

Proteomics and Immunological Analysis of a Novel Shrimp Allergen, Pen m 2¹

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Shellfish are a common cause of adverse food reactions in hypersensitive individuals and shrimp is one of the most frequently reported causes of allergic reactions. A novel allergen from *Penaeus monodon*, designated Pen m 2, was identified by two-dimensional immunoblotting using sera from subjects with shrimp allergy, followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of the peptide digest. This novel allergen was then cloned and the amino acid sequence deduced from the cDNA sequence. The cloned cDNA encoded a 356-aa protein with an acetylated N terminus at Ala², identified by postsource decay analysis. Comparison of the Pen m 2 sequence with known protein sequences revealed extensive similarity with arginine kinase (EC 2.7.3.3) from crustaceans. Pen m 2 was purified by anion exchange chromatography and shown to have arginine kinase activity and to react with serum IgE from shrimp allergic patients and induce immediate type skin reactions in sensitized patients. Using Pen m 2-specific antisera and polyclonal sera from shrimp-sensitive subjects in a competitive ELISA inhibition assay, Pen m 2 was identified as a novel cross-reactive *Crustacea* allergen. This novel allergen could be useful in allergy diagnosis and in the treatment of *Crustacea*-derived allergic disorders. *The Journal of Immunology*, 2003, 170: 445–453.

It has long been recognized that consumption of seafood can produce allergic symptoms in susceptible individuals, and shellfish are one of the most frequently reported causes of allergic reactions (1–4). Sensitized individuals can develop urticaria, angioedema, laryngospasm, asthma, and life-threatening anaphylaxis (5–7). As consumption of seafood increases worldwide, immediate hypersensitivity reactions to seafood have become an important issue; in addition, those involved in seafood processing are at risk from seafood allergy (8–9). In recent years, a number of allergens which stimulate IgE production and cause IgE-mediated disease have been identified. Although considerable information exists on inhaled allergens (dust mites, pollens, and fungi), few food allergens have been identified and studied (10–12). The identification and characterization of clinically relevant seafood allergens are still incomplete, limiting our understanding of their role in the immunopathogenic mechanisms involved in hypersensitivity reactions. Thus, the characterization of the proteins responsible for IgE-mediated food allergies is the main research goal in seafood allergy.

The black tiger shrimp, *Penaeus monodon*, which is widely distributed in the eastern hemisphere, is an economically important fished and farmed shrimp species in many areas of Southeast Asia (13). It is also exported to the U.S. A number of proteins with molecular masses ranging from 8 to 94 kDa that bind serum IgE from atopic patients have been identified immunochemically, but their biological and immunological properties have not been well-

characterized (14–19). To date, only a few major IgE-binding proteins in seafood have been identified. The protein, tropomyosin, first identified as a 36-kDa allergen in shrimp muscle (Pen a 1), is also present in other crustaceans (16), cockroaches (20), and house dust mites (21). A 12-kDa fish allergen, frequently recognized by patients' IgE, has been identified as parvalbumin (22).

Traditionally, the identification and characterization of common allergens requires extensive effort and a large amount of starting material. Newly developed proteomics approaches involving the combined application of separation techniques, mass spectrometry (MS),³ and bioinformatics tools have been proposed for the identification and characterization of proteins in a complex biological mixture in various experimental contexts. Thus, mass fingerprinting of peptides has been used for the rapid identification of proteins in proteomics analysis (23–24). In this study, we describe the use of a proteomics approach, combining two-dimensional (2-D) Western blotting and matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS), in the identification of Pen m 2 allergen from shrimp. Subsequent purification, molecular cloning, and immunological analyses verified its IgE-binding activity and allergenicity in a skin test. This study demonstrates that Pen m 2 allergen is an arginine kinase⁴ and represents a new class of shrimp allergen, which seems to play an important role as a cross-reactive *Crustacea* allergen.

Materials and Methods

Patients' sera and preparation of *P. monodon* extracts

Sera from patients with shrimp allergy were collected in the National Taiwan University Hospital (Taipei, Taiwan) and stored in aliquots at –70°C. The allergic response was confirmed by the clinical history and diagnosis, and characterized using the Pharmacia CAP system (Amersham Pharmacia Biotech, Uppsala, Sweden) for measuring IgE reactivity (25). The initial inclusion of shrimp allergic patients was on the basis of a Pharmacia CAP score for shrimp crude extract-specific IgE Ab >+2. Sera from nonallergic

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³ Abbreviations used in this paper: MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; 2-D, two dimensional; MALDI-TOF MS, MALDI time-of-flight MS; pI, isoelectric point; PSD, postsource decay; PVDF, polyvinylidene difluoride; RACE, rapid amplification of cDNA ends.

⁴ Enzyme Commission nos.: arginine kinase (EC 2.7.3.3), trypsin (EC 3.4.21.4).

individuals were used as controls. Eighteen shrimp-allergic patients had IgE Ab detectable by immunoblotting, and all 18 had a history of atopic disease, with 70% having a history of asthma, 65% of allergic rhinitis, and 25% of atopic dermatitis.

Black tiger shrimp (*P. monodon*) were purchased from a local market. The shrimp muscle was ground in a mortar filled with liquid nitrogen, then extracted for 16 h at 4°C with constant stirring with 50 mM PBS, pH 7.0, containing 0.2 mM DTT and 1 mM PMSF. After centrifugation at $12,000 \times g$ for 10 min at 4°C, the supernatant was dialyzed for 48 h at 4°C against 10 mM sodium phosphate buffer, pH 7.0, then lyophilized to yield the crude extract, which was used to evaluate patients' sera for *P. monodon*-specific IgE reactivity.

SDS-PAGE and immunoblotting with IgE

The crude extract was separated by SDS-PAGE as described previously (26) using a 15% separation gel. For immunodetection of IgE-binding proteins, the separated proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane, which was then blocked with skimmed milk and incubated for 16 h at 4°C with a 1/10 dilution of the shrimp-allergic serum. Bound IgE Abs were detected using alkaline phosphatase-labeled goat anti-human IgE Abs (BD PharMingen, San Diego, CA) and 5'-bromo-4-chloro-3-indolyl phosphatase/nitroblue tetrazolium as the substrate system (26).

2-D immunoblotting

Crude extracts were analyzed by 2-D immunoblotting as described previously (26). Briefly, for the first separation, 0.5 mg of *P. monodon* extract was applied to an immobilized pH gradient gel strip containing pH range of 3–10 ampholytes, and isoelectric focusing was performed in a Multiphor II horizontal electrophoresis system (Amersham Pharmacia Biotech). After isoelectric focusing, the strip was subjected to SDS-PAGE on 12.5% gels. For specific IgE immunodetection, proteins on the 2-D gel were blotted onto a PVDF membrane, and incubated with pooled sera from shrimp-allergic patients 13–18 which showed high IgE binding to the 40-kDa allergen on Western blots, then bound IgE was detected using alkaline phosphatase-conjugated monoclonal anti-human IgE Abs.

N-terminal microsequencing

After 2-D electrophoresis, the blotted proteins were visualized by Coomassie blue staining and the protein spots containing the presumed allergens cut out and subjected to N-terminal sequence analysis in a Procise 494 protein sequencer (Applied Biosystems, Foster City, CA).

Peptide analysis by delayed extraction matrix-assisted laser desorption ionization (MALDI) MS

The protein spots recognized by the pooled sera were excised and subjected to in-gel tryptic digestion as described previously (27). The digests were mixed with saturated α -cyano-4-hydroxycinnamic acid solution in acetonitrile/H₂O and spotted onto a MALDI sample plate, then MALDI MS analysis was performed on a Voyager DE-STR workstation (PerSeptive Biosystems, Framingham, MA) equipped with a 337-nm nitrogen laser. The peptide spectra, acquired in reflectron mode at an accelerating voltage of 20 kV, were the sum of 50 laser shots. The mass spectra were externally calibrated using low mass peptide standards. This procedure typically results in mass accuracies of 50–100 ppm. The peptide mass fingerprint data were compared with those in the National Center for Biotechnology Information nonredundant protein database using the MS-Fit search tool (University of California San Francisco Mass Spectrometry Facility, San Francisco, CA).

Ions of interest for postsource decay (PSD) analysis were obtained after isolation of the appropriate derivatized precursor ions using timed ion selection. The fragment ions were refocused onto the final detector by stepping the voltage applied to the reflectron in the mirror ratios of 1.0 (precursor ion segment), 0.8, 0.77, 0.6, 0.5, 0.4, 0.3, 0.2, 0.15, 0.1, and 0.07 (fragment ion segments). The individual segments were stitched together using software developed by PerSeptive Biosystems, and the PSD mass spectra were searched using the MS-Tag program.

Isolation of mRNA, cDNA cloning, and sequencing

Total RNA was extracted from *P. monodon* muscle using the TRIzol reagent kit (Life Technologies, Eggenstein, Germany) according to the manufacturer's instructions. Poly(A)⁺ RNA was purified by oligo(dT) cellulose chromatography. The rapid amplification of cDNA ends (RACE) method was used to produce cDNA fragments coding for Pen m 2 using a Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA)

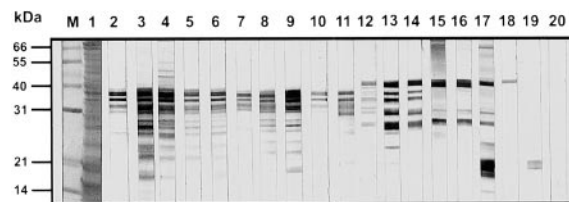


FIGURE 1. Immunoblots showing binding of patients' serum IgE to *P. monodon* crude extract. Lane M, Molecular mass markers; lane 1, Coomassie blue staining of the crude extract; lanes 2–19, immunoblots showing binding of IgE from different serum samples from allergic patients; lane 20, immunoblot using serum from a nonallergic individual.

(28). After MS and PSD analysis, two degenerate oligonucleotides based on the N-terminal sequences and internal sequences were synthesized. The sense primer used was 5'-GCTGACGCTGCTGT(T/C)ATTGA(A/G)AAG-3', encoding the eight N-terminal amino acids (ADAAVIEK), while the antisense primer was 5'-GCGGTC(G/A)TGGTG(A/T)GAGAA(A/G)GGAAT-3', encoding a conserved sequence of amino acids (IPFSHHDR) found in arginine kinases. To obtain the 5' and 3' portions of the Pen m 2 cDNA, the RACE PCR protocol was used as described previously (26). The coding sequence of the Pen m 2 gene was then amplified by PCR, and the amplified product analyzed by electrophoresis and subcloned into the pGEM-T vector, then transformed into *Escherichia coli* strain JM109. After transformation, plasmids from positive clones were subjected to sequence analysis using an ABI 377 sequencer (Applied Biosystems) and the dye terminator cycle sequencing FS Ready reaction.

Purification of arginine kinase

Arginine kinase from shrimp (*P. monodon* or *Metapenaeus ensis*), crawfish, (*Metanephrops thomsoni*), and crab (*Scylla serrata*) was purified by a modification of a previously described protocol (29), purification being monitored by the enzyme activity. Lyophilized seafood (2.5 g) was peeled and ground in liquid nitrogen in a mortar, then the homogenized powder was extracted for 16 h at 4°C with constant stirring using 25 ml of 0.1 M Tris-HCl, 10 mM 2-ME, 1 mM EDTA, 5 μ M NaN₃, and 25 μ M PMSF, pH 8.0, (buffer A). After centrifugation at $12,000 \times g$ for 20 min at 4°C, the supernatant was adjusted to 70% saturation with ammonium sulfate. After centrifugation, ammonium sulfate was added to the supernatant to 90% saturation, then the precipitate was collected by centrifugation, dissolved in 5 ml of 10 mM Tris-HCl, 10 mM 2-ME, and 0.1 mM EDTA, pH 8.0, (buffer B), and dialyzed against the same buffer. The clear supernatant was then applied to a HiTrap Q Sepharose Fast Flow column (Amersham Pharmacia Biotech) pre-equilibrated with buffer B. Fractions with arginine kinase activity were eluted with a 25 ml linear gradient from 0–1 M NaCl in buffer B.

Arginine kinase activity

The arginine kinase assay was a modification of an enzyme-linked creatine kinase assay using arginine phosphate as substrate (30). The incubation

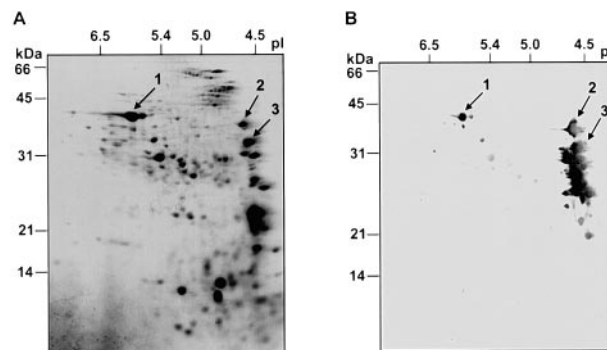
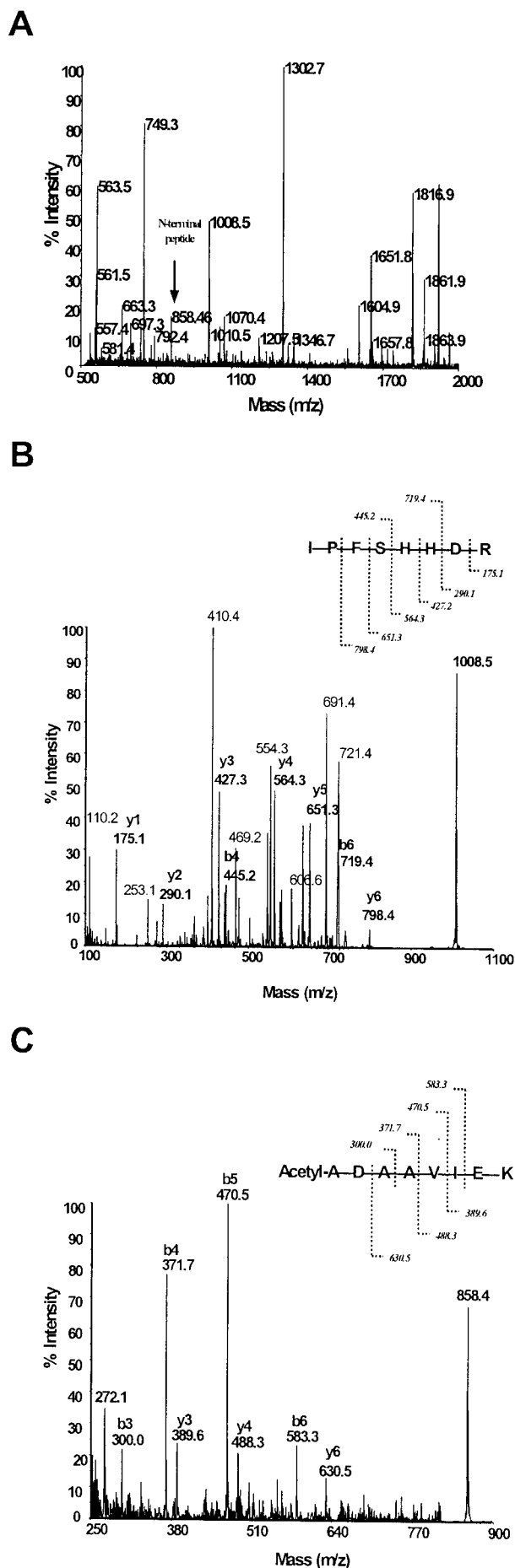


FIGURE 2. 2-D electrophoresis and immunoblot analysis of *P. monodon* allergens. A, Coomassie blue-stained 2-D gel. The approximate M_r and pI values are indicated. B, Immunoblots probed with pooled sera from patients 13–18. The N-terminal sequences and peptide mass fingerprinting of immunoreactive protein spots were determined and are given in the text.



mixture contained 342 mM arginine phosphate, 2.28 mM ADP, 5.7 mM AMP, 22.8 mM *N*-acetyl-L-cysteine, 22.8 mM D-glucose, 11.4 μ M diadenosine 5'-phosphate, 11.4 mM magnesium, 2.28 mM EDTA, 2.28 mM NADP, 2,850 U/L of hexokinase, and 1,710 U/L of glucose-6-phosphate dehydrogenase in assay buffer (Sigma-Aldrich, Steinheim, Germany). Formation of NADPH at 25°C was monitored in an Ultrospec 3000 ultraviolet-visible spectrophotometer at 340 nm (Amersham Pharmacia Biotech). One unit of arginine kinase activity was defined as the amount of enzyme catalyzing the formation of 1 μ mol/L of NADPH per minute under the assay conditions. The protein concentration was determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL), with BSA as the standard. The specific activity was calculated as units minute^{-1} milligram $^{-1}$ protein.

Intradermal skin test

The six patients who underwent skin testing had serum IgE reactive with Pen m 2 in a Western blot. All had a history of shrimp allergy with various clinical manifestations. Eighty-three percent (five of six) had a history of asthma, 50% (three of six) of allergic rhinitis, and 16% (one of six) of atopic dermatitis. Because of its high sensitivity, the intradermal skin test was used as described previously (31). Quantitative intradermal skin tests were performed using 5-fold dilutions of purified Pen m 2 from 2.5×10^{-8} to 1×10^{-9} M, as described previously (32), with 0.9% sodium chloride as the negative control and mite extract as the positive control. Skin reactions (wheals and erythema) were recorded 15 min after injection, with a wheal $>8 \times 8$ mm in diameter being regarded as a positive reaction.

Preparation and testing of polyclonal anti-Pen m 2 antiserum

New Zealand White rabbits were injected s.c. with 500 μ g of purified Pen m 2 in 1.0 ml of PBS emulsified with an equal volume of CFA. After 4 wk, a booster dose of 500 μ g of Pen m 2 emulsified in IFA was given by intradermal injection; this was followed by another injection of 500 μ g of Ag in another 4 wk. The production of specific Abs was monitored by Western blot analysis using purified Pen m 2. Bound Abs were detected using HRP-labeled goat anti-rabbit IgG as secondary Ab and development performed using a substrate solution of acetate buffer containing 3-amino-9-ethyl-carbazole and hydrogen peroxide.

IgE inhibition ELISA

For ELISA cross-inhibition studies, a serum pool from five patients displaying high IgE reactivity to Pen m 2 was used. Microtiter plates (Costar, Cambridge, MA) were coated for 16 h at 4°C with 100 μ l of Pen m 2 (0.01 μ g/ μ l) in PBS, pH 7.9, then probed 16 h at 4°C with aliquots of the serum pool previously incubated with different concentrations of purified arginine kinase from black tiger shrimp (*P. monodon*), sand shrimp (*M. ensis*), crawfish (*M. thomsoni*), crab (*S. serrata*), and lobster (*Homarus gammarus*) (Sigma-Aldrich). BSA was used as the negative control. After washing with 0.02 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.5, the plates were incubated for 1 h at room temperature with alkaline phosphatase-labeled goat anti-human IgE Abs (BD Pharmingen), then color development was performed for 30 min using paranitrophenylphosphate substrate (Sigma-Aldrich), the OD being measured at 405 nm using an ELISA reader (Labsystems, Helsinki, Finland). All assays were performed in triplicate.

Dot blot immunoassay

Dot blots were performed by applying 2 μ g of the arginine kinases purified from black tiger shrimp (*P. monodon*), sand shrimp (*M. ensis*), crab (*S. serrata*), lobster (*H. gammarus*), and crawfish (*M. thomsoni*) onto a PVDF membrane using a Bio-Dot apparatus (Bio-Rad, Richmond, CA). After blocking, the blots were washed, then incubated overnight at 4°C with a 1/5 dilution of serum from Pen m 2-allergic individuals, then for 1 h at room temperature with biotin-labeled goat anti-human IgE Abs (BioSource International, Camarillo, CA) and for 1 h at room temperature with peroxidase-conjugated streptavidin (Endogen, Woburn, MA). They were then washed thoroughly and incubated for 3–5 min at room temperature with

FIGURE 3. MALDI-TOF MS profile of tryptic digests of spot 1. A, Spot 1 was digested in situ with trypsin, the peptides analyzed by MALDI-TOF MS, and prominent mass peaks chosen for database searches. B, PSD spectrum of the derivatized tryptic peptide with a mass of 1,008.5 Da (see A); peptide sequence ions from the N terminus (b-series) and C terminus (y-series) are indicated. C, PSD spectrum of the derivatized tryptic peptide with a mass of 858.4 Da (see A); peptide sequence ions from the N terminus (b-series) and C terminus (y-series) are indicated.

25, 27, and 40 kDa was lower (~33–39%). The remaining IgE-binding components with various molecular masses, such as 22, 17, and 14 kDa, were detected at frequencies of <30%. No IgE binding was seen when serum from a healthy donor was used (Fig. 1, lane 20). The proteins with molecular masses of 32, 34, and 38 kDa and high-frequency IgE-reactive proteins are probably tropomyosin, the well-known shrimp major allergen. We chose to study the 40-kDa protein because it was novel IgE-binding protein recognized at quite high frequency by shrimp allergy sera.

Reactivity of IgE in patients' sera with *P. monodon* crude extract

2-D immunoblotting

To better characterize the *P. monodon* Ags recognized by sera from patients with shrimp sensitivity, the proteins in a crude extract were subjected to 2-D gel electrophoresis followed by immunoblotting. Fig. 2A shows the 2-D gel profile of the *P. monodon* crude extract, in which >100 distinct protein spots were detectable by Coomassie blue staining. To identify spots corresponding to allergens, IgE-binding spots on a 2-D gel were visualized by immunoblotting using a pool of six sera from shrimp allergy patients

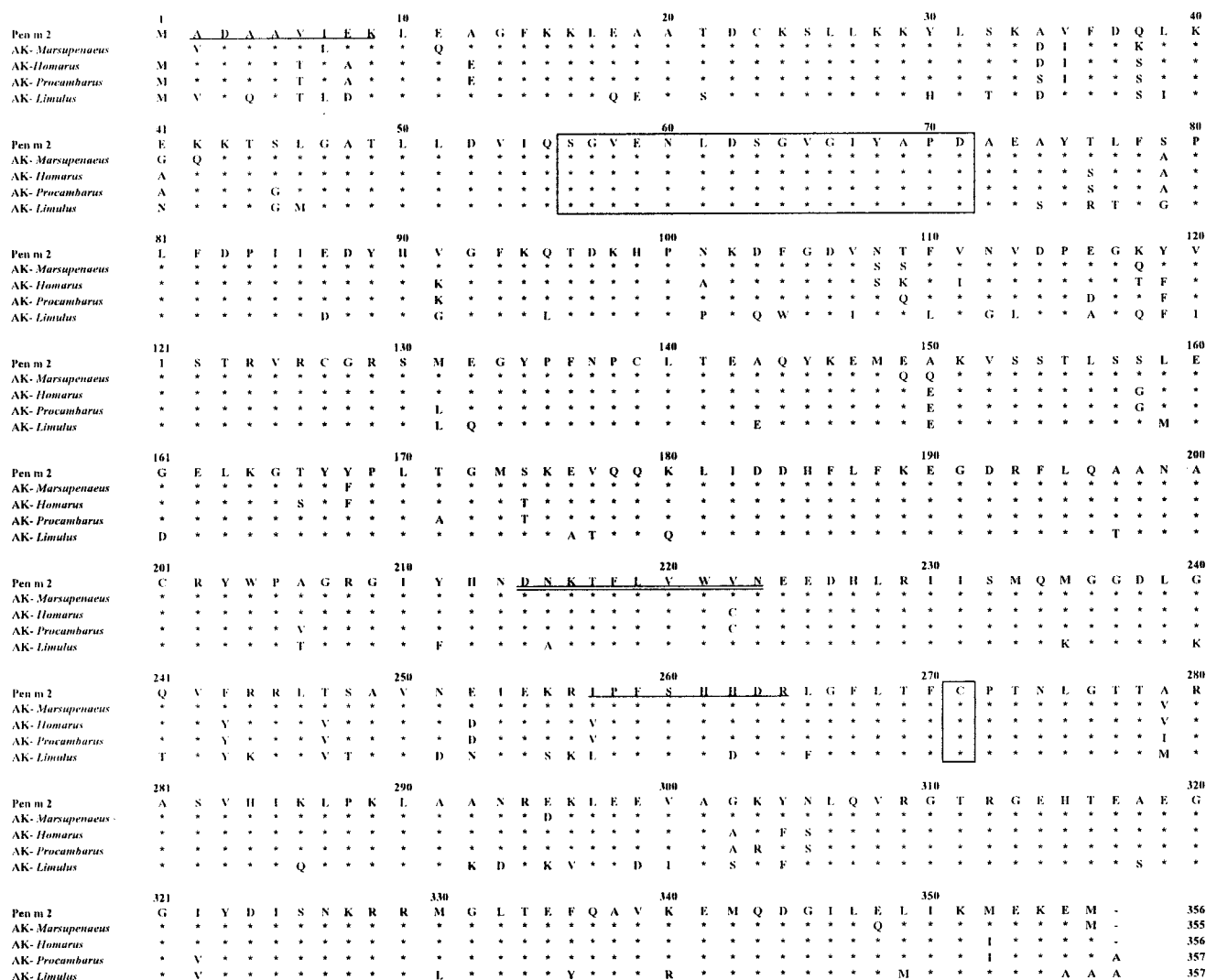


FIGURE 4. Comparison of the amino acid sequence of Pen m 2 with those of various arginine kinases. The accession nos. of the enzymes in the protein database are AK-*Marsupenaeus* (SwissProt: P51545), AK-*Homarus* (SwissProt: P14208), AK-*Procambarus* (prf: locus: 2020435A), and AK-*Limulus* (SwissProt: P51541). The numbering system is based on the *P. monodon* arginine kinase sequence. The gaps are introduced for optimal alignment of, and maximal homology between, all compared sequences. Identical amino acids are indicated by asterisks. The highly conserved amino acid residue at the active site and a possible substrate region, the guanidino specificity region, are shown in boxes. The peptide sequences with masses of 1,008.5 and 858.4 Da (see Fig. 3, B and C) are underlined, and a putative actinin type actin-binding domain is double underlined. The Gene Bank accession no. for the Pen m 2 cDNA is AF479772.

(patients 13–18), and at least 10 different reactive spots with molecular masses of 20–40 kDa and isoelectric point (pI) values ranging from 4.0 to 7.0 were demonstrated (Fig. 2B). Highly reactive protein spots with molecular masses of 30–40 kDa were observed, including one with a molecular mass of 40 kDa and a pI of 6.0, one with a molecular mass of 38 kDa and a pI of 4.7, and another with a molecular mass of 34 kDa and a pI of 4.6. Similar staining was seen using individual sera from allergic patients (data not shown). The arrows in Fig. 2A indicate the protein spots corresponding to the IgE-binding spots in Fig. 2B. No positive spots were detected using serum from nonallergic individuals (data not shown).

N-terminal amino acid sequencing and MS

When the three most prominent immunoreactive protein spots were excised from the Coomassie blue-stained blot (Fig. 2A) and subjected to N-terminal amino acid microsequencing, all three were found to have blocked N termini. To identify the blocked IgE-reactive spots, they were excised, digested in-gel with trypsin, and the resulting peptide mixtures analyzed by MALDI-TOF MS.

The MS profile of the peptides from spot 1, the 40 kDa protein with a pI of 6.0 showed multiple peaks ranging from 500 to 2,000

Da (Fig. 3A); 23 prominent peaks were selected for comparison with established databases, and the protein with the highest correlation with spot 1 (Fig. 3A) was found to be arginine kinase from *Marsupenaeus japonicus* (33), which corresponded to 60% (14 of 23 peaks) sequence coverage. To further characterize the internal sequence of spot 1, the signal at 1008.5 Da was selected for PSD analysis. The generated fragment ion spectrum identified this peptide as IPFSHHDR (Fig. 3B), equivalent to residues 257–264 of arginine kinase (Fig. 4). Based on sequence similarities and comparisons between arginine kinases, the peptide with a mass of 858.4 Da was proposed as the N-terminal peptide after adding the mass of an acetyl group. To further demonstrate posttranslational modification of this peptide, a MALDI-PSD experiment was performed. Fig. 3C shows that on the basis of the fragmented *b*- and *y*-ion series peaks, the 858.4-Da peptide corresponded to the N-terminal sequence (acetyl-ADAAVIEK), showing that the initiation methionine was removed and that the protein was N-terminally acetylated.

The MS profiles of spots 2 and 3 showed high similarity with tropomyosin from shrimp (*M. ensis*) (17), the sequence coverage being 76% (16 of 21 peaks) and 63% (10 of 16 peaks), respectively (data not shown).

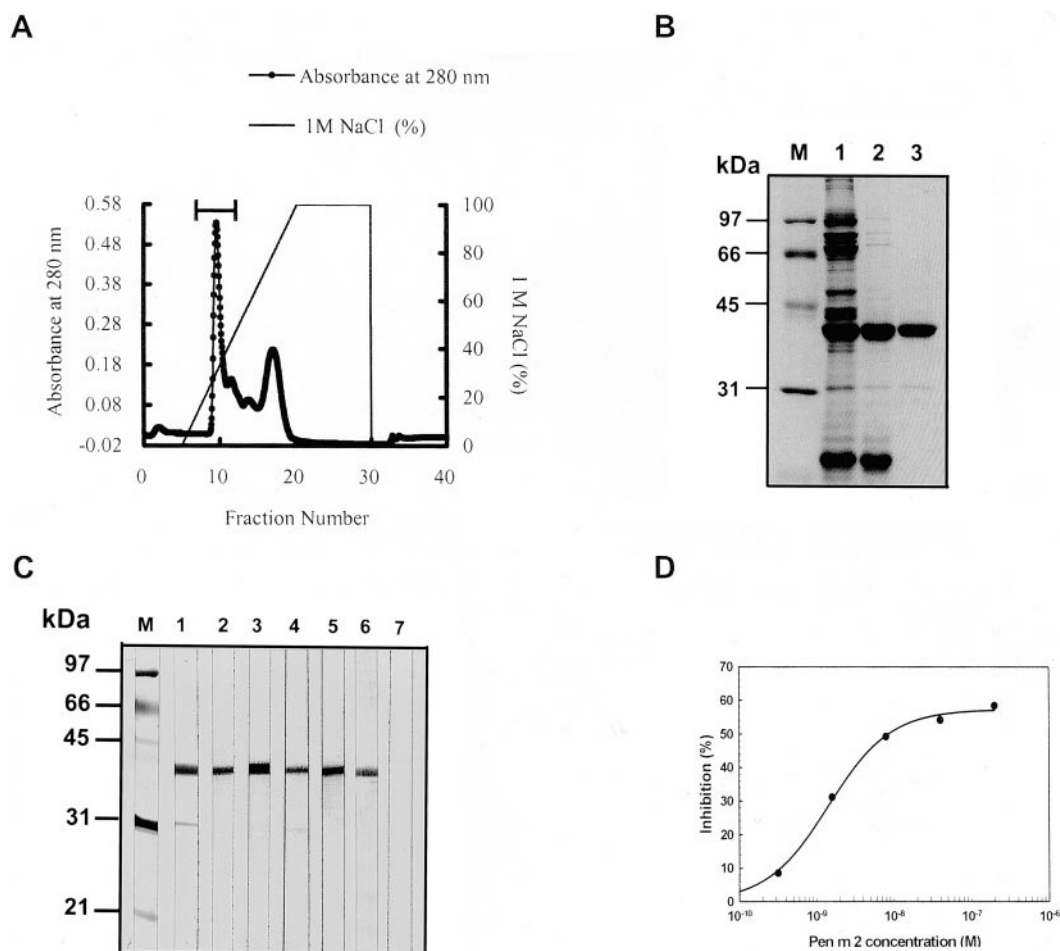


FIGURE 5. Purification and IgE-reactivity of Pen m 2. *A*, Elution profile of Pen m 2 from a HiTrap Q Sepharose Fast Flow column. Fractions with kinase activity were collected (indicated by the horizontal bar). *B*, SDS-PAGE of various purified fractions. *Lane M*, Molecular mass markers; *lane 1*, crude extract; *lane 2*, 70–90% ammonium sulfate cut; *lane 3*, arginine kinase-active fractions from the HiTrap Q Sepharose Fast Flow column. *C*, Immunoblots of purified Pen m 2. *Lane M*, Molecular mass markers; *lane 1*, Coomassie blue-stained Pen m 2; *lanes 2–6*, probed with serum samples from different allergic patients; *lane 7*, probed with nonatopic serum. *D*, ELISA inhibition assay showing inhibition of the binding of human IgE to shrimp crude extract by purified Pen m 2. Five micrograms of shrimp crude extract was applying to ELISA plates. Inhibition experiments were performed by preincubating the serum pool with increasing concentrations of Pen m 2, then applying the mixture to the ELISA plates to test the ability of Pen m 2 to inhibit IgE binding to shrimp allergens.

Table I. Purification of arginine kinase from *P. monodon* muscle^a

Procedure	Total Protein (mg)	Total Units (μmol/min)	Specific Activity (units/mg)	Purification (fold)
Homogenate	331	30,960	93	
70–90% (NH ₄) ₂ SO ₄ cut	38	11,087	292	3.13
Peak 1 from Q Sepharose Fast Flow column	17	8,563	512	5.48

^a One unit of arginine kinase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol/L of NADH per minute under the assay conditions.

Sequence similarities and comparisons between arginine kinases

RT-PCR of *P. monodon* cDNA generated the expected internal fragment, which was cloned into pGEM-T and sequenced. RACE was then performed to obtain the sequence of these PCR-forced regions plus the 5' and 3' coding regions. The cDNA contained 1,071 bp of open reading frame encoding a 356-aa protein with a theoretical molecular mass of ~39.9 kDa and a pI of 6.02. The deduced amino sequence is shown in Fig. 4. The N-terminal sequence of the mature protein started at Ala-2, located two residues from the N terminus of the primary translation product according to the PSD experiment analysis. Using the basic local alignment search tool program, three arginine kinases from shrimp (*M. japonicus*) (33), lobster (*H. gammarus*) (34), and crawfish (*Procambarus clarkii*) (35) were found to have a high similarity (~90% identity) to Pen m 2 (Fig. 4). Arginine kinase from crab (*Limulus polyphemus*) (36) also showed 77% identity with Pen m 2. The active site residue of Pen m 2 was recognized by sequence comparison as Cys²⁷¹. The guanidino specificity region, suggested to be generally conserved in most arginine kinase sequences and with 16 residues (residues Ser⁵⁶ to Asp⁷¹) highly conserved in crustaceans and associated with substrate binding (37), was also found in Pen m 2, as was a putative actinin type actin-binding domain (residues Asp²¹⁴ to Asn²²³) (38).

Isolation and immunogenic reactivity of native Pen m 2

Pen m 2 was purified to homogeneity from *P. monodon* muscle in two simple steps by monitoring arginine kinase activity. After the 70–90% (NH₄)₂SO₄ cut was dissolved in and dialyzed against buffer B, SDS-PAGE analysis showed that it contained two major proteins with molecular masses of 40 and 22 kDa (Fig. 5B). The 22-kDa protein was removed in a second step involving HiTrap Q Sepharose Fast Flow chromatography (Fig. 5A) in which the first peak contained only the 40-kDa protein (Fig. 5B). The protein thus obtained was characterized by immunoblotting and skin testing and was designated "Pen m 2". Kinase activity and relative yields in the various purification steps are summarized in Table I. About 17 mg of Pen m 2 was purified from the muscle of a single shrimp,

the final purification being ~5.48-fold, with a specific activity of 512 U/mg. Purified Pen m 2 reacted strongly in vitro with serum IgE from shrimp-sensitized patients, but not with IgE from non-allergic donors (Fig. 5C). The concentration of Pen m 2-specific IgE Ab, measured by ELISA, was 144 KU/L (data not shown). Pen m 2 also inhibited IgE Ab binding to shrimp crude extract in a dose-dependent manner, the maximal inhibition being ~56% on addition of 4×10^{-8} to 2×10^{-7} M of Pen m 2 (Fig. 5D). To characterize the allergic response of Pen m 2 in vivo, a quantitative intradermal skin test was performed on six selected Pen m 2 allergic patients and six controls (Table II). The results showed that positive skin tests were obtained in all six patients using 100 μl of 1×10^{-9} or 5×10^{-9} M of Pen m 2, while nonallergic controls gave negative skin tests at concentrations up to 2.5×10^{-8} M (data not shown). An example of a skin test with Pen m 2 showing a wheal and flare reaction in one of the sensitized patients (patient JF, see Table II), who had IgE Abs to mite and shrimp, is shown in Fig. 6; no reaction was induced with physiological saline. These results show that Pen m 2 is capable of inducing specific immediate hypersensitivity responses in shrimp allergy patients.

Cross-reactivity of arginine kinase from Crustacea

Purified arginine kinases from the sand shrimp (*M. ensis*), lobster (*H. gammarus*), crawfish (*M. thomsoni*), and crab (*S. serrata*) were tested for antigenic recognition and allergenic cross-reactivity using polyclonal rabbit anti-Pen m 2 Abs and sera from shrimp-sensitive patients. Immunoblotting showed that the rabbit anti-Pen m 2 Abs showed strong reactivity with the 40-kDa protein corresponding to the different crustacean arginine kinases (Fig. 7A, lanes 1–5), and that these proteins were also recognized by sera from shrimp allergy patients (Fig. 7A, lanes 6–10), indicating that common epitopes are present on crustacean arginine kinases. Furthermore, when IgE Ab binding to purified crustacean arginine kinases was tested by dot blot assay using sera from 13 Pen m 2 allergic individuals, the frequency of binding was 100% (13 of 13) for sand shrimp, 92% (12 of 13) for lobster, 85% (11 of 13) for crab, and 85% (11 of 13) for crawfish (Fig. 7B). In addition, when

Table II. Pen m 2 in the immediate skin test and serum IgE Ab tests

Patient	Sex	Age	CAP Class for Shrimp	Serum Total IgE (Ku/L)	Skin Test Using Pen m 2	
					5×10^{-9} M	1×10^{-9} M
JF	Male	38	2	291	18 ^a /65 ^b	15/50
CM	Male	15	3	568	11/45	— ^c
WC	Male	18	3	673	10/30	—
FJ	Male	11	4	750	15/45	—
MF	Female	23	3	734	17/45	15/40
YW	Female	17	2	893	11/20	10/20
Control (n = 6)			≤2	ND	—	—

^a Wheal diameter (millimeters).

^b Erythema diameter (millimeters).

^c Negative response.

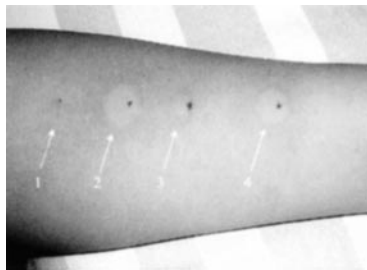


FIGURE 6. Skin test using Pen m 2. One hundred microliters of normal saline (spot 1), 100 AU (100 pg/ml) of mite allergen crude extract (spot 2), a 1×10^{-9} M solution of purified Pen m 2 (spot 3), and a 5×10^{-9} M solution of purified Pen m 2 (spot 4) were injected intradermally into a patient with shrimp allergy and the wheal and flare responses assessed 15 min later.

competitive ELISA experiments were performed by incubating various crustacean arginine kinases with sera containing Pen m 2-reactive IgE before probing with immobilized purified Pen m 2, IgE reactivity with Pen m 2 was inhibited in a dose-dependent manner by shrimp, lobster, crawfish, and crab arginine kinases, but not by BSA (Fig. 7C). These results show that arginine kinase is an allergen common to *Crustacea*.

Discussion

Crustaceans are highly allergenic food sources (1–8). Patients with seafood-induced immediate allergic responses can develop a variety of symptoms affecting the skin, respiratory tract, gastrointestinal tract, and cardiovascular system. To date, only one allergen, tropomyosin, has been identified in the shrimp as a major IgE-reactive component, and its epitopes have been well-characterized (39–40). In the present study, we used proteomics approaches to evaluate the IgE reactivity of a crude *P. monodon* extract and found that the antigenic makeup consisted of a very heterogeneous group of components. On 2-D immunoblots, tropomyosin spots were seen at 34 and 38 kDa, possibly representing two different glycosylated isoforms of the allergen. In addition to tropomyosin, an IgE-binding protein with a molecular mass of 40 kDa and a pI of 6.0 was also noted. On the basis of lectin binding and periodic acid/Schiff staining, the 40-kDa allergen protein was not glycosylated (data not shown). This novel 40-kDa allergen protein, designated Pen m 2, showed high sequence similarity to a previously reported arginine kinase from crustaceans and had arginine kinase activity. About 72% (13 of 18) and 27% (5 of 18), respectively, of shrimp allergy sera showed significant IgE reactivity with tropomyosin and Pen m 2 (Fig. 1), implying that tropomyosin can be considered as the major shrimp allergen, with Pen m 2 being the other important allergenic component in some individuals.

Food allergens are present as major protein components in food, such as seed storage proteins in plants (41), OVA in egg white (42), and parvalbumin in fish (22). The arginine kinase, Pen m 2, is also abundant in shrimp muscle. The high concentration of allergens in foods that cause allergy and the stability of the allergens during processing into specific food products are important factors contributing to the allergenicity of a protein (43–44). The high level of these proteins, together with their resistance to the proteolytic and acid conditions of the human digestive system suggests there is a high probability that many of these proteins will reach the intestinal mucosa after consumption. Thus, consumption of foods containing these major proteins is likely to sensitize an allergic individual.

To the best of our knowledge, this is the first time that arginine kinase has been identified as a food allergen. Arginine kinase has

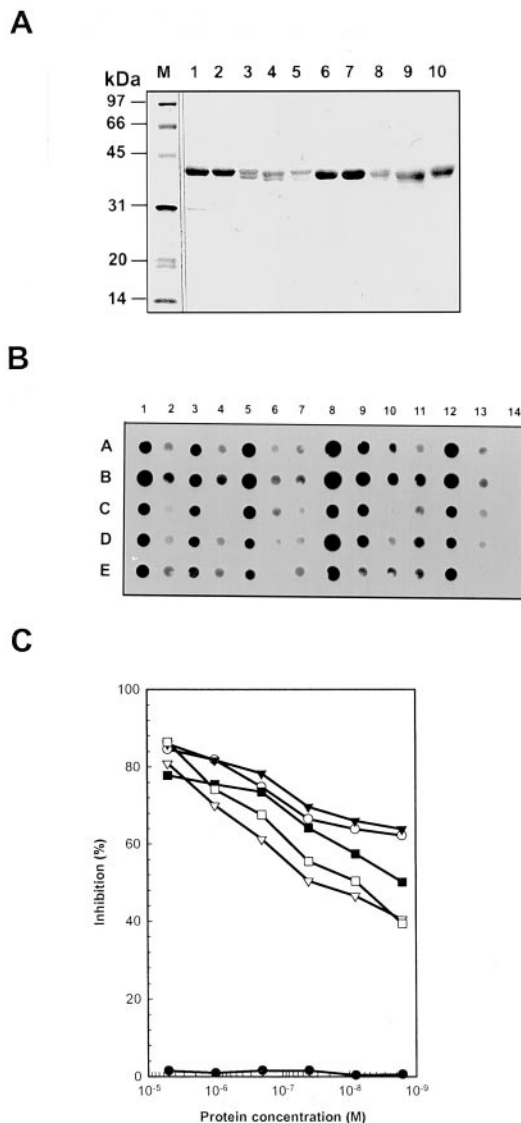


FIGURE 7. IgE cross-reactivity between Pen m 2 and various crustacean arginine kinases. **A**, Reactivity of polyclonal rabbit anti-Pen m 2 antiserum and allergic patients' sera with crustacean arginine kinases. Blots of arginine kinase purified from black tiger shrimp (*P. monodon*) (lanes 1 and 6), sand shrimp (*M. ensis*) (lanes 2 and 7), crab (*S. serrata*) (lanes 3 and 8), lobster (*H. gammarus*) (lanes 4 and 9), and crawfish (*M. thomsoni*) (lanes 5 and 10) were probed with polyclonal rabbit anti-Pen m 2 Ab (lanes 1–5) or pooled sera from allergic patients (lanes 6–10). **B**, Dot-blot assay showing binding of serum IgE from Pen m 2 allergic patients to arginine kinases from crustaceans. Arginine kinases purified from black tiger shrimp (*P. monodon*) (row A), sand shrimp (*M. ensis*) (row B), crab (*S. serrata*) (row C), lobster (*H. gammarus*) (row D), and crawfish (*M. thomsoni*) (row E) were dotted onto PVDF membrane and probed with sera from Pen m 2 allergic patients (lanes 1–13). Serum from a nonallergic subject (lane 14) was used as the control. **C**, Inhibition of the binding of serum IgE from Pen m 2-allergic patients to purified Pen m 2 by arginine kinases from crustaceans. Pen m 2 ($1 \mu\text{g ml}^{-1}$) was coated onto ELISA plates. Inhibition experiments were performed by incubating samples of the Pen m 2-reactive serum pool overnight at 4°C with different concentrations of purified Pen m 2 (*P. monodon* ○), arginine kinases from sand shrimp (*M. ensis* ▼), lobster (*H. gammarus* ■), crab (*S. serrata* ▽), crawfish (*M. thomsoni* □), or BSA (●), then applying the mixture to the ELISA plates.

been described as an allergen in the moth (*Plodia interpunctella*), and recombinant Plo i 1 (the moth arginine kinase) is recognized by sera from 25% of moth-sensitized patients (45). The deduced

amino acid sequence for Pen m 2 showed 81% sequence identity with that of Plo i 1. A number of allergens have been shown to possess transport and regulatory properties and, interestingly, several of these are often encountered as allergens in food allergy. They include plant profilins, involved in actin binding (46), the iron transport protein allergen from egg white (47), animal serum albumins (48), the fish parvalbumin allergen, which has calcium binding properties (49), and the shellfish tropomyosin, involved in actin binding and muscle contraction (50). Arginine kinase catalyzes the reversible transfer of the high-energy phosphoryl group from ATP to arginine, yielding ADP and *N*-phosphoarginine (51). Phosphoarginine is commonly referred to as a phosphagen and represents an intermediate storage and transport form of energy in a wide variety of invertebrates. In addition, a putative actin-binding domain is also found in Pen m 2. Therefore, arginine kinase is a novel food allergen that may have regulatory and/or transport properties.

The mechanism of allergic sensitization to arginine kinase is unknown. Although purified arginine kinase can bind IgE Abs from test sera and induce specific immediate hypersensitivity responses in sensitized patients, the clinical relevance of such responses to Pen m 2 and the functional relationship between its kinase activity and allergenicity require further investigation. Crustaceans include many edible sea creatures, notably shrimp, crab, lobster, and crawfish, which are of particular interest, since a number of studies have demonstrated that they are major seafood allergens. Patients with shrimp hypersensitivity often complain of adverse reactions following ingestion of other shellfish, such as lobster, prawn, crab, and crawfish. This cross-reactivity has been attributed to the crustacean tropomyosin molecule (52–53). In the present study, immunoblot, dot blot, and ELISA inhibition analyses showed that Pen m 2 has a high cross-reactivity with arginine kinase from sand shrimp, lobster, crab, and crawfish, suggesting that this molecule, like tropomyosin, is a common allergen. Further investigations on this class of allergens are underway.

The molecular basis of the interaction between food allergens and the immune system is unclear, and there are a number of unanswered questions concerning the sequence of pathogenic and physiologic events that follows the ingestion of food capable of initiating an IgE Ab response. Standardization of food allergens will be necessary before clinical trials can be conducted; therefore, standardized allergens may be instrumental in determining the molecular basis of the IgE response and in the development of new diagnostic and therapeutic strategies (54–56). Thus far, the proteomics strategy has provided a powerful tool for the identification of allergenic proteins from heterogeneous food sources and should greatly help in further studies addressing the mechanism involved in food allergy sensitization.

References

- Bernstein, M., J. H. Day, and A. Welsh. 1982. Double-blind food challenge in the diagnosis of food sensitivity in the adult. *J. Allergy Clin. Immunol.* 70:205.
- O'Neil, C., A. A. Helbling, and S. B. Lehrer. 1993. Allergic reactions to fish. *Clin. Rev. Allergy* 11:183.
- Daul, C. B., J. E. Morgan, and S. B. Lehrer. 1993. Hypersensitivity reactions to crustacea and mollusks. *Clin. Rev. Allergy* 11:201.
- Daul, C. B., J. E. Morgan, and S. B. Lehrer. 1990. The natural history of shrimp hypersensitivity. *J. Allergy Clin. Immunol.* 86:88.
- Waring, N. P., C. B. Daul, R. D. deShazo, M. L. McCants, and S. B. Lehrer. 1985. Hypersensitivity reactions to ingested crustacea: clinical evaluation and diagnostic studies in shrimp-sensitive individuals. *J. Allergy Clin. Immunol.* 76:440.
- Daul, C. B., J. E. Morgan, N. P. Waring, M. L. McCants, J. Hughes, and S. B. Lehrer. 1987. Immunologic evaluation of shrimp-allergic individuals. *J. Allergy Clin. Immunol.* 80:716.
- Bengtsson, U., L. A. Hanson, and S. Ahlstedt. 1996. Survey of gastrointestinal reactions to foods in adults in relation to atopy, presence of mucus in the stools, swelling of joints and arthralgia in patients with gastrointestinal reactions to foods. *Clin. Exp. Allergy* 26:1387.
- Desjardins, A., J. L. Malo, J. L'Archeveque, A. Cartier, M. McCants, and S. B. Lehrer. 1995. Occupational IgE-mediated sensitization and asthma caused by clam and shrimp. *J. Allergy Clin. Immunol.* 96:608.
- Jeebhay, M. F., T. G. Robins, S. B. Lehrer, and A. L. Lopata. 2001. Occupational seafood allergy: a review. *Occup. Environ. Med.* 58:553.
- Stewart, G. A. 1995. Dust mite allergens. *Clin. Rev. Allergy Immunol.* 13:35.
- Midoro-Horiuti, T., E. G. Brooks, and R. M. Goldblum. 2001. Pathogenesis-related proteins of plants as allergens. *Ann. Allergy Asthma. Immunol.* 87:261.
- Kurup, V. P., H. D. Shen, and B. Banerjee. 2000. Respiratory fungal allergy. *Microbes Infect.* 2:1101.
- Shiau, S. Y., and Y. Chen. 2000. Estimation of the dietary vitamin A requirement of juvenile grass shrimp *Penaeus monodon*. *J. Nutr.* 130:90.
- Hoffman, D. R., E. D. Day, and J. S. Miller. 1981. The major heat stable allergen of shrimp. *Ann. Allergy* 47:17.
- Nagpal, S., L. Rajappa, D. D. Metcalfe, and P. V. Rao. 1989. Isolation and characterization of heat-stable allergens from shrimp (*Penaeus indicus*). *J. Allergy Clin. Immunol.* 83:26.
- Daul, C. B., M. Slatery, G. Reese, and S. B. Lehrer. 1994. Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. *Int. Arch. Allergy Appl. Immunol.* 105:49.
- Leung, P. S. C., K. H. Chu, W. K. Chow, A. Ansari, C. I. Bandea, H. S. Kwan, S. M. Nagy, and M. E. Gershwin. 1994. Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. *J. Allergy Clin. Immunol.* 94:882.
- Lin, R. Y., H. D. Shen, and S. H. Han. 1993. Identification and characterization of a 30 kD major allergen from *Parapenaeus fissurus*. *J. Allergy Clin. Immunol.* 92:837.
- Reese, G., R. Ayuso, and S. B. Lehrer. 1999. Tropomyosin: an invertebrate pan-allergen. *Int. Arch. Allergy Immunol.* 119:247.
- Santos, A. B. R., M. D. Chapman, R. C. Aalberse, L. D. Vailes, V. P. L. Ferriani, and C. Oliver. 1999. Cockroach allergens and asthma in Brazil: identification of tropomyosin as a major allergen with potential cross-reactivity with mite and shrimp allergens. *J. Allergy Clin. Immunol.* 104:329.
- Asturias, J. A., M. C. Arilla, N. Gomez-Bayon, A. Martinez, J. Martinez, and R. Palacios. 1998. Sequencing and high level expression in *Escherichia coli* of the tropomyosin allergen (Der p 10) from *Dermatophagoides pteronyssinus*. *Biochim. Biophys. Acta.* 1397:27.
- Elsayed, S., and J. Apold. 1983. Immunochemical analysis of cod fish allergen M: locations of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *Allergy* 38:449.
- Conrads, T. P., G. A. Anderson, T. D. Veenstra, L. Pasa-Tolic, and R. D. Smith. 2000. Utility of accurate mass tags for proteome-wide protein identification. *Anal. Chem.* 72:3349.
- Peng, J., and S. P. Gygi. 2001. Proteomics: the move to mixtures. *J. Mass Spectrom.* 36:1083.
- Ewan, P. W., and D. Coote. 1990. Evaluation of a capsulated hydrophilic carrier polymer (the ImmunoCAP) for measurement of specific IgE antibodies. *Allergy* 45:22.
- Chow, L. P., N. Y. Su, C. J. Yu, B. L. Chiang, and H. D. Shen. 1999. Identification and expression of Pen c 2, a novel allergen from *Penicillium citrinum*. *Biochem. J.* 341:51.
- Moritz, R. L., J. S. Eddes, G. E. Reid, and R. J. Simpson. 1996. S-pyridylethylation of intact polyacrylamide gels and in situ digestion of electrophoretically separated proteins: a rapid mass spectrometric method for identifying cysteine-containing peptides. *Electrophoresis* 17:907.
- Frohman, M. A., M. K. Dush, and G. R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* 85:8998.
- France, R. M., D. S. Sellers, and S. H. Grossman. 1997. Purification, characterization, and hydrodynamic properties of arginine kinase from gulf shrimp (*Penaeus aztecus*). *Arch. Biochem. Biophys.* 345:73.
- Szasz, G., W. Gruber, and E. Bernt. 1976. Creatine kinase in serum. I. Determination of optimum reaction conditions. *Clin. Chem.* 22:650.
- Kam, K. L., and K. H. Hsieh. 1994. Comparison of three in vitro assays for serum IgE with skin testing in asthmatic children. *Ann. Allergy* 73:329.
- Arruda, L. K., L. D. Vailes, M. L. Hayden, D. C. Benjamin, and M. D. Chapman. 1995. Cloning of cockroach allergen, Bla g 4, identifies ligand binding proteins (or calycins) as a cause of IgE antibody responses. *J. Biol. Chem.* 270:31196.
- Furukohri, T., S. Okamoto, and T. Suzuki. 1994. Evolution of phosphagen kinase (III): amino acid sequence of arginine kinase from the shrimp *Penaeus japonicus*. *Zool. Sci.* 11:229.
- Dumas, C., and J. Camonis. 1993. Cloning and sequence analysis of the cDNA for arginine kinase of lobster muscle. *J. Biol. Chem.* 268:21599.
- Yokota, K., Y. Yazawa, and S. Nakamura. 1994. Purification and molecular cloning of arginine kinase from tail muscle of crayfish *Procambarus clarkii*. *Proc. Jpn. Acad., Ser. B, Phys. Biol. Sci.* 70:48.
- Strong, S. J., and W. R. Ellington. 1995. Isolation and sequence analysis of the gene for arginine kinase from the chelicerate arthropod, *Limulus polyphemus*: insights into catalytically important residues. *Biochim. Biophys. Acta.* 1246:197.
- Zhou, G., T. Smasundaram, E. Blank, G. Parthasarathy, W. R. Ellington, and M. Chapman. 1998. Transition state structure of arginine kinase: implications for catalysis of bimolecular reactions. *Proc. Natl. Acad. Sci. USA* 95:8449.
- Pereira, C. A., G. D. Alonso, M. C. Paveto, A. Iribarren, M. L. Cabanas, H. N. Torres, and M. M. Flawia. 2000. *Trypanosoma cruzi* arginine kinase characterization and cloning: a novel energetic pathway in protozoan parasites. *J. Biol. Chem.* 275:1495.

39. Shanti, K. N., B. M. Martin, S. Nagpal, D. D. Metcalfe, and P. V. Subba-Rao. 1993. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE binding epitopes. *J. Immunol.* 151:5354.
40. Subba-Rao, P. V., D. Rajagopal, and K. A. Ganesh. 1998. B- and T-cell epitopes of tropomyosin, the major shrimp allergen. *Allergy* 53:44.
41. Breiteneder, H. 1998. Plant-food and seafood allergens—an overview. *Allergy* 53:31.
42. Hoffman, D. R. 1983. Immunochemical identification of the allergens in egg white. *J. Allergy Clin. Immunol.* 71:481.
43. Taylor, S. L., R. F. Lemanske, Jr., R. K. Bush, and W. W. Busse. 1987. Food allergens: structure and immunologic properties. *Ann. Allergy* 59:93.
44. Maleki, S. J., R. A. Kopper, D. S. Shin, C. W. Park, C. M. Compadre, H. Sampson, A. W. Burks, and G. A. Bannon. 2000. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J. Immunol.* 164:5844.
45. Binder, M., V. Mahler, B. Hayek, W. R. Sperr, M. Scholler, S. Prozell, G. Wiedermann, P. Valent, R. Valenta, and M. Duchene. 2001. Molecular and immunological characterization of arginine kinase from the Indianmeal moth: *Plodia interpunctella*, a novel cross-reactive invertebrate pan-allergen. *J. Immunol.* 167:5470.
46. Valenta, R., M. Duchene, C. Ebner, P. Valent, C. Sillaber, P. Deviller, F. Ferreira, M. Tejkl, H. Deelmann, D. Kraft, and O. Scheiner. 1992. Proflins constitute a novel family of functional plant pan-allergens. *J. Exp. Med.* 175:377.
47. Ebbelhof, K., A. M. Dahl, H. Frokiaer, A. Norgaard, L. K. Poulsen, and V. Barkholt. 1995. Purification of egg-white allergens. *Allergy* 50:133.
48. Fiocchi, A., P. Restani, E. Riva, G. P. Mirri, I. Santini, L. Bernardo, and C. L. Galli. 1998. Heat treatment modifies the allergenicity of beef and bovine serum albumin. *Allergy* 53:798.
49. Bugajska-Schretter, A., L. Elfman, T. Fuchs, S. Kapiotis, H. Rumpold, R. Valenta, and S. Spitzauer. 1998. Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca^{2+} depletion. *J. Allergy Clin. Immunol.* 101:67.
50. Hitchcock-DeGregori, S. E., Y. Song, and J. Moraczewska. 2001. Importance of internal regions and the overall length of tropomyosin for actin binding and regulatory function. *Biochemistry* 40:2104.
51. Ellington, W. R. 2001. Evolution and physiological roles of phosphagen systems. *Annu. Rev. Physiol.* 63:289.
52. Musmand, J. J., C. B. Daul, and S. B. Lehrer. 1993. Crustacea allergy. *Clin. Exp. Allergy* 23:722.
53. Leung, P. S., Y. C. Chen, and K. H. Chu. 1999. Seafood allergy: tropomyosins and beyond. *J. Microbiol. Immunol. Infect.* 32:143.
54. Platts-Mills, T. A., and M. D. Chapman. 1991. Allergen standardization. *J. Allergy Clin. Immunol.* 87:621.
55. Van Ree, R. 1997. Analytic aspects of the standardization of allergenic extracts. *Allergy* 52:795.
56. Vieths, S., A. Hoffmann, T. Holzhauser, U. Muller, J. Reindl, and D. Haustein. 1998. Factors influencing the quality of food extracts for in vitro and in vivo diagnosis. *Allergy* 53:65.