The FASEB Journal express article 10.1096/fj.02-0872fje. Published online July 18, 2003.

Cross-reactive N-glycans of Api g 5, a high molecular weight glycoprotein allergen from celery, are required for immunoglobulin E binding and activation of effector cells from allergic patients

Merima Bublin,* Christian Radauer,* Iain B. H. Wilson,‡ Dietrich Kraft,* Otto Scheiner,* Heimo Breiteneder,* and Karin Hoffmann-Sommergruber*

*Department of Pathophysiology, University of Vienna A-1090 Vienna, and ‡Institute of Chemistry, University of Agriculture, A-1190 Vienna, Austria

Corresponding author: Karin Hoffmann-Sommergruber, Ph.D., AKH-EBO 3Q, Währinger Gürtel 18-20, A-1090 Vienna, Austria. E-mail: Karin.Hoffmann@akh-wien.ac.at

ABSTRACT

Allergy diagnosis relying on the determination of specific IgE is frequently complicated by the presence of cross-reacting IgE of unclear clinical relevance. Particularly, the anaphylactogenic activity of IgE directed to cross-reactive carbohydrate moieties of glycoproteins from plants and invertebrates has been a matter of debate. In this study, we present the biochemical and immunological characterization of Api g 5, a glycoprotein allergen from celery with homology to FAD containing oxidases. Carbohydrate analysis of the allergen revealed the presence of glycans carrying fucosyl and xylosyl residues, structures previously shown to bind IgE. Chemical deglycosylation of the protein completely abolished binding of serum IgE from all 14 patients tested. Likewise, basophils from a patient allergic to mugwort pollen and celery were stimulated only by native Api g 5, whereas the deglycosylated allergen did not trigger release of histamine. IgE inhibition immunoblots showed that native Api g 5 other than the deglycosylated protein completely inhibited IgE binding to high molecular weight allergens in protein extracts from birch pollen, mugwort pollen, and celery. A similar inhibition was accomplished using the IgE binding oligosaccharide, MUXF, coupled to bovine serum albumin. All these observations taken together confer convincing evidence that IgE directed to cross-reactive carbohydrates is capable of eliciting allergic reactions in vivo.

Key words: type I hypersensitivity • food allergy • pollen allergy • berberine bridge enzyme

Celery (*Apium graveolens*) is a constituent of a variety of food products and can induce immunoglobulin E (IgE) mediated allergic reactions in susceptible individuals ranging from mild oral symptoms to life-threatening anaphylactic shocks (1–3). Celery tubers or stalks are eaten raw or cooked and are widely used for soups and salads. The dried powder serves as a spicing ingredient. In Switzerland, ~40% of patients with food allergy are sensitized to celery tuber (4). Sensitization to celery is frequently associated with birch and mugwort pollinosis; thus the term “birch-mugwort-celery syndrome” has been established (5, 6). These cross-reactivities can be attributed to at least three classes of allergenic proteins: Api g 1, a homologue of the major birch pollen allergen Bet v 1, in birch/celery-allergic patients (7); the

celery profilin Api g 4, a member of a family of highly cross-reactive plant allergens, in both birch/celery and mugwort/celery allergic patients (8); and a group of high molecular weight allergens (3, 6, 9–11).

Little is known about the high molecular weight allergens (45-70 kDa), which are recognized by IgE from 55% of celery allergic patients (12). The high molecular weight allergens appear as a barely resolved cluster of several bands in IgE immunoblots. Until now, two candidates for high molecular weight allergens in celery have been identified: cross-reactive carbohydrate determinants (CCDs; refs 12, 13) and Api g 5, a protein detected as two isoforms of 55 and 58 kDa in IgE immunoblots (14). IgE antibodies directed to CCDs were shown to be cross-reactive not only between different plant-derived glycoproteins but also to glycoproteins from invertebrate animals (e.g., *Hymenoptera*; refs 15–17). The structural analysis of CCDs revealed that they consist of N-linked glycans differing from those found in mammals by the absence of sialic acid and the presence of α1,3-fucosyl and β1,2-xylosyl residues (18–20). Both the α1,3-fucose and β1,2-xylose are responsible for IgE binding of patients' sera to plant glycoproteins (20–22).

Studies from several research groups have confirmed IgE binding to carbohydrate epitopes, but there is a controversial discussion about whether carbohydrate epitopes are able to elicit allergic symptoms (17, 23). Some observations corroborate the hypothesis that the detection of IgE antibodies specific for CCDs has no clinical relevance and may result in false positive diagnosis of allergy (24, 25). On the other hand, it is remarkable that IgE from some patients with celery allergy confirmed by double-blind placebo-controlled food challenge (DBPCFC) recognized CCDs as the only antigenic structure in celery protein extracts (12). In addition, neoglycoproteins containing three to four units of a purified bromelain glycopeptide triggered histamine release by basophils from a celery allergic patient (13).

Previously, allergenic activities of CCDs have been probed by indirect means, such as examining loss of IgE binding to periodate oxidized or chemically deglycosylated whole celery extract. Recently, and more specifically, purified glycoproteins (bromelain, horseradish peroxidase) or glycopeptides coupled to bovine serum albumin have been used as tools to characterize allergenic properties of CCDs (13, 25–27). The relevance of high molecular weight allergens in celery allergy and in the cross-reactivity between celery, mugwort pollen, and birch pollen prompted us to isolate and characterize such an allergen from crude celery protein extract. In the present study, a high molecular weight allergen from celery has been purified for the first time and its biochemical properties, as well as its allergic significance, are presented. Furthermore, it is shown that the CCD-carrying high molecular weight allergen component is identical to the previously described allergen Api g 5.

METHODS

Protein extracts

Celery tuber pieces were homogenized by freezing with liquid nitrogen and grinding to a powder. Proteins were extracted in 10 mM potassium phosphate buffer, pH 7.0, containing 2% (w/v) suspended solid polyvinylpolypyrrolidone, 2 mM ethylene diamine tetraacetic acid, 10 mM sodium dieethyldithiocarbamate, and 3 mM sodium azide by shaking overnight at 4°C. The

extract was cleared by centrifugation (40,000 *g*, 60 min, 4°C) and filtration. Birch and mugwort pollen were obtained from Allergon (Ängelholm, Sweden), and proteins were extracted in 10 mM sodium carbonate buffer, pH 7.0, at 4°C for 20 h and centrifuged (40,000 *g*, 60 min, 4°C). The extracts were dialyzed against 20 mM Tris-HCl, pH 8.0, and freeze-dried. Protein content was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as standard.

Purification of high molecular weight allergens

Ammonium sulfate precipitation was carried out by addition of the salt to the celery tuber extract solution to attain 40% saturation. The precipitate was dissolved in 20 mM Tris-HCl, pH 7.4, and desalted by dialysis against this buffer. Ten milligrams of protein were loaded onto a concanavalin A-Sepharose column (Amersham Biosciences, Freiburg, Germany) equilibrated with binding buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl). Elution of bound proteins was achieved using 0.4 M methyl α-D-mannopyranoside in binding buffer. Eluted proteins were dialyzed against buffer A (20 mM Tris-HCl, pH 8.0) and loaded onto a Resource Q anion exchange column (Amersham Biosciences, Freiburg, Germany). After being washed with buffer A, proteins were eluted by a linear gradient from 0-100% buffer B (20 mM Tris-HCl, pH 8.0, 500 mM NaCl) over 20 min at a flow rate of 1 ml/min.

One milliliter fractions were collected and checked for IgE binding by IgE ELISA and immunoblotting using a pool of celery, mugwort, and birch pollen allergic patients' sera.

Reduction and *S*-alkylation

The purified celery allergen was reduced with dithiothreitol (DTT) and carbamidomethylated with iodoacetamide as follows: 100 µg of the protein were dissolved in 50 µl of 0.3 M Tris/HCl, pH 8.0, containing 8 M guanidine HCl. After 2.5 µl of 1 M DTT were added, the sample was incubated at 50°C for 4 h. *S*-alkylation was performed by incubation with 12.5 µl of 0.4 M iodoacetamide for 1 h in the dark and quenched by adding 2.5 µl of 1 M DTT. The sample was dialyzed against distilled water and subjected to further analysis.

Preparation of deglycosylated allergens

Chemical deglycosylation

Deglycosylation by trifluoromethanesulfonic acid (TFMS; Oxford Glycosystems, Bedford, MA) was carried out according to the instructions of the manufacturer. Briefly, 300 µg of lyophilized protein were dissolved in 50 µl TFMS in toluene and incubated for 4 h at -20°C. The reaction mixture was neutralized with 150 µl pyridine and put on dry ice for 5 min and on ice for 15 min before addition of 400 µl of 0.5% (w/v) ammonium bicarbonate. The deglycosylated protein was dialyzed against 0.01% (w/v) ammonium bicarbonate (pH 7.0) for 12 h.

Periodate oxidation

Periodate oxidation was performed after proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The nitrocellulose strips were incubated in 0.3 M

sodium acetate, pH 4.5, containing 10 mM sodium metaperiodate for 20 h at 4°C in the dark. Strips were washed with water, and IgE binding capacity was analyzed.

Isoelectric focusing

Isoelectric focusing separation of celery tuber proteins was performed on an Ampholine PAGplate, pH 3.5- 9.5 (Amersham Biosciences, Freiburg, Germany) in a flat-bed electrophoretic chamber (Multiphor II Electrophoretic System, Amersham Biosciences, Freiburg, Germany) and run according to the instructions of the manufacturer. Separated proteins were stained with Coomassie Brilliant Blue R-250 or blotted to nitrocellulose membranes.

Amino acid sequencing

N-terminal sequencing of purified proteins was performed with an Applied Biosystems Procise 491 sequencer (Applied Biosystems, Foster City, CA). The proteins were separated by 12% SDS-PAGE and blotted to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol, 1% (v/v) acetic acid. Bands were excised and subjected to sequence analysis using the pulsed liquid cleavage program with the chemistry of the manufacturer version 1.1.1. Sequence data were compared with the protein databases using the BLAST program.

To obtain internal sequences, the lyophilized allergen (500 µg) was dissolved in 50 µl of 50% (v/v) trifluoroacetic acid and 5 µl of 5 M cyanogen bromide. The mixture was incubated overnight at room temperature in the dark, diluted with 500 µl water, and dried. The cleaved fragments were separated by 15% SDS-PAGE, blotted, and sequenced as described above.

Mass spectrometric analysis of N-glycans on the celery allergen

Glycan structures of the purified allergens were analyzed basically as described by Kolarich and Altmann (28). In brief, the proteins were subjected to SDS-PAGE, the upper and lower bands separately excised, washed, reduced, carbamidomethylated, and incubated overnight with 0.05 µg trypsin in 25 mM ammonium bicarbonate and 0.1% (w/v) *n*-octylglucoside. The N-glycans were released by incubation with peptide-N-glycosidase A (PNGase A) and purified using a tri-phasic mini column. Spectra of the tryptic digests of the TFMS-treated allergens and of the tryptic peptides of the native proteins before and after treatment with PNGase A were also recorded and compared. All mass spectra were measured on a Dynamo linear Matrix Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) spectrometer (Thermo Bioanalysis, Santa Fe, NM) with α-hydroxycyanocinnamic acid and 2,5-dihydroxybenzoic acid used as the matrices for peptides and glycans, respectively.

Patients' sera

Fourteen allergic patients' (10 female, 4 male, mean age: 40 yr, range: 15-63 years) sera containing IgE antibodies reacting against allergens above 25 kDa from celery tuber, mugwort, and birch pollen have been selected. Twelve patients had positive skin prick tests and radioallergosorbent tests to birch, mugwort, or grass pollen and reported pollen-induced rhinoconjunctivitis. Eight (patient numbers 1-7 and 9) of these 14 patients had positive case

histories of allergy to raw or cooked celery according to interviews with an allergist in an allergy outpatient department. Celery-related symptoms were confined to the oral allergy syndrome in three patients, one patient had angioedema, and four patients suffered from angioedema and urticaria. A serum pool (pool A) containing sera that show IgE binding exclusively to allergens above 25 kDa in IgE immunoblots (numbers 1, 3, 4, 5, 8, 11, 12, 13, data not shown) was prepared for testing of chromatographic fractions. A second pool (pool B) was prepared using all 14 sera.

Blood from three patients was used for basophil activation assays: patient 5 suffered from rhinoconjunctivitis and asthma related to grass and mugwort pollen and oral allergy syndrome, angioedema, and urticaria after ingestion of celery. Patient 13 had no celery allergy but a latex allergy as well as urticaria and angioedema to banana. An additional patient (no. 15) without sensitization to high molecular weight allergens, but sensitized to Bet v 1 and its homologue, Api g 1, was used for a histamine release assay with Api g 1. The symptoms of the patient comprised rhinoconjunctivitis to birch and grass pollen and oral allergy syndrome to raw fruits and vegetables (apple, peach, cherry, celery, and carrot). A negative control sample was obtained from a nonatopic individual with no history of food allergy.

Immunoblots

Protein extracts of celery tuber, mugwort pollen, and birch pollen as well as purified celery proteins were subjected to denaturing 12% SDS-PAGE. Proteins were then blotted onto nitrocellulose membranes (Protran BA 85, Schleicher and Schuell, Dassel, Germany). Membranes were cut into strips and blocked by incubation in 50 mM sodium phosphate buffer, pH 7.5, containing 0.5% (w/v) bovine serum albumin, 0.5% (v/v) Tween 20, and 0.05% (w/v) sodium azide. For visualization of IgE binding proteins, strips were incubated overnight with patients' sera (diluted 1:4) at 4°C. Bound IgE was detected by incubating the strips with 1:20 diluted ^{125}I-labeled anti-human IgE (MALT Allergy System Isotope Reagent, IBL Hamburg, Germany) overnight at room temperature and exposing them to BioMax MS-1 films (Eastman Kodak, Rochester NY).

N-linked glycan moieties of separated proteins were examined by immunostaining using a rabbit anti-horseradish peroxidase antibody (Sigma Chemical, St. Louis, MO) diluted 1:1000 in Tris-buffered saline containing 0.1% (v/v) Tween 20 and 0.5% (w/v) BSA, which binds to N-linked glycan moieties with the β1,2-xylosyl and α1,3-fucosyl substitutions (20, 29). Bound antibodies were detected by incubating the strips with 1:2000 diluted ^{125}I-labeled anti-rabbit IgG (Amersham Biosciences, Freiburg, Germany).

Immunoblot inhibition

Serum pool B was diluted 1:4 in blocking buffer (described above) and incubated overnight at 4°C with 50 µg/ml of celery allergen as well as with two neoglycoproteins. One conjugate, MUXF-BSA, contained core α1,3-fucosylated and β1,2-xylosylated N-glycans (Fig. 1) from pineapple stem bromelain, whereas the other, MM-BSA, was a nonfucosylated and nonxylosylated control carrying the common core pentasaccharide (Fig. 1). Preparation of these neoglycoproteins, which contained 3-4 mol of glycopeptide per mol of protein according to amino sugar analysis, has been described previously (20). Recombinant birch pollen profilin

(obtained from Biomay, Vienna, Austria), a nonhomologous and nonglycosylated allergen, and buffer without protein were used as controls. Afterward, the remaining IgE binding to celery, mugwort pollen, and birch pollen extracts was tested in an immunoblot assay as described above. Strips were also tested with the serum of a nonallergic subject and with buffer as negative controls.

Additionally, individual sera from three patients (patients numbers 4, 5, 7) displaying IgE binding to high molecular weight allergens in extracts of celery, mugwort pollen, and birch pollen were separately used for cross-inhibition experiments. The sera were preincubated with 50 µg/ml of native or deglycosylated celery allergen or recombinant birch pollen profilin. Thereafter, nitrocellulose blotted celery and mugwort pollen proteins were incubated with these dilutions, and bound IgE was detected as described above.

IgE ELISA

Microtiter plates (MaxiSorp Immuno Plate, Nalge Nunc International, Roskilde, Denmark) were coated with 0.4 µg allergens per well overnight at 4°C. After being blocked with Tris-buffered saline (TBS), 0.05% (v/v) Tween 20, and 1% (w/v) BSA, 1:4 diluted sera (numbers 1-5, 8-14) were applied onto the coated plates and incubated overnight at 4°C. After being washed, the plates were incubated with a 1:1000 diluted alkaline phosphatase-conjugated mouse anti-human IgE antibody (BD PharMingen, San Diego, CA) for 2 h at room temperature. Color development was performed using 0.1% (w/v) disodium *p*-nitrophenyl phosphate substrate (Sigma-Aldrich, Steinheim, Germany), and the optical density was measured at 405 nm (550 nm as reference wavelength) after 30 min.

Basophil histamine release assay

Basophil histamine release assays were performed using a commercial kit (IBL Immunobiological Laboratories, Hamburg, Germany) according to the recommendations of the manufacturer. Briefly, 200 µl of heparinized whole blood were incubated with 200 µl of serial 10-fold dilutions of allergens, 200 µl of buffer alone, and 200 µl of an anti-human IgE antibody for 1 h at 37°C. Final allergen concentration were 100, 10, 1, 0.1, and 0.01 µg/ml. The total histamine content of the blood cells was measured after cell lysis with a hypotonic medium. Cell debris were centrifuged, and histamine concentrations in the supernatants were measured by a competitive enzyme immunoassay. All plasma samples were measured in duplicates.

RESULTS

Biochemical characterization of a high molecular weight celery allergen

Analysis of celery extracts by IgE immunoblot showed a series of poorly resolved bands between 30 and 100 kDa, including a predominant double band of 53 and 57 kDa (Fig. 2*B*, lane 1). To purify these proteins, we enriched them in a first step by ammonium sulfate precipitation. The precipitate was further purified by lectin affinity chromatography on a concanavalin A column, which binds glycoproteins containing mannosyl residues. Final purification was achieved by anion exchange chromatography. Analysis of the chromatographic fractions by IgE ELISA identified proteins that eluted in a wide peak at 250 mM NaCl as the predominant IgE binding species. The fraction contained a mixture of two proteins of 53 and 57 kDa, which was at least

90% pure as determined by Coomassie brilliant blue staining after SDS-PAGE (Fig. 2*A*, lane 2). We obtained 17 mg of purified protein from 800 g of fresh celery tuber (containing 3.5 g total extractable protein). Both proteins bound IgE to a similar extent in immunoblots (Fig. 2*B*, lane 2). Interestingly, isoelectric focusing of the purified allergens showed only one band both in the Coomassie-stained gel and the IgE immunoblot (Fig. 2*D*).

Both the 53 and 57 kDa IgE binding proteins were subjected to N-terminal amino acid sequence analysis. Both proteins yielded identical N-terminal sequences (Table 1), which were identical to the N-terminal sequence of Api g 5, a previously described high molecular weight allergen from celery also isolated as a mixture of two proteins (14). No sequence homologous to this N-terminal peptide was found in the databases (Swiss-Prot and translated EMBL). Cleavage of the protein mixture by cyanogen bromide yielded a large number of fragments. Five predominant ones were sequenced resulting in one unique fragment and two pairs of fragments with identical sequences and molecular weights differing by 2 kDa (Table 1). These sequences showed a high degree of similarity to enzymes belonging to a family of FAD-containing oxygen-dependent oxidoreductases (Fig. 3) encompassing berberine bridge-forming enzymes and carbohydrate oxidases. The ultraviolet absorbance spectrum of purified Api g 5 showed maxima at 350 and 452 nm indicative of flavin (data not shown).

With the intention of confirming the presence of IgE binding N-glycans on Api g 5, purified isoallergens resolved by SDS-PAGE and blotted to nitrocellulose membranes were stained with anti-horseradish peroxidase, an antiserum containing antibodies specific for N-linked glycan moieties containing β1,2-xylosyl and α1,3-fucosyl residues. Both Api g 5 bands gave very strong positive reactions with this antiserum (Fig. 2*C*, lane 1).

Reduction and *S*-alkylation of Api g 5 caused a significant shift in electrophoretic mobility but did not eliminate its heterogeneity, i.e., the reduced protein still migrated as a double band (Fig. 2*A*, lane 4).

Mass spectrometric analysis of native and deglycosylated Api g 5

To prove that the N-glycans were indeed xylosylated and fucosylated, as suggested by the anti-HRP immunoblotting data, the upper and lower bands of SDS-PAGE-separated native and TFMS-deglycosylated forms of Api g 5 were subjected to in-gel tryptic digestion. Spectra of the tryptic peptides derived from native Api g 5 before and after treatment with peptide-N-glycosidase A (PNGase A) were compared with the spectra of the peptides derived from the TFMS-treated allergen. A cluster of peaks, with m/z values of 3015, 3120, 3282, 3488, and 3650, seen only in the spectra of the peptides derived from native Api g 5, was absent from the PNGase A-treated and TFMS-treated samples (compare Fig. 4*A*, *B*, and *C* for the 57 kDa form and Fig. 4*E*, *F*, and *G* for the 53 kDa form).

In both PNGase A-treated samples (Fig. 4*C* and *G*), a major new peak of m/z 2111 appeared consistent with the removal of MUXF and MMXF from the 3120 and 3282 peaks. The peak of m/z 1844 (more prominent in the digest of the 53 kDa form) probably resulted from removal of MMXF from the 3015 peak, whereas the m/z 2478 peak would be derived from removal of MUXF and MMXF from the 3488 and 3650 peaks. TFMS treatment, on the other hand, resulted in new species of m/z 2046, 2313, and 2516. The first peak is consistent with removal of all but

the innermost GlcNAc residue of MMXF from the 3015 peak, whereas the second is consistent with respective removal of all but the innermost GlcNAc residue of MUXF and MMXF from the 3120 and 3282 peaks. The retention of the innermost GlcNAc after TFMS treatment is consistent with previous studies on the use of this reagent (30). However, it is supposed that the 2516 peak corresponds to a partially deglycosylated 3120/3282 peptide retaining a chitobiose, rather than a single GlcNAc, moiety. Other than the aforementioned differences, the peptide maps of the TFMS-treated and the native samples of each form of Api g 5 are qualitatively seemingly identical. In addition, there are some qualitative differences between the peptide maps of the 57 and 53 kDa forms of the native allergen (Fig. 4*A* and *E*), particularly the presence in digests of the former of a species of m/z 2004 in digest of the 53 kDa form, as well as variations in relative peak intensities.

Due to a lack of a complete protein sequence, a full interpretation of the peptide map was not possible. However, the mass of the putative glycopeptide with m/z 3015 is consistent with the addition of MMXF to the tryptic peptide VLWVGNTTQKLEWIR (m/z 1844; one missed cleavage). This sequence forms part of the 12 and 14 kDa CNBr peptides shown in Table 1. Consistent with glycosylation of this peptide, Edman sequencing of the peptide resulted in a blank tenth cycle (an unidentifiable cycle being considered indicative of posttranslational modification), whereas a homologous peptide encoded by an EST derived from *Glomus intraradices*-infected carrot root (GenBank/EMBL/DDBJ BI452005) has an asparagine at this position. Furthermore, as discussed above, peptides with m/z 2046 in the TFMS-treated and with m/z 1844 in the PNGase A-treated allergens indicate that the peptide VLWVGNTTQKLEWIR is glycosylated in both the 53 and 57 kDa forms of Api g 5.

After verification of deglycosylation of the tryptic peptides of the native allergen by PNGase A, the N-glycans were separated from the peptide mixture and analyzed by MALDI. Consistent with the comparative interpretation of the glycosylated and deglycosylated peptide spectra and with the anti-HRP immunoblotting data, two major species, MUXF and MMXF (Fig. 1), were detected (Fig. 4*D* and *H*). There appeared to be no qualitative difference in the glycosylation of the 53 and 57 kDa forms of Api g 5.

Allergenic activities of native and deglycosylated Api g 5

The IgE binding ability of Api g 5 was assayed in an immunoblot with a pool of eight sera containing IgE specific exclusively for high molecular weight allergens (pool A). Both Api g 5 bands bound similar amounts of IgE (Fig. 2*B*, lane 2). Subsequently, the immunoblot was performed with all 14 individual sera of patients sensitized to celery, which resulted in the same binding pattern (data not shown).

To determine the contribution of glycans to its IgE binding capability, blotted Api g 5 was deglycosylated by mild *meta*-periodate oxidation. Periodate oxidation only partially destroyed N-glycan-dependent epitopes, as residual binding of an anti-horseradish-peroxidase antibody specific for N-glycans was observed in an immunoblot (data not shown). Consistent with this, IgE binding activity of *meta*-periodate oxidized Api g 5 bands was significantly decreased but not completely abolished in an immunoblot with serum pool A (data not shown).

Additionally, Api g 5 was deglycosylated by TFMS, which removes O- and N-linked oligosaccharides from proteins. The electrophoretic mobility of both Api g 5 bands changed significantly after removal of glycosyl groups from the protein resulting in a decrease of apparent molecular weights by 12 kDa with both bands still present (compare Fig. 2*A*, lanes 2 and 3). After immunostaining with the glycan-specific anti-HRP antiserum had confirmed that the deglycosylation procedure had been practically quantitative (Fig. 2*C*, lanes 1, 2), deglycosylated Api g 5 was tested for its ability to react with IgE from serum pool A. Indeed, the completely deglycosylated form of Api g 5 lacked detectable binding of IgE (Fig. 2*B*, lane 3). These findings were corroborated by the results of an IgE ELISA with 12 individual allergic patients' sera where only background levels of IgE bound to the deglycosylated protein were detected (Fig. 5). To rule out that the lack of IgE binding to deglycosylated Api g 5 was caused by conformational differences between the native and the deglycosylated protein, reduced and *S*-alkylated Api g 5 was included with the proteins tested in this ELISA. No significant differences between the amounts of IgE bound to native and reduced Api g 5 was detected (Fig. 5).

The allergenicity of native and deglycosylated Api g 5 was further examined by comparing their abilities to release histamine from basophils of a patient allergic to celery, mugwort pollen, and grass pollen (number 5). The patient's serum contained IgE reactive exclusively to high molecular weight allergens, previously determined by IgE immunoblot. Basophils from patient 13, who is allergic to latex and banana but not to celery; patient 15 sensitized to Bet v 1 and its celery homologue Api g 1 (nonglycosylated proteins); and a nonallergic subject were used as negative controls. Cells from all blood samples released histamine when exposed to an anti-human IgE antibody as a positive control. The blood cells from patient 5 gave specific dose-dependent histamine release after stimulation with native Api g 5 but not with deglycosylated Api g 5 (Fig. 6*A*). Similar results were obtained in an independent experiment with a different blood sample from the same patient. In contrast, basophils from patient 13 were stimulated neither by native nor by deglycosylated Api g 5 (Fig. 6*B*). Cells from patient 15 were stimulated with recombinant Api g 1 and released histamine within a similar range of allergen concentrations compared with stimulation of sample 5 with Api g 5 (Fig. 6*C*). The sample obtained from a nonallergic individual was nonreactive to all tested allergens (data not shown).

Allergenic cross-reactivity of Api g 5

We used a pool of sera from 14 patients (pool B) with pollen and/or celery allergy to identify allergens in celery, mugwort, and birch pollen extracts that share IgE epitopes with Api g 5. The serum pool was preincubated with 50 μg of Api g 5, MUXF-BSA (a fucose and xylose-containing glycan coupled to BSA), MM-BSA (a control glycan without fucose and xylose), and birch pollen profilin (a nonhomologous, nonglycosylated control allergen). Subsequently, IgE binding to blotted celery tuber, mugwort pollen, and birch pollen extracts was tested. The serum pool that underwent no preadsorption displayed IgE reactivity to components of 15 and 45-70 kDa in celery extract (Fig. 7*A*, lane 1); to 11, 28, and 35-100 kDa proteins in mugwort pollen extract (Fig. 7*B*, lane 1); and to 17, 35, and 40-70 kDa in birch pollen extract (data not shown). Birch pollen profilin (data not shown) and MM-BSA (Fig. 7*A* and *B*, lane 2) did not significantly inhibit IgE binding to these extracts. Preincubation of the serum pool with Api g 5 almost completely abolished IgE reactivity to the 45-70 kDa allergens in celery extract (Fig. 7*A*, lane 4) and to all IgE reactive bands in mugwort pollen (Fig. 7*B*, lane 4) but only partially inhibited

binding to birch pollen extract (data not shown). Inhibition by MUXF-BSA gave rise to effects equivalent to those achieved with native Api g 5 (Fig. 7*A* and *B*, lane 3).

Cross-reactivity of native and deglycosylated Api g 5 was also tested by inhibition assays using three individual sera of patients with clinically relevant allergy to celery and mugwort pollen and IgE-specificity restricted to high molecular weight allergens in celery. Compatible with the results using the serum pool described above, complete inhibition of IgE binding to celery and mugwort pollen extracts was achieved with native Api g 5, while preincubation with neither deglycosylated Api g 5 nor birch pollen profilin as a negative control resulted in diminution of binding (data not shown).

DISCUSSION

High molecular weight proteins from celery have previously been shown to be one of the major IgE binding components when tested with sera from patients with celery allergy confirmed by double-blind placebo-controlled food challenge (12). IgE specific for these allergens cross-reacts with proteins from birch and mugwort pollen, which in part explains the clinical correlation between birch, mugwort, and celery allergy (6). During recent years, it became clear that IgE directed to high molecular weight allergens binds to glycoproteins carrying N-glycans containing α1,3-fucosyl and β1,2-xylosyl residues, which are found in plants and invertebrates but not in mammals (17, 20–22). The role of N-glycan-specific IgE in the clinical manifestation of allergic diseases has been a matter of debate. The importance of celery as the prevailing food allergen source in central Europe, the cross-reactivity of high molecular weight allergens, and the lack of conclusive data on the clinical significance of CCD-specific IgE prompted us to purify and characterize a naturally occurring glycoprotein allergen from celery.

The purified glycoprotein, which was extracted in large amounts from celery tubers, turned out to be identical to the described previously allergen Api g 5 (14). Glycan structures of the protein were determined by an immunoblot assay using an antiserum reactive to plant-specific N-glycans and by enzymatic removal of N-glycans combined with mass spectrometric analysis of the cleaved sugars. The glycans bound to Api g 5, MUXF and MMXF, two types of fucosylated and xylosylated complex N-glycans, are examples of a range of structures previously found in a total celery extract (31). Api g 5 was purified as a mixture of two polypeptides with their apparent molecular weights differing by ~4 kDa; thus we tried to elucidate the cause of this heterogeneity. Neither irreversible reduction nor deglycosylation eliminated one of the bands. Mass-spectrometric glycan analysis revealed no differences in the glycosylation patterns of the two forms. In contrast, the tryptic digests of the two variants differed by at least one peptide present exclusively in the 53 kDa protein. Furthermore, two of the three peptides obtained after fragmentation of the protein mixture with cyanogen bromide appeared as pairs with identical N-termini and apparent molecular weights differing by 2 kDa. Hence, it can be concluded that the two Api g 5 variants are closely related isoforms most likely differing in their C-terminal sequences. This assumption is corroborated by a sequence comparison of similar sequences found in the databases that shows highly divergent C-terminal sequences with lengths differing by up to 20 amino acids (data not shown).

N-terminal and internal sequencing of Api g 5 revealed that the protein belongs to a family of flavoproteins with diverse functions comprising carbohydrate oxidases from sunflower and

lettuce, berberine bridge-forming enzymes (BBEs) from barberry, and poppy and a drought-inducible protein from cowpea. The biochemical functions of all these proteins are related to the plant's response to pathogen attack and abiotic stress. Interestingly, a very high similarity (76% identity in 25 amino acids) was obtained in comparing Api g 5 to the translation of an EST from the fungus *Glomus intraradices*. As the fungus was grown on carrot roots, it is most likely that the sequence was derived from contaminating carrot RNA. BBEs catalyze the synthesis of (S)-scoulerine, a precursor of several species-specific isoquinoline alkaloids with antimicrobial activities (32, 33). High molecular weight allergens homologous to BBEs have been previously found in Bermuda grass pollen (34) and in oilseed rape pollen (35). The Bermuda grass allergen BG60 was shown to contain fucosylated glycan moieties that take part in IgE binding (36).

A stress-related function of Api g 5 is compatible to the observation that many plant allergens are homologous to pathogenesis-related proteins (37). These protective proteins are accumulated in vulnerable tissues like pollen, storage organs, seeds, fruits, and latex. Their high concentrations in plant tissues predestined to get in contact with the human immune system and their stability may explain, at least to a certain extent, their allergenicity.

The IgE binding capacity of purified Api g 5 was confirmed by Western blotting and ELISA. All 14 celery-allergic patients' sera exhibiting IgE binding to high molecular weight allergens contained IgE reactive to both Api g 5 bands in immunoblots and showed strong reactivity in an IgE ELISA. The chemical nature of the allergenic epitopes on Api g 5 was examined by destroying the covalently linked carbohydrates by periodate oxidation or acid cleavage with TFMS. Periodate oxidation led to a significant reduction of binding capacity for a glycan-specific anti-HRP antibody as well allergic patients' IgE. The residual antigenicity may be explained by incomplete oxidation of the glycan residues. On the other hand, deglycosylation by TFMS in solution was quantitative and affinity for IgE was completely eliminated as determined by immunoblot and ELISA. Possible effects of the TFMS treatment on IgE binding ability via disruption of the protein structure were excluded by additional experiments: mass spectrometric analysis of tryptic digests of native and deglycosylated Api g 5 showed no differences other than those caused by cleavage of the sugar moieties, indicating that TFMS had no effects on the primary peptide structure of Api g 5. Possible disruption of the tertiary structure cannot be excluded, but its influence on the IgE binding capacity of Api g 5 is ruled out by the following argumentation: reduction and *S*-alkylation of Api g 5 irreversibly disrupts its tertiary structure, which can be seen by a shift of the electrophoretic mobility in SDS-PAGE. Nevertheless, reduced Api g 5 retains its IgE binding capability in a native IgE ELISA. Hence, deglycosylated Api g 5 should also retain its carbohydrate-independent IgE binding epitopes. In conclusion, we present strong evidence that IgE binding to Api g 5 is mediated exclusively by its N-glycans. A similar conclusion can be drawn for the cross-reactivity of Api g 5 with other high molecular weight allergens from celery and with proteins from birch and mugwort pollen. IgE immunoblot inhibition experiments showed that native Api g 5 and the fucosylated and xylosylated N-glycan MUXF coupled to BSA completely inhibited IgE binding to high molecular weight proteins from celery, birch, and mugwort in a similar manner. No cross-reactivity was observed with a neoglycoprotein carrying an N-glycan lacking fucose and xylose as well as with deglycosylated Api g 5.

IgE binding capacity and cross-reactivity of plant-specific N-glycans have previously been shown using various pollen and food allergens as well as model compounds like bromelain,

horseradish peroxidase, glycopeptides, and glycans coupled to BSA (reviewed in ref 23). To date, only a few publications have dealt with the clinical significance of carbohydrate-specific IgE. Initially, most researchers shared the opinion that glycan-specific IgE merely leads to false positive allergy diagnosis without being capable of eliciting an allergic reaction. This view was supported by a study of grass pollen-allergic patients with positive peanut RAST, whose peanut-specific IgE exclusively bound to CCDs, even though the patients showed no clinical symptoms of peanut allergy (24). Similar results were obtained after examining a group of 136 pollen allergic patients with positive bromelain CAP but negative skin prick tests to this glycoprotein (25). However, these data can be explained by the fact that bromelain carries only one glycan per molecule and thus may be unable to cross-link cell-bound IgE. On the other hand, binding of carbohydrate-specific IgE to either bromelain glycopeptides coupled to BSA (13), free N-glycans cleaved off from the major olive pollen allergen, Ole e 1 (38), or the cypress glycoallergen, Cup a 1 (39), was shown to be capable of activating basophils derived from allergic subjects. Compatible with these data, our experiments showed that Api g 5 activates basophils derived from a patient allergic to celery and mugwort pollen via binding exclusively to glycan-specific IgE. In contrast, basophils from a CCD-sensitized patient without celery allergy were not responsive to Api g 5. The cross-linking activity of IgE bound to Api g 5 indicates the presence of multiple IgE epitopes, which is consistent with the mass spectrometric data: comparison of untreated and deglycosylated tryptic digests of Api g 5 revealed the presence of at least three glycosylated peptides. The significance of histamine release data has been questioned by some researchers who argued that the affinity of CCD-specific IgE was too low to elicit an allergic reaction in vivo (17, 40). In contrast, maximum histamine release using Api g 5 was achieved at allergen concentrations even lower than those observed in an experiment performed with Api g 1, a nonglycosylated celery allergen.

In conclusion, we present the biochemical and immunological characterization of the first high molecular weight glycoprotein allergen that contributes to the birch-mugwort-celery-spice syndrome. The celery allergen, Api g 5, is capable of binding human IgE and thus activating basophils derived from a mugwort and celery-allergic patient exclusively via its cross-reactive plant-specific N-glycans. The possible clinical relevance of N-glycan-specific IgE may have consequences for the practice of allergy diagnostics. However, it remains to be elucidated if there are additional factors determining the clinical outcome of a glycan-specific IgE response.

ACKNOWLEDGMENTS

The authors thank Friedrich Altmann for the kind gift of the MUXF-BSA and MM-BSA neoglycoconjugates and Katharina Paschinger for preparation of the peptide and glycan samples and for careful reading of the manuscript. Iain B. H. Wilson is the recipient of a Glycoscience Research Award from Neose Technologies, Inc. Karin Hoffmann-Sommergruber is the recipient of a grant "Hertha Firnberg Frauenförderungsstelle" (no. T-70 GEN) from the Austrian Ministry of Science. Otto Scheiner is the recipient of a grant from the "Jubiläumsfonds der Oesterreichischen Nationalbank" (no. 8591)

REFERENCES

1. Forsbeck, M., and Ros, A. M. (1979) Anaphylactoid reaction to celery. *Contact Dermatitis* **5,** 191

2. Pauli, G., Bessot, J. C., Dietemann-Molard, A., Braun, P. A., and Thierry, R. (1985) Celery sensitivity: clinical and immunological correlations with pollen allergy. *Clin. Allergy* **15,** 273–279

3. Jankiewitz, A., Aulepp, H., Fötisch, K., and Vieths, S. (1998) Serological investigation of 30 celery-allergic patients with particular consideration of the thermal stability of IgE-binding celery allergens. *Allergo J.* **7,** 87–95

4. Wüthrich, B. (1993) Zur Nahrungsmittelallergie: Häufigkeit der Symptome und der allergieauslösenden Nahrungsmittel bei 402 Patienten-Kuhmilchallergie-Nahrungsmittel und Neurodermatitis atopica. *Allergologie* **16,** 280–287

5. Wüthrich, B., and Dietschi, R. (1985) Das "Sellerie-Karotten-Beifuss-Gewürz-Syndrom": Hauttest-und RAST-Ergebnisse. *Schweiz. Med. Wschr.* **115,** 358–364

6. Bauer, L., Ebner, C., Hirschwehr, R., Wüthrich, B., Pichler, C., Fritsch, R., Scheiner, O., and Kraft, D. (1996) IgE cross-reactivity between birch pollen, mugwort pollen and celery is due to at least three distinct cross-reacting allergens: immunoblot investigation of the birch-mugwort-celery syndrome. *Clin. Exp. Allergy* **26,** 1161–1170

7. Breiteneder, H., Hoffmann-Sommergruber, K., O'Riordain, G., Susani, M., Ahorn, H., Ebner, C., Kraft, D., and Scheiner, O. (1995) Molecular characterization of Api g 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17-kDa tree pollen allergens. *Eur. J. Biochem.* **233,** 484–489

8. Scheurer, S., Wangorsch, A., Haustein, D., and Vieths, S. (2000) Cloning of the minor allergen Api g 4 profilin from celery (*Apium graveolens*) and its cross-reactivity with birch pollen profilin Bet v 2. *Clin. Exp. Allergy* **30,** 962–971

9. Vallier, P., Dechamp, C., Vial, O., and Deviller, P. (1988) A study of allergens in celery with cross-sensitivity to mugwort and birch pollens. *Clin. Allergy* **18,** 491–500

10. Wüthrich, B., Stager, J., and Johansson, S. G. (1990) Celery allergy associated with birch and mugwort pollinosis. *Allergy* **45,** 566–571

11. Vieths, S., Jankiewicz, A., Aulepp, H., and Haustein, D. (1997) Allergy to heated and processed foods. *Arb. Paul Ehrlich Inst. Bundesamt Sera Impfstoffe Frankf. A. M.* **91,** 250–262

12. Lüttkopf, D., Ballmer-Weber, B. K., Wüthrich, B., and Vieths, S. (2000) Celery allergens in patients with positive double-blind placebo-controlled food challenge. *J. Allergy Clin. Immunol.* **106,** 390–399

13. Fötisch, K., Altmann, F., Haustein, D., and Vieths, S. (1999) Involvement of carbohydrate epitopes in the IgE response of celery-allergic patients. *Int. Arch. Allergy Immunol.* **120,** 30–42

14. Ganglberger, E., Radauer, C., Grimm, R., Hoffmann-Sommergruber, K., Breiteneder, H., Scheiner, O., and Jensen-Jarolim, E. (2000) N-terminal sequences of high-molecular weight allergens from celery tuber. *Clin. Exp. Allergy* **30,** 566–570

15. Aalberse, R. C., Koshte, V., and Clemens, J. G. (1981) Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. *J. Allergy Clin. Immunol.* **68,** 356–364

16. Aalberse, R. C., and van Ree, R. (1997) Crossreactive carbohydrate determinants. *Clin. Rev. Allergy Immunol.* **15,** 375–387

17. van Ree, R. (2002) Carbohydrate epitopes and their relevance for the diagnosis and treatment of allergic diseases. *Int. Arch. Allergy Immunol.* **129,** 189–197

18. Lerouge, P., Cabanes-Macheteau, M., Rayon, C., Fitchette-Lainé, A. C., Gomord, V., and Faye, L. (1998) N-Glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Mol. Biol.* **38,** 31–48

19. Wilson, I. B. H., and Altmann, F. (1998) Structural analysis of N-glycans from allergenic grass, ragweed and tree pollens: core alpha1,3-linked fucose and xylose present in all pollens examined. *Glycoconj. J.* **15,** 1055–1070

20. Wilson, I. B. H., Harthill, J. E., Mullin, N. P., Ashford, D. A., and Altmann, F. (1998) Core alpha1,3-fucose is a key part of the epitope recognized by antibodies reacting against plant N-linked oligosaccharides and is present in a wide variety of plant extracts. *Glycobiology* **8,** 651–661

21. Tretter, V., Altmann, F., Kubelka, V., März, L., and Becker, W. M. (1993) Fucose alpha 1,3-linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals. *Int. Arch. Allergy Immunol.* **102,** 259–266

22. van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J., Milazzo, J. P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R., Rodríguez, R., et al. (2000) Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J. Biol. Chem.* **275,** 11451–11458

23. Fötisch, K., and Vieths, S. (2001) N- and O-linked oligosaccharides of allergenic glycoproteins. *Glycoconj. J.* **18,** 373–390

24. van der Veen, M. J., van Ree, R., Aalberse, R. C., Akkerdaas, J., Koppelman, S. J., Jansen, H. M., and van der Zee, J. S. (1997) Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J. Allergy Clin. Immunol.* **100,** 327–334

25. Mari, A., Iacovacci, P., Afferni, C., Barletta, B., Tinghino, R., Di Felice, G., and Pini, C. (1999) Specific IgE to cross-reactive carbohydrate determinants strongly affect the in vitro diagnosis of allergic diseases. *J. Allergy Clin. Immunol.* **103,** 1005–1011

26. Batanero, E., Villalba, M., Monsalve, R. I., and Rodriguez, R. (1996) Cross-reactivity between the major allergen from olive pollen and unrelated glycoproteins: evidence of an epitope in the glycan moiety of the allergen. *J. Allergy Clin. Immunol.* **97,** 1264–1271

27. Vieths, S., Luttkopf, D., Reindl, J., Anliker, M. D., Wüthrich, B., and Ballmer-Weber, B. K. (2002) Allergens in celery and zucchini. *Allergy* **57,** Suppl **72,** 100–105

28. Kolarich, D., and Altmann, F. (2000) N-Glycan analysis by matrix-assisted laser desorption/ionization mass spectrometry of electrophoretically separated nonmammalian proteins: application to peanut allergen Ara h 1 and olive pollen allergen Ole e 1. *Anal. Biochem.* **285,** 64–75

29. Kurosawa, A., Yano, A., Itoh, N., Kuroda, Y., Nakagawa, H., and Kawasaki, T. (1991) The structure of neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. *J. Biol. Chem.* **266,** 4168–4172

30. Sojar, H. T., and Bahl, O. P. (1987) A chemical method for the deglycosylation of proteins. *Arch. Biochem. Biophys.* **259,** 52–57

31. Wilson, I. B. H., Zeleny, R., Kolarich, D., Staudacher, E., Stroop, C. J., Kamerling, J. P., and Altmann, F. (2001) Analysis of Asn-linked glycans from vegetable foodstuffs: widespread occurrence of Lewis a, core alpha1,3-linked fucose and xylose substitutions. *Glycobiology* **11,** 261–274

32. Rink, E., and Bohm, H. (1975) Conversion of reticuline into scoulerine by a cell free preparation from *Macleaya microcarpa* cell suspension cultures. *FEBS Lett.* **49,** 396–399

33. Dittrich, H., and Kutchan, T. M. (1991) Molecular cloning, expression, and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the response of plants to pathogenic attack. *Proc. Natl. Acad. Sci. USA* **88,** 9969–9973

34. Liaw, S., Lee, D. Y., Chow, L. P., Lau, G. X., Su, S. N., and Chow, L. (2001) Structural characterization of the 60-kDa bermuda grass pollen isoallergens, a covalent flavoprotein. *Biochem. Biophys. Res. Commun.* **280,** 738–743

35. Chardin, H., Mayer, C., Senechal, H., Tepfer, M., Desvaux, F. X., and Peltre, G. (2001) Characterization of high-molecular-mass allergens in oilseed rape pollen. *Int. Arch. Allergy Immunol.* **125,** 128–134

36. Su, S. N., Shu, P., Lau, G. X., Yang, S. Y., Huang, S. W., and Lee, Y. C. (1996) Immunologic and physicochemical studies of Bermuda grass pollen antigen BG60. *J. Allergy Clin. Immunol.* **98,** 486–494

37. Hoffmann-Sommergruber, K. (2000) Plant allergens and pathogenesis-related proteins. What do they have in common? *Int. Arch. Allergy Immunol.* **122,** 155–166

38. Batanero, E., Crespo, J. F., Monsalve, R. I., Martin-Esteban, M., Villalba, M., and Rodriguez, R. (1999) IgE-binding and histamine-release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen, Ole e 1. *J. Allergy Clin. Immunol.* **103,** 147–153

39. Iacovacci, P., Afferni, C., Butteroni, C., Pironi, L., Puggioni, E. M., Orlandi, A., Barletta, B., Tinghino, R., Ariano, R., Panzani, R. C., et al. (2002) Comparison between the native glycosylated and the recombinant Cup a1 allergen: role of carbohydrates in the histamine release from basophils. *Clin. Exp. Allergy* **32,** 1620–1627

40. Aalberse, R. C. (1998) Clinical relevance of carbohydrate allergen epitopes. *Allergy* **53,** 54–57

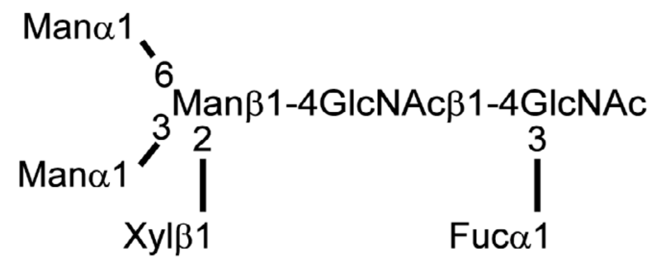
Received January 3, 2003; accepted May 22, 2003.

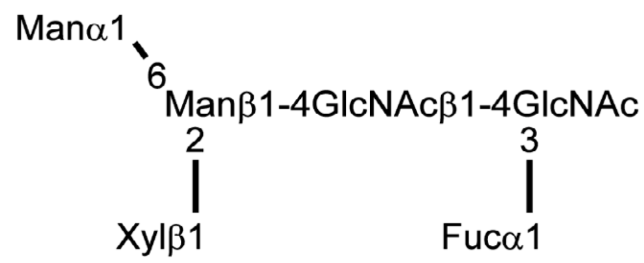
Table 1

Amino acid sequences of Api g 5

N terminus	LPNPS GFVTC LSSIS KSVYT PA
CNBr fragment 1 (26/28 kDa)	INLKA VIADP VAKTA VVQAG ATLGE VYYXI
CNBr fragment 2 (22 kDa)	QSSFF PFFSA
CNBr fragment 3 (12/14 kDa)	IYARV LWVGX TTQKL EWIRS LHDY

Fig. 1





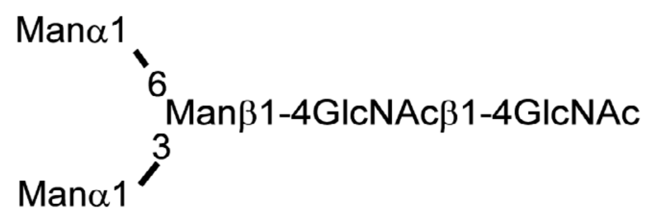


Figure 1. Structures of oligosaccharides mentioned in the text. Man: mannose; GlcNAc: *N*-acetylglucosamine; Xyl: xylose; Fuc: fucose.

Fig. 2

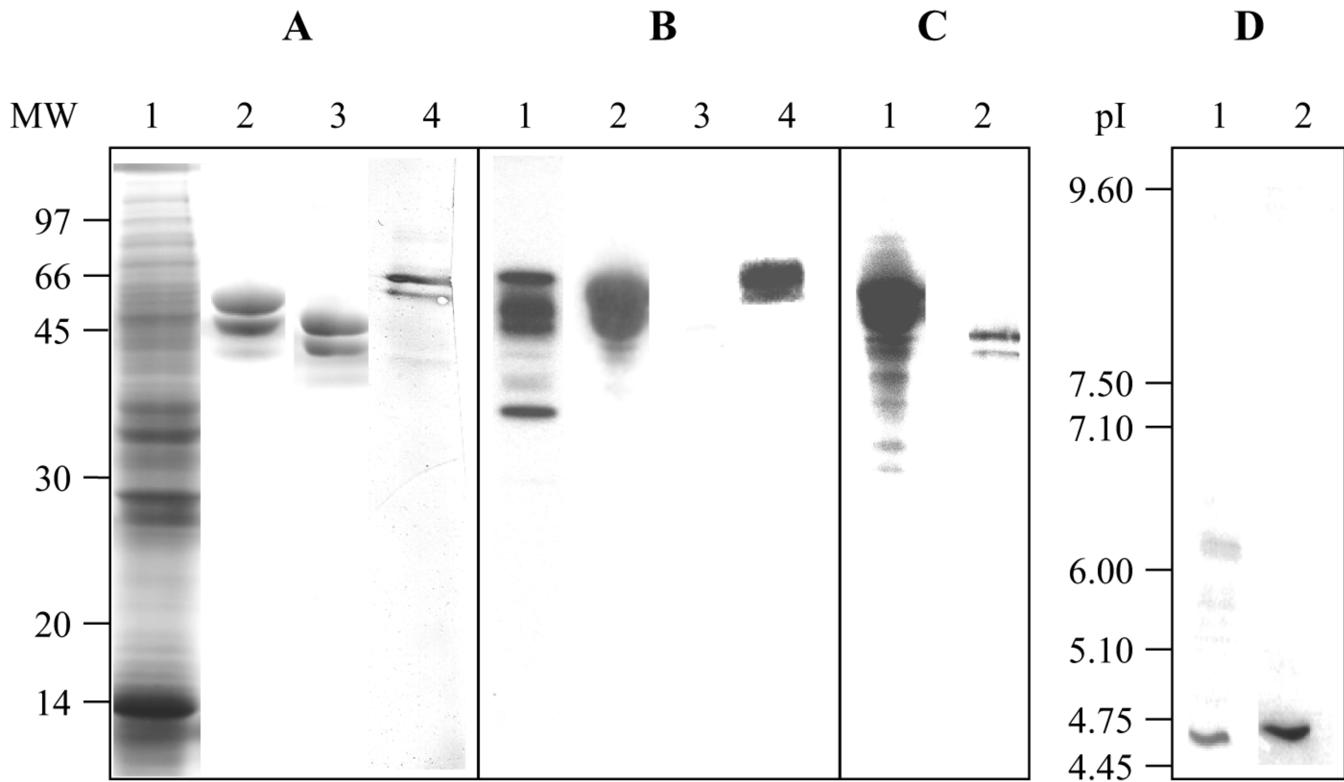


Figure 2. Electrophoretic analysis of purified Api g 5. ***A*)** Coomassie-stained SDS-PAGE of celery tuber protein extract (lane 1), native Api g 5 (lane 2), TFMS deglycosylated Api g 5 (lane 3), and reduced/alkylated Api g 5 (lane 4). ***B*)** Immunoblot with serum pool A showing IgE binding to celery tuber protein extract (lane 1), native Api g 5 (lane 2), TFMS deglycosylated Api g 5 (lane 3), and reduced/alkylated Api g 5 (lane 4). ***C*)** Immunoblot showing anti-HRP binding to native (lane 1) and TFMS deglycosylated (lane 2) Api g 5. ***D*)** isoelectric focusing of native Api g 5: Coomassie stain (lane 1) and IgE immunoblot (lane 2). Fifty micrograms of total protein extracts or 5 µg of purified proteins were applied to the gels. Immunoblots were stained using ^{125}I-labeled secondary antibodies and autoradiography.

Fig. 3

```
N-terminus               1  LPNPSGFVTCLSS--------ISKSVYTPA  22

Q8SA59                  31  RSITDRFIQCLHDRA-DPSFPITGEVYTPG  59
Q8SA60                  30  TSIIDRFTQCLNNRA-DPSFPLSGQLYTPG  58
Q9SA85                  22  SANSETFTQCLTSNS-DPKHPISPAIFFSG  50
Q9SVG3                  32  DSIYESFVQCFSDKTKSPQAQITDNVFSRT  61
Q9AYM8                  25  ADTHENFLQCLYSYP-HNTNSISSVLYTQT  53
Q948L1                   3  ADSTQRFIQCLTKYA-KNSESISQVVFTPA  31
Q8LSM7                  30  TSTSEDFITCLQSNS-NNVTTISQLVFTPA  58
Q9FKU8                  29  ASLQDQFINCVQRNT-HVYFPLEKTFFAPT  57
P30986                  25  --ND--LLSCLTFNGVRNHTVFS----ADS  46
P93479                  25  DVNDNLLSSCLNSHGVHNFTTLS----TDT  50
Q9ZPP5                  21  -VN---LSSCLTSNGVSNFTALS----TSS  42


CNBr fragment 26/28 kD   1  MINLKAVIADPVAKTAVVQAGATLGEVYYXI   31

Q8SA59                 137  MFNLRSINVDIEQETAWVQAGATLGEVYYRI  167
Q8SA60                 136  MFNLRSINVSIEDETAWVQAGATLGEVYYRI  166
Q9SA85                 128  MFNLRSVDVDVASKTAWVQTGAILGEVYYYI  158
Q9SVG3                 137  MSNLRDVSVDIADQSAWISAGATLGEVYYRI  167
Q9AYM8                 127  LLNFREIKVDVENRTAWVQVGATLGELYYTI  157
Q948L1                 107  MINFNRVNIDLKTSTAWVQSGISLGEFYYRI  137
Q8LSM7                 134  LVNMRAIEINVENRTALVQGGALLGELYYTI  164
Q9FKU8                 137  LSKMRQVNINIQDNSAWVQSGATVGELYYRI  167
P30986                 122  LMNLNRVSIDLESETAWVESGSTLGELYYAI  152
P93479                 126  MMNLNRISIDVLSETAWVESGATLGELYYAI  156
Q9ZPP5                 118  LMNLNRISIDLESKTAWVESGATLGEIYCAI  148


CNBr fragment 12/14 kD   1  MIYARVLWVGXTTQK.......LEWIRSLHDY   25

BI452005             (270)  MVYTRVLWVGNTTEK.......LEWIRSLYSY (345)
Q8SA59                 436  KIQYEVNWEDLSDEAE...NRYLNFTRLMYDY  464
Q8SA60                 435  KIQYEVNWDELGVEAA...NRYLNFTRVMYDY  463
Q9SA85                 427  KIQYGANWRD..ETLT...DRYMELTRKLYQF  453
Q9SVG3                 436  KIQYSVTWQENSVEIE...KGFLNQANVLYSF  464
Q9AYM8                 433  HIHYVVVWQEEGDEAT...QRHVNWIRRLYKY  461
Q948L1                 412  MINMAVTLAQN.EEAT......LQWINDLFKY  437
Q8LSM7                 435  QVFKRVDFVDQPSDKTLISLRRLAWLRSFDKT  466
Q9FKU8                 437  KVQYVTSWLD.SDKRP...SRHINWIRDLYSY  464
P30986                 415  MVEYIVAWNQSEQKKK...TEFLDWLEKVYEF  443
P93479                 419  MFEYIIAWNQDEESKI...GEFSEWLAKFYDY  447
Q9ZPP5                 412  MMEYIVAWDRDEDAKS...YEFIGWLHGFYNY  440
```

Figure 3. Alignment of Api g 5 sequences with similar sequences found in the GenBank/EMBL/DDBJ and Swiss-Prot databases. Sequences are designated by their Swiss-Prot/TrEMBL accession numbers: Q8SA59: carbohydrate oxidase (*Helianthus annuus*); Q8SA60: carbohydrate oxidase (*Lactuca sativa*); Q9SA85 and Q9SVG3: berberine bridge enzyme-like proteins (*Arabidopsis thaliana*); Q9AYM8: drought inducible gene CPRD2 (*Vigna unguiculata*); Q948L1: berberine bridge enzyme-like protein (*Daucus carota*); Q8LSM7: carbohydrate oxidase (*Helianthus annuus*); Q9FKU8: putative berberine bridge enzyme (*Arabidopsis thaliana*); P93479: berberine bridge enzyme (*Eschscholzia californica*); P93479: berberine bridge enzyme (*Papaver somniferum*); Q9ZPP5: berberine bridge enzyme (*Berberis stolonifera*); and BI452005 (GenBank/EMBL/DDBJ): *Glomus intraradices* extra-radical mycelium mRNA sequence (base numbers in parentheses, base 309 was omitted in order to obtain an open reading frame). Amino acid residues conserved in at least half of the sequences are shaded, and dashes indicate gaps inserted during the alignment.

Fig. 4

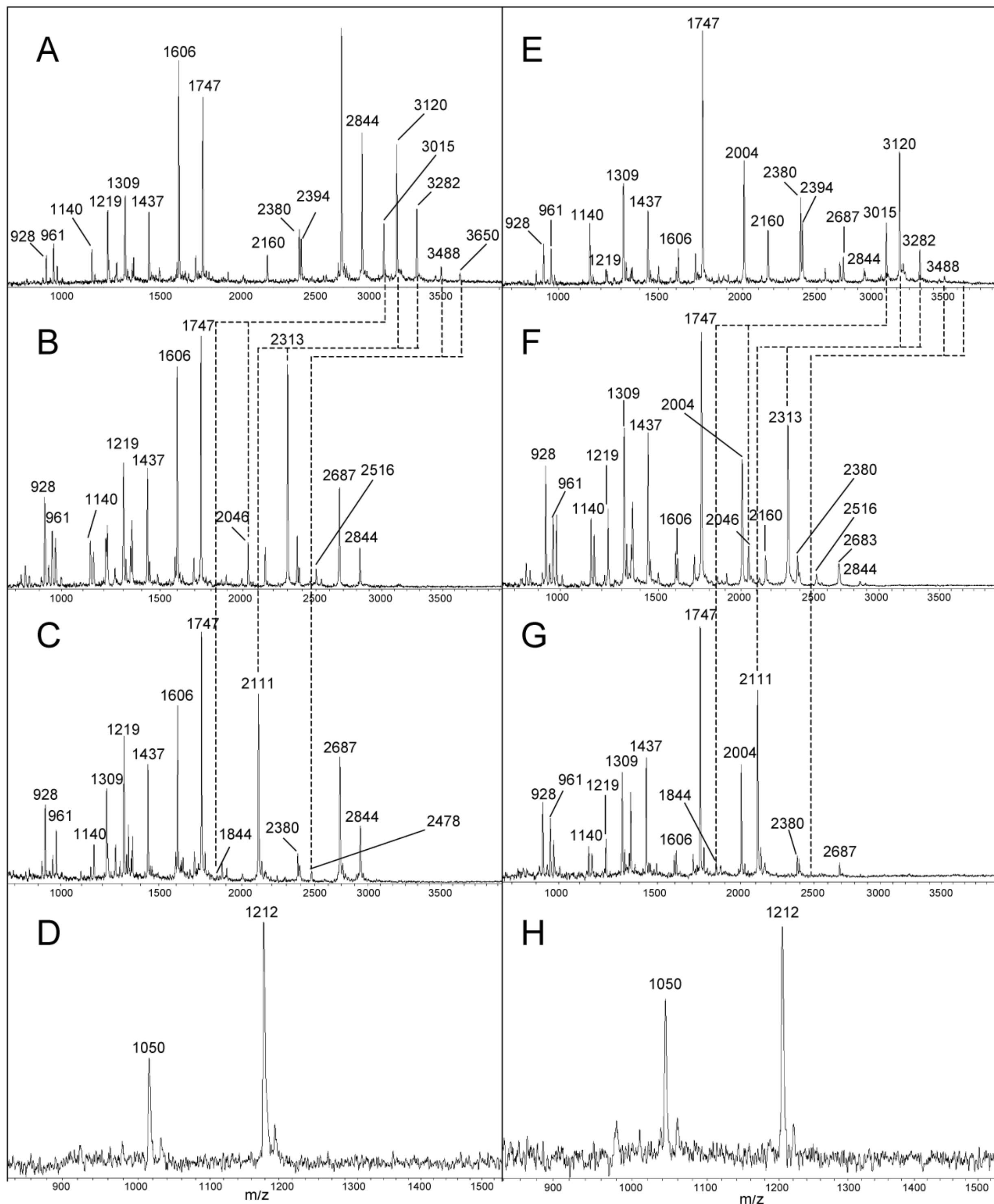


Figure 4. Mass spectrometric analysis of Api g 5 peptides and N-glycans. MALDI-TOF MS spectra of tryptic digests of the 57 and 53 kDa forms, respectively, of Api g 5 (***A*** and ***E***); tryptic digests of the TFMS-treated 57 and 53 kDa forms of Api g 5 (***B*** and ***F***); PNGase A-treated tryptic digests of the 57 and 53 kDa forms of Api g 5 (***C*** and ***G***); the PNGase A-released N-glycans of the 57 and 53 kDa forms of Api g 5 (***D*** and ***H***). The glycans MUXF (m/z 1050) and MMXF (m/z 1212) are present predominantly as $[M+Na]^+$ species. Dashed lines indicate the possible relationships of glycopeptides, TFMS-treated glycopeptides, and PNGase-treated glycopeptides.

Fig. 5

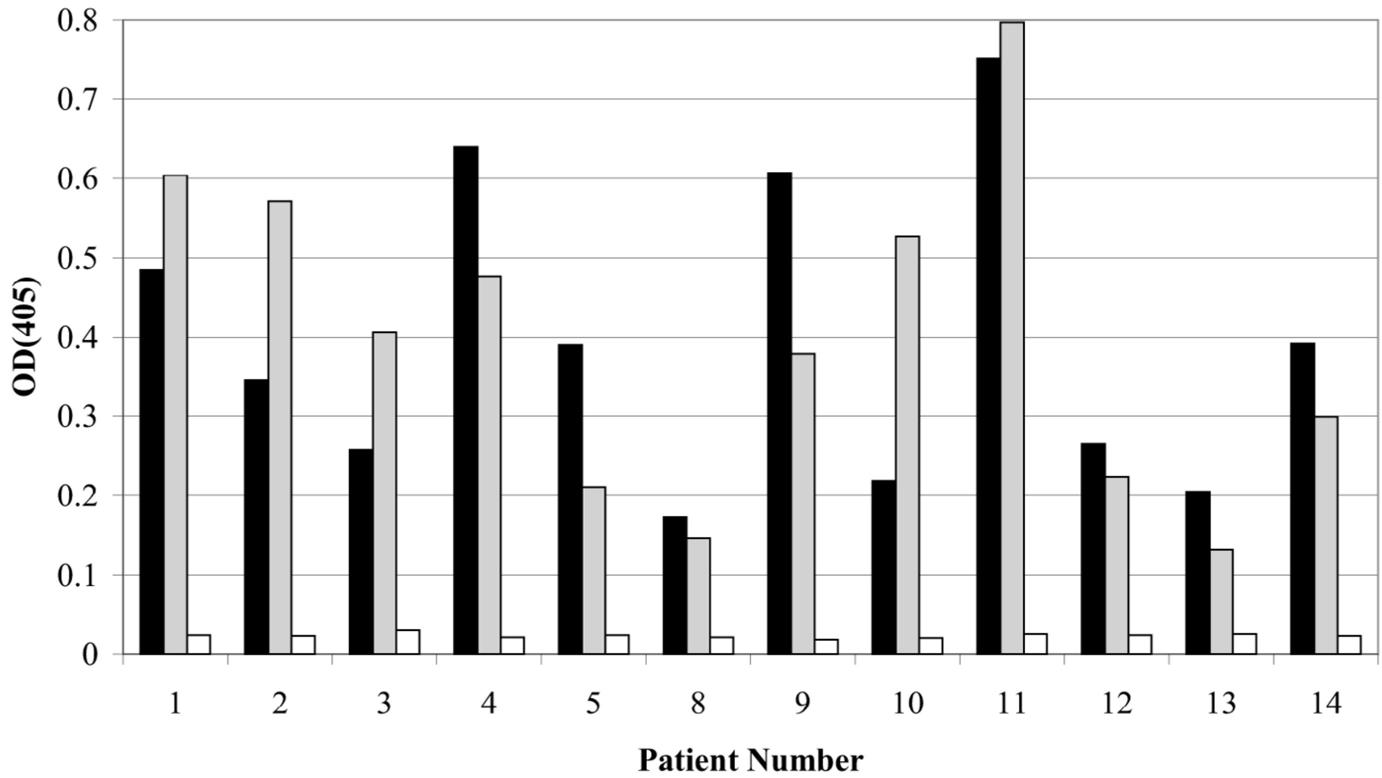


Figure 5. Influence of deglycosylation and alkylation on the IgE binding capacity of Api g 5. Sera of 12 patients were tested for their IgE reactivity to native Api g 5 (solid bars), reduced/alkylated Api g 5 (gray bars), and Api g 5 deglycosylated with TFMS (open bars) by an IgE ELISA. Bound IgE was determined using an alkaline phosphatase-conjugated anti-human IgE in conjunction with *p*-nitrophenyl phosphate staining.

Fig. 6

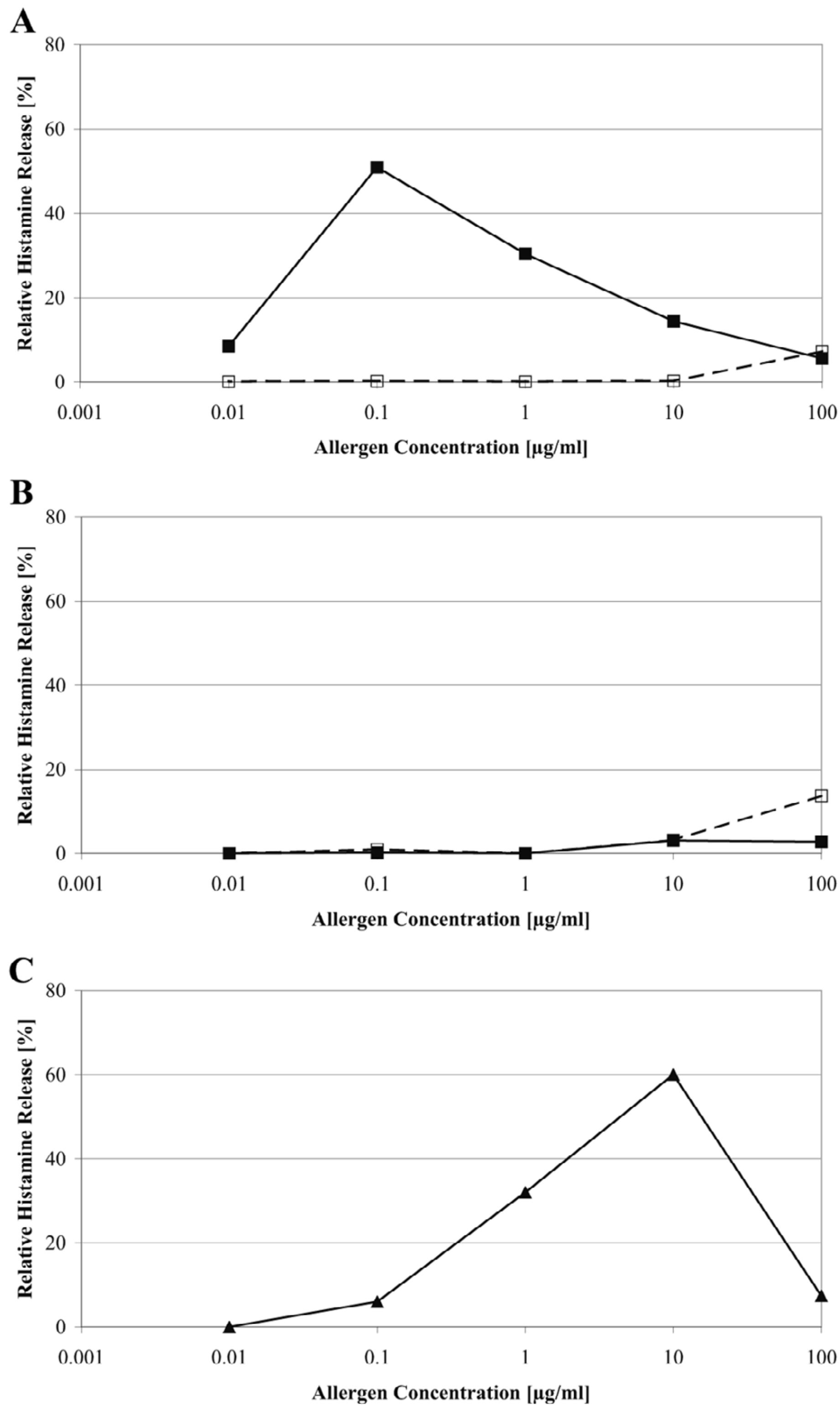


Figure 6. Basophil histamine release. Blood samples were obtained from patient 5, allergic to mugwort and grass pollen as well as celery (***A***); patient 13 with no celery allergy (***B***); and patient 15, allergic to celery and birch pollen (***C***). Basophils were stimulated with purified Api g 5 (filled squares), TFMS deglycosylated Api g 5 (open squares), and recombinant Api g 1 (triangle***s***). Histamine release is expressed as a percentage of total histamine content determined after cell lysis.

Fig. 7

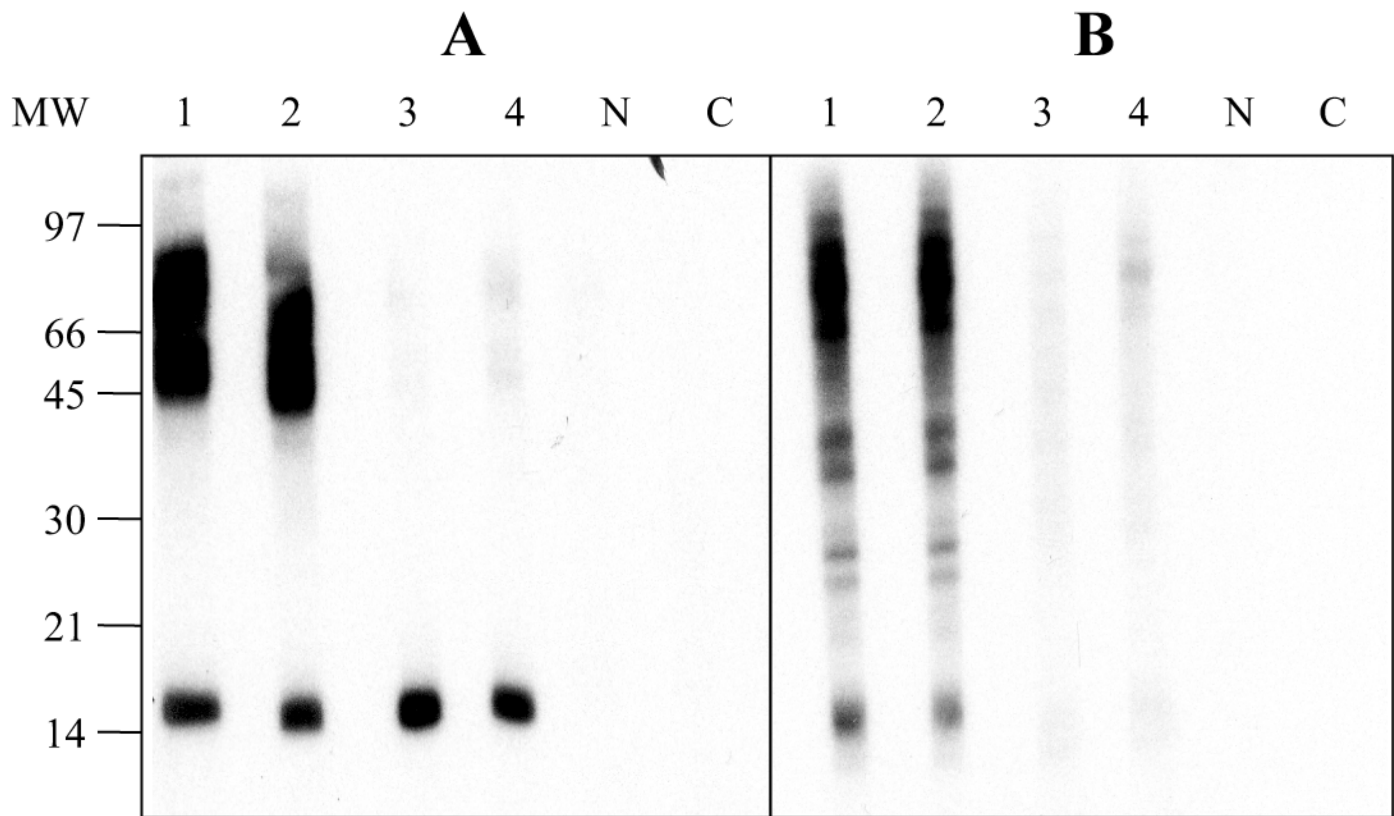


Figure 7. Immunoblot inhibitions experiments. Protein extracts (50 µg) from celery tuber (***A***) and mugwort pollen (***B***) were separated by SDS-PAGE and blotted onto nitrocellulose. IgE binding was determined after preincubation of serum pool B with buffer (lane 1) or 50 µg/ml of MM-BSA (lane 2), MUXF-BSA (lane 3), and Api g 5 (lane 4), respectively. Lane N: serum from a nonallergic donor. Lane C: buffer control. Bound IgE was detected using a ^{125}I-labeled anti-human IgE and autoradiography.