

Purification and characterization of *Lep d I*, a major allergen from the mite *Lepidoglyphus destructor*

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Summary

A major allergen of the storage mite *Lepidoglyphus destructor* (*Lep d I*) has been purified by affinity chromatography using an anti-*Lep d I* monoclonal antibody. The purity of the protein obtained by this procedure was assessed by reverse-phase HPLC. *Lep d I* displayed a molecular weight of 14 kD on SDS-PAGE under non-reducing conditions, and 16 kD in the presence of a reducing agent. Analytical IEF revealed a little charge microheterogeneity, showing three bands with pIs 7.6–7.8. Purified *Lep d I* retained IgE-binding ability, as proved by immunoblotting experiments after SDS-PAGE and RAST with individual sera from *L. destructor*-sensitive patients. Results from the latter technique demonstrated that 87% of *L. destructor*-allergic patients had specific IgE to *Lep d I*, and a good correlation between IgE reactivity with *L. destructor* extract and *Lep d I* was found. In addition, RAST inhibition experiments showed that IgE-binding sites on *Lep d I* are major *L. destructor*-allergenic determinants, since *Lep d I* could inhibit up to 75% the binding of specific IgE to *L. destructor* extract; on the other hand, *Lep d I* did not cross-react with *D. pteronyssinus* allergens.

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Introduction

Isolation of allergens has become a very important goal in allergy, because of their potential utilization for the study of the relationship between molecular structure and activity as well as for diagnosis and therapy of allergic patients. For these reasons, during the last years a number of naturally occurring allergens have been identified and isolated [1].

The storage mite *Lepidoglyphus destructor* has been identified as an important cause of occupational asthma in workers exposed to stored grain [2–4] and, more recently, it has also been demonstrated to be an allergen source in cases of indoor allergy, especially in areas where conditions of temperature and relative humidity are suitable for its growth [5,6]. However, few data on the identification of allergens from this mite have been published so far [7–9].

In a previous work [9], we identified a polypeptide with MW14 kD on SDS-PAGE under non-reducing conditions as the main IgE-binding component of *L. destructor*,

and also, monoclonal antibodies (MoAbs) against this allergen were produced and characterized.

Here we describe the purification of the 14 kD MW allergen by affinity chromatography using one of these MoAbs. The physico-chemical and immunochemical characteristics of this allergen, hereafter named *Lep d I* according to the IUIS-allergen nomenclature system, as well as its capacity to bind human IgE are presented and discussed.

Materials and methods

Preparation of L. destructor extract (LDE)

L. destructor mite bodies (> 99% purity as determined by particle count) were purchased from Biopol Laboratory Inc. (Washington, U.S.A.), and the extract was prepared as described previously [9].

Protein content was estimated by the Folin method [10].

Monoclonal antibody

The anti-*Lep d I* MoAb used in this work (Le5B5) was obtained by fusing P3X63 mouse myeloma cells with

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spleen cells from Balb/c mice immunized with a partially purified *Lep d I* preparation, obtained by ammonium sulphate salt precipitation of LDE. Full details of its production, purification and characterization were published elsewhere [9].

Human sera

Human sera from 24 *L. destructor*-sensitive patients were used throughout the experiments. Patients were selected by clinical history, cutaneous positive reaction and the presence of serum IgE specific to *L. destructor*, using commercial diagnostic kits. Skin prick test was performed with a *L. destructor* extract (Alergia e Inmunología Abelló, Spain) with an activity of 100 BU/ml, that is, an extract with a potency which would produce a geometric mean weal size of 75 mm² when prick tested in a representative sample of sensitive patients [11].

L. destructor-specific IgE levels were determined using Hamlet®-IgE (Alergia e Inmunología Abelló) methodology as described below. All the sera had specific IgE levels of RAST class 3 or 4 to *L. destructor*, and 11 out of the 24 sera also had RAST class 2–3 to *D. pteronyssinus*. For some experiments, a serum pool, made by mixing equal volume aliquots from the individual sera, was used. The resulting pool had RAST-class 3 to *L. destructor*.

Control human sera were obtained from non-atopic individuals among healthy laboratory personnel.

Purification of *Lep d I*

Lep d I was affinity purified using an anti-*Lep d I* Le5B5 MoAb immunosorbent column prepared by coupling 9 mg of purified MoAb to 0.6 g of CNBr-activated Sepharose 4B (Pharmacia).

LDE (6 ml at a protein concentration of 1 mg/ml in PBS) was passed through the column and, after extensive washing with PBS to eliminate the non-retained components, the column was equilibrated with 10 bed volumes of pre-elution buffer (10 mM phosphate, pH 6.8). Bound protein was eluted using 100 mM glycine, pH 2.5, and 1 ml-fractions were collected in tubes containing 50 µl of neutralizing buffer (1 M phosphate, pH 8.0). Fractions were pooled on the basis of protein content (as detected by absorbance at 280 nm), dialysed extensively in a Spectra/Por dialysis tubing with MW cut off 6–8 kD (Spectrum Medical Inc., Los Angeles, California, U.S.A.) against distilled water, lyophilized and stored at 4°C until use. Purity of *Lep d I* obtained was assessed by SDS-PAGE and reverse-phase HPLC. Average recovery of *Lep d I* using the procedure described was estimated to be about 200 µg.

Concentration of purified *Lep d I* samples (after dialysis against distilled water) was measured by the difference between spectrophotometric absorptions at 215 and 225 nm [12].

HPLC

HPLC was carried out on equipment from Waters comprising a Model 680 gradient programmer, two 510 pumps, a U6K injector, and a 490 multiwave-length u.v./visible detector set at 214 nm.

Analytical reverse-phase HPLC of affinity-purified *Lep d I* was performed on a Beckman Ultrapore C3 column (4.6 × 75 mm, 5 µm particle size, 30 nm pore size), using a linear 0–50% gradient of 1-propanol in 0.01 M TFA at a constant flow rate of 1 ml/min.

Amino acid analysis

Protein samples were hydrolysed at 110°C for 24 hr with vapour phase 6 N HCl. The hydrolysates were derivatized with dabsyl chloride and analysed in reverse-phase HPLC using a Dabs-Amino Acid Analysis kit supplied by Beckman (San Ramon, California, U.S.A.), according to the manufacturer's protocol. Analyses were performed in triplicate, and no corrections were made for destruction during hydrolysis or incomplete hydrolysis. The content of tryptophan was not determined.

Electrophoretical procedures

SDS-PAGE was performed with an 8–25% acrylamide gradient in a high molarity Tris buffer system [13]. The following standards from Bio-Rad (Richmond, California, U.S.A.) were used as molecular weight markers: phosphorylase B (92.5 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). Protein detection on the gels was done by Coomassie Brilliant Blue staining.

Electrotransfer of separated polypeptides onto nitrocellulose membranes was carried out by the method of Towbin [14]. For immunodetection of allergenic polypeptides, nitrocellulose sheets were sequentially incubated with *L. destructor*-sensitive human serum pool and Hamlet®-¹²⁵I (Alergia e Inmunología Abelló), a radio-labelled mouse anti-human IgE MoAb [15], at a dilution of approximately 1000 c.p.m./µl. Finally, nitrocellulose membranes were washed, dried and autoradiographed.

Analytical IEF was performed on agarose plates (FMC Isogel, Rockland, Maine, U.S.A.), pH gradient from 3 to 10, according to the manufacturer's instructions. pI markers (FMC Isogel) were run on the same gels.

Immunoassays

Detection of specific IgE was carried out by radioimmunoassay as follows: plastic microtitre wells (Immulon II, Removawells, Dynatech, Alexandria, Virginia, U.S.A.) were coated by incubation with 100 μ l of antigen solutions in distilled water (LDE at 50 μ g/ml, *D. pteronyssinus* at 50 μ g/ml, *Lep d I* at 10 μ g/ml), at 4°C overnight. Unbound protein was then washed three times with PBS containing 0.5% Tween 20. Unoccupied well sites were blocked with 100 μ l of PBS containing 1% bovine serum albumin for 1 hr at room temperature. The plates were afterwards washed three times with PBS-0.5% Tween 20.

The adsorbed antigens were made to react with 100 μ l of individual human sera at 4°C overnight and, after washing, with Hamlet®-¹²⁵I (approximately 100 000 c.p.m./well) for 2 hr at room temperature. Finally, the plates were washed and the binding of specific IgE to the allergosorbents was determined by measuring the radioactivity bound to the wells. IgE values were calculated by interpolation with a standard curve against *L. perenne* (Hamlet®-IgE).

RAST inhibition assays were performed essentially as above, except that the allergosorbent was made to react with a mixture of 50 μ l of *L. destructor*-specific human serum pool (diluted 1:3 with PBS) and 50 μ l of serial dilutions of the samples to be tested.

Results

Purification of *Lep d I*

Lep d I was purified from LDE by affinity chromatography using an anti-*Lep d I* MoAb (Le5B5) coupled to Sepharose. Approximately 3% of the total LDE protein applied on top of the column was recovered as *Lep d I*.

A high degree of purity was achieved with this single-step purification procedure, as it was demonstrated by reverse-phase HPLC (Fig. 1). Affinity-purified *Lep d I* yielded a major sharp peak, and only minor contaminants, counting for less than 2% of total absorbance at 214 nm, could be detected.

The purity of *Lep d I* allergen was also assessed by SDS-PAGE. In Fig. 2, the electrophoretic patterns of LDE employed as the starting material for the purification (lanes a) and pure *Lep d I* (lanes b) are shown. Besides, this technique provided additional information about the molecular structure of *Lep d I*. Thus, the apparent molecular weight of *Lep d I* was estimated to be 14 kD under non-reducing conditions, as it had been previously described [9]; however, the electrophoretic mobility of *Lep d I* was decreased in the presence of a reducing agent, then bringing about a higher apparent molecular weight

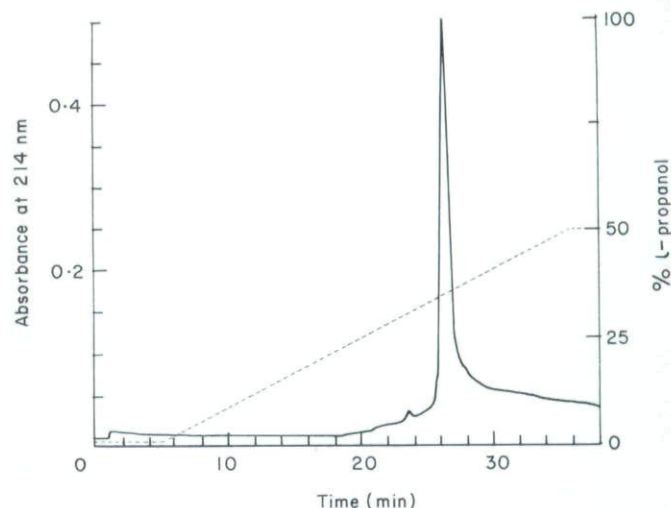


Fig. 1. Analytical reverse-phase HPLC of affinity-purified *Lep d I* on an ultrapore C3 column. Elution was performed as described in Materials and Methods. The dashed line depicts the 1-propanol gradient shape.

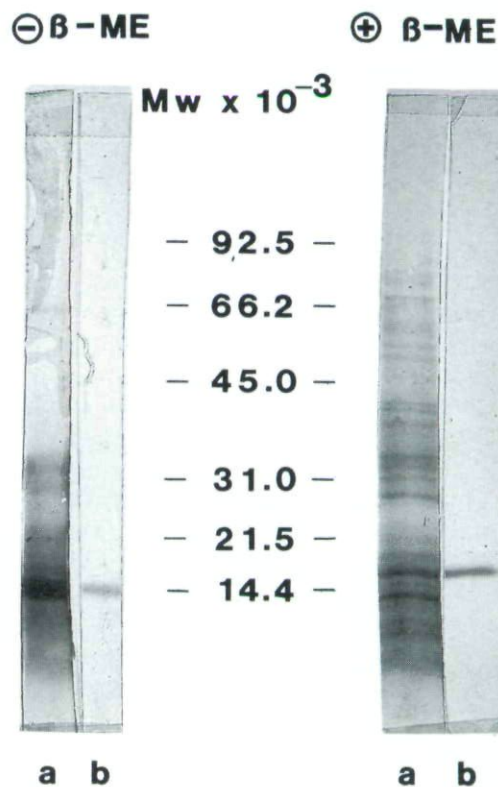


Fig. 2. SDS-PAGE analysis under non-reducing ($-\beta$ -ME) and reducing conditions ($+\beta$ -ME) of LDE (lanes a, 200 μ g per well) and affinity purified allergen *Lep d I* (lanes b, 15 μ g per well).

Table 1. Amino acid composition of *Lep d I* allergen

Amino acid	Per cent*
Asx	7.5
Glx	7.8
Ser	1.6
Thr	9.6
Arg	1.4
Gly	13.7
Ala	9.5
Pro	3.7
Val	9.8
Met	1.7
Ile	6.9
Leu	5.7
Phe	3.5
Cys	2.0
Lys	11.2
His	3.4
Tyr	1.0

* Results are expressed as the number of residues per 100 residues.

(16 kD). This fact is highly indicative of the presence of intramolecular disulphide bonding [16] in the *Lep d I* molecule.

Amino acid composition of *Lep d I* is given in Table 1. This composition did not show any remarkable feature and neither displayed homology to other reported compositions of mite allergens.

Immunodetection after SDS-PAGE with LDE-specific human serum pool (Fig. 3) demonstrated that *Lep d I* retained IgE-binding ability after the purification process. It showed a radiostained band (lanes b) with the same electrophoretic mobility as the most prominent band in LDE-immunoblotting pattern (lanes a), both in the presence (+ β -ME) and absence ($-\beta$ -ME) of a reducing agent. On the other hand, the radiostaining intensity was higher under non-reducing conditions, which seems to indicate that renaturation of *Lep d I* during the transfer process was not complete when the electrophoresis was carried out in the presence of β -ME, and consequently, that some IgE-binding sites on *Lep d I* are conformational.

Charge heterogeneity of *Lep d I*

Although homogeneous when analysed by the preceding techniques, affinity-purified *Lep d I* showed a certain degree of charge heterogeneity (Fig. 4). Two very close

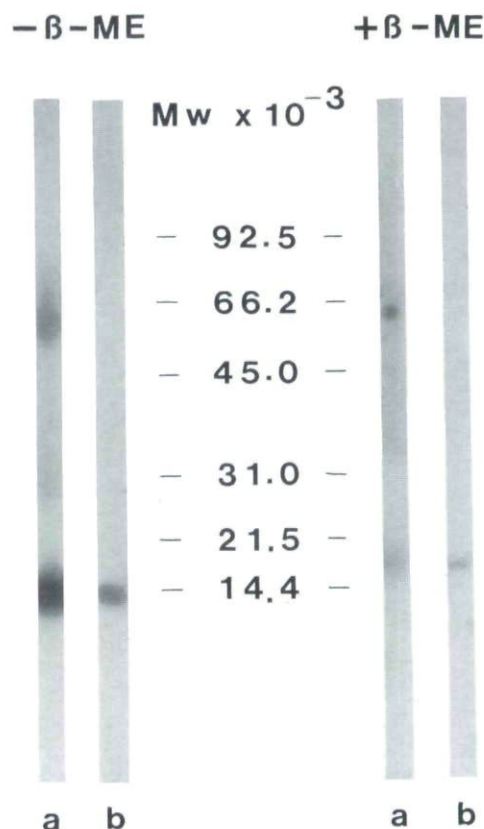


Fig. 3. Immunodetection with *L. destructor*-specific human serum pool of LDE (lanes a) and *Lep d I* (lanes b). Conditions employed for electrophoresis were the same as in Fig. 2

bands, with pI about 7.6, and a third one with pI 7.8 were visualized in *Lep d I* IEF pattern (lane b), that tallied with heavily stained bands in LDE pattern (lane a).

Allergenic significance of *Lep d I*

The prevalence of specific IgE to *Lep d I* in serum from *L. destructor*-sensitive patients was determined by RAST technique. Almost all the sera tested (21 out of 24) clearly had IgE antibodies to the purified allergen and, in addition, a good correlation between IgE reactivity with LDE and *Lep d I* was obtained ($r=0.74$, $P<1\times 10^{-4}$) when results from both RAST were compared by linear regression analysis (Fig. 5).

The allergen activity of *Lep d I* was evaluated by RAST inhibition assay, using a *L. destructor*-sensitive serum pool (Fig. 6). In the same assay, LDE was included for comparison. The inhibition curves show that *Lep d I* was able to inhibit up to 75% the binding of specific IgE to

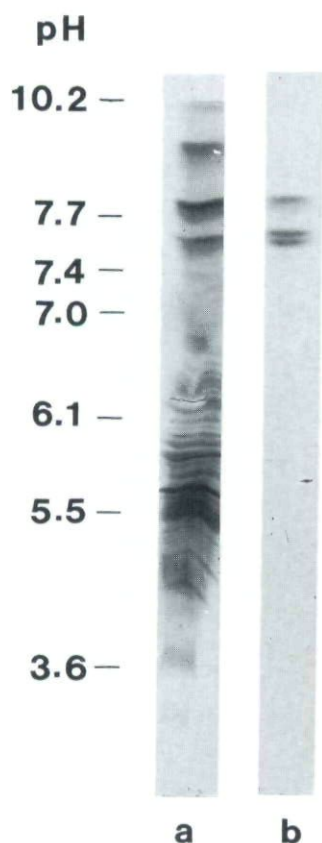


Fig. 4. Analytical IEF on an agarose gel. Two hundred micrograms of LDE (lane a) and 15 μ g of *Lep d I* (lane b) were applied to the gel. Proteins were detected by Coomassie Blue staining.

LDE, then indicating that *Lep d I* bears major allergenic determinants of *L. destructor*.

Also, the allergenic cross-reactivity between *Lep d I* and *D. pteronyssinus* allergens was investigated by RAST-inhibition. As it can be seen in Fig. 6, no significant inhibition of *D. pteronyssinus*-RAST was reached even when a concentration of *Lep d I* 100-fold higher than that needed to inhibit to 75% the *L. destructor*-RAST was used.

Discussion

The complex nature of allergenic extracts, usually ill-defined materials, has long hindered the purification and further characterization of IgE-binding antigens. In fact, only in the last years a significant progress in this field has been made. An important contribution to this progress can be attributed to the application of hybridoma technology to the study of inhalant allergens, such as

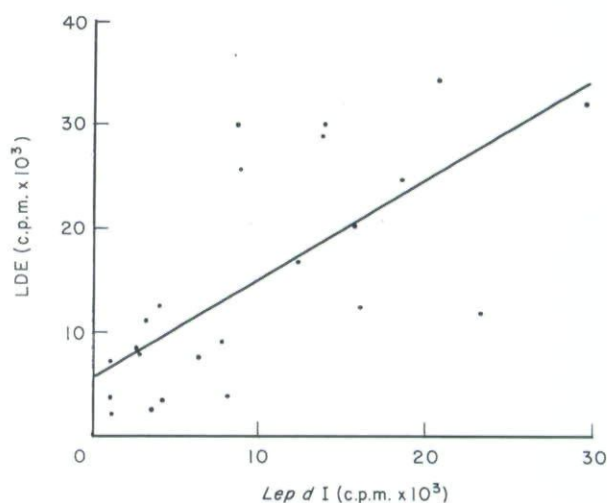


Fig. 5. Correlation analysis of IgE reactivity with *Lep d I* versus LDE as coating antigens. Counts per minute represent ¹²⁵I-anti-IgE MoAb bound to the allergosorbent wells, determined by RAST with 24 individual sera, as described in Materials and Methods.

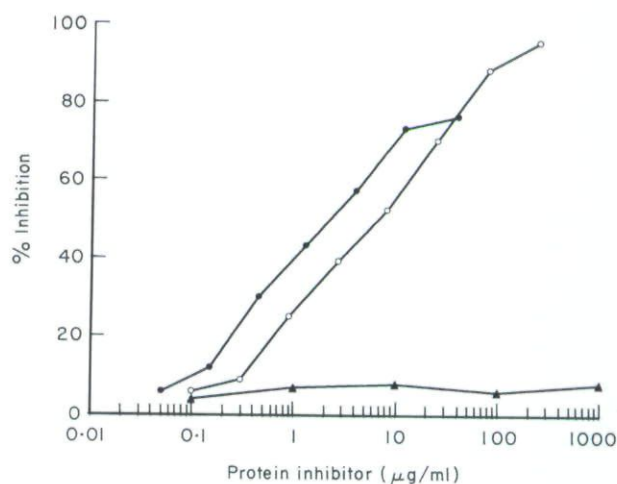


Fig. 6. RAST inhibition curves. The binding of specific human IgE to LDE-coated wells was inhibited using purified *Lep d I* (●), and LDE (○) as competitors. (▲) Inhibition of *D. pteronyssinus*-RAST with pure *Lep d I*. Each point represents the mean value of four different determinations.

those from *Dermatophagoides* mites [17–19], *Felis domesticus* [20–21], *Lolium perenne* [22], etc.

In the present work, monoclonal antibodies have been employed for the purification of the major storage mite allergen *Lep d I*. The purification was achieved by a single-step procedure using affinity chromatography with an anti-*Lep d I* MoAb.

The isolated allergen showed a high degree of purity, and it was homogeneous when analysed by reverse-phase HPLC (Fig. 1) and SDS-PAGE (Fig. 2). However, it displayed a little heterogeneity on analytical IEF (Fig. 4), which seems to be a common characteristic in a number of allergens, for instance, the Group I and Group II allergens from *Dermatophagoides spp.* [23], *Parietaria judaica* allergen I [24], allergens from *Lolium perenne* [25,26], etc.

A structural feature of *Lep d I* that can be inferred from the results presented here is the existence of intramolecular disulphide bridges, since the electrophoretic mobility of *Lep d I* was decreased when electrophoresis was carried out under reducing conditions (Fig. 2), then causing a variation of the apparent MW from 14 kD (in the absence of β -ME) to 16 kD. The latter value tallies with that reported for one of the two main allergenic components from *L. destructor* identified by other authors [7].

On the other hand, *Lep d I* retained its IgE-binding ability upon the purification process, in which rather mild conditions were used. This allergenic activity was demonstrated by immunoblot after SDS-PAGE with a *L. destructor*-specific human serum pool (Fig. 3) and by RAST technique with individual sera (Fig. 5). The fact that 87% of the sera tested in RAST had specific IgE to the purified allergen and, besides, that a correlation between IgE levels to *Lep d I* and LDE were found, allows us to conclude that *Lep d I* is a clinically relevant allergenic component of *L. destructor*.

Moreover, allergenic epitopes on *Lep d I* contribute a high percentage of total IgE-binding capacity of LDE, since the pure allergen could inhibit up to 75% the binding of specific IgE to LDE in RAST inhibition experiments (Fig. 6).

In contrast, *Lep d I* did not inhibit the *D. pteronyssinus*-RAST, which is evidence that *Lep d I* is not responsible for the allergenic crossreactivity between these mites previously described [27]. Furthermore, this result is in agreement with findings recently reported by Johansson *et al.* [28], who demonstrated, by immunoblot inhibition studies, that only two minor allergen components of *L. destructor* showed some crossreactivity with *D. pteronyssinus*.

In conclusion, we have established a simple and rapid chromatographic procedure for the purification of the main allergen of the storage mite *L. destructor* in an allergenic active state. The availability of purified *Lep d I* has enabled us to study some structural features of this protein as well as its allergenic properties, and it could be very useful in future work aimed at the understanding of the relationship between structure and biological activity.

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