

Enzymatic activities of *Alternaria alternata* allergenic extracts and its major allergen (Alt a 1)

Miriam Sáenz-de-Santamaría,¹ Jorge A. Guisantes¹ and Jorge Martínez^{1,2}

¹Department of Immunology, Microbiology and Parasitology, Faculty of Pharmacy, University of the Basque Country, Vitoria and ²Pharmacia Diagnostics, Barcelona, Spain

Summary

Several allergens from *Alternaria alternata* have been isolated allowing some of them to be identified and characterised. Despite the fact that the major allergen of *A. alternata* (Alt a 1) has been extensively produced by recombinant technology, its biological activity still remains unknown. In the present study, extracts from culture filtrates were used to evaluate the intra-specific variability of the enzymes and also as a source for isolating and purifying native Alt a 1. This was purified by affinity chromatography using antibody anti-recombinant Alt a 1 (produced in *Escherichia coli*). Enzyme activities were analysed by the API-ZYM System screening method. Results demonstrated the high variability of enzyme activities among the different strains. Only activities corresponding to phosphatases, esterases and β -glucosidase were expressed by 100% of the strains. Both native and recombinant Alt a 1 showed phosphatase and esterase activities, suggesting that the glucidic moiety of this allergen does not significantly affect its enzyme activity.

Key words: Alt a 1, *Alternaria alternata*, enzymatic activity.

Introduction

The application of recombinant DNA and proteomics to the knowledge of allergens has contributed towards the characterisation and identification of the individual molecular components that cause allergic diseases, and their possible biological and immunological relationships. Therefore, the impact of allergen structure on basic science, taxonomy, phylogeny, standardisation, prevention, diagnosis and therapy of allergic diseases is currently an undeniable fact.¹

Several recombinant allergens from *Alternaria alternata* have been produced, allowing some of them to be identified and characterised,^{2,3} but others, like the major allergen Alt a 1, have still not been defined.⁴

This makes it difficult to complete the characterisation of this protein and, consequently, the study of

homologies with other well-known proteins should also be attempted using immunological or other biochemical approaches.

The aim of this study was to examine the biological role of the major allergen of *A. alternata* (Alt a 1) by means of screening for several enzyme activities.

Materials and methods

Strains

The following strains of *A. alternata* were used:

1 Centraalbureau voor Schimmelcultures (CBS): Strains 106.24, 105.24, 105.49, 104.26, 603.78, 247.85, 795.72, 154.31, 326.65 and 113.41;

2 Spanish Type Culture Collection (CECT): Strain 2662;
3 Municipal Institute for Medical Research, Laboratory of Medical Mycology, Autonomie University of Barcelona, Barcelona, Spain: Strains IMIM 282, IMIM 93039 and IMIM 455-2; and,

4 Faculty of Medicine, Department of Basic Medical Sciences, Microbiology, University Rovira i Virgili, Reus, Spain: Strain FMR 3292.

Correspondence: Jorge Martínez-Quesada, Pharmacia Diagnostics España S.L., Ctra. Rubí, 72-74. 08190-Sant Cugat del Vallès, Barcelona, Spain.
Tel.: +34 699 486 714. Fax: +34 945 013 014.
E-mail: jorge.martinez@ehu.es

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Extracts

Culture filtrate and cellular extracts were obtained from fungal cultures in Czapek broth medium. All strains were incubated for 3 weeks at 25 °C. Fungal extracts were prepared according to Martínez *et al.* [5].

Cellular components were obtained by disruption of the washed mycelium with mechanical homogenising (Virtishear, Virtis, NY, USA) followed by the extraction of the soluble proteins in phosphate-buffered saline (PBS).

Culture filtrate was obtained by filtration in Whatman no. 1, AP and sterilising filter successively. Solutions of soluble material were dialysed by tangential ultrafiltration with a 10 000 Da cut-off, and the retained material was finally freeze-dried.

Preparation of recombinant Alt a 1

cDNA encoding Alt a 1 was cloned as reported previously⁶ using primers designed according to the previously reported sequence by De Vouge *et al.* [4]: A17F, 5'-GCGGATCCCATATGTCCTGCCTGTCACCAC (for N-terminus) and A17R, 5'-GCAAGCTTTTAAGAGCTCTTGGGGAGAG (for C-terminus); and the standard methods described by Sambrook *et al.* [7].

Production of the recombinant protein was produced after transforming *Escherichia coli* BL21 competent cells with the resultant plasmid.

This construction encodes a fusion protein (GST-Alt a 1) consisting of a glutathione S-transferase (GST) from *Schistosoma japonicum* and a six-amino acid peptide spanning thrombin cleavage site, followed by the Alt a 1 polypeptide.

Overnight cultures of *E. coli* BL21 cells containing GST-Alt a 1-encoding plasmid were diluted to an absorbance of 0.2 at 600 nm wavelength with LB medium containing 0.1 mg ml⁻¹ ampicillin.

Cells were grown up to an absorbance of 0.6 and induced with 0.6 mmol l⁻¹ isopropyl-1-thio-β-D-galactopyranoside. After incubation for 3 h, cells were harvested by centrifuging at 4000 *g* for 10 min at 4 °C. The pellet was resuspended in 5 ml of 50 mmol l⁻¹ Tris-HCl pH 8.0, 20 mmol l⁻¹ NaCl per g of cells. Eight milligrams of lysozyme and dithiothreitol (final concentration of 5 mmol l⁻¹) were added and incubated for 30 min at 37 °C.

After mild sonication, Triton X-100 was added to a final concentration of 1%, and incubated at room temperature for 30 min, and the lysate was centrifuged at 5000 *g* for 5 min at 4 °C. The precipitated inclusion bodies were resuspended in 0.1 mol l⁻¹ glycine/NaOH

pH 9.0 containing 8 mol l⁻¹ urea, and chromatographed through a Sephadex G-25 column (Amersham Biosciences, Uppsala, Sweden) equilibrated in 0.1 mol l⁻¹ glycine/NaOH pH 9.0. The eluted protein was stored overnight at 4 °C for refolding. The fusion protein was bound overnight at 4 °C to 1 ml of glutathione-Sepharose 4B (Amersham Biosciences) equilibrated in PBS, 0.1% Triton X-100. After packing and washing with PBS, the fusion protein was cleaved in the column at room temperature for 16 h with 10 U of bovine thrombin mg⁻¹ protein. Recombinant Alt a 1 was eluted from the column with three volumes of PBS.⁶

Rabbit serum anti-rec Alt a 1

Experimental immunosera were raised in adult male white New Zealand rabbits, according to Gallart *et al.*⁸ by subcutaneous injections with 200 µg of rec Alt a 1 in Freund's complete adjuvant. Immunoglobulin fraction containing specific antibodies was obtained by affinity chromatography in protein A (Hi-Trap columns; Amersham Biosciences) following the manufacturer's instructions.

Preparation of native Alt a 1

Native Alt a 1 was purified by affinity chromatography. Rabbit immunoglobulin fraction containing anti-rec Alt a 1 antibodies was covalently coupled to Sepharose-4B BrCN (Amersham Biosciences), according to the manufacturer's instructions. *Alternaria alternata* filtrate culture extract was eluted in a column containing the antibodies bound to the matrix. Retained proteins were eluted with buffer glycine 0.1 mol l⁻¹ pH 2.5 and the fraction obtained was dialysed against PBS and stored at -20 °C.

SDS-PAGE-IgE immunoblotting

Proteins were electrophoresed by SDS-PAGE according to Laemmli⁹ using 12.5% polyacrylamide separating gels.

Separated proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA) according to Towin *et al.* [10]. Sera from a patient allergic to *Alternaria* and from a non-allergic subject were used to characterise the allergenic activity of the recombinant protein. The immune complex was detected according to Shen *et al.* [11].

Enzyme activity

Enzymatic activities were determined by means of the API-ZYM System (Bio Mérieux, Marcy-'Etoile, France) in accordance with the manufacturer's instructions. Two assays were carried out at different times. The samples were analysed in duplicate in each assay.

Either 20 µg of protein of each extract or pure Alt a 1 were added to each well of the Api Zym tray containing the appropriate freeze-dried buffer and naphthol-coupled substrate. The tray was incubated at 37 °C for 4 h. After incubation, the free naphthol released from the substrate was detected by coupling with equal volumes (33 µl) of fast blue BB (0.35% w/v in 2-methoxyethanol) and Tris buffer 2 mol l⁻¹ pH 7.6, containing 10% w/v SDS.

The amount of product formed was semiquantitatively assessed in accordance with the colour chart supplied with the system.¹²

Results

Table 1 shows the enzymatic activities of the extracts obtained from cellular and culture filtrates of 14 different strains of *A. alternata*. Results from each analysed duplicate sample were coincident in all cases.

Enzymatic activities corresponding to acid phosphatase, alkaline phosphatase, phosphoamidase, sterase (C1), sterase lipase (C8) and β-glucosidase were detected

Table 1 Frequency (%) of enzymatic activity detected in cellular and filtrate culture extracts obtained from 15 different strains of *Alternaria alternata*.

Enzymatic activity	Filtrate culture	Cellular extract
Acid phosphatase	100	100
Alkaline phosphatase	100	100
Phosphoamidase	100	100
Esterase (C1)	100	100
Esterase lipase (C8)	100	100
Lipase (C14)	6.7	13.3
Leucine arylamidase	93.3	73.3
Valine arylamidase	46.7	86.7
Cystine arylamidase	6.7	6.7
Trypsin	66.7	0
α-Chymotrypsin	6.7	33.3
α-Galactosidase	33.3	60
β-Galactosidase	26.7	33.3
β-Glucuronidase	6.7	0
α-Glucosidase	86.7	93.3
β-Glucosidase	100	100
N-acetyl-β-glucosaminidase	100	66.7
α-Mannosidase	6.7	6.7
α-Fucosidase	0	0

in all strains and their activities were able to hydrolyse amounts of substrate higher than 20 nmol in all cases.

Lipase, cystine arylamidase, β-glucuronidase, α-mannosidase and α-fucosidase did not show any activity or very scarce activity by two or less strains and with very low hydrolysis capacity (< 10 nmol of hydrolysed substrate).

Only culture filtrate components showed trypsin activity (10 of 15 strains) and none of the cellular extracts demonstrated this activity.

Electrophoretic separation of eluted proteins able to bind specific antirecombinant Alt a 1 antibodies showed the presence of a 15-kDa component, corresponding to a molecular weight described for Alt a 1 subunits. This protein showed a capacity for binding human IgE contained in serum from a patient allergic to *A. alternata* (Fig. 1).

Table 2 shows the enzymatic activities corresponding to the recombinant and native major allergen of *A. alternata*. The screening allowed observation of phosphatase and esterase activities for both recombinant and native Alt a 1.

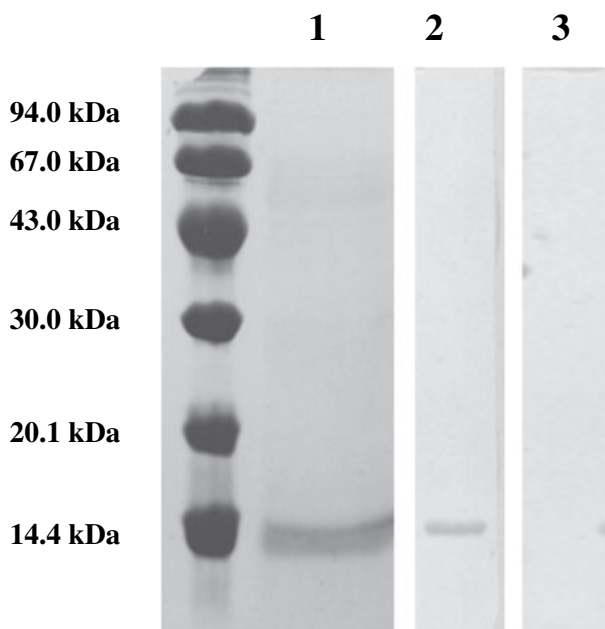


Figure 1 Purification of native Alt a 1 by affinity chromatography. (1) SDS-PAGE of retained fraction by rabbit specific anti-rec Alt a 1 antibodies. (2) SDS-PAGE IgE immunoblotting of the retained fraction (line 1) incubated with serum from a patient allergic to *Alternaria alternata*. (3) SDS-PAGE IgE immunoblotting of the retained fraction (line 1) incubated with serum from a non-allergic subject.

Table 2 Screening of enzymatic activity of recombinant and native major allergen of *Alternaria alternata* (Alt a 1) by means of the API-ZYM System

Enzymatic activity	Recombinant	Native
Acid phosphatase	+	+
Alkaline phosphatase	+	+
Phosphoamidase	+	+
Esterase (C1)	+	+
Esterase lipase (C8)	+	+
Lipase (C14)	–	–
Leucine arylamidase	–	–
Valine arylamidase	–	–
Cystine arylamidase	–	–
Trypsin	–	–
α -Chymotrypsin	–	–
α -Galactosidase	–	–
β -Galactosidase	–	–
β -Glucuronidase	–	–
α -Glucosidase	–	–
β -Glucosidase	–	–
<i>N</i> -acetyl- β -glucosaminidase	–	–
α -Mannosidase	–	–
α -Fucosidase	–	–

+, positive; –, negative.

Discussion

Several authors demonstrated high variability in the antigenic/allergenic composition of different strains of *A. alternata*,^{6,13–15} but data on the enzymatic profile of this mould and its intraspecific variability with regard to enzyme activity are scarce.^{16,17}

Results of this study demonstrate a high intraspecific variability of enzymatic activity. Phosphatase, esterase and β -glucosidase activities were detected in 100% of extracts from cellular and culture filtrates, and *N*-acetyl- β -glucosaminidase activity was present in 100% of extracts from culture filtrates. Leucine arylamidase and α -glucosidase activities were detected in most of the strains (percentages higher than 70% for both types of extracts). Only the activity corresponding to α -fucosidase was not detected in any case. Trypsin activity was expressed by 10 strains, and solely from culture filtrates.

Data corresponding to phosphatase, esterase and β -glucosidase activities in general agree with those reported previously.^{16,17} Enzymatic activity corresponding to trypsin did not agree with the results obtained by Vijay *et al.* [16] who did not find this activity. These differences could be explained by fungal variability, or by differences in methodology used. Several authors demonstrated the influence of carbon source, pH, medium composition and other parameters on enzyme expression.^{18,19}

Martínez *et al.* [5], Mitakakis *et al.* [20] and Green *et al.* [21] agreed that allergen release, including Alt a

1, is higher in culture filtrates and increases after germination of hyphae and spores. Therefore, in the present study, extracts from culture filtrates were used as a source for isolating and purifying the native Alt a 1.

From about 10 well-identified *A. alternata* allergens only seven have been characterised as definite proteins, and, of those, four are enzymes.² Up to now, no biological identity or homologies with other proteins have been described for Alt a 1, using recombinant DNA technology; consequently in the present study, the API-ZYM System was used as a screening method for detecting possible enzymatic activities associated with Alt a 1.

Several authors have used this method to study enzymatic profiles in different mould species.^{16,17,22–24}

Recombinant proteins obtained using *E. coli* as a host usually do not present three-dimensional structures homologous to the native proteins, and no glycosylation post-translational modifications can be made. Hence native proteins should be used.

Taking into account the fact that recombinant Alt a 1 reacts appropriately with the IgE contained in serum from a patient allergic to *Alternaria*, the experimental serum anti-rec Alt a 1 constitutes a valuable tool for obtaining the native protein with a high degree of purity, as demonstrated in this study. Moreover, affinity chromatography using immobilised specific antibodies in the solid matrix allows to obtain high quantities of highly purified proteins.

In the present study enzymatic activity corresponding to phosphatase and esterase could be associated with native and recombinant Alt a 1. These findings suggest that the possible structural differences between the recombinant and the native proteins do not affect the enzymatic activity significantly, nor does glucidic moiety seem to take part in enzyme activity.

The same enzyme activities associated with Alt a 1 were found also in cellular and culture filtrates from all strains and these activities were expressed in 100% of the *Alternaria* strains studied.

The data provided by this screening constitute a significant addendum to the characterisation of Alt a 1 as well as an interesting starting point for further research.

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