Quantification assay for the major allergen of *Cupressus sempervirens* pollen, Cup s 1, by sandwich ELISA

M.C. Arilla, I. Ibarrola, A. Martinez and J.A. Asturias


**ABSTRACT**

**Background:** The Cupressaceae are an important cause of pollinosis, particularly in Mediterranean countries. Cypress pollen allergenic extracts are difficult to produce since they have a low protein and a high carbohydrate content and consequently accurate standardization of these extracts is essential for diagnosis and immunotherapy.

**Method:** Natural Cup s 1 was purified by a combination of hydrophobic interaction, gel filtration and ion exchange chromatographies and its enzymatic activity was analyzed. The allergen was used as reference material in the ELISA standard curve. The assay was based on a specific monoclonal antibody (3D2) immobilized on ELISA plates and used to capture Cup s 1. Bound proteins were detected by a combination of biotinylated specific antisera and peroxidase-conjugated streptavidin.

**Results:** Purified Cup s 1 is a functional pectate lyase enzyme with a specific activity of 750 U/mg protein. The developed ELISA measured Cup s 1 concentrations ranging from 31.25 to 250 ng/ml in the linear portion of the standard curve. The intrain-assay and inter-assay variation coefficients in the working range were less than 8.1 % and 16 %, respectively. The assay was highly sensitive, with a detection limit of 3.8 ng/ml. The dose-response curves obtained with *C. sempervirens* pollen extracts and extracts belonging to other species from the Cupressaceae family showed a good parallelism compared with those obtained using the purified allergen, indicating that the same protein was measured.

**Conclusions:** The assay described is sensitive, specific and reproducible for the quantification of Cup s 1 in *C. sempervirens* pollen extracts for clinical use. This ELISA could also be useful for other Cupressaceae-related pollen extracts.

**Key Words:** *Cupressus sempervirens*. Cup s 1. Major allergen. Quantification. Monoclonal antibody. ELISA. Cupressaceae. Standardization. Pectate lyase.

**RESUMEN**

**Antecedentes:** Las cupresáceas son una importante causa de polinosis, particularmente en los países Mediterráneos. Los extractos alérgicos de polen de ciprés son difíciles de producir ya que tienen bajo contenido de proteínas y alto de carbohidratos, por lo que es esencial una precisa estandarización de estos extractos para su uso en diagnóstico e inmunoterapia.

**Método:** El alergeno natural Cup s 1 fue purificado mediante interacción hidrófoba, tamizado molecular, e intercambio iónico, y se analizó su actividad enzimática. Este alergeno fue utilizado como referencia en la realización de la curva de calibrado del ensayo. El ensayo se basó en un anticuerpo monoclonal (3D2) inmovilizado en la placa y usado para capturar a Cup s 1. Posteriormente un antisuero es-
INTRODUCTION

In the last years the Cupressaceae family plants are undergoing a strong geographical expansion, due to their use as physical barrier or as ornamental motives. Therefore, Cupressaceae pollens are an important cause of pollen allergy in several regions of the world. In Mediterranean countries the prevalence to Cupressaceae pollens can reach until 30% of the pollinosis cases\(^1\). Extensive cross-reactivity of the allergens has been demonstrated between different Cupressus and Juniperus species\(^2\). This fact together with the overlapping of their pollination periods make that the presence of allergic symptoms was continuous from October to April. Cupressaceae allergic patients present different clinical characteristics to other polinosis: the dominant symptom is rhinitis occasionally associated to conjunctivitis, while the asthma incidence is minor than in other pollen allergies; the patients usually have low levels of specific IgE and they develop symptoms older than in other polinosis\(^1,3\).

As a consequence of these low levels of specific IgE in patient sera, the in vitro inhibition studies used at this moment for the standardization of allergenic extracts are difficult to carry out. Moreover, Cupressaceae family pollen extracts are characterized to have a high content in carbohydrates and a low protein content (3%\(^4\)), what has hindered the possibility of producing good standardized extracts for diagnosis and immunotherapy.

Allergen content can be quantified in mass units by using monoclonal (mAb) and/or polyclonal antibodies specific for the allergen. The Cupressus sempervirens major allergen is a 43 kDa protein, named Cup s 1\(^5\) that has been recently purified\(^6\) and cloned (EBI accession number AF7257491), sharing a high homology with Cup a 1. Recently, a mAb-based ELISA has been developed in our laboratory for quantification of Cup s 1\(^7\), but no accurate determination can be obtained for C. sempervirens and other Cupressaceae species. In this work we describe a two-site sandwich ELISA for the quantification of Cup s 1 in C. sempervirens pollen extracts, using a monoclonal antibody as capture and a monospecific polyclonal antibody as detector.

MATERIALS AND METHODS

Allergenic extracts and Cup s 1 purification

In the preparation of the allergenic extracts, pollen of C. sempervirens, C. arizonica, Thuja plicata, and Juniperus communis (Iber-Polen, Málaga, Spain) were defatted and extracted during 4 h at 4 °C in 50 mM phosphate buffer pH 8.0 containing 65 mM NaCl. The obtained extracts were clarified by centrifugation to 5000 xg for 30 min, filtrated through AP glass fiber prefilter (Millipore Corp., Bedford, Madison, USA) and dialyzed by ultrafiltration with a 5000 Daltons exclusion size in a Pellicon System (Millipore). In order to improve Cup s 1 purification, C. sempervirens pollen was washed for 10 min in 10 mM KH\(_2\)PO\(_4\) pH 4.3, followed by an extraction with 40 mM NH\(_4\)HCO\(_3\) at 4 °C\(^9\). 2 M (NH\(_4\))\(_2\)SO\(_4\) was added to the clarified supernatant and was applied to a High Flow Phenyl-Sepharose 16/20 column equilibrated with 20 mM phosphate buffer pH 7.0, containing 2 M (NH\(_4\))\(_2\)SO\(_4\) and applied to the AKTA-prime System\(^10\) (Amersham Biosciences, Buckinghamshire, UK). The bound fraction was eluted with 20 mM phosphate buffer pH 7.0, concentrated and applied to a Superdex S-200 16/60 column equilibrated with phosphate buffer saline (PBS). The fraction containing the 43 kDa protein was collected, and after a dialysis with 50 mM sodium acetate pH 5.0, it was injected in a Mono S 1.6/5 ion exchange column into a Smart System\(^11\) (Amersham Biosciences). The purified al-
leugen was eluted as a unique peak at 100 mM NaCl. The protein content was determined by the Bradford method with the Bio-Rad protein assay kit (Bio-Rad, Hercules, California, USA). Carbohydrate content was measured as previously reported.

Analysis of the pectate lyase enzymatic activity

The pectate lyase activity was analyzed according to Keen et al. Briefly, the reaction mixture contained 0-0.2 % (w/v) of lemon pectin (Poly-D-galacturonic acid methyl ester, Fluka, Buch, Switzerland), 50 mM Tris (pH 8.5), 5 mM CaCl₂ and 5 µg of purified Cup s 1 in 1 ml of total volume. The reaction was carried out at 37 °C for 1 h, and the absorbance was measured at 235 nm. An unit of pectate lyase activity is defined as the amount needed to form 1 µM of product (unsaturated uronides) per minute with a molar extinction coefficient of 4600 M⁻¹ cm⁻¹ at 235 nm.

Production, purification and biotinylation of antibodies

The production and characterization of mAb 3D2, recognizing the major allergen of different Cupres-saceae, has already been described previously. Polyclonal antibodies were obtained in New Zealand rabbits after 5 boosts of 200 µg of the purified Cup s 1 every 2 weeks, emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA). Both, monoclonal and polyclonal antibodies were purified in the lineal portion of the standard curve. The protein content was determined by the Bradford method with the Bio-Rad protein assay kit (Bio-Rad, Hercules, California, USA). Carbohydrate content was measured as previously reported. 1

Quantification of Cup s 1 by a two-site sandwich ELISA

Microwell plates (Greiner, Frickenhausen, Germany) were coated overnight at room temperature with 100 µg/well of the mAb 3D2 at 5 µg/ml in PBS. Next, plates were blocked by adding 200 µg/well of PBS-1 % BSA-0.05 % Tween 20 (PBS-B-T) and incubated 1 h at 37 °C. Afterwards, wells were incubated with 100 µg/well of purified Cup s 1 (2000-2 ng/ml), or with the C. sempervirens pollen extracts (10000-25 ng/ml) in PBS-B-T, followed by a second incubation with streptavidin-peroxidase (0.25 µg/ml) (Sigma). All the incubations were carried out at 37 °C during 1 h, and with 3 washes of 200 µg/well of PBS-T between incubations. Color development was performed at room temperature and in darkness with a solution of o-phenylenediamine dihydrochloride Tablet Sets, Sigma). The reaction was stopped at 30 min with 50 µl/well of 3 M H₂SO₄, and the optical density was measured at 492 nm. Detection limit was calculated as the amount of Cup s 1 which corresponded to the mean plus 3.3 times the standard deviation obtained after 30 measurements of the zero standard. Intrasay variation coefficients were calculated measuring the absorbance of 20 wells at concentrations of 31.25-62.5-125-250 ng/ml. Intrasay variation coefficients were determined in 10 standard curves carried out with Cup s 1 in different days. The extracts were assayed in triplicate and the concentrations were interpolated in the lineal portion of the standard curve.

RESULTS

In the Cup s 1 purification, the pollen was subjected to a quick wash with low ionic content and acidic pH buffer, followed by an extraction at basic pH with the purpose of eliminating most of the non-protein components. The obtained supernatant was supplemented with 2 M (NH₄)₂SO₄ with the purpose of promoting the protein binding to phenyl-sepharose resin. After washing, the bound fraction was eluted with phosphate buffer, and contained a high concentration of proteins with low amounts of carbohydrates (fig. 1A). Further purification was obtained by gel filtration chromatography where Cup s 1 was contained in the 40-50 kDa peak (fig. 1B). This frac-
tion was subjected to a last purification step in a Smart System using a cation exchange column. Cup s 1 was eluted with 100 mM NaCl as a 43 kDa band while the rest of proteins come out in the non-bound fraction at pH 5.0 (fig. 1C). The purification yield was 1.5 % of the total extract proteins.

The identification of the purified protein as Cup s 1 was proven by immunoblotting with specific antibodies, as well as for its pectate lyase activity. Cup s 1 possesses the capacity to degrade pectin with a specific activity of 725 U/mg protein, and this activity is also completely dependent of calcium (fig. 1D). This is the first time it has been demonstrated that Cup s 1 is a functional active pectate lyase enzyme.

For the quantification of Cup s 1 in the two-site sandwich ELISA, the mAb 3D2 was used as capture
The extract dose-response curves showed a good parallelism when compared with the obtained with the purified allergen, indicating that the same protein was measured (fig. 3). The Cup s 1 standard curve was applied to the quantification of Cup s 1-like proteins in T. plicata and J. communis pollen extracts, with an allergen content of 75 and 50 \( \mu g/\text{mg} \) of protein, respectively. Nevertheless, for accurate quantification, the corresponding purified group 1 allergen for each extract should be used as reference in the dose-response curve. The specificity of the assay was tested using extracts of non-plant origin (Alternaria alternata and Dermatophagoides pteronyssinus), and pollen extracts from Olea europaea, Platanus acerifolia, C. sempervirens, C. arizonica, T. plicata, J. communis, P. radiata, and P. vulgaris. No reactivity was detected between 0.25 and 25 \( \mu g/ml \) of protein in any case.

The extract assay was used to quantify the Cup s 1 content in different batches of C. sempervirens pollen extracts, ranging from 73 to 95 \( \mu g/\text{mg} \) of protein.

<table>
<thead>
<tr>
<th>Capture</th>
<th>Detection</th>
<th>Linear portion of the standard curve</th>
<th>Detection limit</th>
<th>Intrassay CV (31.25-250 ng/ml)</th>
<th>Interassay CV (31.25-250 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 3D2</td>
<td>Biotinylated polyclonal antibody (0.31 ng/ml)</td>
<td>31.25-250 ng/ml</td>
<td>3.8 ng/ml</td>
<td>( y = 1.382 + 0.447x )</td>
<td>( R^2 = 0.992 )</td>
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<td></td>
<td></td>
<td>( r = 0.952 (P = 10) )</td>
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<td>(&lt; 8.1 % (n = 20))</td>
<td>(&lt; 16 % (n = 10))</td>
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Figure 2.—Coomassie stained SDS-PAGE (A) and immunoblot incubated with mAb 3D2 (B) or the polyclonal antibody (C) of purified Cup s 1 lane 1, of pollen extracts from C. sempervirens lane 2, C. arizonica lane 3, T. plicata lane 4, J. communis lane 5 and P. radiata lane 6.
The allergenic extract standardization is usually carried out by means of in vitro techniques using allergic patient sera, which are difficult to homogenize. In the case of Cupressaceae allergy, the finding of good sera become more difficult since these patients have low levels of specific IgE to cypress extracts\(^4\). Therefore, the development of an immunoassay for the quantification of Cup s 1 is of great interest as a complementary tool for standardization. The success in the development of this type of procedures is closely linked to the existence of specific antibodies and the availability of a high grade purity standard. The mAb 3D2, used as capture mAb, reacted with Cup s 1, Cup a 1 and a corresponding protein of T. plicata and J. communis pollen extracts. Therefore, it seems to detect a conserved epitope presented in Cupressaceae proteins and becomes a useful tool in the immunoassays for the quantification of major allergen within the Cupressaceae family. Nevertheless, the corresponding purified allergen for each extract should be used for an accurate quantification of the allergen content expressed in mass units. The use of polyclonal antibodies as tracer did not diminish the assay specificity, since this second antibody did not show reaction to any other proteins, even from Pinus or Cedrus extracts, plants from the Pinaceae family included, as the Cupressaceae family, inside the Coniferal order. Using the described assay, allergen concentration of 7.3, 7.5 and 5 % for pollen extracts of C. sempervirens, T. plicata and J. communis pollen seems to be more compatible with data of protein yield after purification and immunoblotting than allergen concentration of 0.5, 1.2, and 1.6 % obtained previously using a sandwich ELISA based on two mAbs\(^\text{10,11}\).

The ELISA could be applicable to the routine analyses of cypress extracts used to specific immunotherapy and diagnostic, given the high sensitivity and specificity requirements, was also fulfilled in the two-site sandwich ELISA, with acceptable intraassay and interassay variation coefficients.

In conclusion, this paper describes a two-site sandwich ELISA procedure for measurement of Cup s 1 in C. sempervirens pollen extracts. This assay results useful due to its specificity, sensitivity and reproducibility, and it can be applicable to the quantification of the other Cupressaceae major allergens.

ACKNOWLEDGMENTS
This work was supported in part by Bial-Aristegui and by Grants FIT-090000-2003-61 from the Plan Nacional de I + D (Programa PRQFIT, Ministerio de...
Ciencia y Tecnología, Spain), and TEI-0163-2002 from the Programa INTEK (Departamento de Industria, Agricultura y Pesca, Gobierno Vasco).

REFERENCES