

Cloning and Expression of Ragweed Allergen Amb a 6

K. M. HILLER, B. C. LUBAHN & D. G. KLAPPER

Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC 27514-7290, USA

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We have cloned the protein coding region of an isoform of short ragweed allergen Amb a 6 (Ra6) and expressed the secreted product in *Pichia pastoris* at mg/l levels. 5' RACE was performed using sequence obtained from a partial Amb a 6 clone. This yielded a product whose deduced protein sequence has a characteristic signal sequence motif at the N-terminus followed by sequence consistent with that previously published for highly purified Amb a 6 [Roebber *et al.* J Immunol 1983;131:706–11]. The region encoding the secreted product was amplified by PCR and cloned into pPICZ α a, an expression vector for the yeast *Pichia pastoris*. Yeast transformed with this vector secrete a protein which migrates near Amb a 6 in SDS–PAGE. This secreted protein reacts with polyclonal anti-Amb a 6 antisera as well as an anti-Amb a 6 monoclonal antibody, and has the N-terminal sequence of Amb a 6. By time-of-flight mass spectrometry, recombinant Amb a 6 has a molecular weight of $9884 \pm 0.2\%$. In addition to the deduced amino acid sequence of an Amb a 6 clone, the amino acid sequence of Amb a 6 protein is reported for comparison. The amino acid sequence was obtained by aligning overlapping tryptic and chymotryptic peptides from enzymatic digests of extensively reduced and alkylated Amb a 6. Sequences from at least three closely related Amb a 6 isoforms are present among these peptides. The amino acid sequence closely matches the deduced amino acid sequence of the Amb a 6 clone.

David G. Klapper, Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC 27514-7290, USA

INTRODUCTION

The past several years have seen a substantial increase in the number of allergens which have been cloned and sequenced using molecular biology technology. A few examples of well-known allergens characterized in this way are ragweed allergens Amb a 1 [1] and Amb a 2 [2], grass pollen allergens Lol p 1 [3, 4] and Lol p 2 [5], house dust mite allergens Der p 1 [6] and Der f 1 [7], peanut allergen Ara h 1 [8], shrimp allergen Met e 1 [9], and latex allergen Hev b 1 [10]. This technology has the potential to be useful in several ways. The sequence information can be used for homology searches in the constantly expanding databases of protein and DNA sequences to determine putative functions for the cloned allergens. In addition, the primary structures of allergens deduced from their DNA sequences are being used in some laboratories to generate overlapping peptides for mapping both B- and T-cell epitopes [11, 12] (see also [32, 33]). Amino acid sequence information has also revealed homology between allergens which has been determined to be a source of cross-reactivity and thus potential cross-sensitization among related

allergens [13]. Cloning has allowed for the expression of recombinant allergens as either independent products or in the context of fusion constructs. These are being evaluated as potential immunotherapeutic materials. Recombinant allergens have also been used to determine the 3-D structure of selected allergens, revealing surface topographic detail for predicting B-cell epitopes [14–17]. This may be especially useful in the case of allergens with multiple, closely related isoforms from which it may be particularly difficult to isolate quantities of any single isoform sufficient for biophysical analysis. Hopefully, the information gained through these studies, along with the recombinant source of allergens, will bring about better, more refined methods of diagnosing and treating allergy.

As an approach toward these goals we have cloned and sequenced ragweed allergen Amb a 6 (from *Ambrosia artemisiifolia*) and expressed it in a yeast system. Amb a 6 was selected not only because it is a ragweed allergen (a principle cause of late summer hayfever in the eastern US and Canada), but also because it has two other useful features. First, as has been found for several other allergens, there exists an association in

allergic humans between hypersensitivity to the allergen and possession of particular class II HLA alleles. Among other allergens known to share this association are Lol p 1, Lol p 2 and Lol p 3, rye grass allergens which associate with DR3 [18, 19]. In ragweed, allergy to homologs Amb a 5 and Amb t 5 is associated with DR2 [20]. Amb a 6, the subject of this paper, also has such an association. In this case, hypersensitivity to Amb a 6 is reported to be associated with DR5 [21]. In one population of subjects, 85% of those with IgE against Amb a 6 were typed as DR5, whereas only 14% of those without anti-Amb a 6 IgE possessed DR5 alleles [21]. Another useful feature of Amb a 6 is the fact that it has an apparent molecular weight of slightly less than 10,000. This makes it an ideal candidate for use in either 2-D NMR or crystallographic approaches to determine 3-D structure. We are interested in investigating further the structural and allergenic properties of Amb a 6 as well as its association with HLA DR5. Recombinant Amb a 6 will be useful for these studies and could potentially find a place in allergy diagnosis and treatment.

Amb a 6 is a basic protein with a molecular weight of ≈ 9900 and a putative glycosylation site at the asparagine in position 35/36. It is considered to be a minor allergen, with 21% of one ragweed hypersensitive population possessing detectable IgE antibody against it. Amb a 6 is thought to exist as at least four isoforms which appear to be both antigenically and allergenically indistinguishable from each other [22]. DEAE chromatography of highly purified Amb a 6 reveals two differentially charged isoforms which elute under different salt concentrations, and each of these possesses two slightly varying N-terminal sequences. While some of the isoforms differentiated by charge are readily separated, the variations between others are apparently subtle and have not allowed purification of any single isoform to homogeneity. Because a pure Amb a 6 isoform cannot be isolated from pollen, detailed analyses of the structure of native Amb a 6 using crystallographic or 2-D NMR approaches have in the past been precluded.

In this paper, we describe the cloning from short ragweed RNA of the coding region of an Amb a 6 isoform and report its sequence. In addition, we report direct protein sequence information in which the majority of the Amb a 6 sequence has been assembled from overlapping Amb a 6 peptides generated by trypsin and chymotrypsin. The deduced Amb a 6 sequence agrees closely with the Amb a 6 protein sequence. For further structural studies, we chose to express this cloned Amb a 6 isoform in a yeast expression system. The *Pichia pastoris* expression system (Invitrogen, San Diego, CA) was used because *Pichia* secretes only a few proteins, allowing for synthesis, secretion and isolation of recombinant Amb a 6 with a relatively simple purification process. In addition, glycosylation is likely to occur in this eukaryotic system. We have generated a clone which expresses recombinant protein in mg quantities per litre of media. The antibody binding activity and physical characteristics of recombinant Amb a 6 are reported.

MATERIALS AND METHODS

Purification and amino acid sequence of Amb a 6. Amb a 6 was isolated from an aqueous extract of short ragweed pollen as described previously [22]. Briefly, the aqueous extract was precipitated with 60% ammonium sulfate and passed through a G-25 molecular sieve equilibrated in 0.015 M Tris at pH 8.0. The excluded fraction was then loaded onto a DEAE column equilibrated in the same buffer and the excluded DEAE fraction (consisting of the basic allergens such as Amb a III, Amb a V and Amb a 6) was then collected for further processing. This fraction was subjected to molecular sieve chromatography over Sephadex G-75 equilibrated in 0.02 M sodium acetate, pH 6.0. Fractions were monitored at an OD of 280 nm and selected fractions were assayed for the presence of Amb a 6 by both ELISA and Ouchterlony analysis using goat polyclonal antiserum to Amb a 6. Amb a 6 positive fractions were pooled and further purified by ion exchange chromatography on a carboxy methyl (CM) resin equilibrated in 0.02 M sodium acetate at pH 6.0, and materials were eluted with a gradient of increasing NaCl in the same buffer. Fractions were assayed with the Amb a 6-specific antisera as well as by agarose slab electrophoresis [22]. Purified Amb a 6-containing fractions were pooled, desalted over a G-25 size exclusion column equilibrated in 0.02 M ammonium acetate (pH 6.5), and lyophilized.

Amb a 6 isolated as described above and consisting of multiple isoforms was extensively reduced (5 M guanidine HCl, 0.1 M Tris, 0.1 M dithioerythritol) at 37°C for 45 min and alkylated (0.22 M iodoacetic acid, Calbiochem, La Jolla, CA) in the dark at room temperature for 1 h. The reaction mixture was desalted over a Sephadex G-25 (Pharmacia, Piscataway, NJ) column equilibrated in fresh 1% ammonium bicarbonate containing 0.01% calcium chloride. The excluded material was divided into two aliquots for immediate enzymatic digestion with either TPCK-treated trypsin (Worthington Biochemical, Freehold, NJ) or chymotrypsin (Worthington) at an enzyme-to-substrate ratio of $\approx 2\%$. The digestion was allowed to proceed at 37°C for 6 h, at which time each digest was lyophilized and stored at -70°C until further use.

Each digest was reconstituted in 0.1% trifluoroacetic acid, spun in a microfuge to remove particulates, and applied to an HPLC column (W-Porex, C₁₈, Phenomenex, Torrance, CA). The column effluent was monitored at 229 nm and peptides were eluted with an increasing acetonitrile gradient and collected manually. Individual peptides were sequenced using an Applied Biosystems (Foster City, CA) model 470 protein sequencer with on-line separation and detection of PTH amino acid derivatives using a Waters HPLC (Milford, MA) and custom designed interface.

Isolation of total RNA. Total RNA was isolated from short ragweed flowers according to the manufacturer's directions with a Qiagen RNeasy Total RNA kit (Qiagen, Chatsworth, CA). Ragweed flowers containing pollen were harvested, frozen immediately on dry ice, and kept frozen at -70°C until they were used for RNA purification. A typical preparation would start with 200 mg of flowers being put into a cold mortar and immersed in liquid nitrogen. The flowers were then ground to a fine powder in the liquid nitrogen. Ten milliliters of buffer RLT (containing guanidinium hydrochloride) plus 100 μl of β -mercaptoethanol (βME) were added to the frozen powder and mixed as thawing occurred. The liquid was then applied to 2 QIA shredder tubes and spun in a microfuge for 2 min. The supernatants were transferred to new tubes and 225 μl of EtOH was added to each and mixed. This solution was applied to an RNeasy spin column and spun for 15 min. RNA bound to the column was then washed with 700 μl of buffer RW1, 500 μl of buffer RPE, and eluted from the column with 40 μl of DEPC (diethyl pyrocarbonate)-treated H₂O. This yielded $\approx 10 \mu\text{g}$ of DNA-free RNA per 100 mg of flowers.

Primers. Primers were synthesized in the laboratory of Dr Clyde Hutchison (Chapel Hill, NC) and by Life Technologies (Gaithersburg, MD).

The following primers were designed using sequence from a previously isolated partial Amba6 clone:

EcoRa6C (ATGAATTCGGCAGGAAATCTGGCTTAAC), used for priming reverse transcription and screening PCR products;

EcoRa6N (ATGAATTCACATGTGATACTGTCAA), used for screening PCR products;

Ra6204 (GACTCTGTGCAACACGC), used for DNA sequencing;

ERa6278 (ATGAATTCGTCAAAACATATTGGCAC), used for southern blotting;

ERa678 (ATGAATTCGCACATTTCTGTAGACAGAAG), used for 5' RACE;

ERa6165 (ATGAATTCACGATCAGCTTTGGTTTTC), used for 5' RACE, southern blotting and DNA sequencing;

BG20 (GAGAATTCGTGACATCGATTTTTTTTTTTTTTTTTT) and

BG21 (GAGAATTCGTGACATCGAT), used for 5' RACE, are the same as primers used for 5' RACE by Ghosh *et al.* [23].

The entire clone was amplified from cDNA using primers SalIFRa6N and E3UTRa6. SalIFRa6N (ACGCGTCGACGAGATCAATTTTCAACTTCAATGG) was designed using upstream sequence generated by 5' RACE. E3UTRa6 (AGTGAATTCATGGTCATGCTATTTC) sequence was derived from the partial Ra6 clone. DNA encompassing the structural gene of Ra6 was generated by PCR using primers KEXRa6 and E3UTRa6. KEXRa6 (ATGCGTCGACAAAAGATCGCAACATGTGATACTGTC) was designed using sequence from the pPIC-ZαA vector and from sequence found by 5' RACE. 5' RACE techniques were based on the method developed by Frohman *et al.* [24, 25]. cDNA was generated from short ragweed total RNA using a modified protocol of the SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies, Gaithersburg, MD). Briefly, 5 µg of total RNA and 30 pmoles of primer EcoRa6C were combined, heated to 70°C for 10 min, and immediately chilled. Synthesis buffer was then added to a final concentration of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂ and 1 µg/µl BSA. To this was added 1 mM dNTPs, 0.01 M DTT and 200 units of SuperScript II RT. This was incubated at room temperature for 10 min, then at 42°C for 50 min. The reaction was terminated by heating at 70°C for 15 min and the reactants were placed on ice. The RNA template was then digested by treating the reactants with 2 units of RNase H for 20 min at 37°C.

EcoRa6C-primed cDNA was then given a 5' poly (A) 'tail' with dATP using terminal deoxy-nucleotidyl transferase (TdT). The cDNA was diluted with 0.1× TE and spun through a Centricon 30 microconcentrator (Amicon, Danvers, MA) several times to wash dNTPs, primers and salts from the cDNA. Tailing was performed in a 40 µl volume of 1× tailing buffer: 0.1 M potassium cacodylate, 2 mM CoCl₂ and 0.2 mM DTT (Life Technologies) with 22.5 units of TdT (Life Technologies) and 1.5 µl of 10 mM dATP for 30 min at 37°C.

Aliquots of the tailed cDNA were used as the template in PCRs with the following primers: ERa678, a nested primer specific for sequence toward the 3' end of the coding region, a limiting amount of BG20, consisting of a sequence with an EcoRI restriction site followed by a poly (T) region to start the PCR amplification, and an excess of BG21, a shorter version of BG20 excluding the poly (T) region. An additional PCR in which ERa678 was replaced with ERa6165, a nested primer specific for sequence in the middle of the coding region, was performed. Each reaction had cDNA template, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.4 mM dNTPs, 2.5 mM MgCl₂, 30 pmoles each of

ERa678 or ERa6165 and BG21, and 6 pmoles of BG20. This was heated to 95°C for 5 min and cooled to 72°C. Taq DNA polymerase (2.5 units, Life Technologies) was added to each tube, and the contents were overlaid with 72°C mineral oil. The initial annealing step was 5 min at 52°C followed by a 40-min extension step at 72°C. Samples were then subjected to 40 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 2 min.

Southern blotting. DNA was run on 1% agarose gels in TAE buffer and gels stained with ethidium bromide in TAE buffer. Gels were treated with 250 mM HCl for 10 min, denatured with 0.5 M NaOH, 1.5 M NaCl for 30 min, and neutralized in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 30 min. DNA was transferred to Hybond N+ nylon membrane (Amersham, Arlington Heights, IL) in 10× SSC by capillary blotting. Membranes were probed using ECL detection and labelling systems (Amersham) according to the manufacturer's instructions. When direct labeling was employed, the partial Amba6 clone was conjugated to horseradish peroxidase (HRP) and used to probe a prehybridized membrane. When indirect labelling was employed, TdT was used to add a 3' fluoro green tail to an Amba6-specific primer. After the primer hybridized to the membrane, the membrane was probed with HRP-labeled anti-fluoro green. In both cases, membranes were developed with ECL substrate to produce bands on autoradiographs.

DNA sequencing. DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility with a Model 373 A DNA Sequencer (Applied Biosystems, Foster City, CA) using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems).

DNA cloning. DNA was generated by PCR using primers with incorporated restriction sites. PCRs consisted of template, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2–0.4 mM dNTPs, 1.5–2.5 mM MgCl₂, 20 pmoles of each primer and 2.5 units of Taq. A typical 35-cycle amplification would consist of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Products were extracted with phenol/chloroform, precipitated in 0.3 M NaOAc pH 5.0, 70% EtOH, and washed with 70% EtOH. DNA pellets were resuspended in appropriate buffers and digested. Digests were typically phenol/chloroform extracted twice, then chloroform extracted once. Quantities of this partially clean DNA were estimated and it was subsequently used in ligations. Ligation reactions combined insert, vector digested with the appropriate enzyme, 1× ligation buffer (66 mM Tris-HCl, 5 mM MgCl₂, 1 mM dithioerythritol, 1 mM ATP, pH 7.5, Boehringer Mannheim, Indianapolis, IN), 1–2.5 units of T4 DNA Ligase (Boehringer Mannheim) and an additional 1 mM ATP. These were incubated overnight at 12–16°C. Fractions of ligation reactions were transformed into competent *Escherichia coli* by incubating the fraction with the cells on ice for 10 min, then heat shocking the bacteria at 42°C for 2 min. Transformants grew at 37°C for 1 h, then were plated onto antibiotic-containing plates. Colonies were screened by boiling bacteria in H₂O and using the 'boilates' in PCRs with Amba6-specific primers.

Expression in Pichia. Amba6 was expressed in the yeast *Pichia* using the *Pichia* Expression Kit (Invitrogen) following the manufacturer's directions. Briefly, the region encoding the Amba6-secreted product was amplified from cDNA using PCR, cloned unidirectionally into plasmid vector pPICZαa, and transformed into DH5α cells. Bacteria were plated onto agar plates containing low salt LB (1% tryptone, 0.5% NaCl, 0.5% yeast extract) and 100 µg/ml Zeocin. Zeocin-resistant colonies were screened for the presence of the Amba6 gene by performing PCR on 'boilates' using the Amba6-specific primers EcoRa6N and EcoRa6C. A positive clone was identified and was confirmed by DNA sequencing of its plasmid to be intact and in frame. The plasmid was transformed into *Pichia* strain GS115 by

electroporation in a GenePulser (Bio-Rad, Hercules, CA) at 1.5 kV, 25 μ F, 200 Ω . Yeast were plated in sorbitol onto YPD plates (1% yeast extract, 2% peptone, 2% dextrose) containing 100 μ g/ml Zeocin and grown for several days at 30°C. Several Zeocin-resistant colonies were then grown overnight in 1 ml of BMGY media (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base with ammonium sulfate, 0.00004% biotin, 1% glycerol). Overnight cultures were then transferred to a larger volume and grown again overnight to log phase. Cells were then spun down and induced to produce the Amb a 6 gene product with MeOH; they were resuspended in BMMY media (0.5% MeOH replaces the glycerol of BMGY media) and grown for several days. Supernatants were screened and a suitable clone was grown in larger volumes of less rich BMGH (initial growth) and BMMH (induction) media, in which yeast extract and peptone are excluded and 0.004% histidine is added.

Purification of recombinant protein. Recombinant yeast supernatant was lyophilized and the powder resuspended in a minimal volume of H₂O. This solution was applied to a G25 molecular sieving column (Pharmacia, Piscataway, NJ) equilibrated in 0.02 M NaOAc. Fractions were screened throughout the purification procedure by SDS-PAGE and Western blot as described below. rAmb a 6-containing fractions were then pooled and applied to a CM Fractogel cation exchange column (Fractogel, EM, Gibbstown, NJ) equilibrated in 0.01 M NaOAc, pH 5.5. Bound proteins were eluted with an increasing NaCl gradient followed by stepwise elutions with 0.2 M and 0.5 M NaCl. The recombinant Amb a 6 was found to be in all fractions of the eluent. The Amb a 6-positive fractions were then highly purified by applying them to a reverse phase HPLC column with a 300 Å pore C₁₈ resin. Protein was eluted with increasing acetonitrile and selected fractions were examined by mass spectrometry for the presence of rAmb a 6.

SDS-PAGE/Western blotting. Supernatants were screened for the presence of recombinant Amb a 6 by SDS-PAGE followed by either silver staining or Western blot analysis, or both. SDS-PAGE was performed by a modification of the method reported by Laemmli [26]. Supernatant (20 μ l) was mixed with 10 μ l of 3 $\times$ sample buffer (0.14 M Tris, pH 6.8, 23% glycerol, 4.7% SDS, 1.6 M β ME) and electrophoresed through a 4% stacking gel and then a 16% resolving gel. Gels were then either silver stained with a Silver Stain Plus kit (Bio-Rad) or transferred to nitrocellulose (MSI, Westboro, MA) according to the method of Towbin *et al.* [27]. Blots were blocked overnight at 4°C in 1% BSA (SIGMA, St. Louis, MO), then incubated in anti-Amb a 6 rabbit serum, H101, diluted 1:200 in PBS Tween (0.15 M NaCl, 0.014 M KCl, 0.01 M Na₂HPO₄·7H₂O, 0.002 M KH₂PO₄, 0.05% Tween) for 75 min. Blots were then washed three times with PBS Tween and incubated in alkaline phosphatase-labeled anti-rabbit IgG (SIGMA), diluted 1:2000 in PBS Tween, for 30 min. After washing three times and rinsing with H₂O, blots were developed in alkaline phosphatase substrate buffer [100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, pH 9.5, with 66 μ l of NBT (Promega, Madison, WI) and 33 μ l of BCIP (Promega) added fresh per 10 ml]. Development was terminated with H₂O.

Alternatively, blots were probed with mouse anti-Amb a 6 monoclonal antibody (MoAb) 3D1 or goat anti-Amb a 6. Blots were blocked with 0.1% BSA as described, then probed with alkaline phosphatase labeled MoAb 3D1 diluted 1:100, or goat anti-Amb a 6 diluted 1:10,000, in PBS Tween. Membranes treated with goat serum were washed and probed with rabbit anti-goat IgG (SIGMA) diluted 1:4000. Blots were then washed and developed as described.

ELISA. ELISAs were performed by immobilizing protein to microtiter wells overnight at 4°C in 1 M NaHCO₃. Plates were then washed three times with PBS containing 1% Tween. Primary antibody diluted in PBS/

Tween (goat anti-Amb a 6 at 1:10,000 or rabbit anti-Amb a 6 at 1:200) was then added and allowed to incubate at 37°C for 75 min. Plates were washed again. Secondary antibodies diluted in PBS/Tween (anti-goat IgG or anti-rabbit IgG) were then added to the wells and incubated for 45 min at 37°C. After a final washing, wells were developed using SIGMA 104 substrate tablets dissolved in diethanolamine buffer. Absorbance was measured with a Bio-Tek (Winooski, VT) microplate autoreader at 405 nm. Inhibition ELISAs were performed in a similar manner except that varying amounts of inhibitor were incubated overnight with goat anti-Amb a 6 (1:5000) before being applied to immobilized Amb a 6 (0.05 μ g) as described above. Inhibition curves were determined by calculating percentage inhibition by the following equation: % inhibition = $[A_0 - A_i]/A_0 \times 100$.

Mass spectrometry. The molecular weight of both native and recombinant Amb a 6 was determined by a MALDI-TOF mass spectrometer (Kratos, Ramsey, NJ). The proteins were loaded onto sample slides and treated with α -cyano-4-hydroxycinnamic acid (Aldrich Chemical Company, Milwaukee, WI) solubilized in 70% acetonitrile. Samples were then ionized to determine molecular weights.

RESULTS

The amino acid sequence of Amb a 6 was determined as described above by sequencing Amb a 6 peptides which were generated from enzyme digests. As can be seen in Fig. 1, a number of peptides gave rise to amino acid sequence information and there are sufficient 'overlapping' peptides to propose an almost complete linear sequence. It should be noted that because there are multiple Amb a 6 isoforms, a number of homologous peptides were found with similar, but not identical, amino acid sequences. Although it is possible to assemble almost the entire sequence of Amb a 6 in this way, there is no evidence that the assembled structure would actually reflect the product of an existing Amb a 6 gene. However, this amino acid sequence proved to be useful in the design of synthetic oligonucleotides which were used as probes in the isolation of a partial Amb a 6 clone from a short ragweed cDNA library.

A clone of ragweed allergen Amb a 6 has been PCR amplified from cDNA using primers which encode the sequence flanking the Amb a 6 coding region. This sequence information was obtained from the partial Amb a 6 clone and by 5' RACE, as described below. Total RNA was purified from pollen-containing short ragweed flowers, with great care being taken to keep the tissue frozen during the initial stages of isolation. Ten micrograms of DNA-free RNA per 100 mg of flowers could be purified using a Qiagen RNeasy Plant Total RNA Kit. This RNA was then used as template in RT-PCR. Preliminary experiments showed that a large segment of Amb a 6 sequence could successfully be amplified from poly (T) primed cDNA (data not shown), indicating that the RNA template was relatively intact. The RNA was subsequently used for 5' RACE.

cDNA was reverse transcribed using Amb a 6-specific primer EcoRa6C. After degradation of the RNA template with RNase H, the cDNA was washed several times with 0.1 $\times$ TE. A poly (A) tail was then added to the 5' end of the transcript using TdT and dATP. PCR was then performed with either ERA678 or ERA6165, BG20 and BG21 as primers. The poly (T) region of BG20

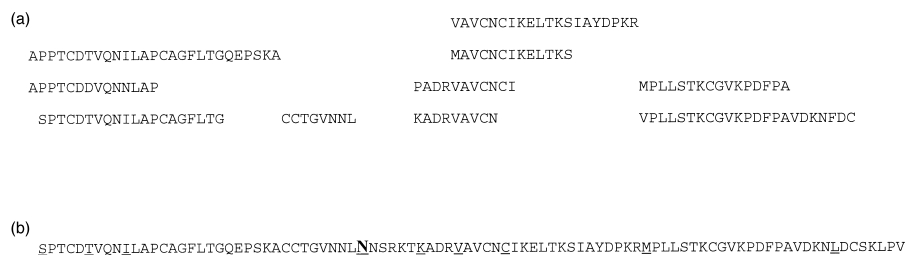


Fig. 1. Comparison of Amb a 6 peptide and cDNA sequences. (A) Amb a 6 was digested with trypsin and chymotrypsin and the peptides were separated by HPLC. N-terminal sequencing was performed on each isolated peptide. Peptides representing at least three different isoforms of Amb a 6 are shown and together provide sequence encompassing almost the entire structural region of Amb a 6. (B) Deduced sequence of Amb a 6 cDNA clone. The putative glycosylated asparagine is highlighted and residues which differ from peptide sequences are underlined.

hybridized with the synthetic poly (A) tail of the transcript and was intended to increase the number of templates for primer BG21, the primer designed for amplification of the target PCR product. PCR products and controls were run on a 1% agarose gel, shown in Fig. 2. EtBr staining revealed a background smearing of DNA with a prominent band just below 564 bp for reactions primed with ERa678 and a band just above 310 bp for reactions primed with ERa6165. This ≈ 100 bp difference in size agreed with the predicted product difference (based on the relative positions of the primer annealing regions) of 87 bp. The ERa678 product hybridized with an ERa6165 probe on a southern blot, demonstrating that it possessed Amb a 6 sequence.

For further confirmation of its identity, the ERa678 product was chosen for sequence analysis. Multiple lanes of the reaction were run on a 1% gel, stained with EtBr, and the prominent band was excised with a razor blade. DNA was eluted using a Qiaquick gel extraction kit (Qiagen) by following the manufacturer's directions, and a small fraction was analyzed for quantitation. Approximately 40 ng of this product was submitted for automated DNA sequence analysis. The sequencing reaction was primed with ERa6165 at a concentration of 2 pmoles/ μ l. The DNA sequence generated was translated to protein sequence and analyzed. The first stretch of sequence corresponded to known Amb a 6 protein sequence (SPTCDT...) preceded by a characteristic signal sequence containing an initiating methionine at position -26, a phenylalanine at position -3, and an alanine at position -1. This is the major sequence as translated by the GCG MAP program, which is based on the nucleotides interpreted by the sequencing program. The raw data, however, revealed several regions of sequence ambiguity, one of which could have possibly translated into another Amb a 6 isoform (APPTCDT...).

In order to investigate the possibility of having more than one isoform in our 5' RACE product, and to generate cleaner sequence, we isolated and sequenced several clones. During 5' RACE, we had utilized PCR primers with *Eco*RI sites at their ends, creating products with ends digestible by *Eco*RI. The ERa6165 product was phenol chloroform extracted, chloroform extracted and digested with *Eco*RI (Life Technologies). It was then ligated into *Eco*RI-cut pACYC184 (New England Biolabs, Beverly, MA). Ligation reactions were transformed by heat

shock into HB101 *E. coli* and plated onto LB plates containing 100 μ g/ml tetracycline (SIGMA). Transformants were selected by loss of resistance to chloramphenicol (SIGMA) and screened by PCR amplifications of colony 'boilates' using EcoRa6N and

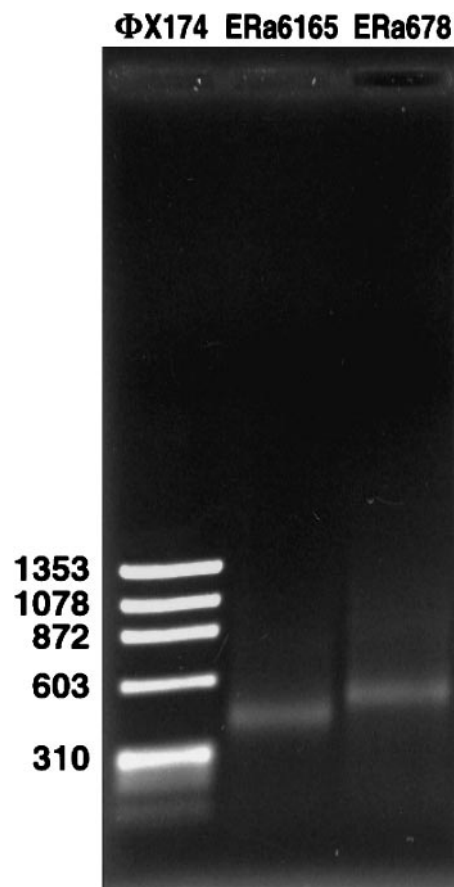


Fig. 2. 5' RACE products. Primers corresponding to different regions of Amb a 6 were used to prime 5' RACE reactions. The product of the reaction primed with ERa6165, located in the middle of the structural gene, is shown in lane 2. The product of the reaction primed with ERa678, located near the 3' end of the structural gene, is shown in lane 3. Lane 1 contains *Hae*III digested PhiX 174 molecular weight markers (Promega).

ERa6165 as primers. PCR products were then screened by southern blot for reactivity to Amb a 6 primer ERa6278. Plasmid DNA was isolated from five positive clones and submitted for sequencing with Amb a 6 primer Ra6204. The data indicate that one of these is truncated with the poly (A) tail beginning at amino acid position -25. The other four are identical from the beginning of the sequence through the ATG codon, although one sequence differs upstream of that region. Regardless of the minor deviations, it appears that the upstream sequence of a dominant Amb a 6 isoform is present in the ERa6165 5' RACE product.

The upstream sequence generated from the 5' RACE clones was used along with previously generated downstream sequence to design primers flanking the Amb a 6 gene. Primers SalIRa6N and E3UT were used to amplify the region encoding the coding region of Amb a 6 from poly (A)-primed cDNA. This was then cloned into vector PHIL-S1 from the *Pichia* expression system and transformed into DH5 α *E. coli*. Bacteria were grown up, and plasmid DNA was isolated from the bacteria and submitted for

DNA sequencing using a vector specific primer. Figure 3 displays the Amb a 6 structural sequence along with its signal sequence. The regions corresponding to primers used in various procedures are shown.

Primers KEXRa6 and E3UTRa6 were used to PCR amplify Amb a 6 from poly (A) primed cDNA. This gave rise to a $\approx$ 400 bp product appearing as a single band on a 1% agarose gel. This product was ligated into *Pichia* vector pPICZ α A and transformed by heat shock into *E. coli* strain DH5 α . Multiple Zeocin-resistant colonies were screened by PCR amplification of 'boilates' using primers EcoRa6N and EcoRa6C. Plasmid DNA from a PCR positive clone was isolated and sequenced, and found to be positioned correctly and in frame. Specifically, Amb a 6 sequence was ligated unidirectionally into *Xho*I, *Eco*RI-cut pPICZ α A. Here, the gene is downstream from the *Saccharomyces cerevisiae* α -factor secretion signal such that rAmb a 6 is produced as part of a fusion protein with α -factor. After being secreted with α -factor, rAmb a 6 is proteolytically cleaved away from it and can be purified from the supernatant. Upstream from the α -factor region is the methanol inducible alcohol oxidase promoter, providing a means to control production of the fusion protein.

The plasmid construct, Z25, was then linearized and electroporated into *Pichia* strain GS115. Cells were plated onto YPDZ plates containing extra sorbitol, then incubated at 30°C for several days. Four colonies, Z251, Z252, Z253 and Z254, along with a colony of yeast transformed with pPICZ α A, were chosen for evaluation. They were first grown in BMGY media to log phase and spun down. Cells were then resuspended in BMMY, a methanol-containing media, to induce fusion protein production. During each of the following 5

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ATGGATTGTATTTCGCACTACTGTGGTCCGTAGCAGTTGGTTTGGTTCATGGAGG
-----+-----+-----+-----+-----+-----+-----+-----+
TACCTAACATAAGCGTATGACACCAGGCATCGTCAACCAACGAAACCAAGTACCTCC
M D C I R I L W S V A V G L L L V S W R

CCAACGATGTTTCGCGCATCGCAACATGTGATACTGTCCAAACATATTGGCACCATGC
-----+-----+-----+-----+-----+-----+-----+-----+
GGTTGCTACAAGCGTCGTAGCGTTGGTGTACACTATGACAGGTTTGTATATAACCGTGTACG
EcoRa6N > > > EcoRa6278 > > >
P T M F A A S P T C D T V Q N I L A P C

          < < < Ra6204
GCAGGTTTCTTAACGGGTCAGGAGCAAGCAAGCGTGTGTCACAGGAGTCAACAATCTT
-----+-----+-----+-----+-----+-----+-----+-----+
CGTCCAAAGAATTGCCAGTCCTCGGTTCTGTCGACGACGTGTCCTCAGTTGTTAGAA
A G F L T G Q E P S K A C C T G V N N L

          < < < EcoRa6165
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-----+-----+-----+-----+-----+-----+-----+-----+
TTATTGAGCTCTTTTGGTTTCGACTAGCACACCGCAGACGTTGACATAGTTTCTTAAC
N N S R K T K A D R V A V' C N C I K E L

          < < < EcoRa678 <
ACAAAATCGATTGCTTACGATCCAAACGTATGCCTCTTCTGTCTACGAAATGTGGCGTT
-----+-----+-----+-----+-----+-----+-----+-----+
TGTTTGTAGCTAACGAATGTAGGTTTTCATACGAGAGACAGATGCTTACACCGCAA
T K S I A Y D P K R M P L L S T K C G V

          < < < EcoRa6C
AAACCGAGATTTTCTGCGTCGATAAGAACCTCGACTGTTCAAAACCTCCAGTATGA
-----+-----+-----+-----+-----+-----+-----+-----+
TTTGGTCTAAAAGGACGGCAGCTATCTTGGAGCTGACAAGTTTGAAGGTCATACT
K P D F P A V D K N L D C S K L P V *

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Fig. 3. Amb a 6 DNA and translated protein sequences. The signal sequence of the translated protein product is underlined. Primer annealing regions of the DNA sequence are indicated by underlining and labeled with the corresponding primers and directions of priming. NCBI GenBank Accession No. U89793.

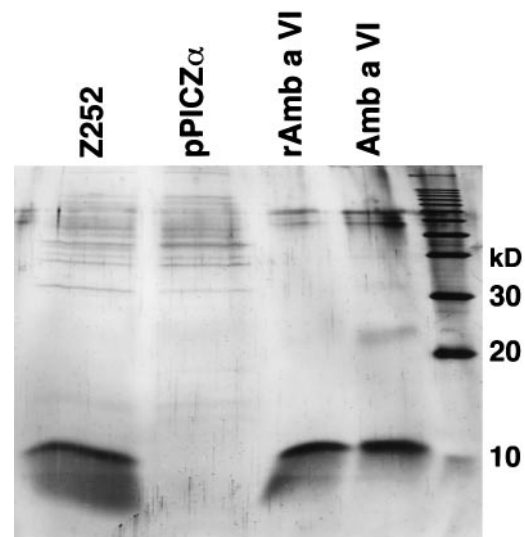


Fig. 4. SDS-PAGE of recombinant Amb a 6. *Pichia* supernatant from clone Z252 (lane 1), as well as from a control clone possessing vector pPICZ α A with no insert (lane 2), were run on a 16% polyacrylamide gel and silver stained. Lane 3 contains the purified recombinant product which migrates close to native Amb a 6 (lane 4). Molecular weight markers are the 10 kDa Protein Ladder (Life Technologies).

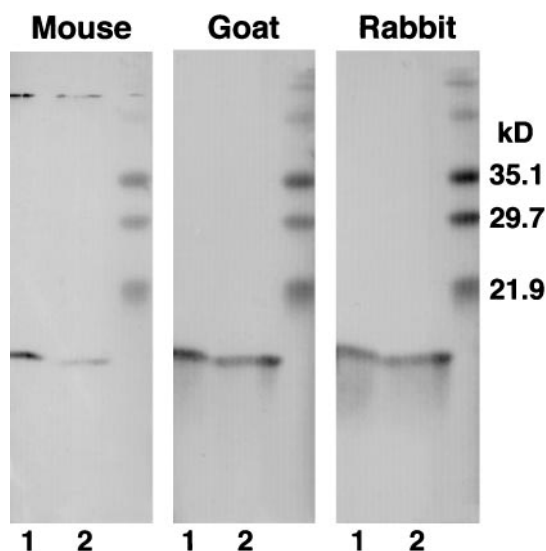


Fig. 5. Western blot analyses of rAmb a 6. rAmb a 6 was run on a 16% polyacrylamide gel next to native Amb a 6, transferred to nitrocellulose, and probed with antibodies from several different species. Lanes labeled '1' contain native Amb a 6. Lanes labeled '2' contain rAmb a 6. Molecular weight markers are prestained low molecular weight standards (Bio-Rad).

days, a sample of supernatant was taken from each flask and 0.5% MeOH was added to each. rAmb a 6 production was monitored by SDS-PAGE and Western blot analysis.

Preliminary induction experiments determined that recombinant protein production is maximal at 4 days of induction (data not shown). Supernatants from day 4 of the four Z25 clones and the pPICZ α A clone were analyzed for the presence of recombinant Amb a 6. As seen in Fig. 4, the Z25 clones produce a protein which migrates near native Amb a 6 on SDS-PAGE. This protein is not present in the pPICZ α A supernatant. Lanes 1 and 2 represent 20 μ l of culture supernatant with 1 $\times$ sample buffer run on a 16% polyacrylamide gel. In a different experiment, equal amounts of the samples, along with native Amb a 6, were separated by SDS-PAGE, transferred to nitrocellulose, blocked with 1% BSA, and probed with an anti-Amb a 6 rabbit serum. This preliminary assay showed that the recombinant protein reacts with anti-Amb a 6, providing evidence that rAmb a 6 was produced by the Z25 yeast clones.

Clone Z252 was grown up in a large volume of BMMH and induced for 5 days. rAmb a 6 was purified from the supernatant as described in Materials and methods. A 700-ml culture yielded $\approx$ 10 mg of recombinant protein. Several analyses were performed on the recombinant protein to determine its antigenic similarity to native Amb a 6. It was first screened with various anti-Amb a 6 antibodies by Western blot. As seen in Fig. 5, the recombinant protein reacts with both polyclonal goat anti-Amb a 6 and with 3D1, an alkaline phosphatase labeled MoAb against Amb a 6, as well as with the polyclonal rabbit anti-Amb a 6 serum. rAmb a 6 was also tested by ELISA for its ability to bind anti-Amb a 6 in a non-denatured state. Table 1 demonstrates that, in ELISA, rAmb a 6 reacts with both rabbit anti-Amb a 6 and goat anti-Amb a 6 in a manner similar to that of native Amb a 6. An inhibition ELISA was performed to show that rAmb a 6 possesses many of the same epitopes as Amb a 6. Figure 6 shows inhibition data in which recombinant Amb a 6 inhibits almost 80% of the binding of goat anti-Amb a 6 to the native protein. The curve is very similar to the control curve in which native Amb a 6 inhibits the binding of the antiserum to native Amb a 6. Equal amounts of bovine cytochrome c (SIGMA) a basic protein with a molecular weight similar to that of Amb a 6, showed no inhibition.

MALDI-TOF mass spectrometry was performed on native Amb a 6 and rAmb a 6 (Fig. 7). These analyses revealed that native Amb a 6, as purified in our laboratory, consists of several species with molecular weights ranging from $\approx$ 9784 to $\approx$ 9879 ($\pm$ 0.2%). Recombinant Amb a 6 was measured to be $\approx$ 9884 ($\pm$ 0.2%). The Amb a 6 preparation from pollen consists of at least three demonstrable isoforms, while the recombinant product appears homogeneous.

DISCUSSION

These studies have been initiated in order to develop a useful system for the investigation of allergic disease. Several attributes of Amb a 6 make it a good candidate for such a system. First, it is an allergen which has been shown to be associated with a particular human HLA allele; there is a correlation between allergic response to Amb a 6 and expression of the human DR 5 allele [21]. In addition, its relatively small size ($\approx$ 9.9 kDa) should make it amenable to 3-D structural analysis using either crystallographic or NMR spectroscopic approaches. It is also a

Table 1. Anti-Amb a 6 binding by ELISA. rAmb a 6, native Amb a 6, and BSA were immobilized on a microtiter plate and probed with antibodies against Amb a 6. The approximate amount of immobilized protein is shown in parentheses for each protein tested. Values are expressed as absorbance at 405 nm

	rAmb a 6 (0.05 μ g)	Amb a 6 (0.05 μ g)	BSA (0.2 μ g)
Goat anti-Amb a 6	0.100	0.135	0.005
Rabbit anti-Amb a 6	0.120	0.202	0.033

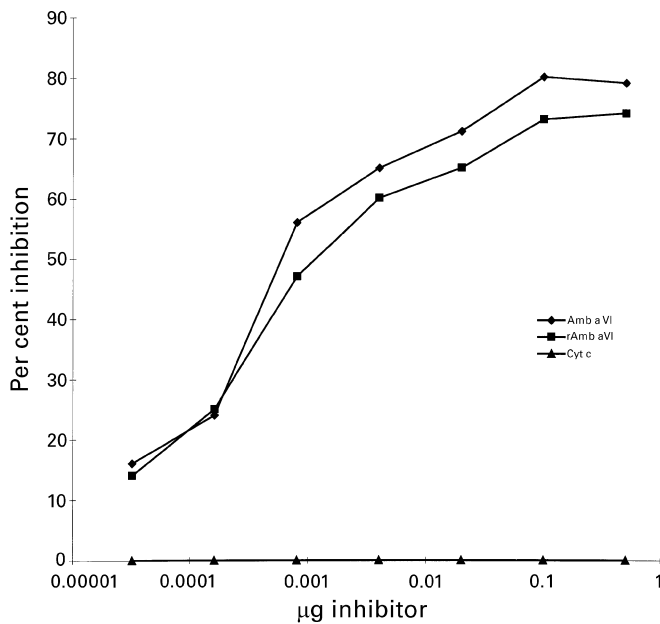


Fig. 6. Inhibition ELISA. ELISAs were performed in which inhibitor was incubated with goat anti-Amb a 6 serum prior to addition of the antiserum to immobilized Amb a 6. Inhibition by recombinant Amb a 6 is comparable to that of native Amb a 6, both of which occur in a dose-dependent fashion. Negative control cytochrome c displays no inhibition.

strongly cationic protein [22] which facilitates its isolation from the vast majority of anionic proteins in pollen extracts as well as from proteins coproduced in eukaryotic expression supernatants. Our initial efforts in developing an Amb a 6 system for studying allergy involved determining its amino acid sequence and obtaining a sufficient amount of highly purified allergen. This latter aspect was complicated by the finding that Amb a 6 represents a minor allergen in short ragweed (1.2 mg/gram of pollen) and that chromatographically isolated Amb a 6 consists of at least four isoforms which are extremely difficult to separate from each other. We have utilized molecular biology technology to clone Amb a 6, deduce the amino acid sequence of a single isoform, and to then subclone the region encoding the secreted protein into a vector for expression. Both the information obtained and the recombinant protein could have important utility in our further understanding of the allergic response as well as in the design of diagnostic and immunotherapeutic materials.

In a previously published initial characterization of Amb a 6 [22], it was concluded that there are at least four isoforms which are antigenically indistinguishable by a hyperimmune anti-Amb a 6 goat antiserum. The isoforms are differentiated by charge (two peaks eluted from an ion exchange column) and by N-terminal amino acid sequence (each 'peak' from the ion exchange column has two related sequences, one beginning with A P P T C D T and the other with S P T C D T). Amb a 6 isolated from ragweed pollen appears to have significantly more of the former sequence than the latter. Conversely, our cloning data

suggest that there is more of the latter sequence message in short ragweed RNA; a partial clone isolated from a cDNA library translates to S P T C D T and all of the cDNA clones generated by PCR (both the incomplete 5' clones and the full clone) also translate to S P T C D T. It is possible that the set generated by 5' RACE was skewed toward the sequence S P T C D T because the primers used were based on the library clone, but PCR conditions were not very stringent (annealing temperature of 52°C) and therefore were unlikely to discriminate between similar isoforms. This phenomenon of the predominant protein isoform not corresponding to the isoform with the predominant message (as determined by our experimental methods) has been observed before in the case of another ragweed allergen, Amb a 1 [1]. While the reason for this discrepancy is unknown, it may be related to our methods in which either the yield of one protein is preferentially enhanced during protein purification or one message is preferentially isolated by molecular biology techniques. It is also possible that one or more isoforms are more rapidly degraded at the protein level. Regardless of the mechanism by which this discrepancy occurs, it is of interest that the phenomenon has been demonstrated for at least two ragweed genes, both of which have been cloned in this laboratory.

The amino acid sequence deduced from the selected clone reported here corresponds closely to the amino acid sequence of peptides derived from enzymatic fragmentation of Amb a 6 isolated from short ragweed. In addition to the N-terminal differences in the peptide pool, this peptide data revealed mostly conservative amino acid substitutions at positions 6, 10, 41, 45, 65 and 85. An NCBI Blast network service search for amino acid sequences homologous to the deduced Amb a 6 revealed a group of sequences coding for plant lipid transfer proteins. Among these are proteins from tobacco (accession no. U14168), barley [34] and wheat [28]. Another homologous sequence which appears to be a member of this family is derived from a major allergen in *Parietaria* [29]. A common feature shared by Amb a 6 and all other members of this lipid transfer protein family which we examined (10 random members of varying homologies) is the presence of eight highly conserved cysteine residues presumably involved in four disulfide bonds. Since Amb a 6 is a molecule with only 92 amino acids, this represents a cysteine content of 8.7%, which is unusually high and reminiscent of Amb a 5, another cysteine-rich allergen from short ragweed pollen [30]. Also of interest and related to the 'blast' homology search, is the finding that some lipid transfer homologs begin with the sequence 'S P T C D T' while others begin with 'A P P T C D T', the alternative N-terminal sequence found among the isotypes of Amb a 6.

The signal sequence of Amb a 6 conforms more or less to the motif established by von Heijne [reviewed in Ref. 31]. Following an initiating methionine residue is a hydrophobic stretch of amino acids characteristic of the h-region found in signal sequences. Positions -1 and -3, or the c-region, are alanine and phenylalanine, respectively. The alanine is a standard small uncharged residue typically found in this position. However, the phenylalanine in position -3 is unconventional; although it is

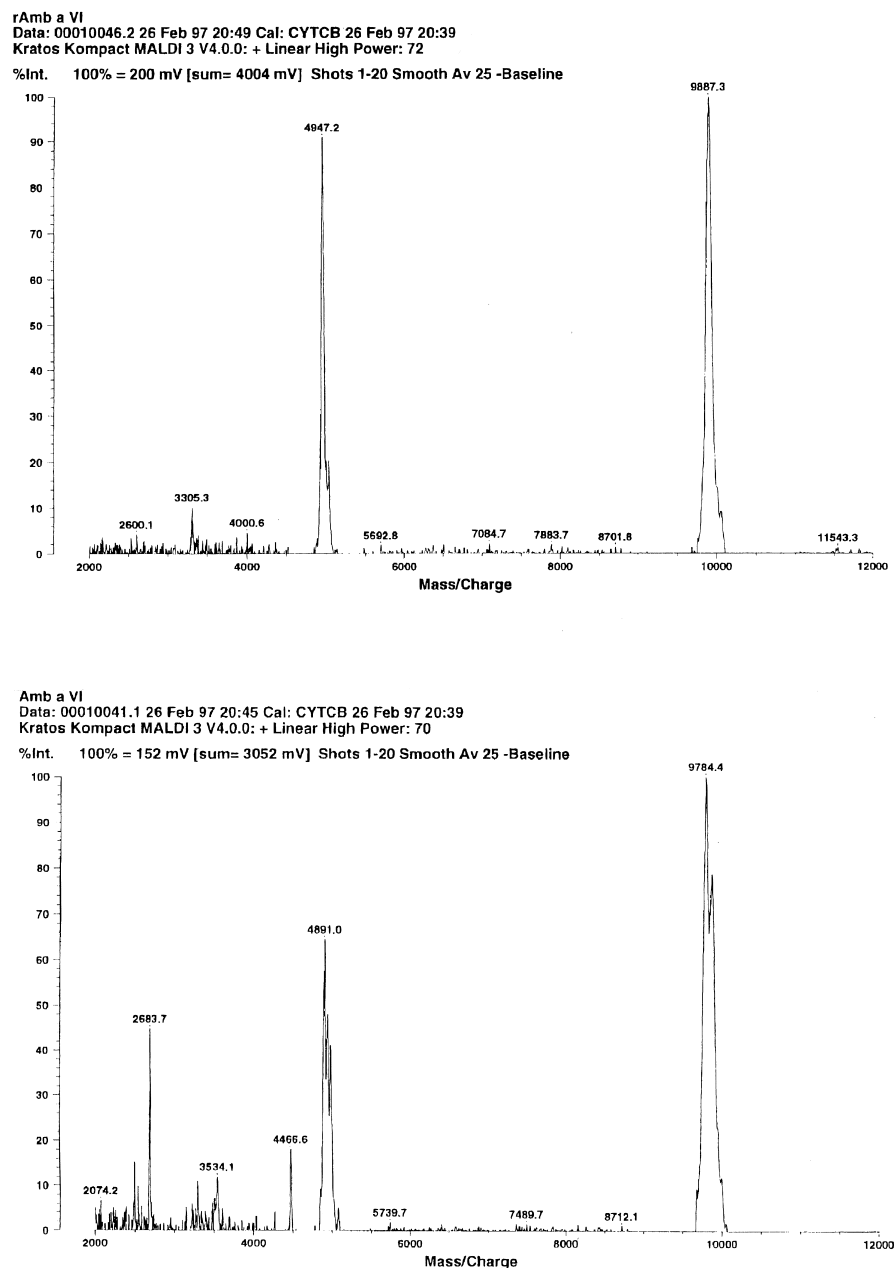


Fig. 7. Mass spectrometric analyses of Amb a 6 and rAmb a 6. Chromatograms representative of an average run are shown. The mass of the unlabelled peak of Amb a 6 is 9784.92. The mass of the trailing shoulder of the largest Amb a 6 peak is 9879.68.

uncharged, it is bigger than amino acids typically found at this position. It is not surprising that the Amb a 6 signal sequence does not conform absolutely to the established motif, as other 'unconventional' plant protein signal sequences have been previously reported. Plants may have different patterns which have yet to be established.

The *Pichia* expression system has proved useful in producing recombinant Amb a 6. As recommended, we analyzed several Zeocin-resistant yeast transformants (Z251, Z252, Z253 and Z254). All of these secreted a protein which migrated near Amb a 6 on SDS-PAGE and reacted with rabbit antiserum to Amb a 6 in Western blot analysis; no apparent differences between clones were observed. The initial media we used for

growth was BMMY, a rich media containing yeast extract and peptone. We found that purifying rAmb a 6 from the many proteins and small peptides contained in this media required multiple steps. We then tried growing yeast in a more minimal media, BMMH. The yeast appeared to grow and secrete recombinant protein just as well in this media, and purification was simplified.

rAmb a 6 appears to be a good representation of naturally occurring Amb a 6. Its molecular weight is compatible with that of our pollen purified Amb a 6 sample. Studies performed to date suggest that it is antigenically very similar to native Amb a 6; it reacts with goat and rabbit polyclonal anti-Amb a 6 in both Western blot and ELISA and also reacts with mouse monoclonal

anti-Amb a 6 in Western blot. In addition, rAmb a 6 inhibits the binding of goat anti-Amb a 6 to native Amb a 6 in a manner very similar to homologous inhibition by its native counterpart. In preliminary experiments (data not shown), rAmb a 6 inhibits the binding of human antibody from some Amb a 6 allergic individuals to highly purified pollen-derived native Amb a 6. The rAmb a 6 N-terminal sequence matches that of one of the previously reported Amb a 6 sequences, and, since our selected clone was introduced into the Z25 plasmid of the expression system, our rAmb a 6 primary sequence is presumably the same as one of the Amb a 6 isoforms. Future studies will further assess the ability of rAmb a 6 to bind to IgE in human ragweed allergic sera and determine whether rAmb a 6 would be a suitable Amb a 6 homolog for extensive structural analysis.

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IN MEMORIAM

We are saddened to note the recent passing of both Dr Marianne Roebber (who made this work possible by defining and isolating Amb a 6 in 1983) and Dr David G. Marsh, a friend and colleague whose laboratory championed the use of highly purified, well-characterized allergens.

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