

Identification and Characterisation of Two Allergens from the Dust Mite *Acarus siro*, Homologous with Fatty Acid-Binding Proteins

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Key Words

Mite · Allergen · *Acarus siro* · Aca s 13 ·
cDNA cloning · Protein expression · Fatty acid-binding
protein

Abstract

Background: Dust mites are a major cause of allergic disease worldwide. The dust mite *Acarus siro* is an inducer of occupational allergy among farmers, but sensitisation has also been found in non-farming populations. **Methods:** A degenerate primer was designed to the N-terminal amino acid sequence of a 15-kD IgE-binding protein in *A. siro* extract. The cDNA sequence was obtained by using reverse transcriptase polymerase chain reaction, standard cloning and sequencing techniques. The protein was expressed in *Escherichia coli* with a 6-histidine tag at its C-terminus. Immunoblotting of the recombinant protein and whole extract was performed using patient sera. **Results and conclusion:** 15 and 17-kD allergens were identified in a fraction of *A. siro* extract. The cDNA of the 15-kD allergen was isolated, cloned and sequenced and the allergen was expressed as a recombinant protein. The calculated molecular weight of the cDNA-encoded protein is 14.2 kD. The predicted amino acid sequence has one potential N-glycosylation site at position 4–6 and a cytosolic fatty acid-binding protein signature at position 5–22. The pro-

tein has 64% sequence identity with Blo t 13, an allergen from the dust mite *Blomia tropicalis*, as well as homology with several other fatty acid-binding proteins (FABPs) from different organisms. The allergen was named Aca s 13 and was recognised strongly by 3 of 13 (23%) of the subjects investigated. The amino acid sequence of the 17-kD protein was partly determined and it also showed high sequence homology with Blo t 13 and FABPs.

Introduction

Dust mites are worldwide the major cause of allergic disease, such as asthma and rhinitis [1]. The dust mite *Acarus siro* is a common and serious pest of stored grain, flour and other cereal products [2]. *A. siro* is an inducer of occupational allergy among farmers [3–5], bakers [6, 7], grain workers [8] and cereal workers [9]. Sensitisation to *A. siro* has also been found in non-farming populations [10–14]. Mite allergens have different structures and functions [15], as has been shown for several allergens from the house dust mite species *Dermatophagoides* [16]. However, very little is known about the allergens produced by *A. siro*. Using SDS-PAGE and immunoblotting, we have previously identified 5 IgE-binding components from *A. siro* extract in the molecular mass range of 15–22 kD [17]. A fraction soluble in 60% saturated ammonium sulphate of *A. siro* extract con-

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Table 1. Primers used for cDNA synthesis, PCR and subcloning for expression

1.	5'-GA(A/G)AA(A/G)TC(A/C/G/T)GA(C/T)AA(C/T)TT(C/T)GA(CT)GC(A/C/G/T)TT(C/T)-3'
Oligo (dT)	5'-GCTGCCTCTGCGTCGACATCTGACAAAGCTTGGTACCTTTTTTTTTTTTTTTNN-3'
2.	5'-GCTGCCTCTGCGTCGACATCTGACA-3'
3.	5'-GAACTCTCGAACGATGGTCACCTC-3'
4.	5'-CGTCTTGACGGTTTTGCCGTCAGCACG-3'
5.	5'-TTCTTCAAATTCTTCGCCAAGCTTGAA-3'
6.	5'-GGCCATATGCAAATCAATGGTAGCTACAAGCTCGAAAAGAGCGACAACCTTTGACGCATTCC-3'
7.	5'-GAGGGTACCTTA(ATG) ₆ CTGGCGCTTGTAAGATCGCACAGA-3'

tained two allergens with approximate molecular weights of 15 and 17 kD. The corresponding ammonium-sulphate-soluble fraction of extracts from other mite species such as *Lepidoglyphus destructor* [18] and *Tyrophagus putrescentiae* [19] contain the mite group 2 allergens Lep d 2 and Tyr p 2, respectively. These group 2 allergens, are major allergens, and are recognised by about 80% of the patients sensitised to *L. destructor* and *T. putrescentiae*, respectively. In this study we cloned, sequenced and expressed the cDNA encoding a 15-kD allergen present in the ammonium-sulphate-soluble fraction of *A. siro* extract and performed amino acid sequencing on the 17-kD allergen. None of these allergens, were homologous to the group 2 allergens, but instead homology was found to Blo t 13 from the dust mite *Blomia tropicalis*, and to several fatty acid-binding proteins (FABPs) from different organisms.

Materials and Methods

Purification and Amino Acid Sequencing

Proteins were extracted from *A. siro* whole cultures in 1/10 (w/v) 0.9% NaCl and 0.01% NaN₃ supplemented with protease inhibitor, Complete™ Mini (Boehringer Mannheim, Germany) overnight at room temperature. The extract was thereafter precipitated with 60% ammonium sulphate. The soluble fraction, which will be referred to as the ammonium-sulphate-soluble fraction, was run on a 15% SDS-polyacrylamide gel and electroblotted to a polyvinylidene-difluoride membrane (PVDF) (Immobilon-PSQ; Millipore, Bedford, Tex., USA). The membrane was protein stained and the pieces containing the 15- and the 17-kD proteins were cut out. The proteins on the membrane pieces were directly subjected to N-terminal amino acid sequence analysis in a Perkin Elmer ABI 494 Procise cLC protein sequencer. The 17-kD allergen was cut out from SDS-polyacrylamide gel and subjected to in-gel trypsin digestion and sequencing of proteolytic fragments.

Isolation of mRNA

A. siro whole cultures were obtained from Allergon (Ängelholm, Sweden). Messenger RNA was isolated from 0.5 g of living mites using a Dynabeads mRNA direct kit (Dynal, Oslo, Norway), following the manufacturer's protocol.

Design of Oligonucleotide Primers for Polymerase Chain Reaction

Reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) techniques were used to determine the cDNA sequence of the 15-kD protein. The primers used in this study are listed in table 1. A degenerate primer, primer 1, was designed to the EKSDNFDFAF region of the N-terminal amino acid sequence of the 15 kD protein. An oligo(dT) primer with a 5' extension was used for preparation of the first-strand cDNA. For the 3' RACE, the degenerate primer, primer 1, and primer 2, which matched the oligo(dT) primer extension, was used. Complementary DNA to be used in the 5' RACE was synthesized with a gene-specific primer, primer 3. The 5' RACE was performed using Abridged Anchor Primer (Gibco/BRL, Paisley, UK) in combination with a gene-specific primer, primer 4, which was designed according to the results from sequencing 3' RACE products. Primer 4 anneals to a region upstream of the annealing site for primer 3. A nested PCR was performed on the diluted primary 5' RACE product using the Abridged Universal Amplification Primer (Gibco/BRL) and primer 5, designed to a region upstream of the annealing site of primer 4.

For cloning into the expression vector, the cDNA was amplified from the 3' RACE template (cloned into the pT7 Blue vector), using primers 6 and 7.

PCR Amplification

RT-PCR was used to amplify the mRNA and to obtain overlapping cDNA fragments. cDNA to be used in 3' RACE was synthesized using oligo(dT) primer as described earlier [19]. The amplification of 3' fragments was carried out using primers 1 and 2 with hot start and thereafter amplification for 40 cycles: 1 min at 94°C, 2 min at 50°C and 2.5 min at 72°C. The final cycle had its polymerisation step extended to 10 min.

First-strand synthesis of the cDNA for the 5' RACE was obtained using the gene-specific primer, primer 3, instead of oligo(dT). Thereafter, the cDNA was denatured and tailed with 0.2 mM dCTP using terminal dideoxytransferase (Gibco/BRL). A primary PCR was performed on the oligo C-tailed cDNA using Abridged Anchor primer and primer 4 for 35 cycles: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The final cycle had its polymerisation step extended to 5 min. The primary PCR product was diluted 1/100 and used in nested PCR using Abridged Universal Amplification Primer (Gibco/BRL) and primer 5 under the same cycle conditions as described above.

Cloning and Screening of PCR Products

Isolated amplified cDNA from the PCR reactions were cloned into the pT7 Blue vector (Novagen, R&D Systems, Abingdon, UK), followed by transformation into competent *E. coli* XL-2 Blue cells

(Stratagene). The recombinant clones were confirmed by restriction enzyme analysis of plasmid DNA isolated from the transformed bacterial colonies.

DNA Sequencing

All DNA sequencing was carried out on an ABI 377 sequencer (Perkin Elmer, Foster City, Calif., USA) using the ABI PRISM Dye Terminator cycle sequencing Ready Reaction kit (Perkin Elmer), according to the instructions of the manufacturer. Both strands of the cDNA, which were cloned into pT7 Blue vector, were sequenced using the T7 promoter sequencing primer and M13 sequencing primer. The pET 17b constructs were sequenced using T7 terminator primer and T7 promoter primer.

Subcloning into pET 17b and Expression

The cDNA encoding the 15-kD allergen was amplified from the 3' RACE template using primers 6 and 7. Primer 6 included a *Nde* I restriction site and a start codon prior to the first complete codon of the cDNA clone. Primer 7 had six histidine codons and a *Kpn* I restriction site included in the primer sequence (table 1). *Nde* I/*Kpn* I-digested PCR products were ligated into the *Nde* I/*Kpn* I-digested expression vector, pET 17b (Novagen). The ligation was initially transformed into a non-expressing host, *E. coli* XL-2 Blue, and thereafter the construct was transformed into expressing cells, *E. coli* BL 21(DE3) pLysS. The expression of the recombinant protein was performed following the protocol provided by the manufacturer. The majority of the recombinant protein was found in insoluble inclusion bodies which were solubilised in 50 mM Tris-HCl, 100 mM NaCl, 8 M urea, pH 8.

Immobilised metal affinity chromatography (Talon, Clontech, Palo Alto, USA) was used to purify the 6-histidine-tagged protein. The recombinant protein was eluted from the affinity resin in PBS buffer, pH 7.4, containing 100 mM imidazole. The eluted recombinant protein was dialysed against PBS.

SDS-PAGE, Western Blot Analysis and Blotting Inhibition

SDS-PAGE was performed under non-reducing conditions and the proteins were electroblotted to PVDF membrane (Immobilon, Millipore). The immunodetection with human sera was performed as described earlier [17].

For the blotting inhibition analysis, patient sera were pre-incubated with recombinant protein (1 and 5 µg, respectively) for 1 h at room temperature and thereafter incubated with *A. siro* whole extract blotted on PVDF membrane. The final dilution of patient sera was 1:4. As a negative control, recombinant Mal f 1, an allergen from the yeast *Malassezia furfur* [20], produced using the same conditions as for the recombinant *A. siro* allergen, was used for pre-incubation (1 and 5 µg) with serum from an *A. siro* RAST-positive patient. Recombinant *A. siro* allergen blotted to PVDF membrane and pre-incubation of a patient serum with recombinant *A. siro* allergen (10 µg) served as a positive control.

Human Sera

Sera from farmers (n = 14) who had previously participated in an epidemiological investigation of respiratory disease [21] were used for immunoblotting. Thirteen of these farmers were RAST positive and one farmer was RAST negative to *A. siro*.

Results

Protein Analysis

The ammonium-sulphate-soluble fraction of *A. siro* extract consisted of at least two allergens in the molecular weight range of 15–20 kD. The 15-kD protein was subjected to N-terminal amino acid sequencing by Edman degradation. The sequence obtained was: GQINGSYKLEKSD-NFDAFLK, and it was unambiguously determined except for the N-terminal position where an alanine and a valine were detected beside the dominant glycine. A degenerate primer was designed to the EKSDNFDAF region and was thereafter used in RT-PCR. The cDNA sequence was in agreement with the N-terminal amino acid sequence.

Homology to FABPs

A partial cDNA encoding the 15-kD protein was determined by cDNA sequencing of 3' and 5' RACE products. The cDNA sequence from the first glutamine encoded to the beginning of the poly (A) tail is 520 bp in length and the 3' untranslated region is 132 bp in length (EMBL nucleotide sequence database, accession number AJ006774). The calculated molecular weight is 14.2 kD, and there is one potential N-glycosylation site (NGS) at position 4–6 in the predicted amino acid sequence (assuming that the glutamine is at position 2 in the amino acid sequence). There is also a cytosolic fatty acid-binding protein signature at position 5–22 as detected by the PROSITE program [22]. The amino acid sequence shows 64% identity with the 14.8-kD allergen Blo t 13 from *B. tropicalis* [16, 23]. The 15-kD *A. siro* allergen also has high sequence homology with several other FABPs from different organisms, e.g. 47% identity with a rat FABP and 38% with a human FABP (fig. 1). Due to its high amino acid sequence identity with Blo t 13 and according to the nomenclature [24], the allergen was named Aca s 13 [16, 23].

Both N-terminal amino acid sequencing and sequencing of internal peptides were performed on the 17-kD allergen and the sequence showed high homology with both Aca s 13 from *A. siro* and Blo t 13 from *B. tropicalis* [23]. Of the 61 amino acid residues analysed from the 17-kD protein, 40 amino acids (66%) were identical to Blo t 13 and 28 out of 61 (46%) were identical to Aca s 13 (calculation based on the alignment of the 17-kD peptides to the *B. tropicalis* and *A. siro* allergens) (fig. 1).

Expression and IgE Binding of Aca s 13

Recombinant (r)Aca s 13 was expressed in *E. coli* with a 6-histidine tag at its C-terminus and metal affinity purified to homogeneity. The yield of rAca s 13 was 2 mg/l bacteri-

Aca s 13	X Q I <u>N G S</u> Y K L E K S D N F D A F L K E L G L N F V T R N L A K S A T P T V E V S V	43
As 17 kD	X Q I D X T Y R Y X M T - - - - - L G V G F M V K - - - T L K P T L E V E T	
Blo t 13	M P I E G K Y K L E K S D N F D K F L D E L G V G F M V K T A A K T L K P T L E V D V	43
FABP rat	D A F V G T W K L V D S K N F D D Y M K S L G V G F A T R Q V A S M T K P T T I I E K	43
FABP human	D A F L G T W K L V D S K N F D D Y M K S L G V G F A T R Q V A S M T K P T T I I E K	43
	* * * * *	
Aca s 13	N G D S Y T I K T A S T L K N T E I S F K L G E E F E E A R A D G K T V K T V V N K E	86
As 17 kD	Q G D T Y - - - - - L F D Q I D E E S - - - - -	
Blo t 13	Q G D T Y V F R S L S T F K N T E I K F K L G E E F E E D R A D G K R V K T V V N K E	86
FABP rat	N G D T I T I K T H S T F K N T E I S F Q L G V E F D E V T A D D R K V K S V V T L D	86
FABP human	N G D I L T L K T H S T F K N T E I S F K L G V E F D E T T A D D R K V K S I V T L D	86
Aca s 13	S D T K F V Q V Q Q G D K E V T I V R E F S D E G L T V T A T V S G V T S V R F Y K R Q	130
As 17 kD	- - - - - E F A G D D V T V T A T V A D V T S V R - - - - -	
Blo t 13	G D N K F I Q T Q Y G D K E V K I V R D F Q G D D V V V T A S V G D V T S V R T Y K R I	130
FABP rat	G G K L V H V Q K W D G Q E T T L T R E L S D G K L I L T L T H G N V V S T R T Y E K E A	131
FABP human	G G K L V H L Q K W D G Q E T T L V R E L I D G K L I L T L T H G T A V C T R T Y E K E A	131

Fig. 1. Multiple sequence alignment of Aca s 13 (deduced from cDNA sequence), peptides from *A. siro* 17-kD protein, Blo t 13, rat FABP and human FABP. Aca s 13 shows 64% identity with Blo t 13. Forty out of the 61 (66%) amino acids analysed from the 17-kD protein are identical to Blo t 13 and 28 out of 61 (46%) to Aca s 13. The potential N-glycosylation site NGS in Aca s 13 is underlined, and the cytosolic FABP signature in the FABPs is indicated by asterisks. The N-terminal sequence of the 17-kD *A. siro* protein could not be determined unambiguously. Dashes represent amino acids not determined in the 17-kD *A. siro* protein and are introduced for alignment purposes. The amino acid residues that could not be determined are shown as X.

al culture. The recombinant allergen was strongly detected by 3 out of 13 (23%) *A. siro* RAST-positive patients investigated, (fig. 2). In addition, 3 patients showed a weak response to r Aca s 13 (fig. 2, lanes 3–5). The 3 patients that reacted strongly, detected not only the approximately 15-kD component, but also a band of approximately 30 kD, which was a dimer of r Aca s 13. Recombinant Aca s 13 was not recognised by a serum RAST negative to *A. siro* (fig. 2, lane 14).

Inhibition Pattern of IgE-Binding

The recombinant allergen inhibited IgE binding of an Aca s 13-positive serum to a 15-kD component in the whole *A. siro* extract. One microgram of rAca s 13 gave complete inhibition of the 15-kD component in the whole extract (fig. 3a). Pre-incubation of patient sera with the unrelated

allergen Mal f 1 [20] did not affect the IgE-binding pattern (fig. 3b). Homologous inhibition of rAca s 13 was demonstrated (fig. 3c).

Discussion

Using SDS-PAGE and immunoblotting, we have previously identified 5 IgE-binding components from *A. siro* extract in the molecular weight range of 15–22 kD [17]. In this study, we have cloned and expressed a 15-kD allergen from *A. siro* as a recombinant protein in *E. coli*. The 15-kD allergen was named Aca s 13 based on recommended nomenclature [24] and its similarity to Blo t 13, a 14.8-kD allergen from the dust mite *B. tropicalis*, homologous to FABPs. Aca s 13 also showed amino acid sequence homology with

FABPs from other species such as the rat and humans. FABPs are involved in the intracellular transport of long-chain fatty acids and their acyl-CoA esters. To further characterise the cloned allergen, the function of Aca s 13 as a FABP could be studied using fluorescent fatty acid analogues. The properties of ABA-1, an allergen from the nematode *Ascaris suum* which is a FABP, has successfully been studied using fluorescent fatty acid analogues [25].

We have performed 3 independent experiments in order to determine the sequence of the 5' end, as well as the upstream non-coding region of Aca s 13 cDNA. We did not find a start codon in any of the clones analysed; instead, the codon for glutamine was found downstream from the poly C-tail. We believe that this glutamine is the second amino acid residue of the protein since it is in agreement with the N-terminal amino acid sequence. The N-terminal residue of the allergen was identified as a glycine by N-terminal Edman sequence analysis, but we believe this to have been caused by contamination often found in the first cycle of sequencing. The SDS-PAGE was carried out in a buffer with a high glycine content which may have led to contamination by glycine. However, besides the glycine, there were also two other amino acids identified in the first sequencing cycle, i.e. alanine and valine. Since we could not determine the N-terminal amino acid residue unambiguously, we chose to amplify Aca s 13 from the 3' RACE template using a primer that introduced an ATG upstream of the glutamine codon. The absence of a start codon in the clones analysed may have been due to the secondary structure of the mRNA preventing the RT progressing further on the mRNA template.

Recombinant Aca s 13 was expressed in *E. coli* and after purification, the protein was subjected to immunoblotting experiments. Three out of 13 (23%) of the *A. siro* RAST-positive patients reacted strongly to r Aca s 13 and also detected a band at approximately 30 kD. The 30-kD protein is probably a dimer of r Aca s 13 since it bound IgE, and the IgE binding could be inhibited by pre-incubation of patient sera with r Aca s 13 (fig. 3c). From the limited number of sera investigated, it is not possible to decide how many *A. siro*-allergic patients react to r Aca s 13. Caraballo et al. [23] found that Blo t 13 was recognised by 11% of the sera tested, suggesting that Blo t 13 is a minor allergen. There is a possibility that rAca s 13, expressed in *E. coli* and recovered from inclusion bodies, has an altered three-dimensional structure as compared to its natural counterpart. This might influence the IgE-binding properties of rAca s 13. However, r Blo t 13 has been expressed both in *E. coli* and in the yeast *Pichia pastoris* and Puerta et al. [26] found that the recombinant allergens from both expression systems have the

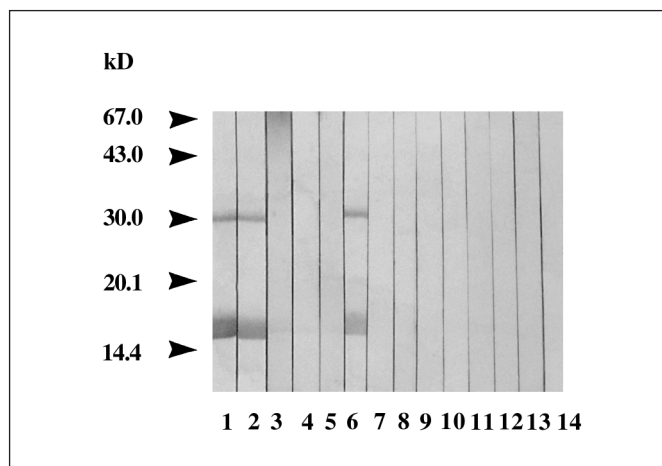


Fig. 2. IgE binding to recombinant Aca s 13 detected on immunoblots by 13 individual sera with a positive RAST to *A. siro* (lanes 1–13) and a serum sample with a negative RAST to *A. siro* (lane 14).

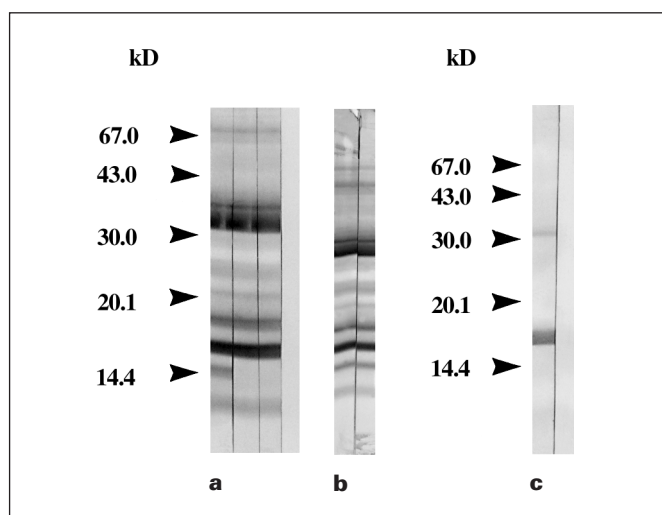


Fig. 3. **a** Inhibition of IgE binding to *A. siro* whole extract. Lane 1: a serum sample RAST positive to *A. siro*, no inhibitor; lane 2: same serum sample as in lane 1, inhibition with 1 µg recombinant Aca s 13; lane 3: same serum sample as in lane 1, inhibition with 5 µg recombinant Aca s 13; lane 4: a serum sample RAST negative to *A. siro*, no inhibitor. **b** Inhibition of IgE binding to *A. siro* whole extract; lane 1: a serum sample with a positive RAST to *A. siro*, no inhibitor; lane 2: same serum sample as in lane 1, inhibition with 10 µg Mal f 1. **c** Inhibition of IgE binding to recombinant Aca s 13. Lane 1: a serum sample RAST positive to *A. siro*, no inhibitor; lane 2: same serum sample as in lane 1, inhibition with 10 µg recombinant Aca s 13.

same IgE epitope composition. The discrepancy with earlier results showing that 7 out of 9 patients had a positive IgE to a protein of approximately 15 kD in *A. siro* extract [17] is probably due to other allergens present in the extract with similar molecular weights. Other mite allergens with molecular weights around 15 kD have been identified, e.g. the homologous group 5 allergens Der p 5 [27] and Blo t 5 [28] in *Dermatophagoides pteronyssinus* and *B. tropicalis*, respectively, Blo t 12 [29] from *B. tropicalis*, and finally the group 2 allergens which have been identified in several other mite species [18, 19, 30–32]. None of these allergens have known functions, and they are not homologous to FABPs. We performed amino acid sequence analysis of a 17-kD protein in *A. siro* (apparent MW estimated from SDS-PAGE) to determine if it corresponded to any of the known allergens from other mite species in this molecular weight range. Interestingly, peptides analysed from the 17-kD protein also had a high sequence homology with Blo t 13 and some regions were completely conserved. The peptide sequences actually showed higher homology to Blo t 13 than Aca s 13 in the regions they aligned to. These results suggest that Aca s 13 and the 17-kD protein are two closely related proteins and are probably members of a family of related FABPs present in mite. FABPs consist of a family of related proteins, and the different FABPs are expressed in a tissue-specific manner. They are all abundant cytoplasmic proteins with molecular weights around 15 kD [33, 34].

FABPs from two mites (*A. siro* and *B. tropicalis*) [23] and a nematode (*A. suum*) [25] have now been shown to be allergens, and thus FABPs may constitute a new family of allergens.

Alternatively, the 15- and 17-kD allergens could be isoforms of the same allergen. However, we find this unlikely since isoforms of allergens, which have been found in other mite species [15, 18, 35], can differ in the amino acid compositions, but the overall size of the protein remains the same. Although Aca s 13 and the peptides from the 17-kD protein are very similar, the Aca s 13 allergen seems to inhibit IgE binding only to a single 15-kD, protein in the *A. siro* extract. Further studies are needed to establish the function as well as the immunogenic and clinical significance of Aca s 13.

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