

Determination of Storage Conditions for Shrimp Extracts: Analysis of Specific IgE-Allergen Profiles

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SUMMARY The consumption of shrimp is a common cause of food hypersensitivity reactions. Shrimp allergy is diagnosed using a skin prick test (SPT) as well as by food challenges. Due to the lack of a wide variety of commercial shrimp extracts for SPTs, we selected various shrimp species for the preparation of local shrimp extracts. However, optimal storage conditions for the shrimp extracts which also maintains allergenic potency has not yet been identified. The objective of the present study was to determine the potency of the shrimp extracts under different storage conditions and durations. Specific IgE-allergen profiles of eight shrimp-allergic patients were investigated by using sera incubated with extracts prepared from lyophilized raw or boiled shrimp, which were stored at 4°C or -20°C for up to 4 weeks. When stored at -20°C, most allergens were preserved after 4 weeks. However, storage at 4°C results in few allergens remaining after 2 weeks. Boiled-shrimp extracts stored at 4°C and -20°C contained higher amounts of IgE-allergen complexes than raw-shrimp extracts. Moreover, in both raw and boiled shrimp extracts, the IgE bound 36-40 kDa allergens constituted the major proteins since they were observed in all IgE-allergen profiles. In conclusion, we recommend that shrimp extracts are stored at -20°C for 4 weeks to prevent the loss of allergens.

Shrimp is a popular inclusion in many diets throughout the world. However, it is also well-known as a common cause of food hypersensitivity reactions. In Thailand, black tiger shrimp (*Penaeus monodon*, *Pm*), a seawater shrimp, is one of the most consumed species. Hypersensitivity to shrimp appears to be a major cause of food allergy in both adults (65%) and children (66%).¹⁻³ Upon contact or ingestion, sensitized individuals develop allergic reactions affecting the skin (oropharyngeal pruritus, 90%; urticaria, 61%; angioedema, 52%), respiratory tract (wheezing, 42%), gastrointestinal tract (abdominal distress, 35%) and the cardiovascular system (syncope, hypotension, anaphylaxis or shock, 10%).^{4,5}

Shrimp-sensitized patients can be screened using a skin prick test (SPT) and confirmed by oral-food challenges. Shrimp extracts are commercially available and are prepared from shrimp such as *Penaeus aztecus*, a species that is consumed in eastern parts of the US.³ Thai-allergic patients are sensitized to *Penaeus monodon* which is consumed in Thailand.³ Thus, commercial extracts may be less specific than those prepared from local shrimp popu-

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lations, possibly resulting in misdiagnosis using SPT.³ For this reason, extracts from local shrimp species are desirable for local allergy centers. However, optimal storage conditions for such extracts that do not lead to a reduction in potency are not known. The aim of the present study was to examine the potency of the lyophilized raw or boiled shrimp extracts stored at 4°C or -20°C at different time intervals. This was done *via* an analysis of the specific IgE-allergen profiles from sera of shrimp-allergic patients.

MATERIALS AND METHODS

Shrimp-allergic patients

Sera from 8 allergic patients, aged 5-15 years, were randomly selected from 40 shrimp-allergic patients, as confirmed by oral-food challenges to the black tiger shrimp. These patients had a positive result to prick tests to boiled *Pm* as well as SPTs to freshly-prepared lyophilized raw *Pm* extracts. Sera were collected and stored at -20°C until analysis. The protocol was approved by the Institutional Review Boards, Siriraj Hospital. Informed consent was obtained from all patients or their parents, as necessary.

Preparation of shrimp extraction solution

Black tiger shrimps were purchased from the local market and the shells and heads were removed. To prepare raw shrimp extract, shrimps were lyophilized and stored at -20°C until use. To prepare boiled shrimp extract, the shrimps were boiled in deionized water for 15 minutes before lyophilization and stored at -20 °C until required. Extracts were prepared by diluting lyophilized raw or boiled shrimps in Coca's solution (29.8 mM NaHCO₃, 86 mM NaCl and 42.5 mM phenol⁶) which was purchased from the Department of Pharmacy, Siriraj Hospital at 1:10 (w/v). The solutions were stirred for 1 hour at 4°C before centrifugation at 17,210 x *g* for 30 minutes. The supernatants were then sterilized by filtering and stored in the absence of protease inhibitors at 4°C and -20°C. Total protein concentrations of all samples were determined using the Bicinchoninic acid (BCA) protein assay. They were subsequently analyzed for specific IgE-allergen profiles on the 1st, 7th and 28th days using human-specific IgE from patient sera *via* immunoblotting.

SDS-PAGE and specific IgE immunoblotting

Fifty micrograms of total protein of individual shrimp extracts from different storage temperatures and times were electrophoresed on 12% SDS-gels under non-reducing conditions. The separated proteins in the gels were stained by 0.08% (w/v) Coomassie brilliant blue G-250 dye. Alternatively, the SDS-PAGE separated proteins were transblotted onto 0.45 µm nitrocellulose membrane. The blotted membrane was blocked with 3% (w/v) non-fat dried milk. The blot was then placed in 1/5-1/10 diluted patient's serum and kept at 4°C for 18 hours. IgE bound proteins were detected by using mouse anti-human IgE horseradish peroxidase (HRP) conjugate (KPL, MD, USA), chemiluminescent HRP substrate (Millipore, MA, USA) and autoradiography.

RESULTS

Shrimp extract - total protein

Lyophilized raw and boiled shrimp extracts were prepared from 100 mg dry weight of the respective shrimp preparations and yielded 17 mg/ml of total protein for raw shrimp extracts and 6 mg/ml of total protein from boiled shrimp extracts. Equal amounts of total proteins of each extract were used for the SDS-PAGE gel and immunoblotting experiments.

4°C-stored lyophilized raw shrimp extract

The Coomassie-stained SDS-PAGE gel of the 4°C-stored lyophilized raw shrimp extract revealed six groups of proteins according to their relative molecular masses. These were: <30, 30-35, 36-40, 50, 60-80 and >80 kDa (Profile s1, Fig 1A). On day 28 of the 4°C-storage, only the 60-80 kDa proteins appeared to be intact. The immunoblotting profiles of specific IgE in sera which bound to the shrimp proteins varied among patients (Profiles w1-w8, Fig 1A). Four of the eight profiles (w1, w3, w4 and w8) produced strong signals at two different sets of proteins, namely, 36-40 kDa and 60-80 kDa (Fig. 1A). Three serum profiles (w1, w3, w4) showed strong signal intensities with three groups of allergens (<30, 30-35, and 36-40 kDa, Fig. 1A). Profile w8 yielded high signal intensity with allergens of different molecular masses, *i.e.* 50, 60-80, and >80 kDa (Fig. 1A). Interestingly, the 50 and 60-80 kDa

allergens remained stable upto 28 days of storage (Fig. 1A). However, most of the low MW allergens (<50 kDa) were substantially degraded after 7 days of the storage (Fig. 1A). The numbers of sera containing specific IgE to allergens in the shrimp extract after 28 days of storage is shown in Fig. 1B. No more than 4 of these groups were detected by any of the patient serum preparations.

4°C-stored lyophilized boiled shrimp extract

SDS-PAGE of this extract shows a single group of proteins at 36-40 kDa is predominant (Profile s1, Fig 2A). The use of specific IgE immunodetection showed that all profiles, with the exception of w7, produced high signals for group 36-40 kDa allergens (Fig 2A). In addition, two other groups of al-

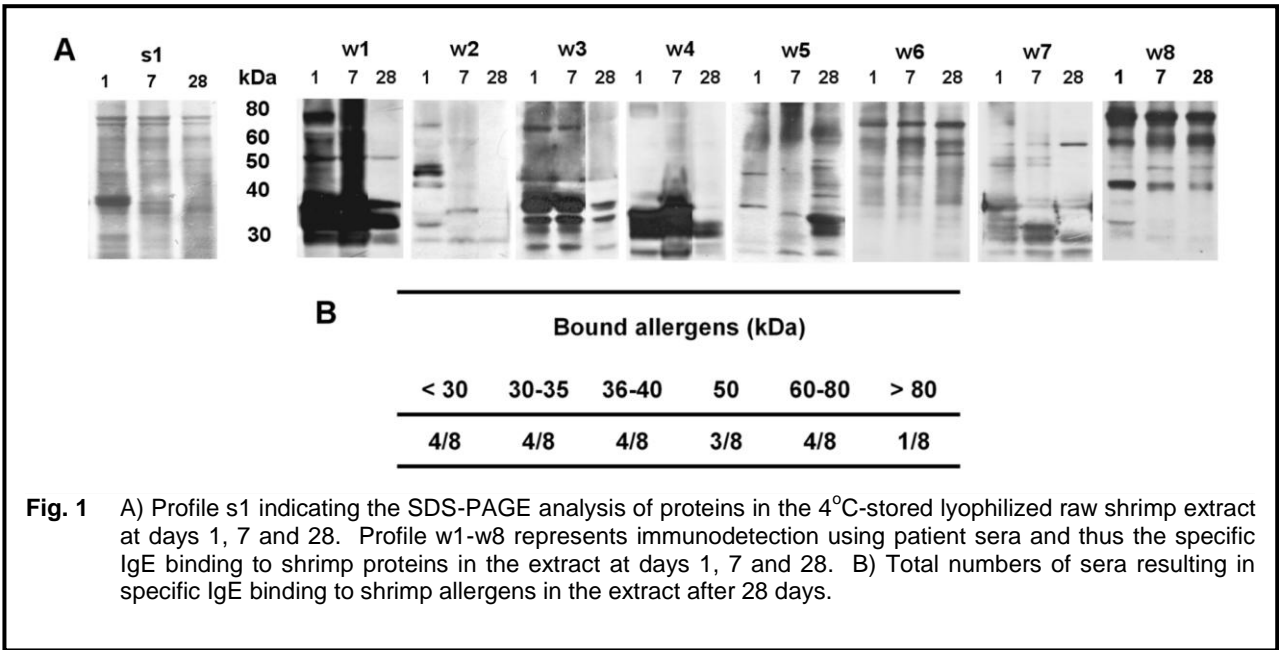


Fig. 1 A) Profile s1 indicating the SDS-PAGE analysis of proteins in the 4°C-stored lyophilized raw shrimp extract at days 1, 7 and 28. Profile w1-w8 represents immunodetection using patient sera and thus the specific IgE binding to shrimp proteins in the extract at days 1, 7 and 28. B) Total numbers of sera resulting in specific IgE binding to shrimp allergens in the extract after 28 days.

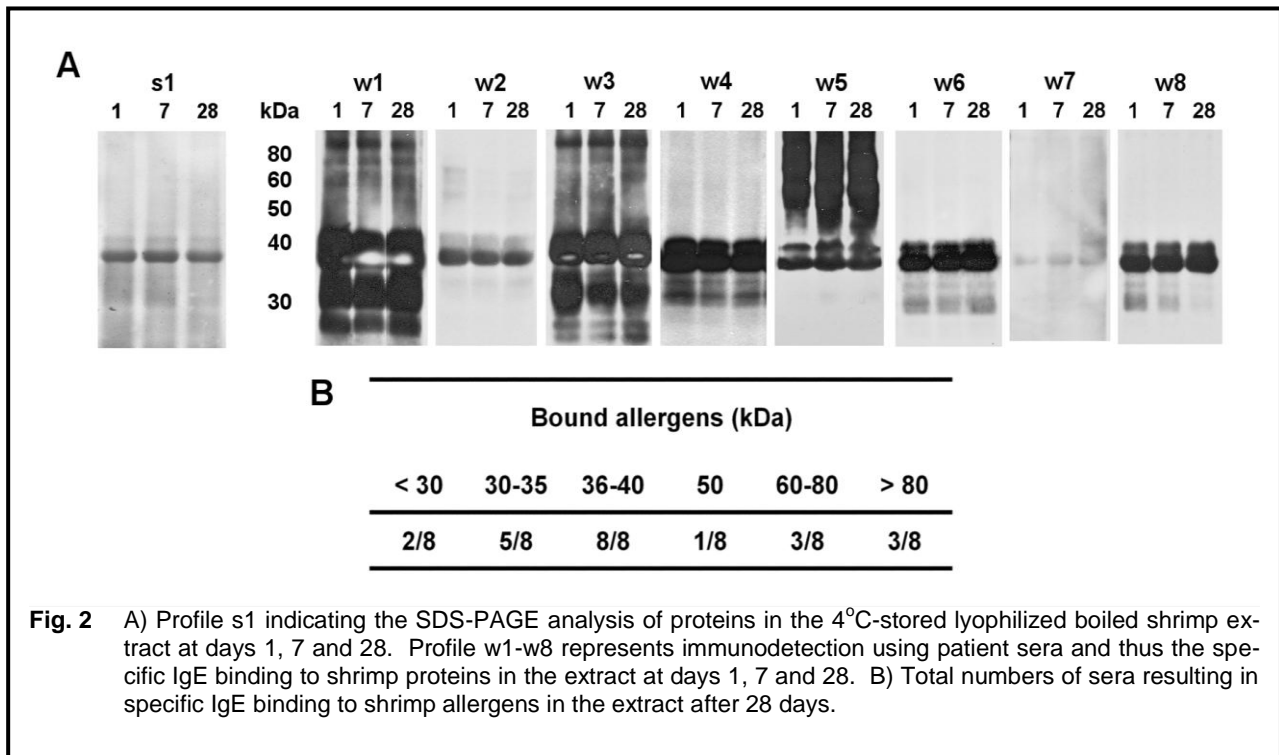
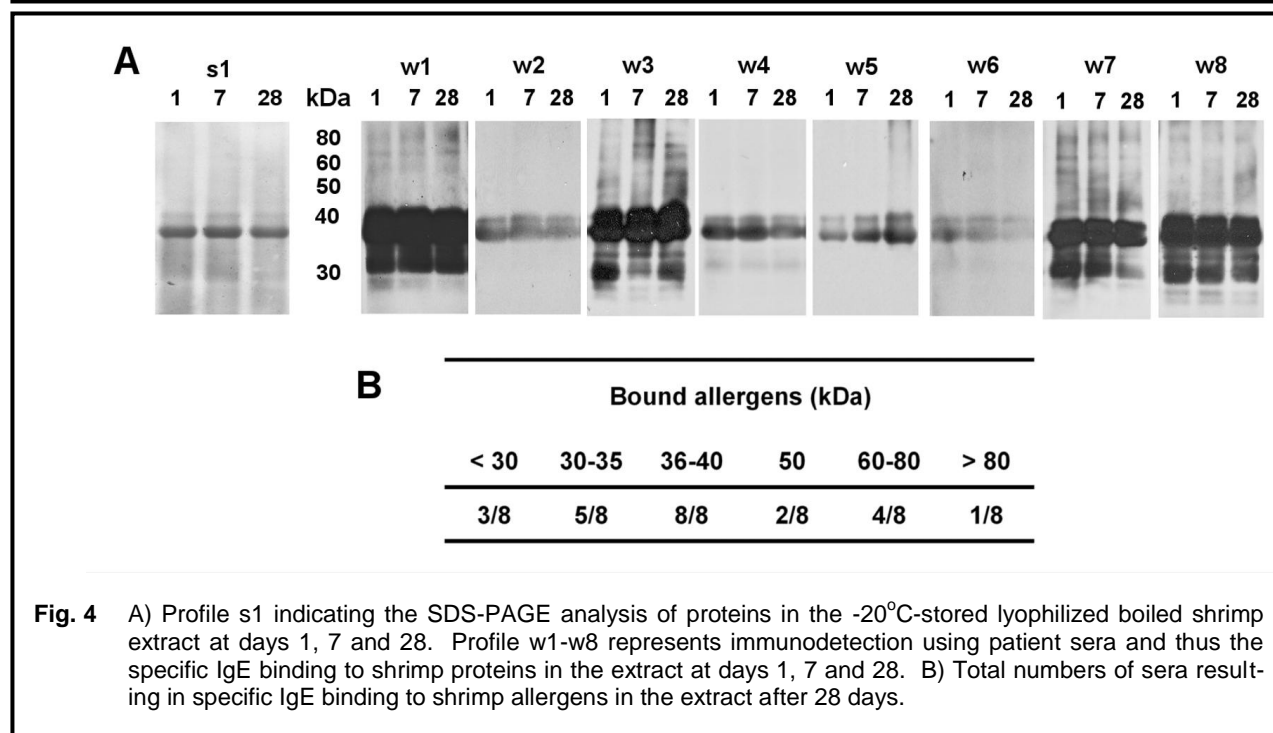
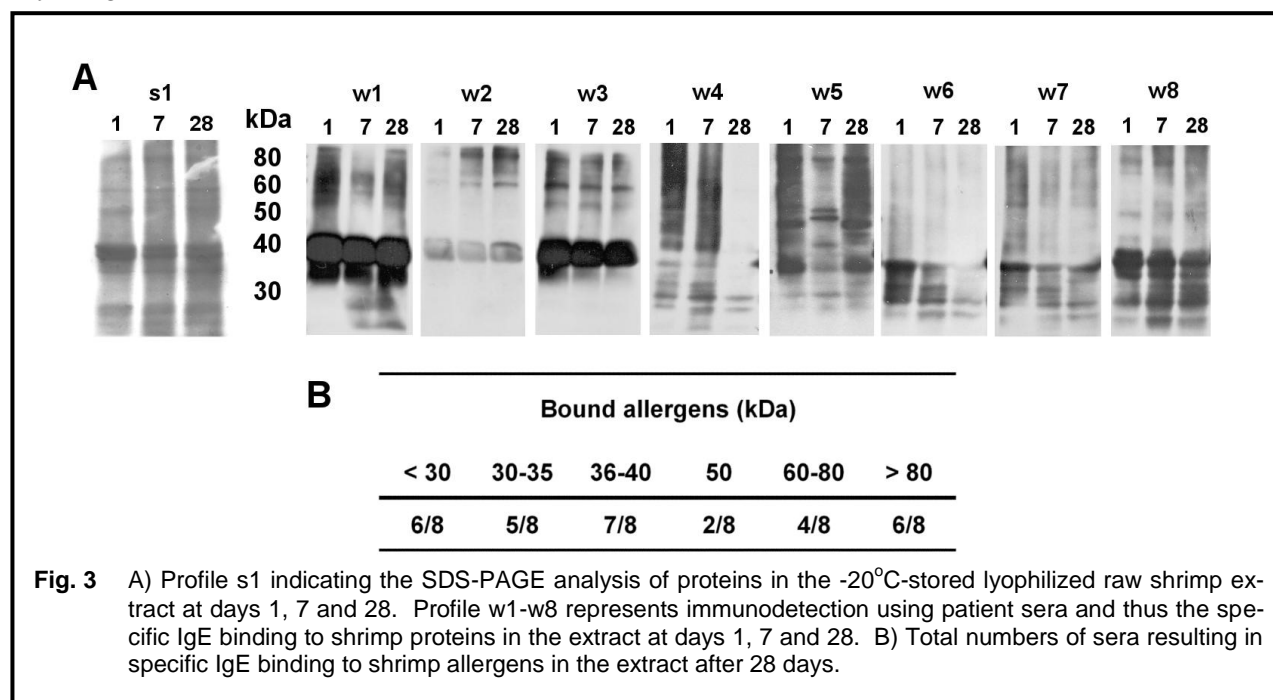


Fig. 2 A) Profile s1 indicating the SDS-PAGE analysis of proteins in the 4°C-stored lyophilized boiled shrimp extract at days 1, 7 and 28. Profile w1-w8 represents immunodetection using patient sera and thus the specific IgE binding to shrimp proteins in the extract at days 1, 7 and 28. B) Total numbers of sera resulting in specific IgE binding to shrimp allergens in the extract after 28 days.

lergens (30-35 and more than 80 kDa) were observed. The 30-35 kDa allergens were observed in profiles, w1, w3, w4, w6, and w8, while the >80 kDa allergens could be detected in profiles, w1, w3 and w5 (Fig 2A). The low MW 30-35 and 36-40 kDa allergens were detected by most of the serum preparations across all 8 profiles after extract storage for 28 days (Fig 2B).

-20°C-stored lyophilized raw shrimp extract

In contrast to the profiles of 4°C-stored lyophilized raw shrimp extract, SDS-PAGE revealed that the -20°C-stored lyophilized raw shrimp extract contained two groups of visible proteins after staining (less than 30 and 36-40 kDa) which persisted for 28 days (Profile s1, Fig 3A). Following immunoblot-



ting, Profiles w1-w8 exhibited specific IgE bound to 36-40 kDa allergens in seven of eight profiles, the exception being w4 (Fig 3A). The 36-40 kDa allergens remained after 28 days (Fig 3A). Five profiles (w4-w8) produced weak signals for the low MW allergens (less than 30 and 30-35 kDa, Fig 3A), while three profiles (w1-w3) produced weak but specific IgE binding to high MW allergens (60-80 kDa, Fig 3A). After 28 days of storage, four of the groups of proteins (less than 30, 30-35, 36-40 and more than 80 kDa allergens) were detected by most of the serum samples from patients (Fig 3B).

-20°C-stored lyophilized boiled shrimp extract

A stained SDS-PAGE gel of the -20°C-stored lyophilized boiled shrimp extract showed a single group of visible proteins (36-40 kDa) which was observed throughout the 28-day period (Profile s1, Fig 4A). Analysis of specific IgE-allergen profiles in the extract showed that only two groups of major allergens, 30-35 and 36-40 kDa, were visible (Fig 4A, B). Four profiles (w1, w3, w7, w8) produced strong signals while the intensity of this signal was moderate for the 36-40 kDa allergens in the remaining four profiles (w2, w4-w6) (Fig 4A). After 28 days, the low MW 30-35 and 36-40 kDa allergens were detected by most of the patient serum samples (Fig 4B), as was also observed for the 4°C-stored lyophilized boiled shrimp extract.

DISCUSSION

Coca's solution has been used for extraction of dust mite⁷ and ragweed⁸ antigens for SPT. However, the use of Coca's solution for the extraction of shrimp allergens has not yet been reported. Since it contains simple components (NaHCO₃, NaCl and phenol) and can be prepared locally, our study examined the allergenicity of shrimp extracts suspended in Coca's solution. SDS-PAGE analysis showed that protein contents of the four extracts varied and were dependant on the cooking process, storage temperatures and storage duration. Immunoblot analysis detected a number of allergens using specific IgE, and in all four shrimp extracts. Therefore, Coca's solution is suitable for the extraction of allergens from both lyophilized raw and boiled shrimp.

Several factors were found to be important in the preservation of allergens in the extracts. Sto-

rage temperature is one of the major determinants of allergen stability in extracts, especially in the absence of a preservative. The extracts stored at -20°C, as well as boiled shrimp extracts, retained most of the allergens after 28 days. In contrast, storage at 4°C diminished the low MW allergens (<50 kDa) in the raw extract after 7 days. Another likely factor that could affect allergen stability is specific proteases present in the extract. Our study shows that the boiled shrimp extract contained all allergen contents, even when stored at 4°C. This may be due to heat-inactivation of proteases in the extract caused by boiling.

In order to determine whether more than one type of extract can result in shrimp sensitization, we examined specific IgE-allergens profiles in both lyophilized raw and boiled shrimp extracts. All profiles produced strong signal intensities corresponding to IgE bound to 36-40 kDa allergens in both the raw and boiled extracts at day 1. However, this remained only in the boiled extracts after 28 days, suggesting that the 36-40 kDa allergens are more stable in boiled rather than raw extracts. The signal caused by specific IgE bound allergens might reflect altered epitopes on allergens that cause an increase in IgE affinity. It has been suggested previously that thermal processing may alter epitopes of allergens leading to enhanced antibody affinities.^{3,16} Thus it is possible that the use of boiled shrimp extracts may be sufficient in diagnosing shrimp allergies.

It is apparent from our findings that the 36-40 kDa allergens in both raw and boiled shrimp extracts are major allergenic components, since 28 of 32 immunoblot profiles identified this group as being bound to the specific IgE preparations. Currently, there are several known allergenic proteins isolated from different species of shrimp. In boiled shrimp extract, two identified major allergens have been documented.^{9,10} One of these is the 38 kDa muscle protein, tropomyosin of *Pm* shrimp, or Pen m 1.⁹ Tropomyosin is a pan-allergen since a specific IgE cross-reacts with a broad range of other invertebrates such as lobster, crab, cockroaches, house dust mites, mollusks, squid, oyster, snail, mussels, clams and scallops.¹¹⁻¹³ Recently, boiled white leg Pacific shrimp (*Litopenaeus vannamei*) extracts were found to contain a 20 kDa muscle protein, myosin light chain, which was identified as an allergen.¹⁰ Two al-

lergens were also identified in raw *Pm* shrimp extracts, a 40 kDa kinase enzyme (arginine kinase or Pen m 2¹⁴) and a 20 kDa sarcoplasmic calcium-binding protein.¹⁵ The 36-40 kDa allergens in -20°C stored raw, 4°C and -20°C stored boiled extracts from our study may possibly contain Pen m 1 and Pen m 2. Further experiments will be required in order to unequivocally establish their identities. Since very few of our immunoblot profiles showed specific IgE bound allergens towards molecular weights less than 30 kDa, the 20 kDa allergens already described above may not feature as shrimp allergenic proteins in Thai individuals.

It is our recommendation from this study that shrimp extracts be prepared from lyophilized boiled shrimp using Coca's solution, and are stored at -20°C to reduce allergen degradation. These extracts may then be stored for up to 28 days without substantial detriment to the samples.

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