

STUDIES ON THE RELATIONSHIP BETWEEN STRUCTURE AND IgE-BINDING ABILITY OF *PARIETARIA JUDAICA* ALLERGEN I*

F. POLO,† R. AYUSO and J. CARREIRA

Dpto Investigación. Alergia e Inmunología Abelló S.A. C/ Miguel Fleita, 19, 28037 Madrid, Spain

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Abstract—The main allergen of *Parietaria judaica* pollen, *Par j* I, is a glycopolypeptide with mol. wt about 10,000. It shows a considerable charge heterogeneity which is mostly due to the carbohydrate prosthetic groups, since treatment with trifluoromethanesulfonic acid yielded a deglycosylated protein with 8500 mol. wt that displayed only a few bands on IEF, in a narrow pH-region around 5.0. Deglycosylated *Par j* I exhibited a specific allergenic activity slightly lower than that of native *Par j* I; however, no allergenic determinants should be located on the sugar moiety since both native and deglycosylated *Par j* I inhibited up to a similar extent the binding of specific human IgE to *P. judaica*-coated wells in ELISA. The decrease of specific allergenic activity following deglycosylation could be ascribed to conformational changes evidenced by CD experiments. On the other hand, fluorescence spectroscopy showed that *Par j* I bears unidentified yellow-brown chromophores strongly linked to the polypeptide chain. These chromophores were not removed by TFMS treatment. Finally, reduction and alkylation caused the complete loss of allergenic activity, showing that disulphide bridges are essential for the IgE-binding ability of *Par j* I.

INTRODUCTION

Pollen of *Parietaria judaica* is known to be one of the main causes of allergic diseases in Southern and Western Europe (Corbí and Carreira, 1984). The major allergen of this pollen, *Par j* I (Ayuso *et al.*, 1988), is a glycopeptide with about 10,000 mol. wt and shows considerable charge heterogeneity. Recently, we have reported a two-step chromatographic procedure which permitted the isolation of *Par j* I in an allergenic active state and with a good yield (Polo *et al.*, 1990).

During the last years a number of naturally occurring allergens have been identified and isolated (Marsh and Norman, 1988). Nevertheless, few studies have been performed to characterize the structural features that are essential for their IgE-binding ability. For instance, many allergens have been demonstrated to be glycoproteins, but the importance of the carbohydrate moiety for the properties of such allergens has been studied only in a few cases, such as Ag-54 allergen from *Cladosporium herbarum* (Sward-Nordmo *et al.*, 1988), Ag-7 from *Artemisia vulgaris*

(Nielsen and Smestad-Paulsen, 1986), and the major feline allergen *Fel d* I (Duffort *et al.*, 1990).

Thus the aim of the present work has been to elucidate the relationship between some structural characteristics of *Par j* I and its IgE-binding ability, mainly, the relevance of the sugar moiety and the disulfide bridges.

MATERIALS AND METHODS

Preparation of the pollen extract

Parietaria judaica pollen (78% purity, <0.5% foreign pollen) was purchased from Allergon AB (Sweden), and the extract was prepared as previously described (Polo *et al.*, 1990).

Obtention of purified Par j I allergen

Purification of *Par j* I from the whole pollen extract was performed according to the previously described procedure (Polo *et al.*, 1990). Briefly, the pollen extract was dissolved in 0.1% aqueous trifluoroacetic acid, injected into a gel filtration HPLC column (Beckman Spherogel TSKG 3000 SW, 30 × 2.1 cm, 13 µm particle size, 250 Å pore size) and eluted with the same solvent at a constant flow rate of 1 ml/min. The fractions containing *Par j* I were pooled and applied onto a reverse-phase HPLC column (Vydac C4, 25 × 1.0 cm, 10 µm particle size, 300 Å pore size). Elution was performed with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a constant flow rate of 1 ml/min. Fractions containing pure *Par j* I were pooled, dialyzed against distilled water and lyophilized.

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†To whom correspondence should be addressed.

Abbreviations: TFMS, trifluoromethanesulfonic acid; 2-ME, 2-mercaptoethanol; GF-HPLC, gel filtration high-performance liquid chromatography; CD, circular dichroism; PAS, periodic acid-Schiff; BCA, 2,2'-bicinchoninic acid.

Deglycosylation of *Par j I*

Par j I was deglycosylated by trifluoromethanesulfonic acid (TFMS) as reported (Sojar and Bahl, 1987). Concisely, 1 mg of protein was dried under vacuum overnight at room temp, and then treated with 150 μ l of anhydrous TFMS. The reaction mixture was incubated at 0°C for 2 hr under nitrogen with occasional shaking. Afterwards, the reaction mixture was neutralized by gradual addition of 400 μ l 60% pyridine in water at -20°C, then dialyzed in a Spectrapor dialysis tubing (Spectrum Medical Inc., mol. wt cutoff 3500) against 0.01% NH_4HCO_3 , and finally against water. Deglycosylated protein was purified by gel filtration HPLC on the Spherogel TSKG 3000 SW column already described, using 0.1 M Na_2SO_4 , 0.02 M NaH_2PO_4 buffer, pH 6.8 as the mobile phase, at a constant flow rate of 1 ml/min. The column had been previously calibrated by injection of a Gel Filtration Standard from Bio-Rad containing thyroglobulin (670,000), gamma globulin (158,000), ovalbumin (44,000) and myoglobin (17,000). The fractions containing deglycosylated *Par j I* were pooled, dialyzed against water and lyophilized.

Reductive cleavage and alkylation of *Par j I*

Par j I (500 μ g) was dissolved in 500 μ l 0.5 M Tris-acetate buffer pH 8.2 containing 7 M guanidine and 2 mM EDTA. Reduction was carried out with 2-ME (final concn 75 mM) for 2 hr at 40°C. Radio-alkylation was accomplished by addition of 10 μ Ci of ^{14}C -iodoacetamide (24.1 mCi/mmol, Dupont, Boston, MA) to the mixture and incubation for 15 min at room temp in the dark. Unlabelled iodoacetamide was then added to a final concn of 150 mM and the reaction mixture was incubated for 2 additional hours. Excess reagents were removed by dialysis against 0.1 M Na_2SO_4 , 0.02 M NaH_2PO_4 buffer, pH 6.8, and the sample injected on a Spherogel TSKG 3000 SW column and eluted with the same buffer at a constant flow rate of 1 ml/min. One ml fractions were collected, and 100 μ l aliquots of each fraction were mixed with liquid scintillation cocktail (Aquasol 2[®], Dupont) and counted in a Packard Tri-Carb 300C System. Finally, the fractions containing reduced and alkylated allergen were pooled, dialyzed against water and lyophilized.

Protein quantitation

Protein content was estimated by the Folin method (Lowry *et al.*, 1951).

Carbohydrate analysis

Two different photometric assays were employed to determine the carbohydrate content of *Par j I* preparations: one of them based on the PAS stain (Mantle and Allen, 1978), using fetuin and ovalbumin as standards; and the other, based on the assay of reducing sugars, obtained by hydrolysis of

glycoproteins, with BCA (Waffenschmidt and Jaenicke, 1987), using D-glucose as a standard.

Allergenic activity evaluation

The allergenic activity of *Par j I* preparations was evaluated by a competitive inhibition ELISA, as previously reported (Polo *et al.*, 1990). Briefly, plastic microtiter wells (Dynatech type II) were coated with 11 μ g per well of *P. judaica* pollen antigens in 0.1 M sodium carbonate buffer, pH 9.6, at 4°C overnight. After blockage of the unoccupied sites with BSA and washing with PBS containing 0.5% Tween 20, the adsorbed antigens were made to react with a mixture of 50 μ l of a *P. judaica*-specific human serum pool (diluted 1:3 with PBS) and 50 μ l of serial dilutions of the samples to be tested, for 2 hr at room temp. Afterwards the plates were washed and incubated with 100 μ l per well of a solution of a mouse anti-human IgE monoclonal antibody, conjugated with β -galactosidase (Hamlet[®], Alergia e Inmunologia Abelló S.A.) for 2 hr at room temp. After washing, 100 μ l of a solution of *o*-nitrophenyl- β -D-galactopyranoside were added per well and allowed to react for 2 hr at 37°C. The colour reaction was stopped with 0.1 M sodium carbonate solution, and the absorbance measured at 405 and 620 nm in an automatic microplate reader.

Electrophoretical procedures

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed with an 8–25% acrylamide gradient in a high-molarity Tris buffer system (Fling and Gregerson, 1986). The following standards were used as mol. wt markers: phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400), and myoglobin (fragment I) (8200).

Protein detection on the gels was done by Coomassie Blue staining. Glycoproteins were detected by a periodic acid-Schiff (PAS) procedure (Zacharius *et al.*, 1969).

Analytical isoelectric focusing (IEF) was performed on a FMC Isogel[®] agarose plate, pH gradient from 3 to 10, according to the manufacturer's instructions. Isogel[®] pI markers were run on the same gel. Transfer of proteins from Isogel agarose plates to a Tris-saline buffer-soaked nitrocellulose membrane was carried out by press blot for 5 min. Further immunodetection of IgE-binding proteins with *P. judaica*-sensitive human serum pool and Hamlet[®]- ^{125}I (Alergia e Inmunología Abelló S.A.), and iodinated mouse anti-human IgE monoclonal antibody (Sánchez-Madrid *et al.*, 1984), was performed as reported (Corbi and Carreira, 1984).

HPLC equipment

All the chromatographic procedures were carried out on HPLC equipment from Waters comprising a

Table 1. Determination of the carbohydrate content of *Par j* I before and after TFMS treatment

Protein	Percentage sugar	
	BCA method ^a	PAS method ^b
Native <i>Par j</i> I	18	15
Deglycosylated <i>Par j</i> I	<2	Undetectable

^aAccording to Waffenschmidt and Jaenicke (1987), 150 µg samples.^bAccording to Mantle and Allen (1978), 300 µg samples.

Model 680 gradient programmer, two 510 pumps, a U6K injector, and a 490 multiwavelength u.v./visible detector set at 214 nm.

Fluorescence spectroscopy

The fluorescence spectra of native and deglycosylated *Par j* I were performed on a Fica 55 MK II spectrofluorometer at 25°C, in 1 cm optical-path cells. Protein solutions (0.5 mg/ml in water) were filtered through a Millipore filter (0.45 µm pore diameter) prior to the spectroscopic determination. For emission spectra, 2.5- and 7.5-nm slit widths were employed for the excitation and emission beams, respectively.

Circular dichroism spectra

CD spectra in the far-UV region were measured in a Roussel-Jouan 185 dichrograph II fitted with a xenon arc lamp at 0.125 nm/s scanning speed. The 10^{-5} ΔE/mm sensitivity scale was used, together with time constants of 4 s. Filtered solutions of proteins (0.5 mg/ml in 10 mM sodium phosphate buffer, pH 7.0) were studied at 22°C, in 0.1 cm optical-path cells. The results are given in terms of mean residue ellipticities (θ mrw), in units of dg cm² dmol⁻¹. These values were calculated on the basis of 108 as the mean residue weight in *Par j* I. Estimates of structural

content were made according to the method of Greenfield and Fasman (1969).

RESULTS

Deglycosylation of *Par j* I

Chemical treatment of native allergen *Par j* I with TFMS resulted in an extensive deglycosylation of the protein. In fact, a negligible amount of sugar was found for the deglycosylated protein using different carbohydrate determination methods (Table 1) and, in addition, it was not stained for carbohydrates on polyacrylamide gels by PAS technique (Fig. 1 lane d). Besides, SDS-PAGE analysis (Fig. 1) showed a reduction in apparent relative molecular mass (from about 10,500 to 8500) similar to the carbohydrate content of native *Par j* I.

In contrast with the SDS-PAGE results, deglycosylated *Par j* I showed an apparent mol. wt greater than that of the native protein (21,000 vs 13,000) when subjected to gel filtration HPLC under non-denaturing conditions (Fig. 2). Evidences for a conformational change following deglycosylation were

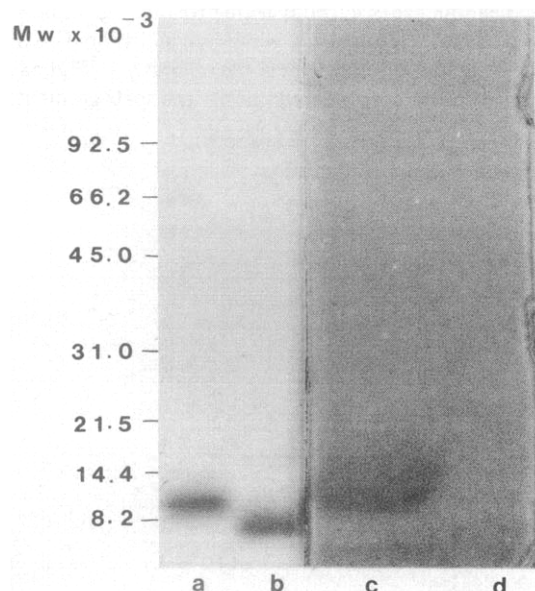


Fig. 1. SDS-PAGE of native (a, c) and TFMS treated (b, d) *Par j* I. (a, b) Coomassie Brilliant Blue staining. (c, d) PAS staining. Protein (15 µg) was applied per lane in a and b, and 40 µg in c and d.

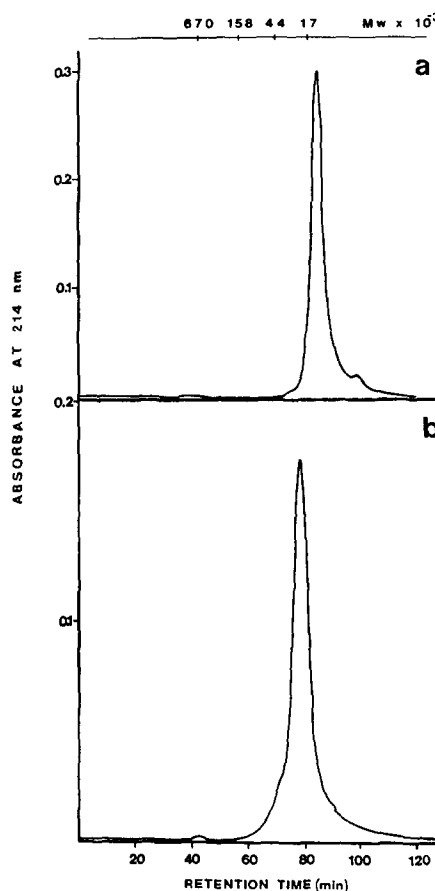


Fig. 2. Analysis of (a) native (250 µg) and (b) deglycosylated (150 µg) *Par j* I on a gel filtration HPLC Spherogel TSKG 3000 SW column. Elution was performed with 0.1 M Na₂SO₄, 0.02 M NaH₂PO₄ buffer, pH 6.8 at a constant flow rate of 1 ml/min. The retention time of the standard proteins are indicated at the top of the figure.

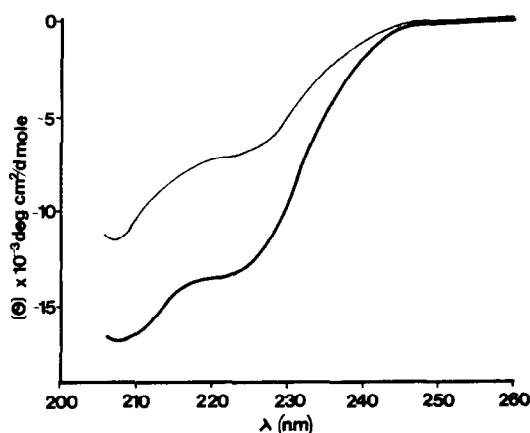


Fig. 3. Circular dichroism spectra of native (—) and deglycosylated (---) *Par j I* in the far UV.

Table 2. Secondary structure estimates for *Par j I* before and after TFMS treatment

<i>Par j I</i>	CD calculated structure ^a		
	% α Helix	β Structure	Random coil
Native	45	0	55
Deglycosylated	26	0	74

^aBased on the method of Greenfield and Fasman (1969): percentage α -helix is estimated by

$$\% \alpha \text{ helix} = \frac{[\theta]_{208} - 4000}{33,000 - 4000}$$

Percentage random coil is estimated to within 10% by comparison with calculated curves at 218 and 222 nm, and the remainder is taken as β structure.

revealed by CD spectra at 200–260 nm (Fig. 3). Estimation of secondary structure from these spectra (Table 2) indicated a significant content of α -helix (45%) for the native protein. It could also be concluded that part of this ordered structure turned into aperiodic conformation by TFMS treatment (26% α -helix for the deglycosylated protein).

The allergenic activity of *Par j I* after deglycosylation was evaluated by a competition ELISA. It

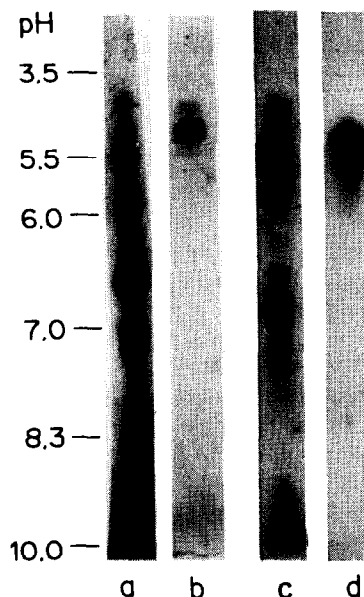


Fig. 5. Analytical IEF on an agarose gel. Fifty micrograms of native (a, c) or deglycosylated (b, d) *Par j I* was applied per lane. After IEF, proteins were transferred to nitrocellulose strips. (a, b) Coomassie Blue staining of the strips. (c, d) immunodetection with *P. judaica*-sensitive human serum and ¹²⁵I-anti IgE.

could be observed (Fig. 4) that the concns of deglycosylated *Par j I* required to reach the same inhibition levels as the native protein were about three-fold higher (1.5 ng vs 0.5 ng for 50% inhibition). However, both of them were able to inhibit the IgE binding to the same extent.

Charge heterogeneity of *Par j I*

The influence of carbohydrate prosthetic groups on the charge heterogeneity was studied by analytical IEF (Fig. 5). Thus, whereas native *Par j I* (Fig. 5a) yielded protein bands all along the pH gradient,

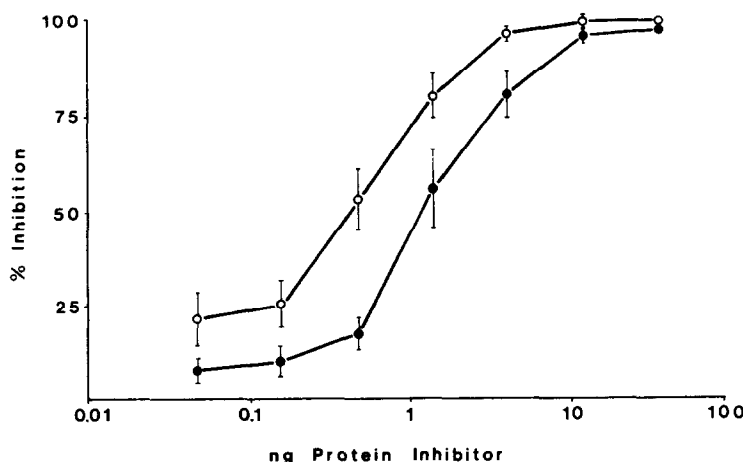


Fig. 4. Competition ELISA curves. The binding of specific human IgE to *P. judaica*-coated wells was inhibited using (—○—) native *Par j I*, and (—●—) deglycosylated *Par j I* as competitors. Each point represents the mean value of four different determinations.

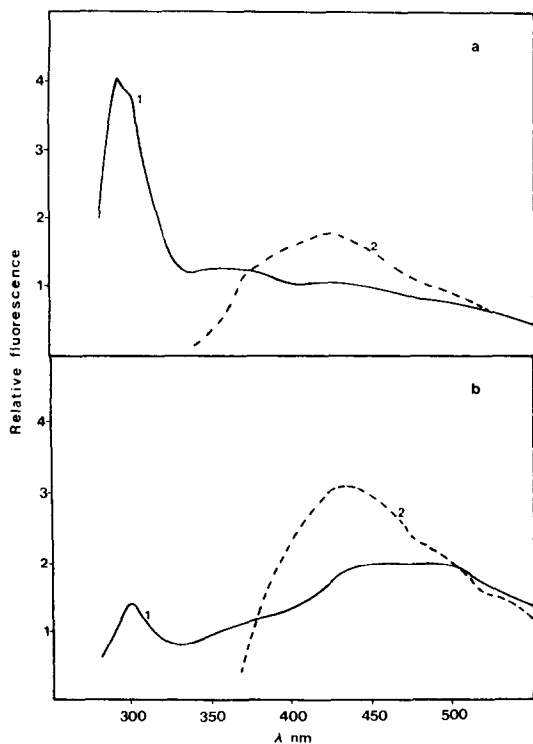


Fig. 6. Fluorescence spectra of (a) native and (b) deglycosylated *Par j* I. (1) Emission spectra for excitation at 280 nm. (2) Emission spectra for excitation at 335 nm. Fluorescence values are plotted in arbitrary units.

deglycosylated *Par j* I (Fig. 5b) displayed only a few sharp bands in a narrow pH-region around 5.0. The same protein patterns were visualized by immunodetection after IEF (Fig. 5c and d). This experiment also confirmed that *Par j* I retained its IgE-binding ability after the deglycosylation procedure.

Fluorescence spectroscopy of *Par j* I

The fluorescence spectra of native *Par j* I are given in Fig. 6a. Upon excitation at 280 nm, a maximum at 300 nm, that must correspond to the emission of the tyrosine residue found in the *Par j* I molecule by amino acid analysis (Polo *et al.*, 1990), and three small and broad bands centered around 370, 425 and 500 nm were observed. These latter maxima do not correspond to any known chromophore of proteins. No tryptophan emission was observed. The emission spectrum for excitation at 335 nm showed a broad band with a maximum at 425 nm and two shoulders at around 375 and 500 nm.

The results obtained for deglycosylated *Par j* I (Fig. 6b) were essentially the same as for the native protein concerning the maxima of the emission spectra. Then, it seems to be evident that TFMS treatment did not remove the chromophore groups. Nevertheless, some differences in the quantum yield of chromophores were observed, that could be due to conformational changes of the protein upon deglycosylation.

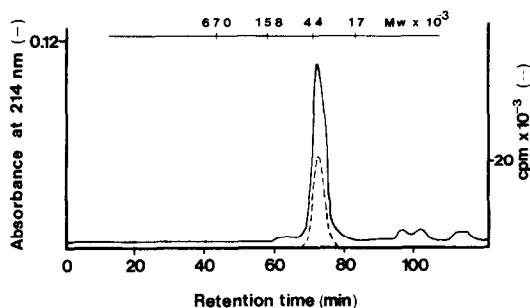


Fig. 7. Gel filtration HPLC of reduced and alkylated *Par j* I. The native allergen was reduced with 2-ME and treated with ^{14}C -iodoacetamide, as described in Materials and Methods. Protein (100 μg) was chromatographed in Spherogel TSKG 3000 SW column. The continuous line shows the absorbance chromatogram at 214 nm, and the dashed line the radioactivity chromatogram.

Reduction and alkylation of *Par j* I

Native *Par j* I allergen was reduced with 2-mercaptoethanol and, subsequently, free thiol groups were alkylated with ^{14}C -iodoacetamide. Gel filtration HPLC (Fig. 7) of the reaction mixture after dialysis showed that complete reduction and alkylation had been accomplished, since the radioactivity peak exactly tallied with the protein absorbance peak.

As well as for deglycosylated *Par j* I (Fig. 2b), the apparent mol. wt showed by reduced and alkylated *Par j* I in GF-HPLC was greater (43,000) than that of native *Par j* I (Fig. 7) what could be explained by the alteration of protein structure after cleavage of disulfide bridges (Withka *et al.*, 1987).

On the other hand, no radioactivity was incorporated into the protein when treatment with ^{14}C -iodoacetamide was carried out without previous reduction of disulfide bridges. This result evidenced that *Par j* I does not bear any free cysteine residue.

Reduction and alkylation caused the complete loss of the allergenic activity of *Par j* I when tested by competition ELISA. Thus, samples up to 0.5 mg/ml protein concn did not appreciably inhibit the binding of specific IgE to *P. judaica*-coated wells.

DISCUSSION

In this work, some structural features of *Par j* I allergen and their implications on the allergenic activity have been studied. Thus, the relevance of the carbohydrate moiety was investigated by deglycosylating the protein, and comparing the results obtained by several physicochemical and immunochemical techniques for both native and deglycosylated allergen.

Since the carbohydrate composition and the linkages to the polypeptide chain are not yet known, and some preliminary experiments of enzymatic deglycosylation were unsuccessful, a chemical deglycosylating agent (TFMS) was employed. This reagent cleaves both *N*- and *O*-linked saccharide structures in

glycoproteins without degrading the polypeptide backbone, besides being more suitable for functional studies than the enzymatic method, because of the greater efficiency and sensitivity of enzymes to the denatured rather than native glycoproteins (Sojar and Bahl, 1987).

In vitro evaluation of the allergenic activity of the deglycosylated protein (Fig. 4) indicated that no allergenic determinants must be located on the carbohydrate moiety of the glycoprotein, since deglycosylated *Par j* I was able to inhibit the specific IgE-binding up to the same extent as the native protein.

On the other hand, the fact that higher concns of deglycosylated *Par j* I were needed to reach inhibition levels similar to those of the native molecule implies a reduction of the affinity of some allergenic determinants to the specific IgE. This result could be explained by conformational changes of the protein, induced following the loss of the stabilizing effect of the carbohydrates residues attached to the polypeptide chain. The fact that conformational changes happened upon deglycosylation would be supported by the behavior of deglycosylated *Par j* I on GF-HPLC (Fig. 2); being that a change to a more extended structure would imply a lesser retention by the gel filtration matrix and, as a consequence, a greater apparent mol. wt (Withka *et al.*, 1987).

Furthermore, the spectral changes observed in CD (Fig. 3) confirmed the alteration of structure. However, the values of estimated percentages for the different structures (Table 2) have to be regarded with some caution because *Par j* I bears one or more unidentified chromophore substances that could contribute to CD spectra. The presence of these chromophore groups, that give the protein its yellow-brown color in solution, was confirmed by recording the fluorescence spectra of *Par j* I (Fig. 6a). This technique also demonstrated that the deglycosylation procedure did not remove the chromophore groups (Fig. 6b) and, consequently, only conformational changes must be responsible for the differences observed in CD spectra.

The deglycosylation experiment also proved that the previously reported charge heterogeneity of *Par j* I (Corbí and Carreira, 1985) is mainly due to the carbohydrate prosthetic groups, as seen from the drastic reduction of the number of bands on analytical IEF (Fig. 5). The microheterogeneity that still remains after deglycosylation could be brought about by the incomplete deglycosylation of the protein or, more likely, by the presence of varying amounts of the chromophores attached to the protein. The nature of these groups as well as the type of the linkage to the polypeptide chain and their contribution to the allergenic activity are unknown so far.

Cleavage of disulphide bridges in native *Par j* I and alkylation of the produced thiol groups caused the complete loss of the IgE-binding ability. This result strongly suggests that IgE-related epitopes

are conformational. Nevertheless, alternative explanations, such as the involvement of cystine residue in a sequential site or the burying of sequential epitopes in the interior of the protein as a result of conformational changes induced by reduction, cannot yet be completely ruled out.

A similar susceptibility to reduction and alkylation has been reported for some other allergens, such as ragweed allergen *Amb a* I (King, 1976), the Group I and Group II allergens from *Dermatophagoides sp.* (Lombardero *et al.*, 1990), and cat allergens *Fel d* I (Duffort *et al.*, 1990). This lability, together with the resistance to a number of proteases exhibited by native *Par j* I in some preliminary experiments anticipates important hindrances in the structural characterization of the IgE-binding sites. In this way, it will be probably needed the design and synthesis of peptides based upon molecular modelling studies to carry out such characterization (Atassi, 1978).

Additional structural information of *Par j* I that could be deduced from the results presented in this work were the lack of tryptophan (the emission fluorescence spectrum for excitation at 280 nm did not show any band around 340 nm), and of free cysteine residues (no incorporation of radioactivity was observed when native *Par j* I was treated with ¹⁴C-iodoacetamide without previous reduction).

In conclusion, it has been demonstrated that the binding sites for human IgE on *Par j* I do not contain carbohydrates although these seem to be important for the conformation of the glycoprotein and thereby influencing the affinity for IgE. Our results also showed that cystine residues are essential for the allergenic activity of *Par j* I.

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