

## IV. 研究成果の刊行物・別冊

# Allergen-specific T-cell Response in Patients with Phenytoin Hypersensitivity; Simultaneous Analysis of Proliferation and Cytokine Production by Carboxyfluorescein Succinimidyl Ester (CFSE) Dilution Assay

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## ABSTRACT

**Background:** Phenytoin can induce diversified adverse reactions including generalized eruptions and the hypersensitivity syndrome. Delayed-type allergic mechanisms have been postulated to underlie these reactions. The tests most widely used to detect T-cell sensitization to drugs are the patch test and the lymphocyte transformation test (LTT), but their sensitivity is not sufficient. Simultaneous assessment of both the frequencies and the cytokine-producing phenotypes of allergen-specific T cells has become possible with the recently introduced carboxyfluorescein succinimidyl ester (CFSE) assay.

**Methods:** Seven patients who presented with phenytoin-induced maculopapular exanthema with and without fever were included in this study. Peripheral blood mononuclear cells (PBMCs) were labeled with CFSE and cultured with phenytoin for seven days. The cells were stained with anti-CD4 and cytokine-specific monoclonal antibodies (MoAbs), and analyzed with FACSCalibur.

**Results:** The phenytoin-specific proliferation of CD4<sup>+</sup> cells in patients was significantly higher than in the four controls exposed to phenytoin, and in seven healthy children with no previous phenytoin intake. A significant difference in the percentages of CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells between patients and the seven healthy children was observed. The sensitivity and specificity of proliferation were 100% and 90.9%, and those of IFN- $\gamma$  secretion were 71.4% and 100%, respectively.

**Conclusions:** Phenytoin-specific proliferation may be detected with greater sensitivity by the CFSE dilution assay than the conventional LTT. The assay revealed that both CD4<sup>+</sup> and CD4<sup>-</sup> T cells proliferated and produced IFN- $\gamma$  and TNF- $\alpha$  after stimulation with phenytoin. The CFSE dilution assay might be useful for the diagnosis and understanding of drug hypersensitivity.

## KEY WORDS

allergen-specific T cell, CFSE dilution assay, IFN- $\gamma$ , lymphocyte transformation test, phenytoin hypersensitivity

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**Table 1** Characteristics of Patients

Case	Age (years)	Sex	Neurological Abnormalities	Hypersensitivity to PHT	
				Symptoms	Interval between Reaction and Analysis (month)
1	2	M	lissencephaly, mental retardation, symptomatic epilepsy	fever, exanthema	19
2	3	F	symptomatic epilepsy	exanthema	1
3	4	M	lissencephaly, mental retardation, symptomatic epilepsy	exanthema	40
4	5	F	frontal lobe epilepsy	exanthema	6
5	11	F	mental retardation, symptomatic epilepsy	exanthema	51
6	11	M	frontal lobe epilepsy	exanthema	50
7	13	M	frontal lobe epilepsy	fever, exanthema	12

## INTRODUCTION

Phenytoin is an effective and widely prescribed anti-convulsant agent used in the treatment of focal and generalized tonic clonic seizures.<sup>1</sup> Phenytoin can induce eruptions that include maculopapular exanthema, the Stevens-Johnson syndrome, generalized exfoliative dermatitis, toxic epidermal necrolysis, vasculitis and fixed drug eruptions. Phenytoin is also linked to a hypersensitivity syndrome that manifests with fever, rash and lymphadenopathy.<sup>2</sup> Allergic mechanisms have been postulated to underlie these diversified adverse reactions.<sup>3</sup>

The clinical signs and symptoms of allergic reactions to drugs are extremely heterogeneous<sup>4</sup> and include different allergic reactions such as immediate-type reactions including anaphylaxis, type II and III reactions such as purpura and hypersensitivity vasculitis, as well as delayed-type reactions.<sup>5,6</sup> For all types of allergic reactions, recognition of the antigen by specifically sensitized T-lymphocytes is a prerequisite. In the case of delayed-type hypersensitivity reactions to aromatic anticonvulsant drugs such as phenytoin, the detection of allergen-specific T cells is especially important for diagnosis because of the absence of allergen-specific IgE.<sup>7</sup>

Currently, both *in vivo* and *in vitro* tests are used for the detection of sensitized T cells. Although an *in vivo* patch test with the suspected compound has been reported to be helpful in determining the cause of drug allergy, the reported sensitivity is relatively low (31.7–50%).<sup>8,9</sup> The test most widely used to detect T-cell sensitization to drugs *in vitro* is a lymphocyte transformation test (LTT),<sup>10,11</sup> which measures <sup>3</sup>H-thymidine uptake of dividing cells. Although it has been in use for more than three decades, many laboratories do not achieve sufficient sensitivity with this test.<sup>10</sup> An alternative for the measurement of T-cell proliferation as a read out of LTT is the determination of antigen-dependent-expressed proteins such as cytokines. IFN- $\gamma$  and IL-5 have been reported to be use-

ful indicators of drug-specific T-cell activation.<sup>11,12</sup>

Recently, Turcanu *et al.* introduced a carboxyfluorescein succinimidyl ester (CFSE) assay to identify food antigen-specific T cells.<sup>13</sup> CFSE is a membrane-permeating dye that binds the amino groups of cytoplasmic proteins with its succinimidyl-reactive group. When cells divide, CFSE-labeled proteins are equally distributed between the daughter cells, thus halving cell fluorescence with each division. Consequently, antigen-specific T cells lose their fluorescence after culture in the presence of the respective antigen (CFSE<sup>low</sup>) and are distinguishable from other cells in culture (CFSE<sup>high</sup>). In this report, we applied this experimental approach to examine both the frequencies and cytokine-producing phenotypes of these phenytoin-specific CD4<sup>+</sup> and CD4<sup>-</sup> T cells simultaneously.

## METHODS

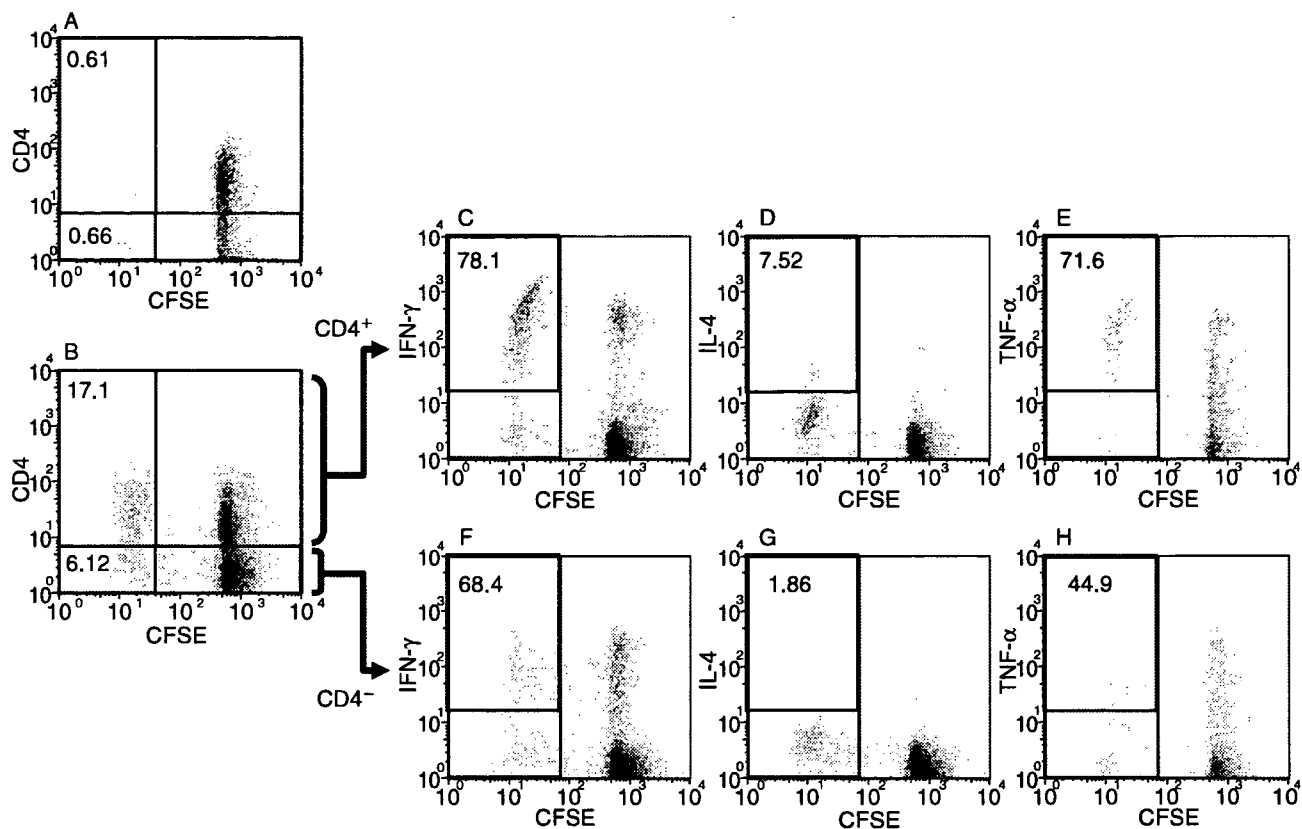
### PATIENTS AND CONTROLS

Seven patients presenting with phenytoin-induced maculopapular exanthema with and without fever were included in this study (Table 1). Informed consent was obtained from all patients participating in this study. The study was approved by the local medical ethics committee.

Two control groups were included in the investigation. In the first control group (control A), four individuals exposed to phenytoin without clinical signs of a drug-allergic reaction were investigated. In the second control group (control B), seven healthy children with no previous phenytoin intake were examined.

### CFSE DILUTION ASSAY

The frequencies and cytokine-producing phenotypes of phenytoin-specific T cells were examined simultaneously using a CFSE dilution assay according to Turcanu *et al.* with some modifications.<sup>13</sup> Briefly, peripheral blood mononuclear cells (PBMCs) isolated from heparinized venous blood by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) were in-



**Fig. 1** Representative results from patient 3. PBMCs from patient 3 were isolated, labeled with CFSE, and cultured in the presence of 50  $\mu\text{g/ml}$  of phenytoin. On day 7, PBMCs were restimulated with PMA and ionomycin in the presence of brefeldin A for 6 hours, then the cytokine production of PC-5-labeled  $\text{CD4}^+$  cells was assessed using intracellular cytokine staining with the PE-labeled MoAbs indicated. **A** represents the background levels of  $\text{CFSE}^{\text{low}}$  cells in  $\text{CD4}^+$  (left upper quadrant) and  $\text{CD4}^-$  cells (left lower quadrant) in culture without phenytoin. **B** represents the percentages of  $\text{CFSE}^{\text{low}}$  cells in  $\text{CD4}^+$  (left upper quadrant) and  $\text{CD4}^-$  (left lower quadrant) cells in culture with phenytoin. **C** to **E** show the percentage of indicated cytokine-positive cells in phenytoin-specific  $\text{CFSE}^{\text{low}}$   $\text{CD4}^+$  cells, **F** to **H** show the percentages of cytokine-positive cells in phenytoin-specific  $\text{CFSE}^{\text{low}}$   $\text{CD4}^-$  cells.

cubated with CFSE (Sigma, St. Louis, MO, USA, labeling concentration of 5  $\mu\text{M}$ ) for 10 minutes at 37°C, and excess dye was washed away. CFSE-labeled PBMCs were then cultured with 50  $\mu\text{g/ml}$  of phenytoin (Aleviatin, Dainippon-Sumitomo, Japan) in RPMI-1640 supplemented with 5% autologous plasma at a concentration of  $1 \times 10^6$  cells/ml under humidified conditions and 5%  $\text{CO}_2$ . After seven days of culture, PBMCs were restimulated with PMA (50 ng/ml) (Sigma) and ionomycin (1  $\mu\text{g/ml}$ ) (Sigma) in the presence of brefeldin A (10  $\mu\text{g/ml}$ ) (Sigma) for six hours. The cells were then stained with PC-5-labeled anti-CD4 antibody, fixed and permeabilized with FIX & PERM cell permeabilization kits (Caltag Laboratories, Burlingame, CA, USA) and stained with PE-labeled IFN- $\gamma$ , IL-4, TNF- $\alpha$  or IL-10 specific monoclonal antibodies (MoAbs; Immunotech Coulter; Miami, FL, USA). Stained cells were acquired with FACSCalibur (BD Biosciences, Milpitas, CA, USA) and the data were analyzed with CellQuest software (BD Bi-

osciences).

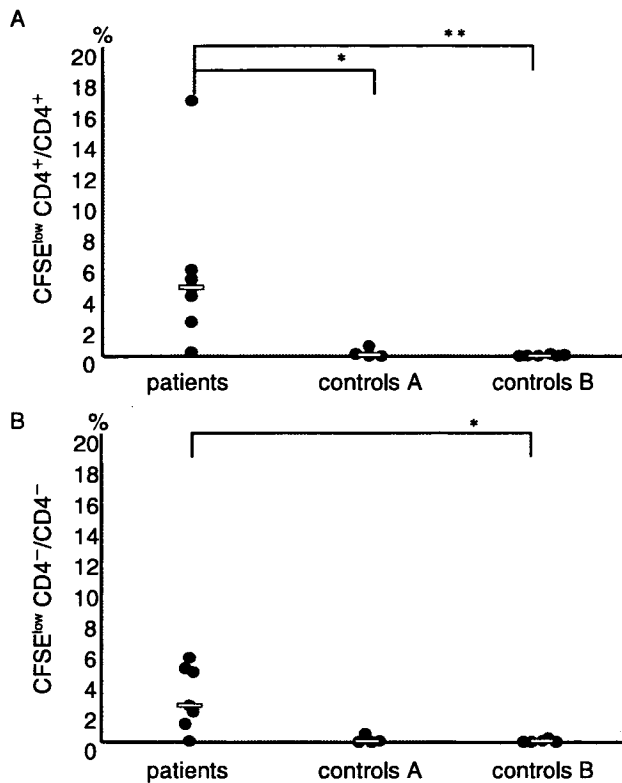
#### STATISTICAL ANALYSIS

Statistical significance was measured by the two-tailed Kruskal-Wallis one-way ANOVA for ranks followed by the Mann-Whitney *U* test with Bonferroni corrections for individual groups using SPSS software (SPSS Inc., Chicago, IL, USA).

#### RESULTS

##### A SIGNIFICANTLY HIGH NUMBER OF $\text{CFSE}^{\text{low}}$ $\text{CD4}^+$ AND $\text{CD4}^-$ T LYMPHOCYTES APPEARED IN PATIENTS AFTER PHENYTOIN STIMULATION

PBMCs from patients were labeled with CFSE and then cultured for seven days in the presence of phenytoin. In Figure 1, representative results from patient 3 are shown. In phenytoin-treated cultures, considerable populations of  $\text{CFSE}^{\text{low}}$   $\text{CD4}^+$  and  $\text{CD4}^-$  T lymphocytes emerged (17.1% and 6.12% of  $\text{CD4}^+$  and  $\text{CD4}^-$  cells, respectively) (Fig. 1B); however, the



**Fig. 2** Proliferation of phenytoin-specific T lymphocytes. PBMCs were isolated, CFSE-labeled, and cultured in the presence of phenytoin for 7 days, then the percentage of antigen-specific CFSE<sup>low</sup> CD4<sup>+</sup> cells in CD4<sup>+</sup> cells (A) and CFSE<sup>low</sup> CD4<sup>-</sup> cells in CD4<sup>-</sup> cells (B) was assessed by flow cytometry. Points represent data from different individuals, while bars show the median values. Statistical significance was measured by the Kruskal-Wallis one-way ANOVA for ranks followed by the Mann-Whiney *U* test with Bonferroni corrections for individual groups (\*\*  $p < 0.01$ , \*  $p < 0.05$ ).

proportions of CFSE<sup>low</sup> cells were small when PBMCs were cultured without phenytoin (0.61% and 0.65% of CD4<sup>+</sup> and CD4<sup>-</sup> cells, respectively) (Fig. 1A).

Phenytoin-specific proliferation was calculated by subtracting the proportion of CFSE<sup>low</sup> cells without stimulation from the proportion after phenytoin stimulation, and these results are presented in Figure 2. Phenytoin-specific proliferation of CD4<sup>+</sup> cells in patients was significantly higher than in Control A exposed to phenytoin (median 4.41%, range 0.26 to 17.1% *vs.* median 0.07%, range 0.0 to 1.83%,  $p < 0.05$ ) (Fig. 2A), and in seven healthy children with no previous phenytoin intake (Control B) (median 4.41%, range 0.26 to 17.1% *vs.* median 0.0%, range 0.0 to 0.12%,  $p < 0.01$ ) (Fig. 2A).

Similar tendencies were seen in the phenytoin-specific proliferation of CD4<sup>-</sup> cells, although statistical significance was weaker. Phenytoin-specific proliferation of CD4<sup>-</sup> cells in patients was significantly higher than in Control B (median 2.36%, range 0.04 to

5.46% *vs.* median 0.04%, range 0.0 to 0.25%,  $p < 0.05$ ) (Fig. 2B), but was not significantly different from that in Control A (median 2.36%, range 0.04 to 5.46% *vs.* median 0.04%, range 0.0 to 0.52%).

### PHENYTOIN-SPECIFIC T CELLS DISPLAY TH1 DOMINANCE

Figures 1C to 1H demonstrate representative results of cytokine production. We expressed the results as a percentage of cytokine-positive cells among CFSE<sup>low</sup> (antigen-specific) lymphocytes: 78.1%, 7.52% and 71.6% of CFSE<sup>low</sup> CD4<sup>+</sup> cells and 68.4%, 1.86% and 44.9% of CFSE<sup>low</sup> CD4<sup>-</sup> cells were stained with PE-labeled antibody to IFN- $\gamma$ , IL-4 and TNF- $\alpha$ , respectively.

We compared the cytokine production of phenytoin-specific CD4<sup>+</sup> cells for all study groups. A significant difference in the percentages of IFN- $\gamma$  cells between patients and Control B (median 59.5%, range 0 to 78.1% *vs.* median 0%, range 0 to 5.65;  $p < 0.05$ ) was observed (Fig. 3A), but the percentage in patients was not significantly different from that in Control A (median 59.5%, range 0 to 78.1% *vs.* median 0%, range 0 to 24.3%). A similar tendency was observed in percentages of IFN- $\gamma$  cells among CD4<sup>-</sup> cells, but the differences were not statistically significant.

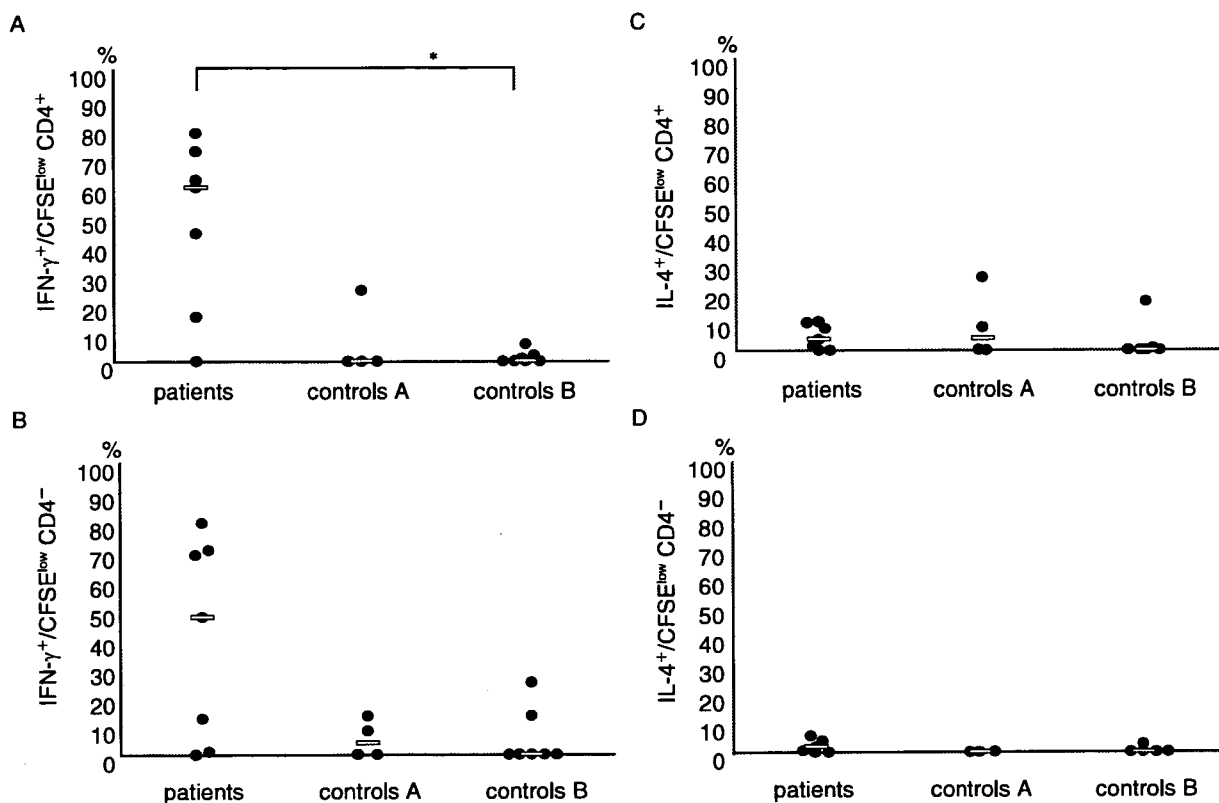
Phenytoin-specific T cells did not seem to secrete considerable levels of IL-4, and there was no significant difference in the percentages of IL-4<sup>+</sup> CFSE<sup>low</sup> cells from patients and controls (Figs. 3C, 3D). TNF- $\alpha$ -positive cells tended to correlate with INF- $\gamma$ -positive cells (data not shown).

### DETECTION OF PHENYTOIN-SPECIFIC PROLIFERATION AND IFN- $\gamma$ SECRETION OF CD4<sup>+</sup> CELLS PROVED SENSITIVE PARAMETERS

The detection of drug-specific proliferation and IFN- $\gamma$  secretion from the CD4<sup>+</sup> cells of the seven patients proved reliable sensitive parameters for the detection of drug sensitization (Table 2). The sensitivity, specificity, positive and negative predictive values (PPV and NPV) were calculated and the best cut-off points (0.26% for proliferation and 24.3% for IFN- $\gamma$  production) were selected as those that maximized the sum of the specificity and sensitivity. Differences in the calculated sensitivity of proliferation (100%) and IFN- $\gamma$  secretion (71.4%) resulted only from a control subject who showed a high percentage of IFN- $\gamma$ -secreted cells. The specificity of the proliferation was relatively low (90.9%) because the low proliferative response of PBMC from a patient decreased the cut off point. This patient had a history of maculopapular exanthema following phenytoin intake six months before this examination.

### DISCUSSION

In the LTT, allergen-stimulated lymphocytes undergo



**Fig. 3** Intra-cytoplasmic cytokine profiles of antigen-specific T lymphocytes. PBMCs were isolated, CFSE-labeled, and cultured in the presence of phenytoin for 7 days, then the cytokine-producing phenotype of antigen-specific (CFSE<sup>low</sup>) CD4<sup>+</sup> (A, C) and CD4<sup>-</sup> (B, D) cells was assessed by flow cytometry. Intra-cytoplasmic cytokine profiles were presented as percentages of CFSE<sup>low</sup> cells that produce IFN- $\gamma$  (A, B) and IL-4 (C, D). Points represent data from different individuals, while bars show the median values. Statistical significance was measured by the Kruskal-Wallis one-way ANOVA for ranks followed by the Mann-Whiney *U* test with Bonferroni corrections for individual groups (\* *p* < 0.05).

blastogenesis and generate lymphokines such as IL-2, followed by a proliferative response that can be measured by the incorporation of <sup>3</sup>H-thymidine during DNA synthesis. Retrospective evaluation of the sensitivity and specificity of the LTT to  $\beta$ -lactam antibiotics revealed a sensitivity of 78% and a specificity of 85%, better than the patch test (64% and 85%, respectively).<sup>14</sup> However, the sensitivity achieved by many laboratories does not achieve such a level, despite the fact that the LTT has been in use for more than three decades.<sup>10</sup> An alternative for the measurement of T-cell proliferation as a read out of the LTT is the determination of antigen-induced proteins such as cytokines. IFN- $\gamma$  has been reported to present a useful indicator of drug-specific T-cell activation.<sup>15</sup> Sachs *et al.* reported that the specific activation of peripheral blood mononuclear cells consistently resulted in IL-5 secretion, and that the sensitivities of the patch test, the LTT, and the assessment of drug-specific IL-5 secretion for the detection of drug sensitization were 55, 75, and 92%, respectively.<sup>12</sup>

In this study we demonstrated that phenytoin-specific proliferation and cytokine production of

PBMC from seven patients with phenytoin hypersensitivity could be detected by the CFSE dilution assay. The determination of phenytoin-specific CD4<sup>+</sup> cell proliferation and INF- $\gamma$  production by this assay resulted in more sensitive parameters (100% and 71.4%, respectively) for the detection of drug-sensitized T cells than the LTT or patch tests. Although both the CFSE and LTT assays detect lymphocyte proliferation, it is possible to examine proliferations of CD4<sup>+</sup> and CD4<sup>-</sup> cells separately but simultaneously by the CFSE assay. Furthermore, true antigen specific cells which divided several times in the culture might be distinguished from bystander cells that divided a few times at the late phase of culture. Thus, our results revealed the potential of the CFSE dilution analysis, although, a study involving a larger number of subjects is necessary to establish the superiority of the CFSE assay to the LTT. As use of this method could be applied to other drugs, the CFSE dilution analysis might provide a non-radioactive, sensitive alternative for the *in vitro* detection of drug sensitization.

It has been shown that in primary cultures of PBMCs both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes are acti-

**Table 2** Diagnostic Accuracy of CD4<sup>+</sup> cell Proliferation and IFN- $\gamma$  Secretion

	Results		Sensitivity (%)	Specificity (%)	PPV* (%)	NPV* (%)
	Positive <sup>†</sup>	Negative <sup>†</sup>				
Proliferation			100	90.9	87.5	100
Patient	7	0				
Control	1	10				
IFN- $\gamma$ secretion			71.4	100	100	84.6
Patient	5	2				
Control	0	11				

<sup>†</sup> Cut-off points were 0.26% for proliferation and 24.3% for IFN- $\gamma$  production.

\* PPV denotes positive predictive value, NPV denotes negative predictive value.

vated by drugs.<sup>16</sup> As T-cell activation with a particular function seems to lead to a specific clinical picture of hypersensitivity, the intensive characterization of drug-specific T cells might contribute to the diagnosis and understanding of drug hypersensitivity. In this respect, the cloning of antigen-specific T cells has proven to be a powerful tool to investigate the immune response to allergens at the T-cell level.<sup>17,18</sup> Nevertheless, several cycles of T cell stimulation in the presence of antigen, feeder cells, and exogenous cytokines are necessary to establish T cell clones. Since this is both time and labor consuming, only a limited number of clones can be studied. Furthermore, human T cells tend to develop a TH2-skewed cytokine-producing phenotype after repeated stimulation *in vitro* under neutral conditions.<sup>19</sup>

In this report, using the CFSE dilution assay we revealed that both CD4<sup>+</sup> and CD4<sup>-</sup> phenytoin-specific T cells existed and that both mainly produced IFN- $\gamma$  and TNF- $\alpha$  (Th1 and Tc1). A small population of CD4<sup>+</sup> cells secreted IL-4 (Th2). Pichler proposed that further classification of delayed-type (type IV) hypersensitivity reactions through the release of certain cytokines and chemokines led to a better understanding of drug hypersensitivity pathogenesis:<sup>7</sup> Type IVa, Th1 cells activate and recruit monocytes; Type IVb, Th2 cells activate and recruit eosinophils; Type IVc, Tc cells develop cytotoxicity; and Type IVd, IL-8 secreting T cells activate and recruit neutrophils. The CFSE dilution assay combined with cytoplasmic IL-8 or perforin staining besides IFN- $\gamma$  and IL-4 might provide further insight into various drug allergies.

In summary, we analyzed phenytoin-specific T-cell proliferation and cytokine production simultaneously using the CFSE dilution assay. Phenytoin-specific proliferation may be detected with greater sensitivity by the CFSE dilution assay than the conventional LTT. Furthermore, the assay revealed that both CD4<sup>+</sup> and CD4<sup>-</sup> T cells proliferated and produced IFN- $\gamma$  and TNF- $\alpha$  after stimulation with phenytoin, suggesting that both Th1 and Tc1 participated in the pathogenesis. The CFSE dilution assay might be useful for the diagnosis and understanding of drug hypersensitivity.

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## ACKNOWLEDGEMENTS

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## A Reliable and Sensitive Immunoassay for the Determination of Crustacean Protein in Processed Foods

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Among food allergens, crustacea such as shrimps, crabs, and lobsters are a frequent cause of adverse food reactions in allergic patients. The major allergen has been identified as a muscular protein, tropomyosin. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of crustacean protein in processed foods was developed using the sample dilution buffer that is added to porcine tropomyosin. The sandwich ELISA method was highly specific for the Decapoda group, apart from minor cross-reactivities to other crustacea and mollusks. The recovery ranged from 85 to 141%, while the intra- and interassay coefficients of variation were less than 2.8 and 8.4%, respectively.

**KEYWORDS:** Crustacea; food allergy; enzyme immunoassay; ELISA; tropomyosin

### INTRODUCTION

In industrialized countries, food allergies have represented an important health problem in recent years, and it is estimated that approximately 8% of children and 2% of adults have some type of food allergy (1, 2). Burks et al. (3) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan, the number of patients with food allergies, especially among young children, is increasing.

To prevent possible life-threatening reactions, the only effective treatment is to strictly avoid the consumption of these allergenic foods. However, various studies have shown that severe allergic reactions can be caused by the accidental intake of food products containing allergenic materials (4, 5). Therefore, sufficient information regarding potentially allergenic ingredients in food products is necessary. The issue of a labeling system for allergenic ingredients in food products has been discussed by international organizations, such as the Codex Alimentarius Commission of the World Health Organization (WHO) and the Food Agriculture Organization (Codex 1998). In 1999, the Joint FAO/WHO Codex Alimentarius Commission Session agreed to label eight kinds of foods that contain ingredients known to be allergens, including soybeans (FAO 1995, 6). In Japan, the Ministry of Health, Labor, and Welfare (MLHW) has enforced a labeling system for allergenic food

material since April 2002 to provide information about these foods to the allergic consumer. In this system, labeling for five food products, including eggs, milk, wheat, buckwheat, and peanuts, is mandatory and is recommended for 20 other food materials, such as soybeans and shrimp. In Japan, it became clear, based on epidemiological investigations, that the number of patients with a crustacean allergy such as to shrimp or crab has increased (7, 8).

In recommendations regarding labeling, Crustacea labeling would be particularly important because of the almost unlimited uses of Crustacea and because the number of patients with an allergy to Crustacea has been increasing, although the crustacean allergy is still less prevalent than the peanut allergy in the food-allergic population (9, 10). Crustacean allergic reactions may occur due to trace amounts of the crustacean protein, and anaphylaxis to Crustacea has been reported (11, 12).

In the present study, we developed a reliable sandwich enzyme-linked immunosorbent assay (ELISA) method with a high sensitivity for Crustacea. We showed that this detection method could be applicable to food-processing products and that the trace amount of Crustacea contained in commercial food products can be detected using the proposed sandwich ELISA method.

### MATERIALS AND METHODS

**Food Samples.** The black tiger prawn (*Penaeus monodon*) was purchased from Integrated Aquaculture Specialist, Inc. (Cebu, Philippines). The common Crustacea and mollusks, namely, northern shrimp (*Pandalus borealis*), Japanese spiny lobster (*Panulirus japonicus*),

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Caribbean spiny lobster (*Panulirus argus*), red king crab (*Paralithodes camtschatica*), swimming crab (*Portunus trituberculatus*), Japanese mantis shrimp (*Squilla oratoria*), euphausia (*Euphausia similis*), opossum shrimp (*Neomysis japonica*), acorn barnacle (*Balanus rostratus*), goose barnacle (*Pollicipes mitella*), common octopus (*Octopus vulgare*), giant octopus (*Paroctopus dofleini*), ocellated octopus (*Octopus ocellatus*), Japanese common squid (*Todarodes pacificus*), spear squid (*Loligo kobeensis*), cuttlefish (*Sepia subaculeata*), common scallop (*Patinopecten yessoensis*), Japanese oyster (*Crassostrea gigas*), bloody clam (*Scapharca broughtonii*), blue mussel (*Mytilus edulis*), short-neck clam (*Tapes japonica*), common freshwater clam (*Corbicula leane*), Japanese hard clam (*Meretrix lusoria*), Sakhalin surf clam (*Spisula sachalinensis*), horned turban (*Turbo cornutus*), Japanese abalone (*Haliotis discus*), and whelk (*Babylonia japonica*), and other ingredients and commercial processed foods were purchased at local stores in Japan.

**Preparation of Model Processed Foods.** The model processed foods were prepared according to a previously reported cooking procedure (13). Specifically, the freeze-dried black tiger prawn muscle powder was mixed with raw foods and then cooked to prepare the processed food items containing the shrimp protein at 10  $\mu\text{g/g}$  as the final concentration. The following were prepared as the model processed foods.

Fish meat sausages were made of minced fish flesh (yellow goatfish, atka mackerel, and walleye pollack), lard, sugar, salt, ice water, and the spiking powder. Lard, salt, sugar, ice water, and the spiking powder were added to the minced fish flesh and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into the fish sausage casings. These were then heated at 121 °C for 15 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5 °C overnight.

The freeze-dried egg soup was made of egg, potato starch, milk sugar (lactose), salt, and the spiking powder. Eggs, potato starch, milk sugar, salt, and the spiking powder were thoroughly mixed. The mixture was dissolved in a plastic tray, frozen in a deep freezer at -80 °C, and then lyophilized for 20 h.

The chicken meatball was made of white meat of chicken, lard, potato starch, sugar, and the spiking powder. Lard, potato starch, sugar, and the spiking powder were added to ground white meat of chicken and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into casings. These were then preserved in a deep freezer at -20 °C.

**Preparation of Black Tiger Prawn Protein Standards.** A 0.1 g sample of the freeze-dried black tiger muscle powder was added to 20 mL of phosphate-buffered saline [10 mM Na-phosphate, 154 mM NaCl (pH 7.4)] containing 5 g/L sodium dodecyl sulfate (SDS), 20 mL/L  $\beta$ -mercaptoethanol, 10  $\mu\text{L/mL}$  protease inhibitor cocktail, and 10  $\mu\text{L/mL}$  0.5 M ethylenediaminetetraacetic acid (Halt protease inhibitor cocktail kit; Pierce, Rockford, IL). The mixture was then shaken for 15 h at room temperature for extraction. After the extraction, the sample was centrifuged at 10000g for 30 min and the supernatant was filtered through a 0.8  $\mu\text{m}$  microfilter paper (DISMIC-25cs; Advantec, Tokyo, Japan) to obtain the extract. The extract was then heated at 100 °C for 10 min. The obtained extracts were analyzed using a 2D Quant Protein Assay Kit (GE Healthcare UK Ltd. NA, England).

**Purification of Black Tiger Prawn Tropomyosin (BTTM), Red King Crab, Swimming Crab, Japanese Oyster, Common Scallop, Japanese Common Squid, and Porcine Tropomyosin (PTM).** The purification of the BTTM was carried out according to the methods reported by Nagpal et al. (14), Ishikawa et al. (15), and Miegel et al. (16), respectively. The black tiger prawn muscles (100 g) were homogenized with 200 mL of a solution containing 20 mM KCl, 1 mM  $\text{KHCO}_3$ , 0.1 mM  $\text{CaCl}_2$ , and 0.1 mM dithiothreitol (DTT). After centrifugation (3000g for 5 min at 4 °C), 200 mL of acetone was added to the precipitant. The suspension was filtered through cheesecloth, and the residue was then washed three times with 200 mL of acetone. Finally, the residue was allowed to dry at room temperature for 2–3 h. The dried powder was extracted overnight at room temperature with 200 mL of 25 mM Tris-HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM  $\text{CaCl}_2$ , and 1 mM DTT. After filtration through cheesecloth, the residue was once more extracted with 200 mL of 1 M KCl. The extracts

were combined and cooled to 4 °C. Ammonium sulfate was added to produce an approximate 30% saturation. After 2 h, the solution was centrifuged (18000g for 60 min at 4 °C) and ammonium sulfate was then added to the supernatant (60% saturation). After 2 h, the solution was centrifuged and the precipitant was dissolved in 20 mL of 5 mM Tris-HCl (pH 7.5) containing 0.1 mM  $\text{CaCl}_2$ , and 0.1 mM DTT and was dialyzed overnight against 6 L of the same solution. The pH was then adjusted to 4.6 by the addition of HCl, and the tropomyosin precipitate was removed by centrifugation. The precipitate was dissolved in 25 mM Tris-HCl (pH 8.0) containing 1 M KCl, 0.1 mM  $\text{CaCl}_2$ , and 0.1 mM DTT and then chromatographed on a HiLoad Superdex 200 pg column ( $\Phi 26 \text{ mm} \times 600 \text{ mm}$ ; GE Healthcare UK Ltd.) equilibrated with the same buffer. Fractions of 5 mL were collected at a flow rate of 2.5 mL/min. The SDS-polyacrylamide gel electrophoresis analyses for all fractions were performed, and the fractions with the band corresponding to 37 kDa were combined (17). The combined fraction was then diluted with an equal volume of 0.2% trifluoroacetic acid and applied to reverse-phase high-performance liquid chromatography on a Wakosil-II 5C18 AR prep column ( $\Phi 10 \text{ mm} \times 250 \text{ mm}$ ; Wako Chemicals, Japan). The column was eluted at a constant flow rate of 2.5 mL/min by a gradient of acetonitrile in 0.1% trifluoroacetic acid. The tropomyosin-containing fractions were collected and lyophilized. The red king crab, swimming crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins were obtained according to the purification procedure of BTTM. Tropomyosin derived from the porcine skeletal muscle (PTM) was obtained using the purification procedure of Greaser et al. (18) and Bailey et al. (19).

**Production of Monoclonal Antibodies and Rabbit Polyclonal Antibodies to BTTM.** The anti-BTTM monoclonal antibodies were generated at Nippon Biotech Laboratories, Inc. (Tokyo, Japan). For the production of the monoclonal antibodies against BTTM, female BALB/c mice were immunized with the purified BTTM. Fusion of the spleen cells was performed according to the method of Kohler and Milstein (20). The cell culture supernatants were screened for specific anti-BTTM antibodies by a direct ELISA with purified BTTM on a solid phase. The positive hybridomas were cloned and subcloned by limiting dilution. The positive hybridoma cells were intraperitoneally administered into BALB/c mice to induce the ascite tumors. The antibody was purified from the ascite fluid using a HyperD Protein A column (Bio Septra Inc., Marlborough, MA). The specificity of the monoclonal antibodies was demonstrated by a direct ELISA method with purified black tiger prawn, red king crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins. The polyclonal antibodies were generated at Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). The rabbit antiserum against BTTM was produced by immunization of New Zealand rabbits with purified BTTM in Freund's adjuvant. Injections were repeated six times at appropriate intervals (7 days). Whole blood was collected, and the serum was separated. The polyclonal antibodies were purified from the serum using a HiTrap Protein A HP column (GE Healthcare UK, Ltd.). The polyclonal antibodies were immunoabsorbed against Japanese common squid purified tropomyosin. The immunoabsorption was performed using the Japanese common squid tropomyosin-coupled column to removed further antibodies to molluscan protein. The specificity of the absorbed polyclonal antibodies was demonstrated by direct ELISA using the various purified tropomyosins.

**Preparation of Sample Solution.** The samples were treated with the Ace AM-4 homogenizer (Nissei, Tokyo, Japan) a few times for 30 s for homogeneity. Nineteen milliliters of 120 mM Tris-HCl (pH 7.4) containing 1 g/L bovine serum albumin (BSA), 0.5 mL/L Tween 20, 5 g/L SDS, and 20 mL/L  $\beta$ -mercaptoethanol (21) was added to 1 g of a homogenized sample, which was then shaken for 12 h at room temperature for extraction. After the extraction, the sample was centrifuged at 3000g for 20 min, and the supernatant was filtered through 5AB paper (Advantec) to obtain the extract.

**Procedure of the Direct ELISA.** Polystyrene 96 well microtiter plates (Nalge Nunc international, Rochester, NY) were coated overnight at 4 °C with 100  $\mu\text{L}$  of purified tropomyosin (0.5  $\mu\text{g/mL}$ ) in coating buffer (50 mmol/L sodium carbonate, pH 9.6). The plates were then washed three times with Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.4, containing 154 mmol/L NaCl). The plates were blocked

for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950 (Supelco, Bellefonte, PA). After the plates were washed six times with TBS containing 0.5 mL/L Tween 20 (TBS-T), diluted monoclonal antibodies or polyclonal antibodies were added to the wells and incubated at 25 °C for 1 h. After the wells were washed with TBS-T, 100  $\mu$ L of horseradish peroxidase-labeled goat antimouse or antirabbit IgG serum was added to each well. After washing, 100  $\mu$ L of the substrate solution containing 3,3',5,5'-tetramethylbenzidine (SureBlue TMB Microwell Peroxidase Substrate; KPL, Gaithersburg, MD) was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100  $\mu$ L/well). The plate was then read on a SPECTRAMax 250 microplate reader (Molecular Devices Corp., Menlo Park, CA) at the wavelength of 450 nm.

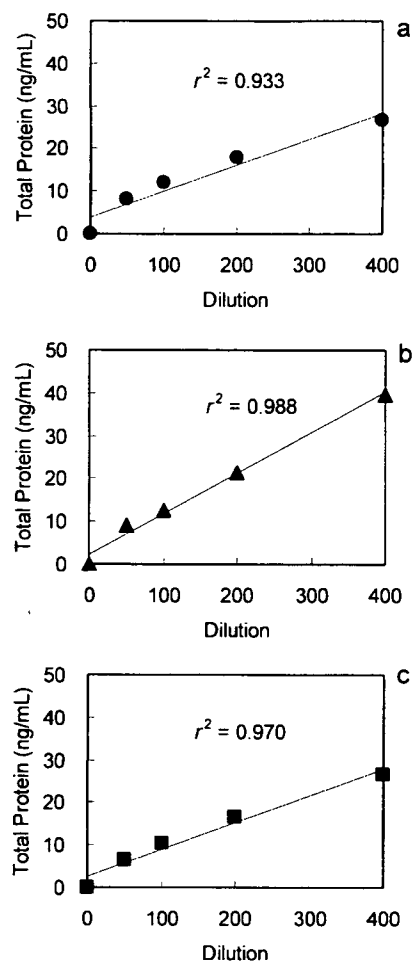
**Procedure of the Sandwich ELISA.** Polystyrene 96 well microtiter plates (Nalge Nunc international) were coated with 100  $\mu$ L/well monoclonal antibodies (Mab #32, 10  $\mu$ g/mL; and Mab #54, 20  $\mu$ g/mL in 50 mmol/L sodium carbonate, pH 9.6) for 18 h at 4 °C. After they were washed three times with TBS, the plates were blocked for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950. After the blocking buffer had been aspirated, the plates were dried in an incubator for 2.5 h at 30 °C, sealed in an aluminum-coated pack with drying agent (I.D. Sheet Desiccant; I.D., Tokyo, Japan), and stored at 4 °C until used. The food sample extracts were diluted 1:20 with the sample dilution buffer [TBS containing 2 g/L BSA, 0.02 g/L PTM, 0.5 mL/L Tween 20, and 0.5 mL/L ProClin 950]. The diluted sample or calibrator (100  $\mu$ L) was added in triplicate to the coated wells, and the plates were then incubated for 90 min at 25 °C. After the plate had been washed four times with TBS-T, horseradish peroxidase-conjugated absorbed polyclonal antibodies (100  $\mu$ L) were added to each well, and the plate was then incubated for 90 min at 25 °C. After another four washes with TBS-T, 100  $\mu$ L of 3,3',5,5'-tetramethylbenzidine solution (SureBlue Reserve TMB Microwell Peroxidase Substrate, KPL) as a substrate was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100  $\mu$ L/well). The plate was then read using a SPECTRAMax 250 microplate reader at a wavelength of 450 nm. Standard curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration.

Eleven crustacean protein extracts and two crustacean purified tropomyosins were tested in the concentration range from 0 ng/mL to 90  $\mu$ g/mL. The concentration-response curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration, and the curves were fitted to a four-parameter logistic equation,  $y = \{ (A - D) / [1 + (x/C)^B] \} + D$ , where  $A$  is the maximum absorbance at infinite concentration,  $B$  is the curve slope at the inflection point,  $C$  is the concentration of the analyte giving 50% responses ( $RC_{50}$ ), and  $D$  is the minimum absorbance for no analyte. The reactivity values were calculated as follows: reactivity % =  $[RC_{50} \text{ of black tiger prawn protein (or tropomyosin)} / RC_{50} \text{ of target crustacean protein (or tropomyosin)}]$ . Seventeen molluskan sample extracts were diluted 1:20 with the sample dilution buffer containing PTM and analyzed using the sandwich ELISA method.

**Evaluation of Assay Variation.** For determination of the intra-assay precision, the mean coefficients of variation (CVs) were based on 10 replicates. The interassay precision was determined as the mean CVs on the basis of triplicate analyses on 10 different days. The limit of detection (LOD) for the sandwich ELISA was calculated as three times the standard deviation (SD) of the buffer blank mean value after 25 experiments. The limit of quantification (LOQ) was calculated as 10 times the SD of the buffer blank mean values after 25 experiments.

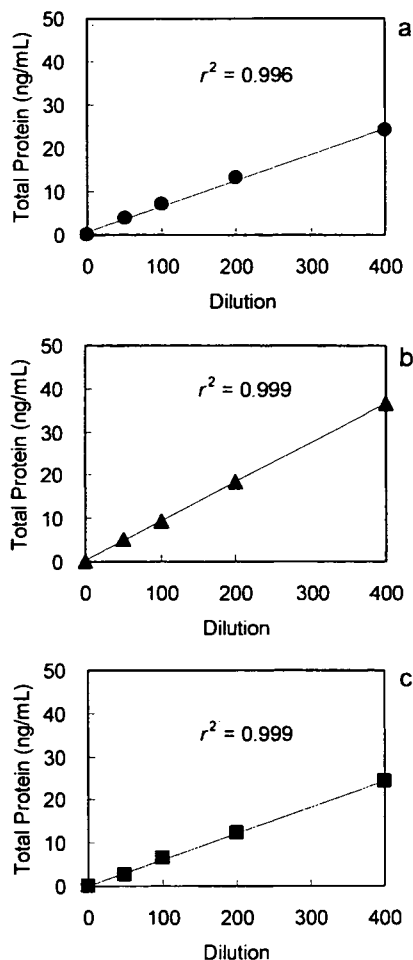
## RESULTS

**Construction of Sandwich ELISA.** To evaluate the characteristics of the absorbed polyclonal antibodies and monoclonal antibodies to the BTTM, we tested the reactivity using a direct ELISA assay. We showed that the absorbed polyclonal antibody could be clearly detected for the crustacean tropomyosin but not for the molluskan tropomyosin. For the preparation of monoclonal antibodies to BTTM, nine monoclonal antibodies



**Figure 1.** Linearity of dilution curves for model processed foods using the sample dilution buffer without PTM. (a) Fish meat sausage (solid circle); (b) freeze-dried egg soup (solid triangle); and (c) chicken meatball (solid square).

were obtained. Of these monoclonal antibodies, Mab #32 and #54 gave a satisfactory specificity and reactivity. In the examination of the different antibody combinations, using Mab #32 and #54 as the capture antibody and the HRP-conjugated absorbed polyclonal antibody as the detected antibody for the sandwich ELISA was found to provide the best results in terms of sensitivity and specificity to determine the total crustacean protein. However, as shown in Figure 1, a satisfactory dilution linearity could not be obtained when the dilution tests were performed using the tentatively constructed sandwich ELISA method and the three model processed foods. These results suggest that the food matrix could affect the dilution linearity in the tentatively constructed ELISA method. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. As shown in Figure 2, the dilution linearity was satisfactorily improved by the addition of the PTM to the sample dilution buffer ( $r^2 = 0.996$ – $0.999$ ), confirming parallelism between the calibrators and the food samples. We statistically compared the two correlation coefficients of the dilution curves obtained using a sample dilution buffer containing PTM and those obtained without PTM for the assay of the model processed foods. A statistical test between the two correlation coefficients was performed using the Z-transformation test.  $P$  values of less than 0.05 were considered statistically significant. In the case of the chicken meatball, there was a statistically significant difference between the two correlation coefficients of the dilution curve ( $P = 0.036$ ). In the case of



**Figure 2.** Linearity of dilution curves for model processed foods using the sample dilution buffer with PTM. (a) Fish meat sausage (solid circle); (b) freeze-dried egg soup (solid triangle); and (c) chicken meatball (solid square).

the fish meat sausage, a dilution curve using a sample dilution buffer to which was added PTM tended to show a good linearity when compared with using a tentative sample dilution buffer ( $P = 0.081$ ). Meanwhile, there was no statistically significant difference in the freeze-dried egg soup ( $P = 0.127$ ). These results suggested that the addition of PTM to the sample dilution buffer significantly improved the dilution linearity. Consequently, we established the sandwich ELISA method using a sample dilution buffer with PTM to minimize the food matrix effects.

**Reactivity and Specificity Test.** Various crustacean proteins, molluskan protein samples, and two crustacean purified tropomyosins (black tiger prawn and swimming crab) were examined to test the reactivity and specificity using the sandwich ELISA method. As shown in **Table 1**, the reactivities of the Decapoda group, which includes prawns and lobsters, are greater than 65.8%, and those of the crabs range between 28.5 and 38.5%. In contrast, the reactivities of the other Crustacea, such as the Japanese mantis shrimp, euphausia, and acorn barnacle, are less than 11.3%. The swimming crab purified tropomyosin demonstrated a reactivity of 154% as compared to the reactivity of black tiger purified tropomyosin. When all of the molluskan samples were tested, all of the levels were determined by the sandwich ELISA method to be less than 1.0 mg/kg (**Table 2**). These results suggest that the sandwich ELISA method has a specific reactivity to the Decapoda group, which includes prawns, shrimps, lobsters, and crabs.

**Table 1.** Reactivity Levels of Various Crustacean Samples in the Sandwich ELISA Method<sup>a</sup>

sample	RC <sub>50</sub> (ng/mL)	reactivity (%)
Decapoda group		
black tiger prawn	9.5	
northern shrimp	14.4	65.8
Japanese spiny lobster	8.4	114.3
Caribbean spiny lobster	9.0	105.6
red king crab	24.6	38.5
swimming crab	33.4	28.5
other varieties of Crustacea		
Japanese mantis shrimp	124.4	7.6
euphausia	799.3	1.2
opossum shrimp	8060.4	0.1
acorn barnacle	83.8	11.3
goose barnacle	166.7	5.7

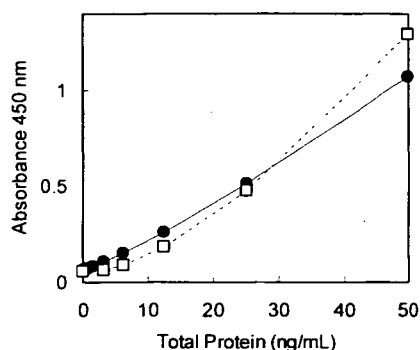
<sup>a</sup> RC<sub>50</sub> is the concentration of analyte giving a 50% OD<sub>max</sub> response. Reactivity % = (RC<sub>50</sub> of black tiger prawn protein/RC<sub>50</sub> of target crustacean protein).

**Table 2.** Cross-Reactivity of Various Molluskan Samples in the Sandwich ELISA Method

sample	cross-reactivity in ELISA (mg/kg)
Cephalopoda group	
common octopus	<1.0
giant octopus	<1.0
ocellated octopus	<1.0
Japanese common squid	<1.0
spear squid	<1.0
cuttlefish	<1.0
Bivalvia group	
common scallop	<1.0
Japanese oyster	<1.0
bloody clam	<1.0
blue mussel	<1.0
short-neck clam	<1.0
common freshwater clam	<1.0
Japanese hard clam	<1.0
Sakhalin surf clam	<1.0
Gastropoda group	
homed turban	<1.0
Japanese abalone	<1.0
whelk	<1.0

**Limit of Detection and Limit of Quantification.** The best model that describes the relationship between the absorbance and the antigen concentration is a four-parameter logistic curve (**Figure 3**). The LOD of the ELISA method determined using the standard proteins is 0.71 ng/mL, equivalent to 0.29 mg/kg samples, and LOQ is 2.25 ng/mL, equivalent to a 0.9 mg/kg sample. Consequently, the practical determination range lies between 1.56 and 50 ng/mL. For the final evaluation of the validation data for the sandwich ELISA and its application, the LOQ for routine analysis was set to 1.0 mg/kg sample. This level was considered to give a safety margin to the majority of consumers with an allergy to peanuts (22).

**Quantification of Crustacean Protein in Model Processed Foods Using the Sandwich ELISA.** To test the applicability of the sandwich ELISA in processed foods, the crustacean protein in three model processed food samples was determined using the sandwich ELISA. As shown in **Table 3**, the mean recoveries for all three model processed food samples ranged from 85 to 141%. The precision data from the three model processed foods are shown in **Table 4**. The interassay precision across all days was 5.3, 6.2, and 8.4% CV for the three model processed foods. The intra-assay precision for the three model processed foods was 2.8, 2.3, and 2.8% CV, respectively.



**Figure 3.** Representative standard curve using the shrimp protein standard in the sandwich ELISA method. The sample diluted buffer with PTM (solid circle); without PTM (open square).

**Table 3.** Recoveries of Crustacean Protein from Three Model Processed Foods

sample	concentration (mg/kg)	recovery (%)
fish meat sausage	25.0	96
	12.5	107
	6.3	114
	3.1	120
freeze-dried egg soup	29.8	124
	14.9	124
	7.4	125
	3.7	141
chicken meatball	25.0	97
	12.5	100
	6.3	105
	3.1	85

**Table 4.** Intra- and Interassay Variances in the Sandwich ELISA Method Using Three Model Processed Foods<sup>a</sup>

sample	concentration (mg/kg)	intra-assay	interassay
fish meat sausage	10	2.8	5.3
freeze-dried egg soup	11.9	2.3	6.2
chicken meatball	10	2.8	8.4

<sup>a</sup> The intra-assay variances were calculated from 10 replicates of the same extract, and the interassay variances were calculated from triplicate analysis of the same extract on 10 different days.

**Application to the Commercial Food Products.** Thirty-two different commercial food samples were analyzed by the sandwich ELISA method. Each commercial food was homogenized, and the extracts were obtained according to the extraction procedure described in the Materials and Methods section. As shown in **Table 5**, 15 commercial foods with a label of shrimp or crab on the ingredients list were clearly detected. In contrast, the levels in products without a label of shrimp or crab on the ingredients list were detected to be less than 1.0 mg/kg. There were no false positives from the no-declaration samples and no false negatives from the declaration samples analyzed in this study. When commercial food products containing shrimp or crab were serially diluted and assayed, each sample gave results close to linearity ( $r^2 = 0.993\text{--}1.000$ ), confirming parallelism between the calibrators and the food samples. These results show that the sandwich ELISA method could appropriately determine the crustacean protein in the processed foods.

## DISCUSSION

We established the sandwich ELISA method for the detection of crustacean protein that has a specific reactivity to the

**Table 5.** Analysis of Various Commercial Food Samples for Using the Sandwich ELISA Method

sample	declaration	substance	quantitative (mg/kg)	regression ( $r^2$ )
bean jammed	+	crab	264	0.998
seafood curry	+	shrimp	1780	0.999
beef curry	-		<1.0	
base of pilaf	+	crab	1100	1.000
cream pasta source	-		<1.0	
croquette	+	crab	404	1.000
croquette	-		<1.0	
croquette	-		<1.0	
dumpling	+	shrimp	77000	0.995
dumpling	+	crab	1040	1.000
dumpling	-		<1.0	
base of fried rice	+	shrimp	653	0.993
base of risotto	+	crab	36.7	0.998
spray-dried soup	-		<1.0	
gratin	+	shrimp	22400	0.995
gratin	-		<1.0	
snack	+	shrimp	100	0.998
cookie	-		<1.0	
Japanese rice cookie	-		<1.0	
fried food (prawn)	+	shrimp	282000	0.995
fried food (chicken)	-		<1.0	
fried food (poke)	-		<1.0	
fried food (oyster)	-		<1.0	
fried food (squid)	-		<1.0	
noodle	+	shrimp	145000	0.998
noodle	-		<1.0	
Japanese wheat noodle	-		<1.0	
steamed fish paste	+	crab	176	0.999
steamed fish paste	-		<1.0	
fried fish paste	+	shrimp	46.4	0.995
terrine	+	shrimp	1560	0.997
fish sausage	-		<1.0	

Decapoda group in Crustacea and applied this method to processed food. Jeoung et al. (23) already reported a determination method for tropomyosin. However, the cross-reactivity to mollusks and the application to processed foods have not yet been sufficiently clarified. Therefore, the reactivity and specificity of the sandwich ELISA method were tested using extracts from various Crustacea, mollusks, and commercial foods. In the test of all of the molluscan sample extracts, the reactivity levels were extremely low. The house dust mite was reported to cross-react with crustacean allergens (24). However, the monoclonal antibodies as the capture antibody do not cross-react with the house dust mite in the Western blot analysis (data not shown). These results suggest that this method would be specific to the Crustacea protein. However, the possibility of a cross-reaction with other less commonly used mollusks or other ingredients, such as crustacean extractants as seasonings, cannot be excluded and remains to be examined. It will be necessary to clarify the applicability of the present method.

The reactivities of lobster and prawn are similar to those of the black tiger prawn. Those of the crab group appear to be lower than those of the black tiger prawn. However, the purified swimming crab tropomyosin showed a high reactivity (154%). These results suggest that the variety of reactivities among the Decapoda group may be involved in the difference of the tropomyosin contents in the sample extracts.

Furthermore, we found that the addition of porcine skeletal tropomyosin to the sample dilution buffer in the sandwich ELISA method can appropriately determine the crustacean protein in processed foods without any food matrix effects.

As described in the Results section, the sample extracts of the model processed foods were serially diluted and assayed using the tentatively constructed sandwich ELISA method, and

a good linearity could not be observed ( $r^2 = 0.936\text{--}0.995$ ). We considered that this result would be due to food matrix effects. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. Consequently, the dilution linearity for model processed food was significantly improved by the addition of the PTM to the sample dilution buffer ( $r^2 = 0.996\text{--}0.999$ ). The addition of troponin or actin failed to improve the dilution linearity (data not shown). These results suggest that tropomyosin may be involved in the food matrix effects, although the food matrix effect mechanism remains unclear. This method offers a new perspective for the determination of various proteins in processed food and is expected to be extremely useful in other protein-measuring methods using ELISA.

To evaluate the sandwich ELISA method for the determination of crustacean protein in processed foods, a recovery study and intra- and interassays were tested using model processed foods. The results of the analysis show that this method has a good accuracy and precision. The sandwich ELISA method's sensitivity was 0.71 ng/mL, corresponding to the 0.29  $\mu\text{g}$  crustacean protein/g food sample weight. This result indicates that the sandwich ELISA method is suitable for detection in the presence of hidden crustacean protein in processed foods.

In conclusion, this sandwich ELISA method is shown to have an acceptable accuracy and precision and no false positive or false negative. This method has been demonstrated to be suitable for the quantitative measurement of the specific crustacean protein in processed foods without food matrix effects.

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## Molecular Cloning of Tropomyosins Identified as Allergens in Six Species of Crustaceans

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Although tropomyosin is known to be a major allergen of crustaceans, its structural information is limited to only five species. In this study, tropomyosin was confirmed to be a major allergen in six species of crustaceans (black tiger prawn, kuruma prawn, pink shrimp, king crab, snow crab, and horsehair crab) by immunoblotting. Then, the amino acid sequences of tropomyosins from these crustaceans were elucidated by a cDNA cloning technique. Sequence data for crustacean tropomyosins including the obtained results reveal that fast tropomyosins are contained in shrimps (or prawns) and lobsters, slow tropomyosins in crabs, and both tropomyosins in crayfishes and hermit crabs. Although fast and slow tropomyosins share a high sequence identity (about 90%) with each other, significant differences are observed in specific regions between both tropomyosins.

**KEYWORDS:** Allergen; cDNA cloning; crab; cross-reactivity; crustacean; prawn; shrimp; tropomyosin

### INTRODUCTION

Crustaceans are widely consumed as delicious foods throughout the world. However, they are simultaneously recognized as one of the most common causes of immunoglobulin E (IgE)-mediated food allergy. Following their ingestion, hypersensitive reactions, such as urticaria, asthma, diarrhea, and anaphylaxis, are immediately induced in allergic individuals. Most edible crustaceans are members of the order Decapoda composed of two suborders, Dendrobranchiata (including shrimps or prawns) and Pleocyemata (including shrimps or prawns, crayfishes, lobsters, hermit crabs, and crabs), and hence previous studies on crustacean allergens have been performed with decapods. So far, the major allergen of crustaceans has been identified at the molecular level as tropomyosin, a 35–38 kDa protein constituting thin myofilaments together with actin and troponin, in the following six species: Indian white shrimp, *Penaeus indicus* (1); brown shrimp, *Penaeus aztecus* (2); sand shrimp, *Metapenaeus ensis* (3); American lobster, *Homarus americanus* (4); spiny lobster, *Panulinus stimpsoni* (4); and red crab, *Charybdis feriatus* (5). The three species of shrimps belong to the suborder Dendrobranchiata and the rest to the suborder Pleocyemata. Although tropomyosin is assumed to be a major allergen in common with decapod crustaceans, no experimental data are available on allergens in shrimps belonging to the suborder Pleocyemata and hermit crabs including commercially important species such as king crab.

Crustacean tropomyosins show IgE cross-reactivity with one another and also with those from various invertebrates including

mollusks, house dust mites, and cockroaches (6–12). Obviously, the first step toward molecular understanding of the cross-reactivity among tropomyosins from various sources is the elucidation of their amino acid sequences. As for crustacean tropomyosins, those from brown shrimp (13), sand shrimp (3), American lobster (14, 15), spiny lobster (4), and red crab (5) have already been clarified for their amino acid sequences. The tropomyosins from two species of shrimps, American lobster and spiny lobster, share an extremely high sequence identity (>98%) with one another. In accordance with this, the eight IgE-binding epitopes proposed for brown shrimp tropomyosin (Pen a 1) (10, 16) are completely conserved in the tropomyosins from the other three species, except that the spiny lobster tropomyosin has one alteration in one epitope. On the other hand, the red crab tropomyosin bears a somewhat different amino acid sequence, and its sequence identity with those of the above four species is about 90%. Importantly, the red crab tropomyosin has as many as six alterations in the region 44–55 corresponding to one of the Pen a 1 epitopes. At present, however, it is unknown whether the amino acid sequence features of the red crab tropomyosin are common to crab tropomyosins.

In view of the circumstances described above, this study was initiated to obtain further evidence that tropomyosin is a major allergen in decapod crustaceans using the following six species widely consumed in Japan: two species of the suborder Dendrobranchiata, black tiger prawn (*Penaeus monodon*) and kuruma prawn (*Penaeus japonicus*), and four species of the suborder Pleocyemata, pink shrimp (*Pandalus eous*), king crab (*Paralithodes camtschaticus*) (a kind of hermit crabs), snow crab (*Chionoecetes opilio*), and horsehair crab (*Erimacrus isenbekii*).

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Table 1. Designations and Nucleotide Sequences of the Primers Used for Molecular Cloning of Crustacean Tropomyosins

designation of primer	nucleotide sequence of primer	corresponding amino acid sequence*
cru-f1	5'-ATCAAGAAGAAGATGCAGGCG-3'	4-IKKKMQA-10
cru-f2	5'-AGAAGGCCAACCCAGCGCGAGGA-3'	212-EKANQREE-219
kuruma-f	5'-AGAAGGCTAACCCAGCGCGAGGA-3'	212-EKANQREE-219
cru-r1	5'-TCTTCGAGCCTGTCGACCTC-3'	251-KEVDRLD-258
cru-r2	5'-GGATGTTAGCCTTCAGCAAGGATT-3'	62-ESLLKANIQ-70
pink-s	5'-CGTTAGAGAGAGCCTTGCC-3'	74-KDKALSNA-81
pink-r	5'-GGTGTGGTTAGCCTTCAGC-3'	64-LLKANTQL-71
snow-f	5'-GGAACAGATCAAGACCCTTGCC-3'	222-KEQIKTLA-229
snow-r	5'-TATGGGTAGCGGCGGACAACCTGCT-3'	62-ESLLKANIQ-70
horsehair-s	5'-CCTCCTCGAGAAGCTGGATG-3'	91-RIQLLEED-98
horsehair-r	5'-CACCTCACCCCTCGGACTTCT-3'	79-SNAEGEV-85
king-f1	5'-CGAGGAGGAGGTTACGGCCCT-3'	39-AEEVHGL-46
king-f2	5'-TGAGGAGGAGATTGCGCTTACC-3'	39-IEEIRLT-46
king-r1	5'-TTGCGTATTCGCCTTAAGCAGGGA-3'	63-SLLKANTQ-70
king-r2	5'-CTTAGTGTAGCCAGAGATAGCTG-3'	63-QLSLANTK-70
AP1	5'-CCATCCTAATACGACTCACTATAGGGC-3'	
AUAP	5'-GGCCACGCGTGCAGTACTAGTAC-3'	
AAP	5'-GGCCACGCGTGCAGTACTAGTACGGIIGGGIIGGGIIG-3'	

\* Refer to Figure 2 for amino acid sequences.

American lobster, for which the major allergen has previously been established to be tropomyosin (4), was used as a reference. Subsequently, amino acid sequences of tropomyosins from the six species of crustaceans were elucidated by a cDNA cloning technique. We report here the identification of tropomyosins as allergens in six species of decapod crustaceans and the primary structural features of their tropomyosins in comparison with the known crustacean tropomyosins.

## MATERIALS AND METHODS

**Crustaceans.** Live specimens of kuruma prawn, pink shrimp, American lobster, and horsehair crab were purchased at the Tokyo Central Wholesale Market. Live specimens of black tiger prawn imported from Vietnam and those of king crab and snow crab caught along the coasts of Hokkaido, Japan, were kindly supplied from Nippon Suisan Kaisha. Abdominal muscle was obtained from each live specimen of black tiger prawn, kuruma prawn, pink shrimp, and American lobster and both leg muscle and chest protection muscle from king crab, snow crab, and horsehair crab. Muscle samples for extraction were stored at  $-20^{\circ}\text{C}$  until used, and those for molecular cloning experiments were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used.

**Preparation of Heated Extracts.** Each muscle sample was homogenized with 4 volumes of 0.6 M KCl in 0.01 M phosphate buffer (pH 7.0). Tropomyosin, a target protein in this study, is heat-stable. Therefore, the homogenate was then heated in a boiling water bath for 10 min and centrifuged at 18000g for 20 min to remove insoluble proteins. The supernatant thus obtained was used as heated extract. Protein concentrations of the heated extracts were estimated according to the method of Lowry et al. (17), using bovine serum albumin as a standard.

**Purification of American Lobster Tropomyosin.** An acetone powder of myofibrillar proteins was prepared from the abdominal muscle of American lobster as reported by Greaser and Gergely (18) and extracted with 25 mM Tris-HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM  $\text{CaCl}_2$ , and 1 mM dithiothreitol. The extract was subjected successively to salting-out with ammonium sulfate (50–60% saturation) and isoelectrical precipitation (pH 4.6) essentially according to the method of Cummins and Perry (19). Finally, small amounts of impurities were removed by reverse-phase HPLC on a TSKgel ODS-120T column (0.46  $\times$  25 cm; Tosoh, Tokyo, Japan), which was eluted at a flow rate of 1 mL/min with a linear gradient of acetonitrile (38.5–49.0% in 50 min) in 0.1% trifluoroacetic acid. The homogeneity of the final preparation was supported by SDS-PAGE. American lobster has previously been reported to contain three types of tropomyosin (fast, slow-twitch and slow-tonic) (14, 15). Because the abdominal

muscle of American lobster is mostly composed of fast muscle fibers (14), our purified preparation was judged to be fast-type tropomyosin.

**Human Sera.** Sera were donated from 10 crustacean-allergic patients. The patients were all diagnosed to be allergic to crustaceans on the basis of the determined capsulated hydrophilic carrier polymer–radioallergosorbent test (CAP-RAST) classes of 2–5 against shrimp or both shrimp and crab as well as on the experiences of immediate hypersensitive reactions after ingestion of crustaceans. Written informed consent was obtained from each patient. In this study, sera from five healthy volunteers without adverse reactions after ingestion of any foods were pooled and used as a control. All sera were stored at  $-20^{\circ}\text{C}$  until used.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE).** SDS-PAGE was performed on a PhastSystem apparatus (GE-Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. Ready-made gels (PhastGel Gradient 8-25) and ready-made buffer strips (PhastGel SDS Buffer Strips) were purchased from GE-Healthcare Bio-Sciences. Prior to electrophoresis, each sample was dissolved in 0.0625 M phosphate buffer (pH 7.5) containing 2.5% SDS and 5% dithiothreitol and heated at  $100^{\circ}\text{C}$  for 10 min. Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA) were run as a reference, along with samples. After running, the gel was stained with Coomassie Brilliant Blue R-250.

**Immunoblotting and Inhibition Immunoblotting.** Immunoblotting was performed as described in our previous paper (20). Briefly, the proteins separated by SDS-PAGE were electrotransferred from the gel to a nitrocellulose membrane, which was reacted successively with primary and secondary antibodies. To detect tropomyosin, an antiserum (diluted 1:4000) raised in rabbits against king crab tropomyosin, which was a kind gift from Dr. H. Ushio of our university, was used as a primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:20000; Kirkegaard and Perry Laboratories, Gaithersburg, MD) as a secondary antibody. On the other hand, patient or control serum (diluted 1:500) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1:10000; Kirkegaard and Perry Laboratories) were used as primary and secondary antibodies, respectively, to detect IgE-binding proteins. Blots were visualized using an ECL Plus Western Blotting Detection System (GE-Healthcare Bio-Sciences) and an ECL Mini Camera (GE-Healthcare Bio-Sciences), as recommended by the manufacturer. For inhibition IgE-immunoblotting, patient serum (diluted 1:250) was preincubated with an equal volume of American lobster tropomyosin solution (20  $\mu\text{g}/\text{mL}$ ) at  $37^{\circ}\text{C}$  for 1 h and used as a primary antibody.

**Primer Design, Polymerase Chain Reaction (PCR), and Nucleotide Sequencing.** Designations and nucleotide sequences of the primers used in this study are shown in Table 1. Both forward (cru-f1) and reverse (cru-r1) primers were designed from the known nucleotide sequences of the crustacean tropomyosin cDNAs. The nucleotide



sequence of *cru-f1* is completely identical with the cDNAs encoding tropomyosins from brown shrimp (accession number of the DDBJ/EMBL/GenBank nucleotide databases: DQ151457) and red crab (AF061783) and three types of tropomyosin from American lobster (AF034954 for fast tropomyosin, AF034953 for slow-twitch tropomyosin, and AY521627 for slow-tonic tropomyosin). On the other hand, the nucleotide sequence of *cru-r1* is identical with the cDNAs encoding tropomyosins from sand shrimp (U08008) and American lobster and spiny lobster (AF030063) but is different by one nucleotide from the cDNAs encoding tropomyosins from brown shrimp and red crab. Except for three primers, AP1 adapter primer (AP1), abridged universal anchor primer (AUAP), and abridged anchor primer (AAP), the other primers were designed on the basis of the determined partial nucleotide sequences. PCR amplifications were all performed using HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 1 min; and 72 °C for 7 min. The PCR products were subcloned into the pT7Blue-2 T-vector (Novagen, Darmstadt, Germany), and at least three clones were analyzed for nucleotide sequences using a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and a PRISM 310 genetic analyzer (Applied Biosystems).

**Cloning Experiments for Tropomyosins of Black Tiger Prawn, Kuruma Prawn, King Crab, and Snow Crab.** Total RNA was extracted from 2 g of each muscle sample with the TRIzol reagent (Invitrogen, Carlsbad, CA), and poly(A)<sup>+</sup> mRNA was purified by affinity chromatography on oligo(dT)-cellulose using an mRNA Purification Kit (GE-Healthcare Bio-Sciences). A small aliquot (1 µg) of the purified mRNA was converted to double-strand cDNA, followed by ligation of an AP1 adapter at both 3' and 5' ends, using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA). The Marathon cDNA library thus constructed was subjected to PCR as a template. At first, intermediate cDNA fragments were amplified using the forward primer (*cru-f1*) and the reverse primer (*cru-r1*). All amplified products were subcloned into the pT7Blue-2 T-vector and sequenced as described above. Then, the remaining 3' region was amplified using the gene-specific forward primer (*cru-f2* for black tiger prawn and snow crab, *kuruma-f* for kuruma prawn, *king-f1* for king crab fast-type isoform or *king-f2* for king crab slow-tonic-type isoform) and the AP1 primer and the remaining 5' region using the gene-specific reverse primer (*cru-r2* for black tiger prawn and kuruma prawn, *king-r1* for king crab fast-type isoform, *king-r2* for king crab slow-tonic-type isoform or *snow-r* for snow crab) and the AP1 primer. In the case of snow crab, nested PCR was additionally performed to amplify the 3' remaining region using the gene-specific forward primer (*snow-f*) and the AP1 primer. All amplified products were subjected to subcloning and sequencing.

**Cloning Experiments for Tropomyosins of Pink Shrimp and Horsehair Crab.** Molecular cloning of pink shrimp and horsehair crab tropomyosins was simply performed by the rapid amplification of cDNA ends (RACE) method using total RNA extracted from each muscle sample, without construction of the Marathon cDNA library. For 3'RACE, first-strand cDNA was synthesized from 5 µg of total RNA using the 3'RACE System for Rapid Amplification of cDNA Ends (Invitrogen) as described in the manufacturer's manual and used as a template. Amplification was carried out using the *cru-f1* primer and the AUAP primer. 5'RACE was performed using the 5'RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 µg of total RNA using the gene-specific reverse primer (*pink-s* for pink shrimp or *horsehair-s* for horsehair crab). The gene-specific reverse primer (*pink-r* for pink shrimp or *horsehair-r* for horsehair crab) and the AAP primer were subjected to 5'RACE reaction using the synthesized first-strand cDNA as a template. All amplified products were subcloned and sequenced.

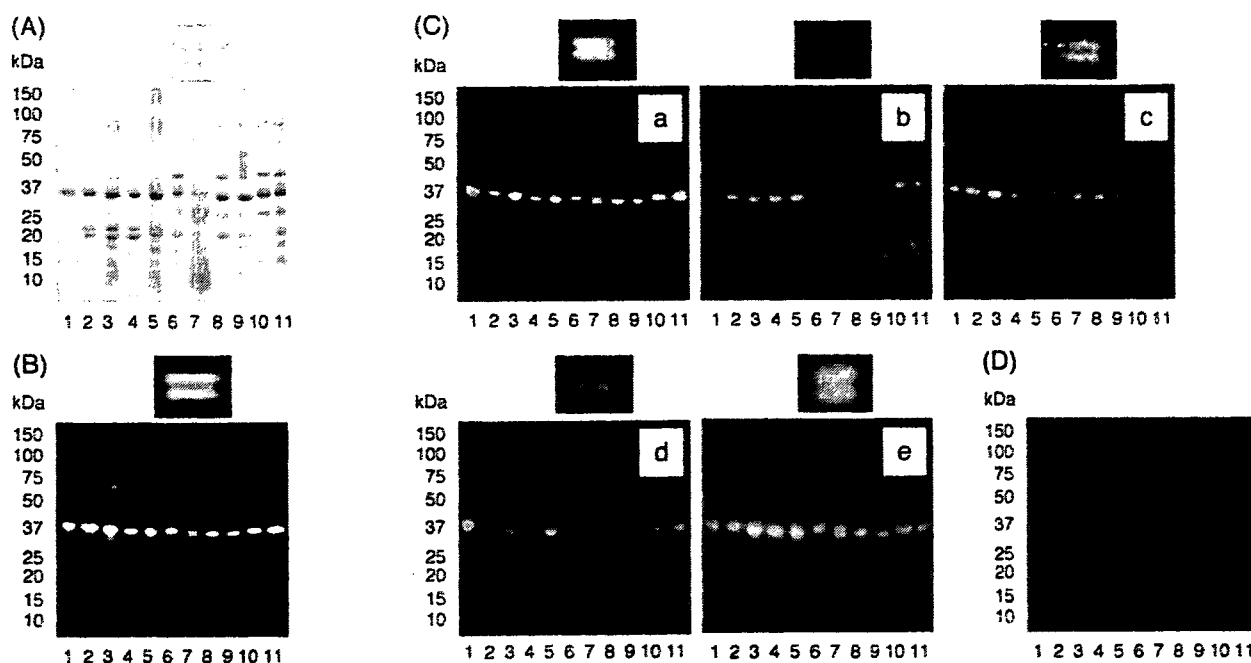
## RESULTS AND DISCUSSION

**Identification of Tropomyosins as Allergens in Crustaceans.** As analyzed by SDS-PAGE, a prominent protein of 37 kDa, which is comparable in molecular mass to American

lobster tropomyosin used as a reference, was detected in all of the heated extracts, except for that from the chest protection muscle of king crab (Figure 1A). Irrespective of the species and the muscle types, the 37 kDa protein reacted with the antiserum against king crab tropomyosin (Figure 1B), allowing us to conclude that the 37 kDa protein is tropomyosin of each crustacean muscle. In the case of the chest protection muscle of king crab, two proteins of about 37 kDa with a considerably weaker staining intensity than tropomyosins in the other samples were observed in SDS-PAGE (Figure 1A). The two proteins also reacted with the antiserum against king crab tropomyosin (Figure 1A). Importantly, two different tropomyosins (fast and slow-tonic tropomyosins) exist only in the chest protection muscle of king crab, as described in detail below. Taken together, it is reasonable to consider that the two proteins of about 37 kDa observed in the chest protection muscle of king crab are tropomyosin isoforms. IgE-immunoblotting, using the pooled serum from 10 crustacean-allergic patients and 4 individual patient sera, showed that all tropomyosins, except for the two tropomyosin isoforms in the chest protection muscle of king crab, are IgE-reactive, regardless of the patient sera (Figure 1C). When the pooled control serum was used, no blots were detected in any of the heated extracts (data not shown). As for the two isoforms in the chest protection muscle of king crab, their IgE reactivity was established by the results with the pooled patient serum and the patient 2 and 4 sera. However, only one blot was observed when patient 1 and 3 sera were used, suggesting that only either one of the two isoforms is reactive with IgE in the patient 1 and 3 sera or that the two blots have appeared as one blot due to poor resolution. Apart from this problem to be clarified in future, it is important to note that tropomyosin seemed to be almost the sole IgE-reactive protein in all of the crustaceans examined, although another IgE-reactive protein of about 30 kDa was also detected in the chest muscle of king crab only when the patient 2 serum was used. In inhibition immunoblotting experiments using American lobster tropomyosin as an inhibitor, the patient sera completely lost their reactivity to tropomyosins from six species of crustaceans (only the results with the pooled patient serum are shown in Figure 1D), demonstrating the cross-reactivity between tropomyosins from American lobster and the other crustaceans.

Our results may be as expected from the current opinion that tropomyosin is a major and cross-reactive allergen in crustaceans (7, 11, 12). However, it should be noted that, of the six species of crustaceans used in this study, pink shrimp belonging to the suborder Pleocyemata and king crab (a kind of hermit crabs) are taxonomically distinct from the crustaceans so far studied on allergens. Therefore, this study is of value in providing experimental evidence that the current opinion is true for almost all crustaceans belonging to the order Decapoda.

**Nucleotide Sequences of Crustacean Tropomyosin cDNAs.** The cDNAs encoding tropomyosins from black tiger prawn, kuruma prawn, king crab, and snow crab were successfully cloned by PCR using the Marathon cDNA library as a template and those encoding tropomyosins from pink shrimp and horsehair crab by both 3'RACE and 5'RACE using total RNA. In the case of the chest protection muscle of king crab, two different cDNAs (encoding fast and slow-tonic tropomyosins as described below in more detail) were obtained; three of the five clones analyzed corresponded to the fast tropomyosin and the rest to the slow-tonic tropomyosin. On the other hand, only one kind of cDNA was cloned from the other samples. The cDNA from the leg muscle of king crab was identical with one



**Figure 1.** Analysis of heated extracts from various crustaceans by SDS-PAGE (A), IgG-immunoblotting (B), IgE-immunoblotting (C), and inhibition immunoblotting (D). Lanes: 1, tropomyosin purified from American lobster; 2, extract from American lobster; 3, extract from black tiger prawn; 4, extract from kuruma prawn; 5, extract from pink shrimp; 6, extract from king crab leg muscle; 7, extract from king crab chest protection muscle; 8, extract from snow crab leg muscle; 9, extract from snow crab chest protection muscle; 10, extract from horsehair crab leg muscle; 11, extract from horsehair crab chest protection muscle. A magnification of the 37 kDa region in lane 7 (extract from king crab chest protection muscle) is shown above each figure in A, B, and C. (A) Heated extracts (0.3  $\mu$ L each) with the following protein concentrations were subjected to SDS-PAGE: 3.6 mg/mL for American lobster, 6.6 mg/mL for black tiger prawn, 3.0 mg/mL for kuruma prawn, 9.8 mg/mL for pink shrimp, 3.8 mg/mL for king crab leg muscle, 7.8 mg/mL for king crab chest protection muscle, 4.2 mg/mL for snow crab leg muscle, 3.2 mg/mL for snow crab chest protection muscle, 3.2 mg/mL for horsehair crab leg muscle, and 5.6 mg/mL for horsehair crab chest protection muscle. (B) Antiserum against king crab tropomyosin was used as a primary antibody. (C) Patient sera were used as a primary antibody: a, pooled serum from 10 patients; b, patient 1; c, patient 2; d, patient 3; e, patient 4. (D) Pooled serum (diluted 1:250) from 10 patients was preincubated with an equal volume of inhibitor (American lobster tropomyosin) solution (20  $\mu$ g/mL) and used as a primary antibody.

(encoding the fast tropomyosin) of the two cDNAs from the chest protection muscle. The same cDNA was found in the leg and chest protection muscles of snow crab, whereas the cDNAs from two types of muscles of horsehair crab differed from each other. These results agreed well with the data obtained in SDS-PAGE and immunoblotting showing the presence of two tropomyosin isoforms only in the chest protection muscle of king crab (Figure 1).

Although not shown in this paper to save space, the determined nucleotide sequences of the full-length cDNAs have been deposited in the DDBJ/EMBL/GenBank databases under the following accession numbers AB270629 for black tiger prawn tropomyosin (1260 bp), AB270630 for kuruma prawn tropomyosin (1299 bp), AB270631 for pink shrimp tropomyosin (1050 bp), AB270632 for king crab fast tropomyosin (1493 bp), AB270633 for king crab slow-tonic tropomyosin (1559 bp), AB270634 for snow crab tropomyosin (1148 bp), AB270635 for horsehair crab slow-twitch tropomyosin (1137 bp), and AB270636 for horsehair crab slow-tonic tropomyosin (1016 bp). Irrespective of the cDNAs, an open reading frame contains 852 bp coding for 284 amino acid residues.

**Amino Acid Sequences of Crustacean Tropomyosins.** The amino acid sequences of eight tropomyosins from six species of crustaceans were elucidated by a cDNA cloning technique. This is the first report of the amino acid sequences of tropomyosins from shrimp (pink shrimp) belonging to the suborder Pleocyemata and hermit crab (king crab), making it

possible to understand the overall features of the amino acid sequences of tropomyosins from edible crustaceans (decapod crustaceans). The known amino acid sequences of crustacean tropomyosins, including our results, are aligned in Figure 2. It is worth mentioning that three types of tropomyosin (fast, slow-twitch, and slow-tonic) have been identified in American lobster (14, 15). As compared to the amino acid sequence of the fast tropomyosin, both slow-twitch and slow-tonic tropomyosins have as many as 15 alterations in the region 39–79 and the slow-tonic tropomyosin has 4 additional alterations in the C-terminal region. On the basis of the sequence features of the three types of tropomyosin from American lobster, the tropomyosins from the abdominal muscle of shrimps (or prawns) and spiny lobster and one tropomyosin isoform from the chest protection muscle of king crab are assignable to the fast type, that from the leg muscle of horsehair crab to the slow-twitch type, and another tropomyosin isoform from the chest protection muscle of king crab, those from the leg and chest protection muscles of snow crab, and that from the chest protection muscle of horsehair crab to the slow-tonic type. The abdominal muscle tropomyosin of pink shrimp has some differences in the region 44–72 compared to the fast-type tropomyosin but is distinguishable from the slow-type tropomyosin. Thus, we tentatively classify the pink shrimp tropomyosin into the fast-type family. In the case of red crab tropomyosin, it is apparently a member of the slow-twitch or slow-tonic type family, although its C-terminal sequence is unclear. Taken together, the relationship

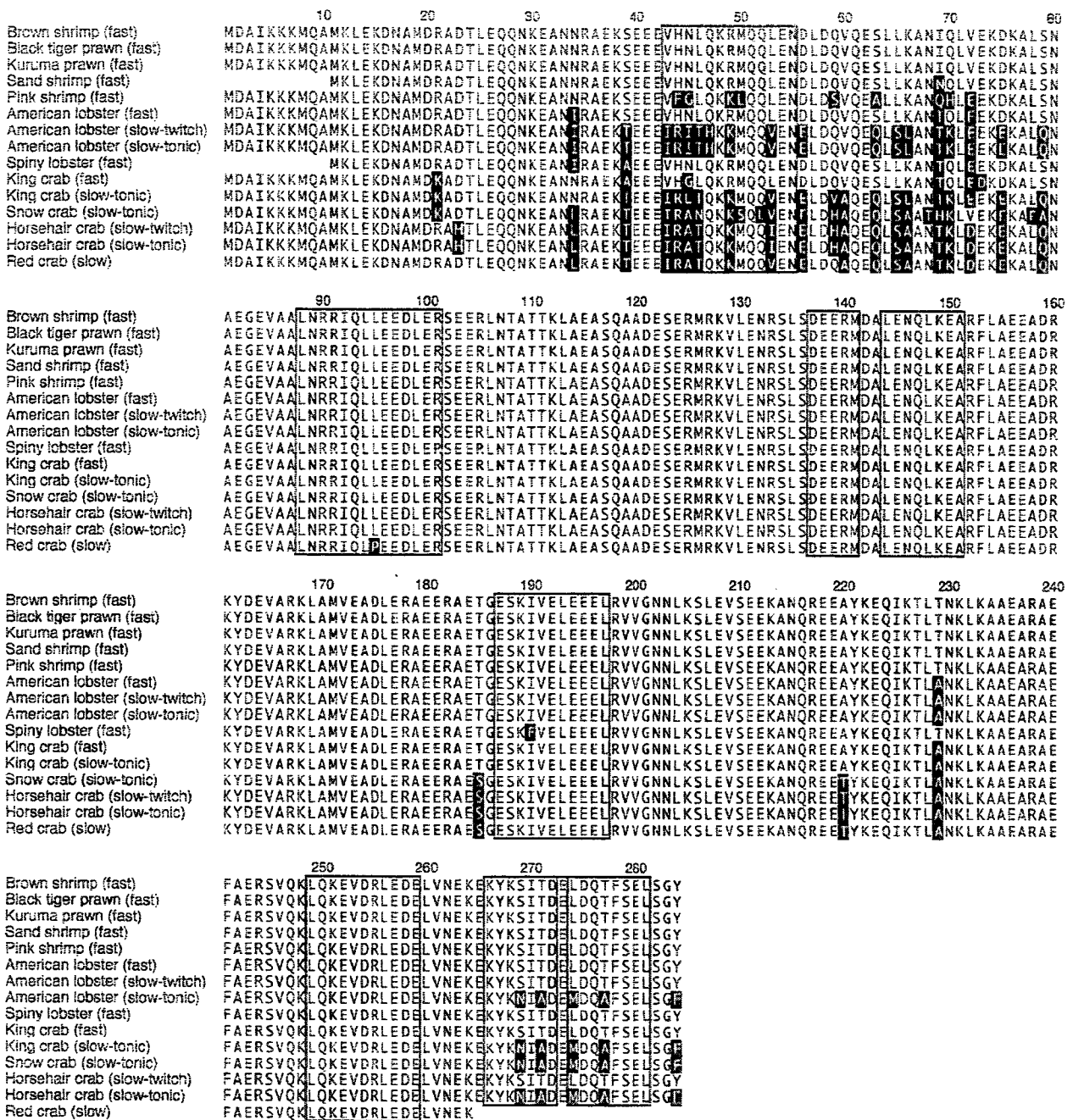


Figure 2. Amino acid sequences alignment of crustacean tropomyosins. Tropomyosin type (fast, slow-twitch, or slow-tonic) is indicated in parentheses after the crustacean name. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases): brown shrimp (*Penaeus aztecus*), DQ151457; black tiger prawn (*Penaeus monodon*), AB270629; kuruma prawn (*Penaeus japonicus*), AB270630; sand shrimp (*Metapenaeus ensis*), U08008; pink shrimp (*Pandalus scus*), AB270631; American lobster (*Homarus americanus*) (fast tropomyosin), AF034954; American lobster (slow-twitch tropomyosin), AF034953; American lobster (slow-tonic tropomyosin), AY521627; spiny lobster (*Penillius simpsoni*), AF030063; king crab (*Paralithodes camtschaticus*) (fast tropomyosin), AB270632; king crab (slow-tonic tropomyosins), AB270633; snow crab (*Chionoecetes opilio*), AB270634; horsehair crab (*Erimacrus isenbeckii*) (slow-twitch tropomyosin), AB270635; horsehair crab (slow-tonic tropomyosin), AB270636; red crab (*Charybdis feriatus*), AF061783. The residues differing from the brown shrimp tropomyosin are shown in white on the black background. The IgE-binding epitopes proposed for the brown shrimp tropomyosin are boxed.

of the major tropomyosin type in edible muscles with the taxonomical position of crustaceans is summarized as follows: fast-type tropomyosins are contained in shrimps (or prawns) and lobsters, slow-type tropomyosins in crabs, and both fast- and slow-type tropomyosins in crayfishes and hermit crabs.

As shown in Table 2, the amino acid sequence identities between fast tropomyosins and between slow tropomyosins are extremely high; even 100% identity is observed among the fast tropomyosins from three species of *Penaeus* shrimp or prawn (brown shrimp, black tiger prawn, and kuruma prawn). The

Table 2. Amino Acid Sequence Identity between Crustacean Tropomyosins

relationship	amino acid sequence identity (%)	
	range	mean $\pm$ SD
between fast tropomyosins	96.0–100.0	98.1 $\pm$ 1.0 ( $n$ = 28)
between slow tropomyosins	92.3–98.5	96.1 $\pm$ 1.4 ( $n$ = 21)
between fast and slow tropomyosins	88.3–94.7	91.4 $\pm$ 1.4 ( $n$ = 56)

sequence identity between fast and slow tropomyosins is also considerably high. These facts seem to provide a molecular basis for the cross-reactivity among crustacean tropomyosins (6, 7, 11, 12). More detailed discussion on the cross-reactivity among crustacean tropomyosins can be derived on the basis of the IgE-binding epitopes proposed for the brown shrimp tropomyosin (Pen a 1) (10, 16). All eight epitope regions (43–55, 88–101, 137–141, 144–151, 187–197, 249–259, 266–273, and 273–281; refer to Figure 2) of Pen a 1 are well conserved in fast tropomyosins, with the exceptions of pink shrimp tropomyosin having four alterations in the region 43–55, spiny lobster tropomyosin having one alteration in the region 187–197, and king crab tropomyosin having one alteration in the region 43–55. This implies that the cross-reactivity among fast tropomyosins is simply realized by the common IgE-binding epitopes. In the case of slow tropomyosins, five of the eight Pen a 1 epitopes are completely or almost completely conserved. However, both slow-twitch and slow-tonic tropomyosins have as many as six to eight alterations in the region 43–55, and slow-tonic tropomyosins have additional two alterations in the two regions 266–277 and 273–281. Moreover, diverse alterations between fast and slow tropomyosins are also observed in the region 56–79, which is not identified as the Pen a 1 epitope. For a better understanding of the cross-reactivity between fast and slow tropomyosins, an examination of how the significant differences in specific regions between fast and slow tropomyosins are associated with the IgE-binding potency is needed.

In general, crustacean-allergic patients are mostly sensitive to both shrimp and crab. This is easily understood by the presence of several common IgE-binding epitopes in shrimp and crab tropomyosins. However, some crustacean-allergic patients specifically recognize only shrimp or crab. This can be realized by two possibilities. One is that both shrimp and crab have specific allergens differing from tropomyosins; for example, arginine kinase has recently been identified as a new allergen in black tiger prawn (21). Another possibility is derived from our finding that there are significant differences in amino acid sequences of specific regions between fast tropomyosins (mainly contained in shrimp) and slow tropomyosins (mainly contained in crab). Namely, it is possible that both shrimp-allergic and crab-allergic patients have IgE specifically binding to the regions with significant differences between fast and slow tropomyosins. In conclusion, the amino acid sequences of crustacean tropomyosins determined in this study will facilitate future molecular study to understand crustacean allergy in more detail.

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