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PURIFICATION AND IMMUNOCHEMICAL CHARACTERIZATION OF RECOMBINANT AND NATIVE RAGWEED ALLERGEN *Amb a* II

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Abstract—The complete sequence of a cDNA encoding *Amb a* II and its relationship to the *Amb a* I family of allergens has recently been described [Rogers *et al.* (1991) *J. Immun.* **147**, 2547–2552; Griffith *et al.* (1991*a*), *Int. Archs Allergy appl. Immun.* **96**, 296–304]. In this study, we present results generated with rabbit antipeptide antisera that recognize *Amb a* II or *Amb a* I, but not both. The specificity of two anti-*Amb a* II antipeptide sera, anti-RAE-50.K and anti-RAE-51.K, was verified on Western blots of recombinant *Amb a* II and *Amb a* I.1. These two sera, directed against separate regions of the *Amb a* II molecule, detected three individual 38-kDa *Amb a* II isoforms on 2D Western blots of aqueous ragweed pollen extract. These *Amb a* II isoforms have pI in the 5.5–5.85 range and can be easily distinguished from *Amb a* I isoforms with pI in the 4.5–5.2 range detected by an anti-*Amb a* I specific peptide antiserum. The *Amb a* II isoforms have also been individually purified from pollen, positively identified as *Amb a* II by amino acid sequencing, and visualized as separate bands on IEF gels. An analysis of *Amb a* II cDNA sequences generated by PCR led to the prediction of three *Amb a* II isoforms with pI of 5.74, 5.86 and 5.97 that are very similar to the pI deduced from 2D Western blot analysis. Recombinant *Amb a* I.1 and *Amb a* II have been expressed in *E. coli*, purified in their denatured form, and examined by ELISA for their capacity to bind pooled allergic human IgE. Purified native *Amb a* and *Amb a* II from pollen were shown to have very similar IgE-binding properties. In contrast, *Amb a* II had a markedly reduced IgE-binding capacity as compared to *Amb a* I.1. These data suggest that recombinant *Amb a* I.1 and *Amb a* II, isolated in a denatured form, differ significantly in their IgE-binding properties whereas the native molecules isolated from pollen do not.

INTRODUCTION

Short ragweed pollen is one of the most important aeroallergens in North America (King, 1976). Ragweed pollen is composed of a complex mixture of at least 52 antigens (Lowenstein and Marsh, 1981), with at least 22 able to bind human IgE (Lowenstein and Marsh, 1983). *Amb a* II (formerly AgK) has been defined as an active allergen in ragweed pollen (King *et al.*, 1967) and has been previously shown to share partial immunological identity with the most important ragweed allergen, *Amb a* I (formerly AgE) (King *et al.*, 1967; Adolphson *et al.*, 1978; Lowenstein *et al.*, 1981). The purification from pollen of these two important allergens has established that these proteins share similar properties including mol. wt, relatively high abundance in pollen, as well as the ability to bind human IgE and trigger histamine release from allergic human leukocytes in *in vitro* assays (King *et al.*, 1964, 1967; King, 1972).

Amb a I has recently been cloned and determined to be composed of a family of four related proteins rather than a single protein (Rafnar *et al.*, 1991; Griffith *et al.*, 1991*a*). This family of proteins, designated *Amb a* I. 1, *Amb a* I.2, *Amb a* I.3 and *Amb a* I.4, according to the IUIS nomenclature, share approximately 80% homology between each family member (Rafnar *et al.*, 1991; Griffith *et al.*, 1991*a*). Importantly, *E. coli* expressed r*Amb a* I.1, I.2, I.3 and I.4 have been shown to bind human allergic IgE and stimulate allergic human T cells in proliferation assays *in vitro* (Bond *et al.*, 1991, unpublished results). Recently, *Amb a* II has been cloned and shown to share 65% identity with *Amb a* I.1, demonstrating that *Amb a* II is a more distant relative of the *Amb a* I group of allergens (Rogers *et al.*, 1991). However, r*Amb a* II primed T cell lines isolated from ragweed allergic patients respond to purified r*Amb a* I.1, suggesting a high level of T cell crossreactivity (Rogers *et al.*, 1991).

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Abbreviations: *Amb a* I.1, I.2, I.3, I.4, and II, allergens from *Ambrosia artemisiifolia* named according to the IUIS approved nomenclature [*Anns Allergy* **60**, 499–504 (1988)]; other allergens are cited according to the IUIS approved nomenclature; mAb, murine monoclonal antibody; r, recombinant; SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; IEF, isoelectric focusing; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; 2D, two-dimensional; TMB, 3,3′,5,5′-tetramethylbenzidine; PBS, phosphate-buffered saline; NTA, nitrilotriacetic acid.

The primary structures of several important pollen allergens have recently been defined by cDNA cloning, including *Bet v* I (Breiteneder *et al.*, 1989), *Amb a* I (Rafnar *et al.*, 1991), *Lol p* I (Perez *et al.*, 1990; Griffith *et al.*, 1991*b*), *Poa p* IX (Silvanovich *et al.*, 1991; Olsen *et al.*, 1991), *Lol p* Ib (Singh *et al.*, 1991) and *Car b* I (Larsen *et al.*, 1992). Several of these pollen allergens have been shown to be comprised of multiple isoforms. The presence of multiple isoforms is not restricted to plant pollen allergens since the major cat allergen, *Fel d* I, and the dust mite allergen, *Der f* II, also have minor sequence polymorphisms (Morgenstern *et al.*, 1991; Griffith *et al.*, 1992; Yuuki *et al.*, 1990; Trudinger *et al.*, 1991). The *Amb a* I and *Amb a* II multigene family is further complicated because of the presence of polymorphic residues within each of the defined individual family members, *Amb a* I. 1, I.2, I.3, I.4 and *Amb a* II (Rafnar *et al.*, 1991; Griffith *et al.*, 1991*a*; Rogers *et al.*, 1991).

In this manuscript we characterize *Amb a* II by: (1) mAb binding specificity and the generation of *Amb a* II and *Amb a* I.1. monospecific anti-peptide antisera, (2) defining the number and the pI of *Amb a* II isoforms in pollen by IEF, 2D SDS–PAGE Western blotting, and nucleotide sequence analysis of *Amb a* II, and (3) examining allergic IgE binding to purified native and recombinant *Amb a* I.1 and *Amb a* II.

Table 1. Predicted pI values for *Amb a* II isoforms[a]

Polymorphic amino acids			Predicted pI
D_{71}	T_{139}	K_{322}	5.74
D_{71}	T_{139}	R_{322}	5.74
D_{71}	K_{139}	K_{322}	5.86
D_{71}	K_{139}	R_{322}	5.86
N_{71}	T_{139}	K_{322}	5.86
N_{71}	K_{139}	K_{322}	5.97
N_{71}	K_{139}	R_{322}	5.97

[a]The potential isoforms for each *Amb a* II family member are identified here by the various combinations of the polymorphic residues. The complete primary structure of *Amb a* II was presented in Rogers *et al.* (1991) and Griffith *et al.* (1991*a*). Each polymorphic residue was detected independently of other polymorphisms. The predicted pI of each *Amb a* II isoform was calculated using software contained in PCGENE (Intelligenetics, Mountain View, USA). The r*Amb a* II protein described in this manuscript has residues N_{71}, K_{139} and K_{322}.

MATERIALS AND METHODS

Expression and purification of Recombinant Amb a *I.1 and* Amb a *II*

Complementary DNA inserts encoding *Amb a* I.1 (Rafnar *et al.*, 1991) and *Amb a* II (Rogers *et al.*, 1991) were inserted into the expression vector pTrc99 (Amann *et al.*, 1988) kindly provided by Dr Egon Amann (Behringwerk AG, Marburg, Germany). These two cDNAs (starting at residue 10) had a polylinker encoding six sequential histidines cloned onto their 5′ ends. The expressed products were purified using a Ni^{2+} chelating support (QIAGEN NTA-Agarase) (Diagen Gmblt, Dusseldorf, Germany) as previously described (Hochuli *et al.*, 1988). A retroregulator stem-loop sequence from *B. thuringiensis* (Skoglund *et al.*, 1990) was inserted downstream from the *Amb a* I.1 and *Amb a* II cDNA to further increase expression levels. The r*Amb a* II described in this manuscript has the sequence presented in Rogers *et al.* (1991) and has the polymorphic residues N_{71} K_{139} K_{322} (see Table 1).

Antibodies

The murine mAbs JB4E3 and JB1E3-4 directed against *Amb a* I (Smith *et al.*, 1988) were produced as ascites. Rabbit anti-*Amb a* I.1 and anti-*Amb a* II peptide antisera was generated by immunizing rabbits with synthetic peptide coupled to KLH. The anti-peptide antisera were raised against the peptide sequences RAE-17.1 (EPGESALSLTSSAGVLSC; residues 375–392 of *Amb a* I.1), RAE-50.K (CLRTGAQEPEWMT; residues 326–338 of *Amb a* II) and RAE-51.K (EPGDMVPQLTMNAGVLTC; residues 375–392 of *Amb a* II) derived from the cloned cDNA sequences (Rafnar *et al.*, 1991; Rogers *et al.*, 1991). These peptide sequences were chosen on the basis of their divergence in sequence (eight different residues out of a total of 18 in the case of RAE-17.1 and RAE-51.K) and the likelihood of generating antibody according to their predicted secondary structure (IntelliGenetics, Inc., Mountain View, CA). Ragweed allergic patient plasma was isolated from heparinized whole blood (received from Dr John Saryan, Lahey Clinic and Dr John Condemi, University of Rochester) and stored at −20°C, prior to analysis.

Immunoblot analysis

The gel electrophoresis and Western blot transfer procedures utilized were as described previously (Towbin *et al.*, 1979; Bond *et al.*, 1991). Isoelectric focusing for 2D gel electrophoresis was performed using the method of O'Farrell (1975). A mixture of 3.5% pH 3.5–10 ampholytes (Pharmacia, Piscataway, NJ) was used in a 7% acrylamide first-dimension IEF gel.

Purification and isoelectric focusing analysis of Amb a *II from Ragweed Pollen*

Fifty grams of defatted short ragweed pollen (Greer Laboratories, Lenoir, NC) was extracted in 500 ml 0.05 M Tris, pH 7.95, containing protease inhibitors (leupeptin, 2.0 μg/ml; pepstatin, 2.0 μg/ml; PMSF, 17 μg/ml; soybeam trypsin inhibitor, 2.0 μg/ml). The extract was depigmented by batch adsorption with Whatman DE-52 DEAE cellulose (150 g dry weight) in the presence of 0.2 M NaCl at 4°C. Unbound material was dialysed against 0.025 M Tris, pH 7.95, with

protease inhibitors. The depigmented sample was applied to an 80 ml DEAE cellulose column (Whatman DE-52) equilibrated in 0.025 M Tris, pH 7.95, containing protease inhibitors. Acidic proteins were eluted with 0.025 M Tris, 0.2 M NaCl, pH 7.95, at 4°C with inhibitors (King *et al.*, 1981). The acidic elution sample was separated into an *Amb a* II enriched fraction by $(NH_4)_2SO_4$ precipitation at 0–45% saturation (4°C). The pellet was solubilized and applied at 0.5 ml/min to a 200 ml Sephadex column in 0.05 M NH_4HCO_3 at 4°C. The partially purified *Amb a* II preparation, with a mol. wt of 38 kDa, was then dialyzed against 0.04 M Tris, pH 8.0. This sample was further FPLC purified by chromatography on an 8 ml Mono Q HR10/10 column (Pharmacia, Piscataway, NJ) in 0.04 M Tris, pH 7.0, at 25°C to separate contaminating *Amb a* I from *Amb a* II. Elution was performed with 0.04 M Tris, pH 8.0, containing 0.08 M NaCl. The major peaks were recovered and analyzed by IEF SDS–PAGE using a mixture of 4.5–5.3 and 3.5–10.0 ampholytes (Pharmacia, Piscataway, NJ) in a 7% acrylamide gel. Protein sequencing analysis confirmed the presence of *Amb a* II in the peak fractions recovered.

Protein sequencing

Protein fractions were subjected to protein sequencing analysis as previously described (Rogers *et al.*, 1991) using an Applied Biosystems (Foster City, CA) model 477A gas-phase sequenator with on-line phenylthiohydantoin derivative analysis (model 120). All known members of the *Amb a* I and *Amb a* II family of proteins have an asp–pro acid sensitive bond near their *C*-terminus (Rafnar *et al.*, 1991; Rogers *et al.*, 1991). Protein samples were subjected to weak acid cleavage and treated in the sequenator with OPA (Brauer *et al.*, 1984) to block all primary amines at the amino terminus of peptides except the proline residue (Rogers *et al.*, 1991).

Sequence analysis and pI prediction of Amb a *II isoforms*

The PCR cloning of *Amb a* II and the use of priming oligonucleotides and sequencing analysis have previously been described (Rogers *et al.*, 1991; Griffith *et al.*, 1991*a*). M13 clones were sequenced (Sequenase kit, U.S. Biochemicals) using the dideoxy chain-termination method of Sanger *et al.* (1977). Computer programs contained in PCGENE (IntelliGenetics, Inc., Mountain View, CA) were used for both protein and nucleotide sequence analysis and pI prediction analysis.

ELISA binding assay

Sandwich ELISA experiments were performed in 96-well microtiter plates as described in Harlow and Lane (1988). Briefly, rabbit anti-*Amb a* II antisera [10 μg/ml (King *et al.*, 1981)] was incubated overnight in PBS at 4°C. The plates were saturated with blocking buffer (5% milk powder dissolved in PBS). Various concs of native and recombinant *Amb a* I and *Amb a* II were added to the antibody-coated wells for 4 hr at room temp. Unbound material was removed by washing with PBS–Tween 20 (0.05%). Pooled ragweed allergic patient plasma (IgG depleted) was serially diluted in blocking buffer and incubated overnight at 4°C. After several washes, biotinylated goat anti-human IgG [0.5 mg/ml (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), diluted 1:10,000 in PBS] was added and incubated for 1 hr at room temp. After rewashing, 1:10,000 of a 0.4 mg/ml solution of horseradish peroxidase-conjugated streptavidin (Southern Biotin, Birmingham, AL) was added and incubated for 30 min at room temp. The plates were rewashed as previously and developed using a TMB-based chromatophoric assay. The reaction was stopped by the addition of phosphoric acid and the absorption at 450 nm was recorded.

RESULTS

Recombinant expression and purification of Amb a *I.1 and* Amb a *II*

Complementary DNA sequences encoding *Amb a* I.1 (Rafnar *et al.*, 1991) and *Amb a* II (Rogers *et al.*, 1991) were inserted in-frame into the pTrc99 vector system (Fig. 1, panel A).This expression system coupled with subsequent metal-ion affinity chromatography using NTA–agarose led to yields of 1–3 mg/l of *E. coli* culture. The Ni^{2+} metal ion chromatography resulted in the isolation of 90% pure r*Amb a* I.1 and r*Amb a* II in a single step as assessed by densitometric analysis of a Coomassie Blue stained SDS–PAGE gel (Fig. 1, panel B, lanes 3 and 4; data not shown). *N*-terminal sequencing analysis of the purified recombinant products verified that the *N*-terminal is intact and not structurally heterogeneous (data not shown). The two recombinant proteins, r*Amb a* I.1 and r*Amb a* II, have a slightly higher than expected apparent M_r of approximately 44 kDa on SDS–PAGE gels (Fig. 1, panel B). This increase in apparent M_r may be due to the presence of the $(his)_6$ purification tag since studies with the recombinant *Fel d* I allergen (Rogers *et al.*, 1993) showed that a leader sequence plus the $(his)_6$ had an anomolous M_r on SDS–PAGE. The recombinant products have nonrelevant residues at the *N*-terminus derived from the *Nco* I cloning site (MG), the polyhistidine purification tag (hexahistidine) and the amino acids EF encoded by the *Eco* RI cloning site (Fig. 1, panel A). The authentic *Amb a* I.1 and *Amb a* II sequences start at residue L_{10}, according to the numbering described previously (Rafnar *et al.*, 1991; Griffith *et al.*, 1991*a*; Rogers *et al.*, 1991). Some faint bands detected above and below the main recombinant product are likely to be aggregates, degradation products and a low level of contaminants (Fig. 1, panel B; Fig. 2).

Antibody reactivity to purified recombinant Amb a *I.1 and* Amb a *II*

Recombinant *Amb a* I.1 and *Amb a* II were Ni^{2+}–NTA–agarose column purified, dialysed against PBS and subjected to antibody binding analysis. Immunoblots were used to determine the specificity of

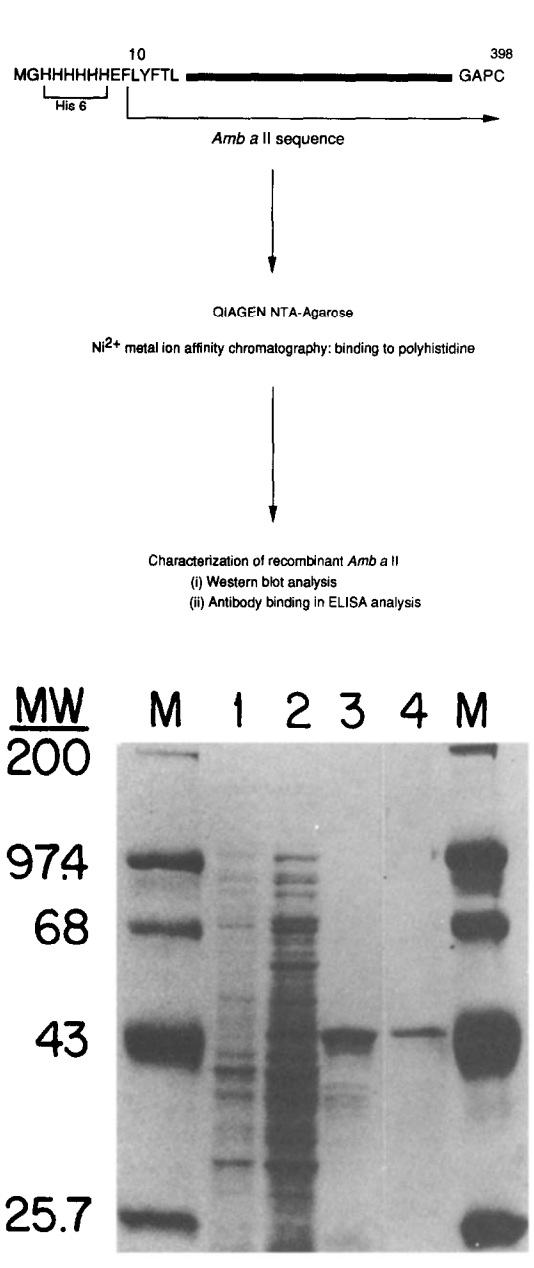


Fig. 1. Expression and purification of *Amb a* II. In panel A, a scheme for the expression and purification of r*Amb a* II is described. The single letter code representing encoded amino acids is used. The amino acid numbering refers to the cDNA encoded sequence reported in Rogers *et al.* (1991). In panel B an SDS–PAGE gel of Ni^{2+} metal-ion affinity purified r*Amb a* I.1 and r*Amb a* II is shown. The samples loaded include: lane M, mol. wt markers; lane 1, uninduced JM109 host cell lysate; lane 2, lysate of JM109 cell harboring the pTrc99–*Amb a* II expression vector after 2 hr of IPTG induction; lane 3, affinity purified *Amb a* II; and lane 4, affinity purified *Amb a* I.1. The 10% acrylamide gel was stained with Coomassie blue.

anti-peptide antisera and mAb to the metal-ion affinity purified recombinants. The metal-ion affinity purified r*Amb a* I.1 and r*Amb a* II proteins, as well as crude pollen extract, were subjected to SDS–PAGE and Western transfer. Six blot sections of these antigens were then probed with several antibody preparations (Fig. 2, panels A–F). Rabbit anti-peptide antisera specific for *Amb a* I.1 (anti-RAE-17.1) and *Amb a* II (anti-RAE-50.K and anti-RAE-51.K) were tested for their reactivity against the purified recombinants (Fig. 2, panels A, B and C, respectively). The peptides used to generate these rabbit anti-peptide antisera are schematically shown in Fig. 3. These three anti-peptide antisera, generated against divergent sequences, are very specific in their reactivity to either r*Amb a* I.1 (Fig. 2, panel A) or r*Amb a* II (Fig. 2, panels B and C). In panel E of Fig. 2, the blot was probed with a murine mAb JE4E3 raised against a denatured *Amb a* I preparation (Smith *et al.*, 1988). The JB4E3 mAb binds to four major species in pollen extract with mol. wts of 38, 35, 26 and 24 kDa. These protein forms have been previously described (King *et al.*, 1981; Bond *et al.*, 1991) and correspond to full-length as well as natural cleavage products of *Amb a* I and *Amb a* II in pollen extract. The 26-kDa band corresponds to the alpha form of *Amb a* I that has been previously described (Smith *et al.*, 1988; King *et al.*, 1981). The JB4E3 antibody binds to an epitope shared by *Amb a* I.1 and *Amb a* II since it shows reactivity to both these purified recombinant proteins (Fig. 2, panel E) and its binding site has been mapped to the peptide RAE-74.1 (KWNWRTNKDVLENGAIFVAS, residues 338–357 of *Amb a* I.1) [Fig. 3 (see below)]. In contrast, mAb JB1E3-4, also raised against the same denatured *Amb a* I preparation as for JB4E3 (Smith *et al.*, 1988), reacts only with *Amb a* I.1 and not *Amb a* II (Fig. 2, panel D).

An overlapping set of peptides based on the *Amb a* I.1 translated cDNA sequence (Rafnar *et al.*, 1991) was probed with the mAb JB1E3-4 and JB4E3 using a solid-phase dot blot assay to define the different observed binding specificities further. Interestingly, the *Amb a* I.1 specific mAb JB1E3-4 binds to the RAE-17.1 but not the RAE.51-K peptide (Fig. 3, data not shown). In contrast, the mAb JB4E3 that binds to both r*Amb a* I.1 and r*Amb a* II (Fig. 2, panel E), was shown to bind to the RAE-74.1 peptide (Fig. 3). The observed binding specificities of these two mAb appear to reflect the degree of shared amino acid sequence (Fig. 3). Therefore, specific binding of JB1E3-4 to *Amb a* I.1 but not *Amb a* II demonstrates the existence of unique non-shared murine B cell epitopes on these two molecules.

Two-dimensional immunoblot analysis demonstrates the presence in pollen of three isoforms of Amb a *II*

Pollen extract proteins were separated by 2D electrophoresis followed by Western transfer to nitrocellulose (O'Farrell, 1975). The *Amb a* II specific anti-peptide antisera, anti-RAE 50.K and anti-RAE 51.K, were used to probe these Western blots and the binding patterns are shown in Fig. 4 (panels A and B). The anti-RAE 50.K and anti-RAE 51.K antisera bind to the same three proteins, which have pI values of approximately 5.5, 5.7 and 5.85. Since both these antisera bind to the same three spots, even though they are directed against different sequences within the *Amb a* II molecule, it is concluded that there are at least three distinct isoforms of *Amb a* II in pollen extract. The previously reported pI values for *Amb a* I and *Amb a* II are 5.1 and 5.85, respectively (King, 1972; King *et al.*, 1981). To clearly

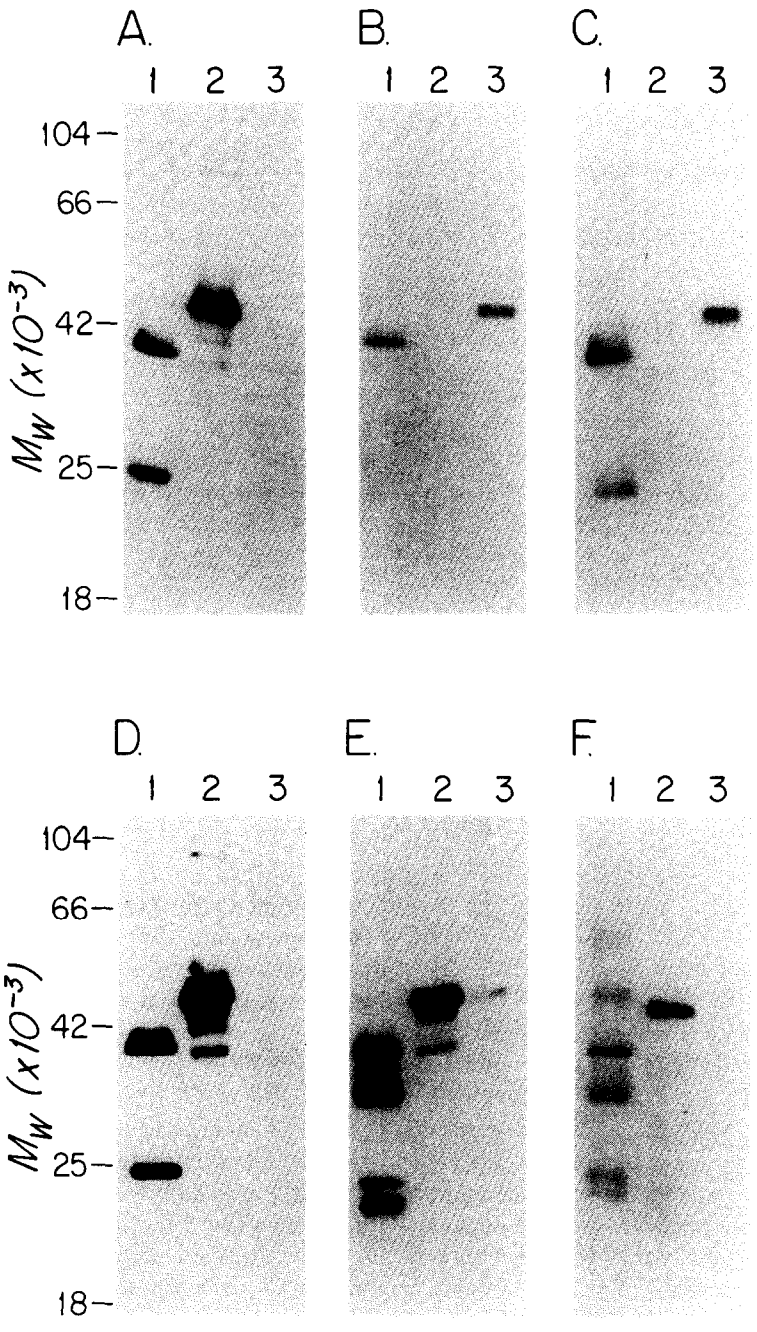


Fig. 2. Immunoblots of affinity purified recombinant *Amb a* I.1 and *Amb a* II. SDS–PAGE immunoblot sections were generated with the following antigens: lane 1; soluble ragweed pollen extract (12 μg/lane), lane 2; affinity purified recombinant *Amb a* I.1 (5 μg/lane), and lane 3; affinity purified recombinant *Amb a* II (5 μg/lane). Panels A–F were probed with various antibody preparations and dilutions as follows: (A) rabbit polyclonal anti-RAE 17.1 (1:2000), (B) rabbit polyclonal anti-RAE 50.K (1:2000), (C) rabbit polyclonal anti-RAE 51.K (1:2000), (D) murine anti-*Amb a* I monoclonal JB1E3-4 (1:2000), (E) murine anti-*Amb a* I monoclonal JB4E3 (1:2000), and (F) ragweed allergic patient IgE pool (1:10 plasma).

differentiate the three *Amb a* II species from multiple *Amb a* I gene products (*Amb a* I.1, I.2, I.3 and I.4), the same blot shown in Fig. 4 (panel B) was re-probed with anti-RAE-17.1, an *Amb a* I.1 anti-peptide antiserum that does not bind to *Amb a* II [see Fig. 2 (panel A)]. A set of at least three *Amb a* I spots were detected that have pI values in the range of 4.5–5.1 (Fig. 4, panel C) and consequently are not overlapping with those detected with anti-RAE-50.K and anti-RAE 51.K.

Amb a *II sequence polymorphism*

PCR analysis of short ragweed flowerhead mRNA revealed that *Amb a* II has three independent sequence polymorphisms at amino acids 71, 139 and 322 (Griffith *et al.*, 1991*a*). Expression of proteins with different combinations of these polymorphisms would generate eight distinct isoforms of *Amb a* II. However, only three distinct pI values can be calculated for

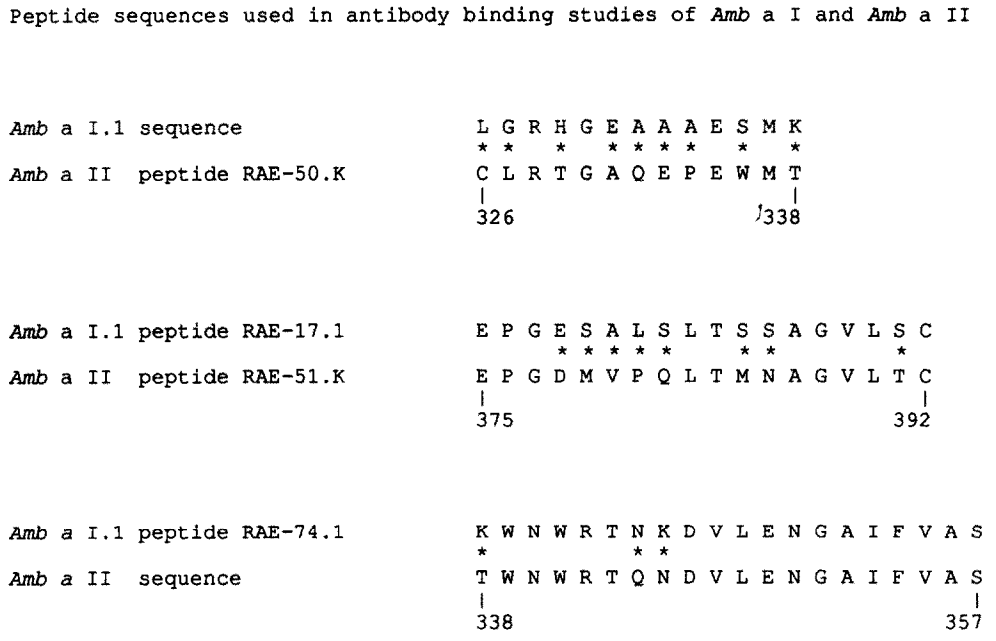


Fig. 3. Peptide sequences used in antibody binding studies of *Amb a* I and *Amb a* II. The peptides used as immunogens to generate the anti-*Amb a* I.1 (RAE-17.1) and anti-*Amb a* II (RAE-50.K and RAE-51.K) specific antipeptide antisera are shown. The peptides recognized by mAb JB1E3-4 (RAE-17.1) and JB4E3 (RAE-74.1) are also shown. The amino acid sequences are represented by the single letter code. Amino acid residues of *Amb a* II that differ from *Amb a* I.1 are indicated by an asterisk. The amino acid numbering is taken from Rafnar *et al.* (1991), Griffith *et al.* (1991*a*) and Rogers *et al.* (1991).

these eight proteins pI 5.74, pI 5.86, and pI 5.97 (Table 1).

Purification of Amb a *II isoforms from pollen*

A native *Amb a* II preparation isolated from short ragweed pollen using previously described methods (King *et al.*, 1967, 1981) was modified and further examined. The modified ion-exchange chromatography revealed that this preparation is comprised of separable bands (Fig. 5, panel A) that upon IEF analysis migrate with pI very similar to those predicted from the primary structure of *Amb a* II [Fig. 5 (panel B) and Table 1]. Samples from the FPLC peaks 4, 12, and 14 (Fig. 5, panel A; IEF sample lanes 2, 5, and 6, respectively, panel B) were subjected to amino acid sequence analysis and correspond to *Amb a* II (Rogers *et al.*, 1991; data not shown). Confirmation is also provided by the binding of anti-*Amb a* II specific antisera (Figs 2 and 4) to proteins in pollen with pI similar to those purified by ion-exchange chromatography (Fig. 5, panels A and B). Consequently, isoforms of *Amb a* II defined by cDNA cloning (Rogers *et al.*, 1991; Griffith *et al.*, 1991*a*; Table 1) can now be correlated with isolated isoforms from short ragweed pollen extract (Fig. 5, panels A and B).

Amino acid sequence analysis of the flowthrough from the FPLC purification unexpectedly led to the identification of a 35-kDa protein species corresponding to *Amb a* I.3 (Fig. 5, panel A, peak at approximately 15 min retention time). This analysis also revealed that the flowthrough is heterogeneous but is devoid of detectable *Amb a* II. Previously, sequence data from purified *Amb a* I samples only identified the presence of *Amb a* I.1 (Rafnar *et al.*, 1991). Therefore, under the different purification procedure described here, *Amb a* I.3 co-purifies with *Amb a* II.

Human ragweed allergic IgE binding to native and recombinant Amb a *II*

ELISA assays were used to measure the extent of ragweed allergic human IgE binding to ragweed pollen extract, purified native *Amb a* I and *Amb a* II, and r*Amb a* I.1 and r*Amb a* II (Fig. 6). The native *Amb a* I and *Amb a* II preparations have very similar IgE binding curves as measured in a Sandwich ELISA (Fig. 6, panel A). Further, each of these antigens bind a substantial proportion of the IgE as compared with whole pollen extract. These results are as expected since these proteins have extensive sequence homology (Rogers *et al.*, 1991) and have been defined as the major allergens in ragweed pollen (King *et al.*, 1964, 1967). Recombinant *Amb a* I.1 and r*Amb a* II also bind human ragweed allergic IgE in ELISA assays but to a markedly reduced extent compared to native antigen isolated from pollen (Fig. 6, panel B). Significantly, r*Amb a* I.1 binds substantially more human IgE than does r*Amb a* II (A_{450} of 0.4 vs 0.15 at 100 μg/ml, respectively) since r*Amb a* II does not bind human IgE to a detectable level in this assay [A_{450} of 0.15 at 100 μg/ml vs a background signal of $A_{450} = 0.08$ (see Fig. 6 panel b)]. This non-detectable IgE binding cannot be attributed to a low level of antigen binding to the solid phase since a rabbit anti-*Amb a* II antiserum binds nearly as well to r*Amb a* II as to r*Amb a* I.1 in a direct-binding ELISA (data not shown).

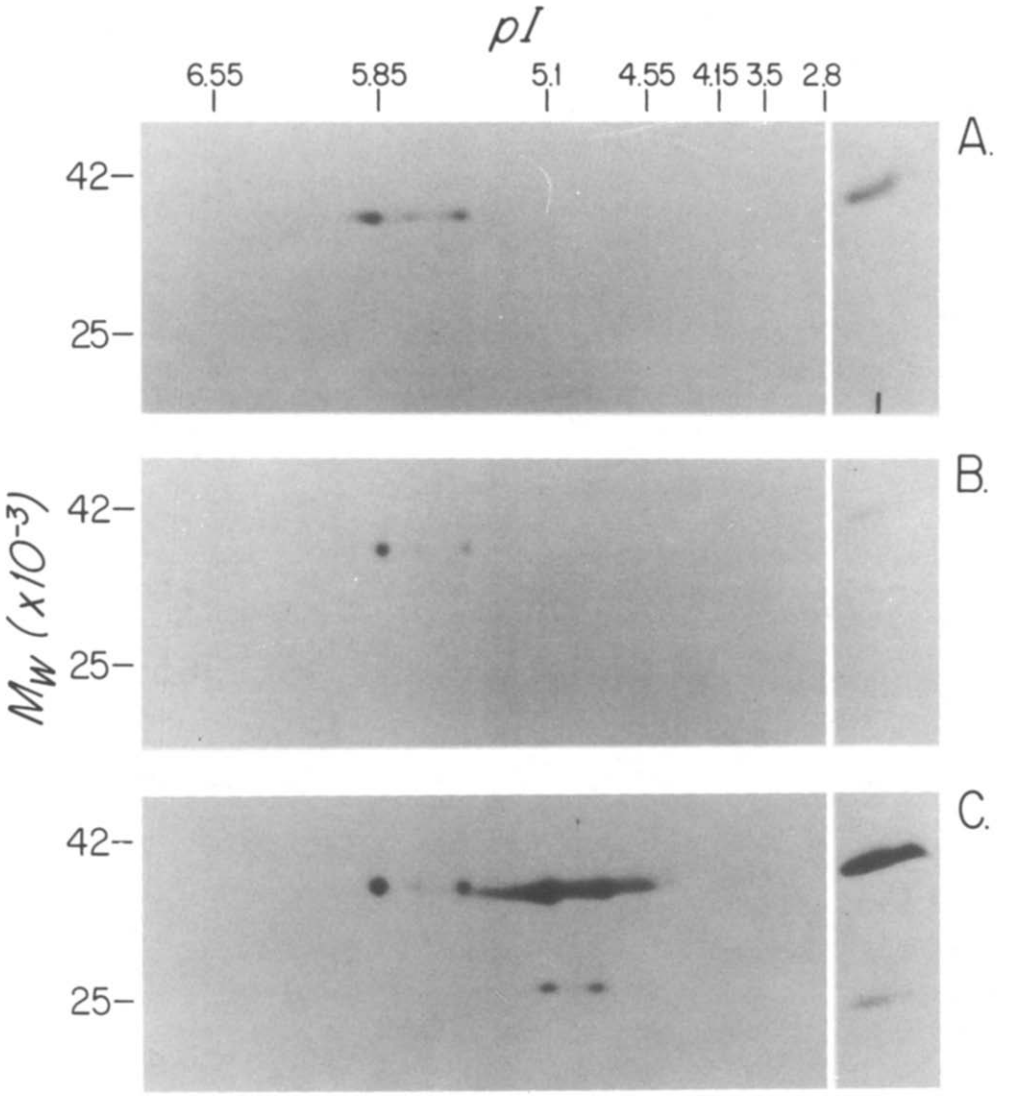


Fig. 4. Analysis of *Amb a* II isoforms in pollen by 2D Western blotting using rabbit anti-*Amb a* II peptide antisera. The tube gel IEF dimension with 30 μg of soluble pollen extract was electrophoresed on 10% acrylamide SDS–PAGE for the second dimension. Molecular weight markers and a lane with 12 μg of pollen extract was included in the SDS–PAGE dimension. The resulting immunoblots were then pooled with: (A) anti-RAE 51.K (*Amb a* II specific) (1:2000), (B) anti-RAE 51.K (*Amb a* II specific) (1:2000). (C) the immunoblot shown in B was preserved and reprobed with anti-RAE 17.1 (*Amb a* I specific) (1:2000).

DISCUSSION

The expression system described herein uses the strong *trp–lac* hybrid-promoter containing pTrc99 plasmid (Amann *et al.*, 1988) in conjunction with Ni^{2+} metal-ion affinity chromatography (Hochuli *et al.*, 1988) of the r*Amb a* II product. Although the expression levels are not particularly high, 1–3 mg/l culture, the Ni^{2+} metal-ion chromatographic method permits the isolation of intact r*Amb a* II with a high level of purity after a single purification step (Fig. 1). Furthermore, the r*Amb a* II has only 10 nonrelevant amino acids at the *N*-terminus (Fig. 1). This highly versatile approach is not restricted to the use of the pTrc99 expression plasmid since other strong-promoter expression systems together with Ni^{2+} chelate affinity chromatographically have recently been described for the high-level expression and purification of *Fel d* I (Rogers *et al.*, 1993) and *Asp f* I (Moser *et al.*, 1992).

Although there is detectable sequence identity between *Amb a* I.1 and *Amb a* II throughout the molecule there are regions where the sequences are significantly different. Regions of extensive sequence divergence include amino acid residues 20–31, 44–49, 150–159, 224–247, 252–274, 326–337 and 378–386 (Rogers *et al.*, 1991). Two anti-peptide antisera, anti-RAE-50.K and anti-RAE-51.K, were generated and shown on Western blots to bind specifically to r*Amb a* II with no detectable binding to r*Amb a* I.1 (Fig. 2, panels B and C). An analogous anti-*Amb a* I anti-peptide antiserum, anti-RAE-17.1 (region 375–392), was used to probe a duplicate blot section and was shown to bind specifically to *Amb a* I.1 but not *Amb a* II (Fig. 2; panel A). These data demonstrate that *Amb a* I.1 and *Amb a* II have unique murine B cell epitopes. This approach of generating anti-peptide antiserum that can differentially bind related allergens may find general utility as

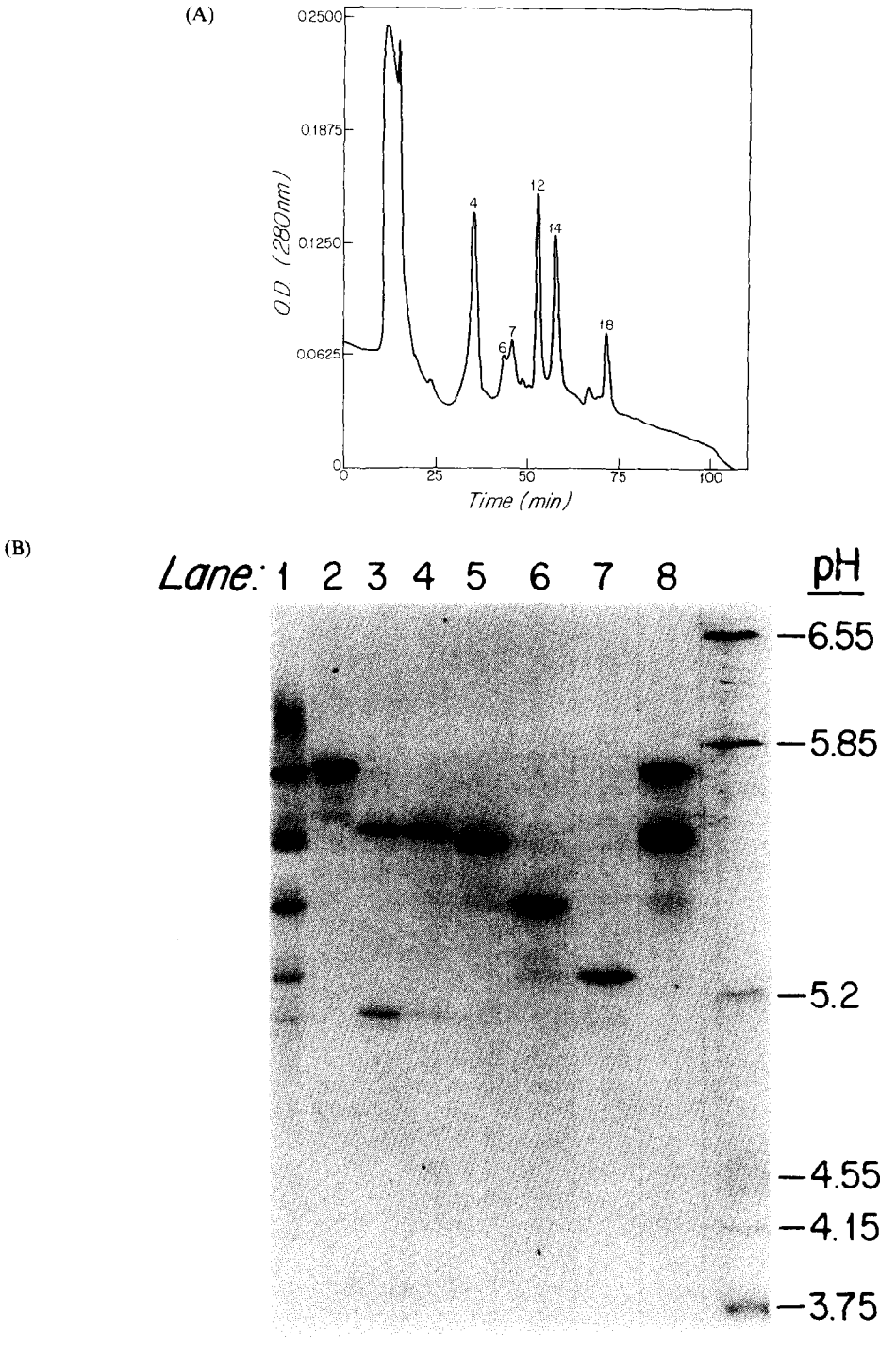


Fig. 5. Detection of isoforms in FPLC purified *Amb a* II isolated from ragweed pollen. Purified *Amb a* II from pollen was subjected to FPLC Mono Q chromatography (panel A) as indicated in Materials and Methods. Clearly discernible peaks detected by A_{280} absorption were recovered and assigned a number as indicated. The major peak fractions were subjected to IEF–PAGE analysis (panel B). The samples loaded include: lane 1, 20 μg of purified *Amb a* II prior to FPLC Mono Q chromatography; lane 2, 9 μg of peak 4 fraction; lane 3, 6 μg of peak 6 fraction; lane 4, 6 μg of peak 7 fraction; lane 5, 8 μg of peak 12 fraction; lane 6, 8 μg of peak 14 fraction; lane 7, 6 μg peak 18 fraction; lane 8, 10 μg of purified *Amb a* II used as a standard (King *et al.*, 1981). Proteins with defined pI were used as standards and are shown in the far-right lane (human carbonic anhydrase B, pI, 6.55; bovine carbonic anhydrase B, pI 5.85; β-lactoglobin A, pI 5.2; soybean trypsin inhibitor, pI 4.55; glucose oxidase, pI 4.15; methylred dye, pI 3.75).

other multigene families of allergenic proteins are defined.

A panel of murine Abs directed against chromatographically purified *Amb a* I, composed predominantly of the *Amb a* I.1 form (Rafnar *et al.*, 1991), has been generated (Smith *et al.*, 1988). One mAb from this panel, JB1E3-4, reacts exclusively with *Amb a* I.1, whereas another, JB4E3, reacts with both *Amb a* I.1 and

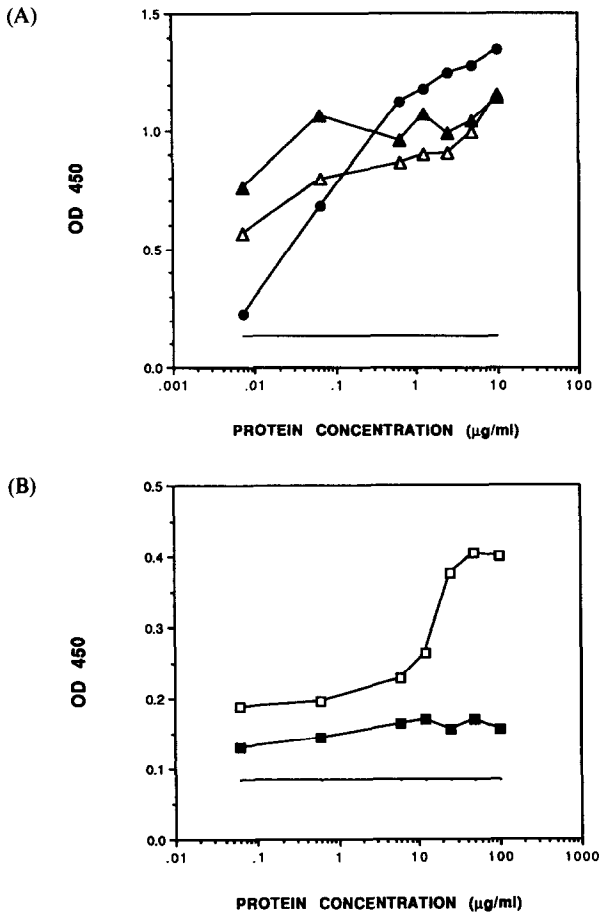


Fig. 6. Antibody binding to purified native and recombinant *Amb a* I.1 and *Amb a* II. The binding of human ragweed allergic IgE to purified native (panel A) and recombinant (panel B) *Amb a* I.1 and *Amb a* II was assessed by Sandwich ELISA. The antigens tested for binding include: soluble ragweed pollen extract (●), biochemically purified native *Amb a* I (△) and *Amb a* II (▲) (King *et al.*, 1981) (see Materials and Methods), and Ni^{2+} metal-ion purified r*Amb a* I.1 (□) and r*Amb a* II (■). The background absorbance observed is indicated by a horizontal line.

Amb a II (Fig. 2, panels D and E, respectively). These data suggest that these two related allergens have shared, partially crossreactive, as well as unique murine B cell epitopes, and this probably is a reflection of the degree of sequence homology between these proteins. This conclusion is supported by comparing the amino acid sequences of the epitopes mapped using mAbs (JB1E3-4 and JB4E3) and peptides based on the primary structure of *Amb a* I.1 (Fig. 3). This finding is also reminiscent of the immunochemical data derived from the related allergens from two species of housedust mite, *Der p* I and *Der f* I, in which both unique and cross-reacting B cell epitopes were identified (Heymann *et al.*, 1986).

Sequence analysis of independent PCR-derived clones showed that *Amb a* II is polymorphic at amino acid residues 71, 139 and 322 (Griffith *et al.*, 1991*a*). Two of three polymorphisms, N_{71}/D_{71} and K_{139}/T_{139}, are non-conservative and involve charge changes, while the third polymorphism, K_{322}/R_{322}, is conservative. All polymorphisms occur independently of each other (Griffith *et al.*, 1991*a*), suggesting that it is possible to generate eight distinct isoforms of *Amb a* II. However, only three distinct pI values can be calculated for these eight proteins: pI 5.74, pI 5.86, and pI 5.97 (Table 1). Since the NH_2-terminus of the mature *Amb a* II protein has not been clearly defined, the calculated pI values can only be compared relative to each other and may not be predictive of actual pI. However, the three predicted pI values are particularly interesting in light of the observation that *Amb a* II can be resolved into three distinct spots on 2D IEF/SDS–PAGE gels with observed pIs ranging from 5.5 to 5.85 (Fig. 4). This strongly suggests that *Amb a* II is a single protein with multiple isoforms, in contrast to *Amb a* I which has multiple family members each composed of several isoforms (Rafnar *et al.*, 1991; Griffith *et al.*, 1991*a*). Individual isoforms of native *Amb a* II have been purified from pollen in the absence of detectable contaminating *Amb a* I (Fig. 5). The observed pI of these *Amb a* II isoforms are very similar to those calculated from the primary structure (Table 1) and observed on 2D Western blots using anti-*Amb a* II antisera as probes (Fig. 4). This correlation between predicted and observed pI would strongly suggest that the observed different species of *Amb a* II are not due to post-translational modification of a single gene product. The presence of multiple related allergens that differ in pI may be characteristic of many pollen derived allergens. For example, *Poa p* I and *Poa p* IX from Kentucky Bluegrass and *Phl p* V from Timothy grass pollen have multiple isoforms that can be detected on 2D Western blots (Ekramoddoullah, 1990; Olsen *et al.*, 1991; Petersen *et al.*, 1992).

It is of interest to examine the reactivity of ragweed allergic human IgE to individual members of this multigene family. Previously, it was shown that IgE from individual ragweed allergic patients differ widely in their binding reactivity to r*Amb a* I.1, I.2 and I.3 proteins (Bond *et al.*, 1991). Nonetheless, although many patients' IgE reacted with more than one member of the *Amb a* I family, most patients' IgE reacted with *Amb a* I.1 (Bond *et al.*, 1991). In Fig. 2, panel F, a pool of IgE from 22 ragweed allergic patients was used to probe a Western blot having as samples purified r*Amb a* I.1 and r*Amb a* II as well as whole pollen extract. Human IgE in this pool clearly reacted with r*Amb a* I.1 that had been reduced and subjected to SDS–PAGE whereas no detectable IgE reactivity was observed with r*Amb a* II (Fig. 2, panel F). In contrast, native *Amb a* I and *Amb a* II purified from pollen have very similar IgE-binding properties when examined by Sandwich ELISA (Fig. 6, panel A). However, the r*Amb a* I.1 and r*Amb a* II isolated under denaturing conditions had very different IgE-binding properties when examined using the same assay (Fig. 6, panel B). These data confirm the r*Amb a* I.1 > r*Amb a* II hierarchy of IgE binding observed using Western blotting (Fig. 2, panel F). The difference in IgE binding observed in the Sandwich ELISA is not due to a difference in the extent of antigen binding to the solid phase since r*Amb a* I.1 and r*Amb a* II were detected to a similar extent by a rabbit anti-*Amb a* I antiserum used

as a control in a direct-binding ELISA (data not shown). Taken together, these data demonstrate that the recombinant forms of related allergens may differ significantly in their IgE-binding properties even though their native counterparts are largely indistinguishable. This observation may be due to a difference in the subset of IgE antibody binding epitopes on the denatured molecule that are not readily detectable when the full spectrum of epitopes is examined on the native structure. This possibility is particularly relevant for allergens that have a markedly reduced IgE binding capacity upon denaturation such as *Amb a* I (King *et al.*, 1974) and *Der p* I (Lombardero *et al.*, 1990).

In summary, this paper describes the expression and purification of r*Amb a* I.1 and r*Amb a* II as well as their immunochemical characterization. These recombinant molecules in conjunction with the defined primary structure of these allergens have been very useful to authenticate the specificity of both antipeptide antisera and mAb. Subsequent use of these antibodies has defined the pI of specific isoforms of native *Amb a* I and *Amb a* II in ragweed pollen. Further work to immunochemically characterize other *Amb a* I family members, *Amb a* I.1, I.2, I.3 and I.4, is presently being pursued.

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