

## Expression of two isoforms of Lep d 2, the major allergen of *Lepidoglyphus destructor*, in both prokaryotic and eukaryotic systems

S. OLSSON\*, M. VAN HAGE-HAMSTEN\*, P. WHITLEY\*, E. JOHANSSON\*, D. R. HOFFMAN†, G. GAFVELIN\* and M. SCHMIDT\*‡

\*Department of Laboratory Medicine, Division of Clinical Immunology, Karolinska Institute & Hospital, Stockholm, Sweden and Departments of †Pathology and Laboratory Medicine and ‡Biology and Physics, East Carolina University, Greenville, NC, USA

### Summary

**Background** The dust mite *Lepidoglyphus destructor* is a major cause of allergic diseases among farmers. We have previously cloned and sequenced two isoforms of the major allergen Lep d 2 (formerly designated Lep d 1) and found significant homology to group 2 allergens of the house dust mite species *Dermatophagoides*. We now report on the production and characterization of recombinant Lep d 2.

**Objective** We have expressed both isoforms in two different expression systems; a eukaryotic system, baculovirus in insect cells and a prokaryotic system, *E. coli*. We have compared the two systems in regard to production yields and immunoreactivity of the recombinant allergens.

**Methods** The complete cDNA including the natural leader sequence was cloned into the pBlueBacIII transfer vector, and the rLep d 2 was produced as a secreted protein in baculovirus. For the expression in *E. coli*, the cDNA was cloned into the pET vector, and the rLep d 2 was produced with six C-terminal histidine residues.

The purified recombinant allergens were tested for immunoreactivity with 10 sera from subjects allergic to *Lepidoglyphus destructor* and were compared with native Lep d 2 using inhibition immunoblotting. The ability of the recombinant allergens to release histamine from basophils was evaluated using a histamine release assay.

**Results** Both expression systems produced immunoreactive recombinant allergens. They inhibited the binding of human sera to native Lep d 2 confirming their retained IgE binding properties. The yield of pure recombinant protein from the prokaryotic system was  $\approx 1$  mg/L compared to the eukaryotic system which produced up to 4 mg/L in an adherent cell culture system.

**Conclusions** We have produced recombinant Lep d 2 in prokaryotic and eukaryotic expression systems which are comparable to the native allergen. Recombinant Lep d 2 might now be included in more extensive clinical studies to confirm its usefulness in the *in vitro* and the *in vivo* diagnosis of *Lepidoglyphus destructor*.

**Keywords:** allergy, baculovirus, dust mite, *E. coli*, isoforms, Lep d 2, *Lepidoglyphus destructor*, recombinant allergen

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## Introduction

Allergy to the non-pyroglyphid mites is a well known problem in farming environments. Since these mite species may also be a part of the house dust fauna in damp dwellings, urban people are also at risk of becoming sensitized [1–3]. *Lepidoglyphus destructor*, a member of the *Glycyphagidae* family, is ubiquitous in Europe, and a predominant cause of storage mite allergy [4–7]. In the USA, Marx *et al.* have shown that seven out of eight Wisconsin dairy farmers have a positive RAST and skin-prick test to *L. destructor* [8].

More than 20 allergens of *L. destructor* have been identified by immunoblotting experiments [9]. In allergic patients, the IgE antibody response is focused on the major allergen *Lep d 2*. Over 90% of these patients react to this particular allergen.

*Lep d 2* has been well characterized [10–12], and recently two isoforms of this protein have been cloned and sequenced [13]. This allergen was firstly named *Lep d 1*, however, the cDNA sequence revealed over 50% homology with the group 2 allergens from the house dust mite *Dermatophagoides* and the protein was thereby redesignated to *Lep d 2*.

So far, only crude allergen extracts of *L. destructor* have been used for serological assays and skin tests. To be able to improve the standardization of the allergen preparations, recombinant allergens can be used.

Based on the isolation and cloning of natural allergens, it has been shown that many allergens from their natural sources consist of several isoforms [13–17]. These isoforms may be as different in their sequence and structure as allergens from different species. It is of importance to determine their differences in allergenicity, and as a result, they might have to be considered as different allergens. The existence of different isoforms of allergens must be considered when recombinant proteins are used in immunotherapy since all allergenic isoforms may need to be present in the evaluation and treatment of patients [18]. Regarding *L. destructor*, we have shown that the two isoforms of the major allergen, *Lep d 2.01* and *Lep d 2.02*, differ at 13 amino acid positions [13].

Many, but not all allergens expressed from cDNA so far have shown a considerable IgE binding reactivity that seems to be comparable to their natural counterparts. Many of these recombinant allergens are produced in *E. coli* [e.g. 19–21], but unfortunately, the amount and/or reactivity is sometimes reduced when the allergen is purified and subjected to immunological and biochemical assays [e.g. 19,22]. To overcome some of these problems eukaryotic expression systems such as yeast and baculovirus in insect cells have been used [23–26]. In this study we describe the production of the two cloned isoforms of *Lep d 2* in two

different expression systems, in *E. coli* as a histidine tagged protein and as a non fusion protein in insect cells using *Autographa californica* nuclear polyhedrosis virus (AcMNPV). We compare the eukaryotic system with the well established *E. coli* expression system concerning the immuno reactivity of the expressed allergens and the production yields obtained. In addition, we compare the two isoforms with respect to their IgE binding with a panel of patient sera.

## Materials and methods

### *Production of recombinant(r)Lep d 2 in insect cells* *Subcloning of Lep d 2.01 and Lep d 2.02*

The plasmids containing the full length *Lep d 2.01* and *Lep d 2.02* cDNA [13] were used as a DNA template. We designed an upstream primer 5'-GAGGGATCCATGATGAAATTCATTGCTCTGTT-3' containing a *Bam*HI site (underlined), and the downstream primer was 5'-GAG-AAGCTTTTATTTCGACTTGTCCGTGGA-3' containing a *Hind*III site (underlined). A polymerase chain reaction (PCR) was performed to generate full length clones, containing the leader sequence, using Pfu polymerase (Stratagene) under the following cycle conditions: an initial denaturation at 94 °C for 8 min, then 5 units of Pfu polymerase and Perfect Match (Stratagene) were added at 80 °C followed by 20 cycles of amplification (1 min at 94 °C, 2 min at 60 °C and 2.5 min at 72 °C). The PCR products were digested with *Bam*HI and *Hind*III (New England BioLabs) and ligated into the baculovirus transfer vector pBlueBacIII (Invitrogen BV, Leek, The Netherlands) and transformed into top 10 *E. coli* cells (Invitrogen). The cDNA sequence of *Lep d 2.01* and *2.02* was then verified by DNA sequencing.

### *Production of recombinant baculovirus*

The recombinant plasmids were cotransfected with linearized wild-type AcMNPV DNA into Sf 9 cells (Invitrogen) derived from *Spodoptera frugiperda* by the technique of cationic liposome mediated transfection [27].

One µg of linear AcMNPV, 3 µg of the recombinant vector and 20 µL of the Cationic Liposome Solution provided in the MaxBac kit (Invitrogen) were added to log phase Sf 9 cells in a 60 mm culture plate and incubated for 4 h at room temperature. Thereafter, the plates were incubated for 48 h at 27 °C in a humid environment. The Sf 9 cells were cultured in Grace's medium (AMS Biotechnology, Täby, Sweden) including 5% fetal calf sera in 25 and 75 cm<sup>2</sup> culture flasks, kept at 27 °C in the dark. A plaque purification assay was performed to detect recombinant clones. Harvested media from the transfections were prepared in 10-fold dilutions, added to freshly seeded Sf 9 cells

in a 100 mm culture plate and incubated for 1 h at room temperature. The cells were then overlaid with 0.7% agarose containing 150 µg/mL of the chromogenic substrate Bluo-Gal (GIBCO/BRL). Blue positive clones were verified to be recombinant by PCR with specific baculovirus primers provided by Invitrogen. To ensure the purity of the clones, further rounds of plaque purification were performed. The recombinant viruses were then transfected into the high producing cell line High-Five (Invitrogen), derived from *Trichoplusia ni*, growing in serum free medium Ex-Cell 400 (AMS Biotechnology). The supernatants were harvested after 4 days and the proteins analysed.

#### *Purification of recombinant Lep d 2 derived from baculovirus*

The supernatants were concentrated 10 times using a SpeedVac centrifuge (Techtum AB, Umeå, Sweden), filtered through a 0.2 µm sterile filter and subjected to a gel filtration on a G50 Sephadex column coupled to a FPLC system (Pharmacia & Upjohn, Uppsala, Sweden). Positive fractions were identified by molecular weight and confirmed by IgE binding in Western blotting. After dialysing and concentrating to a volume of 2.5 mL, the protein solution was fractionated in 0.5 mL amounts on a Vydac C4 column (4.6 × 250 mm) at 45 °C using a gradient of 0–75% of 0.07% trifluoroacetic acid in acetonitrile in 0.1% TFA in water. The Lep d 2 containing fractions were identified by comparison with an authentic sample and SDS-PAGE on a 20–27% gradient gel. The purified fractions were pooled and concentrated using a centrifugal vacuum concentrator.

#### *Production of rLep d 2 in E. coli: subcloning of the cDNA from Lep d 2.01 and Lep d 2.02*

The existing full length clones of Lep d 2.01 and Lep d 2.02 were used as a template for PCR reactions. As an upstream primer we used 5'-GAGCATATGGGCAAGATGACCTTCAA-GGAC-3' containing a *NdeI* site (underlined) and as a downstream primer 5'-GAGAAGCTTAATGATGATGATGATGATGTTTCGACTTGTCCGTGGA – 3' containing 6 histidine codons and a *HindIII* site (underlined). A 20 cycle PCR reaction was performed under the same conditions as for the baculovirus expression. The PCR products which contained no leader sequence, were digested with *NdeI* and *HindIII* and cloned into the pET-17b vector (Novagen, R & D Systems, Abingdon, UK). The recombinant vector was transformed into BL21 *E. coli* cells by electroporation and positive clones were selected by specific restriction digest with *NdeI* and *HindIII* and the cDNA sequence confirmed by DNA sequencing. The selected recombinant plasmids of the two isoforms were transformed into the *E. coli* expression strain BL21(DE3)pLysS cells.

#### *Production and purification of rLep d 2 from E. coli*

Overnight cultures of BL21(DE3)pLysS cells containing the recombinant plasmid were 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 ≈ 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 h at 37 °C. Cells were harvested and inclusion bodies prepared and solubilized according to standard methods [28].

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

#### *Protein concentrations*

Due to the amino acid composition of Lep d 2 with no tryptophan and only one tyrosine, the protein concentration was difficult to measure by absorbance at 280 nm. Instead, an amino acid analysis was performed to determine the molar ratio of the amino acid composition. Hydrolysis was carried out at 110 °C for 24 h in evacuated tubes with 6 M HCl containing 0.5% phenol. Liberated amino acids were determined in a Pharmacia-LKB Alpha Plus 4151. These samples with known concentration were then used as standards on stained SDS-PAGE gels.

#### *Amino acid sequence analysis*

To confirm the cleavage of the leader sequence in the insect cell derived recombinants, the first 10 N-terminal amino acids were sequenced using the Hewlett-Packard G1005 A Protein sequencing system, Routine Method 3.0.

SDS-PAGE was performed with 10 µg of the recombinant protein under reducing conditions (β-mercaptoethanol). After Coomassie Brilliant Blue (CBB)-staining, the zone containing the recombinant was cut out from the gel and eluted in phosphate buffered, 6 M Guanidine-HCl, pH 8. To detect cysteine, the eluted recombinant protein was alkylated in DTT and iodoacetic acid before sequencing.

#### *Patient sera*

Sera from 10 allergic patients with positive RAST (Pharmacia and Upjohn Diagnostics AB) values (3.5–92.5 kU/L) to *L. destructor* were used. These patients are farmers from the island of Gotland in Sweden who are naturally exposed to *L. destructor* and have symptoms of asthma and/or rhinoconjunctivitis. Five of these *L. destructor* positive sera were pooled and used in the initial screening of the recombinant proteins in

Western blotting and thereafter used as a positive control in the dot blots. In dot blots RAST positive sera were used individually and were diluted to  $\approx 2.5$  kU/L. As negative controls a non-atopic patient with a negative RAST value to *L. destructor* and an atopic with a high total IgE value (approximately 1500 kU/L) but negative to *L. destructor* were used. In Western blotting and inhibition blotting a serum with a RAST value of 92.5 kU/L to *L. destructor* was used at a 1/10 dilution.

For the histamine release assay a *L. destructor* allergic patient (8.6 kU/L) and a non-atopic control subject were used. All sera used were stored at  $-20^{\circ}\text{C}$ .

#### Preparation of mite extract

Extract of *L. destructor* was prepared by mixing raw material (Allergon AB, Ängelholm, Sweden) with 0.9% NaCl containing 0.02%  $\text{NaN}_3$  at 1/10 weight/volume. The mixture was incubated overnight at room temperature and subsequently centrifuged at  $6600 \times g$  for 10 min and the collected supernatants were filtered through an  $0.8\text{-}\mu\text{m}$  Miller-HA filter. Lep d 2 is soluble in 60% ammonium sulphate and in order to enrich this allergen the prepared mite extract was treated with 3.3 M ammonium sulphate for 30 min. The soluble fraction was dialysed against PBS and analysed on SDS-PAGE gel and immunoblotting with a *L. destructor* RAST positive patient. This patient reacts to many of the allergens in *L. destructor* as well as to Lep d 2, but in this fraction only Lep d 2 was detected as can be seen in the first lane in Fig. 1(b). This fraction, containing enriched and partly purified Lep d 2, is referred to as native(n)Lep d 2.

#### Sodium dodecylsulphate-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, Richmond, CA, USA). Gels were stained in CBB. The proteins ( $0.5\text{ }\mu\text{g/well}$ ) were separated on 15% polyacrylamide gels and transferred to nitrocellulose (NC) membrane by electroblotting. The immuno staining was performed using a RAST positive serum to *L. destructor* with known reactivity to Lep d 2 (92.5 kU/L) at a dilution of 1/10. We also used the monoclonal antibody (MoAb) 1112D8, previously produced against nLep d 2 followed by a detection method described before [29].

#### Blotting inhibition

A RAST positive serum to *L. destructor* (92.5 kU/L) was preincubated with a mixture of the two recombinant isoforms of Lep d 2 ( $0\text{--}10\text{ }\mu\text{g}$ ) for 1 h at room temperature. The final dilution of the serum was 1/10. The mixture was then incubated with NC membrane strips containing blotted nonreduced nLep d 2. After washing away unbound antibody complexes the reaction was detected as described [29].

#### Dot-blotting

The Dot blot were carried out with a Bio-Dot Microfiltration apparatus from Bio Rad and NC membrane. To each well  $0.5\text{ }\mu\text{g}$  of protein, diluted in a phosphate buffer, was applied using gravity flow for  $\approx 1$  h. The membranes were then taken out of the chamber, blocked with a phosphate buffer

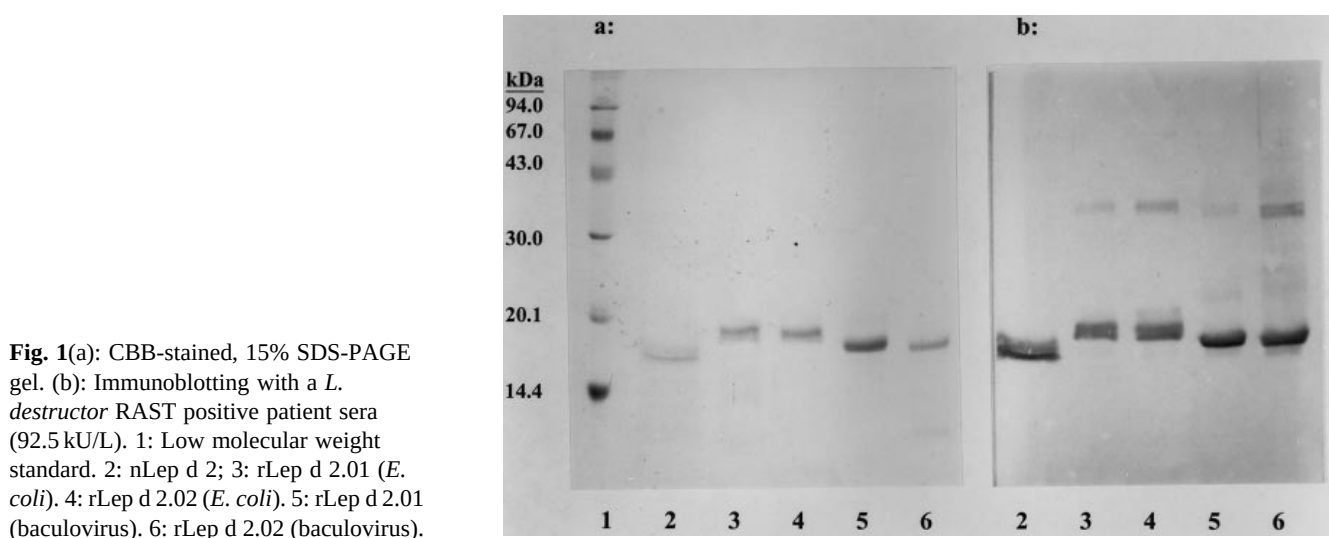


**Fig. 1**(a): CBB-stained, 15% SDS-PAGE gel. (b): Immunoblotting with a *L. destructor* RAST positive patient sera (92.5 kU/L). 1: Low molecular weight standard. 2: nLep d 2; 3: rLep d 2.01 (*E. coli*). 4: rLep d 2.02 (*E. coli*). 5: rLep d 2.01 (baculovirus). 6: rLep d 2.02 (baculovirus).

including BSA and 0.5% Tween 20 for 30 min at 37 °C, and cut into strips. The following incubations were performed each for 2 h under the same conditions as for Western blotting. The sera, diluted in PBS buffer containing 0.5% Tween and 0.2% BSA, were added to the strips. A rabbit-antihuman IgE antibody (Miab, Uppsala, Sweden) was used as a secondary antibody, followed by an incubation with a goat-antirabbit antibody conjugated with alkaline phosphatase (BioRad). The visualization of the reactions was completed with a substrate containing 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (BioRad).

#### Histamine release assay

Whole blood was challenged with various concentrations of rLep d 2, nLep d 2 and polyclonal IgG-anti-IgE (dose range 0.01–10 µg/mL) for 30 min at 37 °C. The released histamine was measured in the supernatant by radio immunoassay (Immunotech, Marseille, France). The total histamine was measured after cell lysis by freezing and thawing twice. The spontaneous histamine release was measured in an unchallenged sample. All measurements were performed in duplicates.

## Results

#### Expression and purification of rLep d 2 in baculovirus

After the transfection into Sf9 insect cells, recombinant clones were isolated by plaque assay with Bluo-gal to detect  $\beta$ -galactosidase producing clones. For each isoform, 11 positive clones were verified to be recombinant using PCR technique. Three clones with a single PCR band at the expected size of 1056 bp for the vector containing our insert were chosen for production analysis. The primers for this study were designed to incorporate the natural 16 amino acid leader sequence of Lep d 2 allowing the protein to be secreted into the medium.

The three selected clones, transfected into High Five cells, were screened for expression of rLep d 2 by SDS-PAGE and for their IgE binding capacity in immunoblotting using the *L. destructor* positive serum pool. One clone of each isoform was selected for further experiments.

The yield from the adhesion cell culture corresponded to a concentration of  $\approx$  2 mg/L for isoform 01 and 4 mg/L for isoform 02. The molar ratios from the amino acid determination were in agreement with the known amino acid composition of Lep d 2, confirming the purity of the recombinant proteins.

#### Expression and isolation of rLep d 2 in *E. coli*

The pET vector was used to express rLep d 2 as a histidine tagged protein in *E. coli*. The vector was cleaved with *Nde* I

which results in the start codon ATG in the restriction site without any additional amino acids. The downstream primer was designed to contribute six extra histidines at the 3' terminus and this tag was used for purification of the expressed protein over a metal affinity resin column. The purified Lep d 2 was eluted at a concentration of 0.5–1 mg/mL corresponding to a concentration of 1 mg/L of cell culture. The purity of the rLep d 2 produced in this system was also confirmed in the amino acid analysis.

#### Characterization of rLep d 2.01 and rLep d 2.02

Fig. 1(a) shows the purified recombinant proteins analysed by SDS-PAGE and CBB staining under reducing conditions. Both isoforms from the two expression systems appear at approximately the same size as nLep d 2, with the *E. coli* derived recombinants slightly higher due to their six extra histidines.

N-terminal amino acid analysis of both isoforms produced in the baculovirus system revealed the sequence GKMTFKDCGH which is consistent with the N-terminal sequence of the nLep d 2 [11,12]. No contaminating sequence or signal sequence were detected, confirming the correct cleavage site of the 16 amino acid long natural leader sequence.

#### Immunoreactivity of rLep d 2

Fig. 1(b) shows the reactivity of IgE to the blotted rLep d 2 compared with nLep d 2. The reaction to rLep d 2 seems comparable to nLep d 2. Some IgE binding can be observed also to what probably are multimers of rLep d 2.

Fig. 2 shows the inhibition of IgE-binding to nLep d 2 when the sera are preincubated with a mixture of the two recombinant isoforms. A strong inhibition was obtained even with the lowest concentration of rLep d 2 (0.1 µg)

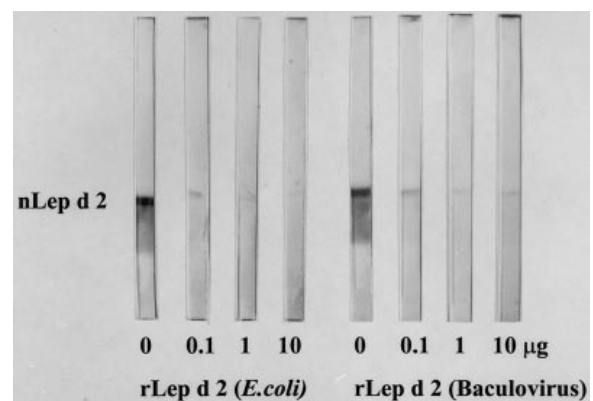


**Fig. 2.** Inhibition of IgE binding from a *L. destructor* RAST positive patient sera (92.5 kU/L) to blotted nLep d 2 with serial dilutions (0–10 µg) of rLep d 2 from *E. coli* and baculovirus.

from both expression systems confirming that the IgE binding capacity of rLep d 2 is similar to nLep d 2. We also performed the inhibition with the two isoforms separately (data not shown) and obtained the same results as for the mixtures.

Concerning the binding of the MoAb directed against nLep d 2, a clear reaction was obtained in Western blotting to all rLep d 2 (results not shown).

Figures 3 and 4 show the reactions of individual sera against rLep d 2.01 and rLep d 2.02 from baculovirus (Fig. 3) and *E. coli* (Fig. 4) in dot blot experiments. The *L. destructor* RAST positive sera (1–10) and the positive serum pool (11) reacted with various intensities to all rLep d 2.

#### Induction of histamine release

To evaluate the capacity of recombinant and native Lep d 2 to induce histamine release, basophils from an allergic patient were challenged with different dilutions (0.01–10  $\mu\text{g/mL}$ ) of nLep d 2 and rLep d 2. 01 and 02 from both baculovirus and *E. coli*. The results expressed as percentage of the total histamine release are shown in Fig. 4. The nLep d 2 gave the highest release, close to 100% at a concentration of 0.1  $\mu\text{g/mL}$ . Both isoforms of rLep d 2 from the two expression systems induced a histamine release of 70–84% at concentrations of 0.1–1  $\mu\text{g/mL}$ .

The spontaneous histamine release was 1% of the total. A

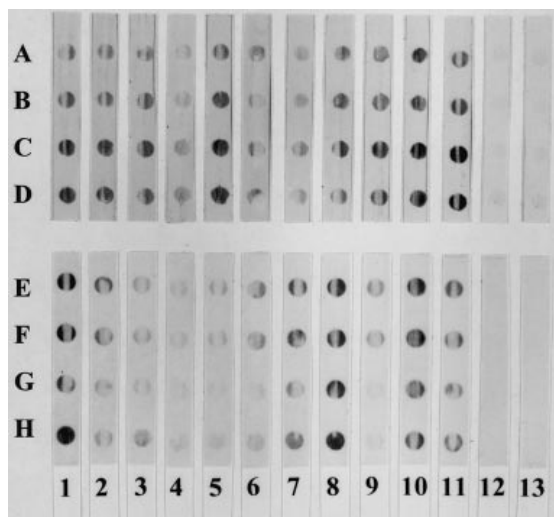


**Fig. 3.** Dot Blots with rLep d 2 from *E. coli* and baculovirus detected by individual *L. destructor* RAST positive sera (1–10), five pooled RAST positive sera (11), high total IgE control, RAST negative to *L. destructor* (12) and a RAST negative sera to *L. destructor* (13). A and B: rLep d 2.01 (*E. coli*); C and D: rLep d 2.02 (*E. coli*); E and F: rLep d 2.01 (baculovirus); G and H: rLep d 2.02 (baculovirus).

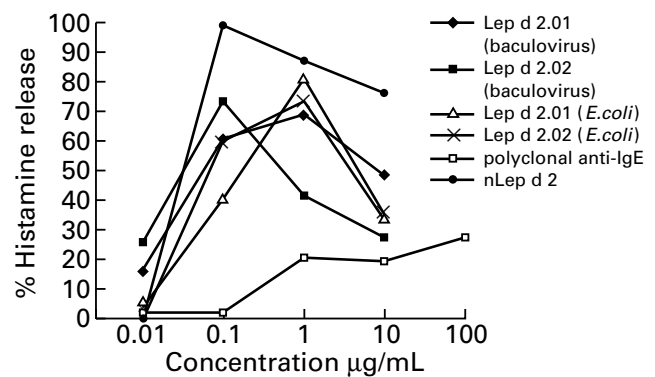


**Fig. 4.** Induction of histamine release from basophils of an allergic patient with different concentrations of nLep d 2, rLep d 2 from *E. coli* and baculovirus (0.01–10  $\mu\text{g/mL}$ ) and a polyclonal anti-IgE control (0.01–100  $\mu\text{g/mL}$ ). Results are expressed as percentage of the total histamine release.

control subject with negative RAST value to *L. destructor* showed no release when challenged with nLep d 2 or rLep d 2.

#### Discussion

To prepare pure recombinant Lep d 2 in a fully immunoreactive form and in significant amounts, we studied the production in two different expression systems, a prokaryotic (*E. coli*) and a eukaryotic system (baculovirus). The *E. coli* system is a well-established system offering many advantages: Easy handling of the bacteria cells and selection of a large variety of vectors using different promoters [30]. Among the disadvantages, overexpressed proteins can be incorrectly folded and may require chemical refolding procedures to obtain the protein in a native, fully active, biological form [31]. We chose to produce rLep d 2 in the pET vector as a histidine tagged protein to facilitate the purification procedure.

The eukaryotic baculovirus expression system is characterized by an extensive array of post-translational processing typical for higher eukaryotic cells. The production of recombinant proteins in this system offers the advantage that secreted proteins are often glycosylated and disulphide-bonded correctly leading to a biologically active conformation [32–34].

The High Five cells we used can be adapted to large scale suspension culture. Thus, it should not be difficult to scale up the production of rLep d 2 and increase the production yield further.

Regarding the immunoreactivity, both isoforms of Lep d 2 produced in the two systems, showed IgE-binding to all sera from the mite allergic patients tested.

It is very important to evaluate the immunoreactivity of the pure rLep d 2 as this will play a role in many

future applications, such as skin prick tests and immunotherapy. We could show that the rLep d 2 from both systems clearly inhibited the binding of IgE to native Lep d2 even in low concentrations. We can conclude that the recombinant allergens have comparable epitope structure as the native form. However, the inhibition was not complete which could indicate that there are some structural differences. Another probable explanation may be the existence of more isoforms in the natural extract that harbour some additional IgE epitopes. We also used a monoclonal antibody raised against native Lep d 2 to detect rLep d 2 and obtained a good reaction in immunoblotting. This further substantiates our conclusion that the rLep d 2 produced in both systems are comparable to the native counterpart.

We could also show that the rLep d 2 is able to bind to IgE receptors on basophils from an allergic patient and trigger them to release histamine in a comparable way as the native allergen preparation.

When comparing the immunological reactivities of rLep d 2 in the two different expression systems, we conclude that they are comparable. The IgE binding capacity of the individual sera varied in both systems and in some cases between the isoforms. This is not surprising and should be expected since they also react differently to this allergen in the crude *L. destructor* extract.

An additional reason that the IgE-binding varies may be differences in the conformation in rLep d 2 when produced in different expression systems that effects the epitope structure. The fact that multimer formation could be observed when rLep d 2 was analysed on gels indicates that there may be some structural alterations.

We have cloned two isoforms of Lep d 2 but more isoforms may exist in nature as is the case for many other allergens [14–17]. The birch pollen allergen Bet v 1 has been shown to consist of over 20 isoforms which differ in their immunoreactivity [18]. It is of great importance to evaluate the number of isoforms and their individual immunogenicity when recombinant allergens are to be used in immunotherapy since the reaction pattern of different patients may vary significantly. This knowledge opens the possibility to create a tailor-made treatment for each individual patient.

Further studies with a large number of patient sera as well as skin-prick tests are necessary to further evaluate the immunological data and especially confirm the usefulness of the recombinant Lep d 2 in the diagnosis of dust mite allergy.

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