
A sensitive two-site enzyme-linked immunosorbent assay for measurement of the major *Alternaria alternata* allergen Alt a 1

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Background: Alt a 1 is the major allergen in *Alternaria alternata*, one of the most important fungi associated with allergic diseases. Mold allergenic extracts show considerable heterogeneity, and thus accurate standardization of these extracts is essential to guarantee their quality.

Objective: To develop an Alt a 1-specific assay and to evaluate the correlation of Alt a 1 content with the IgE-binding activity of *A. alternata* extracts.

Methods: Recombinant Alt a 1 was produced as nonfusion protein from a polymerase chain reaction-cloned complementary DNA Alt a 1 sequence. Natural Alt a 1 was purified from spent culture medium. Monoclonal and polyclonal antibodies directed to Alt a 1 were produced and used to construct a specific Alt a 1 enzyme-linked immunosorbent assay (ELISA).

Results: The ELISA developed was highly reproducible and sensitive, with a detection limit lower than 0.5 ng/mL and a practical working range of 0.5 to 50 ng/mL. The assay was able to detect an Alt a 1-like protein in *Stemphylium* extracts. Identical parallel dose-response curves were observed when natural Alt a 1 and recombinant Alt a 1 were used as standard. A good correlation was obtained between Alt a 1 content of 13 *A. alternata* extracts and their IgE-binding activity. Alt a 1 was responsible for 70% of the IgE-binding activity of the whole extract.

Conclusions: This sensitive and specific Alt a 1 assay allows the quantification of this major mold allergen and represents a useful tool for the standardization of *A. alternata* extracts in mass units. It also provides a reliable indication of the allergenic activity of the whole extract.

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INTRODUCTION

Allergens derived from fungi cause sensitization in 80% of asthmatic patients in the United States¹ and in approximately 20% of Spanish patients with allergies.² *Alternaria alternata* is considered to be one of the most important fungi that cause fungal allergic disease in the United States, and sensitization to *Alternaria* allergens is associated with asthma.^{3,4} Allergen extracts produced from molds show considerable heterogeneity as a result of interstrain differences, culture conditions, and extraction procedures.⁵ Thus, accurate standardization of allergen extracts is essential to guarantee their quality. Current standardization methods are based on in vivo testing of in-house reference (IHR) extracts in allergic volunteers by subcutaneous or intradermal skin tests. Subsequently, production batches are compared with this IHR extract using in vitro IgE-based methods. These procedures are labor intensive and use human serum IgE, which is in short supply. For these reasons, standardization of mold extracts has been a significant problem. Another significant inconvenience with the current standardization protocol is that only an overall allergenic potency is determined on the basis of reactivity with IgE antibodies of pooled serum samples from allergic patients, and extracts with similar potency may have differences in major

allergen ratios.⁶ Recently, considerable progress has been made toward standardization by development of assays to quantify specific allergens, and a monoclonal antibody (mAb)-based assay can be particularly useful for these purposes.

Alt a 1 is the major allergen in *A. alternata* extracts, reacting with serum IgE from 82 to 100% of *A. alternata*-sensitized patients.^{7,8} The allergen is a dimer with a molecular mass of 29 kD, which dissociates into 14.5- and 16-kDa subunits under reducing conditions.⁷ Recently, another major allergen, named Alt a 2, has been cloned.⁹ Alt a 2 was recognized by IgE antibodies in the serum samples of 16 (61%) of 26 individuals allergic to *Alternaria*. Furthermore, three other *A. alternata* allergens (Alt a 6, Alt a 7, and Alt a 10) have been cloned, but these allergens were recognized by less than 8% of *Alternaria* allergic patients, which define them as minor allergens.¹⁰ In this study, we describe the purification of natural Alt a 1 (nAlt a 1) and recombinant Alt a 1 (rAlt a 1), the development of an Alt a 1-specific assay, and its use for improved standardization of *A. alternata* extracts for diagnosis and specific immunotherapy.

MATERIALS AND METHODS

Cloning of Alt a 1—Encoding Complementary DNA and Nucleotide Sequencing

A. alternata CBS 603.78 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was grown in Czapek broth media for 3 weeks at 25°C. Poly(A⁺) messenger RNA (mRNA) was isolated from 130 mg of mycelia using the

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Quick Prep MicroRNA Purification Kit (Amersham Biosciences, Uppsala, Sweden). A total of 1 μ g of poly(A⁺)-enriched mRNA was reverse transcribed using a First-Strand Complementary DNA (cDNA) Synthesis kit (Amersham Biosciences) and random hexadeoxynucleotides as primers. Oligodeoxynucleotide primers for cDNA amplification were designed according to a previously reported sequence⁷: A17F, 5'-GCGGATCCCATATGTCCT GCCTGTCACCAC (for the N-terminus) and A17R, 5'-GCAAGCTTTTAA-GAGTCTTGGGGAGAG (for the C-terminus). *Bam*HI, *Nde*I, and *Hind*III restriction sites, respectively, are underlined. Alt a 1 encoding cDNA was amplified by polymerase chain reaction using 5 μ L of the products of the first-strand cDNA synthesis and the described primers in 100 μ L of final volume under the following conditions: 30 cycles at 94° C (1 minute), 54° C (2 minutes), and 72° C (2 minutes) and a final incubation at 72° C (6 minutes). The predominant band of 400 base pairs (bp) was isolated from 2% agarose gels (GeneClean; Bio101, La Jolla, CA) and, after cutting with *Bam*HI and *Hind*III, cloned into pBluescript II SK (Stratagene GmbH, Heidelberg, Germany). Recombinant DNA techniques were performed following standard methods.¹¹

Nucleotide sequences were determined using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and analyzed on an ABI 373A DNA sequencer (Perkin-Elmer, Foster City, CA). The analysis of the sequences was performed using the GCG Program Package (Genetics Computer Group Inc, Madison, WI) and the BLAST program.¹²

Production and Purification of rAlt a 1

After sequencing, cDNA clone ALT1 was subcloned into the *Nde*I and *Hind*III sites of the expression vector pKN172¹³ (kindly donated by J. M. Arizmendi, Basque Country University, Spain). *Escherichia coli* BL21 (DE3) (Stratagene GmbH) cells containing the pKN172-derived recombinant plasmids were grown in Luria Bertani medium with 200 μ g/mL of ampicillin. When cultures reached an OD₆₀₀ of 0.8, 0.6 mmol/L isopropyl-thio- β -galactoside was added to the culture, and incubation continued for another 3 hours. Cells were harvested by centrifugation, and the pellet, resuspended in 0.05 M Tris hydrochloride, pH 8.0, and 5 mmol/L dithiothreitol, was lysed by lysozyme treatment (100 μ g/mL, 15 minutes, 37° C) and mild sonication (3 times for 20 seconds each). Insoluble material was resuspended in 2 M urea and 0.2% Triton X-100 and sonicated as described herein. After removing urea by step dialysis, rAlt a 1 was purified by ion-exchanged chromatography on a HiTrap Q column (Amersham Biosciences) equilibrated with 20 mmol/L phosphate buffer, pH 7.0, on a ÄKTA-prime System (Amersham Biosciences) using a gradient of 0 to 1.0 M sodium chloride.

Production of Antibodies

Female BALB/c mice (Harlan Interfauna Ibérica, Barcelona, Spain) were immunized by intraperitoneal injection with 20 μ g of purified nAlt a 1 at 2-week intervals. Hybridomas were produced by fusing spleen cells of immunized mouse with

Sp2/0 myeloma cell line (kindly donated by J. Pontón, Basque Country University). Detailed procedures for the fusion and the hybridoma maintenance have been previously described.¹⁴ Isotype detection was performed by IsoStrip kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Polyclonal antibodies were obtained in New Zealand rabbits by immunizing with 5 boosts of 200 μ g of nAlt a 1 every 2 weeks, emulsified in complete Freund adjuvant (Difco Laboratories, Detroit, MI). Serum samples, collected 10 days after the last injection, were tested and stored at -80° C. Both mAbs and rabbit polyclonal antibodies were purified by affinity chromatography with protein G-Sepharose (Amersham Biosciences). The rabbit anti-Alt a 1 serum was labeled with biotin using the ECL protein biotinylation kit (Amersham Biosciences).

Purification of nAlt a 1

A. alternata was grown on Czapeck broth media at 25° C for 3 weeks. The spent culture medium was separated by filtration and then concentrated and diafiltered by a 5,000-kDa cutoff. Purification of Alt a 1 for immunization was achieved by standard chromatographic methods, including anion exchange, gel filtration, and reverse phase. Briefly, concentrated spent culture medium was adjusted to pH 7.0 with 20 mmol/L phosphate buffer and subjected to anion exchange chromatography on a HighFlow Q Sepharose column (Amersham Biosciences) and eluted with a 0- to 200-mmol/L sodium chloride gradient. The nAlt a 1-containing fractions were selected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting incubated with a pool of sera from *Alternaria*-allergic patients. Selected fractions were concentrated and applied to a calibrated Superdex 200 column. Finally, nAlt a 1 was purified by reverse-phase chromatography in a SMART System (Amersham Biosciences) with a μ RPC C2/C18 column and a step gradient of 25 to 40 to 50% acetonitrile in 0.1% trifluoroacetic acid. The nAlt a 1-containing fractions were pooled, dialyzed, concentrated, and kept at -40° C.

For ELISA standard curves and the inhibition experiments, Alt a 1 was immunoaffinity purified using an immunosorbent column prepared by coupling rabbit anti-Alt a 1 polyclonal antibodies to CNBr-activated Sepharose 4B (Amersham Biosciences). *A. alternata*-spent culture medium was passed through the column, and after extensive washing with phosphate-buffered saline (PBS) to eliminate the nonretained components, bound protein was eluted using 100 mmol/L citrate buffer, pH 2.7. Fractions were collected in tubes containing neutralizing buffer (1 M Tris hydrochloride, pH 8.0) and then pooled, concentrated by ultrafiltration, and stored in aliquots at -40° C. Protein content was determined by the Bradford method.¹⁵

Quantification of Alt a 1 by a Two-Site Sandwich ELISA

Microtiter 96-well plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 100 μ L of mAb 1D6 at 5 μ g/mL in PBS overnight at room temperature. After blocking with 200

μL of PBS containing 1% bovine serum albumin and 0.05% Tween 20 (PBS-B-T), they were incubated with 100 μL per well of sample diluted in blocking buffer for 1 hour at 37° C. A standard curve was obtained using dilutions (50, 25, 10, 5, 2.5, 1, and 0.5 ng/mL) of purified nAlt a 1. After washing with 200 μL of PBS containing 0.05% Tween 20 (PBS-T), 100 μL of biotin-labeled rabbit anti-Alt a 1 serum (0.08 $\mu\text{g/mL}$ in PBS-B-T) was added, and the plates were incubated for 1 hour at 37° C. Following further washes, bound antibodies were detected by incubation with 100 μL per well of streptavidin-peroxidase (0.25 $\mu\text{g/mL}$ in PBS-B-T) (Sigma Chemical Co) for 1 hour at 37° C. The plates were finally washed and developed by incubating at room temperature in the dark with 200 μL of freshly prepared enzyme substrate o-phenylenediamine (Sigma). After 30 minutes, color reaction was stopped by adding 50 μL of 3 M H_2SO_4 , and the optical density was measured at 492 nm.

Evaluation of Assay Variation

One standard curve ranging from 0.5 to 50 ng/mL of Alt a 1 was run per day for more than 10 days to evaluate interassay variations. To determinate intra-assay variations, 20 wells were incubated with 1, 5, and 10 ng/mL of purified protein. In both assays, the coefficient of variation (CV), defined as the SD divided by the mean and multiplied by 100, was calculated. The CVs smaller than 20% were considered non-significant. The detection limit was calculated as the amount of Alt a 1 that corresponded to the median plus 3.33 times the SD obtained after 30 measurements of the zero standard. Recovery experiments were performed using *A. alternata* extracts (25, 50, and 100 ng/mL) spiked with 0.5, 1, and 2.5 ng/mL of purified Alt a 1.

Enzyme Allergosorbent Test Inhibition

The allergenic activity (or IgE-binding activity) of each *A. alternata* extract was evaluated by enzyme allergosorbent test (EAST) inhibition. *A. alternata* extract was coupled to paper disks activated with CNBr as described by Ceska et al.¹⁶ Fifty-microliter aliquots of a serum pool prepared with serum samples from *Alternaria*-sensitized patients ($n = 34$, positive response to skin prick tests, and specific IgE levels to *Alternaria* extract greater than EAST class 2 score) were preincubated at 4° C overnight, with 50 μL of 10-fold serial dilutions of each *A. alternata* extract as inhibitor. Allergen disks were added to the tubes and incubated at 37° C for 1 hour. After washing, the disks were incubated 30 minutes at 37° C with 50 μL of alkaline phosphatase-labeled mouse anti-human IgE and developed following the instructions of Hytec-specific IgE enzyme immunoassay (Hycor Biomedical Inc, Kassel, Germany). Relative allergenic activity was calculated on the basis of the 50% inhibition values and expressed in relative allergenic units.

Electrophoresis and Immunoblotting

A. alternata extracts and purified Alt a 1 were separated by SDS-PAGE (12.5% polyacrylamide gels) under reducing and nonreducing conditions using the Laemmli method.¹⁷ Protein

bands were stained with Coomassie brilliant blue. For immunoblot studies, the separated proteins were transferred electrophoretically to polyvinylidene difluoride (Hybond P; Amersham Biosciences). After blocking, membranes were incubated for 1 hour at 37° C with mAbs or rabbit anti-Alt a 1 serum, then washed and bound antibodies were detected with 1:10,000 horseradish peroxidase-conjugated anti-mouse IgG (Sigma) or anti-rabbit immunoglobulins (Dako, Copenhagen, Denmark), respectively. Then the blot was washed and developed by the ECL Western blotting detection system (Amersham Biosciences).

Spore and Dust Sample Extracts

Spores were washed from *A. alternata* growing on Czapeck plates for 1 week. Dust samples were collected from *Alternaria*-allergic patients' houses using the Mitest Dust Collector (Indoor Biotechnologies, Charlottesville, VA) following manufacturer's recommendations. Spore and dust samples were extracted with 0.01 M ammonium bicarbonate, pH 7.5, for 3 hours at 48° C.¹⁸

RESULTS

Nucleotide Sequence

The recombinant DNA insert was 393 bp long and encoded for a protein of 131 amino acids, with a predicted molecular mass of 14,245 Da and a theoretical isoelectric point value of 4.88, which corresponded to the values of the mature Alt a 1 protein described previously.^{7,19} When compared with the published Alt a 1 sequence, four nucleotide differences were found. Two of them have effects on the deduced amino acid sequence: Asp-96 $\Rightarrow$ Asn and Asp-100 $\Rightarrow$ Gly, numbering according to De Vouge⁷ (Fig 1). Two Alt a 1 databank entries (GenBank accession Nos. U86752 and U82633) are identical and share a high homologous structure (98% identical residues) with the sequence described herein. The other Alt a 1 sequence reported (GenBank accession No. U62087)²⁰ shares only 18.4% identical amino acids with them, mainly located at the N-terminal area (data not shown).

Purification of rAlt a 1 and nAlt a 1

The gene encoding Alt a 1 was subcloned into the expression vector pKN172 and expressed as a nonfusion protein. This protein, produced according to the described conditions, represented more than 35% of total bacterial proteins. After lysis, all the recombinant fusion protein appeared mainly as insoluble material and was solubilized in 2 M urea. Renaturation was performed by removing urea by step dialysis at 4° C. The soluble material was purified by ion-exchange chromatography and gave a protein with 96% of purity (Fig 2). The final yield of purified rAlt a 1 protein was approximately 3 mg/L of bacterial culture.

nAlt a 1 was purified from *A. alternata*-spent culture medium by affinity chromatography using polyclonal antibodies coupled to Sepharose. A high degree of purity was achieved with this single-step purification procedure as demonstrated by SDS-PAGE. The electrophoretic pattern of the

M Q F T T I A S L F A A A G L A A A A P 20
ATG CAG TTC ACC ACC ATC GCC TCT CTC TTC GCC GCC GCT GGC CTT GCC GCT GCT GCT CCT 60

L E S R Q D T A
CTC GAG TCT CGC CAG GAC ACC GCA --- --- --- --- --- --- --- --- --- --- 120
ATG TCC TGC CCT GTC ACC ACT GAG GGT GAC TAC GTC TGG
M S C P V T T E G D Y V W 40

--- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- 180
AAG ATC TCC GAA TTC TAC GGA CGC AAG CCG GAA GGA ACC TAC TAC AAC AGC CTC GGC TTC
K I S E F Y G R K P E G T Y Y N S L G F 60

AAC ATC AAG GCC ACC AAC GGA GGA ACC CTC GAC TTC ACC TGC TCT GCT CAG GCC GAT AAG 240
N I K A T N G G T L D F T C S A Q A D K 80

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 300
CTT GAG GAC CAC AAG TGG TAC TCC TGT GGC GAG AAC AGC TTC ATG AAC TTC TCT TTC GGC
L E D H K W Y S C G E N S F M N F S F G 100

AGC GAC CGC AGC GGT CTG CTC CTG AAG CAG AAG GTT AGC GAC GAC ATC ACC TAT GTC GCT 360
S D R S G L L L K Q K V S D D I T Y V A 120

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 420
ACC GCC ACT CTT CCC AAC TAC TGC CGC GCT GGC GGT AAC GGC CCC AAG GAC TTT GTC TGC
T A T L P N Y C R A G G N G P K D F V C 140

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- 474
CAG GGT GTT GCC GAC GCC TAC ATC ACC CTC GTC ACT CTC CCC AAG AGC TCT TAA
Q G V A D A Y I T L V T L P K S S * 158

Figure 1. Nucleotide sequence and deduced amino acid sequence of Alt a 1-encoding complementary DNA fragment. Primer sequences used in the cloning by polymerase chain reaction are underlined. The previous Alt a 1-reported sequence⁵ is shown (italics), indicating only differences. Dashes indicate identity of nucleotides with respect to the upper sequence.

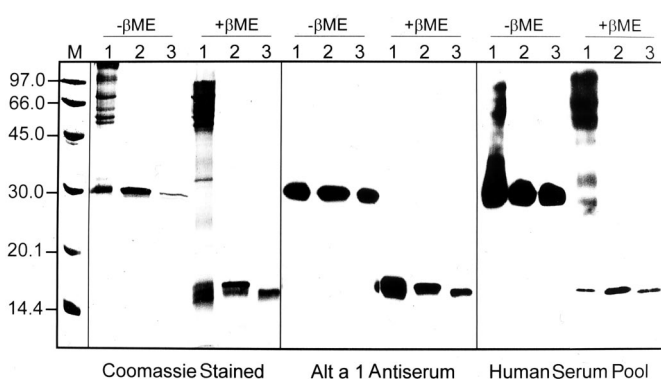


Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots of reduced (+β-mercaptoethanol [+βME]) and nonreduced (-βME) Alt a 1. *Alternaria alternata* extracts (lanes 1) and immunoaffinity purified natural Alt a 1 (lanes 2) and recombinant Alt a 1 (lanes 3) were subjected to SDS-PAGE and immunoblotting. Immunoblots were incubated with a polyclonal anti-Alt a 1 antiserum or with a human serum pool of *Alternaria*-allergic patients. For Coomassie-stained SDS-PAGE and human IgE immunoblots, 10 μg of extract and 1 μg of purified proteins were loaded. For anti-Alt a 1 antiserum immunoblots, 600 and 20 ng were loaded with extract and purified proteins, respectively.

protein secreted into the *A. alternata* culture medium and used as the starting material for the purification of nAlt a 1 is shown in Figure 2. The SDS-PAGE analysis showed that nAlt a 1 exists as a 30-kDa dimer but breaks down into 16.4- and 15.3-kDa subunits under reducing conditions. rAlt a 1 migrates as 15.5 kDa under reducing conditions but tends to dimerize during storage, showing mainly a 30-kDa band under nonreducing conditions (Fig 2). Fresh preparations of rAlt a 1 showed a higher proportion of monomer band than older samples (data not shown). Purified rAlt a 1 showed a slightly faster electrophoretic mobility than the natural form,

probably because of the absence of glycosylation. Reactivity of nAlt a 1 and rAlt a 1 against rabbit anti-Alt a 1 serum was checked by immunoblotting (Figure 2).

ELISA for Alt a 1 Quantification

Immunization with purified nAlt a 1 resulted in 12 IgG mAbs with specificity for nAlt a 1 and rAlt a 1 as judged by ELISA. Eleven of them detected Alt a 1 in immunoblots after SDS-PAGE was run under denaturing conditions but not in ELISA tests using mAbs as capturing antibodies and Alt a 1 in solution. Only 1 of the 12 (mAb 1D6) was able to catch Alt a 1 in ELISA and bind Alt a 1 in immunoblot experiments after SDS-PAGE under nonreduced conditions. The same mAb gave negative results when immunoblots were performed after PAGE of Alt a 1 under reduced conditions, indicating that it is recognizing a conformational epitope. The isotype of the mAb 1D6 was IgG1 with the κ light chain. This mAb was used as a capturing reagent in combination with a biotin-labeled rabbit anti-Alt a 1 serum as detector in a two-antibody sandwich ELISA for Alt a 1 quantitation. The assay was dose-dependent, and the standard dose-response curve revealed a sigmoid shape when plotted on a semilogarithmic scale. The assay had a detection limit lower than approximately 0.5 ng/mL, and it showed a working range between 0.5 and 50 ng/mL (values of concentration with a CV <20%). The correlation was found to be linear between 1 and 10 ng/mL (Fig 3). The intra-assay variation was determined by assaying three concentrations (1, 5, and 10 ng/mL) of purified Alt a 1 for 20 times, and the CV was 13, 8, and 6.8%, respectively. The interassay variation was evaluated by measuring a standard curve per day in 10 different days. The average of interassay CV from the linear portion of the standard curve was 11.7%. These results and the good percentage of recovery obtained (mean ± SD, 98.1% ± 5.8%) supported the validity of the assay. Almost identical parallel

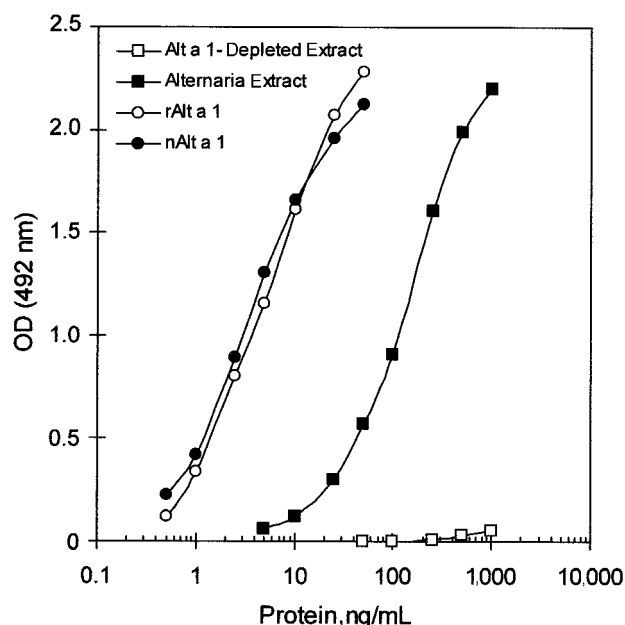


Figure 3. Quantification of Alt a 1 by specific enzyme-linked immunosorbent assay. *Alternaria alternata* extract, Alt a 1-depleted *A. alternata* extract, and purified Alt a 1 (natural [nAlt a 1] and recombinant [rAlt a 1]) were used in the Alt a 1 binding assay.

dose-response curves were obtained with nAlt a 1 and rAlt a 1 (Fig 3).

Quantification of Alt a 1 in Fungal Extracts and Correlation with Allergic Activity

Alt a 1 content was evaluated in 13 *A. alternata* extract batches. Alt a 1 levels ranged from 6 to 26 $\mu\text{g/mL}$, with an average of $15 \pm 7 \mu\text{g/mL}$. Parallel dose-response curves were obtained with nAlt a 1, rAlt a 1, or different *A. alternata* extracts. An immunosorbent column based on polyclonal antibodies was used to obtain an Alt a 1-depleted extract. The amount of allergen remaining after depletion was less than 0.1 $\mu\text{g/mL}$ measured by two-site sandwich ELISA (Fig 3). Alt a 1 levels were determined in seven different *A. alternata* extracts according to the developed assay and a marketed Alt a 1 ELISA kit (Indoor Biotechnologies).²¹ A significant correlation ($R^2 = 0.98$) was observed between the two techniques. However, the levels of Alt a 1 obtained with the marketed assay were approximately fourfold higher than those obtained with the developed assay (data not shown).

The specificity of the assay was tested using other fungal extracts (*Aspergillus*, *Cladosporium*, *Penicillium*, *Curvularia*, and *Stemphylium*) from the Deuteromycetes class and a yeast extract (*Candida*) from the Saccharomycetes class. No signal was found up to a concentration of 1000 ng/mL (10 times the upper limit of the assay range for *A. alternata* extracts). However, a protein sharing a common epitope and molecular mass with Alt a 1 was detected in the *Stemphylium* extract by immunoblotting (data not shown). This extract

contains 32 $\mu\text{g/mL}$ of Alt a 1-like protein, quantified by the developed ELISA.

Eleven different *A. alternata* CBS 603.78 extracts and two Alt a 1-depleted *A. alternata* extracts were assayed by ELISA. The IgE-binding activity was determined by EAST inhibition. A significant correlation ($R^2 = 0.95$) was found between both methods, demonstrating that the Alt a 1 content is highly representative of the overall allergenic activity of the whole *A. alternata* extract. Nevertheless, a residual IgE-binding activity was observed in Alt a 1-depleted extracts (Fig 4). The IgE-binding activity of Alt a 1 in the *A. alternata* extract was estimated by EAST inhibition. Purified nAlt a 1 could inhibit the binding of specific IgE to the whole extract, up to 70% (Fig 5). This demonstrated that Alt a 1 is responsible for most of the IgE-binding activity in *A. alternata* extracts.

Spore and Dust Extracts

The content of Alt a 1 in actively growing *Alternaria* spores and dust samples was evaluated. Alt a 1 was detected at concentrations of 3.5 ng/mL in 6×10^3 spores/mL. Spore concentrations of 6×10^2 contained undetectable Alt a 1 allergen. Alt a 1 was also undetectable in any of the 12 dust samples tested.

DISCUSSION

The standardization of allergenic extracts is essential for a successful diagnosis and immunotherapy of allergic disease.²² The quantification of Alt a 1 is an important method for standardization of extracts because it allows effective quality control of raw material, optimization of extraction proce-

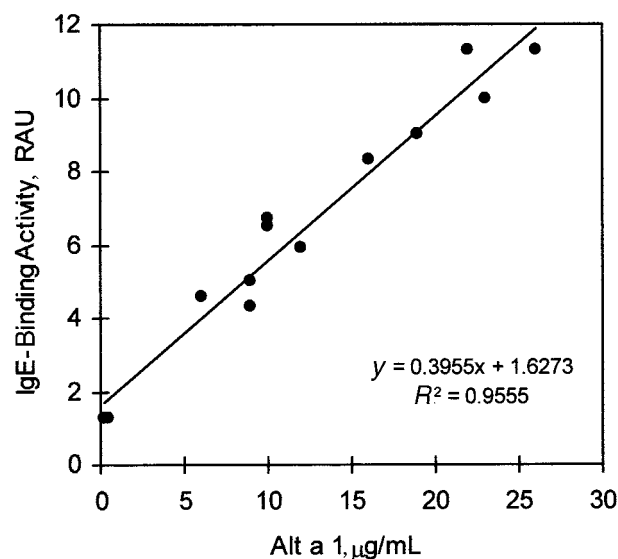


Figure 4. Correlation between IgE-binding activity values expressed in relative allergenic units (RAU) and determined by enzyme allergosorbent test inhibition and Alt a 1 content measured by the two-site enzyme-linked immunosorbent.

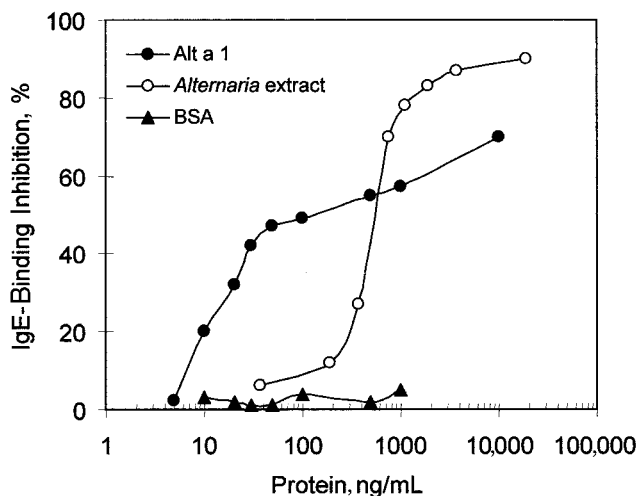


Figure 5. Enzyme allergosorbent test inhibition curves. The binding of specific human IgE to *Alternaria alternata* extract was inhibited using natural Alt a 1, *A. alternata* extract, or bovine serum albumin (BSA) as control.

dures, and comparison of batches with the biologically standardized IHR extract.

The Alt a 1 assay is highly sensitive (0.5 ng/mL of Alt a 1), similar to another recently described assay,²¹ and more than 400-fold higher than the assay reported by Portnoy et al.²³ Since currently no international standards are available for Alt a 1 quantification, the rAlt a 1 could become a certified reference material for the specific Alt a 1 assay because it has several advantages over its natural counterpart. rAlt a 1 is accurately quantified and can be produced in quantities large enough to develop a suitable lyophilization strategy and stability studies. The assay is highly specific for Alt a 1, and only in extracts of *Stemphylium*, which belongs to the same family as *A. alternata* (Pleosporaceae family), has an Alt a 1-like protein been detected. *Stemphylium*, an important fungi in the United States, contains two times more Alt a 1-like protein than *Alternaria* extracts. Similar results have been described by Agarwal et al²⁴ using double-antibody radioimmunoassay.

The described Alt a 1 ELISA could detect the presence of Alt a 1 in extracts produced from spore suspensions only at high concentrations (10⁴/mL) but not in house dust samples. Indoor levels of *Alternaria* spores are 0 to 282 spores/m³, which are much lower than those found outdoors.²⁵ More studies are necessary to evaluate the environmental exposure to *Alternaria* allergens, because low (0.4%) and high (87%) frequencies of Alt a 1 detection have been reported in house dust.^{26,27} The Alt a 1 ELISA could be useful for raw materials screening due to the high interstrain differences in allergen content and the multiple extraction procedures. The presence of Alt a 1 has been found in both fungal mats and culture filtrates,²⁸ and its release is increased during spore germination.²⁹ Therefore, the existence of a sensitive assay for quantification of Alt a 1 in raw material could improve the quality of *Alternaria* extracts.

Alt a 1 reacted with most of *A. alternata*-sensitive patients.^{7,8} In addition, Alt a 1 inhibited by up to 70% the total IgE-binding activity of the *A. alternata* extract, which suggests that allergens not related to Alt a 1 are remarkably less important from a clinical point of view, although they could be recognized by 61% of *Alternaria*-allergic patients.⁹ The good correlation obtained between the allergen content, determined by the Alt a 1 two-site binding assay, and the total IgE-binding activity, determined by EAST inhibition, for 13 extracts of *A. alternata* supports the belief that Alt a 1 is a major allergen and contributes in a high percentage to the total allergenic activity of *A. alternata* extracts.

The trend in allergen standardization is moving toward the measurement of major allergens,^{6,30,31} but the evaluation of the total allergenic potency of an extract should not rely only on the quantification of a single allergen.^{32,33} This point is more important in the case of the high heterogeneity and complexity of *A. alternata* extracts.⁵ van Ree et al^{34,35} have demonstrated that 1 to 3 allergens are necessary for in vitro diagnosis and standardization. Although *A. alternata* extracts were shown to contain only Alt a 1 as the most important major allergen, an allergen, named *Alt a 2*, has recently been cloned, and the rAlt a 2 has been recognized by IgE antibodies of 61% of individuals allergic to *Alternaria*.⁹ This allergen could be a good candidate for developing an Alt a 2 ELISA to complement *A. alternata* extract quality control. More clinical assays on therapeutic efficacy of immunotherapy with standardized *Alternaria* extracts, based on measurement of major allergens content, could establish the optimal Alt a 1 concentration for therapeutic efficacy.

CONCLUSION

We have developed a sensitive and reproducible ELISA to quantify Alt a 1. This ELISA represents a useful tool for the standardization of *A. alternata* extracts in mass units and provides a reliable indication of the allergenic activity of the whole extract.

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