

Quantification of the major brown shrimp allergen Pen a 1 (tropomyosin) by a monoclonal antibody-based sandwich ELISA

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Background: Among 13 allergens found in extracts of cooked brown shrimp (*Penaeus aztecus*) the 36 kd muscle protein tropomyosin has been identified as the only major shrimp allergen (Pen a 1). Cross-reacting molecules with similar molecular weights were detected in other crustacea species such as crab, lobster, and crawfish. Because Pen a 1 and Pen a 1-like allergens are important in crustacea allergy, the aim of this study was to develop a monoclonal antibody (mAb)-based sandwich ELISA to quantify Pen a 1 and to evaluate Pen a 1 levels in four commercial shrimp, crab, and lobster extracts. **Methods:** Two Pen a 1-specific mAbs with different epitope specificities were selected. ELISA plates coated with captured mAb 3.2 were incubated with samples containing Pen a 1. Bound Pen a 1 was detected by a combination of biotinylated mAb 4.9.5 and alkaline phosphatase-labeled streptavidin. **Results:** The optimized sandwich ELISA could detect Pen a 1 concentrations ranging from 4 to 125 ng/ml. Four commercial shrimp extracts demonstrated a 40-fold difference in Pen a 1 levels (24 to 920 µg/ml). Crab and lobster extracts contained detectable levels of Pen a 1-like proteins. No reactivity to cockroach, house dust mite, oyster, codfish, or peanut extracts was detected, which indicates that the developed assay is crustacea-specific.

Conclusion: A sensitive sandwich assay was developed to quantify Pen a 1. This assay will be helpful to standardize shrimp extracts in regard to the content of the major allergen, Pen a 1, and to study cross-reactivities among and evaluate occupational exposure to different crustacea species. (*J Allergy Clin Immunol* 1997;100:229-34.)

Key words: Food allergy, shrimp, crustacea, Pen a 1 monoclonal antibodies, sandwich ELISA, cross-reactivity, quantification

Shrimp is commonly identified as a cause of food hypersensitivity,¹⁻³ and ingestion of shrimp may cause severe allergic reactions such as anaphylactic shock.^{1,2} Furthermore, occupational allergic diseases in shrimp-processing factory workers have also been reported.⁴ Thirteen different allergens were identified in brown shrimp (*Penaeus aztecus*). The only major allergen (36

Abbreviations used

CV:	Coefficient of variation
mAb:	Monoclonal antibody
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBS:	Tris-buffered saline
TMC:	Tulane Medical Center

kd) that reacted to 82% of the subjects' IgE was designated as Pen a 1 according to accepted International Union of Immunological Societies (IUIS) allergen nomenclature.^{5,6} Amino acid sequence analysis of an internal Pen a 1 peptide identified this allergen as the muscle protein tropomyosin.^{7,8}

In general, crude allergen extracts are used for diagnosis and immunotherapy. These extracts are prepared from natural source materials and may contain complex mixtures of proteins of which only a few are allergenic. Because of the lack of a method to measure reproducible allergenic components of extracts, the potency of these extracts may vary from batch to batch.

One way to characterize allergen extracts is to measure their major allergen levels with monoclonal antibodies (mAbs). Allergen-specific mAbs have many advantages for allergen characterization, identification, and quantification^{9,10}; they possess a unique specificity and a high level of selectivity for a single epitope and can be produced in unlimited amounts. In this report we describe the development of an mAb-based, Pen a 1-specific sandwich ELISA; the use of this assay to measure Pen a 1 levels in four commercially available shrimp, crab, and lobster extracts; and the evaluation of crustacea cross-reactivity.

METHODS

Allergen extracts

The Tulane Medical Center (TMC) extract of brown shrimp (*Penaeus aztecus*) was prepared as described previously.^{6,7} Briefly, fresh shrimp were boiled for 15 minutes in ion-depleted water, peeled, and deveined. The meat was homogenized in phosphate-buffered saline. The mixture was stirred overnight at 4° C and centrifuged (15,000 g). The supernatant was concentrated (molecular weight cutoff, 1000 d), recentrifuged (15,000 g), dialyzed (molecular weight cutoff, 3500 d), and stored in 1 ml aliquots at -20° C. Commercial glycerinated (50%) shrimp,

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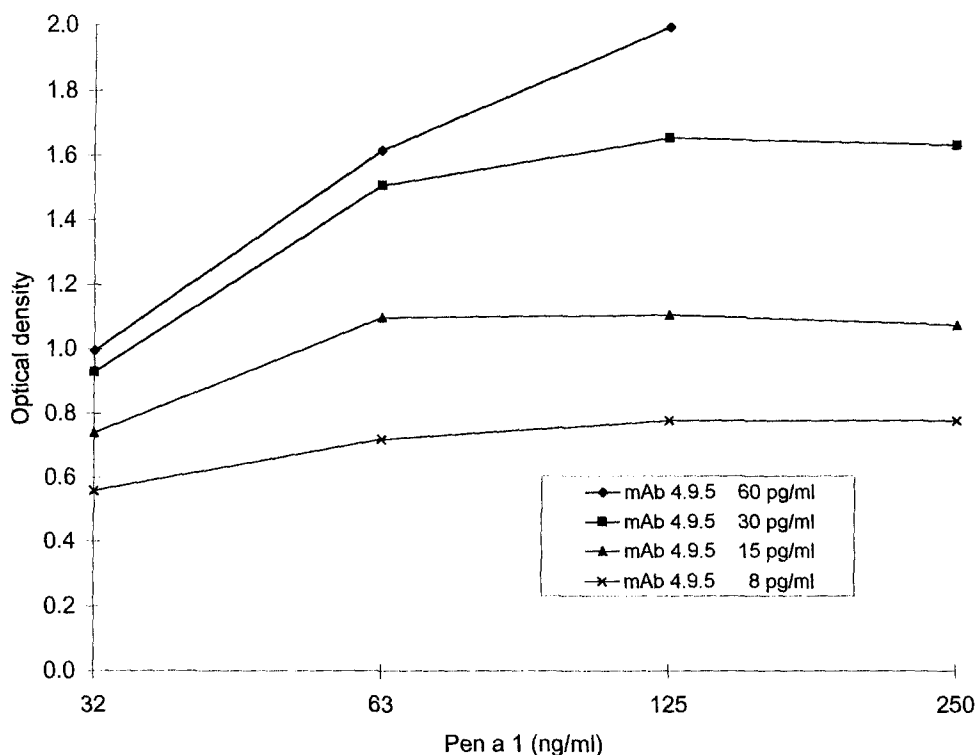


FIG. 1. Reactivity of serial dilution series of mAb 4.9.5 to serial dilutions of Pen a 1.

crab, and lobster extracts were obtained from ALK Laboratories, Inc. (Milford, Conn.), Center Laboratories, Inc. (Port Washington, N.Y.), Greer Laboratories (Lenoir, N.C.), and Bayer Pharmaceuticals (Spokane, Wash.). Cockroach, house dust mite, oyster, codfish, peanut, and buckwheat flour extracts were obtained from Center Laboratories, Inc.

Purification of Pen a 1 by preparative sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Pen a 1 was purified from crude brown shrimp extract by preparative SDS-PAGE (model 491 Prepcell, Bio-Rad).⁸ Briefly, 2 mg of crude shrimp extract was diluted with a 1:2 sample buffer that contained 2% sodium dodecylsulfate and separated by preparative SDS-PAGE (10.5%T, 1.5%C) according to the manufacturer's instructions. Eluted fractions were analyzed by SDS-PAGE and immunoblotting with a serum pool obtained from subjects allergic to shrimp. Pen a 1-containing fractions were pooled, dialyzed, and lyophilized.

Production of Pen a 1-specific mAbs

The production of Pen a 1-specific mAbs has been described previously.⁶ Briefly, 8- to 10-week-old female BALB/c mice were immunized twice with purified Pen a 1 during a 1-week interval; emulsified in complete Freund's adjuvant (cFA) and incomplete Freund's adjuvant (iFA), respectively; and boosted with shrimp extract 3 days before fusion. After 5 weeks, spleen cells were fused with P3X63Ag8.U1 myeloma cells (ATCC CRL 1597) by using the standard polyethylene glycol (PEG) fusion technique.¹¹ Growing hybridomas were screened by ELISA for production of specific antibodies, checked for specificity by immunoblotting, and cloned by limiting dilution. Two mAbs (3.2

and 4.9.5) were selected on the basis of their high affinity to Pen a 1 and their different Pen a 1 epitope specificities and injected into female BALB/c mice primed with Pristane (Sigma, St. Louis, Mo.). Samples of mAb-containing ascites were collected, and mAbs were purified by protein A/G affinity chromatography (Immunopure [A/G] IgG purification column; Pierce Chemical Co., Rockford, Ill.).¹²

Biotinylation of detector mAb 4.9.5

For biotinylation, mAb 4.9.5 (1 mg/ml of phosphate-buffered saline) was incubated with D-biotinyl- ϵ -aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS) (Boehringer-Mannheim) in dimethyl sulfoxide at a molar ratio of 1:400 for 2 hours at room temperature under gentle agitation. Unreacted biotin was removed by Sephadex G-25 chromatography (Boehringer-Mannheim).¹³

Optimization of biotinylated detector mAb 4.9.5

The biotinylated detector mAb 4.9.5 was titrated for optimum binding to Pen a 1. Twofold dilution series of Pen a 1 (32 to 250 ng/ml) were coated on the solid phase, followed by serial dilutions of biotinylated mAb 4.9.5 (7.5 to 60 pg/ml) and 200 pg/ml alkaline phosphatase-labeled streptavidin (Gibco-BRL).

Optimization of coating capture mAb 3.2

Concentrations of capture mAb 3.2 ranging from 5 to 20 μ g/ml were used to coat microtiter plates and incubated with fourfold dilution series of Pen a 1 (4 to 250 ng/ml). Bound Pen a 1 was detected with biotinylated mAb 4.9.5 (optimum concentration: 30 pg/ml) and alkaline phosphatase-labeled streptavidin (200 pg/ml).

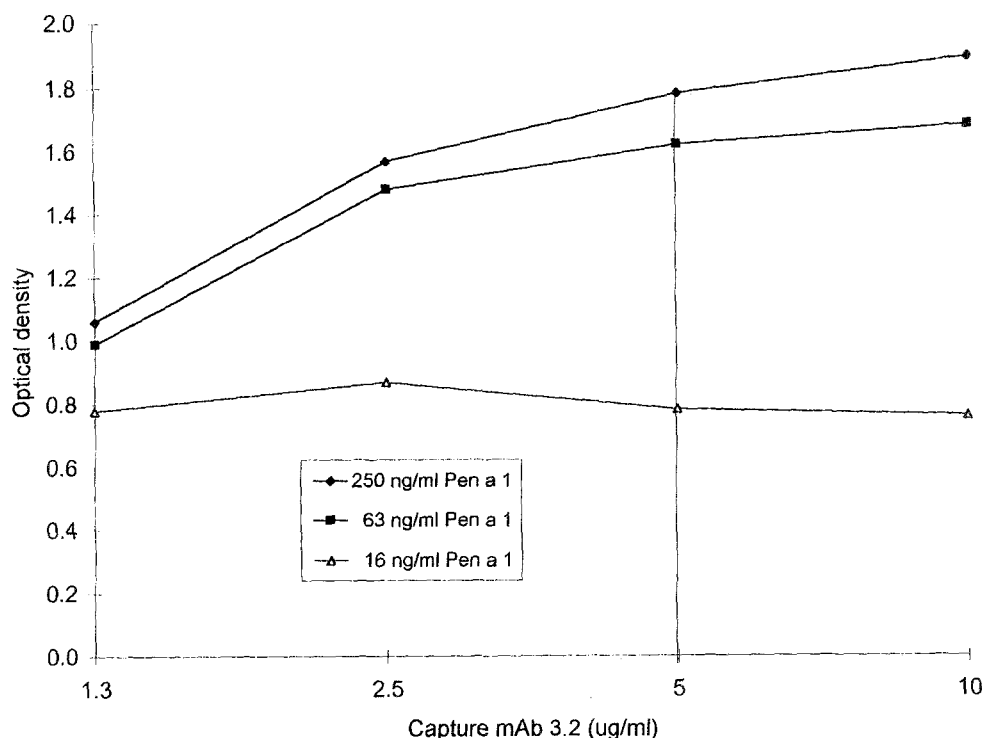


FIG. 2. Optimization of capture mAb 3.2. and reactivity of serial dilutions of purified Pen a 1 to serial diluted capture mAb 3.2. Optimal concentration of detector mAb (30 pg/ml) was used for detection.

Quantification of Pen a 1 by mAb-based sandwich ELISA

Quantification of Pen a 1 was achieved by mAb-based sandwich ELISA as follows. Microtiter plates (Falcon 3911) were coated with capture mAb 3.2 (5 μ g/ml, 100 μ l/well) in 50 mmol carbonate-bicarbonate buffer (pH 9.5) overnight at 4°C. Wells were blocked for 1 hour with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-Tween-20, pH 7.4) and 1% bovine serum albumin (fraction V, Sigma) and incubated for 1 hour at room temperature with 100 μ l of serially diluted (1 to 250 ng/ml) Pen a 1 in TBS-Tween-20 + 1% bovine serum albumin. After three washes with TBS-Tween-20, the wells were incubated for 1 hour at room temperature with 30 pg/ml of biotinylated detector mAb 4.9.5. Then 100 μ l of alkaline phosphatase-labeled streptavidin (200 pg/ml) was added to the wells and incubated for 1 hour at room temperature. After two washes with TBS-Tween-20 and one wash with substrate buffer (100 mmol Tris/HCl, 100 mmol NaCl, and 10 mmol MgCl₂), pH 9.5, the reaction was visualized by addition of substrate p-nitrophenyl phosphate (1 mg/ml), and the optical densities were measured after 45 minutes of incubation at 405 nm. A standard curve was generated with serial twofold dilutions (1 to 250 ng/ml) of purified Pen a 1. Crude shrimp extract and commercial allergen extracts were assayed as dilution series in duplicate. The concentrations of the samples were interpolated from the linear portion of the standard curve.

Evaluation of assay variation

To evaluate intraassay variation, 30 microtiter wells per ELISA plate were incubated with the same concentration of Pen a 1 with Pen a 1 concentrations ranging from 1 to 250 ng/ml. To determine interassay variations, defined wells on each single microtiter well were incubated with serial dilutions of

Pen a 1. This test was repeated 13 times. To quantify assay variations, the coefficient of variation (CV) was calculated. CV is defined as the standard deviation divided by the mean and multiplied by 100. CVs smaller than 20% were considered not to be significant. The ELISA plate-to-plate and day-to-day variations were also validated by testing two standard curves per day for 3 consecutive days. The results were evaluated by linear regression analysis.

RESULTS

Optimization of detector mAb 4.9.5 and capture mAb 3.2

Fig. 1 shows the optimization of biotinylated detector mAb 4.9.5. The 30 pg/ml detector mAb concentration was chosen as the optimal concentration because it yields high yet usable (≤ 2.0) optical densities over a wide range of Pen a 1 concentrations. Fig. 2 shows the reactivity of different coating concentrations of capture mAb 3.2 when serial dilutions of Pen a 1 and the optimal concentration of biotinylated mAb 4.9.5 were used for detection. The concentration of 5 μ g/ml capture mAb 3.2 was found to be optimal for coating because higher concentrations did not yield any significant increase of antibody binding.

Assay variation

Linear regression analysis was used to evaluate the ELISA plate-to-plate and day-to-day variations tested by two standard curves per day for 3 consecutive days. These analyses showed negligible assay variations between plates on a given day (Fig. 3). The correlation

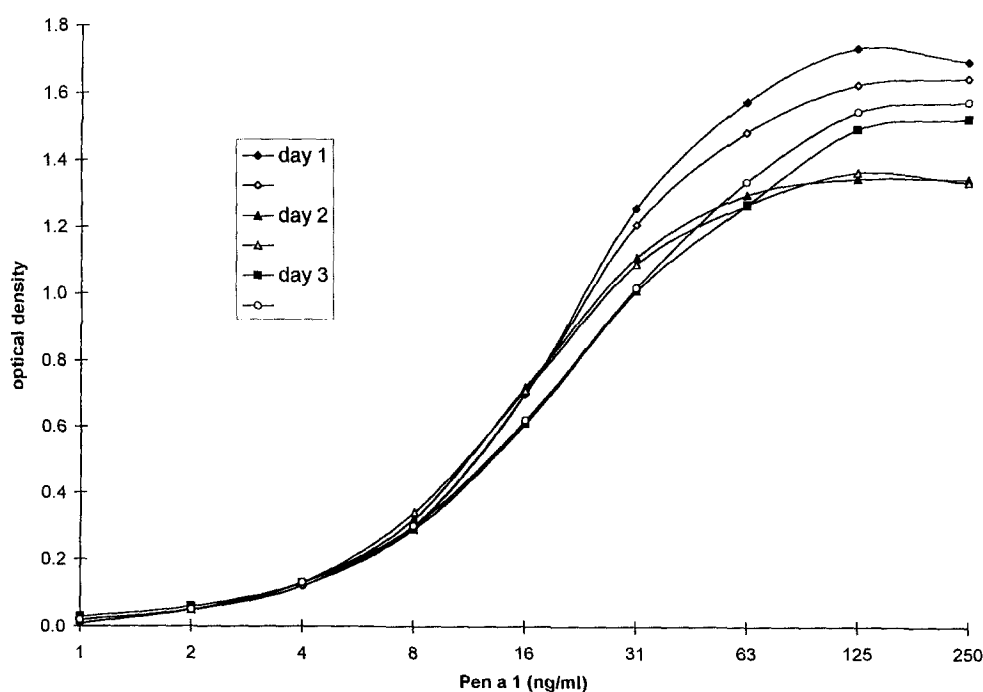


FIG. 3. Day-to-day and plate-to-plate assay variations. Two standard curves were tested on 3 consecutive days.

TABLE I. Evaluation of ELISA plate-to-plate and day-to-day variations

Pen a 1 (ng/ml)	CV between ELISA plates			
	Day 1	Day 2	Day 3	Day 1-3
250	7.1	2.2	2.6	9.0
125	8.8	3.5	2.8	10.2
63	5.5	5.0	6.1	10.5
32	5.5	3.6	2.4	10.8
16	2.6	3.4	3.1	7.3
8	5.7	6.6	5.1	8.1
4	8.6	6.5	6.4	8.1
2	12.6	10.6	11.2	12.9
1	29.5	42.0	12.3	33.1

coefficients were all higher than 0.97, and the regression lines were not significantly different ($p < 0.001$). When assays performed on different days were compared, all regression lines had correlation coefficients higher than 0.98 and were not significantly different ($p < 0.001$). Furthermore, CVs for plate-to-plate and day-to-day variation were smaller than 20% for all Pen a 1 concentrations higher than 2 ng/ml (Table I). The intraassay CV was acceptable at Pen a 1 concentrations as low as 2 ng/ml. Interassay variations showed slightly higher CVs than the intraassay variations, but the average of intraassay CVs from the linear portion of the standard curve, ranging from

4 to 125 ng/ml, was 4.5%. The average interassay CV for the same concentration range was 12.8% (Table II).

Standard curve

The standard dose-response curve for quantification of Pen a 1 revealed a characteristic sigmoidal shape when plotted on a semi-logarithmic scale. The graph is linear for Pen a 1 concentrations between 6 and 60 ng/ml. The statistical analysis showed acceptable assay variations ($CV < 20\%$) for this range. The sensitivity, defined as the antigen concentration that produces a significant positive signal, was determined to be 1 ng/ml. Because the intraassay and interassay CV exceeded the acceptable limit at the Pen a 1 concentrations of 2 and 4 ng/ml, respectively, Pen a 1 concentrations between 4 and 125 ng/ml can be reliably determined.

Pen a 1 levels in crude shrimp extract and commercial allergen extracts

The Pen a 1 contents of the TMC reference extract and four commercial shrimp extracts (S1, S2, S3, and S4) were determined. The Pen a 1 contents of these extracts were lower than that of the TMC extract. The percentage Pen a 1 of total protein in commercial shrimp extracts varied from 2.0% to 16.8%, whereas the TMC extract had a Pen a 1 concentration of 33.5% (Table III). Some crab and lobster extracts showed activities higher than 100%. This result indicates that the developed ELISA is suitable to measure Pen a 1-like molecules in extracts of other crustacea species. However, shrimp tropomyosin is not suitable

TABLE II. Coefficients of variation for intra- and interassay variations

	Pen a 1 concentration (ng/ml)									mean*
	1	2	4	8	16	32	64	125	250	
Intraassay variation (%)	23.0	13.2	4.8	4.4	4.7	4.7	4.0	3.6	5.6	4.5
Interassay variation (%)	52.4	29.0	17.2	19.5	11.1	10.0	11.1	11.4	9.5	12.8

*Mean CV range from 4 to 250 ng/ml.

as the standard, and purified crab or lobster tropomyosin must be used. Cockroach, house dust mite, oyster, codfish, peanut, and buckwheat flour extracts used as specificity controls had negative results (data not shown).

DISCUSSION

Pen a 1 is the only major allergen in brown shrimp (*P. aztecus*) that has been recently identified as the muscle protein tropomyosin. It is detected by sera of more than 80% of all subjects allergic to shrimp and binds up to 75% of all shrimp-specific IgE. Pen a 1 had a molecular weight, amino acid composition, and sequence homology similar to those of other crustacea IgE-binding proteins such as shrimp allergen 11, Sa-11, Par f 1, and Met e 1. This suggests that tropomyosin isoforms and their variants are major crustacea allergens.¹⁴⁻¹⁷

Crude allergen extracts are usually used in the diagnosis and immunotherapy of allergy, although their allergenic potencies and allergen composition may vary. This may be due to the variability of the natural source materials, the different extraction and processing procedures, or both. For example, Chapman¹⁰ reported a 100% difference in Der f 1 content of house dust mite allergen extracts, even though similar extraction protocols were used.

One way to characterize allergen extracts is to measure their major allergen content by using mAbs. Monoclonal antibody-based immunoassays have been used to measure allergen contents in crude extracts of house dust mites *Dermatophagoides pteronyssinus* and *D. farinae* (Der p 1, Der p 2, Der f 1, Der f 2), German cockroach *Blattella germanica* (Bla g 1, Bla g 2), American cockroach *Periplaneta americana* (Cr-P11), and house cat *Felis domesticus* (Fel d 1).¹⁸⁻²² Major allergen quantification is used in the United States for in vitro standardization of short ragweed, cat, and venom extracts.

Different formats are possible for a quantitative assay. The advantage of a sandwich immunoassay over inhibition or direct detection is that nonrelevant proteins, such as other allergens or nonallergens, do not interfere with the detection. This is because a solid phase-bound allergen-specific capture antibody only binds the relevant allergen without competition by other extract components. No allergen purification is needed. A second labeled antibody with different epitope specificity is then used to visualize the binding. For the chosen assay format (sandwich ELISA), detection of detector antibody with alkaline phosphatase-labeled streptavidin, 5 mg/ml capture mAb 3.2 and 30 pg/ml detector mAb 4.9.5 were found to be the optimal concentrations.

TABLE III. Pen a 1 content of allergen extracts

Allergen extract	Manufacturer's concentration (wt/vol)	Protein content (mg/ml)	Pen a 1 content (μg/ml)	Pen a 1 activity (%)
Shrimp				
TMC extract		5.5	2,120	33.5
S1	1:10	6.0	920	15.3
S2	1:10	1.2	24	2.0
S3	1:20	2.8	218	7.8
S4	1:10	3.2	538	16.8
Crab				
C1	1:10	0.8	4,080	523
C2	1:10	2.8	3	0.1
C3	1:20	2.0	3,800	190
C4	1:10	0.8	4,220	563
Lobster				
L1	1:10	2.5	3	0.1
L2	1:10	1.0	844	111
L3	1:20	1.0	6,180	618
L4	1:10	0.6	5	0.9

Three criteria of a good immunoassay are high sensitivity, reproducibility, and specificity. The developed sandwich ELISA has a sensitivity of about 1 ng/ml. This sensitivity is comparable to house dust mite and cat allergen-specific assays, which can detect group 1 and group 2 *Dermatophagoides* allergens ranging from 2 to 5 ng/ml and Fel d 1 as low as 0.25 ng/ml.^{18, 19, 22}

To evaluate assay reproducibility, plate-to-plate and day-to-day assay variations were analyzed by linear regression analysis. To characterize the intraassay and interassay variability, the CVs were calculated. The results of these analyses show that the assay is highly reproducible in the linear portion of the standard curve from 4 to 125 ng/ml.

To test the specificity of this assay, extracts containing tropomyosin were tested. Oyster,²³ cockroach,^{24, 25} and house dust mites²⁶ were reported to cross-react with shrimp allergens. However, no reactivity was observed. This may be due to the epitope specificity and affinity of the mAbs used and the number of epitopes on those allergens. Furthermore, other food extracts, such as peanut and buckwheat flour, caused no reaction at all.

By using this assay to measure Pen a 1 levels in shrimp extracts, four commercial shrimp extracts and the TMC reference extract were compared. The percentage of Pen a 1 fraction of total protein in the TMC shrimp extract was 33.5%. This finding agrees with an earlier preliminary estimation that Pen a 1 makes up at least 20% of the total protein.⁷ The Pen a 1 contents and the

percentages of Pen a 1 in the total extract protein of the commercial shrimp extracts were lower than those in the TMC shrimp extract. The percentage of Pen a 1 in these extracts ranged from 6.0% to 50.1% when compared with the TMC extract. The total Pen a 1 content varied up to about 40-fold (from 24.2 to 920 mg/ml), and the percentage of Pen a 1 in a given extract varied eightfold (from 2.0% to 16.8%).

Assays of four commercial crab and lobster extracts demonstrated that all of those extracts contained detectable levels of Pen a 1-like proteins, which indicates that the developed mAb-based sandwich ELISA is suitable for measurement of Pen a 1-like molecules in extracts of other crustacea species. However, some of the extracts have an activity higher than 100%. This result indicates that the developed ELISA is suitable for measurement of Pen a 1-like molecules in extracts of other crustacea species, but shrimp tropomyosin is not suitable as the standard, although purified crab and lobster tropomyosin may be used.

In summary, we have developed a highly sensitive and reproducible mAb-based sandwich ELISA for the quantification of the major shrimp allergen Pen a 1. This assay will be helpful to standardize shrimp allergen extracts. It is suitable for determining Pen a 1 content in shrimp extracts and correlating the Pen a 1 content of a given extract with its biologic activity and diagnostic value. Because occupational exposure to shrimp has been linked with allergic reactions, this assay may be used to evaluate workplace-related allergies or possible contamination of shrimp allergen in foods in the investigation of unexplained adverse reactions.

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