Cockroach allergen Bla g 2: An unusual aspartic proteinase

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Background: Enzymatic activity of mite, fungal, and bee venom allergens is thought to potentiate their allergenicity. Bla g 2 is a potent cockroach allergen, but despite sharing sequence homology with aspartic proteinases, it contains critical amino acid substitutions that impair proteolytic activity. The biologic function of Bla g 2 remains unclear.
Objective: We sought to investigate the effects of specific amino acid substitutions on enzymatic activity, and the peptide-binding capability of Bla g 2.
Methods: Site-directed mutagenesis was used to produce a recombinant Bla g 2 mutant (Mut) with corrected canonical triads and a flap region. Another mutant (MutF⁻) was expressed after additional mutations in the flap region of Mut. Bla g 2 wild-type (Wt), Mut, and MutF⁻ were assayed for aspartic proteinase activity, and Bla g 2 Wt was tested for pepstatin binding.
Results: Recombinant Bla g 2 Wt and Mut did not show enzymatic activity in a milk-clotting and hemoglobin assay. By using a modified hemoglobin assay, residual activity inhibited by pepstatin was detected for MutF⁻ and Wt at 20 μg/mL, whereas pepsin was active at a 1000-fold lower concentration. Most of Bla g 2 binding to pepstatin-agarose was nonspecific.
Conclusion: Residual proteolytic activity was found for Bla g 2 at concentrations of approximately 4 mM. This weak activity suggests that proteolysis is not the primary function of this allergen and that it is unlikely to contribute to the allergenicity of Bla g 2. Bla g 2 has a cleft that might specifically bind ligands other than pepstatin. (J Allergy Clin Immunol 2005;116:140-5.)

Cockroach infestation in houses has been associated with allergy and asthma. IgE-mediated sensitization and

Abbreviations used
  Mut: Mutant
  PAG: Pregnancy-associated glycoproteins
  Wt: Wild-type

exposure to cockroach allergens has been linked to increased asthma morbidity in the United States, especially among lower socioeconomic groups.[1] Reports in the literature suggest that enzymatic activity might be an important contributor to allergenicity, especially for mite allergens with cysteine and serine proteinase activity.[2-5] Unlike mite allergens, none of the known cockroach allergens is a proteolytic enzyme. Bla g 2 is one of the most potent cockroach allergens, capable of eliciting IgE responses at 10- to 100-fold lower levels than dust mite and cat allergens.[6,7] Despite sharing sequence homology with the aspartic proteinase family of proteolytic enzymes, Bla g 2 lacked proteolytic activity in a standard milk-clotting assay that used casein as a substrate.[8] A molecular model of Bla g 2 showed that the binding cleft differs from the active site of aspartic proteinases. The recently solved crystal structure of recombinant (r)Bla g 2 revealed why critical amino acid substitutions in the canonical triads and in the flap region of the molecule impair enzyme activity.[8-11] Therefore the allergenic potency of Bla g 2 is not related to its proteolytic activity.[7]

However, Bla g 2 has a resemblance to a family of proteins that are expressed in ungulate (hoofed) mammals during pregnancy and are closely related to active aspartic proteinases. Sequence analysis revealed that the pregnancy-associated glycoproteins (PAGs) most closely resemble pepsin (49% to 59% identity). Most PAGs are unable to act as enzymes because of similar amino acid substitutions in the canonical triads and the flap region close to those found in Bla g 2.[12,13] A binding function has been attributed to the PAGs.[14,15]

We investigated the biologic function of Bla g 2 by performing site-directed mutagenesis of amino acids in the catalytic sites. The aim was to determine whether enzyme function could be restored by means of mutagenesis. Two different assays, the milk-clotting assay and the hemoglobin assay were used to study enzymatic activity. In addition, we investigated the ability of rBla g 2 to bind to the aspartic proteinase inhibitor pepstatin, a natural peptide secreted by *Streptomyces* species.

METHODS

Site-directed mutagenesis

Oligonucleotide primers for rBla g 2 Mut were designed to transform the amino acid triads [32]DST and [215]DTS (pepsin numbering) into DTG and the phenylalanine in position 75 from the flap into tyrosine (primer sequences are available on request). The DNA template consisted of Bla g 2 cDNA inserted into the *Pichia pastoris* expression vector pGAPZαC, which allows constitutive expression of the allergen. Site-directed mutagenesis was performed sequentially with QuikChange (Stratagene, La Jolla, Calif), starting at the first triad (DST to DTG), continuing to the second triad (DTS to DTG), and finishing with the flap (F75Y). Another mutant (MutF⁻) was expressed that contained 2 mutations in the flap region, F75Y and a Y75a deletion, and with rBla g 2 Mut DNA as a template. Mutated DNA was transformed into XL1-Blue supercompetent cells, and bacterial clones were sequenced to confirm the desired amino acid substitutions.

Expression, purification, and analysis of Bla g 2 mutants in *P pastoris*

Transformation of *P pastoris* strain KM71 and expression of recombinant proteins were performed as previously described.[16] Recombinant Bla g 2 wild-type (Wt), Mut, and MutF⁻ were purified from culture media by means of affinity chromatography over a 7C11 mAb column.[8] Bound proteins were eluted with 0.1 M glycine and 0.15 M NaCl, pH 2.5. Eluted fractions were neutralized with 2 M Tris Base, pH 8.5, and concentrated with Centricon P-80, MWCO 10,000 (Millipore, Bedford, Mass). Concentrated proteins were dialyzed overnight against PBS and quantitated with Advanced Protein Assay (Cytoskeleton, Denver, Colo). *P pastoris* supernatants and affinity-purified rBla g 2 were analyzed with ELISA.[17]

Milk-clotting assay

Enzymatic activity of rBla g 2 proteins was measured in a milk-clotting assay, as previously described, by using porcine pepsin (Sigma, St Louis, Mo) as a standard.[8] The effect of pH on the assay was compared with the following buffers: 0.1 M sodium acetate, pH 4.5 and 5.5; 0.1 M sodium phosphate, pH 6.5; and 0.1 M Tris-HCl, pH 7.5 and 8.5.

Hemoglobin assay

This assay measures the hydrolysis of denatured bovine hemoglobin by aspartic proteinases.[18-20] Bovine hemoglobin (80 mL of 2.5% wt/vol) in deionized water was denatured by adding 20 mL of 0.3N HCl. Porcine pepsin was used as a positive control for enzymatic activity. Pepsin or rBla g 2 at 5 to 20 μg/mL in 1 mL of 0.01N HCl was incubated with 0.5 mL of hemoglobin for 10 minutes at 37°C. The reaction was stopped by adding 10 mL of 5% trichloroacetic acid. Reactions were cleared by means of centrifugation, and absorbance of the supernatants was recorded at 280 nm. Blanks were run for each individual sample by adding trichloroacetic acid to the hemoglobin substrate before addition of the enzyme sample.

In a modified hemoglobin assay, hemoglobin (300 μL) was denatured by adding 75 μL of 1 M sodium formate, pH 3.5, and incubating for 15 minutes at 37°C. Pepsin or Bla g 2 were added in a volume of 75 μL and incubated for 16 hours at 37°C. The reaction was stopped by adding 700 μL of 4% trichloroacetic acid. Blanks were run as described previously. Recombinant Bla g 4, as well as the supernatant and cell lysate of the *P pastoris* strain KM71, were used as negative controls to rule out contaminating proteolytic activity of the yeast expression system. KM71 supernatant was processed as rBla g 2 samples through the same affinity-chromatography column and with the same elution and concentration conditions.

Pepstatin affinity chromatography

Recombinant Bla g 2 was dialyzed against 50 mM sodium acetate, pH 3.0, containing 0.2 M NaCl and incubated for 2 hours at 4°C with pepstatin-agarose (Sigma) equilibrated in the same buffer. After washing with 10 bed volumes of 50 mM sodium acetate, pH 3.0, and 0.2 M NaCl, bound proteins were eluted with 0.1 M Tris-HCl buffer, pH 8.6, containing 1 M NaCl. A buffer containing SDS (0.22 M Tris-HCl; pH 7.0; 6% SDS; 30% glycerol; and 0.15% bromophenol blue) was used to elute most of the rBla g 2.

Bla g 2 binding to pepstatin was further studied with 0.1 M sodium acetate and 0.35 M NaCl, pH 5.3, as binding buffer. Bla g 2 was applied to pepstatin-agarose (Pierce, Rockford, Ill) and incubated for 5 minutes at room temperature. After washing with 10 bed volumes of binding buffer, retained proteins were eluted with 0.1 M NaHCO₃ and 1.0 M NaCl, pH 9.0.

RESULTS

Expression of rBla g 2 mutants with restored canonical triads

Site-directed mutagenesis was used to create a rBla g 2 mutant (Mut) with the canonical triads DTG, highly conserved in aspartic proteinases, as well as with a modified conserved flap. The complete coding sequences of the mutated clones were obtained and confirmed the desired mutations (Fig 1).

Two consecutive phenylalanine residues are present in the flap of Bla g 2. The sequence alignment of Bla g 2 with pepsin and chymosin, performed for molecular modeling, showed that the second F coincided with Y75 in active aspartic proteinases, an important residue for catalytic activity.[8] Therefore this residue was mutated to Y to create Bla g 2 Mut. Recent data derived from the crystal structure of Bla g 2 revealed that the first F was structurally equivalent to Y75, whereas the second (F75a) was a unique insertion in the flap region.[11] This insertion had not previously been described and does not occur in other aspartic proteinases. The presence of this extra residue changed the conformation of the tip of the flap and the sequence assignment of the subsequent part of the structure, as well as the cleft of the molecule.[11]

As a result, rBla g 2 Mut had a phenylalanine rather than a tyrosine at position 75 and an extra residue at position 75a. An additional mutant (MutF⁻) was expressed containing Y75 and a deletion at 75a to correct the flap region of Mut. The antigenicity of both mutants was compared with rBla g 2 (Wt) and natural Bla g 2 by means of quantitative 2-site mAb ELISA. Superimposable dose-response curves were obtained, indicating that the antibody-binding sites of the mutant proteins were preserved and that the proteins were properly folded (Fig 2).

Effect of Bla g 2 mutations on proteolytic activity

The amino acid substitutions in Mut and MutF⁻ were designed to transform Bla g 2 into an active aspartic proteinase. However, neither rBla g 2 Wt nor Mut showed activity in standard aspartic proteinase assays. Both proteins were inactive in a milk-clotting assay at
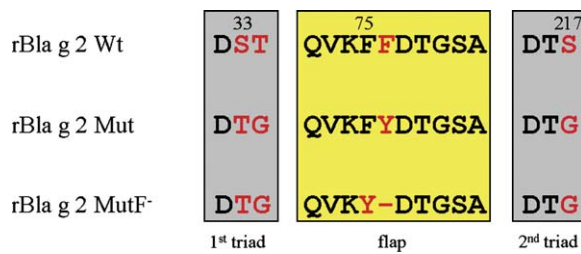
FIG 1. Amino acid sequence alignment of rBla g 2 Wt, rBla g 2 Mut, and rBla g 2 MutF⁻ showing the areas containing the amino acid mutations at positions S33T, T34G, F75Y, S217G, and a F75a deletion. The rest of the 3 sequences were identical.
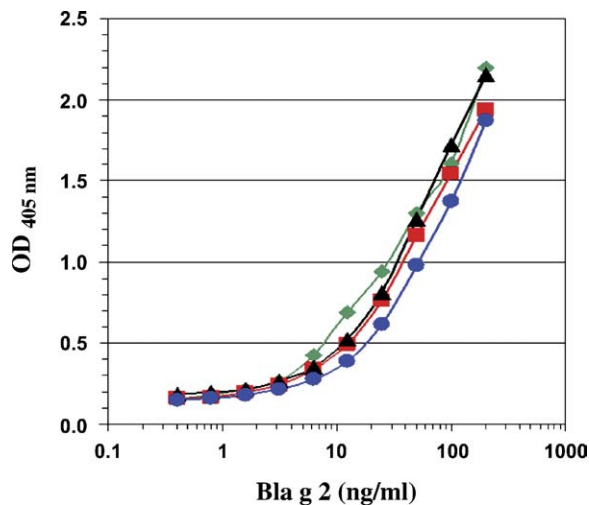

FIG 2. Bla g 2 ELISA. Dose-response curves for natural Bla g 2 (natural Bla g 2 Wt, *diamonds*) extracted from cockroach frass and rBla g 2 (rBla g 2) from *P pastoris* supernatants (rBla g 2 Wt *[squares]*, rBla g 2 Mut *[triangles]*, and rBla g 2 MutF⁻ *[circles]*) assayed in a quantitative, 2-site, mAb-based enzyme-linked immunoassay.

FIG 3. Comparison of the proteolytic activity of rBla g 2 and pepsin in standard aspartic proteinase assays. Proteolytic activity was analyzed by using a milk-clotting assay (**A**) or a hemoglobin assay (**B**).

concentrations as high as 10 μg/mL at pH 5.4, whereas the positive control pepsin was active at less than 0.03 μg/mL (Fig 3, *A*). This assay was also performed at different pH values, ranging from 4.5 to 8.5, with no detectable activity for rBla g 2 Wt or Mut (data not shown).

Recombinant Bla g 2 Wt and Mut were also tested for proteolytic activity in a standard assay that used bovine hemoglobin as a substrate. This assay has been previously used to detect aspartic proteinases from other arthropods, such as the mosquito *Aedes aegypti* and the hard tick *Boophilus microplus*.[18-20] These 2 aspartic proteinases share 33% and 27% amino acid homology to Bla g 2, respectively. Proteolytic activity of rBla g 2 Wt or Mut was not detectable in this standard hemoglobin assay at concentrations as high as 80 μg/mL, whereas pepsin was active at less than 10 μg/mL (Fig 3, *B*).

Recombinant Bla g 2 MutF⁻ was used in place of rBla g Mut for subsequent functional assays because its amino acid sequence of the flap region more closely resembles that of active aspartic proteinases. For functional assays with rBla g 2 MutF⁻, we designed a modified hemoglobin assay with an extended incubation time of 16
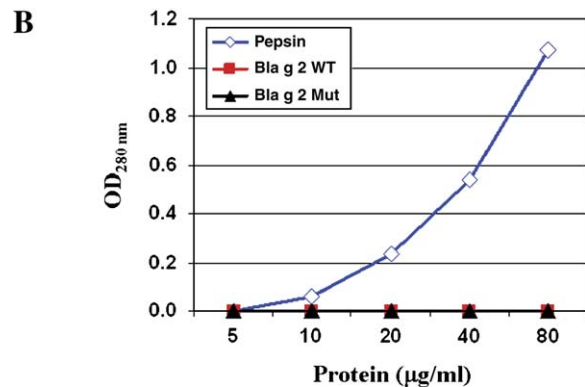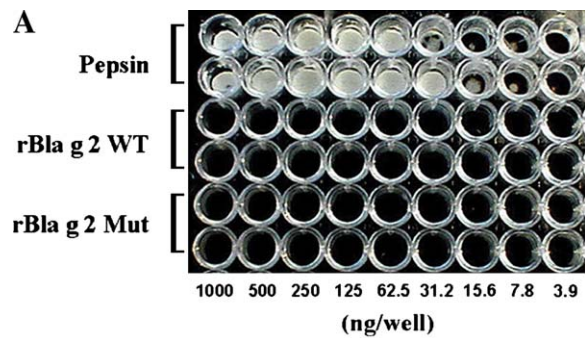
hours versus the 1 hour used in the standard hemoglobin assay. This modification increased the sensitivity of the assay and enhanced detection of trace amounts of proteolytic activity. By using the modified hemoglobin assay, the rBla g 2 Wt showed residual aspartic proteinase activity at 80 μg/mL (Fig 4) and rBla g 2 MutF⁻ at 20 μg/mL (data not shown). In additional experiments, activity of the rBla g 2 Wt was also detected at 20 μg/mL (data not shown). However, pepsin activity was detected at a 1000-fold lower concentration of 80 ng/mL, demonstrating that the activity of rBla g 2 was very low and thus not detectable in standard aspartic proteinase assays. Pepsin and rBla g 2 Wt activity in the modified hemoglobin assay was completely inhibited by pepstatin, a specific 686-Da natural peptide inhibitor of aspartic proteinases, but was unaffected by the serine protease inhibitor phenylmethylsulfonyl fluoride. No activity was detected for the negative controls rBla g 4, a cell lysate of *P pastoris* strain KM71, and KM71 supernatant loaded and eluted from the mAb 7C11 column (KM71 data not shown).

Binding of rBla g 2 to the aspartic proteinase inhibitor pepstatin

We further investigated whether Bla g 2 retained the peptide-binding function of aspartic proteinases. Binding of pepsin and rBla g 2 Wt to pepstatin was investigated by using pepstatin-agarose affinity chromatography. The results showed that 70% percent of the pepsin was
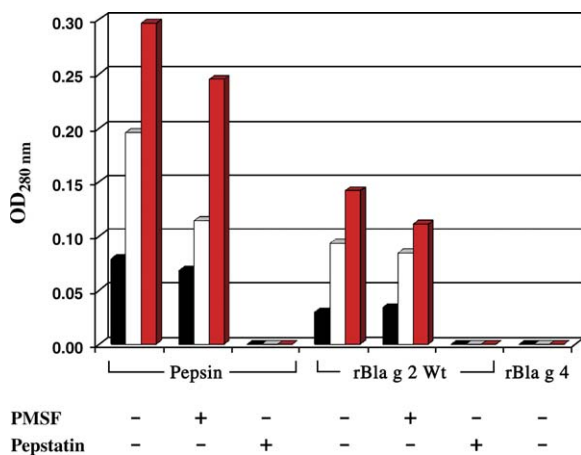
FIG 4. Residual activity of rBla g 2 in a modified hemoglobin assay. Pepsin at 80 ng/mL *(filled columns)*, 160 ng/mL *(open columns)*, and 320 ng/mL *(red columns)*; rBla g 2 Wt; and rBla g 4 at 80 μg/mL *(filled columns)*, 160 μg/mL *(open columns)*, and 320 μg/mL *(red columns)* were analyzed in the presence or absence of phenyl-methylsulfonyl fluoride *(PMSF*; 1 mM) and pepstatin (14.6 μM). Pepsin served as a positive control, and rBla g 4 served as a negative control.

FIG 5. Binding of pepsin and rBla g 2 to pepstatin-agarose. Proteins were loaded in 50 mM sodium acetate and 0.2 M NaCl and eluted with Tris-HCl **(A)** or loaded in 0.1 M sodium acetate and 0.35 M NaCl and eluted with 0.1 M NaHCO₃ **(B)**. Unbound proteins were collected in the flow through *(FT)*. Proteins were quantified by using the advanced protein assay and are expressed as percentage of the load.

absorbed to the column and could be recovered in the eluate (Fig 5, *A*). More than 80% of the loaded rBla g 2 Wt was retained in the column. However, only a small percentage of the absorbed allergen could be eluted. In subsequent binding experiments, we successfully released pepstatin-bound rBla g 2 by boiling the agarose in a buffer containing SDS and visualized eluted proteins in an SDS Phast gel (8% to 25% gradient) after silver staining (data not shown). These results suggested that pepsin and rBla g 2 Wt bind to pepstatin agarose but that rBla g 2 needs a more stringent elution than pepsin under the conditions of the assay.

A loading buffer containing higher salt concentrations was chosen for additional experiments to assess the specificity of Bla g 2 binding to pepstatin. The necessity to include high amounts of salt to prevent nonspecific effects has been previously reported.[21] Under these conditions, the amount of Bla g 2 retained was only 8% to 20%, with 80% of the loaded Bla g 2 being unabsorbed (Fig 5, *B*), whereas pepsin absorption to the column was similar (70% to 90%) to that seen in the previous experiment (Fig 5, *A*).

DISCUSSION

To investigate the function of the cockroach allergen Bla g 2, we studied its enzymatic activity in proteolytic assays and assessed its ability to bind the natural pep-tide pepstatin, which specifically inhibits the majority of aspartic proteinases. The importance of amino acid sub-stitutions in the catalytic site of Bla g 2 and the potential of this allergen to become an active enzyme were inves-tigated by mutating residues that are essential for catalytic activity. The resulting mutants, rBla g 2 Mut and rBla g 2
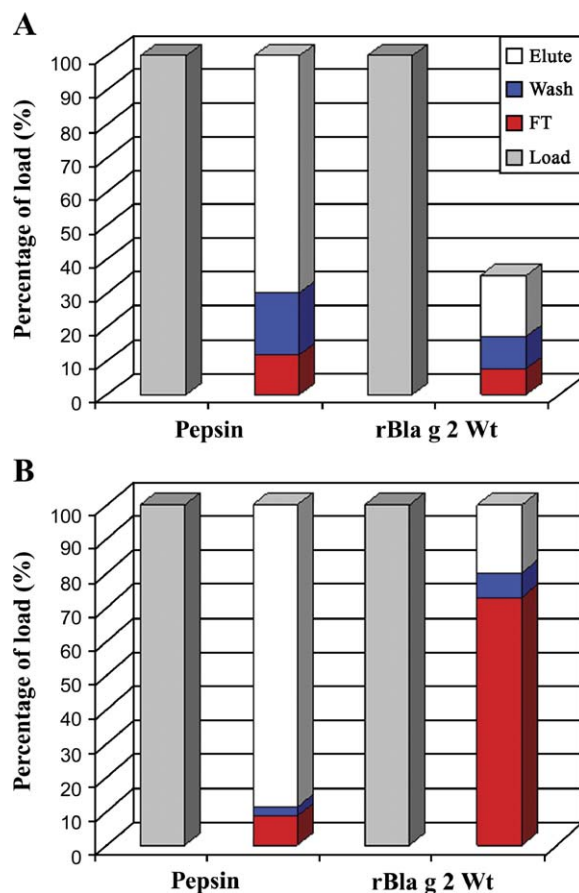
MutF⁻, were expressed in *P pastoris*. Immunologic recognition of rBla g 2 Mut and rBla g 2 MutF⁻ was confirmed by means of affinity purification and mAb binding in ELISA. Whereas standard enzymatic assays with milk casein and hemoglobin as substrates showed that both rBla g 2 Wt and rBla g 2 Mut proteins were inactive, a modified hemoglobin assay with increased sensitivity detected residual activity not only for rBla g 2 MutF⁻ but also for rBla g 2 Wt.

The crystal structure of rBla g 2 Wt (N93Q) revealed a self-inhibited state of the protein, which is due to a unique insertion in the flap region of the molecule, which partially occupies the substrate binding site and thus explains the lack of catalytic activity under standard assay conditions.[11]

A modified activity assay showed that the proteolytic activity of Bla g 2 can be detected at a low level under the assay conditions. We propose that in solution Bla g 2 exists in 2 conformational states, active and self-inhibited, and the ratio between these 2 conformations is signifi-cantly shifted toward the latter. That might explain the low level of catalytic activity and the discrepancy between the

high concentration of the protein needed for detection of activity and the low concentration of pepstatin required for inhibition.

A self-inhibited state of Bla g 2 would not be expected to bind specifically to the pepstatin column because the flap is tightly bound to the catalytic aspartates through hydrogen bonds. Transition to the active state will require significant conformational changes in the active site area, as confirmed by the modeling studies of the inhibitor bound to Bla g 2.[11] Therefore the observed binding of Bla g 2 to pepstatin-agarose must be mostly nonspecific. Nonspecific binding to pepstatin-agarose columns has been previously observed, and a loading buffer containing high salt concentrations (350 mM) was needed to prevent it.[21] In keeping with this, the binding of Bla g 2 to the pepstatin column dramatically decreased when these more stringent conditions were used, whereas pepsin still bound the column as expected. These results further support a nonspecific binding of Bla g 2 to the pepstatin-agarose column. The specificity of the Bla g 2–pepstatin interaction and the conditions for a possible transition between the active and self-inhibited conformations will be analyzed by using surface plasmon resonance. This technique allows the study of biomolecular interactions in real time by passing a putative ligand in solution over a surface with the immobilized protein of study.[22]

Bla g 2 shares only 26% to 28% sequence identity with active aspartic proteinases, such as pepsin and chymosin, and the amino acid mutations performed in this study might not be sufficient to fully restore enzymatic activity. Multiple substitutions during evolution might have transformed the original aspartic proteinase into a protein, Bla g 2, that is far from being an active proteolytic enzyme and that might have another primary function. Enzyme-active sites have evolved as cooperative entities where different mechanisms, besides the direct involvement of side chains in the chemical reaction, collaborate to achieve catalysis.[23] The function of some enzymes is dramatically modified by the accumulation of many seemingly inert mutations of non–active-site residues, and a single amino acid in a distal position to the active site, such as K319 in pepsin, might still be important for catalysis.[24,25] Equine PAG is the only PAG known to be active, which was anticipated because it does have the conserved DTG motif in the canonical triads and the Y75 in the flap region. However, the proteolytic activity observed in a hemoglobin assay was diminished when compared with pepsin and was only detectable after extending the incubation time of the assay to 2 to 4 hours.[26] Therefore it is likely that amino acids other than the ones directly involved in catalysis contribute to proteolytic activity. Additional mutations were performed in rBla g 2 Mut, such as V39W (which might have a role in maintaining Y75 in the correct orientation) and K218T, but did not lead to the expression of a proteolytically active enzyme, as assessed by using standard aspartic proteinase assays (data not shown).[27]

The Bla g 2 natural ligand might have evolved in parallel with Bla g 2 and might have a specific conformation that adapts to the unusual characteristics of the Bla g 2–binding pocket. Thus it might be very different from the structure of milk casein and hemoglobin, which are common substrates for mammalian and insect aspartic proteinases, respectively. Bla g 2, like renin, could have specific surface residues that determine strict substrate requirements.[28]

Members of the aspartic proteinase family that lack or have marginal proteolytic activity include most PAGs and Lma-p54, a cockroach protein with 48.5% amino acid identity to Bla g 2 and that is produced by *Leucophaea maderae*.[29] Lma-p54 has similar amino acid substitutions in the canonical triads and in the flap region. The amino acid substitutions seen in Bla g 2, Lma-p54, and the PAGs appear to explain their residual or lack of enzymatic activity. Molecular modeling and crystallographic studies confirm that, despite the substitutions, Bla g 2 retains a bilobal structure typical of aspartic proteinases, with a cleft for binding ligands.[8,11] A ligand-binding function has been attributed to some PAGs: ovine uterine serpin (ovUS-1) binds ovine PAG and was proposed to be the natural ligand.[14,30] The protein from which Bla g 2 originally evolved might once have been a proteolytic enzyme but might have acquired a new function, possibly as a ligand-binding or carrier molecule, maintaining sequence similarity to active aspartic proteinases.

This study shows that Bla g 2, like PAGs, does not have an enzymatic activity in standard aspartic proteinase assays. Increased allergenicity caused by proteolytic activity has been observed for some mite allergens, but the allergenic potency of Bla g 2 is likely unrelated to its proteolytic activity. Instead, Bla g 2 might function as carrier or ligand-binding molecule. Crystallographic studies of natural Bla g 2 with its bound natural ligand and/or complexes of Bla g 2 with inhibitors should help to identify the natural ligand-binding properties of Bla g 2, which will be an important step to clarify the precise function of this allergen. Other known allergens that function as binding proteins include lipocalins (eg, Bos d 2 and Can f 1), which bind small hydrophobic ligands, such as pheromones and steroids or calcium-binding proteins (eg, Bet v 3).[31-33] Ligand binding is one of the diverse functions attributed to allergens, and it remains to be investigated whether this function contributes to allergenicity.

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