.

from 38% to 47% for jam. These recovery rates were considerably higher than those determined for the extracts prepared without SDS and 2-ME (Table 2).

In addition, ELISA was performed on commercially available food items, in which egg was used as a raw ingredient, by extracting them using Buffer A with and without SDS and 2-ME. As shown in Table 3, all of the samples gave values of egg protein that were 10- to 100-fold higher when the extracts contained SDS and 2-ME. These results confirm the efficiency of the ELISA to accurately detect and quantify egg protein when extracted with a solution containing SDS and 2-ME.

Table 3
Increase caused by SDS and 2-ME in values measured by ELISA

| Processed food | SDS, 2-ME negative | SDS, 2-ME positive |
|---|---|---|
| Hamburg steak | – | 21 |
| Rice gruel | – | 2496 |
| Sandwiches | 27.6 | 2255 |
| Biscuit | 2000 | 15 000 |
| Bread | – | 5.6 |
| Cookies | 290 | 13 000 |
| Fried noodle | – | 170 |

Commercially available food products were extracted with a solution without SDS and 2-ME and the extracts were subjected to ELISA using an anti-native-OVA antibody (SDS, 2-ME negative). The food samples were extracted with a solution containing SDS and 2-ME for ELISA using an anti-SDS-OVA antibody (SDS, 2-ME positive). The extraction and ELISA procedures are described in Materials and methods. Results are representative of three independent experiments.

## 4. Discussion

The addition of SDS and 2-ME to the extraction buffer is very useful for the extraction of proteins from raw eggs, boiled eggs, and fried noodles (Fig. 1A–C). 2-ME cleaves intermolecular and intramolecular S–S bonds formed between cysteine residues of proteins, and SDS helps solubilize proteins by disrupting most of their non-covalent bonds. The amount of protein extraction from the boiled egg and fried noodles with an extraction solution containing more than 1% 2-ME increased as the SDS concentration increased. However, the amount of protein increased little in extracts prepared without 2-ME, even when the SDS concentration was raised. It can be considered that SDS has a sufficient effect when coupled with the action of 2-ME, which cleaves the S–S bonds formed during protein alteration, denaturation, and insolubilization, which can be caused by the heat treatment. Furthermore, a 2-ME concentration of 1% can be considered sufficient for cleaving the intermolecular S–S bonds resulting from processing in the boiled egg and fried noodle samples. As long as the 2-ME concentration was sufficiently high (2% or more), the amount of protein extracted from the raw and boiled eggs was constant and did not change even as the SDS concentration increased; thus, the protein was inferred to have been completely extracted (Fig. 1A,B). On the other hand, the amount of protein extracted from the fried noodles increased with the increase in SDS concentration (Fig. 1C), and it is conjectured that it would keep increasing with further rises in the SDS concentration. Because the fried noodles contain wheat and many wheat proteins are hydrophobic, SDS concentrations higher than those used for the raw and boiled eggs were deemed necessary to increase protein extraction from the fried noodles. It can be concluded that the SDS concentration necessary for maximal protein extraction varies according to the food, because the hydrophobic protein content (including denatured or altered proteins) varies.

As shown by the examples in Table 1, the amount of protein extracted from various raw food ingredients was increased markedly by the addition of SDS and 2-ME to the extracting solution. Thus, SDS and 2-ME are effective for increasing the extraction of protein even from non-processed, raw food ingredients.

ELISA for egg protein treated with SDS and 2-ME must be performed in a system in which an anti-SDS-OVA antibody is used instead of an anti-native-OVA antibody (Fig. 2). The complete lack of a reaction between the anti-native-OVA antibody and the egg protein treated with SDS and 2-ME was deemed to be due to a complete loss of the reactive sites on egg white albumin caused by the SDS and 2-ME treatments. By contrast, the reactivity was sufficiently maintained regardless of treatment time when the anti-SDS-OVA antibody was used (Fig. 2). This shows that the coating antibody did not lose its binding ability by denaturation or alteration with SDS and 2-ME. Furthermore, the anti-SDS-OVA antibody showed a strong, specific reactivity with egg white albumin denatured or altered with SDS and 2-ME. The result of the Western blotting confirmed that the anti-SDS-OVA antibody has specific reactivity only towards egg white albumin (Fig. 3). From these results, it can be concluded that the use of an anti-SDS-OVA antibody has made measurement by ELISA possible in a system containing SDS and 2-ME in the primary reaction.

It is necessary to use sufficient amounts of SDS and 2-ME for protein extraction. The minimum requirements of SDS and 2-ME for protein extraction may vary according to the type of processed food. Color development, dependent on egg protein concentration, was observed even under the conditions of high SDS and 2-ME concentrations at 1% and 7%, respectively, when the anti-SDS-OVA antibody was used (Fig. 4). Thus, it is clear that measurement by ELISA is possible in a system containing high concentrations of SDS and 2-ME in the primary reaction.

The amount of egg protein measured in both the model processed foods and the commercially available food items showed clear differences between the system using extraction solutions without SDS and 2-ME and the anti-native OVA antibody, and the system using extract solutions containing SDS and 2-ME and the anti-SDS-OVA antibody (Tables 2 and 3). SDS and 2-ME have considerably improved dissolution in the denatured or altered OVA, especially because these foods were all heat-treated. This study has demonstrated that the determination of an egg protein content in processed foods is considerably improved by the development of a new ELISA method for egg proteins with a focus on OVA. Processed foods could be measured for other proteins present in milk, wheat,