



ISOLATION AND CHARACTERIZATION OF A NEW BASIC ANTIGEN FROM SHORT RAGWEED POLLEN (*AMBROSIA ARTEMISIIFOLIA*)

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Abstract—A new basic antigen with a molecular weight of 36.5 kDa and a pI of 8.65 was purified from short ragweed pollen using ammonium sulfate precipitation, Q-sepharose chromatography and S-sepharose chromatography. The purified protein, herein referred to as *Ambrosia artemisiifolia* basic antigen (AaBA), migrated as a single band in SDS–polyacrylamide gel electrophoresis and isoelectric focusing, and as a single peak on reverse phase HPLC. AaBA reacted with sera from humans allergic to ragweed pollen including pooled NIH serum. AaBA and two major acidic allergens, *Amb a I* (antigen E) and *Amb a II* (antigen K), displayed immunological cross-reactivity inasmuch as they competed significantly with each other in a competition ELISA. This suggests that at least some major epitopes in all three antigens are similar; however, other possibilities like adjacent location of different epitopes cannot be ruled out at this time. Molecular weight, amino acid composition and sequence data suggest that AaBA is very similar to *Amb a II* whereas it is significantly different from other basic antigens purified from ragweed pollen.

Key words: ragweed pollen, basic antigen, AaBA, *Ambrosia artemisiifolia*.

INTRODUCTION

It is well known that ragweed pollen is composed of a complex mixture of at least 52 antigens, about 20 of which bind specifically to human IgE antibodies (King, 1976; Lowenstein *et al.*, 1981, 1983). Two major allergens from pollen of short ragweed *Ambrosia artemisiifolia*, *Amb a I* (antigen E) and *Amb a II* (antigen K), were isolated by King *et al.* (King and Norman, 1962; King *et al.*, 1964, 1967; King, 1972, 1976; Marsh *et al.*, 1986). Both were acidic proteins with a molecular weight of 38 kDa. Their gene sequences were determined in subsequent studies (Rafnar *et al.*, 1991; Rogers *et al.*, 1991). Several basic allergens have also been isolated: *Amb a III* (Ra3), *Amb a IV* (Ra4 or BPA-R), *Amb a V* (Ra5) and *Amb a VI* (Ra6) with molecular weights in the range of 5–28 kDa and isoelectric points between 8 and 9 (Underdown and Goodfriend, 1969; Griffiths and Brunet, 1971; Lapkoff and Goodfriend, 1974; Mole *et al.*, 1975; Roebber, 1975; Klapper *et al.*, 1980; Goodfriend *et al.*, 1981; Roebber *et al.*, 1982, 1983; Vidusek *et al.*, 1985).

During isolation of *Amb a I* (AgE) and *Amb a II* (AgK) from short ragweed pollen, we noticed a basic fraction which appeared to be a new antigen with immunoreactivity similar to *Amb a I*. Since this fraction had a molecular weight similar to *Amb a I* and *Amb a II* but was basic rather than acidic, we decided to characterize it further. In this paper we describe the purification procedure and

partial characterization of *Ambrosia artemisiifolia* basic antigen (AaBA).

MATERIALS AND METHODS

Materials

Defatted short ragweed (*Ambrosia artemisiifolia*) pollen lot no. 13278 was obtained from International Biologicals Inc. (Piedmont, OK). Q-sepharose, S-sepharose and Sephadex G-15 were obtained from Pharmacia (Uppsala, Sweden). Acrylamide (99.9%), N-N'-Methylene-bisacrylamide, SDS, molecular weight standards and Bio-Rad protein assay reagent were from Bio-Rad (Richmond, CA). Coomassie Brilliant Blue R-250, an isoelectric focusing marker kit, bovine gamma globulin and polyoxyethylenesorbitan monolaurate (Tween 20) were obtained from Sigma (St Louis, MO). Immulon-2 plates were obtained from Baxter (McGaw Park, IL). The Vectastain ABC-AP kit and biotinylated goat antihuman IgE (epsilon chain specific) were from Vector Laboratories (Burlingame, CA). The ELISA amplification system was obtained from Gibco BRL (Grand Island, NY). Spectra/por dialysis membranes with molecular weight cut-offs of 25 and 10 kDa were purchased from Spectrum Medical Industries, Inc. (Houston, TX). Centricon-10 microconcentrators and an Amicon filtration system, model 8050 with YM10 membranes, were from Amicon Inc. (Beverly, MA). Servalyt precast gels (Precotes) 3-10 for isoelectric focusing were obtained from SERVA (Paramus, NJ). Antigens (AgE B + EC;

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AgE B₂, Ra3 and AgK) and pooled human allergic sera were obtained from the National Institute of Allergy and Infectious Diseases (NIAID, Bethesda, MD). Human sera from both allergic and non-allergic individuals were obtained at Cortech. Several serum samples from people allergic to ragweed were also provided by Dr Hunter Smith (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Organic solvents were of HPLC grade. Other chemicals were of analytical grade purity.

AaBA purification

For extract preparation, 5 g of *Ambrosia artemisiifolia* pollen was mixed with 12.5 ml of 0.05 M Tris buffer, pH 7.95, containing 1.1 mM cystine and 0.15 M NaCl for 1–8 hr at 4°C, then centrifuged for 40 min at 27000g. The pellet was then subjected to the same extraction procedure again. Both extracts were pooled and ammonium sulfate was added to 40% saturation at 4°C. The solution was left overnight at 4°C, then centrifuged for 20 min at 3000g. The pellet was dissolved in 0.05 M phosphate buffer, pH 7.1 and desalted on a Sephadex G-15 column (3 × 24 cm) in the same buffer.

A 1.5 × 8.5 cm Q-sepharose column was equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. Approximately 20 ml of protein solution after Sephadex G-15 was dialysed against 2 l of 0.05 M Tris buffer, pH 8.0, using 10 kDa cut-off membranes and was applied to the Q-sepharose column. Proteins were eluted with a 0–0.2 M linear gradient of NaCl in 0.05 M Tris buffer, pH 8.0 and residual protein was eluted with 1 M NaCl. The flow-through fraction containing *AaBA* (total volume 50 ml) was dialysed against 0.05 M acetate buffer, pH 5.35, using 25 kDa cut-off membranes. The dialysed material was applied to a S-sepharose column (2 × 6 cm), equilibrated with 0.05 M acetate buffer, pH 5.35 and the adsorbed proteins were eluted with a 0–0.4 M linear gradient of NaCl in the same buffer. The absorbance of the eluate was monitored at 280 nm with an ISCO (Lincoln, NE) UA-5 Absorbance Detector. *AaBA*-containing fractions were pooled and concentrated using an Amicon filtration system with YM10 membranes.

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out in 15% polyacrylamide gels according to Laemmli (1970). Molecular weight standards were included in each analysis. Proteins were stained with Coomassie Brilliant Blue R-250 or silver stain (Oakley *et al.*, 1980).

Molecular weight determination was performed by SDS-PAGE using the following molecular weight markers (Bio-Rad): soybean trypsin inhibitor (M.W. 20 kDa), trypsinogen (M.W. 24 kDa), carbonic anhydrase (M.W. 29 kDa), glyceraldehyde-3-phosphate dehydrogenase (M.W. 36 kDa) and ovalbumin (M.W. 45 kDa). Four micrograms of each standard and various quantities of *AaBA* (1.2–6 µg) were applied.

Isoelectric focusing

Isoelectric focusing (IEF) was performed using Servalyt precast gels 3-10 (300 µm, 100 × 125 mm).

Anode fluid contained 0.33% L-aspartic acid and 0.37% L-glutamic acid, pH 3.3. Cathode fluid solution consisted of 0.4% L-arginine, 0.4% L-lysine and 12% ethylenediamine. IEF markers (Sigma) were: β-lactoglobulin A (pI 5.1), myoglobin (pI 7.16), β-lactic dehydrogenase (pI 8.55), trypsinogen (pI 9.3) and cytochrome c (pI 10.6). Maximum voltage after 3 hr was 1700 V, maximum current was 5 mA. The proteins in IEF gels were fixed overnight in 20% trichloroacetic acid in ethanol. IEF gels were stained for 15 min in stain solution (0.1% Coomassie Brilliant Blue R-250, 40% ethanol, 10% acetic acid). Destaining of gels was performed in the fixative solution diluted 100 times with water.

Antigen reactivity determination

Reactivity was determined by ELISA. Microtiter plates (Immulon-2) were coated overnight at 4°C with different readout antigens at concentrations of 1 µg/ml. The plates were then incubated with post-coat buffer, containing 0.1% gelatin and 0.01% thimerosal in phosphate buffered saline (PBS) for 1 hr at room temperature. After rinsing with 0.5% Tween in PBS, the plates were incubated for 90 min with allergic or non-allergic sera (dilutions 1:100, 1:150, 1:250, 1:300, 1:500 with serum diluent containing 0.5% bovine gamma-globulin, 0.001% gelatin and 0.5% Tween in PBS). The plates were rinsed with PBS-Tween and incubated with biotinylated goat anti-human IgE antibodies (diluted 1:200 with serum diluent) for 30 min. Following washing with PBS-Tween solution, incubation with avidin DH and biotinylated alkaline phosphatase reagents from the ABC kit was performed for 30 min at room temperature. The plates were rinsed again with PBS-Tween, incubated with substrate (nicotinamide adenine dinucleotide) for 15 min and after addition of amplifier (alcohol dehydrogenase and diaphorase) the absorbance of individual wells was measured on an ELISA reader (Molecular Devices, UV max, Palo Alto, CA) at 490 nm.

Competition experiments

Allergic sera diluted 50–500 times in serum diluent were mixed with 0.3 µg/ml and 1.5 µg/ml competitors and the mixtures were incubated for 1.5 hr at room temperature. Two hundred microliter samples were then added to microtiter wells, which had been coated with the readout antigens. The ELISA was then carried out as described above.

Protein concentration determination

Protein concentration was determined using Bio-Rad (Bradford) Protein Assay. The concentration of the purified material was also estimated from quantitative amino acid analysis. The amino acid analysis indicated concentration values lower than those obtained in the Bradford assay by a factor of 2.6.

Amino acid analysis

Protein samples were hydrolysed under vacuum for 24 hr in 6 N HCl vapor at 110°C. Hydrolysis with methanesulfonic acid (4 M), containing 0.2% 3-(2-

Q-SEPHAROSE

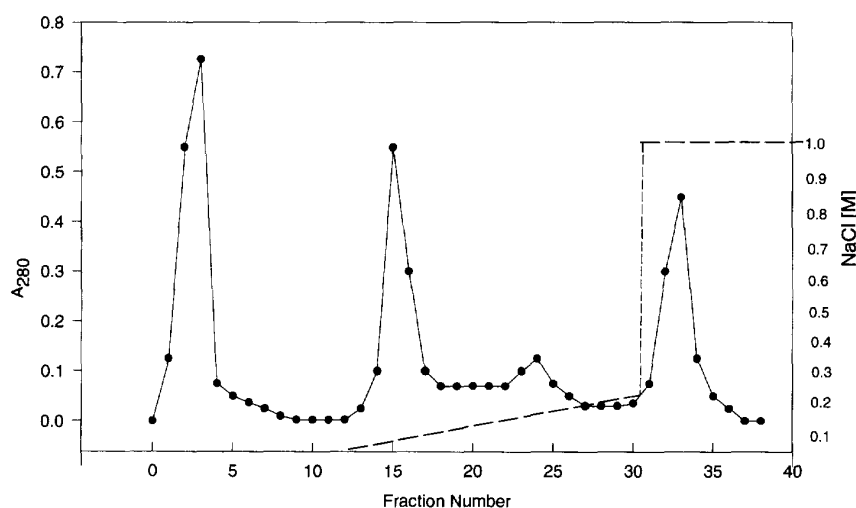


Fig. 1. Purification of *AaBA* by Q-sepharose chromatography. A Q-sepharose column was equilibrated with 0.05 M Tris buffer, pH 8.0 and the dialysed material obtained after ammonium sulfate precipitation was applied (see Materials and Methods). The column was washed with the Tris buffer and developed with a 0–0.2 M linear gradient of sodium chloride in the same buffer. The residual protein was eluted with 1 M NaCl. The non-adsorbed fraction contained mostly *AaBA*.

aminoethyl) indole (Pierce), was used for tryptophan analysis. The acid hydrolysates were analysed using the PICO-TAG system (Waters Associates). Cysteine was determined as cysteic acid using the performic acid oxidation procedure according to Tarr (1986).

Amino acid sequence determination

The sequence of the first 20 amino acid residues of *AaBA* was determined using an Applied Biosystems 473A protein/peptide sequencer coupled to a Microgradient PTH analyser and model 610A Data Analysis system.

Reverse phase high performance liquid chromatography (HPLC)

The Waters HPLC system consisted of two model 510 pumps, an automated gradient controller, a Lambda-Max Model 481 LC Spectrophotometer, and a Model 745 Data module. A 300 Å/5 µm Vydac or Waters Delta-Pak C₄ analytical column (4.6 × 250 mm) was employed with a 30 min CH₃CN gradient (10–90%), containing 0.1% trifluoroacetic acid. The flow rate was 1 ml/min. The absorbance of the eluate was monitored at 214 nm.

RESULTS AND DISCUSSION

Protein purification

The method of *AaBA* purification presented in this paper is straightforward and includes ammonium sulfate precipitation and two chromatography steps: anion-exchange chromatography on Q-sepharose and cation-exchange chromatography on S-sepharose. *AaBA* was initially identified using SDS-PAGE. The antigen reactivity monitoring was introduced after Q-sepharose

chromatography (when *AaBA* was separated from cross-reactive *Amb a I* and *Amb a II*).

AaBA was prepared from defatted pollen of *Ambrosia artemisiifolia*. The antigen was extracted with 0.05 M Tris, 1.1 mM cysteine, 0.15 M NaCl, pH 7.95 and the

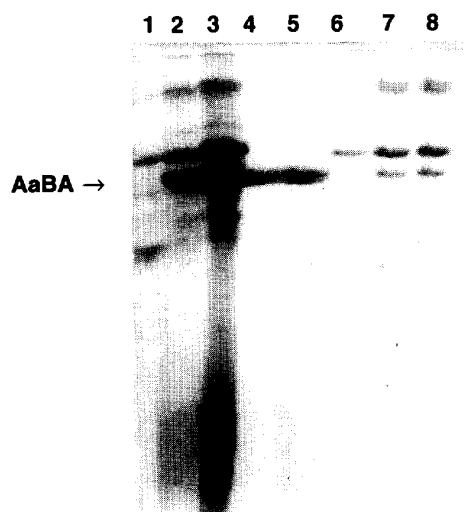


Fig. 2. SDS-polyacrylamide gel electrophoresis of *AaBA* after Q-sepharose. Lane 1: *Amb a I* (NIH AgE); lanes 2 and 3: *AaBA* containing fraction obtained after ammonium sulfate precipitation (see Results and Discussion), 5 and 10 µl, respectively; lanes 4 and 5: non-adsorbed fraction (peak no. 1 containing *AaBA*), 15 µl and 20 µl, respectively; lane 6: pooled flow-through fractions (6–12) collected between peak no. 1 and peak no. 2, 20 µl; lanes 7 and 8: peak no. 2, 15 µl and 20 µl, respectively. Proteins were stained with Coomassie Brilliant Blue R-250.

S-SEPHAROSE

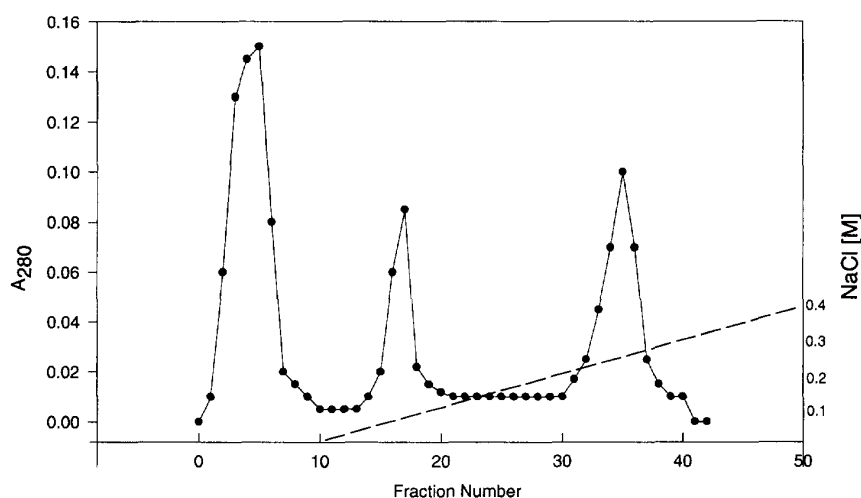


Fig. 3. Purification of *AaBA* by S-sepharose chromatography. A S-sepharose column was equilibrated with 0.05 M acetate buffer, pH 5.35. The non-adsorbed material from Q-sepharose (Fig. 2) was dialysed against 0.05 M acetate buffer, pH 5.35 and applied to the S-sepharose column. The column was developed with a gradient from the equilibrating buffer to 0.4 M NaCl as indicated by the dashed line. *AaBA* was eluted in the third peak.

crude protein material was precipitated with ammonium sulfate (see Materials and Methods). Different concentrations of ammonium sulfate (40%, 60%, 80% and 90% saturation) were tested and the 40% ammonium sulfate saturation fraction was chosen because it contained the largest amount of *AaBA* with the highest purity. We also tried to purify *AaBA* from the extract, omitting the ammonium sulfate step, but this procedure was unsuccessful. The 40% ammonium sulfate fraction was applied to a Q-sepharose column. *AaBA* was found in the

non-adsorbed fraction (Fig. 1). All other proteins, including *Amb a I* and *Amb a II*, were eluted with a linear gradient of NaCl. SDS-PAGE analysis of the eluate is presented in Fig. 2. The *AaBA* fraction contained a doublet band with additional minor contaminants. This doublet sometimes appeared as a single band at higher protein loadings (Fig. 4, lane 6). For further purification, the *AaBA* fraction obtained from the Q-sepharose column was applied to a S-sepharose column (Fig. 3) and the adsorbed proteins were eluted with a linear gradient

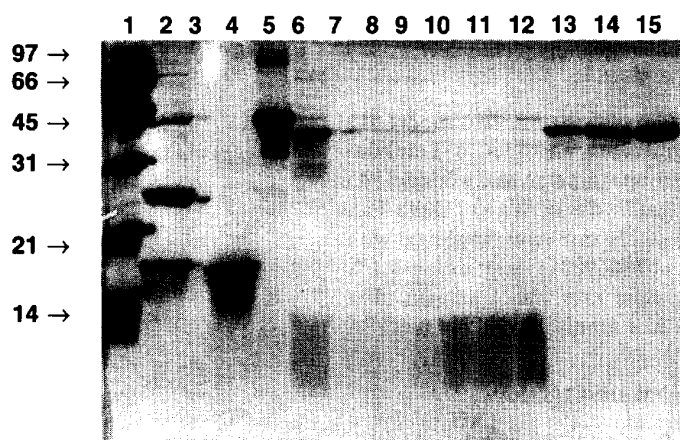


Fig. 4. SDS-polyacrylamide gel electrophoresis of *AaBA* during different stages of purification on S-sepharose. Lane 1: low molecular weight standard; lane 2: *Amb a I* (AgE B + EC); lane 3: *Amb a I* (AgE B₂); lane 4: *Amb a III* (Ra3); lane 5: *Amb a II* (AgK); lane 6: *AaBA* after Q-sepharose; lanes 7–9: non-adsorbed fraction (peak no. 1, Fig. 4) 3, 5 and 10 µl, respectively; lanes 10–12: peak nos 2, 3, 5 and 10 µl, respectively; lanes 13–15: peak no. 3 (*AaBA*), 3, 5 and 10 µl, respectively. The arrows on the left indicate the molecular masses of the standards in kDa. Proteins were stained with Coomassie Brilliant Blue R-250.

<i>AaBA</i>	K	A	X	E	A	H	N	I	I	D	K	X	W	R	X	K	A	D	W	A	N	N	R	Q	A	L
	.	:													:			:								
<i>Amb a II</i>	Q	G	C	E	A	H	N	I	I	D	K	C	W	R	C	K	P	D	W	A	E	N	R	Q	A	L
	.	:	:											.	:								.			
<i>Amb a I</i>	T	S	G	A	Y	N	I	I	D	G	C	W	R	G	K	A	D	W	A	E	N	R	K	A	L	

Fig. 5. Comparison of NH₂-terminal sequence of *AaBA*, *Amb a I* and *Amb a II*. *AaBA* was sequenced in the disulfide form. Solid lines indicate identical residues; colons indicate residues with a similarity index of 0.5 or greater and one dot indicates residues with a similarity index of 0.1 or greater. Values for similarities are from Gribskov and Burgess (1986). X indicates an unidentified residue, most likely cystine. Only background signal was detected in these positions.

of NaCl (see Materials and Methods). *AaBA* was eluted in the last peak. This material migrated as a single band in SDS-PAGE using Coomassie Brilliant Blue (Fig. 4) and silver stain (data not shown). The yield was 0.6 mg of *AaBA* protein (based on amino acid analysis) from 1 g of pollen. Such a high percentage of *AaBA* in pollen suggests that this antigen might be an important allergen in addition to *Amb a I* and *Amb a II*, especially if its solubility and adsorption on biological membranes were greater than those of *Amb a I* and *Amb a II*. However, additional studies are necessary to confirm this.

The molecular weight of *AaBA* was determined by SDS-PAGE in 15% gels under reducing conditions using protein standards and NIH *Amb a I* and *Amb a II* (Fig. 4). *AaBA* was also electrophoresed in the absence of 2-mercaptoethanol (data not shown). In both cases only a single band was observed with a molecular weight of 36.5 kDa.

Table 1. Amino acid composition of *AaBA*: Comparison with *Amb a I* and *Amb a II*

AA ^a	<i>AaBA</i> (Mol%)	<i>Amb a I</i> ^d (Mol%)	<i>Amb a II</i> ^e (Mol%)
Lys	5.88	5.12	4.70
His	3.52	1.83	3.18
Arg	4.28	4.63	4.40
Asp	14.28	14.2	13.47
Thr	5.23	5.12	6.55
Ser	6.01	7.60	5.06
Glu	6.93	7.19	8.70
Pro	4.30	4.39	4.85
Gly	9.55	11.09	9.55
Ala	9.31	9.08	6.34
Cys ^b	3.14	2.09	2.64
Val	6.27	6.99	7.04
Met	2.01	1.98	2.85
Ile	5.85	5.88	6.79
Leu	6.79	6.20	5.25
Tyr	0.83	1.13	1.94
Phe	3.57	3.64	4.00
Trp ^c	2.25	1.78	2.67

^aThe PICO-TAG method was used for all amino acids.

^bDetermined as cysteic acid using performic acid oxidation.

^cAcid hydrolysis was performed in the presence of 4 M methanesulfonic acid.

^dKing *et al.*, 1964.

^eKing *et al.*, 1967.

Isoelectric focusing of *AaBA* showed one strong band at pH 8.65 (data not shown). This pI value is consistent with *AaBA* not being retained on Q-sepharose at pH 8.0. The pI values reported for *Amb a I* and *Amb a II* by King (1972) were in the range of 4.8–5.5 and 5.4–6.0, respectively. These proteins displayed heterogeneity by

Table 2. IgE anti *AaBA* antibody reactivity in human sera

Sera	Relative absorbance (%)
NIH pooled Sera	100 ± 3.5
Male no. 1	13.5 ± 3.4
Male no. 2	24.4 ± 6.3
Male no. 3	83 ± 15
Male no. 4	45 ± 21
Male no. 5	5.6 ± 0.29
Male no. 6	98 ± 14
Female no. 1	12.3 ± 2.9
Female no. 2	18.2 ± 2
Female no. 3	40 ± 13
Female no. 4	62 ± 15
Female no. 5	33 ± 13
Female no. 6	47 ± 14
Female no. 7	13.3 ± 2.4
Male no. 7	0
Male no. 8	0
Male no. 9	0
Male no. 10	0
Female no. 8	0
Female no. 9	0
Female no. 10	0
Female no. 11	0

All allergic sera responses were compared with the NIH pooled sera designated as 100%. The 100% reactivity corresponds to an absorbance of 0.8 ± 0.03 after background subtraction ($0.09 \pm .003$). Standard deviation represents inter-assay variability. Male sera nos 4, 5, 6, 7 and female sera nos 1, 2, 3, 4, 5, 7, 8 and 9 were provided by Dr Hunter Smith from the National Jewish Center for Immunology and Respiratory Medicine. Male sera nos 4, 5, 6 and female sera nos 1–7 were skin-tested positive for *Amb a I*. Male sera nos 1, 2, 3 and female serum no. 6 were from Cortech employees with seasonal allergy. Male sera nos 8–10 and female sera nos 10 and 11 were from Cortech employees without seasonal allergy.

Table 3. Cross-reactivity of *AaBA*, *Amb a I* and *Amb a II* measured by competition ELISA

Sera	<i>AaBA</i> plate	<i>Amb a I</i> plate	<i>Amb a II</i> plate
Diluted sera	100% $\pm$ 8.5%	100% $\pm$ 6.8%	100% $\pm$ 9%
Sera + 1.5 μ g/ml <i>AaBA</i>	4.9 $\pm$ 2.2%	21% $\pm$ 0.33%	37% $\pm$ 7
Sera + 1.5 μ g/ml <i>Amb a I</i>	21% $\pm$ 0.33%	0	31% $\pm$ 7%
Sera + 1.5 μ g/ml <i>Amb a II</i>	44% $\pm$ 1.7%	44% $\pm$ 4.6%	12% $\pm$ 5%

Competition ELISA was carried out as described in Materials and Methods. Reactivity of 100% corresponds to an absorbance of 0.6 ± 0.05 after background subtraction.

The background absorbance was 0.1 ± 0.001 . Standard deviation represents inter-assay variability.

*Similar results were obtained for all three antigens at a concentration of 0.3 μ g/ml.

isoelectric focusing, but all the bands were acidic. *AaBA* was also eluted as a single peak on reverse phase HPLC (C-4 column, data not shown). It is interesting to note that Shen *et al.* (1988), during preparation of monoclonal antibodies against *Amb a I* and *Amb a II*, noticed reactivity with a 36 kDa antigen in the crude extract. It is quite possible that this band corresponds to *AaBA*.

The N-terminal amino acid sequence of *AaBA* is very similar to *Amb a II*, one of the main acidic allergens from pollen (Rogers *et al.*, 1991). The partial sequence shows 73% identity with *Amb a II* and 50% identity with *Amb a I* (Fig. 5). The amino acid compositions of the three antigens (Table 1) are also similar despite the fact that *AaBA* is a basic protein.

However, since the ratio of basic to acidic amino acid residues is about 0.8 in *Amb a I* and *Amb a II*, relatively small changes in the content of basic amino acid residues and changes in the ratios of Asp/Asn and/or Glu/Gln can easily be responsible for the observed differences in the pI values for these proteins. We used a computer program which calculates theoretical pI values for proteins with known molar contents of ionizable amino acid residues. The calculated pI values for *Amb a I* (5.58) and *Amb a II* (6.1) were similar to the experimental values within ± 0.3 pI unit. In the case of *AaBA*, the molar contents of Arg, Lys, His, Asx, Glx and Tyr were estimated from the amino acid composition and the molecular weight of *AaBA*. If one assumes that the number of Asp + Glu residues in an *AaBA* molecule equals 33, the calculated pI value is 8.75. The number of acidic residues in *Amb a I* and *Amb a II* is 42 and 40, respectively. In all three proteins the total numbers of Asx + Glx residues and Arg + Lys + His residues are in the range of 70–80 and 40–50 per protein molecule, respectively.

Antigenic reactivity of *AaBA* was tested with 14 different allergic sera (Table 2), including the NIH standard and five non-allergic sera using ELISA. The allergic sera displayed immunoreactivity with *AaBA*, although the variability between the individual sera was high. As expected, the non-allergic sera were non-reactive.

Competition experiments were performed with *AaBA*, NIH *Amb a I* and NIH *Amb a II*. In every case, each antigen competed best with itself, as expected. However, all the antigens also competed significantly with each other (Table 3). The differences in the immunoreactivities between the antigens could not be evaluated precisely because the exact concentrations of active material in the

samples cannot be simply determined. We measured the total protein concentration only. It is interesting to note that despite the huge differences in charge between *AaBA*, *Amb a II* and *Amb a I*, all the proteins display significant antigenic cross-reactivities.

The competition and sequencing (percentage identity) results suggest that at least some major epitopes are similar; however, other possibilities like adjacent location of different epitopes cannot be ruled out at this time.

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