

High-level expression of cockroach allergen, Bla g 4, in *Pichia pastoris*

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Exposure to cockroach allergens is a risk factor for allergic disease and has been linked to an increase in asthma morbidity among cockroach-sensitive inner-city children. Bla g 4 is a ligand-binding protein (or calycin) that causes IgE antibody responses in 40% to 60% of patients allergic to cockroaches. Recombinant Bla g 4 was expressed in *Escherichia coli* as an 18 kd protein but provided poor yields (only 0.25 mg/L culture). To improve yields, Bla g 4 was expressed in the *Pichia pastoris* yeast system as a 23 kd secreted protein at concentrations of 50 mg allergen/L. By cross-inhibition radioimmunoassay, Bla g 4 expressed in *E. coli* or *P. pastoris* provided overlapping inhibition curves. Both allergen preparations bound comparable levels of serum IgE antibody and showed similar skin test reactivity in individuals allergic to cockroaches (10^{-1} to 10^{-3} µg/ml). Deglycosylation of *Pichia*-expressed Bla g 4 with endoglycosidase F resulted in an 18 to 20 kd doublet, and liquid chromatography-mass spectrometry results suggested that the 20 kd band contained residual sugar residues. Both glycosylated and deglycosylated *Pichia* Bla g 4 showed comparable inhibition of IgE antibody binding in radioimmunoassay. *Pichia*-produced Bla g 4 had the same antigenic reactivity as that produced in *E. coli*, and glycosylation had no effect on IgE antibody binding. The high yield of Bla g 4 obtained in the *Pichia* system will facilitate studies on the structure and function of calycin allergens and on the immune response of asthma patients to cockroach allergens. (J Allergy Clin Immunol 1998;101:274-80.)

Key words: Cockroach, asthma, calycin, allergen, recombinant, *Pichia pastoris*

Exposure to sources of indoor allergens such as dust mites, cockroaches, and animal dander is a risk factor for the production of IgE antibodies and the development of allergic disease.¹⁻⁵ Cockroaches are an important source of allergen in urban and inner-city dwellings, and a recent multi-city study has linked an increase in morbidity among cockroach-sensitive inner-city children to exposure to high levels of cockroach allergen in their homes.²⁻⁸ Allergens produced by both German and American cockroaches have been cloned, and their immunochemical and molecular properties have re-

Abbreviations used

Endo F:	Endoglycosidase F/N-Glycosidase F
MW:	Molecular weight
PCR:	Polymerase chain reaction
RIA:	Radioimmunoassay
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
VEGh:	Human von Ebner's Gland Protein

cently been reviewed.⁹ Principal allergens produced by the German cockroach (*Blattella germanica*), which is common in U.S. homes, include Bla g 1 (Bla g 90 kd), Bla g 2 (an aspartic protease), Bla g 4 (calycin), and Bla g 5 (glutathione transferase).⁹⁻¹⁴ American cockroach (*Periplaneta americana*) allergens that have been cloned include Per a 1 (unpublished) and Per a 3 (arylphorin).¹⁵

The use of recombinant allergens in structural and immunologic studies requires the production of large amounts of allergen that can be easily purified from suitable expression vectors. Previously, we reported the cloning and expression of an important *B. germanica* allergen, Bla g 4, which causes IgE antibody responses in 40% to 60% of patients allergic to cockroaches.¹³ Bla g 4 belongs to a super family of ligand-binding proteins (or calycins), which includes other important allergens such as rodent urinary proteins, β-lactoglobulin, dog, bovine, and equine allergens.^{13, 16-18} Recombinant Bla g 4 (rBla g 4) was initially produced in bacteria as a fusion protein with glutathione-S-transferase. Although the bacterially expressed protein showed excellent allergenic activity, both in intradermal skin testing and serum IgE antibody assays, yields in this system were low, typically only 0.25 mg rBla g 4 per liter of culture.

As an alternative, we investigated the *Pichia pastoris* yeast expression system. Very high yields of recombinant proteins such as tetanus toxin fragment C (a remarkable 12 gm/L) have been reported in *P. pastoris*, and this system has also been used to express pollen and *Alternaria* allergens (Cyn d 1 and Alt a 1).¹⁹⁻²¹ *P. pastoris* is a methylotrophic yeast capable of utilizing methanol as its sole carbon source through two genes encoding alcohol oxidase. The AOX1 gene produces more than 30% of the total soluble protein when the yeast is grown on methanol; AOX2 is expressed at a lower level. In the *P. pastoris* expression system, the gene of interest is cloned behind the inducible AOX1 promoter, and expression of the foreign protein is induced by growth on methanol.

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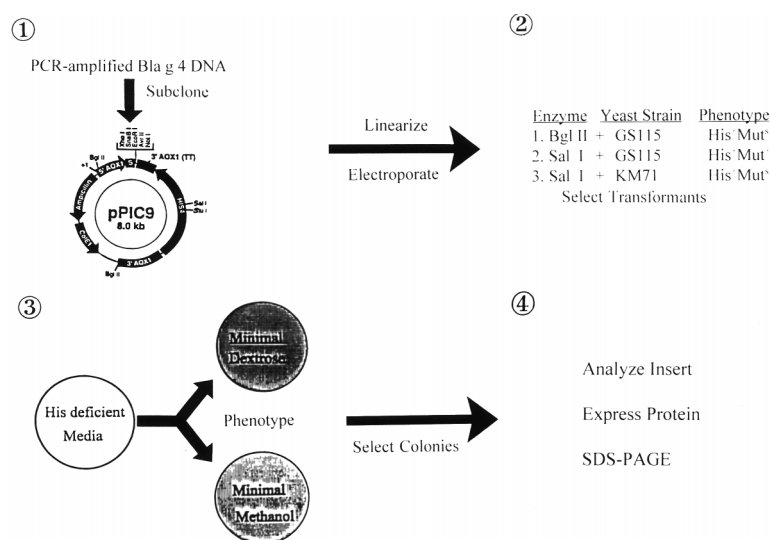


FIG. 1. Schematic outline of protein expression in *P. pastoris*.

An important advantage of the *P. pastoris* system is that the expressed protein is secreted as the single major protein in the culture medium and is easily purified.^{22,23}

In this report we describe high-level expression of recombinant Bla g 4 in *P. pastoris* (50 mg/L) and compare the biologic activity and structure of the yeast-expressed allergen with that produced in bacteria.

METHODS

Production of recombinant Bla g 4 in *P. pastoris*

Several steps were used for the production of rBla g 4 in *P. pastoris* (outlined in Fig. 1).

Subcloning. Bla g 4 plasmid DNA was used as a template to generate a 551bp polymerase chain reaction (PCR) product for cloning into the *P. pastoris* expression vector pPIC9 (Invitrogen, San Diego, Calif.). Primers for PCR were synthesized as follows: 5' CCG GAA TTC ACA GAT ACA TTG GCG AA3' (sense) containing an Eco R1 restriction site and 5' ATA AGA ATG CGG CCG CGT GAC ATG TGG AGT GTA AGT C 3' (antisense) containing a Not 1 site. After an initial denaturation step of 5 minutes at 95°C, PCR incubations were done for 1 minute at 94°C, 1 minute at 55°C, and 3 minutes at 72°C for 30 cycles followed by a final extension at 72°C for 15 minutes. The enzyme-digested PCR product was purified and ligated into the Eco R1 and Not 1 sites of the pPIC9 vector with a Ligation Express Kit (Clontech, Palo Alto, Calif.). Ligation reactions were transformed into competent *E. coli* strain HB101 as described.²⁴ Transformants were verified to contain the Bla g 4 DNA insert by automated DNA sequencing.

Linearization and transformation. Twenty micrograms of pPIC9 containing Bla g 4 DNA was linearized with Bgl II or Sal I and transformed into *P. pastoris* strains KM71 and GS115 to generate different His⁺ phenotypes expressing rBla g 4. Linearizing pPIC9 with Bgl II favored recombination resulting in His⁺Mut⁺ GS115 phenotypes, whereas plasmid linearized with Sal I generated either His⁺Mut⁺ or His⁺Mut⁻ GS115 or KM71 transformants, respectively. Transformation was accomplished by electroporation at 1500V, 25 capacitance, and 200 resistance in a Genepulser (Biorad, Hercules, Calif.).

Selecting transformants and determining phenotype. Transformation reactions were plated onto minimal dextrose media and grown strictly at 30°C to allow colonization of transformed *P. pastoris* cells. After approximately 4 days, 100 GS115 transformants of each linearization were patched onto minimal methanol and minimal dextrose plates to determine phenotype. Mut⁺ clones grew slowly on methanol plates and rapidly on dextrose plates, whereas, Mut⁻ clones grew equally well on both plates. One hundred Bla g 4 KM71 transformants were also selected and patched onto minimal dextrose plates to obtain clonal isolates; however, all were His⁺Mut⁺ because the KM71 strain itself is Mut⁺.

Analyzing transformants for integration of Bla g 4 DNA. Yeast genomic DNA was isolated from selected clones and 5 µl (25 to 30 µg) was used in a PCR containing 10 µl dNTPs (2.5 mmol/L each), 1 µl 3' AOX1 primer (0.1 µg), 1 µl α-factor primer (0.1 µg), 22.5 µl water, 5 µl MgCl₂, 5 µl 10× reaction buffer, and 0.5 µl Taq DNA polymerase (5 U/µl; Perkin Elmer, Branchburg, N.J.) added after the first denaturation step. PCR incubations were done for 2 minutes at 94°C followed by 25 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C with a final extension of 7 minutes at 72°C. PCR products were analyzed on a 1% agarose gel to verify integration of Bla g 4 DNA into the yeast genome.

Expression of rBla g 4 and sample analysis. Initially, small-scale expression cultures were grown for selected clones of each phenotype. Mut⁺ clones were inoculated into 8 ml of buffered glycerol complex medium and grown for 24 to 48 hours at 30°C while shaking (270 rpm) to an OD₆₀₀ greater than 2.0. Cultures were spun down at 2000×g for 5 minutes, and cell pellets were resuspended to an OD₆₀₀ of 1.0 in buffered methanol complex medium. Mut⁻ clones were inoculated into 25 ml buffered glycerol complex medium and grown to an OD₆₀₀ of 2 to 6. Cells were spun down and resuspended in one-fifth volume of buffered methanol complex medium. All cultures were grown for 96 to 120 hours at 30°C while shaking. Samples were taken, and methanol was added to 0.5% final concentration every 24 hours. Samples were analyzed for protein expression by Coomassie-stained sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Pharmacia Phast System).

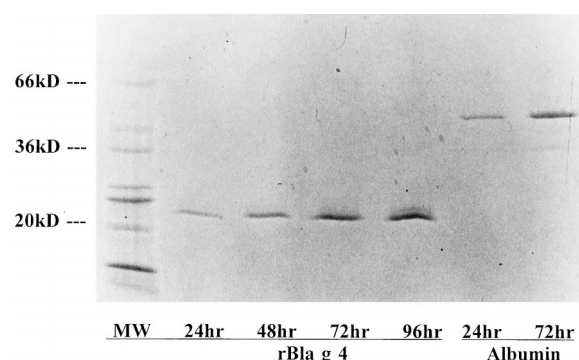


FIG. 2. Time course for expression of rBla g 4. Supernatants from rBla g 4 *P. pastoris* cultures were harvested at different time points and analyzed by Coomassie-stained SDS-PAGE. Cultures expressing albumin were used as positive controls.

Analysis of rBla g 4 by mass spectrometry

Mass spectrometry was used to verify that the recombinant protein produced in *Pichia* yeast was Bla g 4. Briefly, supernatant from two rBla g 4 clones was analyzed on a 15% polyacrylamide gel and the 23 kD Coomassie-stained bands were cut from the gel, reduced with 10 mmol/L dithiothreitol, alkylated in 50 mmol/L iodoacetamide, and digested overnight with 12.5 ng/ μ L trypsin. Tryptic peptides were eluted in 50% acetonitrile/5% formic acid, dehydrated, and analyzed on a tandem quadrupole instrument (Finnigan-MAT TSQ 7000; Finnigan, San Jose, Calif.) interfaced with a 10 cm capillary reversed-phase column. These analyses were carried out in the W.M. Keck Biomedical Mass Spectrometry Laboratory. The molecular weights (MWs) of the peptides were measured, and collisionally activated decomposition spectra were acquired for all peptides detected in the digest.²⁵

Purification of rBla g 4

Pichia-expressed rBla g 4 was purified from culture contaminants over a phenyl Sepharose CL-4B column (Pharmacia). Culture supernatant was dialyzed against 50 mmol/L phosphate buffer (pH 7.0) containing 2 mol/L NaCl and applied to the column at room temperature. Protein was eluted from the column in decreasing salt concentrations with phosphate buffer containing 1.5 mol/L NaCl, 1.0 mol/L NaCl, 0.5 mol/L NaCl, buffer alone, or water. A Superdex 75 HR 10/20 size exclusion column was used as a final purification step, running at 0.4 mL/min in 0.05 mol/L phosphate buffer containing 0.15 mol/L NaCl (pH 7.2). Bacterial rBla g 4 was purified as previously described.¹³

IgE antibody-binding radioimmunoassays

To compare differences in IgE antibody binding between the two allergen preparations, sera from individuals allergic to cockroaches were diluted 1:2 and 1:10 and incubated with 110,000 cpm of either ¹²⁵I-labeled bacterial rBla g 4 or ¹²⁵I-labeled *Pichia* rBla g 4 for 4 hours at room temperature. IgE myeloma serum (1:200 dilution) was used as a carrier, and IgE-allergen complexes were precipitated overnight at 4° C with 50 μ L sheep anti-human IgE (The Binding Site, San Diego, Calif.). Precipitates were washed three times with borate-buffered saline (pH 8.0) and counted in a gamma counter.²⁶

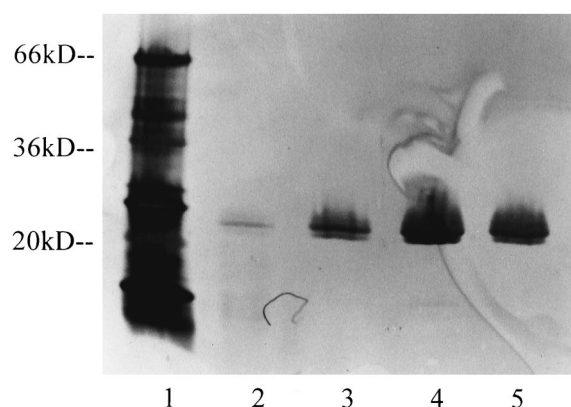


FIG. 3. Purification of rBla g 4 by chromatography on phenyl Sepharose. Lane 1, MW markers; Lane 2, culture supernatant; Lane 3, 1.5 mol/L NaCl eluted pool; Lane 4, 1.0 mol/L NaCl eluted pool; and Lane 5, high-performance liquid-chromatography purified rBla g 4.

Immediate skin testing

Quantitative intradermal skin testing was performed on patients allergic to cockroaches with serial 10-fold dilutions of bacterial or *Pichia*-produced rBla g 4 from 10⁻⁵ to 10⁰ μ g/mL as previously described.^{10,13} An 8 $\times$ 8 mm wheal reaction occurring 15 to 20 minutes after injection was considered positive. Skin testing of human subjects with recombinant allergens was approved by the University of Virginia Human Investigation Committee.

Deglycosylation

Pichia-produced rBla g 4 was deglycosylated with Endoglycosidase F/N-Glycosidase F (Endo F) (Boehringer Mannheim, Indianapolis, Ind.) by incubating 46 μ g of allergen with 2 U of enzyme in 50 mmol/L sodium phosphate, 50 mmol/L ethylenediamine tetraacetic acid, and 0.5% octylglucoside buffer (pH 7.0) overnight at 37° C. Samples were analyzed on an 8% to 25% gradient silver-stained Phast gel (Pharmacia). Effects of deglycosylation on antibody binding were assessed by inhibition radioimmunoassay (RIA). Briefly, serial fourfold dilutions (from 1000 ng/mL to 1 ng/mL) of deglycosylated or untreated *Pichia* rBla g 4 were incubated for 2 hours at room temperature with a 1:4 dilution of serum from an individual allergic to Bla g 4. Radiolabeled *Pichia* rBla g 4 was then added (~115,000 cpm), and tubes were incubated for an additional 2 hours at room temperature, precipitated, washed, and counted as described above.

RESULTS

Ten clones were selected as being representative of the Bla g 4 transformations into *P. pastoris* strains GS115 and KM71. By PCR, seven of 10 clones had Bla g 4 cDNA incorporated into the yeast genome, and four of these transformants secreted rBla g 4 into the medium. Of the four clones expressing protein, two were His⁺ Mut^S phenotypes of the GS115 strain, one was His⁺ Mut⁺ of the GS115 strain, and one was a His⁺ Mut^S transformant from the KM71 *P. pastoris* strain.

Analysis of the time course of allergen expression in culture supernatants showed that a prominent 23 kD

TABLE I. Analysis of *Pichia*-expressed Bla g 4 by mass spectrometry*

Peptide	Measured MW (kd)	Peptide sequence by CAD	Bla g 4 sequence from database
1	749.8	C*WXDR	C*WIDR (749.9)
2	817.4	AXEEDXK	AIEEDLK (816.9)
3	905.8	PNXDYER	PNLDYER (906.9)
4	1015.6	FTXDYNDK	FTIDYNDK (1016.1)
5	1145.0	FSYDDAXVSK	FSYDDALVSK (1145.3)
6	1201.3	FTXDYNDKKGK	FTIDYNDKKGK (1201.3)
7	1797.0	--WXXAAGTSE AXTOYK	GSWIIAAGTSEALTQYK (1797.0)
8	1882.8	· yvef TD TXAN EDC*FR	AVLALC*ATDTXANEDC*FR (1924.0)

*Peptide sequences were obtained from tryptic digests of the 23 kd protein expressed in *P. pastoris* and compared with the published cDNA sequence (Genbank Accession Number U40767).¹³

X, Leucine or isoleucine; C*, carbamidomethyl-modified cysteine; --, a single undetermined amino acid; ·, lower case amino acids are vector sequence; Eco R1 site in bold, cloned Bla g 4 sequence underlined.

band was secreted into the media at 24 to 48 hours with maximum production occurring at 72 to 96 hours (Fig. 2). To confirm that the protein being produced was rBla g 4, the 23 kd band from two clones was cut from a polyacrylamide gel and analyzed by mass spectrometry. Tryptic digests of both clones contained a similar number of peptides with the same MW. Sequence data for seven peptides from one clone showed exact matches to residues predicted from the deduced amino acid sequence of rBla g 4 (Table I). Peptide 8 was not an exact match to the database sequence, but it did match the cloned sequence exactly, containing two amino acids from the vector and two amino acids of the Eco R1 site. Two additional large peptides were detected that were difficult to interpret because of internal charging, but they were completely consistent with peptides from the data base sequence (accounting for an additional 55 amino acids). Overall, the amino acid sequence of the peptides covered 122 of the 182 expected amino acid residues of rBla g 4.

Mass spectrometry confirmed that the *Pichia*-expressed protein was rBla g 4, and the allergen was purified by phenyl Sepharose chromatography and size exclusion high-performance liquid chromatography (Table II). The rBla g 4 eluted with 1.5 mol/L and 1.0 mol/L NaCl with yields of 50 mg/L culture medium. Silver-stained SDS-PAGE analysis showed a single band of rBla g 4 for both the phenyl Sepharose elutions and after gel filtration chromatography, indicating that high-performance liquid chromatography purification may not be necessary for most applications (Fig. 3).

To compare the antigenicity of *Pichia*-expressed rBla g 4 with bacterial rBla g 4 previously produced in the pGEX system, we used *Pichia*- or *E. coli*-expressed rBla g 4 to inhibit binding of either radiolabeled allergen to IgE antibody from an individual allergic to cockroaches.¹³ Both allergens completely inhibited binding of radiolabeled rBla g 4 to IgE antibody and showed identical dose response curves (data not shown). In addition, *Pichia*- and *E. coli*-expressed allergen showed

TABLE II. Purification yield of *Pichia*-expressed rBla g 4*

Experiment no.	Culture volume (ml)	1.5 mol/L pool (mg)	1.0 mol/L pool (mg)	Total yield (mg/L)
1	513	7.5	18.2	50
2	634	12.0	20.3	51

*Yield after chromatography on phenyl Sepharose and elution with 1.5 mol/L or 1.0 mol/L NaCl.

similar reactivity on quantitative intradermal skin testing and in antigen binding RIA. Positive skin test results were obtained in individuals allergic to cockroaches with the same concentrations of *Pichia*- or *E. coli*-expressed rBla g 4 (10^{-1} to 10^{-3} µg/ml), and these patients showed strong IgE antibody binding to both allergens in RIA (Table III). Nonallergic control subjects showed no IgE antibody binding or skin test reactivity to the two allergens. A patient allergic to cockroach who did not have IgE antibody to rBla g 4 also had negative skin test responses.

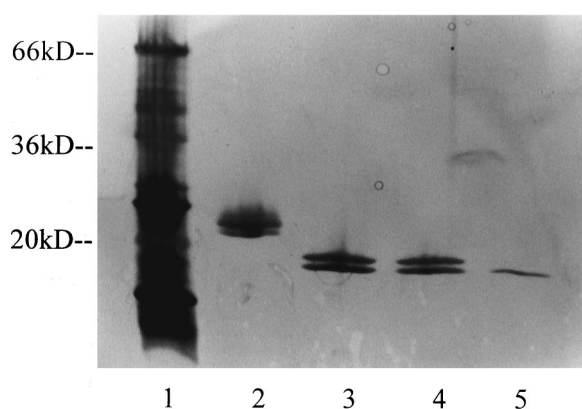
Pichia-produced rBla g 4, has an apparent MW of 23 kd as determined by SDS-PAGE, compared with a MW of 18 kd for *E. coli*-expressed rBla g 4.¹³ To investigate whether the difference in MW was due to glycosylation, we treated *Pichia*-expressed rBla g 4 with Endo F to remove the carbohydrate. The results showed a decrease in apparent MW of 4 to 5 kd as determined by SDS-PAGE, suggesting that the allergen was glycosylated. These deglycosylation experiments consistently showed two bands instead of an anticipated single deglycosylation product, with the lower band having the same MW as the *E. coli*-expressed allergen (Fig. 4). Comparison of deglycosylated *Pichia* Bla g 4 with the glycosylated allergen showed no differences between the two in inhibition RIA. Both preparations inhibited binding of radiolabeled allergen to IgE antibody to the same extent (Fig. 5), showing that the additional carbohydrate had

TABLE III. Skin tests and serum IgE antibodies to recombinant Bla g 4

Patient no.	CR RAST (U/ml)	rBla g 4 in <i>E. coli</i>		rBla g 4 in <i>P. pastoris</i>	
		IgE antibody* (cpm bound)	Skin test (μ g/ml)	IgE antibody* (cpm bound)	Skin test (μ g/ml)
1	450	7,667	10^{-3}	10,331	10^{-3}
2	ND	4,816	10^{-3}	6,774	10^{-3}
3	1,290	11,765	10^{-2}	31,713	10^{-2}
4	1,065	7,396	10^{-1}	8,699	10^{-1}
5	1,080	677	>1	151	>1
Control subjects	<10	$721 \pm 200^\dagger$	>1	$175 \pm 42^\dagger$	>1

ND, Not done.

*Measured by antigen-binding RIA.

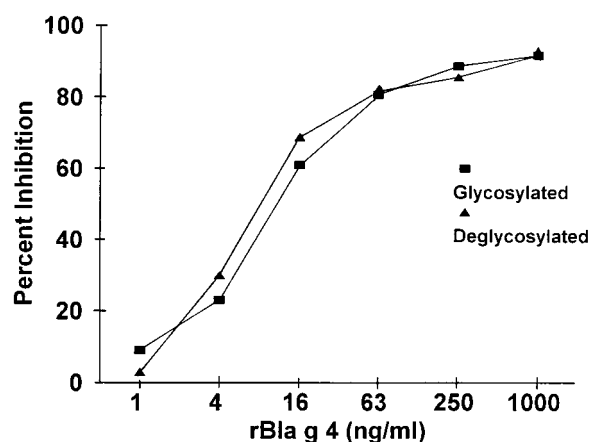
 † Mean $\pm$ 2 SD of three nonallergic subjects.FIG. 4. Effect of deglycosylation on *Pichia*-expressed rBla g 4. Lane 1, MW markers; lane 2, untreated rBla g 4; lanes 3 and 4, Endo F-treated rBla g 4 (1 U and 2 U of enzyme, respectively); and lane 5, *E. coli*-expressed rBla g 4.

no effect on IgE antibody binding and that the IgE binding epitopes were present on protein determinants.

DISCUSSION

In this study high-level expression (50 mg/L) of recombinant Bla g 4 was obtained by using the *P. pastoris* system, and the yeast-expressed allergen had comparable antigenic reactivity to that produced previously in *E. coli* (pGEX system). Recombinant Bla g 4 was the major protein secreted into the yeast culture medium and required only additional purification over phenyl Sepharose to generate a single purified and concentrated product. It was observed that *P. pastoris* transformants that generated large bands when screened for the Bla g 4 DNA insert by PCR were also the clones that produced significant quantities of rBla g 4 in expression cultures (data not shown). Thus PCR verification of the DNA insert may select out high copy transformants, which are noted for producing higher yields of foreign protein.²⁷

One potential problem with eukaryotic systems is the addition of sugars to proteins as they pass through the secretory pathway. However, *P. pastoris* produces much shorter carbohydrate chains (8 to 14 sugars in length

FIG. 5. Inhibition RIA comparing the effect of deglycosylation on IgE antibody binding. Glycosylated or deglycosylated rBla g 4 from *P. pastoris* was used to inhibit binding of ¹²⁵I-labeled *Pichia* rBla g 4 to IgE antibody in serum from an individual allergic to cockroaches.

vs > 50 sugars for *Saccharomyces cerevisiae*), primarily of the N-linked high mannose type. These chains do not have the terminal α 1,3 mannose linkages found in *S. cerevisiae*, which are thought to be antigenic.^{20, 23, 27} Natural Bla g 4 has not yet been isolated, and it is not known whether it is glycosylated. The deduced amino acid sequence of Bla g 4 has one potential N-linked glycosylation site, but the apparent MW on SDS-PAGE is that of a nonglycosylated protein.

The increase in MW of rBla g 4 produced in the *P. pastoris* system compared with that produced in *E. coli* (by 5 kD) was caused by glycosylation. A reduction in MW was obtained on digestion of *Pichia* rBla g 4 with Endo F enzyme; however, two bands were observed instead of a single deglycosylated product. Because eukaryotic organisms glycosylate the same molecule to differing degrees, the upper band detected in our experiment may represent rBla g 4-containing sugar moieties that were not removed by Endo F.^{23, 27} Indeed, mass spectrometry results confirm that both bands are rBla g 4 and could not discern any differences between the two on the basis of tryptic peptides, which further suggests

incomplete removal of carbohydrate from the upper band (data not shown). Glycosylation had no influence on the antigenic reactivity of rBla g 4 because no differences were obtained between the glycosylated or deglycosylated sample in IgE antibody inhibition assays. Even with partial deglycosylation, one would expect to see a shift in binding if the carbohydrate moiety was involved in the binding site. Our results do not completely rule out the possibility that carbohydrate, if present on natural Bla g 4, could react with IgE antibody. This would need to be clarified by using the natural allergen.

Allergen expression in *P. pastoris* avoids several problems associated with bacterial expression systems. In the pGEX system, complete removal of glutathione-S-transferase after thrombin cleavage of the fusion protein can be hard to achieve, leaving trace amounts of glutathione-S-transferase (1%) as a contaminant. In other bacterial systems (e.g. pET) the protein is produced in inclusion bodies and may require extraction in detergents or may be partially denatured and need to be refolded. The *P. pastoris* system enables large quantities of biologically active allergen to be secreted into the medium and requires minimal purification. It is potentially useful for most allergens that have been expressed in bacteria. An 80 kd *Trichophyton* allergen has been expressed in a *Pichia* vector (pPICZA) that carries the gene for zeocin resistance, the myc epitope, and a histidine tag (Dr. Judith Woodfolk, personal communication, 1997). These characteristics allow for easier selection and detection of *P. pastoris* transformants and provide the option for purifying the recombinant allergen using affinity chromatography. Recently, a new *Pichia* vector, which constitutively expresses the gene for glyceraldehyde-3-phosphate at high levels, has been developed. The glyceraldehyde-3-phosphate promoter expresses at comparable levels as P_{AOX1} , but has the advantage that it can use glucose instead of methanol as a carbon source.²⁸

The production of large amounts of biologically active rBla g 4 in *P. pastoris* will enable the structure and function of this allergen to be investigated. It will be particularly useful for crystallographic studies to determine the three dimensional structure of Bla g 4, which may shed light on its biologic function as has recently been demonstrated for the human Von Ebner's gland lipocalin (VEGh). By three-dimensional structural comparison, van't Hof et al.²⁹ showed that the VEGh protein was a cysteine proteinase inhibitor. Structural analysis of Bla g 4 could determine whether it is more closely related to dog allergen (Can f 1), which is homologous to VEGh, or to the pheromone binding protein allergens Rat n 1 and Mus m 1.³⁰ By using *Pichia*-expressed rBla g 4 and other cockroach allergens, it will also be possible to compare T-cell responses in patients allergic to cockroaches. This will allow further investigation of the mechanisms by which cockroach allergens cause inflammation in patients with asthma.

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