



Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite *Lepidoglyphus destructor*

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Abstract

To study the contribution of the 3 disulphide bonds in the major allergen Lep d 2 to the antigenic structure, site-directed mutagenesis was performed. Mutants with one or more cysteine residues altered were constructed with a histidine residue tag for purification purposes and expressed as recombinant proteins in *E. coli*. Seven mutants were analysed: 3 single mutants (Cys 8, Cys 21 and Cys 72), 3 double mutants (Cys 8–117, Cys 21–16 and Cys 72–77) and one mutant with all 6 cysteines altered (6 Cys).

The evaluation of IgE reactivity in 10 allergic patients showed that the disulphide bond formed by cysteine 72 and 77 was the single most contributing bond to IgE binding. Mutants with disruption of the Cys 8–117 bond had a lesser reduction in IgE binding, even though this alteration seemed to influence the compact nature of Lep d 2. However, to abolish the IgE reactivity almost completely, all 6 cysteines had to be altered. A monoclonal antibody previously raised against *Lepidoglyphus destructor* showed a similar binding as human IgE with no reactivity to the Cys 72–77 or the 6 Cys mutant.

Using skin prick test we found no reaction to the 6 Cys mutant at the concentrations tested (1–100 µg/ml) in an *Lepidoglyphus destructor* allergic patient, while the T-cell reactivity was preserved.

The 6 Cys mutant of Lep d 2 may, after further evaluation, be a candidate molecule for improved immunotherapy of *Lepidoglyphus destructor* allergy. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Allergy to dust mites is an increasing problem world wide. Sensitisation to storage mites has previously been considered to be mainly an occupational hazard, e.g. among farmers, grainworkers and bakers (Cuthbert et al., 1979; van Hage-Hamsten et al., 1985; Korsgaard et al., 1985; Terho et al., 1985; Blainey et al., 1989; Marx et al., 1993). However, several reports show that storage mites are also important allergens in house dust in urban dwellings (Wraith et al., 1979; Woolcock et al., 1987; Luczyuska et al., 1990; Arruda et al., 1991; van Hage-Hamsten et al., 1993).

One of the most important storage mites is *Lepidoglyphus destructor* (*L. destructor*). The major allergen in this mite species, Lep d 2, is a group 2 allergen with an apparent molecular weight of 15 kDa according to mobility on SDS-PAGE. We have previously cloned and expressed this allergen in both an *E. coli* system as well

as the eukaryotic baculovirus system (Schmidt et al., 1995; Olsson et al., 1998). We found high immunoreactivity and no large differences in the recombinant allergens produced in the two expression systems.

As many allergens are now available as recombinant proteins they can be produced in large quantities and with high purity. This greatly facilitates studies on their structures as well as aspects of their involvement in the immune response. Recombinant allergens have also become attractive candidates for improving *in vitro* diagnostics and *in vivo* therapy, replacing the use of poorly characterised allergen extracts (Scheiner and Kraft, 1995). However, the problem with unwanted anaphylactic reaction may still remain if recombinant allergens are to be used in immunotherapy since pure allergenic preparations used *in vivo* may be very potent in evoking an IgE response. To reduce this problem, it may be possible to modify allergens in a way that their IgE reactivity is reduced but the T-cell response is unchanged. These modified allergens could lead to improved and safer immunotherapy (Takai et al., 1997; Vrtala et al., 1997).

The disulphide bonding pattern has not been deter-

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Table 1
Oligonucleotides inducing specific mutations at cysteine residues

Mutant-oligonucleotide	Sequence
Cys 8	5'-TTC-AAG-GAC-TCC-GGT-CAC-GGA-3'
Cys 21	5'-ATC-ACG-GGC-TCC-TCT-GGT-GAC-3'
Cys 26	5'-GGT-GAC-ACC-TCT-GTC-ATT-CAC-3'
Cys 72	5'-ACC-GAC-GGT-TCC-AAG-TTC-ATC-3'
Cys 77	5'-TTC-ATC-AAG-TCC-CCA-GTC-AAG-3'
Cys 117	5'-GTC-ATG-GCT-TCC-GGT-ACC-GTC-3'
Cys 21+26	5'-ATC-ACG-GGC-TCC-TCT-GGT-GAC-ACC-TCT-GTC-ATT-CAC-3'
Cys 72+77	5'-ACC-GAC-GGT-TCC-AAG-TTC-ATC-AAG-TCC-CCA-GTC-AAG-3'

mined experimentally for Lep d 2, but due to its high sequence homology with the group 2 allergens Der p 2 (52%) and Der f 2 (57%) from the house dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, respectively, parallels can be drawn. Der f 2 has been shown to consist of 3 disulphide bonds between cysteine residue pairs 21–27, 73–78 and 8–119 (Nishiyama et al., 1993). Furthermore these cysteine residues are conserved in all known group 2 allergens (Eriksson et al., 1998).

A number of studies indicate that IgE binding epitopes of these allergens are highly conformational and that the disulphide bonds play an important role in maintaining the antigenic structure. However there are some discrepancies as to which disulphide bonds are the most important. In Der p 2 the Cys 73–78 disulphide bond appears to be most important for IgE reactivity, while in Der f 2 the Cys 8–119 is the major contributor (Nishiyama et al., 1995; Smith and Chapman, 1996).

In this study the contribution of the disulphide bonds to the antigenic structure of Lep d 2 was investigated by systematically changing the cysteine residues using site directed mutagenesis and expression of mutant variants of Lep d 2.

2. Material and methods

2.1. Oligonucleotide directed mutagenesis

We have previously cloned the cDNA encoding two isoforms of Lep d 2, Lep d 2.01 and Lep d 2.02 (Schmidt et al., 1995). The cDNA for Lep d 2.01 was recloned into the phage vector M13mp19 using restriction enzymes *HindIII* and *XbaI* (New England Biolabs, In vitro AB, Sweden). Oligonucleotides were designed to include altered bases at cysteine codons resulting in serine codons (Table 1). All oligonucleotides were purchased from Medprobe (Norway).

Site specific mutagenesis was performed according to the method of Kunkel (1985) as modified by Geisselsoder et al. (1987). Mutations were confirmed by DNA sequen-

encing on an ABI Prism 310 sequencing apparatus (Perkin-Elmer AB, Sweden). The verified mutant clones were recloned into the expression vector pET-17b (Novagen, Göteborgs Termometerfabrik, Sweden) using restriction enzymes *HindIII* and *NdeI* (New England Biolabs).

2.2. Production and purification of recombinant (r) Lep d 2

The recombinant vectors were transformed by electroporation into the *E. coli* expression strain BL21(DE-3)pLysS (Stratagene, AH Diagnostics, Sweden). Overnight cultures were prepared and diluted 50 fold into prewarmed LB-media containing 100 µg/ml of ampicillin and 30 µg/ml of chloramphenicol and grown to an absorbance at 600 nm of approximately 0.6. The protein expression was induced with IPTG (isopropyl-β-D-thiogalactoside) at a final concentration of 0.4 mM and the culture was incubated for 2–3 h at 37°C. The cells were harvested and sonicated in short repeated intervals. Inclusion bodies were solubilised in 6 M guanidine-HCl Tris-buffer at pH 8.

The purification was then performed on TALON metal affinity resin (Clontech Laboratories, USA) according to instructions from the manufacturer and eluted with PBS containing 100 mM imidazole. The eluted sample was extensively dialysed against PBS.

2.3. Antibodies to Lep d 2

Human sera from 10 allergic patients with positive RAST values (Pharmacia and Upjohn Diagnostics AB, Sweden) to *L. destructor* (6.7–92.5 kU/l) was used in immunoblotting studies. The sera were selected based on a positive reaction to wild-type Lep d 2 in immunoblotting.

In RAST-inhibition studies a pooled serum, including 5 of these RAST positive sera, was used. A serum with RAST negative values to Lep d 2 was used as a control.

In the skin-prick test (SPT) and T-cell stimulation studies a patient with a RAST value of 27.8 kU/l against *L.*

destructor was used. Two non-allergic control patients were included in the SPT.

A monoclonal antibody (MAb), 1112D8, previously raised against native (n) *L. destructor* extract was also used in immunoblotting studies. This MAb (IgG1) reacts to nLep d 2 as well as to wild-type rLep d 2.

2.4. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Electrophoresis and Western blotting procedures were performed with a Mini Protean II equipment according to the instructions of the manufacturer (Bio-Rad Laboratories AB, Sweden). Approximately 5 µg/well of rLep d 2 were separated on 15% polyacrylamide gels under reducing or non-reducing conditions, and transferred to nitro-cellulose (NC) membrane by electroblotting. The immunostaining was performed using RAST positive sera to *L. destructor* at a dilution of 1/5 or the MAb 1112D8 (10 µg/ml), followed by a detection method using alkaline phosphatase substrate as described previously (Olsson et al., 1994). Protein staining of SDS-PAGE gels were performed in Coomassie Brilliant Blue dye.

2.5. RAST inhibition

rLep d 2 was covalently coupled at the optimal concentration to the cellulose solid phase Immuno CAP (Pharmacia and Upjohn Diagnostics AB).

The serumpool containing 5 *L. destructor* RAST positive sera (50 µl) was preincubated with an equal volume of either mutant or wild-type Lep d 2 (0.01, 0.1, 1, 5 and 10 µg) for 2 h at room temperature. 50 µl of each mixture was then further incubated with the rLep d 2 ImmunoCAP. The amount of IgE bound to the rLep d 2 ImmunoCAP was measured under standardised conditions according to the instructions of the manufacturer (Pharmacia and Upjohn). The results are expressed as the minimum amount of protein required to give at least 50% inhibition of IgE binding.

2.6. Skin testing

Skin prick tests were performed according to Pepys (Pepys, 1975) on the volar forearm. Duplicates of wild-type rLep d 2 and the 6 Cys mutant was tested in serial dilutions ranging from 1–100 µg/ml. As positive control 10 mg/ml of histamine chloride (ALK, Denmark) was used and as negative control 0.9% NaCl. The results were assessed after 15 min and a positive SPT was defined as 2+ and at least 3 mm.

2.7. PBMC proliferation assay

Peripheral blood mononuclear cells (PBMC) from one *L. destructor* allergic patient were isolated by gradient

centrifugation on Ficoll Paque® (Pharmacia Biotech, Sweden) and cultured in 96 well culture plates (2×10^5 cells/well) in Iscove's modified Dulbecco's medium supplemented with 10% human AB sera. The PBMC were stimulated with various concentrations of rLep d 2 or the 6 Cys mutant (1–40 µg/ml) and grown for 7 days (37°C, 6% CO₂). As negative controls a recombinant allergen (Mal f 1, 10 µg/ml) (Schmidt et al., 1997), produced using the same conditions as rLep d 2, as well as medium alone were used. Phytohaemagglutinin (PHA) (10 µg/ml) was used as positive control. During the last 18 h of culture, 1.0 µCi/well of [³H]thymidine (Amersham, UK) was added to each well and the thymidine incorporation was determined by scintillation counting. Triplicate cultures were set up and the results are expressed as mean cpm values. The stimulation index (SI) was calculated as specifically induced proliferation divided by background value obtained with media alone.

3. Results

3.1. Generation of Lep d 2 mutants

Oligonucleotides were designed to change the cysteine codons in Lep d 2.01 to serine codons.

Initially 3 mutants were produced, each containing a single mutation at either Cys 8, Cys 21 or Cys 72, respectively. The mutants are designated according to their specific mutation(s), e.g. Cys 8 when mutated at cysteine residue at position 8.

When analysed on CBB stained gels under reducing conditions these mutants all had the same mobility as the wild-type Lep d 2 (Fig. 1, lanes 1–4). However under non-reducing conditions the pattern appeared very different. The majority of the wild-type allergen had an apparent molecular weight of approximately 16–17 kDa

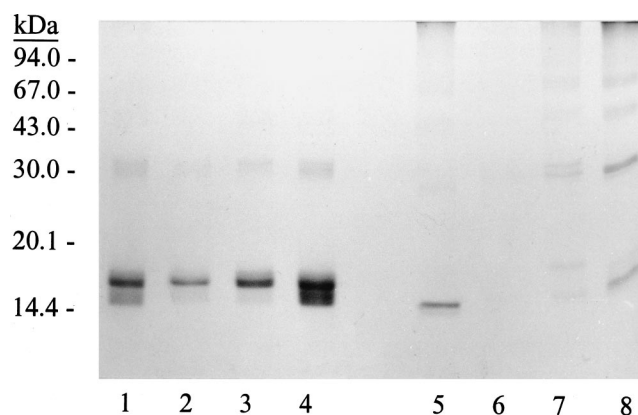


Fig. 1. Lep d 2 single cysteine mutants analysed by SDS-PAGE and CBB staining. Standard molecular weights are indicated at the left side. Lanes 1–4 show the mutants run in reducing conditions and lanes 5–8 in non-reducing conditions. Lanes 1 and 5: rLep d 2.01; lanes 2 and 6: Cys 21; lanes 3 and 7: Cys 72; lanes 4 and 8: Cys 8.

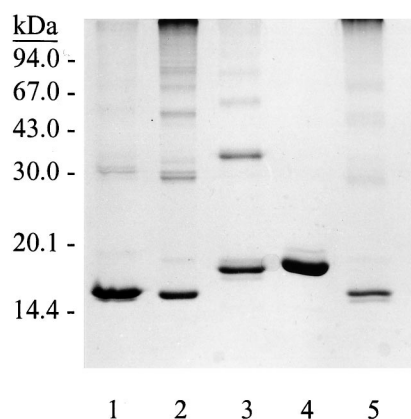


Fig. 2. Lep d 2 mutants analysed by non-reducing SDS-PAGE and CBB staining. Lane 1: Cys 21–26; lane 2: Cys 71–77; lane 3: Cys 8–117; lane 4: 6 Cys mutant; lane 5: rLep d 2.01.

(Fig. 1, lane 5) indicating it is monomeric, while the mutant proteins had various bands with higher molecular weights, over 30 kDa (Fig. 1, lanes 6–8), indicating a large amount of multimer formation. For mutants Cys 21 and Cys 72 Fig. 1 (lanes 6 and 7) almost no bands could be seen, even though repeated in several experiments, indicating formed multimers that could not enter the gel.

To investigate whether the resulting free cysteine in the single mutants were responsible for the extensive multimer formation, 3 additional Lep d 2 mutants were constructed. These were mutated at both cysteines responsible for the disulphide bonding, i.e. Cys 8–117, Cys 21–26 and Cys 72–77. Fig. 2 (lanes 1–3) shows the double-mutants on a CBB-stained gels run under non-reducing conditions. There is a much greater proportion of monomers in the double cysteine mutants than in the single mutants, making them more comparable to the wild-type form of Lep d 2. The Cys 8–117 mutant has a slightly larger apparent molecular weight than the other mutants and wild-type Lep d 2 on non-reducing gel (Fig. 2, lane 3) but not on reducing gel (data not shown). This mobility shift is indicative of the disruption of the Cys 8–117 bond.

In addition, a mutant with all the 6 cysteines changed to serine was produced (6 Cys). This 6 Cys mutant did not show any multimers when analysed on CBB stained gel under non-reducing conditions (Fig. 2, lane 4). The mobility pattern of this mutant is the same as for the monomer Cys 8–117 mutant, supporting the observation that the absence of the cysteine 8–117 bond causes a shift in apparent molecular weight.

3.2. Antibody binding to Lep d 2 mutants

The IgE reactivity to the double cysteine mutants of Lep d 2 was tested in immunoblotting using a serum with

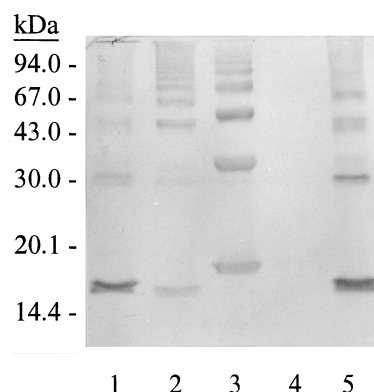


Fig. 3. IgE reactivity to non-reduced Lep d 2 mutants, after immunoblotting, by an *L. destructor* RAST positive serum. Lane 1: Cys 21–26; lane 2: Cys 71–77; lane 3: Cys 8–117; lane 4: Lep 6 Cys mutant; lane 5: rLep d 2.01.

a high RAST value (92.5 kU/l) to *L. destructor* (Fig. 3, lanes 1–3). The Cys 72–77 mutant showed a much lower IgE reactivity compared to the wild-type Lep d 2. The Cys 8–117 mutant showed a slightly lower IgE reactivity and the Cys 21–26 had similar IgE reactivity compared to the wild-type. IgE binding to the 6 Cys mutant was completely abolished with this serum (Fig. 3, lane 4). A further 10 RAST positive sera to *L. destructor* were tested in immunoblotting with similar results (data not shown). None of the sera showed any reaction to the 6 Cys mutant. Five of the 10 sera had no reaction to the Cys 72–77 mutant and the remaining 5 showed a reduced IgE binding as compared to non-mutated Lep d 2. The 10 sera all reacted to the Cys 8–117 mutant, however, a slight reduction in IgE binding as compared to the wild-type Lep d 2 was apparent. The Cys 21–26 mutant showed an IgE reactivity comparable to the wild-type protein in all sera tested.

The results in Fig. 3 show that many of the multimers apparently bind to IgE equally well as their monomeric counterparts.

The recognition of the mutants by MAb 1112D8 is shown in Fig. 4. There is no reaction to the Cys 72–77 or 6 Cys mutants. A slightly reduced reaction is observed to the Cys 8–117 mutant while the reaction to the Cys 21–26, in both its monomeric and multimeric forms, is preserved. These results show a similarity in the binding patterns of the serum IgE tested and this MAb.

3.3. RAST inhibition assay

The results from the RAST inhibition were in agreement with the immunoblotting data. About 10 times more Cys 72–77 (1.0 µg) and 100 times more 6 Cys (10 µg) mutant are needed to give a 50% inhibition of IgE binding than wild-type rLep d 2 (0.1 µg). The amount of the mutant Cys 8–117 required for 50% inhibition were 0.1 µg and the amount of Cys 21–26, 0.01 µg.

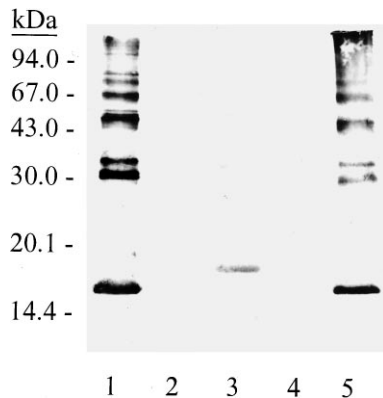


Fig. 4. Reactivity of nLep d 2 directed Mab 1112D8 to blotted non-reduced mutants. Lane 1: Cys 21–26; lane 2: Cys 71–77; lane 3: Cys 8–117; lane 4: 6 Cys mutant; lane 5: rLep d 2.01.

3.4. *In vivo* reactivity of rLep d 2 and the 6 Cys mutant

The patient with positive RAST values to *L. destructor* gave no skin reaction at the lowest concentration tested (1 µg/ml) but strong reactions (>3+) in skin prick test to rLep d 2 at 10 and 100 µg/ml. However, no detectable reaction was observed to the 6 Cys mutant, even at the highest concentration of 100 µg/ml.

No positive test results were obtained in the two control patients for rLep d 2 or 6 Cys when tested up to 100 µg/ml.

3.5. T-cell response to rLep d 2 and the 6 Cys mutant

Whole PBMC were isolated from the rLep d 2 SPT positive patient and stimulated with various concentrations of the wild-type rLep d 2 and the mutant 6 Cys for 7 days in the presence of human AB sera. The optimal concentration to use for comparison of proliferation with both antigens was 10 µg/ml.

The proliferation response was slightly higher for wild-type rLep d 2 than to the mutant, with a SI of 6.5 compared to 4.1. However, both recombinants elicited a specific T-cell response in this patient. No specific stimulation was obtained with the control recombinant protein Mal f 1 (SI = 1.4). The mean cpm values are presented in Table 2.

Table 2
T-cell proliferation in an *L. destructor* RAST positive subject by [³H]thymidine incorporation

Allergen (10 µg/ml)	Mean cpm values
rLep d 2.01	22.925
rLep d 2.6cys	14.335
rMal f 1	4.985
Medium	3.535

4. Discussion

We have systematically disrupted the 3 disulphide bonds in Lep d 2 in order to evaluate the contribution to the structure and IgE binding capacity of this allergen.

Previous studies performed on the group 2 allergens from the house dust mite species *Dermatophagoides* have shown different results. Studies from Smith and Chapman on Der p 2 showed that the two single mutants C78 and C73 showed the greatest reduction in antigenicity with a 100 fold reduction in IgE binding, compared to 10 and 40-fold reduction for the C21 or C27 and C8 or C119, respectively (Smith and Chapman, 1996). Nishiyama and colleagues performed similar studies on Der f 2 showing that the C8 or C119 mutation gave the most marked decrease in IgE binding, while C73 or C78 and C21 or C27 only gave a moderate reduction in IgE binding to Der f 2 (Nishiyama et al., 1995).

Our results for Lep d 2 are more consistent with the results of Der p 2, since the disulphide bond between cysteines at position 72 and 77 seems to be the most important in retaining the IgE binding capacity. However, in order to obtain a close to complete reduction in IgE binding, all 3 disulphide bonds have to be altered in Lep d 2. Consequently, it appears that the major IgE epitopes in Lep d 2 have a conformational nature. Accordingly, Der p 2 has been shown to lose its antigenicity upon complete reduction and alkylation (Lombardero et al., 1990).

The MAb used in this study was not previously characterised concerning the epitope binding. Our results show that this antibody binds to an epitope dependent on the 72–77 disulphide bond, much in the same way as IgE from allergic patients. The epitope could also be located at one of the cysteines altered. Regardless, this MAb could be useful in identifying Lep d 2 variants with reduced IgE binding.

The Cys 8–117 mutant has an altered structure as judged from the mobility in a gel, probably due to a less compact folding since this bond probably stabilises the protein structure. However, this alteration does not seem to reduce the IgE binding capacity to a large extent.

We do not know anything about the formation of the Lep d 2 multimers, but since they were not formed in the 6 Cys mutant it is likely that the disulphide bonding is involved. Nishiyama and colleagues observed the formation of incorrect disulphide bonds and the formation of aggregates in mutants of Der f 2 (Nishiyama et al., 1995).

Altered allergens may be a step towards improved and safer specific immunotherapy by decreasing the risk of anaphylactic reactions. The 6 Cys mutant of Lep d 2 appears to have at least a 100-fold lower IgE reactivity than wild-type rLep d 2. Furthermore we found sustained T-cell reactivity to the 6 Cys mutant in a patient that did not have any detectable SPT reactivity to this mutant. It

shows that it is possible to keep the T-cell epitopes intact even though the IgE binding epitopes are greatly altered in Lep d 2.

Using site-directed mutagenesis, rather than chemical methods such as reduction and alkylation, provides a better controlled system if the resulting molecules are to be used in *in vivo* therapy. There is no risk of reforming cysteine bonds, resulting in retained immunogenicity, or remaining traces of unwanted chemicals in the preparations.

Investigations using a large number of patients are now needed to further evaluate the potential use of the 6 Cys mutant. It might be a candidate molecule for improved and safer *in vivo* therapy of *L. destructor* allergy.

This study has provided us with information that may be useful in our goal to obtain 3-dimensional structural information of Lep d 2. In order to obtain sufficient quantities of protein for structural studies of allergens using nuclear magnetic resonance (NMR) or X-ray crystallography techniques it is necessary to use recombinant proteins. However, recombinant Lep d 2 prepared from *E. coli* using the methods described results in a certain amount of multimer formation. Therefore to obtain a homogeneous population of monomeric Lep d 2 for structural studies extra purification steps such as gel filtration or reduction of disulphide bonds followed by refolding may be necessary.

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