Eur. J. Biochem. **269**, 3086–3092 (2002) © FEBS 2002 doi:10.1046/j.1432-1033.2002.02990.x

IgE reactivity of tandem repeats derived from cockroach allergen, Bla g 1

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Sensitization to cockroach allergens is associated with the development of asthma. Bla g 1 is a German cockroach allergen that shows allergenic cross-reactivity with American cockroach allergen, Per a 1, and has a molecular structure composed of multiple tandem amino-acid repeats. Two consecutive repeats are not identical but form a duplex that constitutes a basic molecular unit of Bla g 1. By molecular mass, purified natural Bla g 1 would contain approximately two duplexes. We investigated the pattern of IgE antibody binding to this repeated structure, and whether one or two duplexes are sufficient for IgE binding. Recombinant (r)Bla g 1 duplexes were expressed in *Escherichia coli* and in *Pichia pastoris*, and analyzed for monoclonal antibody and IgE antibody binding by ELISA and/or immunoblotting. Optimal rBla g 1 expression was obtained using methanol-inducible *P. pastoris* (> 95% pure protein, yield ≈ 48 mg·L^{-1}), and rBla g 1 was produced as multiple molecular forms of molecular mass 43, 32, 21 and 6 kDa, that were the result of proteolytic cleavage. There was an excellent correlation between IgE antibody binding to natural and recombinant Bla g 1 ($r = 0.91$, $n = 29$, $P < 0.001$), and immunoblot analysis showed that a single Bla g 1 duplex was sufficient for IgE antibody binding. The rBla g 1 is suitable for structural studies and a candidate for clinical use in diagnosis of cockroach allergy and development of new forms of immunotherapy.

Keywords: allergen; hypersensitivity; IgE antibody; asthma; cockroach.

Sensitization to cockroach allergens can lead to the development of allergic respiratory diseases, including asthma, in susceptible individuals [1]. In the US, this problem is particularly important in inner city areas where infestation by the German cockroach (*Blattella germanica*) is common [2–5]. Several German cockroach allergens have been cloned including Bla g 1, Bla g 2, Bla g 4, Bla g 5 and Bla g 6 [6–10]. Bla g 1 is the only German cockroach allergen that shows antigenic cross-reactivity with an American cockroach (*Periplaneta americana*) allergen, Per a 1 [7,11–14]. Bla g 1 and Per a 1 share $\approx 70\%$ sequence identity, and the prevalence of IgE antibodies to the group I allergens in cockroach allergic patients is 30–50%. Measurement of Bla g 1 levels in homes has been used to assess environmental exposure to cockroach allergens and exposure to > 2 U·g^{-1} Bla g 1 is a strong risk factor for sensitization [5,15]. A recent report suggested that exposure to Bla g 1 or Bla g 2 at 3 months of age predicted allergen-specific lymphoproliferative responses at 2 years, and repeated wheeze in the first year of life [16].

The novel structural feature of the group 1 allergens is that they comprise multiple tandem repeats of ≈ 100 amino-acid residues and appear to be derived from a ≈ 90 kDa precursor [6,7]. At the DNA level, the degree of homology between alternate amino-acid repeats is higher (91–95%) than between consecutive repeats (44–50%). At the protein level, the degree of homology between alternate amino-acid repeats is also higher (96–98%), but the homology between consecutive repeats is low (26–29%). Two consecutive amino-acid repeats (a duplex) comprise a distinct molecular unit of Bla g 1. Natural Bla g 1 was identified and purified as a protein with a molecular mass consistent with the presence of approximately two duplexes (molecular mass ≈ 42 kDa) [11,12].

Knowledge of the IgE binding epitopes of allergens is important for developing new hypoallergenic products for immunotherapy, as has been carried out for other indoor allergens such as Der p 2 [17]. The nature of IgE epitopes on cockroach allergens has not been studied in detail. In the case of Bla g 1, the first step was to investigate the pattern of IgE binding to the tandem repeat structure of Bla g 1, specifically, whether one or two duplexes were necessary for IgE binding, and whether folding of the duplexes was necessary to create an IgE binding epitope. Bla g 1 proteins were expressed in high level expression systems (*E. coli* or *P. pastoris*) and analyzed for IgE antibody binding. Although the optimal expression was obtained using the methanol-induced *P. pastoris* system, interesting observations about expression of repeated DNA structures were found from *E. coli* expression. The results show that a single Bla g 1 duplex retains epitopes necessary for IgE antibody binding and that recombinant Bla g 1 has comparable IgE reactivity to the natural allergen.

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(Received 15 February 2002, revised 26 April 2002, accepted 9 May 2002)

MATERIALS AND METHODS

Expression of rBla g 1 in *E. coli*

Several rBla g 1 constructs were expressed in *E. coli* using the pET system (Novagen, Madison, WI, USA). Inserts encoding for one, two and seven duplexes were obtained by PCR. Inserts with one and two duplexes were simultaneously amplified using the Bla g 1.0101 cDNA that encodes for two duplexes as template, and an N-terminus primer encoding for the sequence GLTL NAKA (which is the beginning of one duplex) [7]. The inserts were separated in a 1% agarose gel and purified. A full DNA insert containing seven duplexes (3898 bp) was also amplified using the full clone Bla g 1.0102 as template, and with an N-terminal primer encoding for the following sequence that contains the McGleogh cleavage site (▼): MG▼KSIPSTR. Therefore, the insert would encode for the full protein including the N-terminus [6,7]. Vectors pET 22b(+) and pET 21d(+) were digested and inserts were ligated into the *Nco*I and *Xho*I sites. Recombinant Bla g 1 (one duplex) with and without a leader sequence was expressed using the pET 22b(+) and the pET 21d(+) vector, respectively. DNA was transformed to NovaBlue *E. coli* competent cells which are recA$^-$ (Novagen). Expression of rBla g 1 was induced with 1 mM isopropyl thio-β-D-galactoside for 3 h. The protein was expressed in the soluble (cytoplasmic) and insoluble fractions.

Expression of rBla g 1 in *P. pastoris*

Recombinant Bla g 1 was expressed in *P. pastoris* using either the methanol-inducible AOX1 promotor (pPICZαB vector), or the glyceraldehyde-3-phosphate dehydrogenase promotor for constitutive expression (pGAPZαC vector) (Invitrogen, San Diego, CA).

Inducible expression. A DNA insert encoding for two duplexes containing 388 amino acids was amplified using Bla g 1.0101 cDNA as a template (from the amino acid 25 to the stop codon; accession number AF072219). The construct was subcloned into the *Pst*I and *Not*I sites of the pPICZαB vector and linearized using *Bst*XI and electroporated into *Pichia*. Seven Mut^s transformants were obtained from KM17 strain grown on media containing 500 μg·mL^{-1} zeocin, one of which secreted high levels of rBla g 1 after methanol induction (≈ 48 mg·L^{-1}). Cultures were grown as previously described and expression was maintained for 48 h at 28–30 °C [18]. Recombinant Bla g 1 expression was compared by growing *Pichia* in medium equilibrated with 12 M HCl to pH 3, 4, 5 or 6.

Constitutive expression. The Bla g 1.0102 isoform that contained the N-terminal sequence was used as template (accession no. L47595). Inserts were amplified using two primers: one encoding for the start of the N-terminus (MKLAL) and the other for the end of the C-terminus (FGLTH*). The PCR product was analyzed on a 1% agarose gel and the desired bands encoding for one duplex (209 amino acids) or two were purified separately. Inserts were linearized using *Avr*II and subcloned into the pGAPZαC vector using the *Cla*I and *Not*I restriction sites. Two and eight Mut^s transformants expressing one and two duplexes, respectively, were obtained from the KM17 strain grown on media containing 100 μg·mL^{-1} zeocin. Using a single colony, 10 mL of yeast extract/peptone/dextrose medium were inoculated and grown at 28–30 °C in a shaking incubator (250–300 r.p.m.). The following day, 0.1 mL of the overnight culture were used to inoculate 50 mL of medium in a 250-mL baffled flask, and grown for 5 days. Samples (1 mL) were obtained every day to determine optimal expression times. For scale-up, 1 mL of the initial 10 mL culture was used to inoculate 500 mL of medium and grown for 7 days.

Analysis of transformants for integration of Bla g 1 DNA in *P. pastoris* genome.

Yeast genomic DNA was isolated from pPICZαB clones selected by growth under 100 μg·mL^{-1} zeocin (12 clones) and 500 μg·mL^{-1} zeocin (seven clones), using the easy-DNA™ kit (Invitrogen). Negative and positive controls were 1 μL of pPICZαB (100 ng·μL^{-1}) and 1 μL of the recombinant plasmid (182 ng·μL^{-1}), respectively. A PCR was performed with 1 μL DNA as template (7–9 μg·μL^{-1}) from 19 different clones, 8 μL dNTPs (2.5 mM each), 1 μL 5′ AOX1 and 1 μL of 3′ AOX1 primers (100 pmol·μL^{-1}), 32 μL of water, 1.5 μL $MgCl_2$ 50 mM, 5 μL *Taq* 10× reaction buffer, and 0.5 μL Platinum® Taq DNA polymerase (5 U·μL^{-1}, GibcoBRL). The sequences of the primers were: GACTGGTTCCAA TTGACAAGC for 5′ AOX1 and CAAATGGCATT CTGACATCC for 3′ AOX1. PCR incubations were 2 min at 94 °C followed by 24 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, and a final extension for 7 min at 72 °C. PCR products were analyzed on a 1% agarose gel to verify integration of Bla g 1.0101 DNA into the yeast genome.

Purification and sequencing of rBla g 1

Expressed rBla g 1 was purified from culture media by affinity chromatography over a 10A6 monoclonal antibody column as described previously [11] with modifications. Bound rBla g 1 was eluted with 0.05 M glycine in 50% ethylene glycol, pH 10. N-Terminal sequences of purified rBla g 1 (expressed in the methanol-induced *Pichia* expression system) were determined by Edman degradation using a gas phase sequencer (model 470A, applied Biosystems, Foster City, CA, USA) in the Biomolecular Research Facility (University of Virginia). The first eight amino acids were identified.

Analysis and quantification of expressed rBla g 1

Samples were analyzed for protein expression by SDS/PAGE (Pharmacia Phast System) followed by silver staining. Purified rBla g 1 was measured in a quantitative two-site ELISA using mAb 10A6 for allergen capture and polyclonal anti-(rBla g 1) Ig for detection [11].

Measurement of IgE antibody binding to recombinant and natural Bla g 1

'*Chimeric*' *ELISA for Bla g 1-specific IgE*. IgE antibody binding to affinity purified rBla g 1 expressed in *Pichia* (methanol induced) and to natural Bla g 1 was measured by

a two-site ELISA as described previously [19]. Microtiter plates were coated overnight with 1 μg per well of mAb 10A6 and incubated with 100 μL (2 $U \cdot mL^{-1}$) of natural or recombinant Bla g 1 for 1 h. The natural Bla g 1 was a standard prepared from a *Blattella germanica* frass extract and used for ELISA that contains 10 $U \cdot mL^{-1}$ of Bla g 1 (INDOOR Biotechnologies, Inc., Lot #2445). Plates were washed and incubated with serum samples (diluted 1 : 2 and 1 : 10). Bound IgE was detected using biotinylated goat anti-human IgE, followed by streptavidin–peroxidase and a colorimetric substrate [19]. The assay was quantitated using wells coated with anti-Der p 2 mAb αDpx and a chimeric mouse/human IgE anti-(Der p 2) Ig (named 2B12-IgE) to form a control curve [20]. Values for IgE anti-(Bla g 1) were interpolated from the 2B12-IgE control curve [19]. Data were analyzed by linear regression.

Immunoblotting. Recombinant Bla g 1 was separated by SDS/PAGE and blotted onto a poly(vinylidene difluoride) membrane. The membrane was incubated for 2 h with a pool of sera diluted 1 : 2 from five allergic patients with a mean of 1614 U IgE anti-(Bla g 1) per mL measured by a solid-phase radioimmunoassay. The secondary antibody was peroxidase labeled goat anti-(human IgE) Ig (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD, USA) diluted 1 : 10 000. Finally, the SuperSignal® West Pico Chemiluminescent Substrate (Pierce) was used for development of the signal that was detected on film after incubation for 20 s.

RESULTS

Expression of rBla g 1 in *E.coli* and *P. pastoris*

PCR amplification of the inserts. The PCR products obtained in order to express Bla g 1 contained several size amplified inserts, which were consistent with the repeated structure of Bla g 1 (Fig. 1A). Two fragments containing one or two duplexes, respectively, were obtained when Bla g 1.0101 was used as a template, whereas more fragments with different numbers of duplexes were obtained using Bla g 1.0102 as template. By dotplot matrix analysis Bla g 1.0102 contains seven duplexes, and at least five are easily visible in an agarose gel of the PCR products (Fig. 1A) [7]. Bla g 1.0101 was chosen to amplify one or two duplexes because under these conditions the quantity of inserts produced was higher than using Bla g 1.0102 (Fig. 1A).

Recombinant Bla g 1 expressed in *E. coli*

Plasmids with an expression vector plus a ligated insert encoding for Bla g 1 were produced in *E. coli*. However, *E. coli* was unable to maintain a plasmid containing an insert with more than two duplexes despite of the use of a $recA^-$ strain. When inserts containing two or seven (full Bla g 1.0102 clone) duplexes were ligated into the pET 21d(+) vector, the resulting transformant plasmids contained only one or two duplexes. Ligation of more than two duplexes was never achieved (Fig. 1B). Recombinant Bla g 1 (one and two duplexes) was expressed in soluble (cytoplasmic) and insoluble fractions (inclusion bodies) simultaneously. Addition of a leader sequence to the expressed rBla g 1 did not lead the protein to the periplasmic fraction from which it would be easy to purify.

Recombinant Bla g 1 expressed in *Pichia*

Affinity-purified rBla g1 from methanol-induced *Pichia* contained four proteins of 42.6 kDa (two duplexes), 31.9 kDa (one and a half duplex), 21.0 kDa (one duplex) and 6 kDa (Fig. 2A). Reactivity of rBla g 1 by ELISA was higher than to natural Bla g 1, consistent with the polyclonal rabbit antibodies used for detection being raised against rBla g 1 (Fig. 2B). N-Terminal sequencing revealed that the starting sequences of the three main proteins were: NAKASRNL, KYHIRRGV and ASRNLQDD (Fig. 3). The start of these sequences is very close in sequence to the start of natural Bla g 1. Natural Bla g 1 seems to suffer an additional cleavage by trypsin-like enzymes after the arginine residues 34, 131, 132, 226, 323 and 324 [7] (Fig. 3).

Pichia constitutively expressed rBla g 1 at lower levels (from 10 to 200 times lower, depending on the volume of the culture and incubation time) than the *Pichia*-inducible system. Attempts to scale-up constitutive expression from

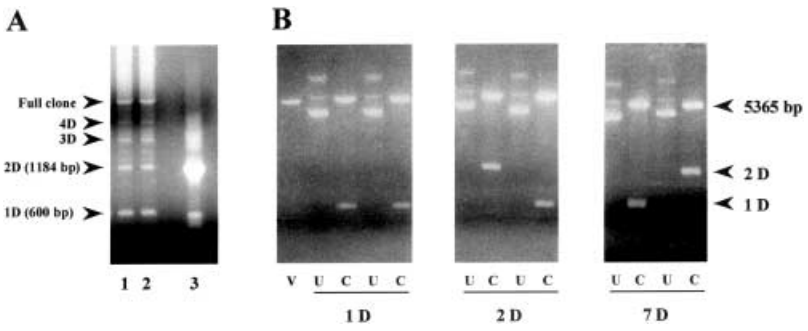

Fig. 1. PCR analysis of rBla g 1 transformants. (A) PCR products of DNA inserts encoding for Bla g 1, using Bla g 1.0102 (lanes 1–2) and Bla g 1.0101 (lane 3) as templates in 1% agarose gel. (B) Analysis of the Bla g 1-transformant plasmids for expression in *E. coli* by 1% agarose gel electrophoresis. The three panels correspond to the results obtained after ligation of one (1 D), two (2 D) or seven (7 D) duplexes to the vector. V indicates the pET 21d(+) vector double digested with *Nco* I and *Xho* I, with a size of 5365 bp. The other lanes correspond to undigested (U) or double digested (C) transformant plasmids. The higher molecular mass band is the vector, and the lower molecular mass bands are the inserts which encode for either one (1 D) or two (2D) duplexes as indicated on the right side of the gel.

5-day cultures of 50 mL to 7-day cultures of 500 mL resulted in a even lower expression when analyzed by SDS/PAGE. Therefore, we focused on the purification and study of the rBla g 1 expressed in methanol-induced *Pichia*.

Origin of expression of rBla g 1 multiple forms

The expression of rBla g 1 in methanol-induced *Pichia* resulted in a mixture of proteins of 42.6, 31.9, 21.0 and 6 kDa (as calculated from the amino-acid sequences). This could have occurred through multiple integrations of expression cassettes in the yeast genome, followed by subsequent recombination events between different repeats and loss of DNA repeats (similar to the recombination observed in *E. coli*). This possibility was discarded by performing PCR amplification of genomic DNA prepared from different clones (selected at 500 and 100 $\mu g \cdot mL^{-1}$ zeocin) using primers flanking the expression cassettes. All the clones showed the same band as the positive control, a transformant plasmid proven to have two duplexes by restriction digestion and used to electroporate *Pichia* (data not shown). Therefore, even if multiple integration took place, recombination did not occur, and all cassettes had two DNA duplexes.

The origin of multiple rBla g 1 forms was also investigated at the protein level by growing the yeast cultures at different pH values. Only at pH 4, expression of rBla g 1 was as expected, with most of the protein containing two duplexes when observed by SDS/PAGE. At pH 3 there was no expression of rBla g 1, and at pH 5 and especially at pH 6, the protein suffered degradation and was broken down to the size of one duplex. Addition of 1 mM phenylmethanesulfonyl fluoride and EDTA to cultures at pH 6 slightly reduced the production of the low molecular mass forms (data not shown).

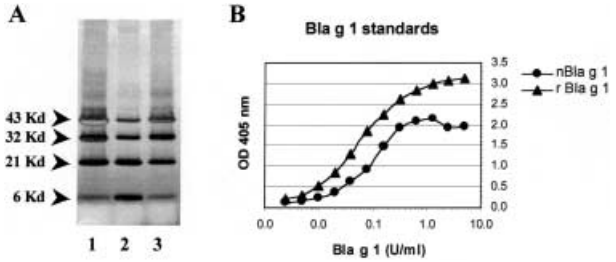

Fig. 2. Recombinant Bla g 1 expressed by methanol-induced P. pastoris. (A) Recombinant Bla g 1 expressed by methanol-induced *P. pastoris* after affinity purification through a 10A6 mAb column. Eluted fractions 7–30 from a culture (lane 1); early (9–14, lane 2); and late (15–30 lane 3), fractions from another culture grown under the same conditions. (B) ELISA activity of rBla g 1 expressed by methanol-induced *P. pastoris* and purified by affinity chromatography compared to the natural allergen.

IgE binding to rBla g 1

A strong correlation was found between binding of IgE antibodies to recombinant (expressed in inducible *Pichia*) and natural Bla g 1, in sera from cockroach allergic patients using a chimeric ELISA ($r = 0.91$, $n = 29$, $P < 0.001$) (Fig. 4). Moreover, Western blot analysis showed that *E. coli*-expressed rBla g 1 containing one or two duplexes, and the *Pichia* expressed rBla g 1 (inducible and constitutive) bound IgE from pooled sera of Bla g 1 allergic patients. Interestingly, a similar pattern of degradation of the two duplexes to one was observed either in *E. coli* or in *P. pastoris* expressed rBla g 1, although *Pichia*-expressed rBla g 1 contained more of the 32-kDa protein (Fig. 5, lanes 2 and 3).

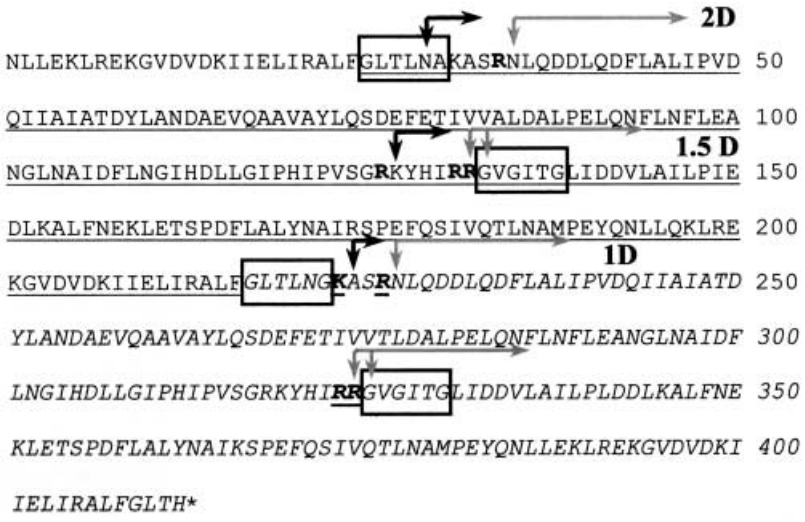

Fig. 3. Myristilation and proteolytic cleavage sites of Bla g 1. The Bla g 1.0101 amino-acid sequence is shown with myristilation sites in boxes that indicate the beginning of the tandem repeats. N-Terminal sequences for recombinant and natural Bla g 1 fragments are shown, commencing at the residues indicated by black and grey arrows, respectively: 29 and 35 for two duplexes (2D), 127 and 132 or 133 for one and a half duplexes (1.5D), and 224 and 227 for one duplex (1D). Basic residues (in bold) are situated before the cleavage sites. One duplex is indicated as underlined sequence and the next one by italics.

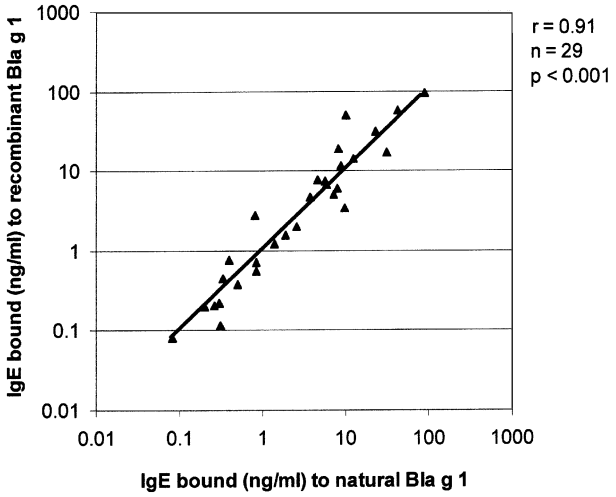

Fig. 4. IgE binding to natural vs. recombinant Bla g 1. Correlation of IgE binding to affinity purified rBla g 1 expressed by methanol-induced *P. pastoris*, compared to natural Bla g 1. Each point represents the serum from a different cockroach-allergic individual.

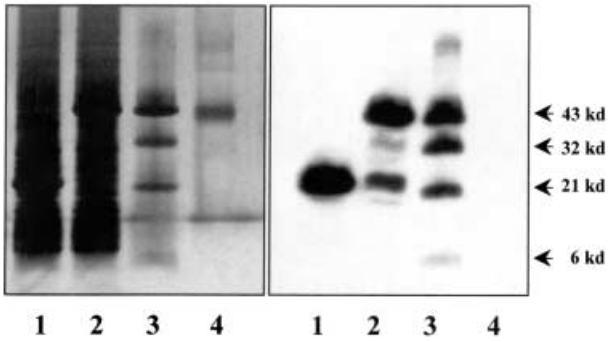

Fig. 5. Western blot analysis of rBla g 1 expressed in *E. coli* and *P. pastoris* using IgE antibodies in a serum pool from Bla g 1 allergic patients (left panel, SDS/PAGE gel; right panel, immunoblot). Cytoplasmic fraction of *E. coli* expressing one (lane 1) and two duplexes (lane 2) of rBla g 1; lane 3, affinity purified rBla g 1 expressed by methanol-induced *P. pastoris*; lane 4, negative control of natural Bla g 2.

DISCUSSION

Bla g 1 was expressed as a recombinant protein in *E. coli* and *P. pastoris*, and bound IgE antibodies from Bla g 1 allergic patients. Optimal expression was obtained using methanol-induced *P. pastoris* system. Using *E. coli*, rBla g 1 was produced in the cytoplasmic and in the insoluble fractions. Attempts to simplify purification by adding a leader sequence that would direct the protein to the bacterial periplasmic fraction failed. Constitutive expression was less productive than the methanol-induced expression in the yeast *P. pastoris*. The added burden of expression has the potential to reduce growth rate so cells that have reduced or switched off expression can grow faster and take over the culture, even though such variants could arise at low frequency (MA Romanos, personal communication). Fortunately, rBla g 1 expressed by methanol-induction in *Pichia* was secreted into the medium and a single mAb affinity purification step was sufficient to obtain > 90% pure allergen. For this reason, methanol-induced *P. pastoris* was the expression system of choice for allergen production and for studies of IgE antibody binding.

E. coli was unable to replicate a plasmid containing more than two Bla g 1 duplexes. There is a strong possibility that this observation is due to a recA independent process called 'replication slippage' by which *E. coli* eliminates repeated DNA in plasmids. When the plasmid replicates, the replication fork 'slips' from one sequence to another because the end of the nascent DNA shares homology with the repeated sequences on the template DNA. A loop of DNA is formed and lost, leading to the formation of a plasmid with fewer repeats [21,22]. This would explain why the full Bla g 1.0102 clone, containing seven duplexes could not be expressed in *E. coli*. Similarly, tandem repeats have been described as a cause for certain unclonable DNA repeated sequences [23,24].

The two other German cockroach allergens that have been expressed in *P. pastoris* (Bla g 2 and Bla g 4) were expressed as single polypeptide chains, whereas rBla g 1 was produced as four discrete proteins [18,25]. N-Terminal sequencing and Western blotting experiments verified that these proteins corresponded to Bla g 1. A similar mixture of proteins has been described for natural Bla g 1 by N-terminal sequencing [7]. The N-terminal sequences of the natural proteins start a few amino acids after trypsin-like cleavage sites in the Bla g 1 sequence, suggesting that natural Bla g 1 undergoes proteolytic digestion resulting in the multiple molecular forms. The presence of multiple molecular forms of Bla g 1 could complicate the interpretation of immunoblotting studies of natural cockroach allergen extracts for allergen identification and characterization. In the case of Bla g 1, the occurrence of multiple bands is not an indication of multiple allergens. From an allergen exposure point of view, it also means that integrity of the allergen is not necessary for allergenicity.

Possible reasons for the generation of multiple molecular forms of rBla g 1 were investigated. PCR analysis of *Pichia* genomic DNA showed that the origin of the multiple forms of the allergen was not at the DNA level. Evidence of recombination that may have led to loss of DNA repeats (as had been seen for DNA introduced in *E. coli*) was not found in the expression cassettes integrated in the *Pichia* genomic DNA. At the RNA level, early termination of protein synthesis from foreign genes is frequent when percentage of A + T in the mRNA is high with AT-rich clusters (> 70%) [26]. However, this was unlikely for Bla g 1 because the percentage of A + T is only 53.9%. Finally, effects at the protein level were explored by studying how pH affects production of Bla g 1. The fact that at pH 4 rBla g 1 is mostly expressed as two duplexes, and that cleavage into multiple forms occurs at higher pH, especially at pH 6, suggests that cleavage may be produced by neutral proteases from *Pichia* that are active at pH 6 and inactive at low pH. The observed cleavage sites in natural and recombinant Bla g 1 occur after a lysine or one or two arginines, which are basic amino acids more susceptible to cleavage. In agreement with this, natural Bla g 1 breaks down into similar molecular mass fragments of 25 kDa and, mostly, 6 kDa on SDS/PAGE [12].

Immunoblot analysis revealed that IgE from Bla g 1 allergic patients binds to all four recombinant Bla g 1 proteins expressed by *P. pastoris*. Strong IgE binding to one duplex expressed either by *E. coli* or by *P. pastoris* clearly indicates that one duplex is sufficient for IgE binding to occur. Therefore, folding of more than one duplex is not necessary to create an IgE binding epitope. Given the repeated structure of Bla g 1, the degree of IgE binding to Bla g 1 fragments will be proportional to the number of duplexes they contain. Interestingly, absence of N-terminus in rBla g 1 did not prevent IgE binding, showing that IgE binding to the duplex does not require the presence of N-terminus in the molecule.

IgE binding studies by 'chimeric' ELISA showed an excellent correlation between antibody binding to natural and to recombinant Bla g 1 expressed in *P. pastoris*. The results suggest that rBla g 1 is a good candidate for studies of allergy diagnosis, when used together with other recombinant cockroach allergens, such as Bla g 2, Bla g 4 and Bla g 5. We have estimated that a cocktail of these four allergens could diagnose > 95% of cockroach allergic patients [9]. Natural cockroach allergenic products contain large amounts of nonallergenic proteins, are prone to form precipitates and may contain proteolytic enzymes. The recombinant cockroach allergens can be formulated at defined concentrations and none of these allergens has proteolytic activity.

The *Pichia* expressed rBla g 1 will enable the three dimensional structure of the allergen to be determined and possible functions of the duplexes to be established. The rBla g 1 will allow further studies of the immune response to cockroach allergens and new immunotherapeutic strategies to be investigated.

ACKNOWLEDGEMENTS

Thanks to Dr Alisa Smith for her advice on the recombinant allergen expression, and to Peter Ngo and Bob Liu for technical assistance. Research described in this article was supported in part by the National Institute of Health Grants AI 32557 and AI 34607, and by Philip Morris Incorporated.

REFERENCES

1. Arruda, L.K., Vailes, L.D., Ferriani, V.P.L., Santos, A.B.R., Pomés, A. & Chapman, M.D. (2001) Cockroach allergens and asthma. *J. Allergy Clin. Immunol.* **107**, 419–428.
2. Call, R.S., Smith, T.F., Morris, E., Chapman, M.D. & Platts-Mills, T.A.E. (1992) Risk factors for asthma in inner city children. *J. Pediatrics* **121**, 862–866.
3. Christiansen, S.C., Martin, S.B., Schleicher, N.C., Koziol, J.A., Hamilton, R.G. & Zuraw, B.L. (1996) Exposure and sensitization to environmental allergen of predominantly Hispanic children in San Diego's inner city. *J. Allergy Clin. Immunol.* **98**, 288–294.
4. Rosenstreich, D.L., Eggleston, P., Kattan, M., Baker, D., Slavin, R.G., Gergen, P., Mitchell, H., McNiff-Mortimer, K., Lynn, H., Ownby, D. & Malveaux, F.N. (1997) The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N. Engl. J. Med.* **336**, 1356–1363.
5. Eggleston, P.A., Rosenstreich, D., Lynn, H., Gergen, P., Baker, D., Kattan, M., Mortimer, K.M., Mitchell, H., Ownby, D., Slavin, R. & Malveaux, F. (1998) Relationship of indoor allergen exposure to skin test sensitivity in inner-city children with asthma. *J. Allergy Clin. Immunol.* **102**, 563–570.
6. Helm, R.M., Cockrell, G., Stanley, J.S., Brenner, R.J., Burks, A.W. & Bannon, G.A. (1996) Isolation and characterization of a clone encoding a major allergen (Bla g 90 kD) involved in IgE mediated cockroach hypersensitivity. *J. Allergy Clin. Immunol.* **98**, 172–180.
7. Pomés, A., Melén, E., Vailes, L.D., Retief, J.D., Arruda, L.K. & Chapman, M.D. (1998) Novel allergen structures with tandem amino acid repeats derived from German and American cockroach. *J. Biol. Chem.* **273**, 30801–30807.
8. Arruda, L.K., Vailes, L.D., Mann, B.J., Shannon, J., Fox, J.W., Vedvick, T.S., Hayden, M.L. & Chapman, M.D. (1995) Molecular cloning of a major cockroach (*Blattella germanica*) allergen, Bla g 2: Sequence homology to the aspartic proteases. *J. Biol. Chem.* **270**, 19563–19568.
9. Arruda, L.K., Vailes, L.D., Hayden, M.L., Benjamin, D.C. & Chapman, M.D. (1995) Cloning of cockroach allergen, Bla g 4, identifies ligand binding proteins (or calycins) as a cause of IgE antibody responses. *J. Biol. Chem.* **270**, 31196–31201.
10. Arruda, L.K., Vailes, L.D., Platts-Mills, T.A.E., Hayden, M.L. & Chapman, M.D. (1997) Induction of IgE antibody responses by glutathione *S*-transferase from the German cockroach (*Blattella germanica*). *J. Biol. Chem.* **272**, 20907–20912.
11. Pollart, S.M., Mullins, D.E., Vailes, L.D., Hayden, M.L., Platts-Mills, T.A.E., Sutherland, W.M. & Chapman, M.D. (1991) Identification, quantitation, and purification of cockroach allergens using monoclonal antibodies. *J. Allergy Clin. Immunol.* **87**, 511–521.
12. Schou, C., Lind, P., Fernández-Caldas, E., Lockey, R.F. & Løwenstein, H. (1990) Identification and purification of an important cross-reactive allergen from American (*Periplaneta americana*) and German (*Blattella germanica*) cockroach. *J. Allergy Clin. Immunol.* **86**, 935–946.
13. Melén, E., Pomés, A., Vailes, L.D., Arruda, L.K. & Chapman, M.D. (1999) Molecular cloning of Per a 1 and definition of the cross-reactive Group 1 cockroach allergens. *J. Allergy Clin. Immunol.* **103**, 859–864.
14. Wu, C.H., Wang, N.M., Lee, M.F., Kao, C.Y. & Luo, S.F. (1998) Cloning of the American cockroach Cr-PII allergens: evidence for the existence of cross-reactive allergens between species. *J. Allergy Clin. Immunol.* **101**, 832–840.
15. Pollart, S.M., Smith, T.F., Morris, E.C., Gelber, L.E., Platts-Mills, T.A.E. & Chapman, M.D. (1991) Environmental exposure to cockroach allergens: analysis with monoclonal antibody-based enzyme immunoassays. *J. Allergy Clin. Immunol.* **87**, 505–510.
16. Finn, P.W., Boudreau, J.O., He, H., Wang, Y., Chapman, M.D., Vincent, C., Burge, H.A., Weiss, S.T., Perkins, D.L. & Gold, D.R. (2000) Children at risk for asthma: home allergen levels, lymphocyte proliferation, and wheeze. *J. Allergy Clin. Immunol.* **105**, 933–942.
17. Smith, A.M., Chapman, M.D., Taketomi, E.A., Platts-Mills, T.A.E. & Sung, S.S. (1998) Recombinant allergens for immunotherapy: a Der p 2 variant with reduced IgE reactivity retains T-cell epitopes. *J. Allergy Clin. Immunol.* **101**, 423–425.
18. Vailes, L.D., Kinter, M.T., Arruda, L.K. & Chapman, M.D. (1998) High-level expression of cockroach allergen, Bla g 4. *Pichia pastoris*. *J. Allergy Clin. Immunol.* **101**, 274–280.
19. Ichikawa, K., Iwasaki, E., Baba, M. & Chapman, M.D. (1999) High prevalence of sensitization to cat allergen among Japanese children with asthma, living without cats. *Clin. Exp. Allergy* **29**, 754–761.
20. Schuurman, J., Perdok, G.J., Lourens, T.E., Parren, P.W., Chapman, M.D. & Aalberse, R.C. (1997) Production of a mouse/human chimeric IgE monoclonal antibody to the house dust mite allergen Der p 2 and its use for the absolute quantification of allergen-specific IgE. *J. Allergy Clin. Immunol.* **99**, 545–550.

21. Bierne, H., Vilette, D., Ehrlich, S.D. & Michel, B. (1997) Isolation of a dnaE mutation which enhances RecA-independent homologous recombination in the *Escherichia coli* chromosome. *Mol. Microbiol.* **24** (6), 1225–1234.
22. Saveson, C.J. & Lovett, S.T. (1997) Enhanced deletion formation by aberrant DNA replication in *Escherichia coli*. *Genetics* **146**, 457–470.
23. Kang, H.K. & Cox, D.W. (1996) Tandem repeats 3′ of the IGHA genes in the human immunoglobulin heavy chain gene cluster. *Genomics* **35**, 189–195.
24. Muller, J.P., le Maire, M., le Hegarat, J.C. & Randsholt, N. (1985) Cloning of the newt *Pleurodeles waltlii* chromosomal DNA. *Gene* **35**, 209–215.
25. Vailes, L.D., Ichikawa, K., Pomés, A., Smith, A.M., Best, E., McDermott, M.J., Jacquet, A. & Chapman, M.D. (2001) Validation of recombinant allergens: ELISA reactivity, IgE antibody binding, and skin test reactivity. *J. Allergy Clin. Immunol.* **107**, S219.
26. Romanos, M.A., Makoff, A.J., Fairweather, N.F., Beesley, K.M., Slater, D.E., Rayment, F.B., Payne, M.M. & Clare, J.J. (1991) Expression of tetanus toxin fragment C in yeast: gene synthesis is required to eliminate fortuitous polyadenylation sites in AT-rich DNA. *Nucleic Acids Res.* **19**, 1461–1467.