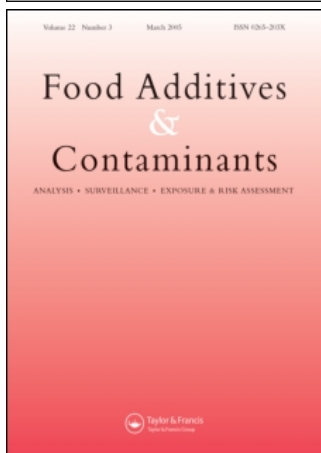


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Food Additives & Contaminants

Publication details, including instructions for authors and subscription information:

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Online Publication Date: 01 September 2007

To cite this Article: Motoyama, Kanna, Hamada, Yuki, Nagashima, Yuji and Shiomi, Kazuo (2007) 'Allergenicity and allergens of amphipods found in nori (dried laver)', Food Additives & Contaminants, 24:9, 917 - 922

To link to this article: DOI: 10.1080/02652030701305454

URL: <http://dx.doi.org/10.1080/02652030701305454>

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Allergenicity and allergens of amphipods found in nori (dried laver)

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(Received 11 October 2006; revised 12 February 2007; accepted 26 February 2007)

Abstract

Gammaridean and caprellid amphipods, crustaceans of the order Amphipoda, inhabit laver culture platforms and, hence, are occasionally found in nori (dried laver) sheets. Amphipods mixed in nori may cause allergic reactions in sensitized patients, as is the case with other crustaceans, such as shrimp and crab, members of the order Decapoda. In this study, dried samples of amphipods (unidentified) found in nori and fresh samples of gammaridean amphipod (*Gammarus* sp., not accurately identified) and caprellid amphipod (*Caprella equilibra*) were examined for allergenicity and allergens using two species of decapods (black tiger prawn and spiny lobster) as references. When analyzed by ELISA, sera from crustacean-allergic patients reacted to extracts from amphipod samples, although less potently than to the extracts from decapods. In IgE-immunoblotting, a 37-kDa protein was found to be the major allergen in amphipods. Based on the molecular mass and the cross-reactivity with decapod tropomyosin evidenced by inhibition ELISA and inhibition immunoblotting, the 37-kDa protein was identified as amphipod tropomyosin.

Keywords: Allergen, allergenicity, amphipod, cross-reactivity, crustacean, dried laver, nori, tropomyosin

Introduction

Nori (dried laver) is made from the red alga *Porphyra yezoensis* and is generally available in ready-to-use sheets. In Japan, about nine billion sheets are produced each year and used in sushi-making and as condiments in other foods, such as soups and salads. Since nori is rich in various nutrients, particularly minerals (especially calcium and iron), vitamins (especially vitamins A and C) and fibers, its popularity and consumption is increasing in many countries.

Gammaridean amphipods (Figure 1a) and caprellid amphipods (Figure 1b), crustaceans belonging to the order Amphipoda, are found around the platforms used for the culture of *Porphyra* spp. Therefore, amphipods become accidentally mixed with the seaweed during harvest and, when dried, are occasionally found in nori sheets, resembling small

stones (Figure 1c). Nori sheets containing dried amphipods are ranked 'shrimp grade' and traded at a much lower price than those devoid of them. Although no poisoning incidents due to amphipods have so far been recorded, queries on the safety of dried amphipods in nori sheets have been received by nori-producing companies from many consumers.

Commercial edible crustaceans, such as shrimp and crab (members of the order Decapoda) are frequently involved in immunoglobulin E (IgE)-mediated food allergies (Lehrer et al. 2003; Chu et al. 2005; Wild and Lehrer 2005). Ingestion, even at very low doses, immediately causes allergic reactions, such as urticaria, asthma, diarrhea and anaphylaxis, in sensitized patients. The major allergen of decapod crustaceans has been identified as tropomyosin, a myofibrillar protein of

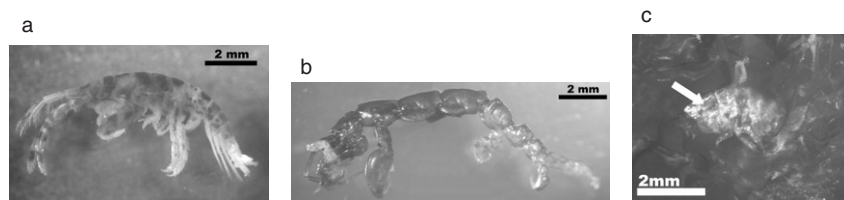


Figure 1. Photographs of a gammaridean amphipod (*Gammarus* sp.) (a), a caprellid amphipod (*Caprella equilibra*) (b) and an amphipod (indicated by an arrow) in nori (c).

35–38 kDa, in shrimp (Shanti et al. 1993; Daul et al. 1994; Leung et al. 1994; Motoyama et al. 2007), crayfish (Leung et al. 1998a), lobster (Leung et al. 1998a), hermit crab (Motoyama et al. 2007) and crab (Leung et al. 1998b; Motoyama et al. 2007). Moreover, cross-reactivity of tropomyosin has also been established not only among crustaceans but also between crustaceans and other invertebrates, such as mollusks, house-dust mites and cockroaches (Leung et al. 1996; Reese et al. 1999; Santos et al. 1999; Chu et al. 2000; Ayuso et al. 2002; Lehrer et al. 2003). In view of these findings, it is possible that crustacean-allergic patients could also be sensitive to amphipod tropomyosin. If so, hypersensitivity reactions could be induced in crustacean-allergic patients following ingestion of nori sheets infested with amphipods. In this study, dried amphipods mixed in nori sheets and fresh specimens were assessed for allergenicity and allergens, using two species of edible decapod, black tiger prawn *Penaeus monodon* and Japanese spiny lobster *Panulirus japonicus*, as references.

Materials and methods

Crustacean and tropomyosin samples

Specimens of the gammaridean amphipod (*Gammarus* sp., not accurately identified) and the caprellid amphipod (identified as *Caprella equilibra* by Dr. I. Takeuchi, Faculty of Agriculture, Ehime University) were collected at a laver culture platform off Futtsu, Chiba Prefecture, in March 2003. Samples were immediately frozen and transported to our laboratory. The dried amphipod samples were carefully removed from nori sheets (shrimp grade) and, although not correctly counted due to the difficulty in identification, the nori amphipods were assumed to be composed of gammaridean and caprellid amphipods at a ratio of about 9:1. Fresh specimens of black tiger prawn and Japanese spiny lobster were purchased at the Tokyo Central Wholesale Market. A tropomyosin preparation, purified from the abdominal muscle of Japanese spiny lobster by a combination of isoelectrical precipitation and salting-out with ammonium

sulfate, was a kind gift from Dr. T. Ojima, Graduate School of Fisheries Sciences, Hokkaido University. All crustacean and tropomyosin samples were stored at -20°C until use.

Preparation of heated extracts

Samples (0.5 g each) of gammaridean and caprellid amphipods were homogenized separately in 3 vols of 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). The manufacture of nori sheets includes a heating process, but tropomyosin, the target protein in this study, is known to be thermostable. Therefore, the homogenate was heated in a boiling water bath for 10 min and, after centrifugation, the supernatant was used as a heated extract. Similarly, heated extracts were prepared from the abdominal muscles (1 g each) of black tiger prawn and Japanese spiny lobster. In the case of nori amphipods (0.2 g), 10 vols of the solvent were used to prepare the heated extract. Protein concentrations of the heated extracts were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Human sera

Sera were donated from 10 crustacean-allergic patients with documented clinical histories of immediate hypersensitivity reactions after ingestion. The patients were also diagnosed to be allergic to crustaceans based on the CAP-RAST (capsulated hydrophilic carrier polymer-radioallergosorbent test) classes 2–5 against shrimp or both shrimp and crab. Written informed consent was obtained from each patient. The sera were used individually or pooled. In this study, pooled sera from eight healthy volunteers without adverse reactions after ingestion of any foods was used as a control. All sera were stored at -20°C until use.

ELISA and inhibition ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed using a flat-bottomed polystyrene plate with 96 wells (Type H multi-well plate for ELISA; Sumitomo Bakelite, Tokyo, Japan), as reported previously (Ishikawa et al. 1997). In brief, each

heated extract of an appropriate dilution was coated on the plate and immunoreacted with patient serum (50-fold dilution), followed by horseradish peroxidase-conjugated goat anti-human IgE antibody (2500-fold dilution; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The enzyme reaction was carried out using substrate solution (0.1% *o*-phenylenediamine and 0.03% hydrogen peroxide in 0.05 M phosphate-citrate buffer, pH 5.0) and the developed colour was measured at 490 nm on a kinetic microplate reader model 550 (Bio-Rad Laboratories, Hercules, CA, USA). For inhibition ELISA, the pooled patient serum (25-fold dilution) was incubated with an equal volume of spiny lobster tropomyosin solution (20 $\mu\text{g ml}^{-1}$) or the heated extract (50-fold dilution) from gammaridean amphipods at 37°C for 2 h. A 100 μl aliquot of the mixture was then added to a microtiter plate that had previously been coated with each heated extract (1000-fold dilution). Subsequent procedures followed that for the ELISA described above. All ELISA (including inhibition ELISA) were performed in triplicate and the data were expressed as mean values.

SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a PhastSystem apparatus (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) 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 loading, each sample was dissolved in 0.01 M phosphate buffer (pH 7.2) containing 2.5% SDS and 5% dithiothreitol and heated at 100°C for 10 min. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Precision Plus protein standards (Bio-Rad Laboratories) were run along with samples as references.

Immunoblotting and inhibition immunoblotting

Immunoblotting was performed as described in our previous paper (Motoyama et al. 2006). Briefly, the proteins separated by SDS-PAGE were electrotransferred from the gel to a nitrocellulose membrane, which was reacted successively with a primary antibody (500-fold dilution of pooled patient serum) at 37°C for 1 h and a secondary antibody (5000-fold dilution of horseradish peroxidase-conjugated goat anti-human IgE antibody) at 37°C for 1 h. Antigen-antibody binding was visualized using an ECL Plus Western blotting detection system and an ECL mini-camera (GE Healthcare Bio-Sciences), according to the manufacturer's instructions. For inhibition

immunoblotting, the pooled patient serum (250-fold dilution) was incubated with an equal volume of inhibitor solution (20 $\mu\text{g ml}^{-1}$ spiny lobster tropomyosin solution or 50-fold dilution of the heated extract from gammaridean amphipods) at 37°C for 2 h and used as a primary antibody.

Results

Allergenicity of amphipods and decapods

The protein concentrations of the heated extracts were determined to be 5.0 mg ml^{-1} for nori amphipods, 7.5 mg ml^{-1} for gammaridean amphipods, 7.5 mg ml^{-1} for caprellid amphipods, 7.8 mg ml^{-1} for black tiger prawn and 7.5 mg ml^{-1} for Japanese spiny lobster. Since the nori amphipod sample was in the dried state, it was extracted with a larger volume of the solvent compared to the other fresh samples. Nevertheless, the heated extract from nori amphipods was comparable in protein concentration to those from the other samples. Thus, the difference in allergenicity between the heated extract from nori amphipods and those from the other samples were compared without considering the difference in solvent volume used.

ELISA showed that all the heated extracts from amphipods and decapods reacted with IgE in the pooled patient serum in a dose-dependent manner (Figure 2). Obviously, IgE reactivity was high in the heated extracts from the two species of decapods (black tiger prawn and Japanese spiny lobster),

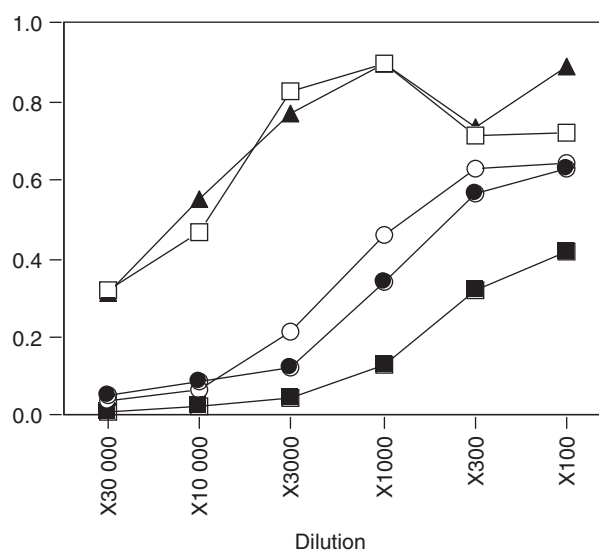


Figure 2. Reactivity of pooled patient serum to the heated extracts from amphipods and decapods by ELISA. Heated extracts: (●) nori amphipod; (○) gammaridean amphipod; (■) caprellid amphipod; (□) black tiger prawn; (▲) Japanese spiny lobster.

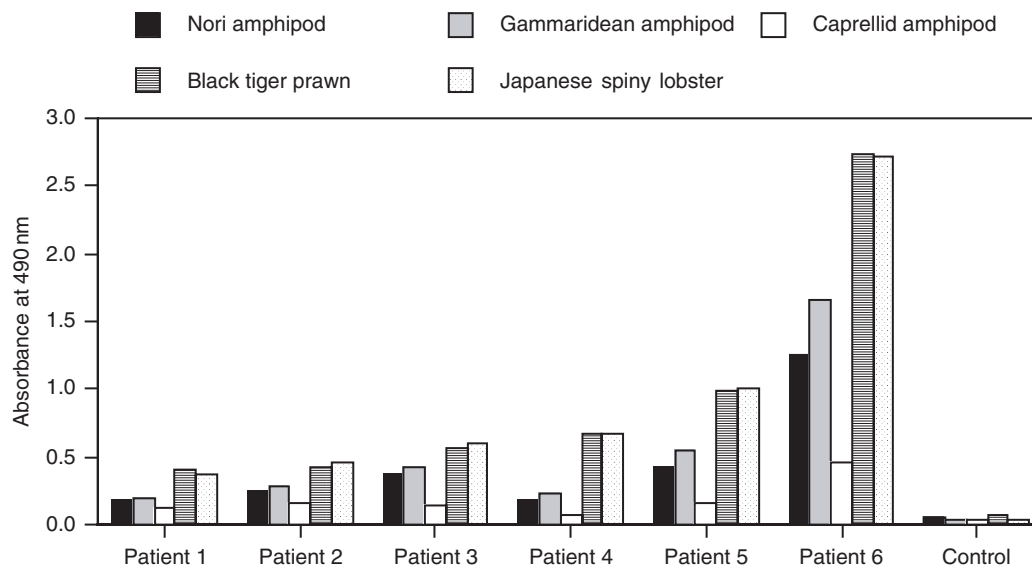


Figure 3. Reactivity of six patient sera to the heated extracts from amphipods and decapods by ELISA. A 1000-fold dilution of each heated extract was subjected to ELISA.

moderate in those from nori and gammaridean amphipods and low in that from caprellid amphipods. The same tendency was also recognized when six patient sera were individually evaluated for reactivity to a 1000-fold dilution of each extract by ELISA, although reactive potencies varied among the sera (Figure 3). In this study, the heated extracts were prepared from whole amphipods, while only muscle samples containing tropomyosin at high levels were used from decapods. This difference seems to account for the observed variation in allergenicity between amphipods and decapods.

As summarized in Table I, the reactivities of the pooled patient sera to the heated extracts from amphipods and decapods were all significantly reduced by preincubation of the serum with either Japanese spiny lobster tropomyosin or the heated extract from gammaridean amphipods, demonstrating the cross-reactivity between allergen molecules from amphipods and decapods. Regardless of the inhibitors, IgE reactivities to the heated extracts from amphipods were inhibited at higher levels compared to those from decapods. This can be understood by assuming that allergen levels are lower in amphipods than in decapods.

Allergens of amphipods and decapods

On SDS-PAGE, a prominent protein band of about 37 kDa was detected in both heated extracts from black tiger prawn and Japanese spiny lobster; this protein was identified as tropomyosin when compared to Japanese spiny lobster tropomyosin (Figure 4a). In contrast, all heated extracts from amphipods provided only obscure bands. However,

Table I. Reactivity inhibition between pooled patient serum and heated extracts of Japanese spiny lobster tropomyosin and heated extract from gammaridean amphipods.

Heated extract	Inhibition (%)	
	Japanese spiny lobster tropomyosin	Heated extract from gammaridean amphipod
Nori amphipods	94	86
Gammaridean amphipods	100	97
Caprellid amphipods	95	91
Black tiger prawn	87	67
Japanese spiny lobster	78	70

Pooled patient serum (250-fold dilution) was incubated with an equal volume of inhibitor solution ($20 \mu\text{g ml}^{-1}$ spiny lobster tropomyosin solution or 50-fold dilution of the heated extract from gammaridean amphipods) at 37°C for 2 h and used as a primary antibody in ELISA. Inhibition (%) was calculated by comparing the reactivity between pooled patient serum (500-fold dilution) and each heated extract (1000-fold dilution).

a protein band of about 37 kDa, probably ascribable to tropomyosin, was faintly found in the heated extracts from nori and gammaridean amphipods, but from caprellid amphipods.

In immunoblotting, the pooled patient serum reacted strongly to tropomyosins in black tiger prawn and Japanese spiny lobster, moderately to the 37-kDa protein in gammaridean amphipods and weakly to nori amphipods (Figure 4b-1). In addition to these blots, smeared blots were observed in the range 75–150 kDa in nori amphipods, blots of 100 and 150 kDa in gammaridean amphipods and a blot of about 100 kDa in Japanese spiny lobster, while no IgE-reactive bands were detected in caprellid amphipods. Reactivity to the 100-kDa protein of

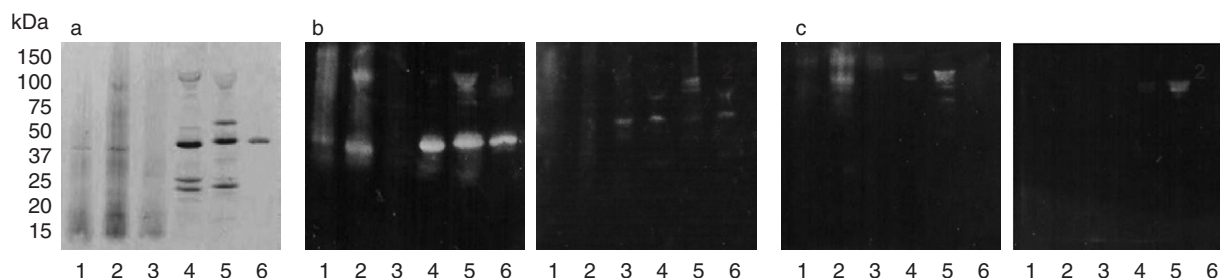


Figure 4. Analysis of the heated extracts from amphipods and decapods by SDS-PAGE (a), immunoblotting (b) and inhibition immunoblotting (c). Lanes: 1, extract from nori amphipods; 2, extract from gammaridean amphipods; 3, extract from caprellid amphipods; 4, extract from black tiger prawn; 5, extract from Japanese spiny lobster; 6, Japanese spiny lobster tropomyosin. (a) Stained with Coomassie Brilliant Blue R-250. (b) Sera: 1, pooled patient serum; 2, control serum. (c) Inhibitors: 1, Japanese spiny lobster tropomyosin; 2, extract from gammaridean amphipods.

Japanese spiny lobster was considered to be non-specific, since positive reactivity was also displayed by control serum (Figure 4b-2). When preincubated with either Japanese spiny lobster tropomyosin or the heated extract from gammaridean amphipods, the pooled patient serum lost its reactivity to the 37-kDa protein in nori and gammaridean amphipods, as well as to tropomyosins of black tiger prawn and Japanese spiny lobster (Figure 4c).

Discussion

ELISA data showed that amphipods are allergenic to sensitized patients, although less potently than the two species of decapods, black tiger prawn and Japanese spiny lobster, used as references. Of the two species of amphipods examined, gammaridean amphipods clearly shows higher allergenicity than caprellid amphipods. Thus, it should be noted that nori amphipods are slightly lower in allergenicity than gammaridean amphipods but significantly higher than caprellid amphipods. This conforms well to the fact that gammaridean amphipods occupy about 90% of the nori amphipod samples used and, importantly, suggests that the allergenicity of amphipods is not reduced during nori production, including the drying and heating processes.

Immunoblotting experiments revealed that the allergen in both nori and gammaridean amphipods is a 37-kDa protein, which is comparable in molecular mass to Japanese spiny lobster tropomyosin. Moreover, cross-reactivity of the 37-kDa protein with Japanese spiny lobster tropomyosin was confirmed by inhibition immunoblotting. Although no IgE-reactive proteins were detected in caprellid amphipods by immunoblotting, the caprellid amphipod allergen was shown to cross-react with Japanese spiny lobster tropomyosin by inhibition ELISA. Due to the difficulty to collect sufficient amphipod samples, more definite molecular evidence concerning amphipod allergens was not obtained in

this study. However, based on our data, it is reasonable to conclude that the major allergen of amphipods is tropomyosin, as previously demonstrated for some species of decapod crustaceans (Shanti et al. 1993; Daul et al. 1994; Leung et al. 1994, 1998a, 1998b).

Amphipods are potentially allergenic to crustacean-allergic patients; however, only several mg of dried amphipods are mixed in a sheet (about 3 g) of shrimp-grade nori. Ingestion of nori containing such a small amount of amphipods may rarely cause hypersensitive reactions. In fact, to the best of our knowledge, no incidents of amphipod allergy have been recorded to date. However, it should be noted that processed foods containing specifically allergenic food materials (such as egg and milk) and other allergenic food materials, including shrimp and crab, have a recommended and regulated level, respectively, of $>10 \mu\text{g protein g}^{-1}$, to be labeled on packaging or bottles by the Ministry of Health, Labour and Welfare of Japan. From the protein concentration (5.0 mg ml^{-1}) of the heated extract from nori amphipods and the volume (10 vol of the sample weight) used to prepare the heated extract, the protein content of nori amphipods in nori is evaluated as $\sim 50 \mu\text{g g}^{-1}$, which considerably exceeds the Japanese regulatory value. Therefore, it should be considered that amphipods in nori have the potential to cause food allergy as a "hidden allergen", particularly in severely sensitized crustacean-allergic patients. To avoid accidental incidence of food allergy by amphipods in nori, labeling, indicating the presence of a small number of crustaceans, is recommended on packages of shrimp grade nori (containing amphipods).

Acknowledgements

The authors thank Drs. N. Shimojo and Y. Kohno, Department of Pediatrics, Chiba University, Drs. R. Shibata and S. Nishima, National Minami-Fukuoka Chest Hospital, and Drs. H. Ohsuna and

Z. Ikezawa, Department of Dermatology, Yokohama City University, for providing patient sera. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, a grant from the Ministry of Health, Labour and Welfare of Japan and a grant from the Japan Society for the Promotion of Nori Culture.

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