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Assignment of Disulphide Bridges in Par j 2.0101, a Major Allergen of *Parietaria judaica* Pollen

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Par j 2.0101, a major allergen of the *Parietaria judaica* pollen, was expressed in *E. coli*, purified to homogeneity and fully characterised both at the structural and the functional level. The recombinant rPar j 2.0101 protein showed an allergenic activity in histamine release, skin prick tests and capacity to bind IgE, almost identical to that of the native allergens purified from aqueous pollen extract. The complete pattern of S-S bridges of rPar j 2.0101 was determined by enzymatic digestion with endoproteinase Lys-C followed by mass spectrometric analysis of the resulting peptide mixtures. The eight cysteines occurring in the allergenic protein were found to be paired into the following four disulphides: Cys35-Cys83, Cys45-Cys60, Cys61-Cys106 and Cys81-Cys121. This structural information probes Par j 2.0101 to attain a 3-D fold consistent with that of the non-specific lipid transfer protein (ns-LTP) family and it represents an effective molecular basis to develop modified antigens by selective site-directed mutagenesis for immunotherapy.

Key words: Allergen / Disulphide bridges / Mass spectrometry / Non-specific lipid transfer protein.

Introduction

Immunotherapy with allergen extracts, termed according to the World Health Organization (WHO) therapeutic allergy vaccines (Bousquet *et al.*, 1998), has been widely used since its introduction by Noon in 1911 (Noon, 1911). The clinical efficacy of these vaccines has been reported (Des Roches *et al.*, 1997; Jacobsen *et al.*, 1997; Bousquet *et al.*, 1998) even though the underlying immunological pathways are not yet completely defined. A reliable therapeutic allergy vaccine must fulfil the following criteria: (i) absence of symptomatic effects since the risk of IgE-mediated anaphylactic side effects increases with the amount of allergen injected and (ii) capability of interfering with the ongoing allergic disease by acting at the level of the helper T cells. As far as the safety of the vaccine is concerned, the anaphylactic reaction constitutes the most important and dangerous side effect that must be avoided by keeping the allergenic activity of the extracts close to zero. On exposure to a specific multivalent allergen, the IgE molecules fixed on basophils and mast cells are cross-linked by the allergen, leading to the release of inflammatory mediators (Stanworth, 1988). If the goal is to reach an allergenic activity close to zero, it is essential to modify the IgE epitopes shared by the allergenic molecule. Most of the IgE epitopes occurring on allergens are discontinuous, *i.e.* they consist of amino acid residues brought together by the folding of the polypeptide chain. Knowledge of the three-dimensional structure (3-D) of an allergen is then of the utmost importance in order to design the appropriate modifications to impair cross-linking of the cell fixed IgE.

The pollen of the weed *Parietaria judaica* (Pj) causes the most diffuse allergic reaction in the Mediterranean area, affecting approx. 10 million people (Colombo *et al.*, 1998). The pollen extract contains several allergens (Geraci *et al.*, 1985; Ford *et al.*, 1986) and two of them, Parj 1.0101 and Parj 2.0101, have already been cloned and sequenced (Costa *et al.*, 1994; Duro *et al.*, 1996). These two allergens show a certain degree of homology with the members of a protein family named non-specific lipid transfer proteins (ns-LTPs). A 3-D model for Par j 1.0101 has been proposed by homology modelling (Colombo *et al.*, 1998b), showing an overall α-α-α-α-β secondary structure arrangement with the four α-helices held together by four disulphide bonds. This model was supported by site-directed mutagenesis of Cys45 and Cys60 residues leading to the destruction of an immunodominant IgE epitope.

The design of site-directed mutagenesis experiments, based on the attempt to reduce IgE binding activity of the allergens, has been performed using several strategies, like the destruction of disulphide bonds. In addition, it has already been published that native allergenic proteins are often a mixture of closely related isoforms. In fact,

cDNA cloning described isoforms from different allergenic sources like *Ambrosia artemisiifolis*, *Poa pratensis*, *Phleum pratense*, the pan-allergen profilin and *Parietaria judaica* pollen (Rafnar *et al.*, 1991; Silvanovich *et al.*, 1991; Staiger *et al.*, 1993; Petersen *et al.*, 1994). For these reasons, we decided to perform the complete assignment of the disulphide bridge pattern of the Parj 2 major allergen using a recombinant isoform previously isolated (rPar j 2.0101) (EMBL accession number X95865). The allergenic activity of rParj 2.0101 was indistinguishable from the native protein in histamine release, skin prick tests and binding capability with IgE from Pj allergic patients. The pairing of the four disulphide bridges was elucidated by combining protein chemistry procedures with mass spectrometric methodologies and resulted in Cys35-Cys83, Cys45-Cys60, Cys61-Cys106 and Cys81-Cys121. This result fully validates the previously proposed 3-D model (Colombo *et al.*, 1998b) and allows the design of modified antigens that might be considered as the starting point towards a new and safe approach to immunotherapy.

^{32}
Met-Glu-Glu-Ala-Cys-Gly-Lys-Val-Val-Gln-
Asp-Ile-Met-Pro-Cys-Leu-His-Phe-Val-Lys-
Gly-Glu-Glu-Lys-Glu-Pro-Ser-Lys-Glu-Cys-
Cys-Ser-Gly-Thr-Lys-Lys-Leu-Ser-Glu-Glu-
Val-Lys-Thr-Thr-Glu-Gln-Lys-Arg-Glu-Ala-
Cys-Lys-Cys-Ile-Val-Arg-Ala-Thr-Lys-Gly-
Ile-Ser-Gly-Ile-Lys-Asn-Glu-Leu-Val-Ala-
Glu-Val-Pro-Arg-Lys-Cys-Asp-Ile-Lys-Thr-
Thr-Leu-Pro-Pro-Ile-Thr-Ala-Asp-Phe-Asp-
Cys-Ser-Lys-Ile-Gln-Ser-Thr-Ile-Phe^{129}

Fig. 1 Amino Acid Sequence of the rPar j 2.0101 Allergen Met(32–129).

Results and Discussion

Cloning and Expression of Recombinant Par j 2.0101

The recombinant form of the major allergen from *Parietaria judaica*, rParj 2.0101, was expressed in *E. coli* and fully characterised both at the structural and functional levels. The cDNA coding for the mature allergen lacking the signal peptide (sequence 32–129 plus the initial methionine as reported in Figure 1) was amplified by PCR and subcloned into the pET-11a plasmid. The recombinant protein was then expressed in *E. coli* BL21 (DE3) following induction with IPTG and after a single purification step a discrete amount of pure and water-soluble rPar j 2.0101 was obtained.

The molecular mass of HPLC-purified rPar j 2.0101 was measured by ESMS as 10957.4±0.9 Da, 28 Da higher than that expected on the basis of the predicted Met(32–129) rPar j 2.0101 amino acid sequence (10928.7 Da). The complete sequence of rParj 2.0101 was then verified by MALDI mass fingerprinting as shown in Figure 2. The structural discrepancy was located within the fragment 90–104 (measured mass value 1582.2 Da, see Table 1) and was eventually identified as Lys104→Arg (Δm=28.1 Da). This substitution might be related either to an error in the initial sequence or to a mutation induced by the PCR amplification procedure that escaped cDNA se-

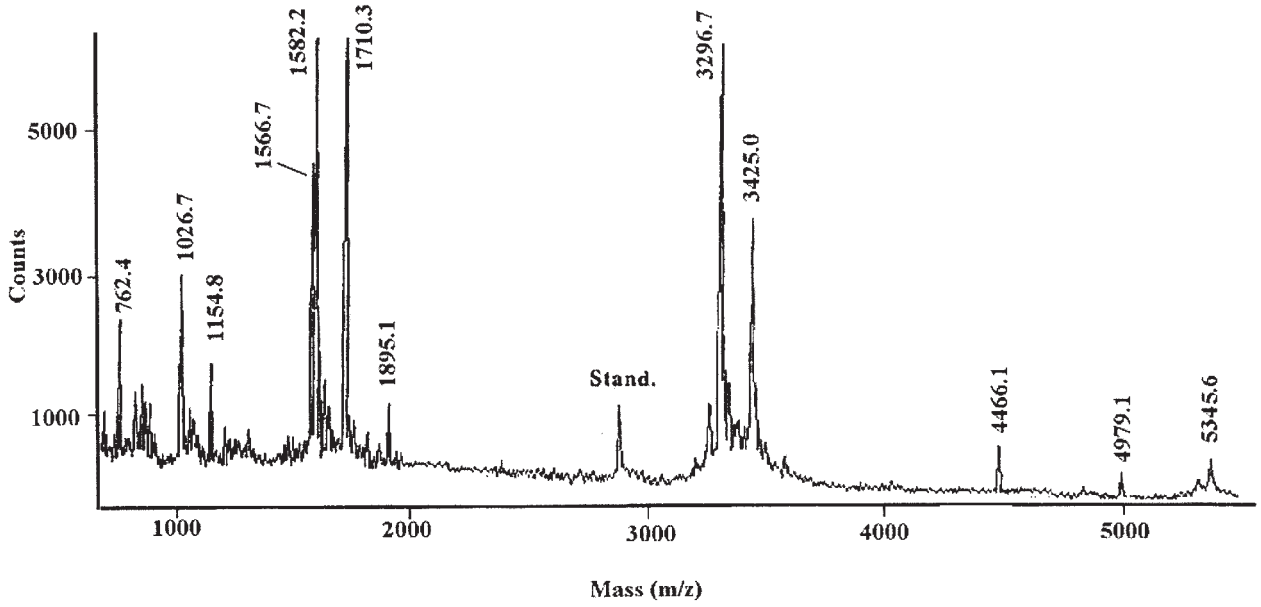

Fig. 2 MALDI-MS Spectrum of the Reduced and Alkylated rPar j 2.0101 Digested with Trypsin.
The signals recorded in the spectrum were assigned to the corresponding peptides within the protein sequence on the basis of their molecular masses. The interpretation of the mass values is reported in Table 1.

Table 1 MALDI-MS Analysis of the Reduced and Carboxymethylated rPar j 2.0101 Digested with Trypsin.

MH^+	Peptide	MH^+ (theor.)	Note
5345.6	38 – 82	5344.9	
4979.1	67 – 109	4950.9	Δm=28
4466.1	90 – 129	4438.2	Δm=28
3425.0	38 – 66	3425.6	
3296.7	38 – 65	3297.1	
1895.1	79 – 95	1894.7	
1710.3	90 – 105	1682.5	Δm=28
1582.2	90 – 104	1555.7	Δm=28
1566.7	110 – 123	1567.1	
1154.8	96 – 105	1126.3	Δm=28
1026.7	96 – 104	999.1	Δm=28
762.4	73 – 78	762.8	

Table 2 Histamine Release Activity of rPar j 2.0101.

Patients	Stimuli		
	Crude extract	rPar j 2.0101	aIgE
R.P.	14	30	18
G.C.	27	19	23
G.D.	42	49	33
V.M.	30	29	38
I.D.	26	9	35
D.G.	5	4	25

The concentration of rPar j 2.0101 and of the crude extract was 1 µg/ml; that of anti-human IgE was 10 µg/ml. D.G. represents a non-allergic subject. The percentage values of histamine release were calculated as follows:
% = (stimulus – spontaneous release) / (total release – spontaneous release) × 100.

Table 3 Biological Activity of rPar j 2.0101.

Patients	Intradermal skin test			
	Par j 2	W21	H	G
C.G.	4	6	6	1
C.A.	4	5	6	1
C.F.	5	7	8	0
D.D.	5	7	5	1
F.C.	4	6	8	0

rPar j 2.0101 was freshly dissolved in a 0.9% sterile NaCl solution (w/v) at concentrations of 10 µg/ml; W21: positive control; H: positive control (1 mg/ml histamine-HCl); G: negative control (glycerinated saline). The area of the wheal was read after 20 min, the largest diameter (D) and the diameter perpendicular to it (d) were measured in millimetres and the reaction was calculated as D+d/2.

quencing. Taking into account the substitution Lys→Arg, the measured molecular mass for the protein is in excellent agreement with the theoretical value.

rPar j 2.0101 Protein Binds IgE and Is Allergenically Active

The purified recombinant allergen was analysed for its capability to bind IgE. The nitrocellulose-blotted allergen was tested for IgE binding by using sera from 60 patients allergic to the Pj pollen while 5 sera from non-allergic subjects were used as control. All the sera from allergic patients (100%) showed a specific IgE-binding property, whereas the control sera gave negative results. The allergenic activity of rPar j 2.0101 was further analysed in the histamine release assay by using basophils from 5 out of the 60 Pj allergic patients. The results reported in Table 2 show that the recombinant allergen is capable of inducing degranulation and release of histamine from the basophils of all the allergic patients tested. The negative value obtained with basophils from a non-allergic subject (D.G.) proved that the histamine release observed was not due to a non-specific toxic effect.

Similar results were obtained when the allergenic activity of rPar j 2.0101 was tested by skin prick test (SPT) on the forearms of 5 patients allergic to the Pj pollen. Table 3 reports the activity of the allergen; no wheal was observed when the SPT was performed on the forearms of a non-allergic subject, confirming that the recombinant allergen was not toxic. The glycerinated saline solution used as control gave no positive reaction, while the histamine solution provided a positive wheal reaction in all the 5 subjects analysed.

rPar j 2.0101 Is Immunologically Related to the Wild-Type Allergen

The immunological correlation between rPar j 2.0101 and the wild-type allergen was analysed by CIE and CLIE tests. A rabbit polyclonal antiserum, prepared by injecting the recombinant Parj2 allergen, was used against a total crude extract of Pj pollen. Immunoprecipitate arcs were obtained (Figure 3), demonstrating an antigenic correlation between the recombinant allergen and the native Parj2 allergen contained in the crude extract. The presence of several arcs may be explained by the presence of related native Parj2 isoforms (G. Duro, unpublished results) and an immunological cross-reactivity among different Pj allergens (Colombo *et al.*, 1998b). The latter conclusion is supported by the homology at the nucleotide level already reported for at least two of the Pj allergens (Baud *et al.*, 1993; Duro *et al.*, 1996). The result of the CLIE test (Figure 3), performed by addition of the rPar j

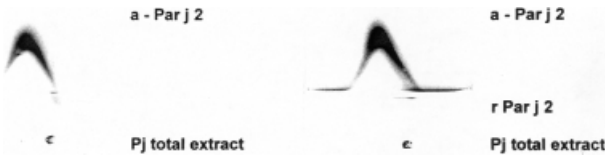

Fig. 3 CIE and CLIE Tests Demonstrating the Immunological Correlation between the rPar j 2.0101 Allergen and the Native Protein.

2.0101 to the intermediate gel, supports the specificity of the analysis.

Assignment of the S-S Bridge Pattern

The complete pattern of S-S bridges in rPar j 2.0101 was established by enzymatic digestion of the native protein with endoproteinase Lys-C followed by MALDI mass spectrometry analysis of the resulting peptide mixture. Figure 4 shows the MALDI mass spectrum of the Lys-C digest where each mass signal was assigned to the corresponding peptide within the rPar j 2.0101 sequence on the basis of its mass value and the specificity of the enzyme. The results are summarised in Table 4.

Direct mass spectral analysis of the Lys-C digest led to the identification of two disulphides, Cys35-Cys83 and Cys81-Cys121. The two mass signals at m/z 2700.9 and 2113.0 were, in fact, assigned to the peptide 110 – 123

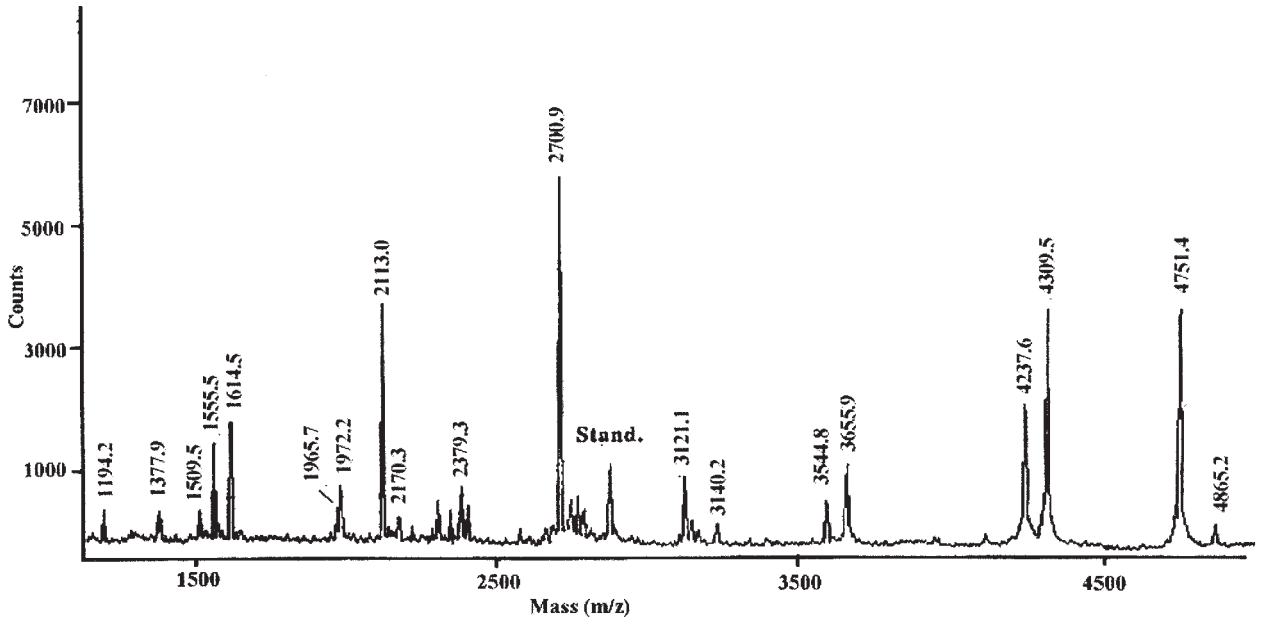

Fig. 4 Partial MALDI-MS Spectrum of Native rPar j 2.0101 Digested with Endoproteinase Lys-C.
Three signals in the spectrum could not be assigned to any linear fragment on the basis of the amino acid sequence only. The two mass signals at m/z 2700.9 and 2113.0 were, in fact, assigned to the peptide 110 – 123 linked to the fragments 73 – 82 and 78 – 82, respectively, by the S-S bridge involving Cys81 and Cys121. The signal at m/z 1555.5 was identified as originated by the peptide pair (31 – 37)+(83 – 89) joined by the cysteine pairing Cys35-Cys83.

Table 4 MALDI-MS Analysis of rPar j 2.0101 Digested with Endoproteinase LysC.

MH^+	MH^+ (theor.)	Peptide	Notes
4865.2	4864.7	79 – 123	Cys81,Cys83,Cys106,Cys121
4751.4	4751.5	(38 – 58)+(59 – 65)+(96 – 109)	Cys45,Cys60,Cys61,Cys106
4309.5	4310.1	(38 – 54)+(59 – 65)+(96 – 109)	Cys45,Cys60,Cys61,Cys106
4237.6	4238.1	(31 – 37)+(73 – 89)+(110 – 123)	Cys35,Cys81,Cys83,Cys121
3655.9	3656.1	(38 – 50)+(59 – 77)	Cys45,Cys60,Cys61
3544.8	3544.2	(38 – 50)+(59 – 72)+(106 – 109)	Cys45,Cys60,Cys61,Cys106
3140.2	3139.7	(38 – 50)+(51 – 65)	Cys45,Cys60,Cys61
3121.1	3121.9	38 – 65	Cys45,Cys60,Cys61
2700.9	2701.0	(73 – 82)+(110 – 123)	**Cys81-Cys121**
2379.3	2380.9	(38 – 50)+(59 – 65)	Cys45,Cys60,Cys61
2170.3	2170.6	90 – 109	
2113.0	2113.4	(78 – 82)+(110 – 123)	**Cys81-Cys121**
1972.2	1972.3	38 – 54	
1965.7	1966.3	73 – 89	
1614.5	1614.9	96 – 109	
1555.5	1555.9	(31 – 37)+(83 – 89)	**Cys35-Cys83**
1509.5	1509.7	110 – 123	
1376.5	1377.1	78 – 89	
1194.2	1194.3	73 – 82	

linked to the fragments 73–82 and 78–82 respectively, by the S-S bridge involving Cys81 and Cys121. The signal at m/z 1555.5 was identified as originating from the peptide pair (31–37) + (83–89), thus demonstrating the occurrence of a second disulphide bridge joining Cys35 and Cys83. These findings were confirmed both by reduction of the peptide mixture with DTT and by manual Edman degradation steps followed by MALDI-MS re-examination of the truncated peptide mixtures producing a shift of the mass signals to lower mass values due to the removal of the N-terminal residue from each peptide (Morris and Pucci., 1985). Moreover, in order to support these assignments, the peptide mixtures were fractionated by HPLC and all the disulphide-containing fractions were submitted to automated N-terminal degradation, thus confirming the assignments of the S-S bridges inferred by mass spectral data.

In the high mass region of the MALDI spectrum, the signals at m/z 4751.4 and 4309.5 were tentatively assigned to species containing three-peptide clusters, including fragments 59–65, 96–109 and 38–58 or 38–54, respectively, bridged by two disulphide bonds involving the remaining four cysteines, Cys45, Cys60, Cys61 and Cys106. The occurrence of these clusters was due to the presence of two juxtaposed cysteines at positions 60 and 61 that could not be separated by the enzymatic hydrolysis, leaving uncertainty as to whether Cys60 was linked to Cys45 and Cys61 to Cys 106 or *vice versa*. Therefore, on the basis of the mass value alone, these components could be associated with two isomeric structures differing for the cysteine pairings. The situation here was similar to that found in a number of cases previously reported (Poerio *et al.*, 1991; Orrù *et al.*, 1997). The correct assignment of the remaining two disulphides was then dependent on the ability to cleave the peptide bond Cys60-Cys61 in order to discriminate between the two isomeric structures. This could only be achieved by chemical methods since the Cys-Cys bond proved to be resistant to proteases. However, as Cys60 is close to the N-terminus of the peptide 59–65, whereas Cys45 and Cys106 are further inside the sequence of their respective fragments, the structural ambiguity could be solved by subjecting the unfractionated peptide mixture to two consecutive steps of manual Edman degradation followed by MALDI analysis of the truncated peptides according to the strategy already described (Morris and Pucci, 1985; Nitti *et al.*, 1995). The Edman reaction provided the chemical method to cleave between the two juxtaposed cysteines (Cys60 and Cys61) leaving the S-S bridges intact. The first cycle of the Edman degradation removed the N-terminal residue from each of the peptides within the clusters, causing the mass signals to be shifted to lower mass values according to the amino acids released. As an example, the MALDI analysis of the truncated peptide mixture following a single Edman degradation step showed that the signal at m/z 4751.4 moved to m/z 4409.6 due to the removal of Val38, Glu59 and Asn96. This signal was accompanied by a satellite peak at m/z 5021.8 corresponding to the same peptide cluster in which the lysines at positions 50, 105 had been modified by the Edman reagent into phenylthiocarbamoyl derivatives. After the first cycle of Edman reaction, the two clusters consisted of fragments 60–65, 97–109 and 39–58, or 39–54, respectively, with Cys60, located at the N-terminus of the peptide 60–65, involved in a disulphide bridge. Removal of Cys60 and disruption of the corresponding S-S bridge following the second Edman step led to the collapse of the peptide clusters, with the release of a single peptide and a fragment pair linked by the remaining S-S bridge. The MALDI spectrum of the truncated peptide mixture clearly showed the occurrence of a new mass signal at m/z 1861.0, which was assigned to the peptide pairs 61–65 and 98–109 still linked by the S-S bridge between Cys61 and Cys106. The last S-S bridge in rPar j 2.0101 was then inferred by exclusion as Cys45-Cys60. The complete pattern of S-S bridges in rPar j 2.0101 was eventually defined as Cys35-Cys83, Cys45-Cys60, Cys61-Cys106 and Cys81-Cys121.

This S-S bridges pattern is identical to that found in a large number of orthologue plant ns-LTPs for which the structures were solved by either NMR or X-ray crystallography (Poznanski *et al.*, 1999), with the eight cysteines paired according to an 'abbcdacd' scheme. A 3-D structure of the homologous Par j 1.0101 allergen has been proposed (Colombo *et al.*, 1998b) by structural homology modelling using the crystal structure of the soybean LTP as a template (Baud *et al.*, 1993). This model showed that Par j 1.0101 consists of a single compact domain having an α-α-α-α-β-like fold with the four disulphide bridges holding together the four α-helices (H1 through H4). The experimental elucidation of the cysteine pairings validates the proposed 3-D model and confirms that the native conformation of the major allergens from *Parietaria judaica* is reminiscent of that of the members of the ns-LTP family. In particular, the two disulphide bonds, Cys35-Cys83 and Cys81-Cys121 hold together the N- and the C-terminus of the protein that are located on the opposite sides of helix H3, whereas the remaining two S-S bridges, Cys45-Cys60 and Cys610-Cys106, link the C-terminus of helices H1 and H4 to the N-terminus of helix H2.

Particular claims have been made on the contribution of the intramolecular disulphide bridges to the allergenicity of proteins (Smith *et al.*, 1996; Takai *et al.*, 1997; Olsson *et al.*, 1998; Takai *et al.*, 2000). Deletion mutants and site-directed mutagenesis experiments demonstrated that the disruption of the three-dimensional architecture yields proteins with reduced or absent capacity to bind IgE but with retained immunogenicity.

Conclusions

Specific immunotherapy (SIT) has been recognised by the WHO as the only treatment capable of interfering with

the course of the allergic disease. Despite being used in clinical application, SIT needs to overcome a few problems like allergen standardisation, purity of the allergenic extract and the risk of anaphylactic reactions. The current approach to the treatment of allergic diseases consists in the design of non-IgE binding allergens with retained immunogenicity toward helper T cells.

The recombinant DNA technology allowed the isolation of most of the major allergens (Olsson *et al.*, 1998) and represents a powerful tool to 'engineer' innovative derivatives of allergenic proteins for specific immunotherapy. The knowledge of the 3-D structure of allergens and/or the intramolecular interactions governing their folding represents a key step towards the design of new antigens. The rPar j 2.0101 described here shows an immunological activity similar to its native counterpart in the capability of inducing immediate skin reaction, triggering release of histamine and binding human IgE, establishing that rPar j 2.0101 is a *bona fide* Pj allergen. By sequence comparison, it shows a degree of homology with the nsLTP protein family, a large group of plant proteins whose three-dimensional solution structures have been recently solved (Duro *et al.*, 1997; Bufe 1998). They show a highly conserved general folding and define a class of proteins formed by four α-helices. The experimental data provided in the present work, together with the 3-D model proposed for the Parj1 allergen, prove that the two major Parj1 and Parj2 allergens of the *Parietaria judaica* pollen are members of the plant nsLTP and that the data presented here may form an effective molecular basis for the development of a safe therapeutic allergy vaccine.

Materials and Methods

Cloning of Par j 2.0101 in pET-11a and cDNA Sequencing

The cDNA coding for the mature form of Parj 2.0101 starting from Glu32 and containing the initial methionine was obtained by PCR amplification of the Par j 2.0101 cDNA clone (Duro *et al.*, 1996). Sequence numbering used here is consistent with the protein sequence deposited in the SwissProt database (accession number: P55958).

The following oligonucleotides primers (obtained from M-Medical, S.r.l. – Genenco Life Science) were used in the amplification reaction: 5'-GTA CAT ATG GAG GAG GCT TGC GGG AAA GTG GTG CAG-3' and 5'-GCG GGA TCC CTA ATA GTA ACC TCT GAA AAT AGT ACT-3'. The *Nde*I and *Bam*HI restriction sites incorporated in the primers are underlined. One ng of the Parj 2 clone was subjected to 30 cycles at the following conditions: 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s. The PCR products were purified, digested with *Nde*I and *Bam*HI restriction enzymes and cloned in frame in the *Nde*I and *Bam*HI sites of the pET-11a vector (Stratagene, Cedar Creek, USA). The clone was sequenced by the dideoxy chain termination method by using a Sequenase Kit (Amersham, New York, USA) and the open reading frame confirmed.

Expression and Purification of Recombinant Par j 2.0101

The recombinant clone was grown at 37 °C to a density of 0.5 – 0.6 OD_{600} in Terrific Broth (tryptone 12 g/l, yeast extract 24 g/l, glycerol 4 ml/l) in 50 mM potassium phosphate pH 7.2 and induced for 3 hours by adding 0.5 M isopropylthio-β-D-galactoside (IPTG). The cells were harvested by centrifugation at 4000 *g* for 20 min, the pellet dissolved in PBS (10 mM sodium phosphate pH 7.2, 200 mM NaCl, 1 mM EDTA and 1 mM NaN_3) and lysed by sonication using a Heat System Ultrasonic, W-385. The cell debris was removed by centrifugation at 9000 *g* for 30 min. The recombinant protein was purified by affinity chromatography using a rabbit Parj2 specific polyclonal antibody bound to CNBr-activated Sepharose (Pharmacia Biotech, Uppsala, Sweden).

For structural studies, the recombinant protein was further purified by RP-HPLC on a Vydac C4 column (25×0.46 cm, 5 µm) using 0.1% trifluoroacetic acid (Sigma, St. Louis, USA) as solvent A and 0.07% trifluoroacetic acid in 95% acetonitrile (Baker) as solvent B. A linear gradient of solvent B from 5% to 65% in 60 min at flow rate of 1 ml/min was employed. The UV absorbance of the eluent was monitored at 220 nm. The purity of the protein preparation was tested by electrospray mass spectrometry (ES-MS) analysis.

Enzymatic Hydrolyses

The recombinant protein was reduced, denatured and alkylated as previously reported (Amoresano *et al.*, 1996). Endoproteinase Lys-C and trypsin hydrolyses of the native and/or alkylated rParj 2.0101 were both carried out in 50 mM ammonium bicarbonate, at pH 8.5 and 37 °C, for 18 h using an enzyme-to-substrate ratio of 1/50 (w/w). Digestion with carboxypeptidase B was performed in 50 mM ammonium bicarbonate, pH 8.5, at 37 °C, for 20 min by using an enzyme-to-substrate ratio of 1:1000 (w/w).

Amino Acid Sequence Determination

The N-terminal sequence of HPLC-purified peptides was determined by using an Applied Biosystem 477A pulsed-liquid protein sequencer equipped with an Applied Biosystem 120A HPLC apparatus for phenylthiohydantoin (PTH)-amino acid identification.

Sera

Sera (n=60) were collected from patients (40 males and 20 females) with seasonal allergy towards the pollen of Pj, with a clear clinical history and a high level of Pj-specific IgE (RAST 4). The most diffuse symptoms among these patients were rhinitis (80%) and asthma (15%). The selected patients received no hyposensitization therapy. Sera from non-allergic subjects (n=5) were also collected and used as a control.

IgE-Binding Capacity of Recombinant Par j 2.0101

The IgE-binding capability of the recombinant protein was tested by using 60 sera from Pj-allergic subjects diluted 1:20 with IXPBS buffer containing 0.25% BSA, 0.1% Tween-20 and 0.02% NaN_3. Filters were then incubated overnight with the single sera. After washing with IXPBS buffer containing 0.1% Tween-20, the filters were incubated for 45 min with horseradish peroxidase-conjugated rabbit anti-human IgE (Sigma). The final reaction was developed with an Electro ChemiLuminescence (ECL) system (Amersham).

Histamine Release Assay

The histamine release assay was performed by an enzymatic isotopic microassay using sensitised human leukocytes obtained from patients allergic to Pj pollen. The enzyme histamine

methyltransferase was prepared from kidney of male rats as described (Shaff *et al., 1979).* S-adenosyl L-methionine-[^{3}H] (1 mCi) purchased from the Radiochemical Centre (Amersham) was used as labelling reagent. Total blood histamine was detected by measuring the radioactivity of 100 µl of blood diluted with 100 µl of 0.05 M phosphate buffer pH 7.9 after boiling for 10 min. Spontaneous release was calculated by incubation of the blood with buffer alone. The amount of histamine released on challenge with the rPar j 2.0101 was expressed as percentage of total histamine after subtraction of spontaneous release in the absence of the stimulus.

Skin Tests

Skin prick tests were performed by placing 20 µl of each solution on the forearms of patients with unequivocal clinical history of allergy to Pj pollen. rPar j 2.0101 was freshly dissolved in a 0.9% sterile NaCl solution (w/v) at concentrations of 1 µg/ml. Histamine-HCl [1 mg/ml] and 0.9% NaCl solutions were used as positive and negative controls respectively. The area of the wheal was read after 15 min, the largest diameter (D) and the diameter perpendicular to it (d) were measured in millimetres and the reaction was calculated as D+d/2 according to Marsh (1975). Wheals of D+d/2 = 3 were recorded as negative. Wheals derived from the histamine prick were used as a reference for the reaction produced by the allergenic fractions.

Crossed Immunoelectrophoresis (CIE) and Laurell Immunoelectrophoresis (CLIE)

The immunoelectrophoretic methods were performed as described elsewhere (Geraci *et al.*, 1985).

Mass Spectrometric Analyses

ESMS analyses were carried out using a BIO-Q triple quadrupole mass spectrometer equipped with an electrospray ion source (Micromass, Manchester, UK). In a typical experiment, 10 µl of the HPLC peak were directly injected into the ion source *via* a Harvard syringe pump, at a flow rate of 5 µl/min. Spectra were recorded by scanning the first quadrupole at 10 s/scan. Data were acquired and elaborated by the MassLynx software (Micromass, Manchester, UK). Mass scale calibration was performed by means of multiply charged ions from a separate injection of horse heart myoglobin (average molecular mass 16951.5 Da).

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analyses were carried out using a Voyager DE mass spectrometer (Applied Biosystems, Boston, USA) equipped with the delayed extraction device. The mass range was calibrated using bovine insulin (average molecular mass 5734.6 Da) and a matrix peak (379.1 Da) as internal standards. Samples were dissolved in 0.2% TFA to a final concentration of about 10 pmol/µl and 1.0 µl was applied to a sample slide and mixed with 1.0 µl of an α-cyano-4-hydroxycinnamic acid solution (10 mg/ml) in CH_3CN/0.2% TFA 70:30 (v/v) before air-drying. Mass spectra were generated from the sum of 50 laser shots.

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References

Amoresano, A., Siciliano, R., Orru, S., Napoleoni, R., Altarocca, V., De Luca, E., Sirna, A. and Pucci, P. (1996). Structural characterisation of human recombinant glycohormones follitropin, lutropin and choriogonadotropin expressed in Chinese hamster ovary cells. Eur. J. Biochem. *242*, 608 – 618.

Baud, F., Pebay-Peyroula, E., Cohen-Addad C., Odani, S. and Lehmann, M. S. (1993). Crystal structure of hydrophobic protein from soybean; a member of a new cysteine-rich family. J. Mol. Biol. *231*, 877 – 887.

Bousquet, J., Lockey, R. and Malling, H. J. (1998). Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J. Allergy Clin. Immunol. *102*, 558 – 562.

Bufe, A. (1998). The biological function of allergens: relevant for the induction of allergic diseases? Int. Arch. Allergy Immunol. *117*, 215 – 219.

Colombo, P., Duro, G., Costa, M. A., Izzo, V., Mirisola, M., Locorotondo, G., Cocchiara, R. and Geraci, D. (1998a). An update on allergens. *Parietaria* pollen allergens. Allergy *53*, 917 – 921.

Colombo, P., Kennedy, D., Ramsdale, T., Costa, M. A., Duro, G., Izzo, V., Salvadori, S., Guerrini, R., Cocchiara, R., Mirisola, M. G., Wood, S. and Geraci, D. (1998b). Identification of an immunodominant IgE epitope of the *Parietaria judaica* major allergen. J. Immunol. *160*, 2780 – 2785.

Costa, M. A., Colombo, P., Izzo, V., Kennedy, H., Venturella, S., Cocchiara, R., Mistrello, G., Falagiani, P. and Geraci, D. (1994). cDNA cloning, expression and primary structure of Par jI, a major allergen of *Parietaria judaica* pollen. FEBS Lett. *341*, 182 – 186.

Des Roches, A., Paradis, L., Menardo, J. L., Bouges, S., Daures, J. P. and Bousquet, J. (1997). Immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract. VI. Specific immunotherapy prevents the onset of new sensitizations in children. J. Allergy Clin. Immunol. *99*, 450 – 453.

Duro, G., Colombo, P., Costa, M. A., Izzo, V., Porcasi, R., Di Fiore, R., Locorotondo, G., Mirisola, M. G., Cocchiara, R. and Geraci, D. (1996). cDNA cloning, sequence analysis and allergological characterization of Par j 2.0101, a new major allergen of the *Parietaria judaica* pollen. FEBS Lett. *399*, 295 – 298.

Duro, G., Colombo, P., Assunta Costa, M., Izzo, V., Porcasi, R., Di Fiore, R., Locorotondo, G., Cocchiara, R. and Geraci, D. (1997). Isolation and characterization of two cDNA clones coding for isoforms of the *Parietaria judaica* major allergen Par j 1.0101. Int. Arch. Allergy Immunol. *112*, 348 – 355.

Ford, S. A., Baldo, B. A., Geraci, D. and Bass, D. (1986). Identification of *Parietaria judaica* pollen allergens. Int. Arch. Allergy Appl. Immunol. *79*, 120 – 126.

Geraci, D., Billesbolle, K. B., Cocchiara, R., Lowenstein, H. and Ipsen, H. (1985). Immunochemical characterization of antigens of *Parietaria judaica* pollen. Identification of allergens by means of crossed radio immunoelectrophoresis. Int. Arch. Allergy Appl. Immunol. *78*, 421 – 428.

Jacobsen, L., Nuchel Petersen, B., Wihl, J. A., Lowenstein, H. and Ipsen, H. (1997). Immunotherapy with partially purified and standardized tree pollen extracts. IV. Results from long-term (6-year) follow-up. Allergy *52*, 914 – 920.

Marsh, D. G. (1975). Allergens. In: The Antigens, M. Sela, ed. (New York, USA: Academic Press), pp. 271 – 359.

Morris, H. R. and Pucci, P. (1985). A new method for rapid assignment of S-S bridges in proteins. Biochem. Biophys. Res. Commun. *126*, 1122 – 1128.

Nitti, G., Orru, S., Bloch, C., Jr., Morhy, L., Marino, G. and Pucci, P. (1995). Amino acid sequence and disulphide-bridge pattern

of three γ-thionins from *Sorghum bicolor*. Eur. J. Biochem. *228*, 250–256.

Noon, L. (1911). Prophylactic inoculation against hay fever. Lancet *1*, 1572–1573.

Olsson, S., van Hage-Hamsten, M. and Whitley, P. (1998). Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite *Lepidoglyphus destructor*. Mol. Immunol. *35*, 1017–1023.

Orrù, S., Scaloni, A., Giannattasio, M., Urech, K., Pucci, P. and Schaller, G. (1997). Amino acid sequence, S-S bridge arrangement and distribution in plant tissues of thionins from *Viscum album*. Biol. Chem. *378*, 989–996.

Petersen, A, Becker, W.M., and Schlaak, M. (1994). Epitope analysis of isoforms of the major allergen Phl p V by fingerprinting and microsequencing. Clin. Exp. Allergy *24*, 250–256.

Poerio, E., Caporale, C., Carrano, L., Pucci, P. and Buonocore, V. (1991). Assignment of the five disulfide bridges in an α-amylase inhibitor from wheat kernel by fast-atom-bombardment mass spectrometry and Edman degradation. Eur. J. Biochem. *199*, 595–600.

Poznanski J, Sodano P, Suh S.W., Lee J.Y., Ptak M., and Vovelle F. (1999). Solution structure of a lipid transfer protein extracted from rice seeds. Comparison with homologous proteins. Eur. J. Biochem. *259*, 692–708.

Rafnar, T., Griffith, I. J., Kuo, M. C., Bond, J. F., Rogers, B. L., and Klapper, D. G. R. (1991). Cloning of Amb a I (antigen E), the major allergen family of short ragweed pollen. J. Biol. Chem. *266*, 1229–1236.

Shaff, R. E. and Beaven, M. A. (1979). Increased sensitivity of the enzymatic isotopic assay of histamine: measurement of histamine in plasma and serum. Anal. Biochem. *94*, 425–430.

Silvanovich, A., Astwood, J., Zhang, L., Olsen, E., Kisil, F., Sehon, A., Mohapatra, S., and Hill, R. (1991). Nucleotide sequence analysis of three cDNAs coding for Poa p IX isoallergens of Kentucky bluegrass pollen. J. Biol. Chem. *266*, 1204–1210.

Smith, A. M. and Chapman, M. D. (1996). Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. Mol. Immunol. *33*, 399–405.

Staiger, C. J., Goodbody, K. C., Hussey, P. J., Valenta, R., Drobak, B. K., and Lloyd, C. W. (1993). The profilin multigene family of maize: differential expression of three isoforms. Plant J. *4*, 631–641.

Stanworth, D.R. (1988). Inter-receptor relationships in effector cell triggering, with particular reference to the mast cell. Mol. Immunol. *25*, 1213–1215.

Takai, T., Ichikawa, S., Yokota, T., Hatanaka, H., Inagaki, F. and Okumura Y. (2000). Unlocking the allergenic structure of the major house dust mite allergen Der f 2 by elimination of key intramolecular interactions. FEBS Lett. *484*, 102–107.

Takai, T., Yokota, T., Yasue, M., Nishiyama, C., Yuuki, T., Mori, A., Okudaira, H. and Okumura, Y. (1997). Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. Nature Biotechnol. *15*, 754–758.

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