

Clinical aspects of allergic disease

Immunologic and physicochemical studies of Bermuda grass pollen antigen BG60

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Background: In a previous study we showed that antigen BG60 of Bermuda grass pollen contains isoallergens. Because the yield of purified isoallergens was low when a chromatofocusing technique was used, it was difficult to carry out further studies, such as determination of carbohydrate composition and structure.

Objective: The aim of this study was to establish a procedure to purify antigen BG60 proteins as a group and to characterize this group's physicochemical and immunologic properties.

Methods: A combination of chromatographic techniques (ion-exchange, gel filtration, blue gel affinity, and reverse-phase high-performance liquid chromatography) was used for the purification of BG60. Immunoblot and ELISA techniques were used to study BG60-specific IgE and IgG antibodies in patients' sera. The role of the carbohydrate moiety in antigenicity and allergenicity was examined with monoclonal antibodies and allergic sera by using periodate-treated BG60. Its carbohydrate composition was analyzed by high-performance anion-exchange chromatography with a pulsed amperometric detector.

Results: Homogeneity of BG60 was demonstrated by a single sharp peak in reverse-phase high-performance liquid chromatography, a single band in sodium dodecylsulfate-polyacrylamide gel electrophoresis, and only one band stained by anti-BG60 monoclonal antibody. BG60-specific IgE and IgG antibodies were shown to be present in allergic sera. Six plant lectins were found to react with BG60. On periodate treatment, BG60 reduced binding toward its monoclonal antibody and human IgE and IgG. Carbohydrate composition analysis showed that BG60 contains three kinds of sugars: mannose, N-acetylglucosamine, and fucose (in a ratio of approximately 3:2:1) and a minute amount of xylose. The carbohydrate content is approximately 7.5%, and peptide content is about 92.5%.

Conclusion: A procedure was established for the purification of a large quantity of the BG60 antigen. The results suggest that the carbohydrate moiety of antigen BG60 may play an important role in the immune response. (*J Allergy Clin Immunol* 1996;98:486-94.)

Key words: Pollen, allergen, purification, carbohydrate

Bermuda grass (*Cynodon dactylon*) pollen has been shown to cause allergic reactions and positive skin test results in allergic patients.¹⁻³ It contains a

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Abbreviations used

BGP:	Bermuda grass pollen
HPAEC:	High-performance anion-exchange chromatography
mAb:	Monoclonal antibody
PBS:	Phosphate-buffered saline
RP-HPLC:	Reverse-phase high-performance liquid chromatography

very complex mixture of at least 53 antigens.⁴ More than 12 protein molecules have been demonstrated to bind specific human IgE and IgG by immunoblot techniques.⁴⁻⁷ Many studies^{5, 8-15} have been done to isolate and characterize its allergenic

components. However, only few antigens have been purified and partially characterized. Recently, we showed that one of the protein molecules with an apparent molecular weight of 60 kd (designated BG60) contains a group of IgE-specific binding molecules.^{12, 13} Two of the components, BG60a and BG60b, share immunologic identity.¹² Our data (unpublished) also showed that the third component of BG60 also manifests immunologic activity identical to that of the other two molecules.

Although we were able to purify the isoallergens of BG60 from the pollen, the yield of each allergen was low, and it would be very difficult to study its physicochemical and immunologic properties thoroughly. Therefore the aim of this study was to establish a procedure to purify a large quantity of BG60 and to characterize its physicochemical and immunologic properties. With the availability of a large quantity of purified BG60, studies of the role of the carbohydrate moiety in immune response and carbohydrate composition and structure analysis became feasible.

METHODS

Preparation of A2

Fraction A2 was prepared as described previously.¹² All experiments were carried out at 4° C unless otherwise indicated. Briefly, pollen was extracted by phosphate-buffered saline (PBS), and the extract was precipitated by 90% ammonium sulfate. Precipitate was collected by centrifugation and dissolved in distilled water, and undissolved material was removed by centrifugation again. Clear brownish supernatant was applied to a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) to remove small compounds and pigments. The breakthrough fraction (designated G1) was treated with solid ammonium sulfate, and the precipitate was collected, dissolved, and dialyzed against phosphate buffer, then applied to a cation exchange column. Fraction C1, eluted from the column with buffer containing 0.1 mol/L NaCl, was concentrated and applied to an Ultrogel AcA 44 column (IBF Biotechnics, France). Antigen BG60 was found to reside in the major peak, designated A2. Fraction A2 was treated with 70% ammonium sulfate, and the precipitate was collected and dialyzed extensively against Tris-HCl (25 mmol/L, pH 8.4).

Affinity chromatography on a blue gel column

Fraction A2 obtained from a previous step was applied to a blue gel column (Bio-Rad Laboratories, Life Science Group, Hercules, Calif.) (2.5 × 15 cm) equilibrated with Tris-HCl (25 mmol/L, pH 8.4). Elution was carried out at a flow rate of 64 ml/hr by stepwise increase of NaCl concentration. The fraction obtained from the

blue gel column, designated B2, was treated with 70% ammonium sulfate; and the precipitate was collected, dissolved, extensively dialyzed against distilled water, and then lyophilized.

Reverse-phase high-performance liquid chromatography (RP-HPLC)

Dried sample, fraction B2 (10 mg), obtained from blue gel column was dissolved in 1 ml of 0.1% trifluoroacetic acid and applied to a semi-preparative RP-HPLC column (8 × 100 mm, Delta-Pak C18; Waters Chromatography, Milford, Mass.) equilibrated with 0.1% trifluoroacetic acid. Elution was carried out with a continuous gradient of increasing acetonitrile from 0% to 100% for 60 minutes at a flow rate of 1 ml/min.

Protein determination

Protein content was determined by using a bicinchoninic acid reagent¹⁶ with bovine serum albumin as standard.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Highly purified BG60 was used for SDS-PAGE according to the method of Laemmli¹⁷ with 5% stacking gel and 10% separating gel. After electrophoresis, protein was either stained by Coomassie blue or transferred¹⁸ to a nitrocellulose sheet (BioRad) for reaction with IgG and IgE or lectins with the staining procedures described by Shen et al.⁷

Lectin-binding reactivity

After BG60 (0.5 µg/strip) was transferred to nitrocellulose strips, blocked, and washed, a panel of six biotinylated lectins—*Ricinus communis* agglutinin, concanavalin A, wheat germ agglutinin, *Phaseolus vulgaris* erythroagglutinin, *Datura stramonium* agglutinin, and peanut agglutinin (Lectin-Link kit; Genzyme, Boston, Mass.)—was added and incubated for 1 hour with constant shaking at room temperature. The binding of lectins to BG60 was detected by using premixed avidin and biotinylated alkaline phosphatase reagent according to the manufacturer's instructions.

Periodate oxidation

The effect of periodate oxidation of BG60 on the binding of monoclonal antibodies (mAbs) and allergic serum was studied. Periodate oxidation of the antigen was performed as described by Pazur.¹⁹ Briefly, BG60 (20 µg) was oxidized in 100 µl of sodium periodate (20 mmol/L, pH 4.5) at 4° C for 18 hours and dialyzed against distilled water; then sodium borohydride was added to a final concentration of 50 mmol/L, and the BG60 solution was incubated at 22° C for 30 minutes and dialyzed against distilled water extensively before it was coated onto a 96-well microtiter plate. Control samples were treated the same way except without

periodate oxidation. The binding of two-hybridoma culture soup (3-537-03 and 3-537-91) and pooled allergic sera to BG60 and oxidized BG60 was measured by ELISA as described below.

ELISA

ELISA was carried out essentially as described by Voller et al.²⁰ In general, five washings with distilled water were performed between each step unless otherwise indicated. Fifty microliters of antigen BG60 (2 µg/ml), diluted in carbonate buffer (15 mmol/L, pH 9.6), was coated onto a 96-well microtiter plate (Costar, Cambridge, Mass.) at 4° C overnight. The wells were blocked with blocking solution (1% normal goat serum in PBS-Tween 20) at 37° C for 35 minutes. Patients' sera were diluted 1000-fold for IgG and 10-fold for IgE with blocking solution and were allowed to react at 4° C overnight. Alkaline phosphatase-conjugated mouse anti-human (IgE, IgG) antibody was diluted 1000-fold for IgE assay and 5000-fold for IgG assay according to the manufacturer's suggestion (Pharmingen, San Diego, Calif.). Horseradish peroxidase-conjugated goat anti-mouse IgG antibody was diluted 5000-fold for mAb assay. Alkaline phosphatase activity was determined by using disodium p-nitrophenyl phosphate as a substrate. Horseradish peroxidase activity was measured by using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and by measuring the absorbance at 405 nm between 10 and 30 minutes.

Human serum, rabbit antiserum, and mAb

Patients were referred to the allergy clinic at the University of Florida because of history of seasonal allergic rhinitis for at least 1 year, and they had never received Bermuda grass pollen (BGP) immunotherapy. Their ages ranged from 15 to 35 years. Serum samples were obtained when patients visited the clinic for the first time and were stored in 1 ml aliquots at -20° C until use. Rabbit antiserum against BGP was raised by an intramuscular injection of the crude extract as described by Haroe and Ingild.²¹ Rabbit antiserum was treated with 33% in ammonium sulfate; and the precipitate was collected by centrifugation, dissolved in distilled water, and extensively dialyzed against distilled water. The precipitate was removed by centrifugation, adjusted to the original volume of the serum, and stored in aliquots at -20° C until use. Murine mAb was prepared as described previously.¹³

Skin tests

Skin tests were applied to each patient's back and included the standardized allergenic extracts of bahia, Bermuda, Johnson, red top, and timothy grass pollens (Miles Laboratories, Spokane, Wash.). Wheal reactions of greater than 5 mm over albumin-saline controls were considered positive. Classification of the skin reaction ranged from 0 to 4+. Eighteen patients included in this study had registered at least 3+ (15 mm induration over

control) reaction by the prick test with the BGP extract. Three normal sera (nonatopic) were used as negative controls and showed very low absorbance values (0.05 to 0.08), which was considered no reactivity. Two patients with asthma who were allergic to BGP were subjected to skin testing by subcutaneous injection with 25 µl of the purified BG60 antigen (2 µg/ml) in PBS. Wheal reading was taken within 15 minutes after the injection, and a wheal greater than 8 mm over the PBS control was considered a positive response.

Amino acid and N-terminal analyses of the antigen BG60

About 1 nmol of BG60 was hydrolyzed with 100 µl of 4 mol/L methane-sulfonic acid containing 0.2% tryptamine in a capillary tube at 150° C for 90 minutes as described previously.²² Amino acids were analyzed with a Beckman model 120 amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) with both long (for neutral and acidic amino acids) and short (for basic amino acids) columns. N-terminal analysis was carried out as described previously.²³

Carbohydrate composition analysis

Carbohydrate composition analysis was carried out by high-performance anion-exchange chromatography (HPAEC) with a Dionex BioLC (Dionex Corp., Sunnyvale, Calif.) in tandem with a PAD-II electrochemical detector.²⁴ A small amount of sample (about 20 nmol protein) was hydrolyzed either in 2 mol/L trifluoroacetic acid (for neutral sugars) for 6 hours at 100° C or in 4 mol/L HCl (for amino sugars) for 6 hours at 100° C.²⁵ The individual hydrolysates were evaporated under vacuum, and the residue was dissolved in 200 µl of water and evaporated again under vacuum. The process of dissolution and evaporation was repeated once more to ensure complete removal of the acid. The residue was taken up in 200 µl of distilled water, and aliquots were used for analysis by HPAEC by using isocratic elution of a Carbo-Pac PA-1 (4 × 250 mm) column (Dionex Corp.) with 1 mmol/L NaOH-0.03 mmol/L sodium acetate. This condition enables separation of xylose from other sugars. Rhamnose was used as an internal standard. The reference sugars used for identifying unknown compounds were fucose, 6-methylgalactose, galactose, N-acetylgalactosamine, N-acetylglucosamine, glucose, xylose, and mannose.

RESULTS

Purification of antigen BG60

When the fraction A2 was chromatographed on the blue gel column, BG60 was so tightly bound to the column as to be eluted with a buffer containing 0.25 mol/L NaCl. This fraction was designated B2 (Fig. 1). Fraction B2, containing mostly BG60 as determined by SDS-PAGE (data not shown), was then further purified by RP-HPLC (Fig. 2), in which only one peak was found. The purified

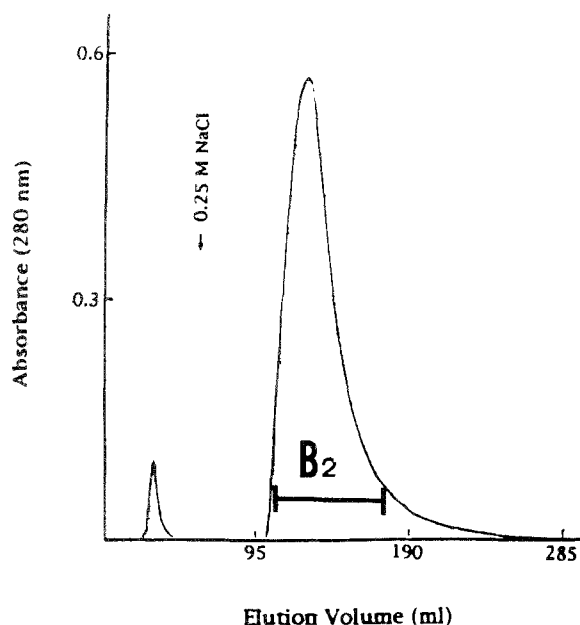


FIG. 1. Chromatogram of fraction A2 (50 mg) on a blue gel affinity (2.5×15 cm) column. Fractions containing BG60 were collected as indicated by the black bar and designated B2.

material produced one broad band, corresponding to an apparent molecular weight of 55 to 60 kd as determined by SDS-PAGE (Fig. 2, inset). BG60 also showed one diffuse band when stained with anti-BG60 mAb (data not shown).

Antigen BG-60 and lectin binding

Lectins are carbohydrate-binding proteins and have been used for purification of many glycoproteins. Because BG60 is a glycoprotein, the binding of different lectins to BG60 was examined. As shown in Fig. 3, all six lectins (*R. communis* agglutinin, concanavalin A, wheat germ agglutinin, *P. vulgaris* erythroagglutinin, *D. stramonium* agglutinin, and peanut agglutinin) were able to bind to BG60 as indicated by the arrow. The lectin-binding reactivity to BG60 strongly confirmed the presence of the carbohydrate in BG60. Furthermore, the binding of IgE and IgG antibodies to BG60 was also inhibited by lectin mixture (data not shown). The binding specificity of these lectins suggests that BG60 may contain sugars such as mannose, glucose, galactose, and N-acetylglucosamine in its carbohydrate moiety.

Periodate oxidation

The possibility of the involvement of a carbohydrate moiety in the antigenic determinant site of

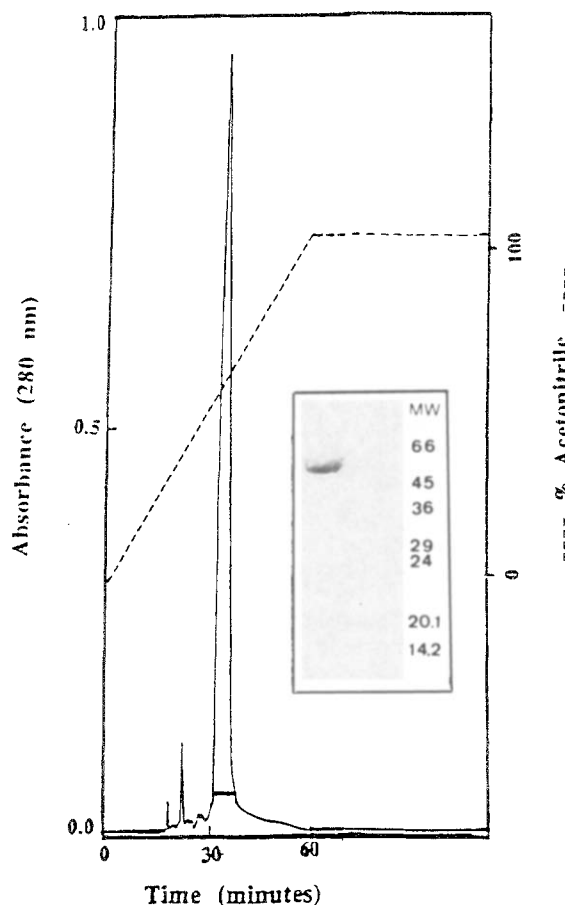


FIG. 2. Chromatogram of fraction B2 (10 mg) on a semi-preparative RP-HPLC (10×100 mm). Sample (8 μ g) obtained from HPLC major peak (indicated by bar) was loaded for SDS-PAGE (10% gel) and stained with Coomassie blue (inset). Seven standard proteins were included. MW, Molecular weight.

BG60 was investigated by periodate oxidation (20 mmol/L). Two mAbs used for the binding study are shown in Fig. 4, A. The loss of oxidized BG60-binding activity to mAbs (3-537-03 and 3-537-91) was between 60% and 70%, indicating that the carbohydrate moiety of BG60 is involved in the binding of the mAbs. Furthermore, the reduced binding activity of oxidized BG60 to human IgE and IgG antibodies was also demonstrated, in a dose-dependent manner, by ELISA (Fig. 4, B, IgE) and immunoblot experiments (IgG, data not shown). As shown in Fig. 4, B, the oxidized BG60 decreased its binding activity to IgE from 20% to 65% as the concentration of periodate increased from 1 mmol/L to 10 mmol/L. Both of these results provided evidence for the involvement of the carbohydrate moiety of BG60 in immune responses.

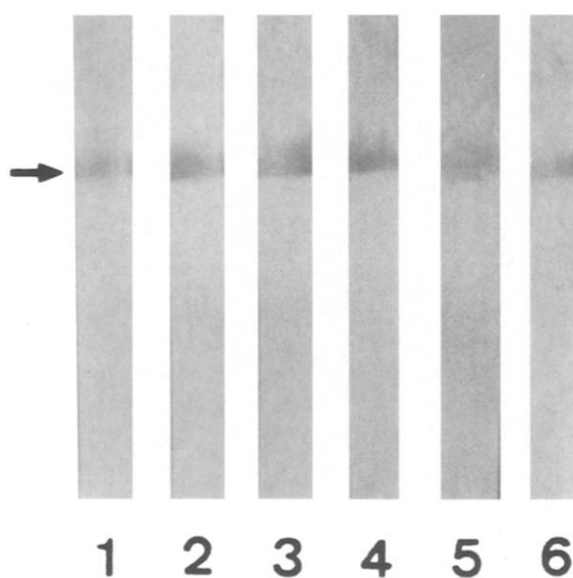


FIG. 3. Lectin-binding reactivity of BG60 (indicated by arrow). The binding of lectins to BG60 strips (0.5 μ g/strip) was detected by a lectin-link kit. Lane 1, *R. communis* agglutinin; lane 2, concanavalin A; lane 3, wheat germ agglutinin; lane 4, *P. vulgaris* erythroagglutinin; lane 5, *D. stramonium* agglutinin; and lane 6, peanut agglutinin.

Antigenic and allergenic properties of antigen BG6

The specific human IgG and IgE antibodies against BG60 were demonstrated by ELISA with allergic sera. As shown in Table I, all sera contained specific IgG and IgE antibodies to BG60. All sera contained a high titer (4+) of IgG antibody. However, among these sera, two showed a low level (1+), six showed a medium level (2+), and 10 showed a high level (3+) of specific IgE antibody to the antigen. These studies indicated that BG60 is a strong immunogen in human beings. Furthermore, the allergenicity of the BG60 was also confirmed by a positive skin test reaction in two patients with asthma who were allergic to BGP (data not shown).

Amino acid and N-terminal analysis of the antigen BG60

The amino acid composition of BG60 is summarized in Table II. A total of 186.5 μ g of BG60 was hydrolyzed, and 170 μ g was recovered as a sum of amino acid residues, accounting for 91% recovery. BG60 contains most of the natural amino acids and is rich in lysine and arginine. The value for tryptophan was not corrected for destruction during hydrolysis, and cysteine was not determined. We failed to detect N-terminal amino acid in BG60 by using a solid-phase sequencer, suggesting the possible blockage of the N-terminal amino acid.

Carbohydrate composition analysis

The chromatograms of the sugar analyses are shown in Fig. 5. As shown in Fig. 5, A, there are three major peaks (corresponding to fucose, N-acetylglucosamine, and mannose) and a fourth peak, labeled *Rha*, which was added as an internal standard to the hydrolysate of BG60. However, a minute amount of xylose was also present as indicated by a small peak. Fig. 5, B, is the elution profile of the standard sugars, which was run in parallel. A total of 186.5 μ g of BG60 was hydrolyzed, and 13.8 μ g of sugars was recovered as calculated from the peak of sugar residues (Fig. 5), which accounts for 7.4% of the BG60. The results of the sugar analyses are summarized in Table III. According to the ratio of sugars and the results of the lectin-binding study and on the basis of other known structures of oligosaccharides in the plant glycoproteins, the structure of the major oligosaccharide may be proposed as shown in Fig. 6. Other types of oligosaccharide structures, especially those containing xylose, may also exist. The exact structures of the oligosaccharides are under investigation.

DISCUSSION

In a previous study we showed that BGP contains a highly reactive allergen component known as BG60, with an apparent molecular weight of 55 to 60 kD, which contains three or four separable protein molecules. Using chromatofocusing, we were able to purify two of the components, which showed total immunologic identity.^{12, 13} Our recent data (unpublished) showed that the third component of the BG60 also manifests total immunologic identity to two other components. However, the yield of individual isoallergen purified by chromatofocusing was too low to accumulate sufficient quantity with which to study the nature of carbohydrate in detail. Because BG60 contains three or four protein molecules that possess similar physicochemical and immunologic properties, we decided to establish a purification procedure for the whole group of BG60 molecules without separating individual isoallergens.

In this study we were able to obtain about 20 mg of purified BG60 from 800 gm of BGP by a combination of ion-exchange chromatography, gel filtration, blue gel affinity chromatography, and RP-HPLC. The homogeneity of BG60 was demonstrated by a single sharp peak on RP-HPLC chromatogram, one band on SDS-PAGE, and one band stained with anti-BG60 mAb (data not shown). BG60 was previously estimated to contain

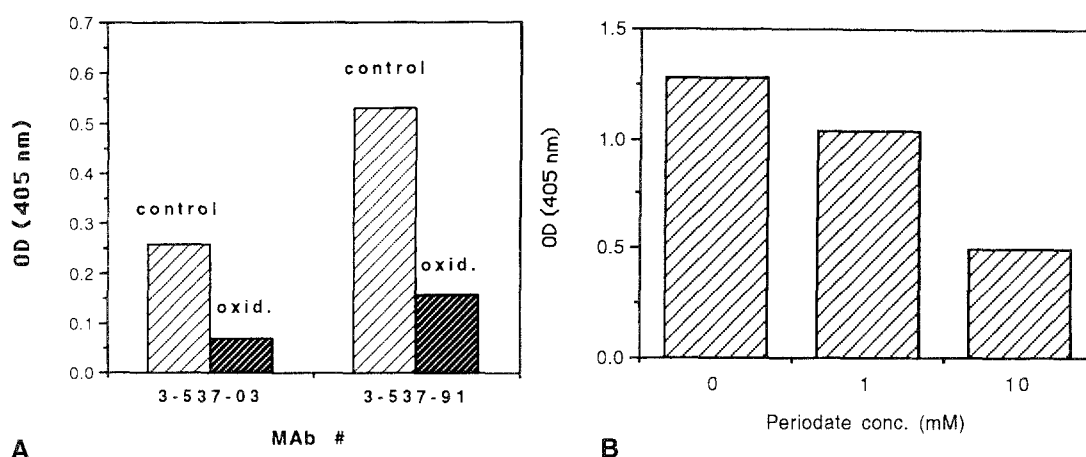


FIG. 4. Effect of periodate oxidation on binding of BG60 to antibodies. **A**, Binding of two mAbs (3-537-03 and 3-537-91) to BG60 treated with 20 mmol/L sodium periodate. **B**, Binding of human IgE to BG60 treated with different concentrations of periodate.

TABLE I. Results of skin tests with crude extract and IgG*-IgE-binding reactivity of BG60 in patients allergic to BGP (rhinitis and bronchial asthma)

Serum No.	Skin test score (+) (crude extract)	ELISA (IgE, +) (BG60)
102	3	3
114	3	3
139	3	3
146	3	3
159	3	3
173	3	1
203	3	3
226	3	3
267	3	3
293	3	3
299	3	2
319	3	2
332	3	2
362	3	3
384	3	1
401	3	2
401	3	2
421	3	2

Skin reactivity was recorded according to the manufacturer's recommendation. The positivity scale for ELISA was arbitrary, taken for the net value of the serum as: 0.2 < 1+ < 0.5, 0.5 < 2+ < 0.9, 0.9 < 3+ < 1.5, and 1.5 < 4+.

*All sera showed 4+ of IgG reactivity.

about 28% carbohydrate as determined from the difference between the weight of the lyophilized material and the amount of protein measured by colorimetry.¹³ However, on the basis of results of compositional analysis in this study, the amino acid

TABLE II. Amino acid composition of antigen BG60

Amino acid	Molar ratio
Asp	16.7
Thr	10.5
Ser	12.5
Glu	11.0
Pro	10.8
Gly	17.1
Ala	14.6
Val	10.4
Met	2.1
Ile	6.5
Leu	10.9
Tyr	7.6
Phe	7.2
Lys	8.5
His	2.3
Arg	2.4

content was about 92.5%, and the carbohydrate content was about 7.5%. The reason for different values obtained by two techniques is not clear at present. One possible explanation is that lyophilized protein may still contain salts, although it had been dialyzed for a week against deionized water. Thus the salt remaining in the sample was misrepresented as carbohydrate by deduction of the protein assayed by colorimetry.¹³

The binding of IgE and IgG to BG60 was not abolished by the unfolding of the protein molecule on SDS-PAGE under reducing conditions, which suggests that both antigenicity and allergenicity are not totally dependent on the peptide conforma-

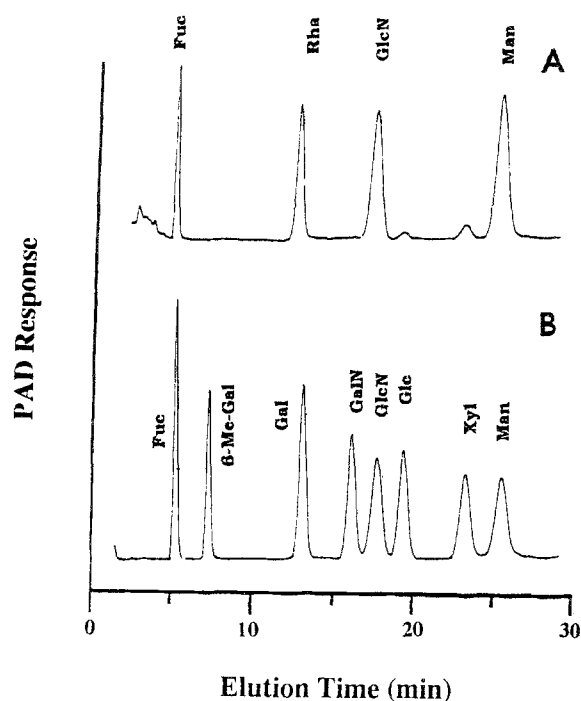


FIG. 5. HPAEC for carbohydrate analysis. **A**, Chromatogram of BG60 hydrolysates. Rha was added to sample as an internal standard. **B**, Chromatogram of the standard sugars for identification of the unknown sugar. *Fuc*, Fucose; *GlcN*, N-acetylglucosamine; *Man*, mannose; *6-Me-Gal*, 6-methylgalactose; *Gal*, galactose; *Glc*, glucose; *Xyl*, xylose.

tional structure. This characteristic seems quite common in grass pollen, as pointed out by Singh et al.²⁶ The retention of the BG60 binding to antibodies under SDS-PAGE conditions led us to examine the role of the carbohydrate moiety in the antibody response. The role of the carbohydrate in allergenicity or antigenicity has been controversial. Previous studies^{27, 28} have suggested that the carbohydrate moiety is not essential for allergenicity of the pollen allergens. However, the involvement of carbohydrate in antigenicity or allergenicity of the allergen has been reported.²⁹⁻³⁴ Recently, Hansen et al.³⁵ reported that two allergens (90 kd and 38 kd) of *Aspergillus fumigatus* partially lost their IgE-binding abilities on periodate oxidation and suggested that the carbohydrate is involved in IgE binding. Also, N-glycans of plant glycoprotein have been shown to play an important role in mammalian immune responses.³⁶⁻⁴¹

In this study the binding of mAb and allergic sera to oxidized BG60 was reduced to 30% to 40% of the unoxidized BG60, suggesting that the carbohydrate moiety of BG60 is involved in the mAb and human IgE and IgG binding domain. How-

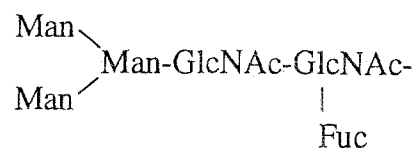


FIG. 6. Structure of major oligosaccharide of BG60.

TABLE III. Monosaccharide composition of antigen BG60 as determined by Dionex-BioLC after trifluoroacetic acid hydrolysis

Sugar	Molar ratio
Fucose	0.95
N-acetylglucosamine	2.23
Mannose	3.0
Xylose	—

ever, to prove that the carbohydrate moiety of BG60 plays a major role in immune response, direct evidence must be obtained. We are in the process of performing a binding-inhibition assay with the pronase-digested product of BG60, and preliminary results indicate that the carbohydrate plays an important role in the immune response.

Several investigations have shown that BGP has little cross-reactivity with other grass or tree pollen antigens.⁴²⁻⁴⁸ However, we have shown that an mAb of BG60 cross-reacts with some other grass pollen antigens with similar molecular weight.⁴⁹ Furthermore, our recent studies also showed that an mAb of BG60 cross-reacts with bromelain and horseradish peroxidase (unpublished data). Therefore the notion that BGP lacks antigenic cross-reactivity may be due to the use of different techniques. Alternatively, it may be explained by the different antibody preparation with varying specificities (murine mAbs, human allergic sera, rabbit or mouse polyclonal antibodies) used in the studies.

In conclusion, we have established a purification procedure that enables us to obtain a large quantity of BG60. The binding of BG60 to the mAb and human IgE can be inhibited by periodate oxidation of the antigen, indicating that the carbohydrate moiety of the antigen plays an important role in the immune response.

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