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Quantitative Sandwich ELISA for the Determination of Tropomyosin from Crustaceans in Foods

MARIANNE T. WERNER,* CHRISTIANE K. FÆSTE,* AND ELIANN EGAAS

National Veterinary Institute, P.O. Box 8156 Dep., N-0033 Oslo, Norway

The ubiquitous muscle protein tropomyosin has been identified as the major shrimp allergen and is suggested to be a cross-reacting allergen. Previously, only a few methods for the detection of tropomyosin in food have been published. A quantitative sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of tropomyosin from crustaceans in foods has been developed and validated. A polyclonal rabbit antitropomyosin capture antibody and the biotinylated conjugate of the same antibody for detection were the basis for the ELISA, which was specific for crustaceans. The ELISA was able to quantitate tropomyosin in various food matrixes, had a detection limit of 1 μg/g, and cross-reacted to some extent with cockroach. Recoveries ranged from 63 to 120%, and the intra and interassay coefficients of variation were <6 and <14%, respectively.

KEYWORDS: Allergen; ELISA; food allergy; tropomyosin

INTRODUCTION

Shellfish is a generic term including all aquatic animals that have a shell or shell-like exoskeleton and can in general be separated into two categories; crustaceans and mollusks. Hypersensitivity reactions to ingested crustaceans, such as shrimp, lobster, crayfish, and crab, as well as mollusks such as squid, octopus, snails, clams, mussels, oyster, and scallops, count among the most frequent causes of food-induced allergic reactions (*1*, *2*) and can cause various clinical symptoms such as urticaria, asthma, diarrhea, and anaphylaxis (*3*). In contrast to allergies toward milk and egg that often emerge in early childhood, allergies toward crustaceans more commonly appear at a somewhat later stage of life and are less likely to be outgrown (*4*, *5*).

The molecular identity of crustacean allergens, especially the shrimp allergens, was described during the 1980s and 1990s. Two heat-stable IgE reactive proteins of 38 kDa from raw and cooked shrimp (unreported species), termed antigens I and II, were purified and described by Hoffman et al. (*6*) in 1981. Later, a heat-stable allergen (Sa-II) from the shrimp *Penaeus indicus* was isolated and characterized (*7*), and later on a similar protein of 36 kDa from *Penaeus aztecus* (Pen a 1) was isolated (*8*). Shanti et al. (*9*) demonstrated that the major shrimp allergen biochemically conformed to the muscle protein tropomyosin (Sa-II, also referred to as Pen i 1). Comparison of the amino acid sequences of Pen a 1, antigen I, and Sa-II revealed that they were homologous, (*8*). Tropomyosin is present both in muscle and nonmuscle cells in all vertebrate and invertebrate species.

* Authors to whom correspondence should be addressed [(M.T.W.) e-mail marianne.werner@vetinst.no, telephone +47 23 21 62 21, fax +47 23 21 62 01; (C.K.F.) e-mail christiane.faste@vetinst.no, telephone +47 23 21 62 32, fax +47 23 21 62 01].

Sera from patients allergic to shrimp may react with other crustaceans and mollusks (*10*), and several studies have shown that shrimp may also cross-react with arthropods such as house dust mites (Arachnida) (*11*) and cockroaches (*11–13*). Tropomyosin is considered as a possible cause for cross-reactivities between food and respiratory allergens of animal origin. Exposure and sensitization to crustacean allergens may thus lead to sensitization to certain respiratory allergens (*11*), and sensitization to mite tropomyosin may induce food allergy (*14*). In 2003, Fernandes et al. published a study about orthodox Jews, who lived by the strict kosher dietary laws that prohibit all consumption of shellfish. Nevertheless, a sensitization to shrimp tropomyosin was discovered in this collective and explained by sensitization to inhalant allergens such as mite (*15*).

Seafood is not only an important food allergen but has also been identified as a cause of occupational induced reactions (*16*). Workplace exposure by handling seafood or the inhalation of aerosols or vapors, particularly those generated during cooking, can lead to hypersensitivity reactions such as dermatitis, urticaria, rhinitis, conjunctivitis, asthma, and pneumonitis (*17*).

Possible sources for the involuntary intake of seafood proteins can be foods from shared production lines where carry-over and cross-contamination may take place between shellfish-containing and shellfish-free products, inappropriately labeled foods such as Asian dishes with crustacean stuffing, or flavors in seafood imitations. In Europe, crustaceans (e.g., shrimp, crab, crayfish, and lobster) and products thereof and mollusks (e.g., snails, clams, mussels, squids) and products thereof have been included in a list of 13 major food allergens and sulfite by the European Commission (*18*). These food components must be declared in a product's ingredient list, independently from other labeling policies.

For the quantitation of allergens in foods, specific and reliable methods of analysis are essential. The sensitivity required has

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to be evaluated in connection with the data that exist about threshold levels for food-allergic patients. For the time being, the enzyme-linked immunosorbent assay (ELISA) is probably the most utilized method for the detection of food allergens, as the method easily can be used on a routine basis, and has demonstrated high precision and good potential for standardization (*19*). Compared to other food allergens, there are only a few methods described for the detection of crustacean proteins in food. Crustaceans incorporate a large number of species, and for detection purposes it is necessary to decide whether to try testing for one definite species or crustaceans as a group. From the food industry's perspective, the second approach is probably more worthwhile, seeking to generally detect crustacean residue as a total, disregarding species or type of allergenic proteins. A sandwich ELISA using monoclonal antibodies was developed by Jeoung et al. (*20*), for the purpose of quantitating tropomyosin in commercially available crustacean extracts intended for skin testing of patients. The detectable protein levels in these clinically used extracts ranged between 4 and 125 ng of tropomyosin/mL of extract. An ELISA method, designed for the detection of shrimp in food using antibodies raised against shrimp tropomyosin, was described by Ben Rejeb et al. (*21*). The assay's limit of detection (LOD) was approximately 2.5 mg/kg. However, this method has been described only in an abstract and with very few details. The first commercially available ELISA for the quantitative detection of crustaceans in food claimed to have a LOD of 0.05 mg/kg. Recently, a quantitative ELISA kit for the detection of shellfish protein in food based on a polyclonal antibody produced against tropomyosin from prawn (*Penaeus latisulcatus*) has been released (*22*). Its quantitation range is 1–20 mg/kg and the LOD, 0.1 mg/kg of shellfish protein. Nevertheless, considering that assay applicabilities can vary due to validation criteria, specificities, and matrixes, we decided to develop an ELISA designed according to our requirements.

The aim of the current study was therefore to develop and validate a sensitive, quantitative, and specific sandwich ELISA method for the detection of tropomyosin traces from crustaceans in a number of representative foods.

MATERIALS AND METHODS

Preparation of the Tropomyosin Standard. Crude muscle meat from shrimp (*Pandalus borealis*) was homogenized in 0.1 M tris(hydroxymethyl)aminomethane (Tris)/0.5 M glycine buffer (pH 8.7)/1 mM dithioerythriol (DTE) (Sigma-Aldrich) extraction buffer (w/v 1:2) with a rod homogenizer (Braun Vario, Kronberg, Germany). After overnight extraction at 45 °C in a shaking water bath (OLS 200, Grant, Cambridge, U.K.) and centrifugation for 15 min at 4 °C and 18000*g* (J2-MC, Beckman Instruments, Palo Alto, CA), the supernatant was transferred to a glass beaker and precipitated with 25% ammonium sulfate (Merck, Whitehouse Station, NJ), previously pounded in a mortar, at room temperature (RT) under continuous stirring. After further centrifugation (15 min, 4 °C, 10000*g*), the supernatant was precipitated for a second time with ammonium sulfate, reaching a final concentration of 75%. The pellet obtained by a final centrifugation step (15 min, 4 °C, 10000*g*) was resuspended in 50 mM Tris (pH 8.0)/1 mM DTE and separated by size exclusion centrifugation (15 min, 4 °C, 1500*g*), using a Centriprep 30 column (Millipore, Billerica, MA). The supernatant was extensively dialyzed against water in a dialysis tube with 6–8 kDa μm pore size (Spectra/Por, Spectrum Medical Industries, Los Angeles, CA) and freeze–dried (Heto, Allerød, Denmark). The protein was resuspended in phosphate-buffered saline (PBS) (pH 7.4). The total protein content was determined with the Lowry Protein Assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to 2 mg/mL. Analysis by sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) (Mini Protean 3 cell, Bio-Rad) at 200 V for 40 min in 25 mM Tris/192 mM glycine buffer (pH 8.3) under reducing conditions using 12% gels and ProSieve 10–190 kDa protein marker (Cambrex, Karlskoga, Sweden) showed one dominant protein band at about 36 kDa containing the purified shrimp tropomyosin and one very weak contamination at 17 kDa (data not shown). Subsequently, the purified shrimp tropomyosin was used as antigen in rabbit immunization and as standard in the ELISA.

Purification and Labeling of Antitropomyosin Antibody. A polyclonal antiserum was raised against the tropomyosin standard in a rabbit using the same procedure as previously described (*23*). The proteins (5 mg) in the shrimp extract were covalently coupled to NHS-activated (Sepharose) HP columns (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The rabbit serum was desalted on PD-10 columns (Amersham Biosciences) and then passed through the previously tropomyosin-coupled (Sepharose) affinity column to bind tropomyosin-specific antibodies. Bound antibodies were eluted in fractions from the column with 100 mM glycine (pH 2.5), neutralized, and stored at 4 °C. The eluted fractions were tested for binding activity using indirect ELISA, and total protein was determined by the Lowry method. The purity of the IgG in the collected fractions was tested on SDS-PAGE under reducing conditions. The purest antibody fractions were pooled prior to buffer exchange to 0.1 M sodium phosphate, 0.15 M NaCl, and 0.1% sodium azide (pH 7.4), using a PD-10 column. In total 4.4 mg of IgG had been obtained from 10 mL of crude rabbit serum. Finally, the antibody stock solution was further concentrated using Vivaspin 6 concentrators with a 10000 molecular weight cutoff membrane (Vivascience, Hannover, Germany) and stored in aliquots containing 1 mg/mL IgG at −80 °C until use.

For covalent conjugation with biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (Sigma-Aldrich, Steinheim, Germany), the purified antibodies were dialyzed against 0.05 M carbonate-bicarbonate buffer (pH 9) overnight at 4 °C with a 3500 molecular weight cutoff membrane (Pierce, Rockford, IL) and afterward adjusted to 1 mg/mL. The biotin salt was then dissolved in deionized H_2O to a concentration of 1 mg/mL by adding 1 part of the biotin salt solution to 6.67 parts of the antibody solution. The mixture was vortexed and rotated at ambient temperature for 4 h and subsequently neutralized with 1 M NH_4Cl under rotation at RT for 10 min The total protein concentration of the biotinylated antitropomyosin was measured after a buffer change to 0.1 M sodium phosphate, 0.15 M NaCl, and 0.1% sodium azide (pH 7.4), using PD-10 columns, and the antibody was stored at −20 °C until use.

Extraction of Proteins and Food Sample Preparation. Retail food items for use as sample models during the method validation were obtained from local stores and seafood markets in Oslo, Norway. Cultures of house dust mite (*Dermatophagoides pteronyssinus*) and dried fruit mite (*Carpoglyphus lactis*) were purchased from Central Science Laboratory (CSL, York, U.K.), and samples of German cockroach (*Blattella germanica*) were received as a gift from Anticimex (Oslo, Norway). Prior to extraction with 0.1 M Tris and 0.5 M glycie (pH 8.7), the samples were homogenized in a mechanical blender (Retsch GmbH& Co., Haan, Germany). A portion of the homogenized samples (2 g) was added to 10 mL of the extraction buffer and extracted overnight at 45 °C in a shaking water bath (Grant Instruments, Cambridge, U.K.). The resulting extracts were centrifuged at 39200*g* for 25 min at 4 °C. The supernatants were removed and, if necessary, filtered through glass wool to remove fat and coarse particles from the matrix, and total protein concentrations were determined. The resulting supernatants were stored at −20 or 4 °C or were used freshly. Prior to analysis using the sandwich ELISA, extracts were diluted at least 1:20 in PBS (Oxoid, Basingstoke, U.K.) containing 1% bovine serum albumin (BSA).

Western Blotting. The NuPage Gel System (Invitrogen, Carlsbad, CA) was used for electrophoretic separation of protein samples by SDS-PAGE, in accordance with the manufacturer's instructions. All protein samples were applied in equal amounts (1 μg). Samples were prepared with lithium dodecyl sulfate (LDS) sample buffer and dithiothreitol (DTT) reducing agent (all from Invitrogen). Separation was performed under reducing conditions for 40 min at 200 V in 2-(*N*-morpholino-)ethanesulfonic acid (MES) SDS running buffer, using 4–12% Bis-Tris gels and SeeBluePlus2 prestained reference standard with a range

of 3–188 kDa. Proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane (Bio-Rad) for 60 min at 30 V with transfer buffer in an XCell II Blot Module (Invitrogen). Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.6) was used as washing buffer, and TBS-T containing 1% BSA was used as blocking and assay buffer for the Western blots. After blocking for 30–60 min, the blots were incubated at 4 °C overnight, with the unlabeled antitropomyosin (1 mg/mL) diluted 1:100000 in assay buffer. The blot was washed (3 × 15 min) and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat antirabbit secondary antibody (Zymed, San Francisco, CA) diluted 1:5000 in assay buffer. After washing (3 × 10 min), the membrane was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Zymed) until bands of satisfactory intensity appeared (2–10 min). All washing and incubation steps were performed under gentle shaking at ambient temperature.

Sandwich ELISA Procedure. Ninety-six-well flat-bottom polystyrene microtiter plates (Corning Inc., Corning, NY) were coated overnight at 4 °C with 100 μL/well of purified rabbit antitropomyosin polyclonal antibody diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) (Sigma-Aldrich) to 2.5 μg/mL. Then, the plates were washed with PBS containing 0.05% Tween-20 (PBS-T, pH 7.6) three times, using a programmable automatic plate washer (Skatron Instruments, Lier, Norway). All consecutive washing steps performed between each operation in the tropomyosin sandwich ELISA were the same. PBS containing 1% BSA was used as blocking and assay buffer for the ELISA. All dilutions, except for the initial coating step, were performed in assay buffer, and the plates were sealed with plate-sealing film during the incubation. Unsaturated binding sites on the polystyrene surface of the microtiter plate were blocked by incubation with 250 μL/well of the blocking/assay buffer for 1 h at RT. After washing, a 2-fold serial dilution of the previously described tropomyosin standard was added in the concentration range from 0.38 to 390 ng/mL. Incubation of standards, buffer blanks, and sample extracts, at a minimum dilution of 1:20, was performed in triplicate on each plate, for 1 h at RT using an electronic plate shaker (IKA-Werke, GmbH& Co. KG, Staufen, Germany) at low speed. After washing, bound tropomyosin was detected by adding 100 μL/well of biotinylated rabbit antitropomyosin antibody diluted 1:100000 and incubated for 1 h at RT under gentle shaking. Following the next wash, the plates were incubated for 1 h at RT with 100 μL/well of HRP-streptavidin conjugate (Zymed) at 1:5000 dilution. After a final wash, each well was incubated with 75 μL of K-Blue TMB substrate (Neogen, Lexington, KY). After 20 min, the color development was stopped by the addition of 50 μL/well of 2 M H_2SO_4. Absorbance was read at 450 nm using a 1420 VICTOR (*2*) multilabel plate counter (Wallac, Turku, Finland).

Assay Validation: Specificity, Accuracy, Precision, Limit of Detection, and Limit of Quantitation. The in-house validation of the tropomyosin sandwich ELISA generally followed the recommendations in the "Harmonized guidelines for single-laboratory validation of methods of analysis" (*24*). The specificity of the assay was evaluated through cross-reactivity studies using extracts of different seafood species and some potentially food-contaminating arthropods. The extracts had been prepared as described previously. Extracts from shrimp (*Pandalus borealis*), edible crab (*Cancer pagurus*), blue mussel (*Mytilus edulis*), king scallop (*Pecten maximus*), giant tiger prawn (*Penaeus monodon*), common cockle (*Cerastoderma edule*), European brown snail (*Helix aspersa aspersa*), mackerel (*Scomber scombrus*), Atlantic herring (*Clupea harengus*), plaice (*Pleuronectes platessa*), bluefin tuna (*Thunnus thynnus*), European eel (*Anguilla anguilla*), pollock (*Pollachius virens*), halibut (*Hippoglossus hippoglossus*), Atlantic wolffish (*Anarhichas lupus*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), Norway lobster (*Nephrops norvegicus*), American lobster (*Hommarus americanus*), German cockroach (*Blattella germanica*), squid (*Loligo vulgaris*), European house dust mite (*Dermatophagoides pteronyssinus*), and dried fruit mite (*Carpoglyphus lactis*) were all diluted 1:20 in ELISA assay buffer, which corresponds to a portion of 100% in a typical food matrix, prior to the ELISA analysis.

The accuracy of the method was assessed by performing recovery studies. Five different tropomyosin-free food items (surimi, breaded codfish, fish cakes, fish sauce, and mayonnaise) were homogenized as described earlier and spiked with the tropomyosin standard at levels of 1, 10, and 100 μg of tropomyosin/g of sample in a total volume of 5 mL of extraction buffer. After vortexing and 15 min of incubation at RT, an additional portion of 5 mL of extraction buffer was added, and the extraction procedure was performed as previously described. For the evaluation of recovery rates, extractions were performed in triplicate, the resulting extracts were analyzed by the tropomyosin sandwich ELISA, and the mean values for the recoveries and the standard error of the mean were calculated.

The precision of the method was evaluated as within (intra-assay) and between (interassay) assays, by using extracts from three different food items (breaded codfish, fish sauce, and fish cake) containing either different levels of crustaceans after spiking with shrimp meat and tropomyosin standard or crustaceans as an ingredient. After extraction, the samples were stored in aliquots at −20 °C, and each analysis was performed with a freshly thawed aliquot. The intra-assay precision was determined as the mean coefficient of variation (CV) of 7 replicates in one assay. The interassay precision was calculated as the mean coefficient of variation (CV) on the basis of triplicate analyses on 12 different days.

The limit of detection (LOD) of the tropomyosin sandwich ELISA was initially calculated as the mean of the measured content of 15 buffer blank samples plus 3 times the standard deviation (SD) of the mean value. In later studies, using five tropomyosin-free food matrixes (surimi, fish sauce, breaded codfish, fish cakes, and mayonnaise) diluted 1:20 in assay buffer, the LOD was assessed similarly and on the basis of 11 experiments and considering the sample dilution in the assay. The limit of quantitation (LOQ) was calculated as the mean of the measured content of 15 buffer blank samples plus 10 times the standard deviation (SD) of the mean value.

Tropomyosin Sequence Alignment. Publicly available amino acid sequences of tropomyosin from different species of shellfish, mollusks, and insects were obtained from the bioinformatics search engine Entrez at NCBI (www.ncbi.nlm.nih.gov/entrez) and compared to elucidate the theoretical basis of cross-reactivity. Amino acid sequences were aligned, and previously studied allergenic shrimp tropomyosin epitopes were highlighted (*12*, *25*). The following species were included in this comparison (scientific name and accession number in parentheses): shrimp (*Penaeus aztecus*, AAZ76743), German cockroach (*Blatella germanica*, AAF72534), American lobster (*Hommarus americanus*, AAC48288), crab (*Charybdis feriatus*, Q9N2R3), spiny lobster (*Panulirus stimpsoni*, O61379), common octopus (*Octopus vulgaris*, BAE54433), blue mussel (*Mytilus edulis*, AAA82259), brown snail (*Helix aspersa*, O97192), and house dust mite (*Dermatophagoides pteronyssinus*, AAB69424).

RESULTS

Detection of Tropomyosins. The protein-binding characteristics of the polyclonal antitropomyosin antibody were determined by Western blot analysis, using the tropomyosin standard, a crude extract from *P. borealis*, and extracts from several seafoods and insects (**Figure 1**). In the tropomyosin standard (lane 2), one protein band of major intensity at approximately 38 kDa was detected; two additional bands at 6 and 17 kDa showed considerably weaker IgG binding. The protein pattern detected in the crude shrimp extract (lane 13) was similar to that of the purified tropomyosin standard; however, additional proteins with approximately 14, 20, and 32 kDa were detected. In the other arthropod, mollusk, and fish extracts tested, the antitropomyosin antibody detected potential tropomyosin bands at molecular masses ranging from about 35 to 45 kDa. For three extracts of common cockle, Atlantic cod, and German cockroach, no visible bands were detected.

Sandwich ELISA Standard Curve. The tropomyosin standard was used to produce a standard curve with concentrations ranging from 0.38 to 390 ng/mL. The working range of the assay was defined as the linear part of the curve with a correlation coefficient of (R (*2*)) > 0.99 when linear regression

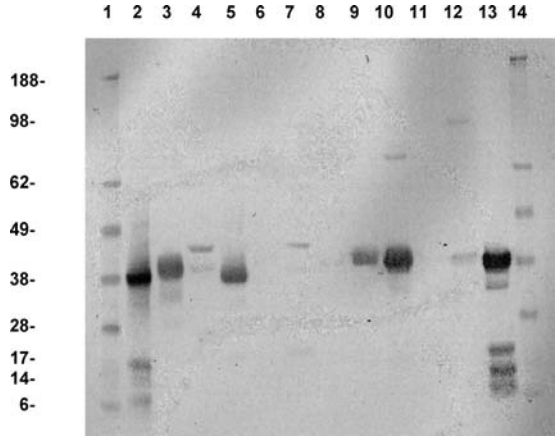

Figure 1. Western blot analysis of various potential cross-reacting species of seafood and insects using purified polyclonal antitropomyosin antibody: lanes 1 and 14, molecular weight marker, protein sizes (kDa) are indicated on the left side of the gel; lane 2, tropomyosin standard; lane 3, edible crab; lane 4, king scallop; lane 5, giant tiger prawn; lane 6, common cockle; lane 7, European eel; lane 8, Atlantic cod; lane 9, Norway lobster; lane 10, American lobster; lane 11, German cockroach; lane 12, squid; lane 13, shrimp extract.

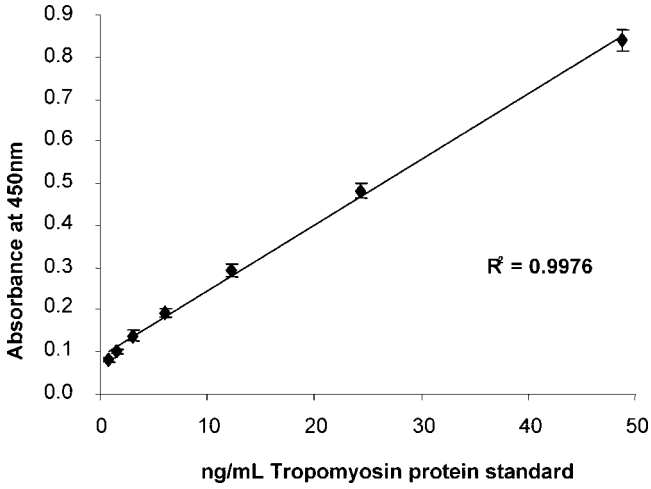

Figure 2. Representative linear seven-point calibration curve obtained using the tropomyosin standard in the sandwich ELISA. The curve shows the average values of triplicate measurements, the standard deviation in each point of the curve, and the linear regression curve and coefficient R^2.

was applied. A seven-point calibration curve typically ranged from 0.38 to 49 ng/mL (**Figure 2**). For the evaluation of possible matrix effects, standard curves were prepared from different dilutions of blank extracts of surimi, breaded codfish, fish cakes, fish sauce, and mayonnaise. The obtained results were compared to those of the standard curve prepared in the assay buffer. Undiluted matrix extracts seemed to suppress the read-off signal and, at the same time, increased the background noise at low concentrations so that the signal-to-noise ratio worsened considerably. However, at a 1:20 dilution no difference to the buffer standard curve was observed (data not shown). Consequently, all food samples were prediluted at least 1:20. For the quantitation of tropomyosin concentrations in food items, serial dilutions of extracts were performed if necessary, typically with unknown sample material. Dilutions having optical density (OD) values ranging in the mid area of the linear part of the standard curve were preferably used to calculate the tropomyosin concentrations.

Table 1. Cross-Reactivity of Selected Species of Crustaceans, Insects, Mollusks, Fish, Arachnids, and Other Animals in the Tropomyosin Sandwich ELISA

food ingredient[a]	total protein[b] (mg/mL)	equivalent tropomyosin (μg/g)	response in ELISA[c]
German cockroach	15.0	15.5	1.03
American lobster	0.8	7.7	9.6
giant tiger prawn	4.7	7.7	1.6
edible crab	3.7	6.6	1.8
Norway lobster	1.5	6.1	4.1
Atlantic cod	2.0	0.9	<0.45
squid	6.0	0.7	<0.12
common cockle	3.0	0.7	<0.23
European eel	2.9	<0.2	<0.07
blue mussel	4.3	<0.2	<0.05
king scallop	4.8	<0.2	<0.04
European brown snail	4.2	<0.2	<0.05
mackerel	4.3	<0.2	<0.05
herring	3.0	<0.2	<0.07
plaice	3.0	<0.2	<0.07
bluefin tuna	4.6	<0.2	<0.04
pollock	1.2	<0.2	<0.17
halibut	3.0	<0.2	<0.07
Atlantic wolffish	1.0	<0.2	<0.20
Atlantic salmon	3.0	<0.2	<0.07
house dust mite	25.0	<0.2	<0.008
dried fruit mite	31.0	<0.2	<0.006
chicken	11.3	<0.2	<0.02
cattle	9.1	<0.2	<0.02
pig	9.8	<0.2	<0.02

[a] All food ingredients were extracted 1:5 (w/v) as described under Materials and Methods and diluted 2-fold starting at 1:20 (minimum dilution in ELISA). [b] Values were obtained using the Lowry protein determination method. [c] Responses were calculated as ratios of measured tropomyosin equivalents and total protein concentrations.

Specificity. An initial cross-reactivity survey with typical food ingredients, using a nonoptimized competitive ELISA, was performed with extracts from hazelnut, peanut, Brazil nut, almond, walnut, cashew, pea, chickpea, pine nut, lentil, lupin, soy, casein, ovalbumin, ovomucoid, salmon, pollock, wolffish, and cod. None of the above-mentioned extracts with concentrations of total protein concentrations up to 40 μg/mL showed any inhibition, whereas the tropomyosin standard at the same concentration showed total inhibition (data not shown).

A further investigation of potentially cross-reacting species was performed with the tropomyosin sandwich ELISA, using extracts from different species of crustaceans, mollusks, fish, insects, arachnids, birds, and mammals under real sample conditions (**Table 1**). None of the 1:20 diluted protein extracts gave rise to ELISA results exceeding a concentration equivalent to 0.2 μg/g of the tropomyosin standard, with the exception of Atlantic cod, squid, and common cockle. These produced minor ELISA signals corresponding to tropomyosin levels of, respectively, 0.7 and 0.9 μg/g. An especially concentrated sample of whole body extract of German cockroach was run in the tropomyosin ELISA, showing considerable cross-reactivity. However, the same extract did not produce visible bands in Western blot analysis (**Figure 1**), and on SDS-PAGE only weak protein bands >50 kDa were produced (data not shown). In contrast, crude shrimp extract led to almost the same response in the tropomyosin ELISA as the standard protein. Other crustaceans such as lobster, giant tiger prawn, crab, and Norway lobster in the tropomyosin ELISA revealed, as expected, considerable reactivities corresponding to about 6–7 μg/g of tropomyosin protein. Western blot analysis (**Figure 1**) demonstrated similar binding patterns as for the shrimp tropomyosin standard and the crude shrimp extract. With the aim of

Table 2. Recovery of Tropomyosin from Various Blank Food Samples Spiked with 1, 10, or 100 μg/g of Tropomyosin Standard

	recovery (%) at indicated amountof tropomyosin standard		
blank food matrix	1 μg/g	10 μg/g	100 μg/g
surimi	77 ± 7	66 ± 3	88 ± 4
breaded codfish	73 ± 5	68 ± 2	83 ± 8
fish cake	63 ± 4	74 ± 3	87 ± 5
fish sauce	94 ± 12	79 ± 6	86 ± 6
mayonnaise	112 ± 8	102 ± 8	120 ± 15

[a] Values represent the average of three spiking experiments and are reported as mean ± standard error of the mean (SEM).

Table 3. Intra and Interassay Variances Determined for the Tropomyosin Sandwich ELISA Using Foods Containing Tropomyosin as an Ingredient or after Spiking with Shrimp Meat and Tropomyosin[a]

blank food	tropomyosin (mean, μg/g)	intra-assay variance (%CV, $n = 7$)	interassay variance (%CV, $n = 12$)
breaded codfish	6	6	14
fish sauce	464	6	9
fish cake	2717	5	13

[a] The breaded codfish were spiked with tropomyosin standard, the fish sauce contained crustaceans as an ingredient, and the fish cake was spiked with shrimp meat. [b] The intra-assay variances were calculated from 7 replicates of the same extract, and the interassay variances were calculated from triplicate analyses of the same extracts on 12 different days.

normalizing the different protein contents in the different extracts, the individual responses in the ELISA were calculated as ratios of measured tropomyosin equivalents and total protein concentrations. Using this approach, the American lobster produced the most intense signal, reaching a value of 9.6, Norway lobster obtained a value of 4.1, crab of 1.8, and giant tiger prawn of 1.6. From the noncrustacean species, German cockroach reached a response value of 1.03, common cockle of 0.23, squid of 0.12, blue mussel of 0.05, brown snail of 0.05, and house dust mite of 0.008. The cross-reactivity of Atlantic cod was 0.45.

Accuracy. Recovery studies were performed to assess the accuracy of the sandwich ELISA. Five blank commercial food items, surimi, breaded codfish, fish cake, fish sauce, and mayonnaise, spiked with three different amounts of tropomyosin standard before sample extraction were used to determine the recovery rates (**Table 2**.). The recoveries of spiked products ranged between 63 and 94% for surimi, breaded codfish, fish cake, and fish sauce, whereas for mayonnaise the recovery was between 102 and 120%. The slight differences seen in the recovery ranges between low and high levels of tropomyosin concentration were in the range of the standard errors and therefore rated as not reliable. All blank matrix extracts without tropomyosin gave results below the LOD at 0.2 μg/g.

Precision. An assessment of the intra and interassay precision for the tropomyosin sandwich ELISA was performed by analyzing three representative food items containing tropomyosin at different concentration levels, that is, 5–2700 μg/g, intrinsically or after spiking with either shrimp meat or tropomyosin standard (**Table 3**). The intra-assay precision, expressed as the coefficient of variation (%CV), was 6% in breaded codfish, 6% in fish sauce, and 5% in fish cake. Interassay precision was 14% in breaded codfish, 9% in fish sauce, and 13% in fish cake. Hence, this indicates that the precision was independent of the tropomyosin concentration.

Limit of Detection and Limit of Quantitation. The LOD of the ELISA corresponded to 0.002 μg/mL tropomyosin standard, equivalent to 0.2 μg/g of food, considering the sample dilution in the assay. The LOQ was 0.003 μg/mL tropomyosin standard in the assay and 0.3 μg/g of tropomyosin in food. Additionally, the LOD was assessed in five different blank food matrixes resulting in varying LODs in the different matrixes. In mayonnaise, the LOD was 0.2 μg/g, in fish sauce, breaded codfish, and fish cake, the LOD was 0.3 μg/g, and in surimi the LOD was 0.9 μg/g.

Tropomyosin Sequence Alignment. Tropomyosins from different crustacean, mollusk, insect, and arthropod species were compared by amino acid sequence alignment, using data published in the library of the National Center for Biotechnology Information (NCBI). When species from the Nordic environment were not found in the reference sequence database, near taxonomic relatives were used (**Figure 3**). The homology rate among the tropomyosins analyzed is high. When 106 amino acids of the five potential allergenic epitopes in shrimp tropomyosin (Pen a 1) are used for correlation, the lobster tropomyosin Hom a 1 had the greatest congruity with 100%, followed by spiny lobster tropomyosin Pan s 1 with 99%. Other tropomyosins with relatively good homology were Der p 1 from house dust mite with 86% and BLAGE from German cockroach with 84%, whereas the tropomyosins from octopus with 76%, Cha f 1 from crab with 75%, and Hel as 1 from brown snail with 74% were less congruent to Pen a 1. The least homologous tropomyosin was from mussel, with only 60%.

DISCUSSION

Food allergy is a considerable health problem, and the occurrence of allergic reactions to food has constantly increased in recent years. Presently, strict avoidance of the offending food is the only possible treatment. Contamination of food is probably the main cause of ingestion of hidden allergens by allergic individuals. Routines for food manufacturing and hygiene practices vary. Hidden allergens can make their way into food products via unintended routes, such as improper cleanup procedures, shared equipment and production lines, and cross-contamination by dust or by food remains in the processing system, or simply by accidental introduction during production (*26*). In the present study, a sensitive and specific sandwich ELISA for the detection of tropomyosin crustaceans in foods was developed and validated with five representative food matrixes. Using this ELISA method, extractable tropomyosins were successfully detected and quantitated from both spiked samples and commercially available food products.

The novel sandwich ELISA presented in this study was constructed using an affinity-purified polyclonal antishrimp tropomyosin antibody as capture antibody and a biotin conjugate of the same antibody for detection. Although the use of monoclonal antibodies could potentially have given higher specificity, a polyclonal antibody has the advantage to recognize various epitopes on different proteins. This versatility was considered to be favorable for designing an ELISA assay for the purpose of detecting crustacean tropomyosin in foods. On the one hand, the chance of spotting tropomyosins from different species was increased, and, on the other hand, possible alterations in the tropomyosin structure caused by different food-processing techniques, destroying specific epitopes or exposing formerly hidden ones due to protein unfolding, would be less likely to have an effect on the assay results (*27, 28*).

The specificity of the antitropomyosin antibody was tested in ELISA as well as Western blot, using extracts from different crustaceans, mollusks, fish, mites, cockroach, and additionally one avian and two mammal species. The test conditions used

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Pen a1     1 mdaikkkmqa mklekdnamd radtleqqnk eannraekse eevhnlqkrm qqlendldqv
BLAGE      1 ********** ********** **llc***ar d**i****a* **ars***ki **i*******t
Hom a1     1 ********** ********** ********** ***i****** ********* **********
Cha f1     1 ********** ********** ********** ***l****t* **irat**k* **v**e***a
Pan s1     1            ********** ********** ***i****a* ********* **********
Octopus    1 ********l* **m*relat* k*eqtd*klr dtednkn*l* *dltt***kf sn****f*na
Mussel     1 ********v* **m**e**l* **eq***klr *teeaka*i* ddyns***ks i*t*****nt
Hel as1    1 ********l* **m**e**l* **eqv**klr dcecnkn*v* *dln****kf ai****f*si
Der p10    1 *e******** ********i* **eia**kar d**l****** ***ra***ki **i**e****

Pen a1    61 qesllkaniq lvekdkalsn aegevaalnr riqlleedle rseerlntat tklaeasqaa
BLAGE     61 m*q*mqv*ak *d******q* **s******* ********** ******a*** a*********
Hom a1    61 ********t* *e******** ********** ********** ********** **********
Cha f1    61 **q*sa**tk *d**e***q* ********** ****p***** ********** **********
Pan s1    61 ********t* *e******** ********** ********** ********** **********
Octopus   61 k*q*ae**qk *etse*rvge c*s*i*g*** ********** ******s**q ***d***k**
Mussel    61 *tq*qdvqak yette*qiae h***iqs*t* k*sm****im k****yt**a s**e***k**
Hel as1   61 n*q**d**tk *ease*knae i*s*t*g*q* ********** ******qs** e**e***k**
Der p10   61 **q*sa**tk *e**e***qt ***d****** ****i***** ******ki** a**e****s*

Pen a1   121 desermrkvl enrslsdeer mdalenqlke arflaeeadr kydevarkla mveadlerae
BLAGE    121 *****a**i* *skg*a**** ********** ***m*****k ********** **********
Hom a1   121 ********** ********** ********** ********** ********** **********
Cha f1   121 ********** ********** ********** ********** ********** **********
Pan s1   121 ********** ********** ********** ********** ********** **********
Octopus  121 *****g**** ****qg**** i*l**k**e* *kwi**d*** *f**a***** it*v******
Mussel   121 *****n**** **lncgnd** i*q**k**t* *kwi*****k **e*a***** it*v******
Hel as1  121 *****g**** *s***a*d** l*g**a**** *kyi**d*e* *f**a***** it*v******
Der p10  121 ********m* *h**it**** *eg******* **mm**d*** ********** **********

Pen a1   181 eraetgeski veleeelrvv gnnlkslevs eekanqreea ykeqiktltn klkaaearae
BLAGE    181 ********** ********** ********** *****l***e **q*****nt r**e******
Hom a1   181 ********** ********** ********** ********** ********a* **********
Cha f1   181 ****s***** ********** ********** *********t ********a* **********
Pan s1   181 *********f ********** ********** ********** ********** **********
Octopus  181 a*l*aa*a** *******k** ***m****i* *qe*s***ds *e*t*rd**h r**e**n**a
Mussel   181 a*l*aa*a*v id***q*t** **in*t*q*q ndq*s***ds *e*t*rd*** r**d**n**t
Hel as1  181 a*l*aa*a** l******k** ***m****i* *qe*s***ds *e*t*rd**q r**d**n**s
Der p10  181 ********** ********** ********** ****q***** heq**rim*t ***e******

Pen a1   241 faersvgklq kevdrledel vnekekyksi tdeldqtfse lsgy
BLAGE    241 ********** ********** *h******y* c*d**m**t* *i*n
Hom a1   241 ********** ********** ********** ********** ****
Cha f1   241 ********** ********** ****
Pan s1   241 ********** ********** ********** ********** ****
Octopus  241 e***t*s*** ********** la***r**a* s*******a* *a**
Mussel   241 e***t*s**r ********** lt******a* s****a**a* la**
Hel as1  241 e***t*s*** ********** la***r**at s****s**a* *a**
Der p10  241 ********** ********** *h******** s*******a* *t**
```

Figure 3. Comparison of potentially cross-reacting and allergenic tropomyosins by alignment of amino acid sequences: *Penaeus aztecus* (shrimp), Pen a 1; *Blattella germanica* (German cockroach), BLAGE; *Hommarus americanus* (American lobster), Hom a 1; *Charybdis Pagurus (*crab), Cha f 1; *Panulirus stimpsoni* (spiny lobster), Pan s 1; *Octopus vulgaris* (common octopus), octopus; *Mytilus edulis* (blue mussel), mussel; *Helix aspersa* (brown snail), Hel as 1; *Dermatophagoides pteronyssinus* (house dust mite), Der p 1. Sequence homologies with Pen a 1 are marked with an asterisk, and binding epitopes previously described by Ayuso et al. (*25*) are underlined.

in the sandwich ELISA to assess the specificity of the assay were based on the protein concentrations of the raw food ingredients, thus clearly overestimating the real situation in commercial food products, which usually consist of a considerable number of mixed ingredients.

The aim of this study was to develop an assay for the general detection of crustacean protein traces in foods. As earlier described (*12*, *29*, *30*), cockroach is known to cross-react to a certain extent with crustaceans. However, the observed cross-reactions of cockroach in the ELISA were still considerably lower than for all the other crustaceans included in this study.

The binding patterns for the respective crustacean species obtained in Western blot analysis using the antitropomyosin antibody confirmed the ELISA data. Cross-reactions between different crustaceans have also been described by other authors and are most probably caused by highly conserved epitopes on homologous proteins in the different species (*10*, *11*, *31*). The clinical relevance of these cross-reactions is believed to be significant and has been demonstrated (*31*, *32*). The amino acid sequence alignment of some relevant tropomyosins performed in this study showed that lobster tropomyosins had the greatest homology within the five allergenic epitopes compared to the Pen a 1 shrimp tropomyosin, which was used to develop the ELISA method. Consistent with this theoretical data, we found that American lobster also produced the most intense signal on Western blot and had the highest response in the tropomyosin sandwich ELISA. Likewise, less homologous tropomyosins resulted in lower cross-reactivity responses in the ELISA and

weaker Western blot signals. For instance, the observed ELISA-based cross-reactivities to Atlantic cod, squid, and common cockle found in the ELISA could not be confirmed on the Western blot.

Cross-reactivity between shrimp and German cockroach has been demonstrated by Crespo et al. (*29*) in in vitro studies using serum from shrimp allergic patients. Additional studies (*15, 30*) support the suggestion of clinically significant cross-reactivity between arthropods such as cockroach and crustaceans. In the novel tropomyosin sandwich ELISA presented here, a concentrated crude whole body extract of German cockroach showed relatively high cross-reactivity, reaching absorbance values equivalent to 20–30% of the tropomyosin standard in a comparison of the titration profiles for the cockroach extract at corresponding concentrations. However, this could not be confirmed by Western blot analysis using the same antitropomyosin antibody, as no visible bands were observed. It has been suggested that the major allergen in German cockroach is most probably Bla g II, which has a molecular weight of approximately 36 kDa (*33*). An SDS-PAGE (data not shown) of the cockroach extract used in this study did not detect any protein bands in this region. The stability of the Bla g II allergen was discussed by Pollart et al. and Schou et al. (*34, 35*), who showed that degradation and loss of immune reactivity occurred at temperatures exceeding 56 °C and under reducing conditions, as, for example, under SDS-PAGE analysis. Considering the high OD values obtained during analysis of the cockroach extract, it seems unlikely that the observed response in the assay should be false, even though the analytical conditions were challenging due to the very concentrated sample. The relevance of cross-reactivities between tropomyosins from different arthropod species such as crustaceans and insects in regard to food analysis could be questioned, because for food manufacturing processes the batch-to-batch carry-over is a greater issue than contaminations with miscellaneous proteins at the production site because the general hygiene is generally well attended to.

The key parameters and characteristics of the tropomyosin sandwich ELISA were further evaluated and determined by an in-house validation. The accuracy of the ELISA method was studied in recovery experiments, adding tropomyosin standard at three different concentration levels to five different blank food matrixes. The recovery rates determined for surimi, breaded codfish, and fish cake were good although a bit too low, were relatively similar (63–88%), and showed generally low variances. In a more complex matrix, such as fish sauce, the variance was slightly higher, especially at the lowest spiking level. This might be due to lower sample homogeneity. In contrast to the other matrixes tested, the recovery rates in mayonnaise were >100% at all concentration levels, resulting possibly from the high fat content in the samples. The extraction procedure used for all samples in this method has not been fully validated so far, and it is possible that the recoveries achieved for some matrixes and concentration levels could be improved. However, the presented recoveries for the tropomyosin sandwich ELISA were considered to be satisfactory for the intended application of the method. At the moment, there are no available reference materials, proficiency tests, or collaboratively tested and standardized analytical methods that could be used for the evaluation of the real extraction efficiency and trueness of the analytical results in the tropomyosin sandwich ELISA.

The method precision was evaluated as repeatability (intra-assay variance) and reproducibility (interassay variance), by using extracts from three relevant and different foods containing different levels of tropomyosin. The tropomyosin contents detected ranged from 6 μg/g in breaded codfish to almost 3000 μg/g in fish cake. The repeatability of the sandwich ELISA was <6%, and the reproducibility was <14%. The method worked reliably also for the lowest concentration range tested and was shown to be robust with respect to minor changes.

The LOD and LOQ were evaluated on the basis of buffer blank samples and five blank food matrixes. The LOD determined in blank buffer was equivalent to 0.2 μg of tropomyosin/g of sample, and the corresponding LOQ was 0.3 μg/g; these parameters represent approximately the background noise in the assay. However, the detection of allergens in food products can be rather challenging due to potential matrix effects caused by various ingredients in multicomponent foods masking or increasing the analyte signal. Therefore, the LOD and LOQ of the tropomyosin sandwich ELISA were additionally determined under more realistic conditions in relevant food matrixes. The LODs obtained ranged from 0.2 μg/g in mayonnaise to 0.3 μg/g in fish sauce, breaded codfish, and fish cake and to 0.9 μg/g in surimi. The results obtained were used for a final evaluation and balancing of the technically feasible detection limits and the observed cross-reactivities in the assay against each other. We concluded that it was reasonable to operate with an LOD of 1 μg/g for commercial and routine analysis because this would reduce the probability of false-positive results and increase the reliability of the assay.

Although the sensitivity of the tropomyosin sandwich ELISA would be lower than 1 μg/g in most cases, this operative LOD is in agreement with the assay specifications considered to be necessary in general. As discussed by Poms et al. (*19*), the sensitivity finally required for methods in allergen analysis is still an open question, but at the moment there is a kind of consensus that LODs in food matrixes should be somewhere between 1 and 10 μg/g.

In conclusion, a novel sandwich ELISA for the detection of tropomyosin in crustaceans was successfully developed and validated with five different representative food matrixes. This is one of only a few quantitative methods for the detection of tropomyosin suited for food analysis. The tropomyosin sandwich ELISA could be used for the analysis of raw materials and end products and can be used for quality control during food production and for governmental food surveys.

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