Sequencing Analysis of cDNA Clones Encoding the American Cockroach Cr-PI Allergens

HOMOLOGY WITH INSECT HEMOLYMPH PROTEINS*

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A previous article described the isolation of several Agt22A cDNA clones expressing the American cockroach (Periplaneta americana) Cr-PI allergens recognized by both human atopic IgE antibodies and anti-Cr-PI monoclonal antibodies (Wu, C. H., Lee, M. F., and Liao, S. C. (1995) J. Allergy Clin. Immunol. 96, 352–359). This article presents the nucleotide and deduced amino acid sequence of two cDNA clones encoding major allergens of P. americana. Clones C12 and C20 encode proteins of 685 and 631 amino acids with two potential N-glycosylation sites each. The predicted molecular weights for C12 and C20 cloned proteins are 79,300 and 75,500 with isolectric point values of 6.26 and 6.63, which are compatible with the determined sizes (M, 78,000 and 72,000) and isolectric point value (6.2) of the Cr-PI allergens of P. americana. A high degree of identity (69.1%), including several overlapped predicted central antigenic determinant residues, was found between two allergens. The anti-fusion protein antibody-based enzyme-linked immunosorbent assay was able to detect crude American cockroach extract, Cr-PI, recombinant proteins, and commercial cockroach extracts, which provides further evidence that two allergens share common antigenic determinants. Recombinant allergens of clones C12 and C20 both showed 47.4% skin reactivities on 19 cockroach-sensitive asthmatic patients. Unexpectedly, both clones were antennated by the fact that the inhalant vespid, shrimp, and German cockroach (Blattella germanica) allergens have recently been cloned and sequenced (23–29). We recently reported the construction of a Agt22A cDNA library and the subsequent identification of several clones recognized by anti-Cr-PI mAb. Clones C12 and C20 with 2.6- and 2.4-kilobase inserts, respectively, were recognized by both anti-Cr-PI mAb and human-specific IgE antibodies and were selected and subcloned into expression vectors and used to transform Escherichia coli. Immunoblot analysis of clones C12 and C20 revealed fusion proteins of M, 78,000 and 72,000, respectively, recognized by both anti-Cr-PI mAb and human-specific IgE antibodies (30). In this article we report the complete nucleotide and deduced amino acid sequences of these two cDNA clones encoding the major American cockroach allergens. Unexpectedly, both clones were found to have remarkable sequence similarity to insect storage proteins, insect juvenile hormone-suppressible proteins, and arthropod hemocyanins.

EXPERIMENTAL PROCEDURES

Sequencing of cDNA—IgE-binding and anti-Cr-PI mAb-reactive cDNA clones C12 and C20 were isolated from a Agt22A cDNA library derived from the American cockroach mRNA (22, 30). Phage DNAs were isolated by a polyethylene glycol precipitation method (31). The DNA insert released by NotI and EcoRI digestion was subcloned into the plasmid pSPORT 1 (Life Technologies, Inc.). DNAs were prepared from the transformed E. coli DH5α colonies, and deoxynucleotide sequenc-
ing (32) was performed using the Multi-Plex DNA Sequencing System (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer’s directions. A set of nested deletions was created using the Erase-a-base Ex II/S1 nuclease system (Promega, Madison, WI). 3′-Labeled products were analyzed on 6% polyacrylamide gels and autoradiographed.

Computer Analysis—Sequence data were analyzed using the PC/GENE software (IntelliGenetics, Inc., Mountain View, CA), and the FASTA program (33) was used for the sequencing homology search. The antigenic index was calculated according to the algorithm of Jameson and Wold (34).

Expression and Purification of Fusion Protein—DNA was prepared from a plasmid phage using the phage plaque purification method. A phage insert was ligated into the multiple cloning sites of the pET system (Novagen, Inc., Madison, WI) and used to transform E. coli BL21(DE3) (30, 35–37). Recombinant proteins were recovered under denaturing conditions from inclusion bodies of the host cells, and further purification of fusion proteins under denaturing conditions was performed using rapid affinity column chromatography with the pET His-Tag system as described by Novagen. Refolding was performed using dialysis with gradual removal of the guanidine in 0.1 M Tris-HCl buffer, pH 8.0, and lyophilization. The purified fusion protein in a range of 1.0–2.0 mg/ml was finally dialyzed in 0.1 M Tris-HCl buffer, pH 9.8.

Preparation of Antiserum to Fusion Protein—The fusion protein was used as an immunogen, and polyclonal antibodies were produced from immunized rabbits as described below. A class-specific anti-rabbit IgG (P. americana) was purchased from Zymed Laboratories, Inc. (South San Francisco, CA).

Cockroach Extracts and Preparation of CRA-A and Cr-PI—Cockroach skin extracts were obtained from American cockroaches, Periplaneta americana, Decatur, IL, of eight test heads. PBS containing 50% (v/v) serum of 5% SDS, and the absorbance was determined at 415 nm on an EAR 400 AT Easy Reader (SLT Labinstruments, Salzburg, Austria).

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RESULTS

Sequence Analysis—A 12.2-kb cDNA library derived from the Periplaneta americana mRNA was screened initially with anti-CrA-A polyclonal antibody, and 29 cDNA clones were isolated. Subsequently, several clones recognized by anti-Cr-PI or anti-Cr-PI mAb and human IgE antibodies were identified. Recombinant proteins of M, 78,000 and 72,000 from clones C12 and C20, respectively, were recognized by both anti-Cr-PI mAb and human-specific IgE antibodies (30). The cDNA sequences from C12 and C20 inserts are presented in Fig. 1. The complete sequences of C12 and C20 were shown to be 2418 and 2274 bases long. The C12 insert contains a 7-base pair (bp) 5′-proximal end sequence and a 2055-bp open reading frame that begins with a methionine codon (ATG) and ends at the nucleotide position 2063–2065 (TAA stop codon). The 338-bp 3′-untranslated region includes an AATAAA polyadenylation signal and an 18-bp poly(A) tail. The C20 insert includes a 1-bp 5′-proximal end sequence and an 1895-bp open reading frame that begins with a aspartic acid codon (GAT) and terminates at the nucleotide position 1895–1897 (TGAG stop codon), a 354-bp 3′-end noncoding region, three AATAAA polyadenylation signals, and a 23-bp poly(A) tail. The alignment of the complete nucleotide sequence reveals 75.65% identity between the C12 and C20 clones (Fig. 1). Sequence data on the other P. americana allergen clones isolated from the cDNA library will be reported elsewhere.

Fig. 2 presents the alignment of the complete deduced amino acid sequence of mature C12 and C20. The comparison of deduced amino acid sequence of mature C12 with C20 reveals 69.1% (436 of 631) identities. The complete C12 and C20 inserts correspond to 685- and 631-amino acid polypeptides of calculated molecular weights of 81,175 and 75,511 and the predicted isoelectric point values for mature C12 and C20 are 6.26 and 6.63. The C12-derived protein appears to possess a leader peptide that is indicated in lower case characters in Fig. 2. The 16-amino acid putative leader of C12 (residues 1–16) has features typical of a eukaryotic signal peptide, and the cleavage site conforms to the −3 (valine), −1 (alanine) rule (39–41).

This predicted signal peptide also includes consensus residues alanine, phenylalanine, alanine, and valine at −2, −4, −5 and −6.
The calculated molecular weights of mature C12 (excluding leader sequences) and C20 are 79,300 and 75,500, respectively, compatible with the $M_r$ 78,000 and 72,000 of Cr-PI major allergens determined by SDS-polyacrylamide gel electrophoresis (12). The predicted isoelectric point values of 6.26 and 6.63 for mature C12 and C20, respectively, are also compatible with

**Fig. 1.** Nucleotide sequence of cDNA clones C12 and C20. Sequences are aligned with the translated region, and gaps (—) are introduced to optimize the alignment. $+$, homology with clone C12. The regions encoding potential N-glycosylation sites, the TAA stop codon, and the AATAAA polyadenylation signal sequences are underlined.

**Fig. 2.** Alignment of the deduced amino acid sequences. The single-letter amino acid code is used, and gaps (—) are introduced to optimize the alignment. The initiation methionine is lacking for clone C20. The putative signal peptide of C12 is in lower case characters. The potential $N$-glycosylation sites, NFT and NTT, are underlined. $+$, homology with clone C12. The potential central antigenic determinant residues as described in the text are underlined and in lower case characters.
the determined isoelectric point value of 6.2 for Cr-PI (20). The deduced amino acid sequence of C12 contains three cysteines and two potential N-glycosylation sites, Asn-Phe-Thr and Asn-Thr-Thr, encoded by nucleotides 599–607 and 704–712, respectively. Clone C20 contains four cysteines and two potential N-glycosylation sites, Asn-Phe-Thr and Asn-Thr-Thr, encoded by nucleotides 425–433 and 530–538, respectively. Table I presents the amino acid compositions of the two proteins derived from the sequences, and it can be seen that these allergens are rich in aromatic amino acids containing 16.5–17.3% phenylalanine and tyrosine.

Structure Predictions—A computer-assisted analysis of antigenic sites with the deduced primary amino acid sequences was performed. The peaks reaching an antigenic index in the 1.3–1.7 area represent potential central epitopes recommended by the methods of Eisenberg et al. (42) and Rao and Argos (43), and no region could be found to satisfy the criteria recommended by those authors.

Amino Acid Sequence Homologies—A computer-assisted homology search of the data base (SWISS-PROT, release 29) revealed that C12 and C20 sequences have significant degrees of identity with several insect storage proteins (20.1–33.9%), insect juvenile hormone-suppressible proteins (30.9–36.4%), and arthropod hemocyanins (29.7–34.6%). Sequencing search results also indicated 29.7% and 31.5% identities in 283- and 92-amino acid overlaps of C12 and C20, respectively, with the fat body specificity of yolk protein (Fbp1) in Drosophila melanogaster (fruit fly). No similarity was found to other known allergens. The percentages of identity between the cockroach allergens and the sex-specific storage protein 1 (Ssp1) and 2 (Ssp2) precursors of the silk moth, the arylphorin α (Arya) and β (Aryb) subunit precursors of the tobacco hawkmoth, the arylphorin A4 (Ary1) and C223 (Ary2) precursors of the blue blowfly, the acidic (Jsp1) and basic (Jsb1 and Jsb2) juvenile hormone-suppressible proteins of the cabbage looper, the hemocyanins of the American tarantula (A, D, and E chains) and California spiny lobster (A–C chains), and the hemocyanin II of horseshoe crab (Hcy2) are given in Table II. Alignment of representative amino acid sequences of Ary2, Jsb2, the hemocyanin E chain (Hcye) of the American tarantula, C12, and C20 is presented in Fig. 3.

Detection of Cr-PI by Anti-fusion Protein Antibodies—A polyclonal antibody-based ELISA using anti-C12 and -C20 was performed for the detection of Cr-PI allergens in the cockroach extracts and recombinant proteins. The response of Cr-PI to all anti-fusion protein antibodies in the ELISA was dose dependent, and no Cr-PI allergens were detected when preimmunized mouse serum was substituted for anti-fusion protein antibodies as the coating antibody for the ELISA (data not shown). Each of the polyclonal antibodies to the different recombinant fusion proteins was able to detect CRA-A, Cr-PI, and other recombinant proteins. All commercial cockroach extracts contained detectable Cr-PI, although there were differences between the quantity of allergens in the extracts, and no Cr-PI allergens were detected in the German cockroach extract (Table III).

Allergy Skin Test and IgE Antibodies—Thirty-nine allergic patients (16–57 years old) with asthma and 10 nonallergic adults were skin tested with the commercial American cockroach extracts and Cr-PI allergens. Of 39 patients, 60% (23 of 39) had CRA-A-positive immediate skin reactions. Among these cockroach-positive patients, 82.6% (19 of 23) were sensitive to Cr-PI allergens. Skin tests were performed on these CRA-A- and Cr-PI-positive patients with recombinant proteins, and C12 and C20 both showed positive skin reactions in 47.4% (9 of 19). In the nonallergic group, positive reactions were found in

### Table I

| Deduced amino acid composition (mol %) of clones | C12  | C20  |
|-----------------------------------------------|------|------|
| Glycine                                       | 5.11 | 5.07 |
| Alanine                                       | 5.40 | 4.44 |
| Valine                                        | 8.32 | 6.61 |
| Leucine                                       | 5.99 | 6.50 |
| Proline                                       | 5.40 | 5.39 |
| Serine                                        | 4.38 | 4.75 |
| Threonine                                     | 3.50 | 3.80 |
| Phenylalanine                                 | 5.69 | 6.81 |
| Tyrosine                                      | 10.80| 10.46|
| Tryptophane                                   | 0.58 | 0.63 |
| Lysine                                        | 5.99 | 7.45 |
| Arginine                                      | 4.82 | 5.39 |
| Histidine                                    | 5.11 | 5.71 |
| Aspartate                                    | 7.45 | 7.77 |
| Asparagine                                   | 5.36 | 3.96 |
| Glutamate                                    | 5.26 | 6.18 |
| Glutamine                                    | 3.50 | 2.85 |
| Cysteine                                     | 0.44 | 0.63 |
| Methionine                                    | 3.21 | 1.74 |

### Table II

| Protein Source | Percent identity of amino acid sequences between the American cockroach allergens, insect arylphorin-type storage proteins, juvenile hormone-suppressible proteins, and arthropod hemocyanins | C12 | C20 |
|---------------|-------------------------------------------------------------------------------------------------|-----|-----|
| Ssp1-Bommo    | B. mori (silk moth)                                                                             | 31.3 (339) | 33.9 (448) |
| Ssp2-Bommo    | B. mori (silk moth)                                                                             | 20.1 (666) | 24.4 (599) |
| Arya-Manse    | M. sexta (tobacco hawkmoth)                                                                     | 20.9 (703) | 29.4 (647) |
| Aryb-Manse    | M. sexta                                                                                       | 22.5 (708) | 27.1 (646) |
| Ary1-Calvi    | Calliphora vicina (blue blowfly)                                                                | 29.0 (587) | 29.3 (570) |
| Ary2-Calvi    | C. vicina                                                                                      | 30.7 (587) | 31.1 (572) |
| Jsp1-Trini    | Trichoplusia ni (cabbage looper)                                                                | 35.2 (339) | 32.4 (487) |
| Jsb1-Trini    | T. ni                                                                                          | 30.9 (463) | 31.9 (464) |
| Jsb2-Trini    | T. ni                                                                                          | 36.3 (485) | 36.4 (486) |
| Hoya-Eurca    | Euryplema-californicum (tarantula)                                                              | 29.7 (637) | 33.4 (598) |
| Hycy-Eurca    | E. californicum                                                                                 | 31.0 (632) | 33.3 (597) |
| Hycy-Eurca    | E. californicum                                                                                 | 33.1 (626) | 34.6 (594) |
| Hoya-Panin    | Panulirus interruptus (spiny lobster)                                                           | 31.0 (638) | 33.1 (625) |
| Hycy-Panin    | P. interruptus                                                                                 | 30.9 (647) | 33.0 (625) |
| Hycy-Panin    | P. interruptus                                                                                 | 30.9 (666) | 31.9 (624) |
| Hcy2-Limpo    | Limulus polyphemus (horseshoe crab)                                                             | 30.8 (637) | 34.6 (601) |
10% (1 of 10) of the subjects. CRa-A- and CR-PI-specific IgEs were also determined in these 19 patients by the fluoroallergosorbent test. All patients were found to contain specific IgE to CRa-A and CR-PI, ranging from 0.47 to 2.30 and 0.52 to 35.3 IU/ml, whereas control subjects showed no cockroach-specific IgE ([132x285],[139x285] 0.35 IU/ml). Results are summarized in Table IV.

**DISCUSSION**

The complete nucleotide sequences of cDNAs encoding the American cockroach allergen CR-PI are presented in this study. The most common and major domiciliary cockroaches are the American and German cockroaches both in Taiwan (11, 12) and the United States (28, 29). The existence of common IgE-binding components shared in whole-body extracts of the American and German cockroaches has been suggested (13, 15–17). Two important allergens of B. germanica have recently been cloned and sequenced. Bla g 2 is a Mr 36,000 protein sharing sequence homology to the aspartic proteases, and no Bla g 2 mRNA was detected in the American cockroach (28). Bla g 4, a Mr 21,000 allergen, is a ligand-binding protein or calycin, and its mRNA was unable to be transcribed in the American cockroach (29). It was suggested that the expression of Bla g 4 was species specific. The alignment of Bla g 2 and Bla g 4 with sequences reported here revealed that no sequence similarities were found among them. These results are consistent with our previous anti-Cr-PI mAb studies on immunoblotting and ELISA, which showed mAb unable to bind the crude German cockroach extract (21), and in the present studies, which showed that no CR-PI allergens were detected in the commercial German cockroach extract by anti-fusion protein antibody-based ELISA. It appears that CR-PI also is a group of species-specific allergens of the American cockroach.

Several classes of important insect hemolymph proteins, such as storage proteins, juvenile hormone-suppressible proteins, and arthropod hemocyanins, have been identified and sequenced. Holometabolous insect storage proteins are synthesized by the larval fat body and released into the hemolymph. These are either stored in the hemolymph or selectively sequestered from the hemolymph by the fat body cells and stored there in the form of protein granules (44–47). The function of storage proteins has not been fully elucidated. However, evidence suggests that they may function as amino acid stores required at later stages for the development of adult tissues (48–50). There are two classes of insect storage proteins with molecular weights of about 500,000, composed of six subunits of approximately Mr 72,000–80,000 each, which may or may not be identical. One class is arylphorin, a protein rich in aromatic amino acids (47), and another class is methionine-rich, female-specific proteins (51, 52). Hemocyanins occur in the hemolymph of many arthropods containing a binuclear copper site for binding molecular oxygen. The hexameric arthropod hemocyanins are composed of individual subunits with molecular weights of about 50,000, composed of six subunits of approximately Mr 72,000–80,000 each, which may or may not be identical. One class is arylphorin, a protein rich in aromatic amino acids (47), and another class is methionine-rich, female-specific proteins (51, 52). Hemocyanins occur in the hemolymph of many arthropods containing a binuclear copper site for binding molecular oxygen. The hexameric arthropod hemocyanins were proposed by Telfer and Massey (56), and the high percentage identities (24–60%) between them have been reported (53–55, 57, 58). A mAb prepared against the American tarantula hemocyanin cross-reactive with the arylphorin from the blowfly has been...
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Table III

| Cockroach allergen | Anti-C12 | Anti-C20 |
|--------------------|---------|---------|
| CRa-A              | 0.713   | 0.979   |
| Cr-PI              | 0.975   | 1.182   |
| Cr-PII             | 0.018   | 0.014   |
| C12                | 1.639   | 1.389   |
| C13                | 1.471   | 2.380   |
| C20                | 1.171   | 1.611   |
| C28                | 0.471   | 0.886   |
| A (Cockroach mix)  | 0.093   | 0.098   |
| B (American cockroach) | 0.107 | 0.154 |
| C (American cockroach) | 0.128 | 0.110 |
| D (American cockroach) | 0.312 | 0.375 |
| E (American cockroach) | 0.594 | 0.717 |
| F (German cockroach) | 0.014 | 0.018 |

Table IV

| Patient | CRa-A | CR-PI | C12 | C20 | CRa-A | CR-PI |
|---------|-------|-------|-----|-----|-------|-------|
| I1/μl   |       |       |     |     |       |       |
| 1       | 0.47  | 0.62  | 0.62| 2.56| 0.65  | 0.98  |
| 2       | 0.52  | 0.85  | 0.96| 1.32| 1.71  | 1.86  |
| 3       | 0.62  | 2.56  | 0.96| 1.32| 1.71  | 1.86  |
| 4       | 0.58  | 0.56  | 1.12| 9.72| 0.62  | 2.56  |
| 5       | 0.85  | 0.62  | 1.12| 9.72| 0.47  | 0.52  |
| 6       | 0.75  | 3.29  | 0.75| 3.29| 0.65  | 0.98  |
| 7       | 0.64  | 1.36  | 0.64| 1.36| 1.08  | 1.52  |
| 8       | 0.58  | 2.56  | 0.96| 1.32| 1.71  | 1.86  |
| 9       | 0.58  | 1.36  | 0.64| 1.36| 1.08  | 1.52  |
| 10      | 0.65  | 0.98  | 0.65| 0.98| 1.71  | 1.86  |
| 11      | 1.25  | 1.75  | 1.71| 1.86| 1.71  | 1.86  |
| 12      | 1.52  | 2.56  | 1.71| 1.86| 1.71  | 1.86  |

Fluorolipid test.

In contrast to the insect storage protein genes, the hemocyanin gene lacks a sequence coding for a signal peptide. Other than the lack of copper in arylphorin, and differences in biosynthesis, release, secretion (61–63), gene structure, and function, hemocyanins and arylphorins share remarkable amino acid sequence similarities. It was suggested that these two types of proteins have evolved from a common ancestor (54, 56). Two classes (acidic and basic) of juvenile hormone-suppressible proteins ranging in size from Mr 78,000 to 76,000 have been identified, and 19–39% sequence identities to arthropod hemocyanins and arylphorins have been reported (64–66). Acidic Jsp1 of the cabbage looper is located to the arylphorins as well as to the arthropod hemocyanins (65). Basic Jsb1 and Jsb2 of the cabbage looper are more closely related to the female-specific, methionine-rich storage proteins than to the arylphorins, Jsp1, or arthropod hemocyanins (66). Metamorphosis-associated acidic and basic hormone-suppressible proteins, insect storage proteins, and arthropod hemocyanins have been suggested to be within the hemocyanin superfamily (66).

Sequence analysis revealed that two cockroach allergen clones share a remarkable degree of identity (20.1–36.4%) to the above proteins. Taken together, the results presented here strongly suggest that the these American cockroach allergens are ancestrally related to insect hemolymph proteins and represent a new group of hemocyanin superfamily proteins. The majority of insect storage protein genes thus far characterized are arylphorins generally containing 18–25% phenylalanine and tyrosine (47, 53, 54). Deduced sequences of the American cockroach allergens are also aromatic amino acid rich, containing 16.5–17.3% phenylalanine and tyrosine. In the American cockroach allergens from Manduca sexta, a strong bias toward the use of tyrosine in the third position of codons was noted for Phe (84–91%) and Tyr (79–81%), and TTC (Phe) and TAC (Tyr) together accounted for about 16% of all codon usage (54). Similarly, in C12 and C20 genes, the TTC or TAC codons account for 97.4 or 85.1% and 79.1 or 77.3%, respectively. In C12 and C20 genes, the TTC and TAC together account for 14.7% and 13.5% of all codon usage, respectively, which indicates that the corresponding tRNAs must be abundant to support the biosynthesis of these allergens. Thus, it appears from sequence homologies that these aromatic, amino acid-rich allergens are more closely related to the arylphorins than to the methionine-rich, female-specific proteins, juvenile hormone-suppressible proteins, or arthropod hemocyanins. Allergen sequence similarity to the insect hemolymph proteins has not been reported until now. Nevertheless, peanut allergen Ara h 1, a Mr 68,000 protein, has recently been cloned and sequenced, and significant homology (64–66%) to the vicilin seed storage protein family found in most higher plants was reported (67).

No information concerning arthropod hemocyanin-specific, juvenile hormone-suppressible protein-specific, or insect storage protein-specific IgE have previously been reported in atopic patients until recently. Interestingly, in 1995, Suzuki et al. (68) reported that a high positive frequency of silkworm moth-specific IgE using the CAP-RAST system (Pharmacia) was detected in atopic patients. A total of 267 sera from patients with allergic rhinitis were tested against 15 commercially prepared allergens (Pharmacia) without cockroach allergens. The highest positive rate was J apus picea (73.8%), followed by mite (53.3%), and lower rates were found with Candida and Alternaria. The positive frequencies for silkworm moth (M. mori) and adult chironomid midge were 32.1% and 19.4%, respectively. Because recombinant proteins of clones C12 and C20 share 20.1–33.9% identities (Table II) to M. mori storage proteins, these results open the possibility that some insect hemolymph proteins may have a tendency to cause atopic diseases and are thus worthy for further investigation.

Our previous studies indicated that Cr-PI elicits 73% skin reactivity in cockroach-sensitive atopic patients, and the components of M. mori 78,000 and 72,000 in Cr-PI were identified as the major allergens of the American cockroach by immunoblotting (12). The present study revealed a high prevalence of IgE antibody to Cr-PI (82.6%), and both fusion proteins of clones C12 and C20 share 20.1–33.9% identities (Table II) to M. mori storage proteins, these results open the possibility that some insect hemolymph proteins may have a tendency to cause atopic diseases and are thus worthy for further investigation.

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allergic patients (69). Therefore, recombinant allergens and sequence data will provide for structural studies and epitope mapping and may be valuable for diagnostic and therapeutic purposes.

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