

An enzyme-linked immunosorbent assay (ELISA) for the major crustacean allergen, tropomyosin, in food

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Abstract

Shellfish are a common cause of food reactions in hypersensitive individuals and are among the eight foods that account for over 90% of food allergies. At present, the only way to prevent these serious consequences of food allergies is to avoid the foods that trigger the reactions. A sandwich-ELISA kit has been developed for the detection of crustacean meat in food, based on the major heat-stable shellfish allergen, tropomyosin. Tropomyosin was purified from whole prawn (*Penaeus latissulcatus*) and used to immunize rabbits after confirming its identity by MALDI-TOF MS. A sandwich-ELISA based on the rabbit antibodies takes less than 2 h to perform, including the food extraction, and has a detection limit of 1 ppm crustacean (prawn, lobster), without detectable cross-reactivity with fish or mammalian meat.

Keywords: *Tropomyosin, ELISA, allergen, prawn, detection, food.*

Introduction

It is estimated that food allergy affects 1–2% of adults and 5–7% of children. Food allergy can be triggered by trace amounts of protein allergens in food and may result in life-threatening reactions, such as anaphylaxis. It is thought that the most common reason for allergic individuals coming into contact with a hidden allergen is through contaminated food (Steinman 1996). Organizations such as the US Food and Drug Administration have helped to improve labelling of food products and educate manufacturers about issues such as cross-contamination, but strict legislation is difficult to enforce. The availability of well-defined, commercially-available detection methods will mean that legislation will be easier to enforce and also may encourage manufacturers to adopt adequate decontamination methods instead of using the advisory “may contain” labels which prevent allergy sufferers from consuming foods that may actually be harmless.

Shellfish are a common cause of food reactions in hypersensitive individuals and are among the eight foods that account for over 90% of food allergies. In the US alone,

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more than 250,000 individuals are at risk of developing hypersensitivity responses upon ingestion of shellfish such as shrimp, lobster and crab (Leung & Chu 1998).

The major heat-stable shrimp allergen was first described by Hoffman et al. (1981) and later identified as tropomyosin by Shanti et al. (1993). The 34 kDa protein was capable of provoking IgE-mediated hypersensitivity after ingestion and had 86% homology with tropomyosin from *Drosophila melanogaster*. Following this experiment, another two groups confirmed that tropomyosin is the major heat-stable shrimp allergen by screening the sera of shrimp-allergic patients (Daul et al. 1994, Leung et al. 1994).

Using a similar approach, it was confirmed that tropomyosin was the major heat-stable allergen in other crustaceans, such as crab and lobster, and molluscs, such as mussel and squid (Hefle et al. 1995, Leung et al. 1996, 1998a, 1998b, Leung & Chu 2001).

Pre-absorption of shrimp-allergic patient's sera with recombinant shrimp tropomyosin appeared to remove IgE reactivity to the crustaceans and molluscs in the panel and so it was concluded that tropomyosin is the major heat-stable and cross-reactive allergen found in shellfish (Leung & Chu 1998).

Immunological-based techniques to identify allergens in food have been described including an enzyme-linked immunosorbent assay (ELISA) to detect the major peanut allergen *Ara h 1* in foods (Pomes et al. 1998). Whilst a monoclonal antibody-based ELISA has been described for the quantification of *Pen a 1* (Brown shrimp tropomyosin) in shellfish extracts, it was not validated for the detection of shellfish in cooked and raw food extracts (Jeoung et al. 1997). Here, we describe the development of an ELISA assay using antibodies against Western King prawn tropomyosin, to detect both raw and cooked shellfish in food, with no detectable cross-reactivity with fish. The assay has a sensitive detection limit of about 1 ppm and takes less than 2 h to perform.

Materials and methods

Purification of Western King prawn tropomyosin

Tropomyosin was partially purified according to the method of Spudich and Watt (1971). Uncooked, frozen Western King prawns (*Penaeus latissulcatus*) were homogenized. The homogenate was washed 4 times with low salt buffer (20 mM KCl, 1 mM KHCO₃, 0.1 mM CaCl₂ and 0.1 mM DTT) (two volumes) and filtered through four layers of fine muslin between each wash. The residue was then re-suspended in cold 95% ethanol (2 volumes), left to stand for 10 min and filtered as before. This was repeated twice and the filtrate was discarded. The residue was then washed twice with cold anhydrous diethyl ether (2 volumes) in the same way. The residue was extracted in an equal volume of high salt buffer (1M KCl, 25 mM Tris-acetate pH 8.0, 1 mM CaCl₂ and 1 mM DTT) overnight at 25°C. The solution was filtered as before and the filtrate was precipitated with ammonium sulphate (208 g/litre) at 4°C for 30 min before centrifugation at 10,000 g for 10 min. The precipitate was re-dissolved in 5 mM Tris-acetate pH 7.5 (1/2 volume) and dialyzed against the same buffer overnight at 4°C. The dialysed prawn extract was applied to a DE52 ion-exchange column (Whatman, UK) equilibrated in 50 mM Tris-acetate, pH 7.5, and eluted with a 150–350 mM NaCl gradient in the same buffer. Fractions containing tropomyosin were identified by SDS-PAGE. The identity of the purified prawn extract was

confirmed as tropomyosin by MALDI-TOF MS analysis of tryptic digests on a Voyager DE Pro (Applied Biosystems, Warrington, UK).

Immunization protocol

Two New Zealand White rabbits were immunized, one with purified prawn tropomyosin and a second with heat-treated purified prawn tropomyosin (100°C for 15 min). Both rabbits were given a subcutaneous injection with 200 µg immunizing antigen in 0.5 ml of complete Freund's adjuvant. Five boost injections followed at 14-day intervals using 200 µg immunizing antigen in 0.5 ml incomplete Freund's adjuvant.

Screening of the rabbit anti-tropomyosin antibodies by ELISA

The immunizing antigens (purified prawn tropomyosin and heat-treated purified prawn tropomyosin) were diluted to 5 µg/ml in PBS (4.7 mM Na₂HPO₄, 1.25 mM NaH₂PO₄, 150 mM NaCl, dH₂O). The diluted purified prawn tropomyosin was added to half of the microtitre plate (100 µl in each well) and the diluted heat-treated purified prawn tropomyosin was added to the remaining half of the microtitre plate (100 µl in each well). The microtitre plate was washed 3 times with PBS, post-coated with (300 µl per well) 1% Byco A/dH₂O (Croda Colloids, Cheshire) for 90 min at room temperature and dried overnight at 37°C. Rabbit antisera diluted in working wash (1.5M NaCl, 32 mM Tris base, 68 mM Tris-HCl, 40 mM Tween 80) were added to the wells of the coated microtitre plate (100 µl) for 10 min. The wells were washed 5 times with working wash and HRP-conjugated swine anti-rabbit Ig (1:300; DAKO A/S Denmark) was added (100 µl to each well) for 10 min. The wells were then washed 5 times with working wash solution and TMB "One-Step" substrate solution (DAKO A/S Denmark) was added (100 µl) and for 10 min. Finally, acid stop solution (25% H₃PO₄) was added (50 µl) for 10 min. The A₄₅₀ was determined using a Multiskan NS (Labsystems) plate reader. Statistical analysis was performed on the A₄₅₀ readings using INSTAT (Graphpad software, San Diego, USA).

Protein-A purification and biotinylation of the rabbit anti-tropomyosin antibodies

Rabbit antisera were purified on protein-A sepharose according to the manufacturer's recommendations (Sigma, Poole, UK). The protein-A purified antibodies were dialysed overnight against 30 mM NaH₂PO₄, 90 mM Na₂HPO₄, 1.5M NaCl, pH 7.2. Carbonate/bicarbonate buffer (0.2M Na₂CO₃ /0.2M KCl pH 8.8) was added (375 µl) to the rabbit anti-tropomyosin antibodies (5 mg). NHS-d-Biotin solution (50 µl of 16.7 mg/ml in DMSO) was added for 30 min at room temperature with gentle mixing. The biotinylated anti-tropomyosin antibodies were desalted on a PD10 column (Amersham Pharmacia Biotech, UK).

ELISA development

The protein-A purified rabbit anti-tropomyosin was diluted in PBS and microtitre plates were coated with it and post-coated according to the method described for the antibody testing. The optimal post-coat was found to be 10% lactitol, 3% peptone, 0.01% thiomersal in PBS. Purified prawn tropomyosin was diluted in working wash

and 100 μ l was added to each well for 15 min without shaking, followed by 5 washes with working wash. Biotinylated antibodies were tested at different dilutions (50 μ l to each well) and incubated for 15 min at room temperature (later changed to 30 min to increase sensitivity). The microtitre plate was washed 5 times with working wash solution, followed by the addition of streptavidin-peroxidase (50 μ l: Pierce, UK) and incubated without shaking at room temperature. The microtitre plate was washed 5 times and developed by the addition of TMB substrate solution (100 μ l) at room temperature for 15 min. The reaction was stopped by the addition of 25% $\text{H}_3\text{PO}_4/\text{dH}_2\text{O}$ (50 μ l).

Sample extraction procedure

A food sample (5 g) was extracted in 25 ml of extraction buffer (2M NaCl, 50 mM Tris-HCl, pH 8.0) and homogenized in a stomacher machine (Seward 400, Seward, Worthing, UK) for 2 min. The homogenized samples were centrifuged at 13000 g for 2 min and the supernatant was diluted to 1/10 before testing on the optimized ELISA.

Western blotting

A 12.5% polyacrylamide gel was electro-transferred overnight to a nitro-cellulose membrane which was then blocked with 4% skimmed milk powder in incubation buffer (0.1% Triton X-100 in PBS) for 1 h at room temperature on a rocker. The blot was then washed 3 times in PBS and incubated with primary antibody in dilution buffer (0.1% bovine serum albumin, 1% horse serum, 1% foetal calf serum in incubation buffer) for 1 h at room temperature on the rocker. The blot was washed 3 times using PBS before being developed by VECTASTAIN ABC kit (Vector Laboratories, Peterborough, UK) and biotinylated goat anti-rabbit Ig (1:500), according to the manufacturer's instructions. Finally, the blot was washed 3 times with PBS and developed with DAB substrate.

Results

Confirmation of antibody specificity

Figure 1 shows a total prawn protein extract, prepared by the ELISA sample preparation method and subjected to SDS-PAGE and western blotting with rabbit

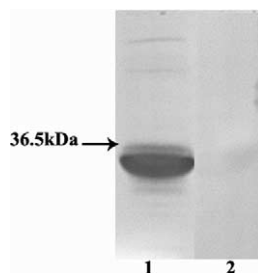


Figure 1. Specificity of rabbit antibodies for prawn tropomyosin. A Western blot of prawn extract incubated with anti-tropomyosin serum (lane 1) or pre-immune sera (lane 2) at 1/20,000 dilution. Pre-stained Mr markers (Sigma) included triosephosphate isomerase from rabbit muscle (36.5 kDa). A single major band consistent with the known Mr of tropomyosin was observed.

anti-tropomyosin antibodies. The antibodies recognize a clear band of about 34 kDa, the same size as the original immunogen. The immunogen band was electro-eluted from a separate Coomassie Blue-stained gel, cleaved with trypsin and the resulting peptides were analysed by MALDI-TOF mass spectrometry. The peptide masses were submitted to the SwissProt protein database using MASCOT software and searched against all known proteins. Crustacean tropomyosins were the top matches, with tropomyosin from the Greasy Back shrimp (*Met e 1*) (Swiss-Prot Q25456) being the top hit. A combination of overnight and 4 h trypsin digests enabled 83% sequence coverage of *Met e 1*, while the V8 protease peptide matches increased the coverage to 86%. Chymotryptic peptide matches enabled 100% coverage of the published amino acid sequence of *Met e 1* (data not shown).

ELISA development

There is strong evidence that tropomyosin is heat-stable (Shanti et al. 1993). In order to test this, and to see if the heat-treated tropomyosin cross-reacts with untreated tropomyosin in ELISA, each antigen was assayed for activity against dilutions of rabbit anti-tropomyosin and rabbit anti-heat-treated tropomyosin antisera between 1/10,000 and 1/100,000. Pre-immune sera were used as negative controls. The results are shown in Figure 2. The anti-tropomyosin antibodies reacted equally well with both tropomyosin and heat-treated tropomyosin (unpaired *t*-test *p* value 0.1436). The anti-heat-treated antibodies also reacted equally well with both tropomyosin and heat-treated tropomyosin (unpaired *t*-test *p* value 0.3067). From Figure 2, it appears that the reactivity of the anti-heat-treated tropomyosin antibodies against both antigens is higher than that of the anti-tropomyosin antibodies, though this could be due to a difference in immune response between the two rabbits. Heating tropomyosin at 100°C for 15 min clearly does not affect its immunological reactivity. Only the rabbit

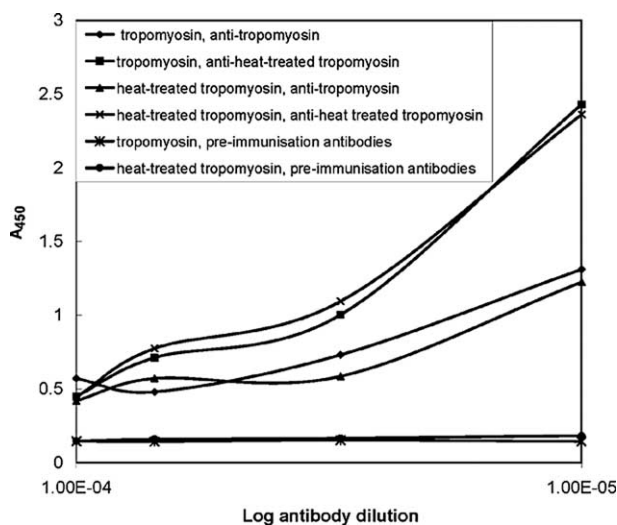


Figure 2. Effect of heat treatment of the tropomyosin antigen in a direct ELISA. Microtitre wells were coated with tropomyosin or heat-treated tropomyosin at 5 µg/ml. Anti-tropomyosin or anti-heat-treated tropomyosin antisera were used to detect the coated antigens.

antisera against untreated tropomyosin was used to develop the shellfish ELISA further.

To optimize the coating antibody concentration, a microtitre plate was coated with 5 µg/ml, 2 µg/ml or 1 µg/ml purified rabbit anti-tropomyosin antibodies. Tropomyosin at concentrations from 15,000 ng/ml to 75 ng/ml was used as a bridge in the sandwich ELISA. The sample diluent was used as a negative control. Bound tropomyosin was detected with biotinylated rabbit antibodies as described in the Methods section and the results are shown in Figure 3. The plates coated with 2 µg/ml rabbit anti-tropomyosin antibodies gave best signal-to-background and were chosen for future work.

To optimize the biotinylated antibody concentration, dilutions (1/100, 1/150 and 1/200) were tested against a range of tropomyosin standard concentrations (from 15–0.3 ng/ml). The sample diluent was used instead of tropomyosin as a negative control. The incubation time with the biotinylated antibodies was increased to 30 min to increase assay sensitivity. A graph of the A_{450} against tropomyosin dilutions is shown in Figure 4. The biotinylated antibody diluted 1/150 gave the best standard curve, with a low background to noise ratio. The longer incubation time of 30 min with the biotinylated antibodies was used as standard format for the following experiments.

Lowest limit of prawn detection

The limit of tropomyosin detection by ELISA was determined as 0.75 ng/ml for an A_{450} of 1.5 times the background control (data not shown). A positive A_{450} was seen when a raw prawn extract (1 g in 4 ml buffer) was further diluted to 1/1,000,000 (wet weight). Thus 1 µg/g, or 1 part per million (ppm), is the limit of detection (wet prawn). This corresponds to a dry prawn content of 0.2 ppm or less, since 80% of the prawn weight was lost by freeze drying overnight (data not shown).

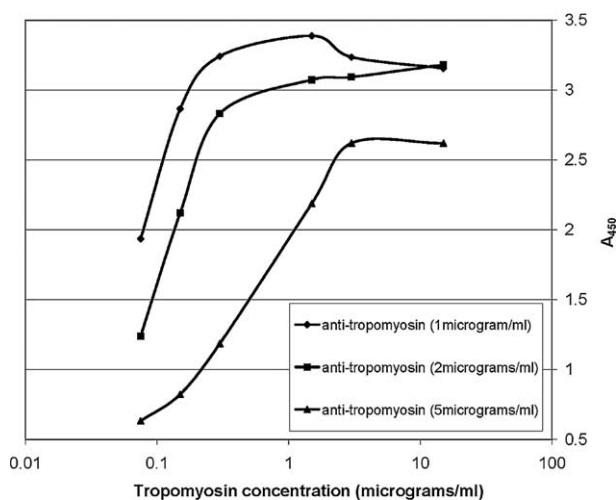


Figure 3. Optimization of coating antibody concentration in the sandwich ELISA. The tropomyosin concentration (µg/ml) is plotted on a log scale against the A_{450} obtained by ELISA for three different coating antibody concentrations. The mean A_{450} of the negative control was 0.086.

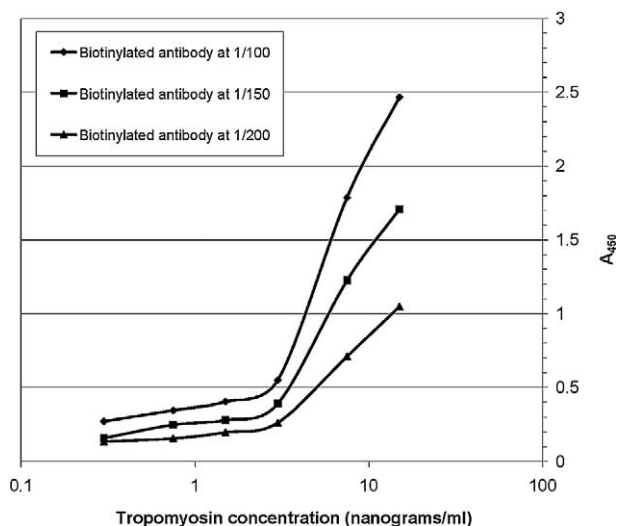


Figure 4. Optimization of biotinylated antibody concentration in the sandwich ELISA. The tropomyosin concentration (ng/ml) is plotted on a log scale against the A_{450} obtained by ELISA with three different biotinylated antibody dilutions. A_{450} of the experimental controls (sample diluent) were 0.161, 0.122 and 0.111 at biotinylated antibody dilutions of 1/100, 1/150 and 1/200 respectively.

Cross-reactivity of the rabbit anti-tropomyosin antibodies

Fish, shellfish and vertebrate meat samples were extracted by the same method for testing on the optimized ELISA. A purified tropomyosin standard curve from 0.1 $\mu\text{g/ml}$ to 0.4 ng/ml was included as a reference. The results with shellfish are shown in Figure 5.

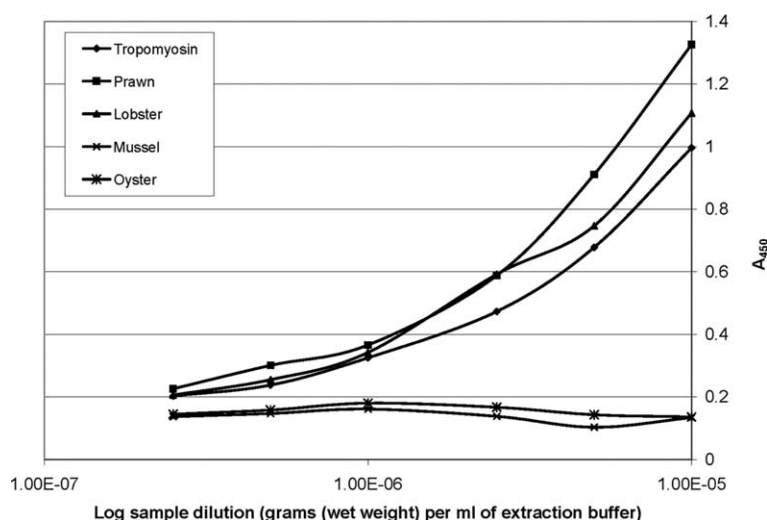


Figure 5. Cross-reaction of lobster, but not mussel or oyster, in the prawn sandwich ELISA. The shellfish sample dilutions (log scale) are plotted against the A_{450} obtained on the optimized ELISA. The average A_{450} of the experimental control (sample diluent) was $0.123 (\pm 0.011)$. The final sample dilution took into account the initial 1 in 5 extraction of the samples, e.g. shellfish (5 g wet weight) was extracted in extraction buffer (25 ml) followed by a 1/20,000 dilution; giving a final dilution of 1/100,000.

Other meat extracts including pork, beef, salmon, cod, mackerel and pollock showed no cross-reactivity at all in the present assay (Table II). The assay is specific for crustaceans, since the fish, vertebrate meat and mollusc extracts (mussel and oyster) did not react. The prawn and lobster extracts reacted similarly to the tropomyosin standard. This shows that common crustacean species are detectable in the assay at low levels.

Spike detection using the ELISA

A range of different food types were spiked with prawn to give a final prawn content of 7.5 ppm. The samples were then extracted by the same method as described for the cross-reactivity testing. Samples were also spiked with the same amount of prawn extract and cooked for 10 min at 100°C before extracting by the same method. The extract supernatants were diluted 1/10 before testing on the optimized shellfish ELISA, with a purified tropomyosin and prawn extract standard curve as a reference. The results are shown in Table I. On average, there was a 98% recovery of tropomyosin in the 10 food samples. The highest background (non-spiked food extract) was 1.45 times the experimental control (sample diluent). This result demonstrates that minor prawn contaminants in different foods are easily detected in this assay.

Tropomyosin was also measured in positive controls using two foods known to have prawn content; prawn crackers and prawn fishcakes (Sainsbury Supermarkets, UK). Compared with a whole prawn value of 0.32 mg/ml, prawn crackers were found to contain 0.06 mg/ml, which is consistent with their stated average prawn content of 17%. The prawn fishcakes had a content of 0.63 mg/ml which corresponds to nearly 200% compared with a stated value of 31% prawn content. However, the fishcakes also contained an additional unstated amount of “concentrated shellfish stock” which likely accounts for the very high measured value.

Discussion

A sandwich-ELISA to detect the major brown shrimp allergen, *Pen a 1* (tropomyosin), has been described previously (Jeoung et al. 1997). This ELISA used a monoclonal

Table I. Detection of tropomyosin in raw and cooked food samples spiked with prawn extract. The A_{450} obtained by ELISA with the spiked samples was compared to the A_{450} of the equivalent dilution of prawn extract alone. The A_{450} obtained from the non-spiked food controls were compared to the background control (sample diluent buffer only) which had an average A_{450} of 0.136 (± 0.009) (raw) or 0.139 (± 0.006) (cooked). For a positive result, the A_{450} has to be at least 1.5x that of the control.

Spiked food	Percentage spike recovery		Non-spiked control/ background control	
	Raw	Cooked	Raw	Cooked
Soy sauce	87%	76%	1.01	1.10
Tuna and sweetcorn spread	117%	143%	1.04	0.86
Pilau rice	76%	117%	1.01	1.08
Vegetable balti	86%	67%	1.07	0.97
Quiche	112%	41%	1.04	1.00
Thai crackers	140%	99%	1.45	1.00
Ocean pie	74%	84%	1.05	1.00
Lemon and dill sauce	85%	124%	1.04	1.06

Table II. Lack of detectable cross-reaction of mammalian, avian and fish extracts.

Sample	Ratio sample/control A ₄₅₀
Sheep	0.973
Cow	1.046
Pig	0.877
Chicken	1.095
Pollock	1.158
Cod	1.046
Coley	1.025

The A₄₅₀ obtained when the vertebrate and fish extracts were tested on the crustacean ELISA were divided by the A₄₅₀ obtained from the control (sample diluent buffer alone). A ratio of 1.5x the control is considered positive according to the ELISA specifications.

antibody and was designed for quantification of *Pen a 1* and standardization of shrimp extracts. The *Pen a 1* ELISA was found to be suitable for measuring the presence of *Pen a 1* like proteins in crustaceans other than the Brown Shrimp. Here, we have described the development of a polyclonal antibody-based sandwich ELISA that has been validated for the detection of crustaceans in both cooked and raw food. We chose to produce polyclonal antibodies against tropomyosin, rather than monoclonal antibodies, for two reasons. Whilst it is widely accepted that tropomyosin is a heat-stable crustacean allergen (Leung et al. 1996), some monoclonal antibodies may recognize conformational epitopes that are altered during the heating process. Secondly, the ELISA was designed to detect tropomyosin in all crustaceans and whilst tropomyosin has large regions of homology throughout crustacean species, minor sequence differences could restrict the range of species detected with a monoclonal antibody. This second problem did not arise in the ELISA described by Jeoung et al. (1997).

The main criteria of a commercial immunoassay include sensitivity, specificity and a short extraction and performance time. The assay described here has a sensitivity of about 1 ng/ml (1 ppm dry weight of prawn) and is performed in less than 2 h (including the sample extraction). In the *Pen a 1* ELISA (Jeoung et al. 1997), which took twice as long to perform, the sensitivity was the same (1 ng/ml detection limit of the *Pen a 1*). The assay we describe here appears to be specific for crustaceans and, despite reports that molluscs cross-react with shrimp allergens (Leung & Chu 1998), no cross-reactive epitopes were evident in this assay or in the *Pen a 1* ELISA of Jeoung et al. (1997). Sequence alignments show that *Met e 1* (tropomyosin from the Greasy Back Shrimp) has a 94–98% homology with other crustaceans. However, *Met e 1* only has a 54–67% homology with mollusc tropomyosin and a 51–57% homology with some vertebrate tropomyosin including human, mouse and chicken. The homology difference between crustaceans and molluscs may be sufficient to cause the lack of specificity seen when using antibodies raised against crustacean tropomyosin.

At present, the only way to prevent food allergies is to avoid foods that trigger the reactions. However, even the most diligent ingredient checkers will be inadvertently exposed to an allergen that will elicit a response at some point. Unlabelled allergens and misleading labels have caused concern to regulatory bodies such as the European Union (EU), which has resulted in an amendment to the directive 2003/89/EC, concerning food allergen labelling, to be fully implemented by the end of 2005.

This new legislation means that commercial interest in testing for allergens in food will be increased.

We have now described the development of a sensitive and specific immunoassay for the detection of crustacean tropomyosin in food. Whilst organizations such as the EU are helping to improve labelling of food and educate manufacturers about issues such as cross-contamination, strict legislation is difficult to enforce as allergen detection methods remain to be fully standardized. This ELISA has potential applications for food manufacturers to check cleaning efficiencies and for retailers to check food for the presence of hidden allergens.

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