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 antiserum 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 intra-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.


ORIGINAL ARTICLES

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


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 hidrofóbica, tamizado molecular, y intercambio iónico, y se analizó su actividad enzimática. El 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-
pacifico contra Cup s 1 marcado con biotina unido a estreptavidina conjugada con la enzima peroxidasa sirvieron como detectores del alergeno unido.

Resultados: Se determinó que Cup s 1 es una enzima funcional con actividad pectiato liasa y una actividad específica de 750 U/mg. La parte lineal de la curva estándar del ELISA fue considerada la zona óptima de ensayo y se situaba entre 250 y 31,25 ng/ml, siendo en menor que en otros polen alérgicos, y del 16 % respectivamente. Además de reproducible, el ensayo resultó sensible con un límite de detección de 3,8 ng/ml. Las curvas obtenidas con los extractos de C. sempervirens y otros extractos de polen de géneros de la familia Cupressaceae fueron paralelas a la del alergeno purificado indicando que se estaba midiendo la misma proteína.

Conclusiones: Se describe un ensayo que resulta específico y de gran sensibilidad para la cuantificación de Cup s 1 en extractos de polen de C. sempervirens de uso clínico, y que puede ser aplicable a otros géneros de la familia Cupressaceae.


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 polinosis cases.1-3

Extensive cross-reactivity of the allergens has been demonstrated between different Cupressus and Juniperus species.5-6 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 present different clinical characteristics to other pollinosis; the patients usually have low levels of specific IgE and they develop symptoms older than in other polinosis.6-7

As a consequence of these low levels of specific IgE in pollen, the in vitro inhibition studies used at this moment for the standardization of allergenic extracts are difficult to carry out. Moreover, Cupressacae family pollen extracts are characterized to have a high content in carbohydrates and a low protein content (3 %), 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 that has been recently purified and cloned (EBI accession number AF7257491). sharing a high homology with Cup a 1. Recently, a mAb-based ELISA has been developed in our lab for quantification of Cup s 1, 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 7.0 containing 65 mM NaCl. The obtained extracts were clarified by centrifugation to 5000 xg for 30 min, filtered through AP glass fiber prefilter (Millipore Corp., Bedford, Madison, USA) and dialyzed by ultrafiltration at 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 KH2PO4 pH 4.3, followed by an extraction with 40 mM NH4HCO3 at 4 °C.2 M (NH4)2SO4 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 (NH4)2SO4 in the AKTA-prime System (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 1.6x5 ion exchange column into a Smart System (Amersham Biosciences). The purified al-

Allergol et Immunopathol 2004;32:6:319-25 18
The proteins were transferred to Hybond-P and recognized by the major allergen of different Cupressaceae, has already been described previously. The major allergen 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, Bush, Switzerland), 50 mM Tris (pH 8.5), 5 mM CaCl$_2$, and 5 µg of purified Cup s 1 in 1 ml of total volume. The reaction was carried out at 37 °C for 1 hour, 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 Cupressaceae, 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 by affinity chromatography in HiTrap Protein G beads (Amersham Biosciences). 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 by affinity chromatography in HiTrap Protein G resin. After washing, the bound fraction was eluted at 37 °C during 1 h, and with 3 washes of 200 µl/well of PBS-T between incubations. Color development was performed at room temperature and in darkness with a solution of o-phenylenediamine (Sigma-Fast 3-Diaminobenzidine Tablet Sets, Sigma). The reaction was stopped at 30 min with 50 µl/well of 3 M H$_2$SO$_4$, 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 3.33 times the standard deviation obtained after 30 measurements of the zero standard. Intraassay variation coefficients were calculated measuring the absorbance of 20 wells at concentrations of 31.25-62.5-125-250 ng/ml. Interassay 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 linear portion of the standard curve.

### Electrophoresis and Immunoblotting

The protein bands of the pollen extracts and of Cup s 1 were detected with Coomassie brilliant blue after being separated by SDS-PAGE at 12.5 % acrylamide under reducing conditions according to Laemmli method. For the immunodetection assay, the proteins were transferred to Hybond-P membranes (Amersham Biosciences) and blocked for 1 h at room temperature with PBS containing 0.1 % Tween-20. Next, the membranes were incubated under similar conditions with mAb 3D2 (0.5 µg/ml) or with anti-Cup s 1 rabbit antiserum (diluted 1:50,000). After washing, blots were incubated with the corresponding peroxidase-labeled secondary antibody and developed by the ECL western blotting detection system (Amersham Biosciences).

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

Microwell plates (Greiner, Frickenhausen, Germany) were coated overnight at room temperature with 100 µl/well of the mAb 3D2 at 5 µg/ml in PBS. Next, plates were blocked by adding 200 µl/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 µl/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 biotin-labeled anti-Cup s 1 rabbit polyclonal antibody (0.31 µg/ml) and finally 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 µl/well of PBS-T between incubations. Color development was performed at room temperature and in darkness with a solution of o-phenylenediamine (Sigma-Fast o-phenylenediamine dihydrochloride Tablet Sets, Sigma). The reaction was stopped at 30 min with 50 µl/well of 3 M H$_2$SO$_4$, and the optical density was measured at 492 nm. Intra- and interassay variation coefficients were calculated measuring the absorbance of 20 wells at concentrations of 31.25-62.5-125-250 ng/ml. Interassay 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 linear 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 (NH$_4$)$_2$SO$_4$ 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/μg/g de 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, Betula verrucosa, Pinus radiata, Cedrus atlantica, Dactylis glomerata, Helianthus annuus, Parietaria judaica, Artemisia vulgaris and Plantago lanceolata. No reactivity was detected between 0.25 and 25/μg/ml of protein in any case.

and the biotin-labeled anti-Cup a 1 polyclonal antibody as detector. Both were specific to a 43 kDa protein presented in pollen extracts from Cupressaceae family (Cupressus, Thuja, Juniperus), but these antibodies did not react with proteins from other taxonomically related pollen extracts (Pinus and Cedrus) (fig. 2). Purified Cup s 1 was used as reference material in a standard curve with concentrations between 2000 and 2 ng/ml. The working range of the assay was defined as the lineal portion of the curve, between 250 and 31.25 ng/ml. The detection limit was calculated as 3.8 ng/ml. The assay was reproducible, since the intrassay variation coefficients at 250, 125, 62.5, and 31.25 ng/ml were of 7.6, 7.0, 8.1, and 6.7 %, respectively. The interassay variation coefficients for the same points were always minor than 16 % (table I).

This assay was used to quantify the Cup s 1 content in different batches of C. sempervirens pollen extracts, ranging from 73 to 95/μg/g de protein.

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 μg/g de 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, Betula verrucosa, Pinus radiata, Cedrus atlantica, Dactylis glomerata, Helianthus annuus, Parietaria judaica, Artemisia vulgaris and Plantago lanceolata. No reactivity was detected between 0.25 and 25 μg/ml of protein in any case.

<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 302</td>
<td>Biotinylated polyclonal antibody (0.31 μg/ml)</td>
<td>31.25-250 ng/ml</td>
<td>3.8 ng/ml</td>
<td>&lt; 8.1 % (n = 20)</td>
<td>&lt; 16 % (n = 10)</td>
</tr>
</tbody>
</table>

Table I
Two-site sandwich ELISA features for the quantification Cup s 1
DISCUSSION

The cypress pollen extracts are characterized by their high carbohydrate and low protein content, due to the physical-chemical properties of the pollen grains. Efforts have been made to establish the optimal conditions for pollen extraction and to provide qualitative definition of the relevant allergenic components in these extracts.

The *C. sempervirens* major allergen purification was carried out by the same method than the one described for *Cup a 1* [11,17], but including an additional step based on a cation exchange chromatography at pH 5.0. This step purified the protein from the low molecular weight proteins present in the *C. sempervirens* extract. *Cup s 1* had more than 98% purity and was used as standard in the two-site sandwich ELISA. The allergen had high homology with other cypress group 1 allergens (*Cry j 1, Jun a 1, Cha o 1, Cup a 1*) ranging from 80% identical amino acids with *Cry j 1* to 96% with *Jun a 1*. Cypress group 1 allergens have been described as enzymes with pectate lyase activity dependent of Ca$^{2+}$ ion, but functional enzymatic activity have been only demonstrated for *Cry j 1* and *Cup a 1* [11,17]. Here, we have shown that purified *Cup s 1* has pectate lyase activity with a specific activity of 725 U/mg protein, comparable to ones described for *Cup a 1* (1200 U/mg) and *Cry j 1* (350 U/mg) [11,17].

The allergic 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. 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 [17].

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 FT-090000-2063-61 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

10. Arilla MC, Barris-I, Garcia R, de la Hoz B, Martinez A, Asturias JA. Quantification of the major allergen from cypress (Cupressus arizonica) pollen, Cup a 1, by monoclonal antibody-based ELISA. Int Arch Allergy Immunol 2004;134:10-6.

23

Allergol et Immunopathol 2004;32(6):319-25