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Allergenicity of crustacean extractives and its reduction by protease digestion

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Abstract

This study was undertaken to evaluate and reduce the allergenicity of crustacean extractives that are added to a variety of processed foods as seasonings. Tropomyosin, the major crustacean allergen, was detected in only one of the six kinds of crustacean extractives by SDS–PAGE and immunoblotting. However, all the extractives were shown to be considerably allergenic by inhibition ELISA using sera from crustacean-allergic patients. Analyses by gel filtration HPLC suggested that tropomyosin is mostly degraded to peptide fragments during manufacturing of the extractives but at least some of the fragments are still IgE-reactive. On digestion with proteases, the allergenicity of the extractives was almost completely lost. The effectiveness of protease digestion to reduce the allergenicity of tropomyosin was also confirmed in model experiments using the heated extracts from four species of crustaceans.

Keywords: Allergenicity; Crustacean; Enzyme digestion; Extractive; Inhibition ELISA; Tropomyosin

1. Introduction

Crustaceans such as shrimp and crab are among the most popular causes of food allergy mediated by IgE antibodies. As clearly demonstrated in shrimp (Daul, Slattery, Reese, & Lehrer, 1994; Shanti, Martin, Nagpal, Metcalfe, & Subba Rao, 1993), prawn (Miyazawa et al., 1996), lobster (Leung, Chen, Mykles, Chow, Li, & Chu, 1998b) and crab (Leung, Chen, Gershwin, Wong, Kwan, & Chu, 1998a), the major crustacean allergen is tropomyosin, a myofibrillar protein of about 35 kDa. Moreover, previous immunoblotting studies have suggested that not all but many crustacean-allergic patients are IgE specific only for tropomyosin (Leung, Chow, Duffey, Kwan, Gershwin, & Chu, 1996; Lin, Shen, & Han, 1993; Musmand, Daul, & Lehrer, 1993). In order to develop hypoallergenic crustacean foods, therefore, it is essential to reduce allergenic activity of tropomyosin. Accordingly, food irradiation technology was recently applied to shrimp and shown to be considerably effective in reducing its allergenicity (Byun, Kim, Lee, Park, Hong, & Kang, 2000). However, irradiated shrimp may have side effects and hence its utilization should await a careful risk assessment to human health.

Crustacean extractives are added as seasonings to a variety of processed foods such as soup, confectionery, pickles and kamaboko. They are usually manufactured by boiling raw crustaceans, followed by concentrating the boiled water. Judging from several previous papers concerning the purification and identification of tropomyosin as the major allergen in crustaceans, tropomyosin is extractable by heating and importantly heat-denatured tropomyosin still maintains its original allergenic activity (Daul et al., 1994; Leung et al., 1994; Shanti et al., 1993). It is, therefore, possible that significant amounts of tropomyosin with allergenic activity exist in crustacean extractives. Even if tropomyosin is degraded to peptide fragments during manufacturing of crustacean extractives, some of the peptide fragments may have IgE-binding epitopes. These circumstances

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lead us to assume that crustacean extractives, as "hidden allergens" (Hefle, 2001; Steinman, 1996), can cause adverse reactions in crustacean-allergic patients who ingest processed foods without noticing the addition of crustacean extractives. In this study, therefore, some crustacean extractives manufactured for the food industry were evaluated for their allergenicity. Attempts were also made to reduce their allergenicity by digestion with proteases.

2. Materials and methods

2.1. Samples

Four kinds of shrimp extractives (1–4) and two kinds of crab extractives (1 and 2) were supplied from MARUHA Co. The species names of shrimp and crab used as raw materials were unidentified. Also, no information about the detailed manufacturing processes of the extractives was available. Fresh specimens of Japanese spiny lobster *Panulirus japonicus*, sakura shrimp *Sergia lucens*, horsehair crab *Erimacrus isenbeckii* and tanner crab *Chionoecetes opilio* were purchased at a local fish shop in Tokyo. The tropomyosin preparation purified from Japanese spiny lobster was a gift from Dr. T. Ojima, Graduate School of Hokkaido University.

2.2. Extraction

Liquid samples (shrimp extractives 1 and 4 and crab extractive 1) were directly centrifuged at 18,000*g* for 10 min, while paste samples (shrimp extractives 2 and 3 and crab extractive 2) were diluted with nine volumes of distilled water and then centrifuged. The supernatants were individually filtered through a 0.45 μm Dismic membrane (Advantec Toyo, Tokyo, Japan) and the filtrates were used as the extracts of crustacean extractives. Specimens of Japanese spiny lobster, sakura shrimp, horsehair crab and tanner crab, without dissecting, were separately immersed in three volumes of boiling water containing 3% NaCl and heated for 15 min. The boiled water was cooled, centrifuged and filtered as in the case of extractives. The filtrate was used as the extract from raw materials.

2.3. Protein determination

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard.

2.4. Human sera

Sera were obtained from four patients with a documented clinical history of immediate hypersensitivity reactions, such as urticaria and diarrhea, after ingestion of crustaceans. All patients had been diagnosed at hospitals to have elevated serum IgE (CAP-RAST class $\geqq$ 4) to shrimp and/or crab extracts. As negative control, serum from one subject without adverse reactions after ingestion of any food was used. All sera were stored at −20 °C until used.

2.5. SDS–PAGE and immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a PhastSystem apparatus (Amersham Bioscience, Piscataway, NJ), using ready-made slab gels (PhastGel Gradient 8–25) and ready-made buffer strips (PhastGel SDS Buffer Strips). Prior to electrophoresis, each sample was dissolved in 0.01 M Tris–HCl buffer (pH 6.8) containing 2.5% SDS, 20% glycerin and 5% dithiothreitol and heated in a boiling water bath for 10 min. Running and staining of the gel with Coomassie Brilliant Blue R-250 were carried out according to the manufacturer's instructions. A LMW electrophoresis calibration kit (Amersham Bioscience), containing phosphorylase b (94 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa), was used as a reference.

Immunoblotting was also carried out on the PhastSystem apparatus with a PhastTransfer as described in the manufacturer's manual. The proteins separated by SDS–PAGE were electrotransferred from the gel to a polyvinylidene difluoride membrane. The membrane was washed with Tween–PBS (0.01 M phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.05% Tween 20) and blocked with 2% skimmed milk in Tween–PBS at 37 °C overnight. After washing with Tween–PBS, the membrane was reacted successively with patient serum (diluted 1:500 with Tween–PBS) at 37 °C for 3 h and peroxidase-conjugated goat anti-human IgE antibody (Cosmo Bio, Tokyo, Japan; diluted 1:1000 with Tween–PBS) at 37 °C for 3 h. Antigen–antibody binding was visualized using an ECL Plus Western blotting detection system and an ECL Mini Camera (Amersham Bioscience), according to the manufacturer's instructions.

2.6. ELISA and inhibition ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed as reported previously (Ishikawa, Shimakura, Nagashima, & Shiomi, 1997). Briefly, samples immobilized on a polystylene microtitre plate with 96-wells (ELISA plate H type; Sumitomo Bakelite, Tokyo, Japan) were immunoreacted with pooled patient serum (diluted 1:50), followed by peroxidase-conjugated goat anti-human IgE antibody (diluted 1:2500). Enzyme reaction was performed in 0.05 M phosphate–citrate

buffer (pH 5.0) containing 0.1% *o*-phenylenediamine (Sigma, St. Louis, MO) and 0.03% H_2O_2 and arrested by addition of 1 M sulfuric acid. The developed colour was measured by absorbance at 490 nm with a microplate reader (Model 450; Bio-Rad Laboratories, Hercules, CA).

For inhibition ELISA experiments, pooled patient serum (diluted 1:25) was incubated with an equal volume of inhibitor solution (see Fig. 4 below for protein concentrations) at 37 °C for 2 h. A 0.1 ml portion of the reaction mixture was then added to a microplate that had previously been coated with Japanese spiny lobster tropomyosin at 0.1 μg/ml. The subsequent procedure was the same as described for ELISA.

All ELISA experiments were performed in triplicate and the data were given in mean values.

2.7. Gel filtration HPLC

The extracts from crab extractives 1 and 2 were separately applied to gel filtration HPLC on a TSKgel G2000SW (0.75 × 30 cm; Tosoh, Tokyo, Japan), which was eluted with 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. Proteins were monitored at 280 nm with a UV detector. Fractions of 1 ml were collected and subjected to inhibition ELISA to detect allergens.

2.8. Enzymatic digestion

Three kinds of proteases, trypsin (Wako Pure Chemical Industries, Osaka, Japan), α-chymotrypsin (Sigma) and protease P (Amano Enzyme, Nagoya, Japan), were used. Enzymatic degradation was performed at an enzyme–substrate ratio of 1:50 (w/w) at 37 °C for 12 h. Then, the reaction mixture was heated in a boiling water bath for 5 min to inactivate the enzyme and evaluated for allergenicity by inhibition ELISA.

3. Results and discussion

3.1. Identification of allergens in crustaceans

In SDS–PAGE, all the extracts from Japanese spiny lobster, sakura shrimp, horsehair crab and tanner crab afforded a band of 37–38 kDa that corresponded to the purified tropomyosin from Japanese spiny lobster (Fig. 1). Immunoblotting experiments revealed that four patient sera were all reactive not only to Japanese spiny lobster tropomyosin but also to one protein in each extract with the same molecular mass as Japanese spiny lobster tropomyosin (Fig. 2). It is relevant to conclude that the IgE-reactive protein is tropomyosin in common with the four species of crustaceans tested, as previously demonstrated for some crustaceans at the molecular level (Daul et al., 1994; Leung et al., 1994, 1998a, 1998b; Miyazawa et al., 1996; Shanti et al., 1993). Furthermore, all the four patient sera are considered to recognize only tropomyosin in crustaceans, similar to many crustacean-allergic patients (Leung et al., 1996; Lin et al., 1993; Musmand et al., 1993).

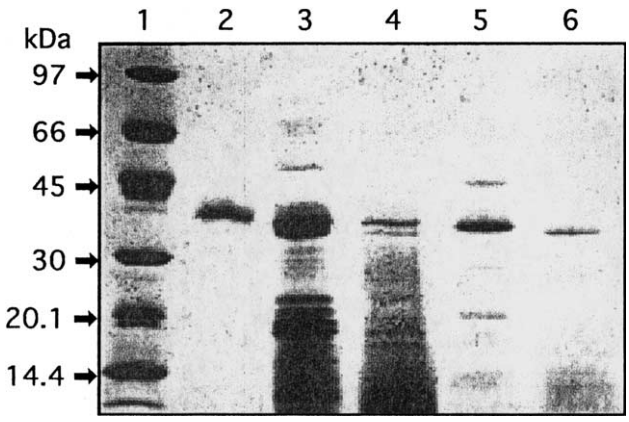

Fig. 1. SDS–PAGE of Japanese spiny lobster tropomyosin and extracts from four species of crustaceans. Lanes: 1, reference proteins; 2, Japanese spiny lobster tropomyosin; 3, Japanese spiny lobster extract; 4, sakura shrimp extract; 5, horsehair crab extract; 6, tanner crab extract.

3.2. Allergenicity of crustacean extractives

Of the six kinds of crustacean extractives, only crab extractive 1 gave a protein band corresponding to Japanese spiny lobster tropomyosin in SDS–PAGE (Fig. 3). This protein was confirmed to be IgE-reactive by immunoblotting using patient sera (data not shown) and hence identified as tropomyosin. In accordance with these results, the allergenicity of crab extractive 1 was also demonstrated by ELISA using pooled patient serum (Fig. 4). In the case of the other extractives, no distinct protein bands were observed in SDS–PAGE (Fig. 3) and also no IgE-reactive bands in immunoblotting (data not shown). Nevertheless, the results of ELISA showed that shrimp extractive 1 was highly allergenic at almost the same extent as crab extractive 1 and shrimp extractive 2 and crab extractive 2 were moderately allergenic, although no allergenicty was yet recognized in both shrimp extractives 3 and 4 (Fig. 4).

Much more important results were obtained by inhibition ELISA; the IgE reactivity of pooled patient serum to Japanese spiny lobster tropomyosin was more or less inhibited by all the crustacean extractives (Fig. 5). Since crustacean tropomyosins are known to be antigenically cross-reactive with each other (Reese, Ayuso, & Lehrer, 1999), the result with crab extractive 1 is easily realized by the cross-reactivity between Japanese spiny lobster tropomyosin and tropomyosin contained in crab

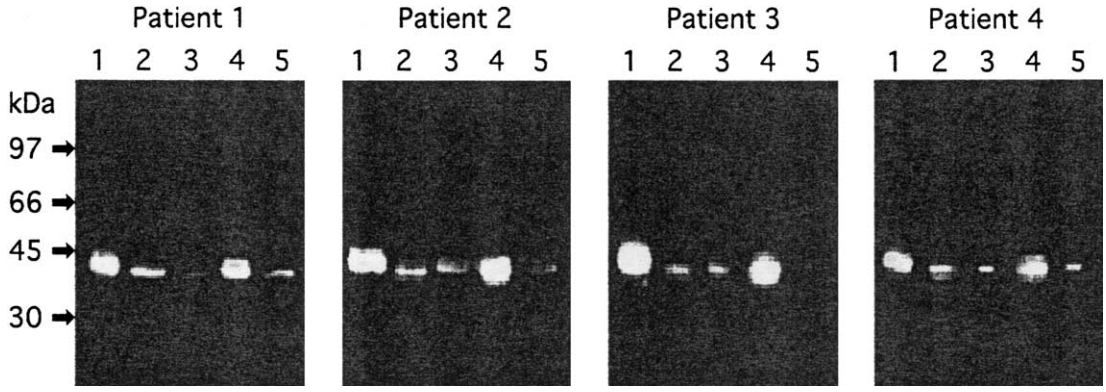

Fig. 2. Immunoblotting of Japanese spiny lobster tropomyosin and extracts from four species of crustaceans. Samples were separated by SDS–PAGE, electrotransferred to a membrane and immunoreacted with sera from crustacean-allergic patients. Lanes: 1, Japanese spiny lobster tropomyosin; 2, Japanese spiny lobster extract; 3, sakura shrimp extract; 4, horsehair crab extract; 5, tanner crab extract.

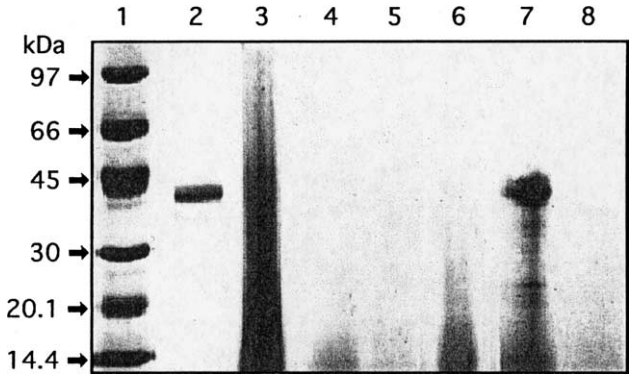

Fig. 3. SDS–PAGE of extracts from crustacean extractives. Lanes: 1, reference proteins; 2, Japanese spiny lobster tropomyosin; 3, shrimp extractive 1; 4, shrimp extractive 2; 5, shrimp extractive 3; 6, shrimp extractive 4; 7, crab extractive 1; 8, crab extractive 2.

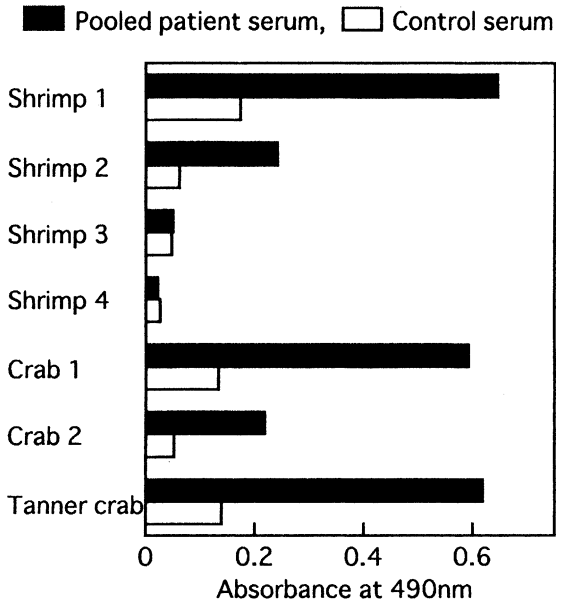

Fig. 4. Analysis by ELISA of the IgE reactivity to extracts from crustacean extractives and tanner crab. The extracts from crustacean extractives and tanner crab were diluted 1:1000 and 1:2000, respectively, and subjected to ELISA using pooled patient serum or control serum. The protein concentrations of diluted samples (μg/ml) were as follows: 6.7 for shrimp extractive l, 2.4 for shrimp extractive 2, 1.8 for shrimp extractive 3, 5.3 for shrimp extractive 4, 9.2 for crab extractive 1, 3.7 for crab extractive 2 and 7.5 for tanner crab.

extractive 1. On the other hand, tropomyosin was undetectable in the other crustacean extractives as analyzed by SDS–PAGE and immunoblotting but the presence of IgE-reactive substances having cross-reactivity with Japanese spiny lobster tropomyosin was confirmed by inhibition ELISA. It is possible that the IgE-reactive substances are low molecular weight peptide fragments with IgE-binding epitopes derived from tropomyosin during manufacturing of crustacean extractives. In order to ascertain this possibility, the extracts from crab extractives 1 and 2 were individually subjected to gel filtration HPLC and IgE-reactive substances were monitored by inhibition ELISA. In the case of crab extractive 1, containing tropomyosin, IgE-reactive substances were eluted only in high molecular weight fractions as expected (Fig. 6(a)). However, the majority of IgE-reactive substances in crab extractive 2 appeared in low molecular weight fractions, suggesting the presence of IgE-reactive peptide fragments derived from tropomyosin (Fig. 6(b)). Small amounts of allergenic proteins observed in high molecular weight fractions are ascribable to tropomyosin that was not detected by either SDS–PAGE or immunoblotting.

Based on the results described above, we assume that crustacean extractives used in the food industry are all allergenic. Some crustacean extractives contain tropomyosin and others contain not tropomyosin but its degraded peptide fragments with IgE reactivity. It should be noted that unlike proteins, small peptides are hardly immobilized on the ELISA plate. This is the reason why no allergenicity was recognized in both shrimp extractives 3 and 4 by ELISA. To avoid false-negative results,

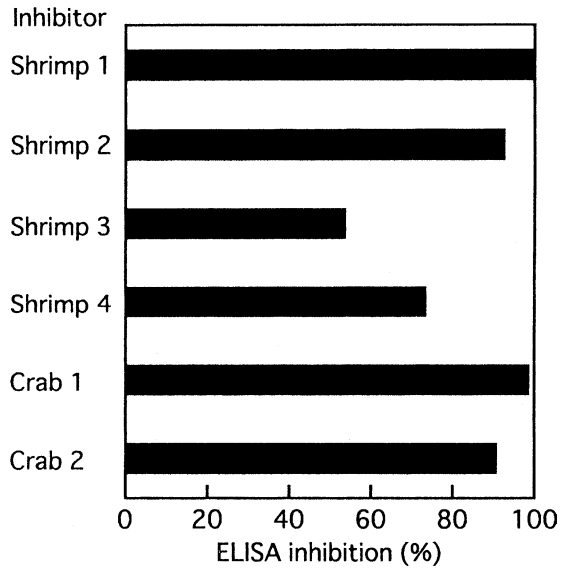

Fig. 5. Inhibition of the IgE reactivity to Japanese spiny lobster tropomyosin by extracts from crustacean extractives. The extracts from crustacean extractives were diluted 1:500 and used as inhibitor solutions. Pooled patient serum (1:25 dilution) was incubated with an equal volume of inhibitor solution at 37 °C for 2 h. The final protein concentrations of inhibitor solutions were the same as in Fig. 4. A 0.1 ml portion of the mixture was reacted with Japanese spiny lobster tropomyosin previously immobilized on a microplate at a concentration of 0.1 μg/ml.

therefore, the allergenicity of crustacean extractives should be evaluated by inhibition ELISA, not by immunoblotting and/or direct ELISA, as in this study.

3.3. Reduction of allergenicity by protease digestion

The effectiveness of protease digestion to reduce the allergenicity of Japanese spiny lobster tropomyosin, the extracts from four species of crustaceans and the extracts from six kinds of crustacean extractives was evaluated by inhibition ELISA. As shown in Fig. 7, irrespective of proteases, protease digestion resulted in complete or almost complete loss in the allergenicity of all samples. In addition to the cross-reactivity between Japanese spiny lobster tropomyosin and the crustacean extractives described above, the results of inhibition ELISA showed that tropomyosins from sakura shrimp, horsehair crab and tanner crab are cross-reactive with Japanese spiny lobster.

In summary, this study demonstrates that crustacean extractives used in the food industry are allergenic since they have tropomyosin and/or its degraded peptide fragments with an ability to bind IgE. Therefore, crustacean extractives currently added to a variety of processed foods can act as "hidden allergens". However, this study further demonstrates that protease digestion is very effective in reducing the allergenicity of crustacean extractives. Judging from the results in model experiments using four species of crustaceans, protease digestion appears to be applicable to the reduction of allergenicity of all crustacean extractives, whatever raw materials are used. Protease digestion is a widely adopted technique in food processing but sometimes produces bitter peptides that lower the quality of foods. Future

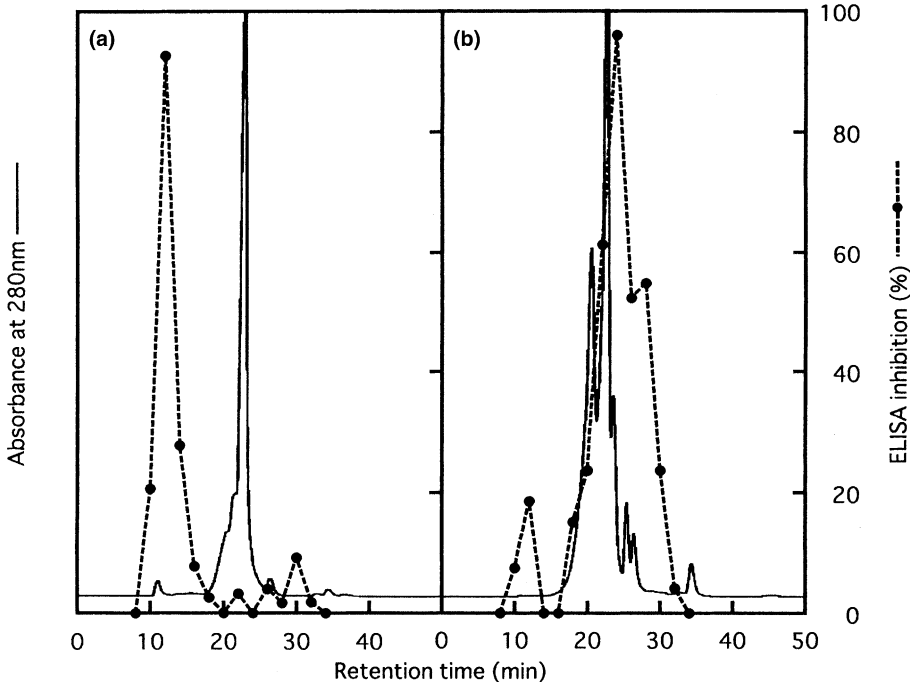

Fig. 6. Gel filtration HPLC of extracts from crab extractives 1 (a) and 2 (b). Column, TSKgel G2000SW (0.75 × 30 cm); solvent, 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0); flow rate, 0.5 ml/min. Fractions of 1 ml were collected, diluted 1:5 and subjected to inhibition ELISA as in the legends for Fig. 5.

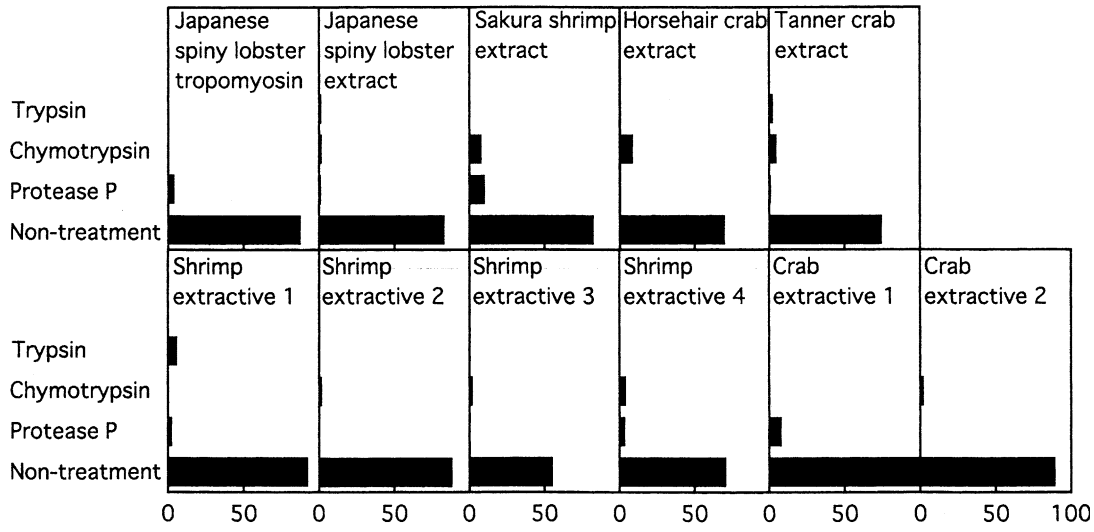

Fig. 7. Effects of protease digestion on the reduction of allergenicity of Japanese spiny lobster tropomyosin and extracts from crustaceans and crustacean extractives. Enzyme digestion was performed at an enzyme–substrate ratio of 1:50 (w/w). After enzymatic and heating treatments, each sample was subjected to inhibition ELISA. Pooled patient serum (1:25 dilution) was incubated with an equal volume of inhibitor solution at 37 °C for 2 h. A 0.1 ml portion of the mixture was reacted with Japanese spiny lobster tropomyosin previously immobilized on a microplate at a concentration of 0.1 μg/ml.

study is needed to set up the most desirable conditions in protease digestion to reduce the allergenicity but not to lower the quality of crustacean extractives. Finally, it should be emphasized that our results are helpful in designing a method to reduce the allergenicity of molluscan extractives, since tropomyosin has been identified as the major allergen in several mollusks (Chu, Wong, & Leung, 2000; Ishikawa et al., 1997; Ishikawa, Ishida, Shimakura, Nagashima, & Shiomi, 1998a; Ishikawa, Ishida, Shimakura, Nagashima, & Shiomi, 1998b; Ishikawa, Suzuki, Ishida, Nagashima, & Shiomi, 2001) and the cross-reactivity between crustacean and molluscan tropomyosins has also been demonstrated (Leung et al., 1996; Miyazawa et al., 1996; Reese et al., 1999).

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