

Cloning of three new allergens from the dust mite *Lepidoglyphus destructor* using phage surface display technology

Tove L. J. Eriksson¹, Omid Rasool¹, Sonia Huecas², Paul Whitley³, Reto Crameri⁴, Ulrich Appenzeller⁴, Guro Gafvelin¹ and Marianne van Hage-Hamsten¹

¹Unit of Clinical Immunology and Allergy, Karolinska Institute and Hospital, Stockholm, Sweden; ²Department of Biochemistry and Molecular Biology, University of Madrid, Spain; ³Department of Biology and Biochemistry, University of Bath, UK;

⁴Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

The dust mite *Lepidoglyphus destructor* is a common species in Europe and a major cause of dust mite allergy in rural surroundings, but it also contributes to dust mite allergy in urban areas. One major allergen, Lep d 2, has been expressed as a recombinant protein and evaluated both *in vivo* and *in vitro* and shown to detect 60% or more of *L. destructor*-sensitized subjects. Additional recombinant allergens are needed to obtain a reliable diagnostic tool for *L. destructor* allergy. The aim of this study was to clone and express new allergens from *L. destructor* and determine their recognition frequency among sensitized individuals. A phage display cDNA expression library was constructed and screened with sera from *L. destructor*-sensitized

individuals. The cDNAs encoding the allergens were cloned into the pET17b vector and subsequently expressed in *Escherichia coli* as C-terminal His₆-tagged proteins. Immunoblotting of the recombinant proteins was performed using sera from 45 subjects allergic to *L. destructor*. Three new allergens from *L. destructor*, Ld 5 (originating from a partial Lep d 5 clone), Lep d 7 and Lep d 13, were identified and recognized by 4/45 (9%), 28/45 (62%) and 6/45 (13%) sera from *L. destructor*-sensitized subjects, respectively.

Keywords: Ld 5; Lep d 13; Lep d 7; *Lepidoglyphus destructor*; phage display.

All over the world, dust mites are a major cause of allergic disease [1]. The mite allergens have different structures and functions as has been shown for the house dust mite species *Dermatophagoides* [2,3]. The properties of mite allergens have been reviewed by Thomas & Smith [3], and the group 1, 3, 4, 6, 8 and 9 allergens are enzymes while the functions of group 2, 5, 7 and 12 allergens are unknown. Group 10, 11 and 13 allergens are known to be tropomyosins, paramyosins and fatty acid-binding proteins, respectively. However, less is known about the molecular nature of the allergens produced by the dust mite *Lepidoglyphus destructor*. Non-pyroglyphid mites such as *L. destructor* are known to cause sensitization among farmers [4–7], bakers [8,9] and grain workers [10,11]. In recent years, however, evidence for sensitization in urban populations has emerged [12–16] especially in regions with damp housing conditions [14,17]. Thus, sensitization is not restricted to individuals with occupational exposure. At least 20 IgE-binding components have been identified by SDS/PAGE and immunoblotting in whole extract of *L. destructor* [18].

One major allergen, Lep d 2, has been cloned, sequenced [19] and expressed [20]. Recombinant Lep d 2 has also been coupled to ImmunoCAPs, and 73% of patients with a positive radioallergosorbent test (RAST) to *L. destructor* could be identified with this single allergen in the fully automated ImmunoCAP IgE-detection system [21]. In the skin prick test, rLep d 2 could detect 59% of the *L. destructor*-sensitized subjects [22]. Therefore, additional recombinant allergens are needed to obtain a reliable diagnostic tool for *L. destructor* allergy based on recombinant allergens. Diagnostic tests based on recombinant allergens can be thoroughly standardized, in contrast with those based on whole extracts, which may be subject to large batch-to-batch variations [23].

In this study, we used a strategy that allows expression and display of cDNA libraries on the surface of a filamentous phage [24] to identify and clone additional allergens from *L. destructor*. This strategy covalently links gene products displayed on the phage surface, as a fusion protein to the phage pIII coat protein, to the cDNAs integrated into the phage genome. The physical linkage between phenotype and genotype allows selective enrichment of phage displaying proteins able to interact with a ligand. This is in contrast with the commonly used λ phage cloning systems, in which genetic information integrated into the phage genome and gene product expression exploiting the machinery of the host cell during the lytic process are not physically linked [24]. Several allergens from the mould *Aspergillus fumigatus* [25], the yeast *Malassezia furfur* [26] and peanut [27] have successfully been cloned using the pJuFo system. In this study, we have modified the method previously used for selection of IgE-binding proteins and cloned three new allergens from *L. destructor*. The new

Correspondence to T. L. J. Eriksson, Unit of Clinical Immunology and Allergy, Karolinska Hospital, S-171 76 Stockholm, Sweden.

Fax: + 46 8 33 57 24, E-mail: tove.eriksson@ks.se

Abbreviations: RAST, radioallergosorbent test; PVDF, poly(vinylidene difluoride).

Note: the cDNA sequences of partial Ld 5 and full-length Lep d 7 and Lep d 13 have been submitted to the EMBL nucleotide sequence database and are available under accession numbers AJ250278, AJ271058 and AJ250279, respectively.

(Received 27 September 2000, revised 7 November 2000, accepted 7 November 2000)

allergens showed homology with other mite allergens of group 5, 7 and 13. The group 5 allergen was named Ld 5 because it originates from a partial clone, lacking the 5' end. The allergens formally termed Ld 5, Lep d 7 and Lep d 13 were expressed in *Escherichia coli* and tested for their capacity to bind IgE in sera from *L. destructor*-sensitized individuals.

MATERIALS AND METHODS

Isolation of mRNA

L. destructor whole cultures were obtained from Allergon AB (Ängelholm, Sweden). mRNA was isolated from 0.5 g living mites using the Dynabeads mRNA direct kit (Dyna, Oslo, Norway), according to the manufacturer's protocol.

cDNA synthesis, adaptor ligation and PCR amplification

cDNA was synthesized with the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) using an oligo(dT) primer with an *Asp*718 restriction enzyme site (Table 1) according to the manufacturer's protocol. After second-strand synthesis, the cDNA was phenol/chloroform extracted and ethanol precipitated before adaptor ligation. The adaptor was generated by annealing adaptor-oligonucleotide 1, which contained an *Xba*I site (5'-CTAATACGACTCACTATAGGGCTCTAGAGCGGCCG-CCCGGGCAGGT-3') and adaptor-oligonucleotide 2 (5'-PO₃ACCTGCCCCNH₂-3') in equal concentrations, 10 µM. The adaptors were ligated in 1 × DNA ligation buffer with 5 U T4 DNA ligase (Boehringer, Mannheim, Germany) at 16 °C overnight. The adaptor-ligated cDNA was amplified using primer 1, which matched the adaptor sequence, and primer 2, which matched the oligo(dT) primer, in 1 × Klen *Taq* PCR buffer containing 0.2 mM dNTPs and Advantage Klen *Taq* polymerase mix (Clontech). All primers are listed in Table 1. PCR was carried out for 25 cycles: 1 min at 94 °C, 2 min at 57 °C and 2.5 min at 72 °C. The amplified PCR products were phenol/chloroform extracted, ethanol precipitated, and digested with *Asp*718 and *Xba*I (Boehringer). The digested sample was run on an agarose gel and fragments < 300 bp were removed as described previously [26].

Construction of library and isolation of phage

For the construction of an *L. destructor* cDNA library, the pJuFo vector [24] and the cDNA were digested with *Asp*718 and *Xba*I, and then 1.5 µg of the digested cDNA was ligated to 1.9 µg of the digested vector using T4 DNA ligase (Boehringer). Construction of the library and precipitation of phage were carried out as previously described in detail [26].

Human sera used for screening of proteins displayed on phage surfaces (biopanning) and immunoblotting

Sera from four subjects, containing 92.5, 37.9, 20.4 and 13.0 kU·L⁻¹ *L. destructor*-specific IgE, were used individually in the biopanning procedure. These four sera were chosen because of their ability to recognize a broad spectrum of different allergens in *L. destructor* whole extract.

Sera from 45 *L. destructor* RAST-positive subjects, who had previously participated in an epidemiological investigation of respiratory disease [29] were used for immunodetection. One non-atopic serum sample that was RAST negative to *L. destructor* was used as a negative control.

Biopanning

Washed Dynabeads Pan Mouse IgG (Dyna; 100 µL) was mixed with 5 µg mouse anti-human IgE monoclonal antibody designated CGM9 [28] in 400 µL NaCl/P_i containing 1% BSA. The beads were incubated end over end at room temperature for 1 h, washed twice in NaCl/P_i, and resuspended in blocking buffer (NaCl/P_i, pH 7.4, containing 0.05% Tween 20 and 2% milk powder). After 1 h of blocking and two washes in NaCl/P_i, the beads were resuspended in 500 µL of undiluted patient sera and incubated end over end for 1 h at room temperature. This was followed by two washes in NaCl/P_i. The washed beads, coated with human serum IgE, were mixed with 100 µL of the *L. destructor* phage library and incubated for 2 h at room temperature end over end. The beads were then washed once with water, 10 times with 5 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20, and finally twice with water to remove unbound phage. Bound phage

Table 1. Primers used for cDNA synthesis, PCR amplification, PCR + 1 and subcloning into the expression vector.

Primer	Sequence
Oligo(dT)	5'-GCTGCCTCTGCGTCGACATCTGACAAAGCTTGGTACCTTTTTTTTTTTTTTTNN-3'
Primer 1	5'-CCATCCTAATACGACTCACTATAGGGC-3'
Primer 2	5'-GCTGCCTCTGCGTCGACATCTGACA-3'
Primer 3	5'-CTGTTCCAATTGGCGAACTGTTC-3'
Primer 4	5'-GAGAAGCTTTTA(ATG) ₆ TTTGGTTTCGATGGCCTT-3'
Primer 5	5'-GGCCATATGGACGATTTCGGTAACGAATT-3'
Primer 6	5'-GGAGATCTGACGATTTCCGTAACGAA-3'
Primer 7	5'-GAGGGATCCTTAGTGATGGTGGTGATGATGTTTGGTTTCGATGGCCTTG-3'
Primer 8	5'-CACCATATGCATAAGCCAGCCTACTACGATGAC-3'
Primer 9	5'-CACAAGCTTA(ATG) ₆ GTTCAATTAAAGTTTGTGATCTCTTCGA-3'
Primer 10	5'-GGCCATATGGCAAACATCGCTGGACAA-3'
Primer 11	5'-GAGAAGCTTTTA(ATG) ₆ AGCTCGCTTGTA-3'

were eluted twice with 100 µL elution buffer (0.1 M HCl, 0.1% BSA, adjusted to pH 2.2 with solid glycine). The eluate was immediately neutralized with 6 µL 2 M Tris base, and the eluted phagemide (200 µL) were used to infect 4 mL freshly grown *E. coli* XL-2 cells for 15 min at room temperature. Prewarmed NZY medium (1% casein hydrolysate, 0.5% yeast extract, 10 mM NaCl, 12.5 µM MgSO₄, 12.5 µM MgCl₂, 20 µM glucose, pH 7.5) supplemented with antibiotics (20 µg·mL⁻¹ ampicillin, 10 µg·mL⁻¹ tetracycline) was added to the bacteria and 100, 10 and 1 µL of the culture was immediately plated on Luria–Bertani medium/agar plates (100 µg·mL⁻¹ ampicillin) to determine the number of packaged phagemide eluted. Further culture incubation, phage precipitation and titration were performed as described previously [26]. The precipitated phage were stored at -20 °C until used in the next round of biopanning. In total, five rounds of biopanning were performed. Twenty-four individual colonies from each round were grown in liquid culture, and plasmids were prepared, digested and sequenced.

Sequence analysis

DNA sequencing was carried out on an ABI 377 Sequencer (Perkin–Elmer, Foster City, CA, USA) using the ABI PRISM dRhodamine terminator cycle sequencing kit. cDNA strands cloned into pJuFo were sequenced using forward and reverse primers. The sequences of the isolated clones were analysed from the BCM search Launcher using WUBLASTX mode 2.0 search of the NCBI non-redundant protein database. T7 promoter and M13 reverse sequencing primers were used to sequence 5'-RACE products and PCR + 1 products which were cloned into pT7 Blue vector (Novagen, R & D systems, Abingdon, Oxon., UK). The pET17b constructs were sequenced using T7 terminator and T7 promoter primer.

Amplification of 5' end and PCR + 1 reactions for the group 5 allergen

In an attempt to obtain the complete sequence of the group 5 allergen, 5'-RACE was performed on adaptor-ligated cDNA using a gene-specific primer, primer 3, in combination with primer 1. The concentration used for both primer 1 and primer 3 was 0.2 µM, and the PCR was carried out in 50 µL Advantage 2 PCR buffer containing 0.2 mM dNTPs and Advantage Klen *Taq* polymerase mix.

PCR + 1 reactions for the group 5 allergen were carried out using gene-specific primers, 4, 5, 6 (Table 1), which were designed according to results from sequencing of clones from the biopanning and 5'-RACE products. The PCR + 1 method was used to avoid sequence artefacts associated with isoforms. Quasi-homologous sequences will contain miss-matches, and, when cloned into bacteria, heteroduplexes will be repaired randomly before replication. This generates sequences that are difficult to determine correctly. The addition of a third primer containing a restriction site at its 5' end for only one cycle ensures that only homoduplexes will contain the specific restriction site [30]. The first part of the PCR + 1 reaction was carried out in *Pfu* buffer, containing cDNA, primers 4 and 5 each at 0.2 µM, and 0.2 mM dNTPs. *Pfu* polymerase (Stratagene) was added under hot start conditions. The PCR was carried

out for 35 cycles: 1 min at 94 °C, 2 min at 60 °C and 2.5 min at 72 °C. The second part of the PCR + 1 reaction contained 25 µL of the first reaction product, a single primer at 2.6 µM, primer 6 (including a *Bgl*II site), and 0.2 mM dNTPs in 1 × *Pfu* buffer. *Pfu* polymerase was added under hot start conditions and a single cycle was performed under the same cycle conditions as for the first part of PCR + 1. Two independent reactions were carried out and the PCR + 1 clones were screened by *Bgl*II digestion and sequencing.

Subcloning into pET17b, expression and immunoblotting

The allergens were amplified using gene-specific primers before cloning into the expression vector. All primers are listed in Table 1. Ld 5 was amplified from the PCR + 1 template using primers 5 and 7. Primer 5 includes an *Nde*I restriction site and a start codon, and primer 7 has six histidine codons before the stop codon and a *Bam*HI restriction site included at the 3' end (Table 1). Lep d 7 and Lep d 13 were amplified from the respective pJuFo templates using primer 8 in combination with primer 9 and primer 10 together with primer 11, respectively. Primers 8 and 10 contain an *Nde*I site and a start codon and primers 9 and 11 include a His₆ tag, a stop codon, and a *Hind*III site. PCR products digested with the respective enzymes were ligated into the appropriately restricted expression vector, pET17b (Novagen). The ligations were initially transformed into a non-expressing host, *E. coli* XL-2 Blue, for sequence confirmation. Correct constructs were transformed into expressing cells, *E. coli* BL21(DE3)pLysS (Novagen). The recombinant proteins were found in insoluble inclusion bodies, which were solubilized in buffer containing 20 mM Tris, 100 mM NaCl and 6 M guanidine hydrochloride, pH 8. Immobilized metal affinity chromatography TALON® resin (Clontech) was used to purify the His₆-tagged proteins which were eluted from the affinity resin in NaCl/P_i, pH 7.4, containing 100 mM imidazole. The eluted recombinant proteins were dialysed against NaCl/P_i, subjected to non-reducing SDS/PAGE, and electroblotted on poly(vinylidene difluoride) (PVDF) membrane (Immobilon, Millipore, Bedford, MA, USA). Immunodetection with human sera was performed as described previously [18].

Blotting inhibition

For blotting inhibition analysis, patient sera were preincubated with 10 µg of the respective recombinant allergens for 1 h at room temperature and then incubated with *L. destructor* whole extract blotted on PVDF membrane. The *L. destructor* extract was prepared as described previously [18]. The PVDF strips were evaluated by densitometry scanning using a BioRad model GS-670 Densitometer with a Molecular Analyst Software version 2.1. The inhibition was calculated using the formula:

$$\% \text{ inhibition} = 100 - [100 \times (X - B)/(A - B)]$$

where *X* is the absorbance value obtained for inhibited protein bands on PVDF strips. The absorbance value for extract incubated with serum only (without inhibitor) was taken as 100% binding of IgE (*A*) and *B* is the background value (0% IgE binding).

RESULTS

Construction of phage display cDNA library

The pJuFo cDNA phage display library of *L. destructor* consisted of 1.4×10^6 independent clones. After amplification with helper phage, a titre of 1×10^{11} colony forming units (c.f.u.) μL^{-1} was obtained. Before the biopanning, 14 individual c.f.u. were picked and plasmid DNA was subjected to restriction analysis. Twelve (85%) of the colonies had inserts with sizes between 250 and 750 bp in length. This library was used for selective enrichment of phage displaying IgE-binding proteins.

Selective enrichment and characterization of IgE-binding clones

The *L. destructor* phage display library was subjected to five rounds of biopanning against individual sera from four *L. destructor*-allergic patients. After each round of selection, plasmid DNA from 24 colonies was prepared and the cDNA inserts were sequenced. Sequence analysis revealed the presence of three different clones with significant homology to other known mite allergens of group 5, 7 and 13. Ld 5 and Lep d 13 first appeared in biopanning round number 4, and Lep d 7 was recovered after biopanning round 5. One of the clones showed homology (36% amino-acid identity) with Der p 5 from the house dust mite *Dermatophagoides pteronyssinus* [31] as well as with

Blo t 5 (46% identity) from *Blomia tropicalis* [32,33] and was named Ld 5 because it lacked the 5' end. The partial clone of Ld 5 used for expression spans 333 bp, encoding 110 amino acids with a predicted molecular mass of 12.5 kDa. Based on homology to Der p 5 and Blo t 5, there are four N-terminal amino-acid residues missing in the partial Ld 5 expressed. Sixteen clones were analysed from two independent PCR + 1 reactions and at least five isoforms of Ld 5 were detected. The isoforms showed amino-acid substitutions in eight positions of the 110 amino acids (7%). One isoform seems to be dominant and was present in 9/16 (56%) of the clones analysed and was used for expression. A sequence alignment of the mite group 5 allergens is shown in Fig. 1A.

The other two allergens were designated Lep d 7 and Lep d 13 because of their sequence similarity to other mite allergens and according to the nomenclature [34]. Lep d 7 showed homology with Der p 7 and Der f 7 from *Dermatophagoides farinae*, 29% and 27% identity, respectively [35,36]. The multiple sequence alignment of the group 7 allergens is shown in Fig. 1B. The ORF of the isolated clone coding for Lep d 7 is 648 bp in length, encoding a 197-amino-acid protein with a deduced molecular mass of 22 kDa. There are no cysteine residues and, in contrast with Der p 7 and Der f 7, there are no potential N-glycosylation (N-X-S/T) sites in the predicted amino-acid sequence. In addition, the protein has a hydrophobic N-terminal region which may represent a signal sequence [37].

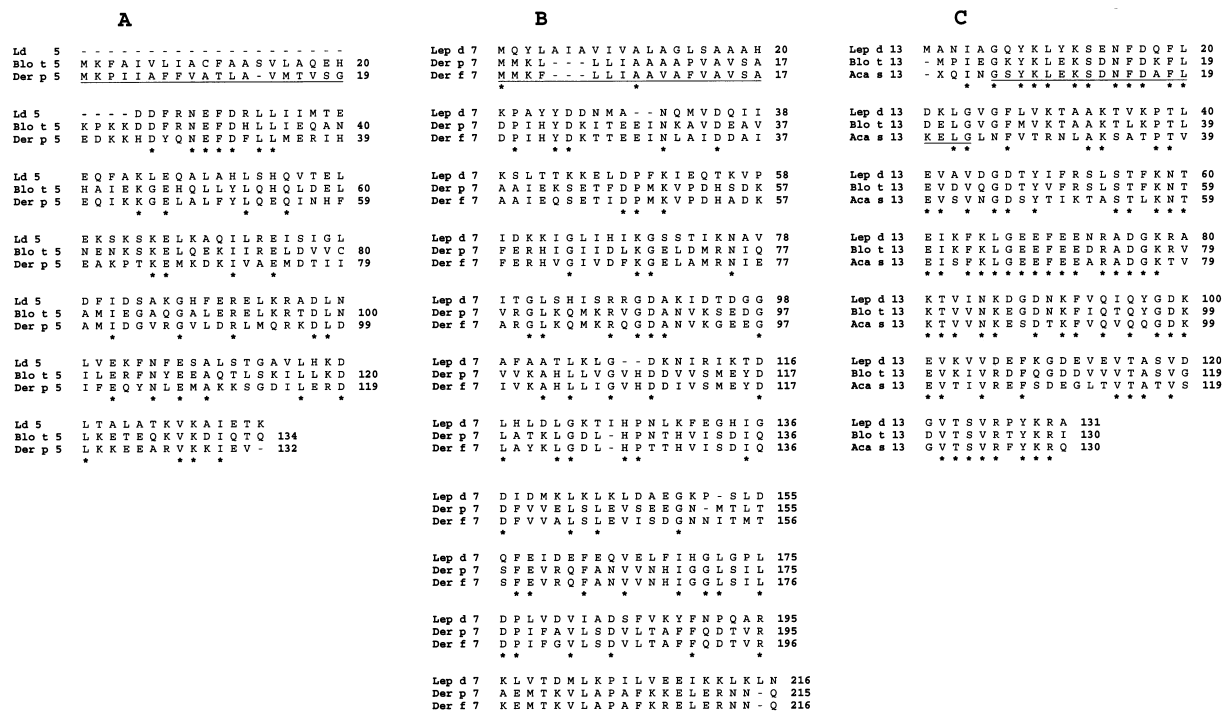


Fig. 1. Multiple sequence alignment. The amino acids conserved between the species are indicated by asterisks, and dashes are introduced for alignment purposes. (A) Multiple sequence alignment of the group 5 mite allergens. A partial clone of Ld 5, starting with an aspartic acid, shows 46% identity with Blo t 5 and 36% identity with Der p 5. The signal sequence of Der p 5 is underlined. (B) Multiple sequence alignment of the group 7 mite allergens. Lep d 7 shows 29% identity with Der p 7 and 27% identity with Der f 7. The signal sequence of Der f 7 is underlined. (C) Multiple sequence alignment of the group 13 mite allergens. Lep d 13 shows 78% identity with Blo t 13 and 60% identity with Aca s 13. The cytosolic fatty acid-binding protein signature is underlined.

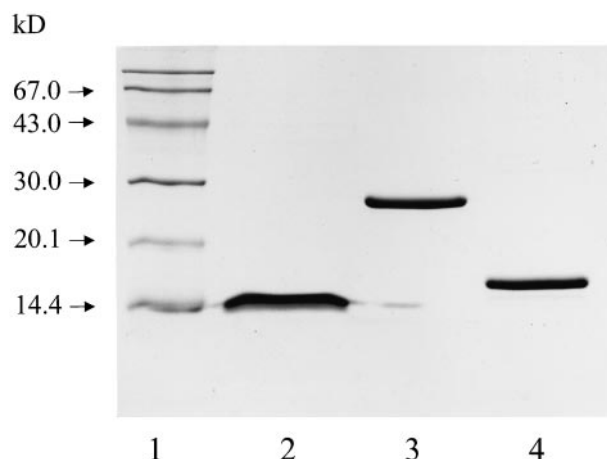


Fig. 2. SDS/PAGE analysis of the purified recombinant allergens. Lane 1, molecular-mass marker; lane 2, rLd 5; lane 3, rLep d 7; lane 4, rLep d 13. The proteins were denatured by boiling under reducing conditions, separated by SDS/PAGE (15% gel), and stained with Coomassie brilliant blue R. The amount of each allergen loaded was 1 μ g per mm of gel.

Lep d 13 showed significant homology with Blo t 13 [38] and Aca s 13 from *Acarus siro* [39], with 78% and 60% sequence identity at the amino-acid level, respectively. In addition, Lep d 13 also showed high sequence homology to other fatty acid-binding proteins from different species. A sequence alignment of the group 13 allergens is shown in Fig. 1C. The ORF encoding Lep d 13 is 393 bp, predicting a protein of 131 amino acids with a calculated molecular

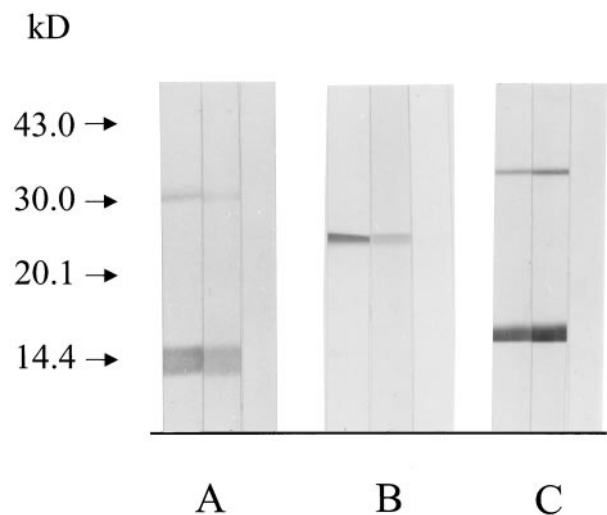


Fig. 3. Immunoblot analysis of the recombinant allergens, rLd 5, rLep d 7 and rLep d 13. (A) Lanes 1 and 2, IgE binding to rLd 5 detected on immunoblots by two individual sera with a positive RAST to *L. destructor*. Lane 3, one serum sample with a negative RAST to *L. destructor*. (B) Lanes 4 and 5, IgE binding to rLep d 7 detected on immunoblots by two individual sera with a positive RAST to *L. destructor*. Lane 6, one serum sample with a negative RAST to *L. destructor*. (C) Lanes 7 and 8, IgE binding to rLep d 13 detected on immunoblots by two individual sera with a positive RAST to *L. destructor*. Lane 9, one serum sample with a negative RAST to *L. destructor*. Full results are summarized in Table 2.

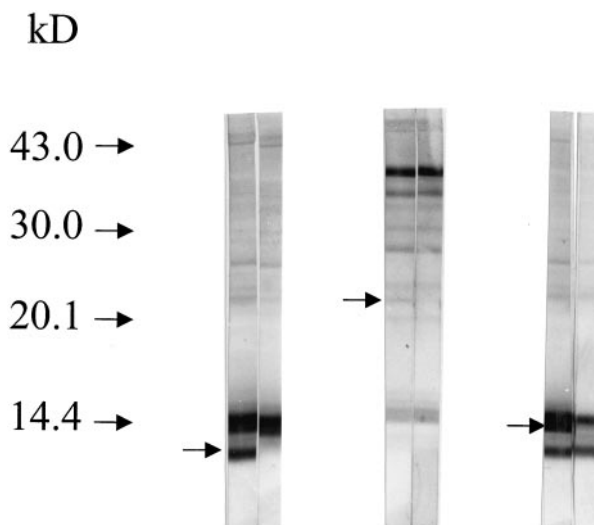


Fig. 4. Inhibition of IgE binding to *L. destructor* whole extract. Sera RAST positive to *L. destructor* incubated without inhibitor (lanes 1, 3 and 5), with 10 μ g rLd 5 (lane 2), with 10 μ g rLep d 7 (lane 4) and with 10 μ g rLep d 13 (lane 6). The corresponding native allergens are indicated with arrows.

mass of 14.6 kDa. There is a cytosolic fatty acid-binding protein signature [40] at position 6–23, no cysteine residues and no potential N-glycosylation sites in the predicted amino-acid sequence. Sequence analysis of eight plasmids with cDNAs encoding Lep d 13 indicated that isoforms are not present.

Production of recombinant proteins and immunoblotting

The cDNAs encoding Ld 5, Lep d 7 and Lep d 13 were subcloned into the expression vector pET17b and the proteins were expressed as His₆-tagged proteins in the *E. coli* strain BL21(DE3) pLysS. The recombinant proteins were found in insoluble inclusion bodies which were solubilized and then purified to homogeneity. No impurities were detected when the recombinant proteins were subjected to SDS/PAGE and Coomassie staining (Fig. 2). The recombinant allergens had apparent molecular masses comparable with their calculated molecular masses. The yields were 4 mg·L⁻¹ bacterial culture of recombinant (r) Ld 5, 25 mg·L⁻¹ rLep d 7, and 19 mg·L⁻¹ rLep d 13 (Table 2).

Pure recombinant proteins were subjected to immunoblotting experiments, and rLd 5 was recognized by 4/45 (9%), rLep d 7 by 28/45 (62%) and rLep d 13 by 6/45 (13%) of patients who were RAST positive to *L. destructor*. None of the recombinant allergens were detected by the *L. destructor* RAST-negative serum. Examples of immunoblots with sera recognizing the respective recombinant allergens are shown in Fig. 3 and the complete results are summarized in Table 2.

Inhibition of IgE binding to native (n) allergens in *L. destructor* extract

All the recombinant allergens had, to different degrees, the ability to inhibit IgE binding to their native counterparts in

Table 2. Characteristics of the three IgE-binding proteins isolated from an *L. destructor* phage surface cDNA library.

Allergen	Calculated molecular mass (kDa)	Number of <i>L. destructor</i> RAST positive subjects recognizing the recombinant protein	Amount of pure recombinant protein recovered per litre of <i>E. coli</i> culture (mg)	EMBL accession number
Ld 5 ^a	12.5 ^a	4/45 (9%)	4	AJ250278
Lep d 7	22.0	28/45 (62%)	25	AJ271058
Lep d 13	14.6	6/45 (13%)	19	AJ250279

^a Expressed from a partial clone.

L. destructor whole extract. A 10-μg sample of rLd 5 gave 86% inhibition of binding to an ≈ 13-kDa band in whole *L. destructor* extract. A 27% inhibition of binding to an ≈ 23-kDa band in the extract was achieved by preincubation of serum with 10 μg rLep d 7. The IgE binding to nLep d 7 in the whole extract was very weak. With regard to rLep d 13, 10 μg gave 50% inhibition of binding to an ≈ 15-kDa band in whole extract. The inhibition pattern of IgE binding is shown in Fig. 4.

DISCUSSION

Our aim is to develop a standardized diagnostic tool for *L. destructor* IgE using recombinant allergens. In this study, we used phage display technology to clone and characterize additional allergens from *L. destructor*. We modified the protocol previously used for biopanning [25,26]. Instead of performing biopanning in a microtitre well, we captured the IgE-binding phage with antibody-coated magnetic beads. With this method, the surface able to interact with the phage can be considerably increased compared with that available in a microtitre plate. Three new allergens were isolated by this approach, two of them, Lep d 7 and Lep d 13, as full-length clones, while the third, Ld 5, lacked the 5' end.

The partial Ld 5 clone that we have expressed was only recognized by 9% of the *L. destructor*-sensitized individuals. In contrast, Der p 5 has been shown to be recognized by 50% [31] and Blo t 5 by 47% [32] of patients sensitized to *D. pteronyssinus* and *B. tropicalis*, respectively. It is possible that the IgE-binding frequency determined in this study is affected by the fact that we used a truncated protein. The discrepancy in IgE binding compared with other group 5 mite allergens could also be due to sub-optimal refolding of the *E. coli*-expressed Ld 5. However, rLd 5 was able to inhibit 86% of IgE binding to an ≈ 13-kDa component in whole *L. destructor* extract. Analysis of PCR + 1 clones of Ld 5 showed amino-acid substitutions at eight positions, suggesting that isoforms of Ld 5 are present. Interestingly, Lin *et al.* [31] found that different Der p 5 clones only had one amino-acid substitution, which is in contrast with several other major allergens for which isoforms often are present [3]. The PCR + 1 method developed by Boriello & Krauter [30] was used for Ld 5 in order to avoid sequence artefacts associated with isoforms. The addition of a third primer containing a restriction enzyme site at its 5' end for only one cycle, ensures that only homoduplexes of the DNA will contain the specific restriction enzyme site. The PCR + 1 method cannot overcome problems with artefacts introduced by the

polymerase itself. However, we used *Pfu* polymerase, which has 3' to 5' endonuclease activity and a low error rate. The function of the group 5 allergens is unknown and the only significant homologies found for Ld 5 were related to the mite group 5 allergens.

Lep d 7 was recognized by 62% of the *L. destructor*-sensitized patients, which is more in agreement with the IgE-binding frequency for other group 7 allergens, i.e. 46% for Der p 7 [35] and 53% for Der f 7 [36]. Three bands of 24, 27 and 29 kDa, corresponding to Der p 7, have been identified in *D. pteronyssinus* whole extract, and the different sizes of the protein may be due to different degrees of glycosylation [35]. Der p 7 contains one N-glycosylation site, in contrast with Lep d 7, which lacks potential N-glycosylation sites. Only one band of about 23 kDa in *L. destructor* extract could be inhibited by rLep d 7, suggesting that nLep d 7 is not present in variants of different sizes. We cannot exclude the possibility that Lep d 7 could be present in isoforms in mite extract, as we only analysed one clone selected by the biopanning procedure. However, isoallergenic variation has not been reported for Der p 7 [35] or Der f 7 [36]. The biochemical function of the group 7 mite allergens is also unknown, and for Lep d 7, no significant homologies to proteins other than the group 7 mite allergens were found.

Lep d 13 was recognized by 13% of the *L. destructor*-sensitized patients investigated. Blo t 13 has been shown to be recognized by 11% [38] and Aca s 13 by 23% [39] of patients sensitized to *B. tropicalis* and *A. siro*, respectively. Recombinant Lep d 13 gave 50% inhibition of an ≈ 15-kDa band in the extract. Double inhibition with both rLep d 13 and rLep d 2, an allergen with similar molecular mass, has also been performed (data not shown), but this double inhibition did not completely inhibit IgE binding to proteins in the 15-kDa region. This indicates that additional allergens in this molecular-mass region are present in *L. destructor* extract. Lep d 13 and the other group 13 mite allergens [38,39] show sequence homology with fatty acid-binding proteins from different organisms. These proteins are involved in the intracellular transport of lipids.

Phage display is an elegant tool for identifying new allergens. However, like many other techniques, this strategy has limitations. We discovered that the cDNA library we used contained the cDNAs encoding Lep d 2 and Lep d 10 (with homology to tropomyosins; G. Gafvelin, unpublished work). These allergens were not isolated by the biopanning procedure but could be isolated from our library using gene-specific primers. There could be several reasons why we could not isolate these two allergens during the

biopanning procedure. Lep d 10 forms a coiled-coil dimeric protein [41], and Lep d 2 forms internal disulfide bonds which are essential for optimal IgE binding [42]. Therefore, if expressed and displayed on the phage surface, the two proteins may not be able to refold correctly. However, we cannot exclude, at least not in the case of Lep d 10, the possibility that the sera used for biopanning lack the critical amount of allergen-specific IgE required for successful enrichment of specific phage according to mass law considerations [43].

Several of the known mite allergens, such as group 1–3, 5–7 and 9, are secreted proteins containing secretion signal sequences. As there is one signal sequence encoded by Pel B in the pJuFo vector and one encoded by the mite cDNA, it is possible that the signal sequence from the mite protein acts as a stop transfer signal. If this occurs, the mite proteins could not be displayed on the phage surface, preventing successful biopanning.

Today, allergy diagnostic tests are mainly based on crude extracts. These extracts are subjected to batch-to-batch variations related to the extraction procedure used, the protease content, and stability in storage. Furthermore, the extracts contain several different proteins which can cause new sensitizations when used for *in vivo* diagnosis or therapy [23]. Therefore, recombinant allergens have many advantages and may in the future replace crude extracts for diagnosis and treatment of allergic diseases.

In an earlier study, we investigated how many subjects positive to *L. destructor* extract could be identified by rLep d 2 coupled to ImmunoCAP [21]. In fact, of those positive to *L. destructor* extract but negative to rLep d 2, 10 new subjects were identified using rLep d 7. In our study population, it should be possible to detect as many as 96% of the *L. destructor*-sensitized subjects using a combination of rLep d 2, rLd 5, rLep d 7 and rLep d 13 coupled to ImmunoCAP. Further studies are needed to investigate if this holds in other populations.

ACKNOWLEDGEMENTS

Mouse anti-human IgE monoclonal antibody (CGM9) was a gift from C.G.M. Magnusson, Department of Medicine, Karolinska Institutet and Hospital, Stockholm, Sweden. This work was supported by grants from the Swedish Medical Research Council (grant 13497), the Swedish Foundation for Health Care Sciences and Allergy Research, the Hesselman Foundation, the Swedish Asthma and Allergy Association, the Swedish Society of Medicine, the Consul Th.C. Berghs Foundation, the Trygg-Hansa Fund, the Magnus Bergvalls Foundation, the King Gustaf V 80 Birthday Foundation and the Karolinska Institute, Stockholm, Sweden.

REFERENCES

- Chapman, M.D., Smith, A.M., Vailes, L.D. & Arruda, L.K. (1997) Recombinant mite allergens. New technologies for the management of patients with asthma. *Allergy* **52**, 374–379.
- Stewart, G.A., (1995) Dust mite allergens. *Clin. Rev. Allergy Immunol.* **13**, 135–150.
- Thomas, W.R. & Smith, W. (1998) House-dust-mite allergens. *Allergy* **53**, 821–832.
- Cuthbert, O.D., Brostoff, J., Wraith, D.G. & Brighton, W.D. (1979) 'Barn allergy': asthma and rhinitis due to storage mites. *Clin. Allergy* **9**, 229–236.
- van Hage-Hamsten, M., Johansson, S.G.O., Höglund, S., Tüll, P., Wirén, A. & Zetterström, O. (1985) Storage mite allergy is common in a farming population. *Clin. Allergy* **15**, 555–564.
- Tehro, E.O., Husman, K., Vohlonen, I., Rautalahti, M. & Tukiainen, H. (1985) Allergy to storage mites or cow dander as a cause of rhinitis among Finnish farmers. *Allergy* **40**, 23–26.
- Marx, J.J., Twigg, J.T., Ault, B.J., Merchant, J.A. & Fernandez-Caldas, E. (1993) Inhaled aeroallergen and storage mite reactivity in a Wisconsin farmer nested control study. *Am. Rev. Respir. Dis.* **147**, 354–358.
- Revsbech, P. & Dueholm, M. (1990) Storage mite allergy among bakers. *Allergy* **45**, 204–208.
- Tee, R.D., Gordon, D.J., van Hage-Hamsten, M., Gordon, S., Nunn, A.J., Johansson, S.G.O. & Newman Taylor, A.J. (1992) Comparison of allergic responses to dust mites in U.K. bakery workers and Swedish farmers. *Clin. Exp. Allergy* **22**, 233–239.
- Blainey, A.D., Topping, M.D., Ollier, S. & Davies, R.J. (1989) Allergic respiratory disease in grain workers: the role of storage mites. *J. Allergy Clin. Immunol.* **84**, 296–303.
- Armentia, A., Martínez, A., Castrodeza, R., Martínez, J., Jimeno, A., Méndez, J. & Stolle, R. (1997) Occupational allergic disease in cereal workers by stored grain pests. *J. Asthma* **34**, 369–378.
- Gaig, P., Botey, J., Pena, M., Marín, A. & Eserverri, J.L. (1993) Study of sensitisation to storage mites in a pediatric population in Barcelona. *J. Invest. Allergol. Clin. Immunol.* **3**, 151–155.
- Ebner, C., Feldner, H., Ebner, H. & Kraft, D. (1994) Sensitization to storage mites in house dust mite (*Dermatophagoides pteronyssinus*) allergic patients. Comparison of a rural and an urban population. *Clin. Exp. Allergy* **24**, 347–352.
- Vidal, C., Chomón, B., Pérez-Carral, C. & González-Quintela, A. (1997) Sensitization to *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, and *Acarus siro* in patients allergic to house dust mites (*Dermatophagoides* spp). *J. Allergy Clin. Immunol.* **100**, 716–718.
- van der Heide, S., Niemeijer, N.R., Hovenga, H., de Monchy, J.G.R., Dubois, A.E.J. & Kauffman, H.F. (1998) Prevalence of sensitization to the storage mites *Acarus siro*, *Tyrophagus putrescentiae* and *Lepidoglyphus destructor* in allergic patients with different degrees of sensitization to the house-dust mite *Dermatophagoides pteronyssinus*. *Allergy* **53**, 426–430.
- Warner, A., Boström, S., Möller, C. & Kjellman, N.I.M. (1999) Mite fauna in the home and the sensitivity to house-dust and storage mites. *Allergy* **54**, 681–690.
- Iversen, M. & Dahl, R. (1990) Allergy to storage mites in asthmatic patients and its relation to damp housing conditions. *Allergy* **45**, 81–85.
- Johansson, E., Johansson, S.G.O. & van Hage-Hamsten, M. (1994) Allergenic characterisation of *Acarus siro* and *Tyrophagus putrescentiae* and their cross-reactivity with *Lepidoglyphus destructor* and *Dermatophagoides pteronyssinus*. *Clin. Exp. Allergy* **24**, 743–751.
- Schmidt, M., Olsson, S., van der Ploeg, I. & van Hage-Hamsten, M. (1995) cDNA analysis of the mite allergen Lep d 1 identifies two different isoallergens and variants. *FEBS Lett.* **70**, 11–14.
- Olsson, S., van Hage-Hamsten, M., Whitley, P., Johansson, E., Hoffman, D.R., Gafvelin, G. & Schmidt, M. (1998) Expression of two isoforms of Lep d 2, the major allergen of *Lepidoglyphus destructor*, in both prokaryotic and eukaryotic systems. *Clin. Exp. Allergy* **28**, 984–991.
- Johansson, E., Eriksson, T.L.J., Olsson, S., Kronqvist, M., Whitley, P., Johansson, S.G.O., Gafvelin, G. & van Hage-Hamsten, M. (1999) Evaluation of specific IgE to the recombinant group 2 mite allergens Lep d 2 and Tyr, p. 2 in the Pharmacia CAP system. *Int. Arch. Allergy Immunol.* **120**, 43–49.
- Kronqvist, M., Johansson, E., Magnusson, C.G.M., Olsson, S., Eriksson, T.L.J., Gafvelin, G. & van Hage-Hamsten, M. (2000) Skin prick test and serological analysis with recombinant group 2

- allergens of the dust mites *L. destructor* and *T. putrescentiae*. *Clin. Exp. Allergy* **30**, 670–676.
23. Scheiner, O. & Kraft, D. (1995) Basic and practical aspects of recombinant allergens. *Allergy* **50**, 384–391.
 24. Cramer, R., Janussi, R., Menz, G. & Blaser, K. (1995) Display of expression products of cDNA libraries on phage surfaces: a versatile screening system for selective isolation of genes by specific gene-product/ligand interaction. *Eur. J. Biochem.* **226**, 53–58.
 25. Cramer, R. (1998) Cloning of *Aspergillus fumigatus* allergens, from nucleotide sequences to clinical applications. *Int. Arch. Allergy Immunol.* **115**, 99–114.
 26. Lindborg, M., Magnusson, C.G.M., Zargari, A., Schmidt, M., Scheynius, A., Cramer, R. & Whitley, P. (1999) Selective cloning of allergens from the skin colonizing yeast *Malassezia furfur* by phage display technology. *J. Invest. Dermatol.* **113**, 101–106.
 27. Kleber-Janke, T., Cramer, R., Appenzeller, U., Schlaak, U. & Becker, W.-M. (1999) Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int. Arch. Allergy Immunol.* **119**, 265–274.
 28. Magnusson, C.G.M., Aalberse, R.C. & Johansson, S.G.O. (1986) Monoclonal antibodies against human IgE. Identification of determinants restricted to IgE of the lambda light chain type. *Int. Arch. Allergy Appl. Immunol.* **80**, 329–332.
 29. Kronqvist, M., Johansson, E., Pershagen, G., Johansson, S.G.O. & van Hage-Hamsten, M. (1999) Increasing prevalence of asthma over 12 years among dairy farmers on Gotland: storage mites remain dominant allergens. *Clin. Exp. Allergy* **29**, 35–41.
 30. Borriello, F. & Krauter, K.S. (1990) Reactive site polymorphism in a murine protease inhibitor gene family is delineated using a modification of the PCR reaction (PCR+1). *Nucleic Acids Res.* **18**, 5481–5487.
 31. Lin, K.-L., Hsieh, K.-H., Thomas, W.R., Chiang, B.-L. & Chua, K.-Y. (1994) Characterisation of Der p 5 allergen, cDNA analysis, and IgE mediated reactivity to the recombinant protein. *J. Allergy Clin. Immunol.* **94**, 989–996.
 32. Arruda, K., Fernández-Caldas, E., Naspitz, C., Montealegre, F. & Chapman, M. (1995) Identification of *Blomia tropicalis* allergen Blo t 5 by cDNA cloning. *Int. Arch. Allergy Immunol.* **107**, 456–457.
 33. Caraballo, L., Avjioglu, A., Marrugo, J., Puerta, L. & Marsh, D. (1996) Cloning and expression of complementary DNA coding for an allergen with common antibody-binding specificities with three allergens of the house dust mite *Blomia tropicalis*. *J. Allergy Clin. Immunol.* **98**, 573–579.
 34. WHO/IUS Allergen Nomenclature Subcommittee (1995) Allergen nomenclature. *Clin. Exp. Allergy* **5**, 27–37.
 35. Shen, H.-D., Chua, K.-Y., Lin, K.-L., Hsieh, K.-H. & Thomas, W.R. (1993) Molecular cloning of a house dust mite allergen with common antibody binding specificities with multiple components in mite extract. *Clin. Exp. Allergy* **23**, 934–940.
 36. Shen, H.-D., Chua, K.-Y., Lin, K.-L., Hsieh, K.-H. & Thomas, W.R. (1995) Molecular cloning and immunological characterisation of the house dust mite allergen Der f 7. *Clin. Exp. Allergy* **25**, 1000–1006.
 37. von Heijne, G. (1985) Signal sequences, the limits of variations. *J. Mol. Biol.* **184**, 99–105.
 38. Caraballo, L., Puerta, L., Jiménez, S., Martínez, B., Mercado, D., Avjioglu, A. & Marsh, D. (1997) Cloning and IgE binding of a recombinant allergen from the mite *Blomia tropicalis*, homologous with fatty acid-binding proteins. *Int. Arch. Allergy Immunol.* **112**, 341–347.
 39. Eriksson, T.L.J., Whitley, P., Johansson, E., van Hage-Hamsten, M. & Gafvelin, G. (1999) Identification and characterisation of two allergens from the dust mite *Acarus siro*, homologous with fatty acid-binding proteins. *Int. Arch. Allergy Immunol.* **119**, 275–281.
 40. Bairoch, A., Bucher, P. & Hofmann, K. (1997) The PROSITE database: its status in 1997. *Nucleic Acids Res.* **24**, 217–221.
 41. Reese, G., Ayuso, R. & Lehrer, S.B. (1999) Tropomyosin: an invertebrate pan-allergen. *Int. Arch. Allergy Immunol.* **119**, 247–258.
 42. Olsson, S., van Hage-Hamsten, M. & 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.
 43. Levitan, B. (1998) Stochastic modelling and optimisation of phage display. *J. Mol. Biol.* **277**, 893–916.