

Identification and Characterization of an Arginine Kinase as a Major Allergen from Silkworm (*Bombyx mori*) Larvae

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Key Words

Arginine kinase • RT-PCR • *Bombyx mori* • Immunoblot • ELISA • Cross-reactivity

Abstract

Background: The silkworm, *Bombyx mori*, is an important insect in the textile industry and its pupa are used in Chinese cuisine and traditional Chinese medicine. The silk, urine and dander of silkworms is often the cause of allergies in sericulture workers and the pupa has been found to be a food allergen in China. Recent studies have focused on reporting cases of silkworm allergies, but only a few studies have addressed the specific allergens present in the *B. mori* silkworm. **Methods:** We collected sera from 10 patients with a positive skin prick test to silkworm crude extract (SCE) and analyzed these samples by Western blot and ELISA. The cDNA of arginine kinase from the *B. mori* silkworm was also cloned and expressed in high yield in *Escherichia coli*. Allergenicity and cross-allergenicity of the recombinant *B. mori* arginine kinase (rBmAK) were investigated by ELISA inhibition assay. **Results:** Collected sera all reacted to a 42-kDa protein in a Western blot with SCE as the antigen. Preincubation of sera with rBmAK eliminated the reactivity of the patients' sera to this 42-kDa band. All patient sera also exhibited pos-

itive reactivity to SCE in an ELISA assay. BmAK also demonstrated cross-reactivity with a recombinant AK from cockroach. **Conclusion:** Arginine kinase from the *B. mori* silkworm is a major allergen and crossreacts with cockroach AK.

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Introduction

China has a sericulture industry that has developed over thousands of years [1] to produce silk from silkworms. Silkworm pupa are also included as a protein-rich delicacy in Chinese cuisine [2] as well as in traditional Chinese medicine for the treatment of hypertension and fatty liver [3]. Sensitization to silkworms frequently develops in those who work in sericulture. Silk, excrements, dander of silkworm and even silk products can cause allergic reactions [4–7]. Similarly, the presence of silkworm pupa in food can also cause hypersensitivity [2]. Although case reports of silkworm allergies are documented and reported, the specific allergens from silkworms that cause allergic reactions are poorly characterized. Therefore, the goal of this study was to identify an allergen in the *Bombyx mori* silkworm for characterization.

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Arginine kinases (AKs) are a group of enzymes that are involved in energy catabolism and are exclusively found in invertebrates [8]. Several AKs have recently been characterized as allergens [9–12], and subsequently have been proposed to be a type of panallergen [12]. Therefore, sera from 10 skin prick test (SPT)-positive patients reactive to silkworm crude extract (SCE) were tested for their reactivity to AK from *B. mori* (BmAK). The cDNA of BmAK was cloned and expressed as a His-tagged fusion protein. Its allergenicity and cross-allergenicity were investigated by ELISA inhibition assays. Our results show that rBmAK reacts with sera from patients manifesting a reaction to SCE on a SPT and cross-reacts with the AK from the cockroach *Periplaneta americana*, rPaAK. Therefore, we hypothesize that BmAK is a major allergen from silkworm that requires further study.

Materials and Methods

Preparation of SCE

One gram of *B. mori* larva was homogenized and suspended in acetone at 4°C overnight. The extract was then lyophilized and resuspended in coca's buffer [13]. The resulting supernatant was dialyzed against distilled water, lyophilized and stored at –20°C. All the protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Hercules, Calif., USA).

Human Serum

SPT against SCE (10 µg/ml) were conducted in the People's Hospital of Haikou, Hainan province, China. Histamine (10 mg/ml) was used as the positive control and saline solution was the negative. The SPT results were recorded 15 min later. People with wheal response diameters larger than 5 mm were regarded as positive to the test. 150 patients who claimed allergy to silkworm or silk products participated in the SPT and 38 (25.3%) of these people demonstrated positive reactions to the tests. Sera from 10 positive patients (5 male, 5 female, average age 24 years old, range 11–48) were collected. The sera of 3 healthy people (aged 7, 18 and 65) were used as negative controls in the following assays. All the manipulations were approved by the patients as well as the local medical and ethics authorities.

Cloning of cDNA of BmAK

Total RNA was extracted by using RNeasy mini kit (Qiagen) from 30 mg of *B. mori* larva muscle, which was purchased from a local vendor. Two specific primers were designed based on the sequence of BmAK (No. DQ272299.1) in GenBank. The sequences of the forward primer (F1) and reverse primer (R1) were as follows: F1 5'-ATGATGGTCGACGCCGCA AC-3'; R1 5'-CTTTT-ACAGAGACTTCTCGATCTTGATG-3'. The first strand of the cDNA was synthesized using RNA as the template by a first-strand synthesis kit (Bio Basic Inc.). The PCR parameters were as follows: denaturation at 94°C for 4 min, then 36 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, and then a final extension at 72°C for 10 min. The resulting product was cloned into

pMD 18 T-vector (Takara Inc.) and sequenced. The sequence of BmAK was then aligned with other AK allergens using software ClustalX (1.8) [14].

Expression of BmAK

A pair of primers (F2/R2) which include Nde I and Hind III restriction sites were synthesized as follows (restriction sites underlined): F2 5'-GAACATATGATGGTCGACGCCGCAAC-3' and R2 5'-GAAAGCTTTTACAGAGACTTCTCGATCTTGATG-3'. The primers were used to clone the open reading frame of BmAK cDNA. The PCR reaction was optimized as follows: denaturation at 94°C for 4 min, then 30 cycles of 94°C for 30 s, 58°C for 1 min and 72°C for 1 min, and then elongation for 10 min at 72°C. The cloned gene was cloned into pET-28a (+) Vector (Novagen) and transformed into *Escherichia coli* BL21. Cells were grown to an OD₆₀₀ of 0.6–0.8 at 37°C. BmAK expression was induced with 1 mM IPTG and the cells were harvested 3 h later.

Purification of Recombinant BmAK (rBmAK)

The harvested cells were resuspended in cell lysis buffer 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 8.0) and subjected to sonication for 5 × 10 s with 10 s intervals. The cell lysate was clarified by centrifuge at 15,000 g for 20 min and the supernatant was loaded to a Ni-NTA column (Amersham Pharmacia Inc.) equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.2) and 200 mM NaCl. After exhaustive washing with the same buffer, the column was eluted with 2 column volumes (40 ml) of 200 mM imidazole in the equilibration buffer. The elution peak was collected, dialyzed against 1× PBS and the homogeneity was analyzed by 12% SDS-PAGE [15].

Immunoblot and Immunoblot Inhibition Assay of SCE

SCE was first applied to SDS-PAGE (150 µg per-well) and then electrophoretically transferred to nitrocellulose membrane. The membrane was then cut into small strips of about 4 mm wide and blocked in blocking buffer (Tris-buffered saline solution containing 5% BSA and 0.1% Tween 20, TBST, pH 7.6). After blocking for 1 h, the blots were incubated with sera from patients (1:5 diluted in TBST) as well as control sera from 3 healthy people. After being washed 3 times, the biotin-labeled goat anti-human IgE antibody (Katzenbach Partners LLC; 1:3,000 diluted in TBST) and horseradish peroxidase-conjugated streptavidin (Katzenbach; 1:2,000 diluted in TBST) were sequentially added. Lastly, the bands were visualized by using 3,3',5,5'-tetramethyl-benzidine (TMB; Katzenbach) as substrate [16]. For the immunoblot inhibition assay, 150 µg silkworm crude extract was blotted into the nitrocellulose membrane and then reacted with pooled sera (1:5 diluted from patients 1, 2 and 3), which was preincubated with 10 µg purified rBmAK for 1 h at room temperature.

ELISA and ELISA Inhibition Assay of rBmAK

In ELISA assay, 0.1 µg SCE was coated into a 96-well microtiter plate containing 200 µl PBS and incubated at 4°C overnight. After being blocked with BSA in PBS at 4°C overnight and washed 3 times, 100 µl of sera from patients 1–10 and 3 healthy controls (1:10 diluted with PBS) were added and incubated overnight at 4°C. The bound IgE was then reacted with 100 µl biotin-labeled goat anti-human IgE (1:3,000) followed by 100 µl horseradish peroxidase-conjugated streptavidin (1:2,000). TMB was used as the substrate and the reaction was stopped by 100 µl of 2 M H₂SO₄.

BmAK	1	MVDAATLEKLEAGFSKLQGS-DSKSLKKYLTRVFDLKNKKT-SFGSTLLDCIQSGVE	58
Plo i 1	1	MVDAATLEKLEAGFSKLQGS-DSKSLKKYLTRVFDLKNKKT-SFGSTLLDCIQSGVE	58
PaAK	1	MVDAAVLEKLEAGFAKLAAS-DSKSLKKYLTKVFDNLKTKKTPSFGSTLLDVIOQSGLE	59
LvAK	1	MADAADVIEKLEAGFKKLEAATDCKSLKKYLTKVFDKLDKKT-SLGLATLLDVIOQSGVE	59
Pen m 2	1	MADAADVIEKLEAGFKKLEAATDCKSLKKYLSKAVFDQLKEKKT-SLGLATLLDVIOQSGVE	59
BmAK	59	NLD SGVGIYAPDAEAYVVFADLFDPIIEDYHNGFKKTDKHPKKNUGDVEDTLGNLDPAGEF	118
Plo i 1	59	NLD SGVGIYAPDAEAYVVFADLFDPIIEDYHNGFKKTDKHPKKNUGDVEDTLGNLDPAGEF	118
PaAK	60	NLD SGVGIYAPDAEAYVVFADLFDPIIEDYHNGFKKTDKHPKKNUGDVEDTLGNLDPAGEY	119
LvAK	60	NLD SGVGIYAPDAEAYTLFAPLFDPIIEDYHNGFKKTDKHPKKNUGDVEDTLGNLDPAGEY	119
Pen m 2	60	NLD SGVGIYAPDAEAYTLFAPLFDPIIEDYHNGFKKTDKHPKKNUGDVEDTLGNLDPAGEY	119
BmAK	119	VVSTRVRCGRSLEGYFPNCLTESQYKEMEDKVSSTLSLEGELKGTFFPLTGMSKETQQ	178
Plo i 1	119	VVSTRVRCGRSLEGYFPNCLTESQYKEMEDKVSSTLSLEGELKGTFFPLTGMSKETQQ	178
PaAK	120	VVSTRVRCGRSLEGYFPNCLTESQYKEMEDKVSSTLSLEGELKGTFFPLTGMSKETQQ	179
LvAK	120	VVSTRVRCGRSLEGYFPNCLTESQYKEMEDKVSSTLSLEGELKGTFFPLTGMSKETQQ	179
Pen m 2	120	VVSTRVRCGRSLEGYFPNCLTESQYKEMEDKVSSTLSLEGELKGTFFPLTGMSKETQQ	179
BmAK	179	QLIDDHFLFKEGDRFLQAANACRFUPTGRGIYHNDNKTFLVWVNEEDHLRIISMOMGGDL	238
Plo i 1	179	QLIDDHFLFKEGDRFLQAANACRFUPTGRGIYHNDNKTFLVWVNEEDHLRIISMOMGGDL	238
PaAK	180	KLIDDHFLFKEGDRFLQAANACRFUPTGRGIYHNDNKTFLVWVNEEDHLRIISMOMGGDL	239
LvAK	180	KLIDDHFLFKEGDRFLQAANACRFUPTGRGIYHNDNKTFLVWVNEEDHLRIISMOMGGDL	239
Pen m 2	180	KLIDDHFLFKEGDRFLQAANACRFUPTGRGIYHNDNKTFLVWVNEEDHLRIISMOMGGDL	239
BmAK	239	QGVYKRLVSAVNEIEKKIPFSSHEDRLGFLTFCPTNLGTTVRASVHIKLPKLAADKKKLEE	298
Plo i 1	239	QGVYKRLVSAVNEIEKKIPFSSHEDRLGFLTFCPTNLGTTVRASVHIKLPKLAADKKKLEE	298
PaAK	240	QGVYKRLVSAVNEIEKKIPFSSHEDRLGFLTFCPTNLGTTVRASVHIKLPKLAADKKKLEE	299
LvAK	240	QGVYKRLVSAVNEIEKKIPFSSHEDRLGFLTFCPTNLGTTVRASVHIKLPKLAADKKKLEE	299
Pen m 2	240	QGVYKRLVSAVNEIEKKIPFSSHEDRLGFLTFCPTNLGTTVRASVHIKLPKLAADKKKLEE	299
BmAK	299	VASKYHLOVRGTRGEHTEAEGGVYDISNKRRMGLTEYDAVKEMYDGIKLEKSL	355
Plo i 1	299	VASKYHLOVRGTRGEHTEAEGGVYDISNKRRMGLTEYDAVKEMYDGIKLEKSL	355
PaAK	300	VAGKYHLOVRGTRGEHTEAEGGVYDISNKRRMGLTEYDAVKEMYDGIKLEKSL	356
LvAK	300	VAGKYHLOVRGTRGEHTEAEGGVYDISNKRRMGLTEYDAVKEMYDGIKLEKSL	356
Pen m 2	300	VAGKYHLOVRGTRGEHTEAEGGVYDISNKRRMGLTEYDAVKEMYDGIKLEKSL	356

Fig. 1. Alignment of AKs from different sources. Plo i 1 = *Plodia interpunctella* AK; PaAK = *Periplaneta americana* AK; LvAK = *Litopenaeus vannamei* AK; Pen m 2 = *Penaeus monodon* AK.

The OD₄₅₀ of each well was measured using an ELISA microplate reader (Bio-Rad). The cutoff value for positive ELISA results was set as the mean value plus 10 standard deviations (SD) of the sera from the 3 healthy controls. For the inhibition assay, pooled sera from patients 4, 5 and 6 were pre-incubated with inhibitors (rBmAK, rPaAK [17] and SCE) at different concentrations (1, 10, 50, 100, 500 and 1,000 µg/ml) for 2 h at 37°C and then reacted with the coated rBmAK (0.1 µg/well) or SCE (0.1 µg/well) as antigen. Data from 3 measurements were accumulated. The inhibition rates were calculated according to the following formula: inhibition (%) = (OD₀ - OD_{inhibitor}) / (OD₀ - OD_{BSA}), where OD₀ is the optical density of antigen without any inhibitor, OD_{inhibitor} is the optical density after adding an inhibitor and OD_{BSA} is the optical density with only BSA in the plate.

Results

Cloning and Expression of BmAK cDNA

The cDNA of the arginine kinase from *B. mori* was obtained by RT-PCR and the sequence was confirmed by

comparison with the AK GenBank entry (accession number DQ272299.1). The amino acid sequence of BmAK was deduced from the 1,065-bp open reading frame and 7 consecutive amino acids (²⁷⁰CPTNLGT²⁷⁶) establish the catalytic site of the enzyme. Three residues of the 7 amino acid motif, C271, D61 and R192, are essential for enzyme activity [18–20]. Figure 1 displays the alignment results of BmAK compared to AKs from other invertebrates. The highest identity for BmAK was found to *Plodia interpunctella* AK (Plo i 1; 92%) from the Indianmeal moth. AKs derived from cockroach and 2 shrimp AKs were found to contain 1 more amino acid than AKs derived from 2 insect AKs, which all showed less identity to BmAK than Plo i 1, but maintained identities greater than 80%: 87% for *P. americana* AK (PaAK); 83% for *Litopenaeus vannamei* AK (LvAK), and 81% for *Penaeus monodon* AK (Pen m 2). The cDNA encoding AK from silkworm was cloned and expressed as a 6-histidine-tagged protein, and SDS-PAGE analysis of affinity chromatog-

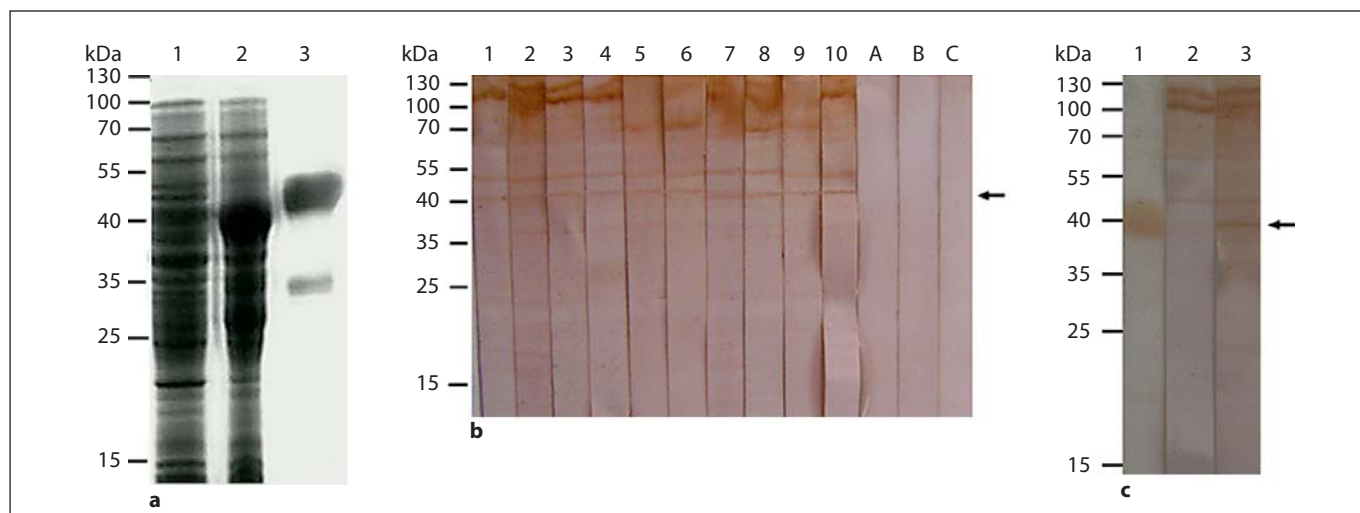


Fig. 2. Characterization of rBmAK and patient sera. **a** SDS-PAGE analysis of BmAK before and after affinity chromatography purification. Lane 1 shows sample before IPTG induction; lane 2 shows sample after IPTG induction, and lane 3 shows purified BmAK with His tag. **b** Western blot of rBmAK with sera from 10 patients and 3 healthy controls. Immunoblot with SCE to allergic patients

and healthy control. Lanes 1–10 show patients who were sera positive by SPT; A, B and C show sera from healthy individuals. Arrow indicates native BmAK. **c** Immunoblot inhibition to SCE with pooled sera from patients 1, 2, and 3. Lane 1 shows rBmAK; lane 2 shows SCE inhibited by rBmAK, and lane 3 shows SCE. Arrow points to rBmAK. All sera were diluted 1:5 in PBS.

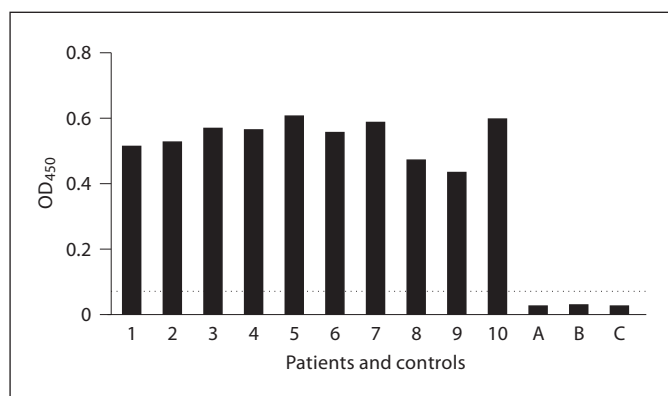


Fig. 3. ELISA assay of human sera IgE binding to SCE. 1–10 are sera from patients positive for reactivity to SCE by SPT; A, B and C are sera from healthy individuals. All sera were diluted 1:10 in PBS. The dotted line represents the cut-off value.

raphy purification identified an apparent molecular weight of approximately 42 kDa for rBmAK and a minor impurity present at 35 kDa (fig. 2a).

IgE/Inhibition Immunoblot Assay

Sera from 10 SCE-SPT-positive patients (5 female and 5 male) were collected. Sera from 3 healthy individuals

were also collected as negative controls. Western blot analysis of patient sera was used to identify patterns of IgE reactivity to SCE (fig. 2b). While all patient sera demonstrated reactivity toward bands at 50 and 42 kDa, subsets of patients showed differences in IgE reactivity. For example, patients 2–4, 7 and 10 reacted with 2 bands, at 110 and 120 kDa. Patient 1 reacted with a 110-kDa band, while patients 5, 6 and 9 reacted with a 70-kDa band. Patient 8 was reactive to bands at 70, 110 and 120 kDa. In contrast, all sera samples from healthy individuals exhibited no reactivity to SCE. Based on sequence analysis of BmAK, we hypothesized that the 42-kDa band detected in Western blot analysis of patient sera represented the panallergen AK. We tested this hypothesis with an immunoblot inhibition assay using preincubation of patient sera with rBmAK. Pooled sera reacted to SCE with bands at 42, 50, 110 and 120 kDa (fig. 2c, lane 3). Following preincubation of the pooled sera with rBmAK, the IgE-binding ability of the 42-kDa band was inhibited, thereby identifying the 42-kDa band as BmAK (fig. 2c, lane 2).

ELISA and ELISA Inhibition Assay

The reactivity of patient sera to SCE was also examined by ELISA assay (fig. 3). Of the 10 patient sera samples tested, all showed binding of SCE by ELISA assay, even when our cutoff value was set as the mean of 3 healthy

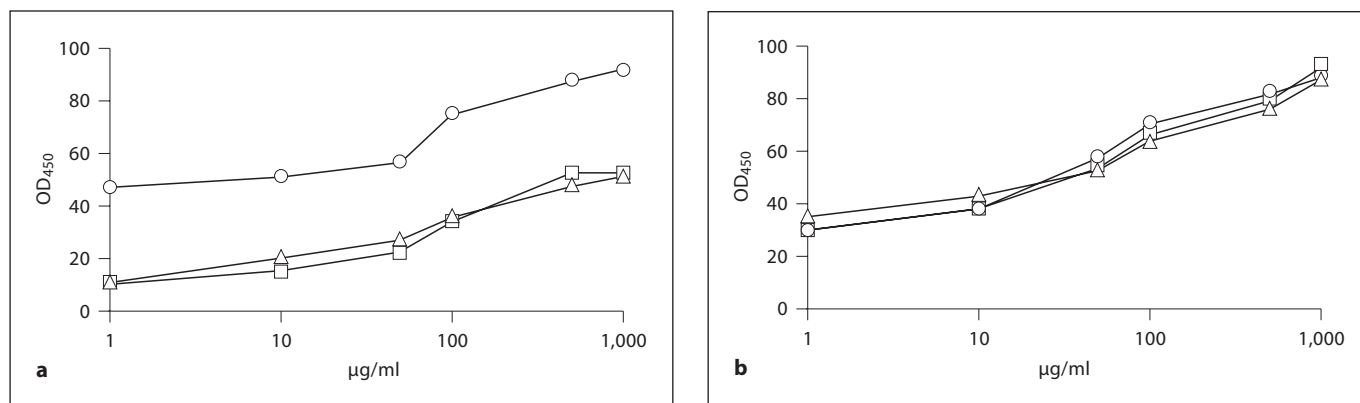


Fig. 4. IgE inhibition experiment. Pooled sera from patients 4, 5, and 6 (diluted 1:10 in PBS) were preincubated with inhibitors at varying concentrations (1, 10, 50, 100, 500 and 1,000 $\mu\text{g/ml}$) and then reacted with coated SCE allergen (**a**) or *rBmAK* (**b**). Inhibitors included SCE (circles), *rBmAK* (rectangles) and *rPaAK* (triangles).

people plus 10 SD. In contrast, the 3 healthy controls gave negligible values on ELISA.

An ELISA inhibition assay was used to investigate the possible cross-reactivity between *BmAK* and another AK derived from cockroaches, PaAK, also prepared in our laboratory (fig. 4). SCE completely inhibited itself at a concentration of 1,000 $\mu\text{g/ml}$, while *rBmAK* and *rPaAK* showed sizable inhibition towards SCE and reached a plateau of inhibition slightly greater than 50% at 500 $\mu\text{g/ml}$. All 3 inhibitors were able to completely abrogate the IgE-binding ability of *rBmAK* at 1,000 $\mu\text{g/ml}$ and showed similar inhibition profiles.

Discussion

China produces half the world supply of raw silk [21], and as a result this industry has a large number of workers exposed to silkworm allergens. Cases of hypersensitivity to silkworms and their products have been reported, and silk farmers and workers in silk filatures who are occupationally exposed to silkworm allergens have been found to develop asthma [4, 5]. Non-occupational exposure to silk can also result in sensitization. For example, allergic reactions can be caused by inhaling or touching allergens from silk products such as quilts and clothes or eating pupa-containing cuisine [2, 6, 7]. However, to our knowledge, no specific allergen has been ascribed to silkworm hypersensitivity until now.

Using sequence alignment analysis, we determined that *BmAK* shares significant similarity (ranging from 81 to 92%) with other AKs that have been associated with

allergenicity. By running FASTA in the Structural Database of Allergenic Proteins, we found that the sequence of *BmAK* shares remarkable similarity with 2 known allergens, Plo i 1 from the Indianmeal moth and Pen m 2 from the black tiger shrimp. The comparison of *BmAK* with Plo i 1 and Pen m 2 was associated with extremely low e-values ($4.3\text{e-}133$ and $1.6\text{e-}119$, respectively), which, as proposed by Schein et al. [22], is indicative that *BmAK* is an allergen. Correspondingly, in data from this study, *BmAK* was shown to react with SCE-positive patient sera and crossreact with *rPaAK*. The combination of these data suggest that *BmAK* is a major silkworm allergen.

In the analysis of sera from 10 patients positive for silkworm allergies, 5 IgE-reactive components were detected with molecular weights of 42, 50, 70, 110 and 120 kDa. This is in sharp contrast to a report in 1997 [23] in which bands of 72, 76, 79 and 82 kDa were detected. This inconsistency could be the result of differences in detection methods used, the population tested and differences in protein preparation. Our inhibition studies, however, did confirm that the detection of a 42-kDa band represented reactivity with *BmAK*.

For workers in sericulture and customers of silk products who are allergic to silkworm allergens, sensitization is probably mediated by skin or mucosal contact, during which the allergens of silkworms are largely able to maintain their native structure. In contrast, during the preparation of silkworm pupa as an ingredient for Chinese cuisine or Chinese medicine, allergens have to withstand extended boiling and the denaturing conditions of the gastrointestinal system. The high IgE-binding frequency of *BmAK* that was maintained under analogous treat-

ment conditions during preparation of *BmAK* for SDS-PAGE analysis supports the robust allergenicity that *BmAK* can maintain under otherwise denaturing conditions. This was specifically evident in analysis of IgE-binding patterns by patient sera of SCE in both Western blot and ELISA analyses. However, it should be noted that different epitopes can be involved in these assays. Preparation of samples for Western blot can damage protein tertiary structure, while sample preparation for ELISA assays largely conserves a protein's native structure.

rPaAK demonstrated cross-reactivity with r*BmAK* in that it was able to completely remove its IgE reactivity and showed comparable profiles with those of r*BmAK* in ELISA inhibition assays. r*BmAK* only attained greater than 50% inhibition towards SCE, indicating the presence of IgE-reactive molecules other than *BmAK*. Given the comparable profiles of SCE with r*BmAK* in inhibition assays, these data imply that the native form of *BmAK* has similar allergenicity to the recombinant one. Cross-reactivity between invertebrate proteins has frequently been attributed to homologous allergens such as tropomyosins [24–26] and AKs [11, 12], although the latter remain largely unexplored. We cloned a tropomyosin from silkworm larva and expressed it as a fusion protein. When tested in an immunoblot assay, the tropomyosin fusion protein reacted with less than 12% of the 18 patient sera tested. The cross-reactivity of *BmAK* and PaAK detected may be relevant to reports of cockroach and silkworm al-

lergies, although additional studies would be needed to confirm whether there is a correlation between them or if there is a primary cause of sensitization.

As observed by Wang et al. [27], gene expression of *BmAK* increases as the larva matures and the gene for *BmAK* is also expressed in pupa, although at relatively low levels. In the present study we only examined the allergenicity of AK from silkworm larva. Additional studies are needed, and have been initiated in our laboratory, to investigate the allergenicity of silkworm larvae at different developmental stages versus silkworm pupae, silkworm moths and silk products. In conclusion, we have demonstrated that silkworm allergies are IgE-mediated and that a cloned AK from *B. mori* silkworms represents a potential allergen. Additionally, this protein showed cross-reactivity with another allergic cockroach AK. To our knowledge, this is the first study to characterize an allergen from silkworm and our results provide valuable insight into the potential for diagnosis and treatment of patients with silkworm allergies.

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