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## Bla g 3: a novel allergen of German cockroach identified using cockroach-specific avian single-chain variable fragment antibody

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### ABSTRACT

**Background:** The IgE response to cockroach allergens is thought to be associated with asthma. German cockroach (GCr) allergen extract is a complex mixture of allergens, and the identification and characterization of immunodominant allergens is important for the effective diagnosis and treatment of GCr-induced asthma.

**Objective:** To characterize a novel GCr allergen homologous to the American cockroach allergen Per a 3.

**Methods:** GCr-specific avian monoclonal antibodies were used for direct immunoprecipitation of specific targets from whole-body GCr extract. Precipitated protein was identified by mass spectrometry and sequence analysis. Putative recombinant protein also was expressed, purified, and used for determination of allergenicity, determined by IgE enzyme-linked immunosorbent assay with serum from 61 GCr-allergic patients. The identified target also was analyzed for heat stability using a bead-based assay.

**Results:** The immunoprecipitated target of monoclonal antibody 2A1 was identified as a novel allergen of GCr homologous to American cockroach allergen Per a 3. This homolog, designated Bla g 3, has an apparent mass of 78 kDa, can be measured in GCr extract using antibody 2A1, and is a heat-stable protein. Screening of 61 serum samples from GCr-allergic patients showed a 22% prevalence of Bla g 3-specific IgE.

**Conclusion:** Bla g 3 is a GCr allergen with structural homology to American cockroach allergen Per a 3.

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### Introduction

Cockroach allergen sensitization and exposure is a major cause of asthma and rhinitis in US cities. Skin testing, bronchial challenges, and serum IgE determinations have indicated that approximately 60% of US inner-city residents with asthma have IgE to cockroach allergens<sup>1–4</sup> and that most inner-city children in the United States are sensitized to cockroach allergens.<sup>5–9</sup> Although US patients are sensitized to German cockroach (GCr; *Blattella germanica*), 40% to 60% of patients with asthma residing in urban areas outside the United States are sensitized to American cockroach (*Periplaneta americana*).<sup>10–12</sup>

German cockroach is a highly complex extract containing multiple allergens. These allergens from saliva, skin casts, egg cases, and feces are present in aqueous extracts, and the presence of proteases may contribute to extract complexity and instability.<sup>13–15</sup> Currently there are 7 *B. germanica* allergens listed by the World Health Organization's International Union of Immunological Societies. Bla g 1, Bla g 2, Bla g 4, and Bla g 5 have been reported to be major allergens,<sup>5,16–18</sup> but other studies have suggested that this

may not be the case.<sup>17,19,20</sup> Recently, arginine kinase and vitellogenin, among others, have been identified as new allergens using a proteomic approach.<sup>21</sup>

In a previous study, the authors used single-chain variable fragment (scFv) monoclonal antibodies to characterize GCr allergen extracts.<sup>22</sup> In the present study, they report on the identification of a novel 78-kDa GCr allergen, designated Bla g 3.01, a member of the hemocyanin family and a homolog of American cockroach allergen Per a 3.

### Methods

#### Preparation of GCr Extract

Defatted whole-body GCr powder (Greer Laboratories, Lenoir, North Carolina) was extracted overnight at 4°C in phosphate buffered saline (PBS; pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich, St Louis, Missouri). The supernatant was collected by repeated centrifugation at 4°C for 30 minutes at 10,000g, passed through a 0.45-μm filter, and freeze dried. The aliquots were stored at –20°C and reconstituted with deionized water containing 50% glycerol for further analysis. Total protein concentration of the extract, designated E6Cg, by bicinchoninic acid assay (Thermo Scientific, Pittsburgh, Pennsylvania) was estimated to be 20 mg/mL. Similarly prepared lyophilized American and Oriental cockroach extracts were reconstituted in 50%

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glycerin-dH<sub>2</sub>O for immunoblot analysis and enzyme-linked immunosorbent assay (ELISA) screening.<sup>23</sup>

#### Generation of scFv Antibodies

An avian scFv antibody library was generated as previously described<sup>24</sup> using whole-body GCr extract as the immunogen. Antibody sequences were cloned into pCOMB3X plasmid containing a His tag at the N-terminus and a hemagglutinin (HA) tag at the C-terminus.<sup>24,25</sup> *E. coli* were transformed by vector in the presence of M13 helper phage; then, the antibody was expressed on the phage surface as a PIII coat fusion protein. After bio-panning with E6Cg, selected scFv antibodies were expressed in *E. coli* Top 10 cells (Invitrogen, Grand Island, New York) and purified from the bacterial lysate by nickel chelation chromatography (His-select Nickel, Sigma Life Sciences, St Louis, Missouri).<sup>26</sup>

#### Expression and Purification of Recombinant Bla g 3

Plasmid containing the putative recombinant Bla g 3 (rBla g 3) isoform 2 sequence was provided by 1 of the authors (F.T.C.). Large-scale alignment was performed on expressed sequence tag libraries of *B. germanica* (GenBank accession numbers FG125509.1 to FG128349.1; GenBank, Bethesda, Maryland) against the existing Per a 3 sequence from GenBank (GU726839.1). The expressed sequence tag clones aligned to Per a 3 were identified and sequenced, but all clones thus isolated lacked a 5' end, so 5' rapid amplification of cDNA ends polymerase chain reaction was performed (SMART RACE cDNA Amplification Kit, Clontech, Mountain View, California). Total RNA was isolated from live flash-frozen *B. germanica* ground tissues and poly A+ mRNA was obtained from total RNA. Reverse transcription polymerase chain reaction performed on poly A+ mRNA yielded cDNA. The resulting cDNA was sequenced, restriction sites were added, and inserts were cloned into the pET-32a expression plasmid system (Novagen, Darmstadt, Germany). Recombinant protein was expressed in *E. coli* BL21 (DE3) after overnight culture and induction with 1 mmol/L of isopropyl-1-thio-β-galactopyranoside. Recombinant protein was purified from soluble fractions by nickel chelation chromatography and analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

#### Production and Screening of Polyclonal Antibodies

New Zealand white rabbits were immunized with 100 µg of E6Cg or rBla g 3 in Freund complete adjuvant followed by 3 doses of 100 µg of E6Cg or rBla g 3 in incomplete Freund adjuvant at 21-day intervals (Spring Valley Labs, Woodbine, Maryland). Direct ELISA was used for screening of terminal sera. E6Cg (1 µg/mL) or rBla g 3 (1 µg/mL) was coated on microwell plates overnight at 4°C. After blocking, serially diluted serum (1:100 starting dilution) was added to duplicate wells. Antibody binding was detected using horseradish peroxidase (HRP)-conjugated antirabbit IgG (1:1000) and TMB peroxidase substrate (KPL, Gaithersburg, Maryland).

#### Purification of Native Bla g 3

The Bla g 3-specific rabbit polyclonal serum was cross-linked to cyanogen bromide-activated Protein A-Sepharose beads (Sigma Life Sciences, St Louis, Missouri). E6Cg extract was mixed with the beads and incubated overnight at 4°C. Native Bla g 3 (nBla g 3) was eluted with 0.2 mol/L of glycine buffer (pH 2) and analyzed by SDS-PAGE. Identity was confirmed by peptide mass fingerprinting (not shown).

#### scFv Antibody ELISA

The scFv antibody was tested for allergen binding by ELISA. Polystyrene 96-well plates (Nunc, Thermo Fisher Scientific) were coated overnight with serially diluted cockroach extracts at 4°C.

Wells were washed and blocked, and antibody 2A1 (1.0 µg/mL in blocking solution) was added for 2 hours at room temperature. After washing, bound antibody was detected with HRP-conjugated anti-HA (1:1,000 [v/v] in 3% bovine serum albumin [BSA] in PBS; Roche Diagnostics, Indianapolis, Indiana) and TMB peroxidase substrate (KPL).

#### SDS-PAGE and Western Blot

Proteins were separated by SDS-PAGE on 4% to 12% Bis Tris gels under reducing conditions and visualized with Coomassie Brilliant Blue. For immunoblotting, proteins were transferred to a polyvinylidene difluoride membrane (pore size 0.2 µm; Invitrogen). After blocking, the membrane was incubated with scFv antibody (1.0 µg/mL), and bound antibody was detected with HRP-conjugated anti-HA (1:1,000 in PBS containing 3% BSA), which was visualized with a chemiluminescent peroxidase substrate (SuperSignal, West Pico, Thermo Scientific).

#### Immunoprecipitation and Mass Spectrometric Analysis

Target protein recognized by antibody 2A1 was immunoprecipitated from E6Cg using the Direct Immunoprecipitation Kit (Thermo Scientific) and was analyzed by SDS-PAGE. After destaining, the target protein was excised from the gel, and protein in the gel slice was reduced and alkylated with iodoacetamide. Trypsinization was performed with sequencing grade modified trypsin (Promega Corp, Madison, Wisconsin). The extracted peptides were lyophilized and reconstituted in 0.1% formic acid before analysis by liquid chromatographic mass spectrometry (Facility for Biotechnology Resources, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Maryland).

#### Coupling of Carboxylated Microspheres and Bead-Based Assay

Purified scFv antibody 2A1 was coupled to polystyrene carboxylated beads (Bio-Rad Laboratories, Hercules, California).<sup>26</sup> Microbeads were primed with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and N-hydroxy-sulfo-succinimide. Primed beads (2.5 × 10<sup>6</sup>) were coupled with antibody (10 µg), washed, and stored in PBS with 0.1% BSA, 0.02% Tween, and 0.05% sodium azide.

For the bead-based assay, 96-well filter bottom plates (Millipore, Billerica, Massachusetts) were rinsed twice with wash buffer (PBS containing 1% BSA). Antibody-coupled beads were incubated in the presence of serially diluted E6Cg for 1 hour at room temperature with gentle mixing. Bound allergen was detected by sequential incubation with polyclonal rabbit anti-E6Cg serum (1:500), biotinylated antirabbit IgG (H + L; 1:1,000), and streptavidin-R-phycoerythrin (1:250). After the final incubation, beads were resuspended in wash buffer and median fluorescence intensities were measured using Bio-Plex 200 (Bio-Rad Laboratories). The data were exported to GraphPad Prism 5.0 (GraphPad, San Diego, California) for analysis.

#### Heat-Stability Studies

Fifty microliters of E6Cg (20 mg/mL), nBla g 3 (100 µg/mL), and rBla g 3 (100 µg/mL) were aliquoted into thin-walled microcentrifuge tubes and heated at 40°C to 80°C for 2 minutes in a heating block with water added to the wells. After heating, the extract was immediately chilled on ice, diluted, and analyzed using the bead-based assay.

#### Patient Sera and IgE ELISA

ELISA was performed using sera from 61 cockroach-allergic adults with asthma<sup>27</sup> and a serum pool (S1Cr) collected from 16 additional cockroach-allergic patients<sup>19</sup> to determine the prevalence of IgE to rBla g 3 isoform 2 and nBla g 3. Microtiter

Microtiter plate wells were coated overnight at 4°C with 100 µL of E6Cg (10 µg/mL in PBS). Before use, the 1:10 diluted S1Cr serum pool was inhibited with various concentrations of rBla g 3 or E6Cg.

After washing, the wells were blocked, inhibited human serum was added to duplicate wells, and the plate was incubated overnight at 4°C. Biotinylated goat antihuman IgE antibody (1:1,000; KPL), HRP-conjugated streptavidin (1:1,000), and TMB substrate solution, in sequence, were used for detection of IgE antibodies. The assay was repeated 3 times and mean percentages of inhibition are reported.

## Results

### scFv Antibodies from Avian Library

Eighty unique anti-GCr clones selected by phage ELISA were isolated (sequence data not shown) and 42 soluble anti-GCr antibodies were purified, 14 of which had strong affinity to E6Cg by ELISA.<sup>22</sup> No cross-reactivity with nonspecific allergenic extracts (cat hair and ragweed extracts) was observed during ELISA screening (data not shown). All 14 unique clones were expressed in *E coli* Top 10 cells and antibodies were purified from soluble fractions.<sup>22</sup> Antibody 2A1 was selected based on western blot screening of the E6Cg extract. The remaining 13 clones recognized additional proteins of GCr extract (not shown).

### Purification and Identification of Target Protein of Antibody 2A1

Direct immunoprecipitation experiments were performed to identify the target allergen of antibody 2A1. SDS-PAGE of the immunoprecipitated product displayed a major band with an apparent molecular mass of 78 kDa. Liquid chromatographic mass spectrometry indicated a protein of 78.9-kDa molecular weight, the sequence analysis of which yielded a close match with putative Bla g 3 isoform 2 (GU086323 and ACY40651.1). The isoforms of Bla g 3

share 91% identity and 96% similarity at the amino acid level. The deduced amino acid sequence of Bla g 3 isoform 2 is shown in Figure 1, with mass spectrometric peptide sequences from the 2A1 target underlined. Sequence alignment showed 68% identity and 75% similarity between Bla g 3 and Per a 3, a storage protein of the hemocyanin superfamily and a known allergen of the American cockroach.<sup>28,29</sup>

### Purification of rBla g 3 Isoform 2 and Immunoblot Analysis

Coomassie-stained SDS-PAGE of the expressed putative Bla g 3 isoform indicated a highly pure product with an apparent molecular mass of 78 kDa (Figure 2A). Antibody 2A1 recognized rBla g 3 (Figure 2B) and homologous proteins in other domestic cockroach species (Figure 2C). In E6Cg (Figure 2C, right lane), aside from the dominant 78-kDa target, a 66-kDa signal was observed that might be another isoform or a Bla g 3 degradation product. Antibody 2A1 bound to Bla g 3 and its homologs in other cockroach extracts in a dose-dependent manner (Figure 2D).

### Affinity Purification of nBla g 3 from E6Cg

Affinity-purified nBla g 3 was identified by peptide fingerprint and compared with rBla g 3 fractions by SDS-PAGE. A small difference in migration pattern was observed between nBla g 3 and rBla g 3 (eFigure 1).

### Thermostability

When E6Cg extract was heated for 2 minutes, Bla g 3 was found to be stable up to 60°C, with a 30% decrease in 2A1 binding at 70°C

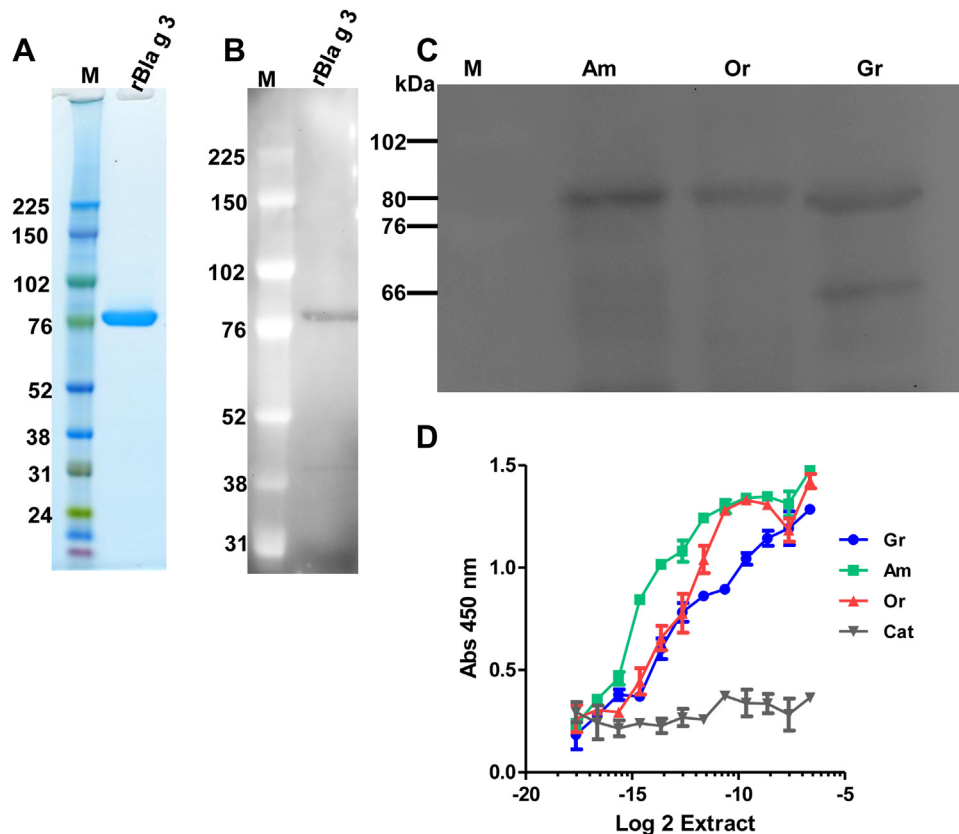


**Figure 2.** (A) Purification of recombinant Bla g 3 (rBla g 3) expressed in *Escherichia coli* BL21. (B) Purified rBla g 3 analyzed by immunoblot using 2A1 antibody. (C) Western blot of various cockroach extracts (Am = American; Or = Oriental; Gr = German cockroach extract [E6Cg]). The primary antibody was 2A1 (1.0 µg/mL) and horseradish peroxidase-conjugated antihemagglutinin (1:1,000) was used for detection using a chemiluminescent substrate. (D) Dose-dependent enzyme-linked immunosorbent assay of cockroach extracts. Wells were coated with E6Cg, extracts of American or Oriental cockroach, or cat hair extract as a negative control. The primary antibody was 2A1 (1.0 µg/mL), and horseradish peroxidase-conjugated antihemagglutinin (1:1,000) was the secondary antibody followed by TMB peroxidase substrate. Abs, absorbance; M, molecular weight markers.

and a 90% decrease at 80°C. In contrast, pure rBla g 3 and nBla g 3 were stable up to 50°C, with more modest but still statistically significant decreases in 2A1 binding at 60°C and above (eFigure 2).

#### Prevalence of Specific IgE to rBla g 3 and nBla g 3

In preliminary testing, the S1Cr serum pool of 16 cockroach-allergic patients was shown to contain IgE specific to rBla g 3 using microtiter wells coated with 62.5 to 1,000 ng of rBla g 3 (data not shown). Based on the dose-response of S1Cr, the sera from 61 GCr-allergic patients were tested for rBla g 3 and nBla g 3 binding and 14 (22%) of these contained IgE specific for rBla g 3 and nBla g 3 (10 sera had IgE to nBla g 3 and rBla g 3; Figure 3A, B).

#### rBla g 3 Inhibits Specific IgE Binding to GCr Extract

In an ELISA in which wells were coated with E6Cg and S1Cr was preabsorbed with different concentrations of E6Cg or rBla g 3, E6Cg extract showed a maximum self-inhibition of 87%. At the same concentration (100 µg/mL), rBla g 3 inhibited IgE binding to GCr extract by 38% (Figure 4).

#### Discussion

German cockroaches produce potent indoor inhaled allergens responsible for triggering IgE-mediated antibody responses and allergic diseases.<sup>5,9,16,30</sup> Allergen extracts produced from GCr are highly complex and nonstandardized. Previous reports have indicated that allergic individuals from various U.S. cities recognize a variety of well-characterized GCr allergens and show unique IgE-binding patterns.<sup>5,16,17,27</sup> In addition, in a prior report, the authors observed that overall potency of the GCr extract, as analyzed using a human serum pool, was not significantly altered when Bla g 1 or Bla g 2 was quantitatively depleted.<sup>19</sup> These studies suggest that other proteins may contribute to GCr allergenicity. Although many GCr allergens are known, the immunodominance of some of them remains uncertain, and there is a need to identify and characterize additional allergenic components.

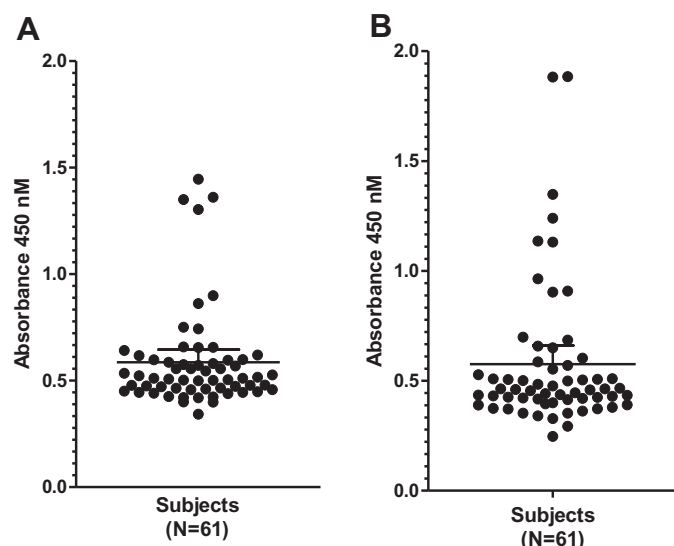


**Figure 3.** Prevalence of (A) anti-recombinant Bla g 3 IgE and (B) anti-native Bla g 3 IgE. Ninety-six-well plates were coated with recombinant Bla g 3 (250 ng/well) and native Bla g 3 (500 ng/well) overnight at 4°C and blocked. Serum from 61 patients allergic to German cockroach (1:10) was added to each well. Specific IgE binding was determined after sequential addition of biotinylated goat antihuman IgE (1:1,000), horseradish peroxidase-conjugated streptavidin (1:1,000), and TMB substrate. Mean values for each subject are reported; horizontal lines represent the mean and 95% upper limit of all values, calculated on log-transformed absorbances.

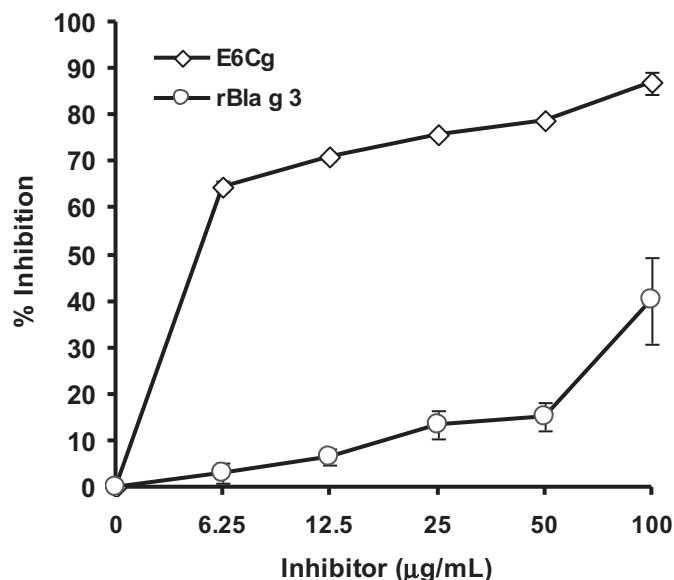


**Figure 4.** Inhibition of IgE binding to German cockroach by recombinant Bla g 3 (rBla g 3). Microtiter plate wells were coated 1 µg of German cockroach extract (E6Cg) and blocked. The S1Cr serum pool (1:10) was preabsorbed with rBla g 3 or E6Cg. Preabsorbed S1Cr was added and incubated overnight at 4°C. Bound IgE was detected with biotinylated goat antihuman IgE (1:1,000), horseradish peroxidase-conjugated streptavidin (1:1,000), and TMB substrate solution, in sequence. Percentage of inhibition was calculated as (control – inhibited)/control. Values are reported as mean ± SEM.

This study shows that scFv antibodies can be used as a tool for the isolation and identification of proteins from a complex allergenic extract. In the course of studying the targets of these avian antibodies, the authors report the isolation of allergen Bla g 3, a homolog of the American cockroach allergen Per a 3.<sup>28,31–33</sup> Bla g 3 contains similar IgE epitope sequences to those identified previously in Per a 3.<sup>29,33,34</sup> Bla g 3 and Per a 3 are hexamerins, members of the hemocyanin super family, which are present in large amounts in insect hemolymph. Hemocyanins are known for their strong antigenic properties and resistance to degradation under different conditions.<sup>34</sup>

Immunoblot of the aqueous GCr extract E6Cg with antibody 2A1 showed a strong protein signal at 78 kDa, approximating the mass spectrometric estimate of Bla g 3 molecular mass from peptide fingerprinting and the calculated molecular mass of the deduced Bla g 3 sequence. An additional 2A1-immunoreactive band in GCr extract at about 66 kDa could be another isoform or degradation product of Bla g 3 (Figure 2C); the peptide sequences obtained from mass spectrometric analysis matched Bla g 3 isoform 2 (not shown). Two isoforms of Per a 3 (72 and 78 kDa) have been identified as potent allergens of American cockroach.<sup>28,33</sup> Based on the human IgE responses (Figure 3) and homology to Per a 3, the World Health Organization and International Union of Immunological Societies subcommittee has designated this allergen Bla g 3.0101.

To further characterize nBla g 3, the thermal stability of the 2A1 epitope was determined by heating E6Cg and purified nBla g 3 and rBla g 3. Based on its 2A1 binding, Bla g 3 appeared to be relatively thermostable (eFigure 2), a feature that has been reported for Per a 3.<sup>34</sup>

The authors expressed rBla g 3 as a soluble bacterial expression product and purified nBla g 3 from GCr extract, which allowed them to estimate a 22% prevalence of IgE anti-Bla g 3 in 61 GCr-allergic patients (Figure 3). These same sera had an overall 28% seroprevalence of IgE antibody to Bla g 1, Bla g 2, Bla g 4, and Bla g 5 when tested by CAP ELISA.<sup>27</sup> Inhibition data suggested that as much as

38% of pooled serum IgE binding to aqueous GCr extract may be attributable to IgE–anti-Bla g 3 (Figure 4).

### Acknowledgments

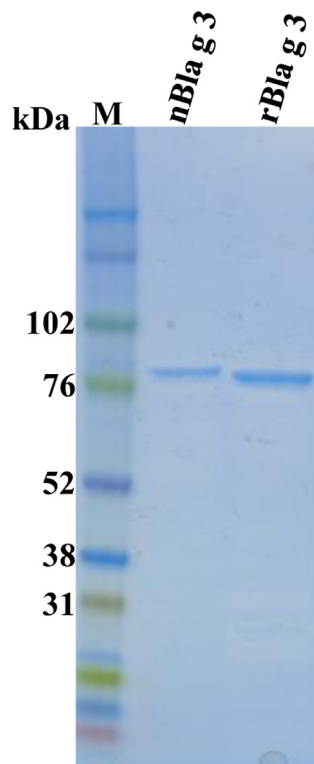
The authors thank Dr Nga Yen Nguyen (Center for Biologics Evaluation and Research, Food and Drug Administration) for performing mass spectrometric analyses and Dr Judith A. Woodfolk (University of Virginia) for sharing CAP IgE data for cockroach-allergic individuals. They also thank Ekaterina Dobrovol'skaia for technical assistance.

### Supplementary Data

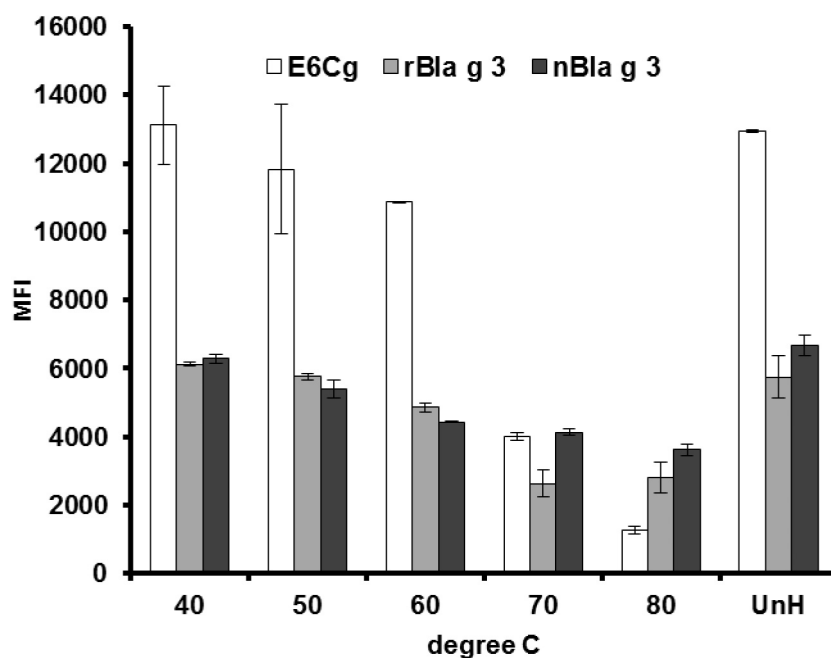
Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.janai.2013.11.007>.

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**eFigure 1.** Sodium dodecylsulfate polyacrylamide gel electrophoresis analyses of purified recombinant Bla g 3 (rBla g 3) and native Bla g 3 (nBla g 3). Samples were electrophoretically separated onto 4% to 20% Tris-glycine gel. The gel was stained with Coomassie blue.



**eFigure 2.** Thermostability of Bla g 3 in German cockroach extract (E6Cg), recombinant Bla g 3 (rBla g 3), and native Bla g 3 (nBla g 3). Samples were heated at 40°C to 80°C for 2 minutes and cooled rapidly on ice. Heated and unheated extracts (UnH) were incubated with 2A1 antibody-coated beads for analysis. The experiment was performed in duplicate and data are represented as mean  $\pm$  SD. MFI, median fluorescence intensity.