Methods of the Major House Dust Mite Allergen Der p 2*

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Expression and Secondary Structure Determination by NMR

There exists a strong correlation between asthma and sensitization to indoor allergens. This study reports on the secondary structure of the major house dust mite allergen Der p 2, determined using heteronuclear NMR methods. The DNA was subcloned from the yeast expression vector pSAY1 into the high yield bacterial expression vector pET21a, resulting in yields of 50 mg/liter. The recombinant protein was shown to have immunoreactivity comparable with that of the natural mite protein using competitive inhibition enzyme-linked immunosorbent assay (ELISA) and a modified monoclonal radioallergosorbent test (RAST). The secondary structure was determined by examining chemical shifts, short and long range NOE SSYs, JHN-HA coupling constants, and amide exchange rates. From these data, it is clear that Der p 2 is composed of β-sheets and random coil. Based on long range distance constraints, a number of β-strands were aligned into two three-stranded, antiparallel β-sheets.

Sensitization to inhalant allergens of the house dust mite Dermatophagoides sp. is commonly associated with asthma, atopic dermatitis, and allergic rhinitis (1, 2). Indeed up to 85% of patients with asthma have IgE Ab1 to house dust mite allergens that are low molecular weight proteins or glycoproteins (3). To date, the cDNA encoding allergens from ten different protein groups from the house dust mite have been cloned and sequenced (4). Based on sequence homology, four of these allergens are related to known proteases, and one is related to amylase. Given this high incidence of lytic enzymes, it has been hypothesized that the allergenic response is related to the enzymatic function (5). The Group 2 allergens (Der p 2, Der f 2, Lep d 2, etc.) exhibit a 35% sequence identity to a human epididymal gene product (HE1), suggesting that they may play a role in mite reproduction (6). However, in contrast to many of the other allergens, there is no known homologous structure or enzymatic function. The Group 2 allergens are considered to be major allergens because 80–90% of mite-allergic individuals produce humoral and cellular responses to these allergens (7). While the structure and function of the Group 2 allergens is unknown, there have been numerous studies on the antigenic nature of Der p 2 and Der f 2. B-cell epitopes of the Group 2 allergens were heat and pH-resistant yet were lost when the protein was subjected to reduction and alkylation (8). Studies with peptides showed minimal IgE reactivity, confirming that the epitopes on Der p 2 are dependent on the tertiary structure of the protein, i.e. they are discontinuous epitopes (9–11).

This was supported by Nishiyama et al. (12) using both deletion and alanine-scanning mutagenesis of the homologous Der f 2 allergen to show that disruption of any of the three disulfide bonds had significant effects on IgE binding. More recently site-directed mutagenesis was used to disrupt each of the three disulfide bonds of Der p 2 and to study the effects of these changes on intradermal skin tests as well as binding by human IgE (13). While the results of the two studies differ in the hierarchical importance of individual disulfide bonds, it is clear that establishing a structural basis for these sometimes subtle effects needs to be determined.

Despite the importance in understanding B-cell epitopes and especially IgE binding, there are few examples of structural studies of allergens. The structures of three pollen allergens, the minor ragweed allergens Amb t 5 and the related Amb a 5 and the birch pollen allergen Bet v 1, have been determined (14–16). Models of Der p 1 and the cockroach allergen Bla g 4 have been reported (17, 18). However, there have been no structures determined de novo for any of the indoor allergens that are strongly implicated as a cause of asthma (1). Here, we report the expression and purification, together with the immunochmical and secondary structure characterization, of the recombinant Group 2 house dust-mite allergen from Dermatophagoides pteronyssinus, Der p 2.

EXPERIMENTAL PROCEDURES

Oligonucleotide Mutagenesis and Subcloning of rDer p 2—The coding sequence for Der p 2 was amplified from the pSAY1 yeast vector using standard polymerase chain reaction techniques (19). The 5′ primer contained the appropriate codons to mutate Asp-1 to Ser (designated D1S) to promote efficient cleavage of the N-terminal Met residue after expression in this bacterial system (20, 21). The coding sequence was ligated into the pET-21a expression vector (Novagen Inc., Madison, WI) and subsequently sequenced to confirm the entire correct sequence including the D1S mutation.

Expression of rDer p 2 (D1S)—The Escherichia coli strain BL21-pLysE (Novagen Inc.) was used to express rDer p 2 (D1S). Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture.
lactopyranoside when the culture $A_{	ext{opt}}$ reached 1.0, and the cells were harvested after 5.5 h.

Isotopically enriched rDer p 2 (DIS) was prepared by growing the bacteria in M9 minimal media with $^{13}$C-glucose (5 g/liter) and $^{15}$N-ammonium sulfate (1 g/liter) as the sole carbon and nitrogen sources. Soluble fractions of rDer p 2 (DIS) were prepared using the *E. coli* trans-aminase-deficient strain DL-39. This strain was transfected with DE3 phage to supply endogenous T7 polymerase for expression of rDer p 2 (DIS). The M9 media was supplemented with 200 mg/liter of $^{13}$C-glucose in combination with either $^{15}$N-valeine or $^{15}$N-leucine, similar to that described by Penington and Rule (22).

**Isolation of rDer p 2 (DIS)/from Inclusion Bodies—** Extraction of the protein was adapted from a protocol used to isolate Der f 2 from inclusion-like bodies in bacteria (23). The cell pellet was frozen for 3–4 h at $-20 \, ^\circ\text{C}$ and subsequently thawed and resuspended to one-twentieth of the original culture volume in TE (100 mM Tris, pH 8.5, 10 mM EDTA) and sonicated for 2 min on ice. The sonicate was centrifuged (GS-34 rotor, 12,000 rpm for 20 min), and the supernatant was discarded. The pellet was dissolved in one-fifth of the original culture volume of 6 M guanidine-HCl and dialyzed against 20 mM Tris, pH 8.5, 1 mM EDTA without stirring overnight at room temperature, followed by a buffer change and an additional 4 h of dialysis. After dialysis, NaCl was added to 100 mM concentration, and the sample was centrifuged (GS-34 rotor, 15,000 rpm for 20 min). Note that at no time did the extraction or refolding buffers contain any disulfide exchange reagents, e.g., dithiothreitol, and glutathione. Therefore, the disulfide bonds present in the final folded recombinant molecule most probably were formed during synthesis and not during the isolation procedure.

**Isolation of rDer p 2 (DIS)—** The supernatant containing the refolded rDer p 2 (DIS) was applied to a mAb affinity column consisting of the mAb dDx coupled to Affi-Gel-10 (Sigma) (24). The protein was eluted with 50 mM glycine, 150 mM NaCl, pH 2.3. The pH of the eluate was neutralized by dialysis against phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 100 mM NaCl), concentrated, and loaded onto a size-exclusion column (Sephadex G-75, 500-ml column, 2.5 cm x 100 cm in phosphate-buffered saline). Der p 2 containing fractions were pooled, and the purity of the sample was assessed by SDS-polyacrylamide gel electrophoresis using the PhastSystem (Pharmacia Biotech Inc.) and stained with Coomassie Brilliant Blue. Protein concentration was determined by $A_{280}$ using an extinction coefficient (e) of 0.72 l/(g·cm).

**Isolation of Natural Mite Der p 2—** *D. pteronyssinus*-spent mite culture medium, provided by Dr. Larry Arlian (Wright University State, OH, USA) was the source of natural mite Der p 2 (15). The extract was passed over the dDx-affinity column, and the Der p 2 eluate was described above.

**Antibodies—** The murine mAb used in this study were previously described (26). The mAbs were mixed with increasing concentrations of inhibitor, either natural mite Der p 2 or rDer p 2 (DIS). The mixtures were added to natural mite Der p 2 coated wells, and subsequently, bound Ab was detected using horseradish peroxidase-conjugated goat-anti-mouse IgG and substrate. Results were reported as a percent inhibition with respect to the reaction in the absence of inhibitor.

**Competitive Inhibition ELISA—** The competitive inhibition ELISA was performed as described previously (26). The mAbs were mixed with increasing concentrations of inhibitor, either natural mite Der p 2 or rDer p 2 (DIS). The mixtures were added to natural mite Der p 2 coated wells, and subsequently, bound Ab was detected using horseradish peroxidase-conjugated goat-anti-mouse IgG and substrate. Results were reported as a percent inhibition with respect to the reaction in the absence of inhibitor.

**Immunoreactivity of rDer p 2 (DIS)—** The immunoreactivity of rDer p 2 (DIS) and the natural allergen were compared using competitive inhibition ELISA and a modified monoclonal RAST assay. The results for the competitive inhibition ELISA are shown in Fig. 1, arranged in clusters based on previous studies which showed that the panel of Group 2 specific monoclonal antibodies recognized four distinct and non-overlapping antigenic regions on the surface of Der p 2 (25). Monoclonal antibodies recognizing the three antigenic regions defined by 15E11, dDx, 7A1, 13A4, and 6D6 did not distinguish between natural Der p 2 and rDer p 2 (DIS) in that they gave overlap-

![Figure 1. Competitive inhibition ELISA](image)

*Fig. 1. Competitive inhibition ELISA.* Varying concentrations of natural mite Der p 2 (●) or rDer p 2 (DIS) (▲) are used to inhibit the binding of a given mAb to natural mite Der p 2 bound to a plate. The monoclonal antibodies are grouped according to the regions they define: region 1, mAb 15E11 and mAb dDx; region 2, mAb 7A1 and mAb 13A4; region 3, mAb 6D6; and region 4, mAb 2B12, mAb 4G7, and mAb 1D8. The heteronuclear experiments were virtually identical to those described by Briercheck et al. (27). The chemical shifts were assigned using the following triple resonance experiments: HNCA (28), CA(CO)NH (29), HN(CC)NA (30), H(CO)NH (33), and (CO)NH (36). To provide additional sequential information, two double-label preparations ($^{15}$N-Leu + $^{13}$C-Gly, and $^{15}$N-Val + $^{13}$C-Gly) provided residue-specific assignments. NMR spectra were referenced as described by Jerala et al. (37).

The intensity of proton NOESY cross-peaks was measured in the $^{15}$N-NOESY-HSQC (38), CH-H-NOSEY (39), and H-N-NOESY using a mixing time of 120 ms. To definitively assign the protein cross-peaks, the data were analyzed in conjunction with the corresponding heteronuclear experiment, either the NHH-N-NOSEY (40) or the CN-NOSEY (27).

**Determining $J_{\text{HN-CA}}$ Coupling Constants—** The fraction of magnetization transferred from the H$_{\alpha}$ to H$_{\beta}$ proton was used to determine the three-bond coupling constant $J_{\text{HN-CA}}$ using the HNHA experiment (41).

**Amide Exchange—** To evaluate the protection of amide residues, the protein was dissolved in a D$_2$O buffer with the same salts as described above and a pH of 6.0. After 1 week of exchange, a $^{15}$N-$^\text{H}$-HSQC spectra was obtained (42). Peaks that remained were classified as strongly protected.

**RESULTS**

**High Level Expression of rDer p 2 (DIS)—** The expression and isolation protocol reported here consistently yielded 50 mg or more of purified protein per liter of media. The final protein preparation gave a single band on SDS-polyacrylamide gel electrophoresis. N-terminal amino acid sequencing confirmed the purity of the protein sample and also confirmed the correct processing of the initiator methionine and the serine substitution (data not shown).

**Immunoreactivity of rDer p 2 (DIS)—** The immunoreactivity of rDer p 2 (DIS) and the natural allergen were compared using competitive inhibition ELISA and a modified monoclonal RAST assay. The results for the competitive inhibition ELISA are shown in Fig. 1, arranged in clusters based on previous studies which showed that the panel of Group 2 specific monoclonal antibodies recognized four distinct and non-overlapping antigenic regions on the surface of Der p 2 (25). Monoclonal antibodies recognizing the three antigenic regions defined by 15E11, dDx, 7A1, 13A4, and 6D6 did not distinguish between natural Der p 2 and rDer p 2 (DIS) in that they gave overlapp-

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2 D. M. Briercheck and G. S. Rule, unpublished observations.
peaks in the $^{15}$N/$^1$H-HSQC spectra (Fig. 2). Many of the $^{15}$N/$^1$H HSQC peaks were associated with the same side chain resonances in $^2$H/$^1$H-NMR three-dimensional experiments, thus confirming the assignment of the multiple peaks to a single residue.

Panels A and B show examples of residues with multiple $^{15}$N/$^1$H peaks in comparison with other peaks.

Panning inhibition curves. In contrast, mAb recognizing a fourth antigenic region could distinguish between the two preparations of Der p 2. Monoclonal antibodies 4G7 and 1D8 did not react with the recombinant Der p 2, whereas mAb 2B12 consistently reacted with a 10-fold greater affinity for rDer p 2 (DIS) than the natural allergen preparation.

A modified monoclonal RAST measured IgE Ab binding to natural mite Der p 2 and rDer p 2 (DIS) (data not shown). Statistically, the results show an excellent correlation ($r = 0.79, p < 0.001$) between patient sera IgE Ab binding to the recombinant Der p 2 and probably identical to natural mite Der p 2.

**Resonance Assignments**—The resonances of rDer p 2 (DIS) were completely assigned. These assignments may be obtained upon request to the authors. The presence of residues with multiple conformations were detected based on the number of peaks in the $^{15}$N/$^1$H-HSQC spectra (Fig. 2). Many of the $^{15}$N/$^1$H peaks were associated with the same side chain resonances in three-dimensional experiments, thus confirming the assignment of the multiple peaks to a single residue. Panels A and B of Fig. 2 are expanded regions of the $^{15}$N/$^1$H-HSQC spectra that highlight the differences in the morphology of the peaks corresponding to residues with multiple peaks (e.g., Glu-25 and His-22) versus residues with unique peaks. These peaks are presumed to reflect multiple conformations of a given residue. These residues are: Leu-17, Gly-20, Cys-21, His-22, Gly-23, Glu-25, Leu-61, Ile-68, Asp-69, Val-94, and Ile-97.

**Secondary Structure Determination**—The secondary structure of rDer p 2 (DIS) was determined by analysis of chemical shift, short and long range NOEYs, and $J_{HN-HA}$ coupling constants (Fig. 3). Using the Chemical Shift Index, the deviations of the chemical shift for $C_{a}, C_{b}, CO$, and $H_{N}$ were analyzed for each residue, and the type of secondary structure was predicted (42). The protein is predicted to be composed solely of $\beta$-sheet and random coil (Fig. 3a).

The intensity of certain short range NOEYs are indicative of local secondary structure. For example, $\beta$-sheet residues show weak $\delta_N\delta_N$-NOEs and strong $\delta_{NN}$-NOEs while $\alpha$-helical residues show the opposite trend. Regions predicted by the chemical shift index to have a $\beta$-sheet conformation tend to have weak $\delta_N\delta_N$-NOEs and strong $\delta_{NN}$-NOEs (compare Figs. 3, a–c). Regions with strong $\delta_{NN}$-NOEs correlate well with the chemical shift index prediction of random coil.

The $J_{HN-HA}$ coupling constant for each residue is shown in Fig. 3d. Coupling constants of 8–10 Hz generally correspond to $\beta$-sheet residues and 4–7 Hz generally correspond to $\alpha$-helical residues. In general, regions predicted to be in sheet by the Chemical Shift Index show high coupling constants. Fig. 3e shows which amides were classified as strongly protected. Strongly protected amides are indicative of hydrogen bonding and/or residues involved in a form of stable secondary structure. The protected residues appear to be involved in the formation of $\beta$-strands (Fig. 4).

**Alignment of $\beta$-strands**—The alignment of $\beta$-strands as shown in Fig. 4 was based on the analysis of long range NOE-
SYs including H\textsubscript{N}-H\textsubscript{N'}, H\textsubscript{N}-H\textsubscript{\alpha}, and H\textsubscript{\alpha}-H\textsubscript{\alpha} NOEs that are displayed as dotted lines. Ovals encircle a strongly protected amide and its potential hydrogen bonding partner. A majority of the residues predicted to be involved in \(\beta\)-strands are shown to have long range interactions with another strand. The amide protection data correlate well with the long range NOESYs to define the \(\beta\)-strand interactions.

**DISCUSSION**

The recombinant expression system reported here provides ample quantities of rDer p 2 (D1S) for labeling with heavy isotopes preparatory to NMR analysis. In addition, immunological analyses showed the recombinant protein to have the same conformation as the natural mite allergen.

In multiple competitive inhibition assays, mAb recognizing three of four antigenic regions did not distinguish between the natural and recombinant Der p 2 forms. This strongly suggests that rDer p 2 (D1S) has an overall native conformation. In contrast, mAb that recognize epitopes of the fourth region did see differences. However, this is likely due to the isoform specificity of these three antibodies. For example, when the same isoform is expressed in yeast, without the N-terminal mutation, there was similar lack of reactivity with the 1D8 and 4G7 mAb.\(^3\) Therefore, the lack of reactivity of these two mAb with the rDer p 2 (D1S) protein used in the present study was not due to the D1S change. Furthermore, site-directed mutants that mimicked substitutions found in a Der p 2 isoform were made, and the mAb 2B12 was found to react better with the Asp-114 isoform than it did with the Asn-114 isoform.\(^3\) Our study used the Asp-114 isoform, whereas the natural allergen stock used as a competitive inhibitor presumably contains all possible isoforms since it was isolated with the mAb aDpX, which has not been shown to be isoform-specific. Consequently, the results showing that mAb 2B12 reacts more strongly with the recombinant allergen are likely due to the isoform specificity of mAb 2B12 and not to any overt conformational difference in this region.

Similarly, the results of the RAST assay show a good correlation between the reactivity of human IgE antibody with the

\(^3\) G. A. J. Hakkaart, unpublished observations.
recombinant and natural forms of Der p 2. These results strongly suggest the rDer p 2 (D1S) is in the same conformation as the natural mite Der p 2 allergen.

The heteronuclear resonances of the rDer p 2 (D1S) protein were assigned, and the secondary structure was characterized. The majority of resonance assignments of each residue fall in the normal range of resonances associated with that particular residue type.

The NMR data strongly suggest that the protein is composed predominantly of β-sheet and random coil. The secondary structure results, long range NOEys, and strongly protected amides show an excellent correlation. These data show that Der p 2 is composed of two three-stranded β-sheets involving residues 15–17, 34–48, 51–57, 79–94, 105–111, and 118–123 as shown in Fig. 4. The secondary structure of these strands can be described as regular β-sheet, except in the region of Gly-83. In this region, the interstrand interactions appear to be broken, suggesting the presence of a β-bulge.

Since there appear to be two three-stranded anti-parallel β-pleated sheets, a search of several structural data bases was initiated. Only a single potential structural homologue, the α-amylase inhibitor (1hoe.pdb), was found. The alignment of the sheets relative to the preliminary model of Der p 2 is shown in Fig. 5. The arrangement of secondary structural elements in α-amylase inhibitor is clearly unlike that of Der p 2. An examination of other strand-loop-strand motifs in the data base revealed that the two-dimensional model of Der p 2 appears similar to that of the immunoglobulin superfamily, also shown in Fig. 5. Strand 4 of the immunoglobulin superfamily has been replaced with an extended loop that comprises residues 59–78 of Der p 2. However, a crucial element of the immunoglobulin superfamily is the Greek-key motif, and Der p 2 is lacking the strand that would define a Greek-key. Whether the Der p 2 fold is a new fold or a distant relative of the α-amylase inhibitor or the immunoglobulin superfamily remains to be determined.

What is clear is that the structure is not similar to that of the avian lysozymes as was previously suggested (44). Hen egg white lysozyme contains several α-helices that span residues clearly defined as β-sheet in Der p 2.

In conclusion, we report here the expression, purification, and characterization of the secondary structure of the major mite allergen Der p 2. The data from the competitive inhibition ELISA and the serum IgE RAST indicate that the recombinant is largely indistinguishable from the natural mite protein, thus meeting an important criteria for using the recombinant allergen in structural studies. The secondary structure analysis using chemical shift, short and long range NOEys, and strongly protected amide protection corroborate that the protein is composed of β-sheet and random coil. Efforts now are being directed at determining a well refined solution structure of the protein.

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