

Characterization of the N-glycans of recombinant bee venom hyaluronidase (Api m 2) expressed in insect cells

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ABSTRACT

Honeybee venom hyaluronidase (Api m 2) is a major glycoprotein allergen. Previous studies have indicated that recombinant Api m 2 expressed in insect cells has enzyme activity and IgE binding comparable with that of native Api m 2. In contrast, Api m 2 expressed in *Escherichia coli* does not. In this study, we characterized the carbohydrate side chains of Api m 2 expressed in insect cells, and compared our data with the established carbohydrate structure of native Api m 2. We assessed both the monosaccharide and the oligosaccharide content of recombinant Api m 2 using fluorophore-assisted carbohydrate electrophoresis and HPLC. To identify the amino acid residues at which glycosylation occurs, we digested recombinant Api m 2 with endoproteinase Glu-C and identified the fragments that contained carbohydrate by specific staining. Recombinant Api m 2 expressed in insect cells contains N-acetylglucosamine, mannose, and fucose, as well as trace amounts of glucose and galactose, and the oligosaccharide analysis is consistent with heterogeneous oligosaccharide chains consisting of two to seven monosaccharides. No sialic acid or N-acetylgalactosamine were detected. These results are similar to published data for native Api m 2, although some monosaccharide components appear to be absent in the recombinant protein. Analysis of proteolytic digests indicates that of the four candidate N-glycosylation sites, carbohydrate chains are attached at asparagines 115 and 263. Recombinant Api m 2 expressed in insect cells has enzymic activity and IgE binding comparable with the native protein, and its carbohydrate composition is very similar.

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Key words: Allergen, bee venom allergy, honeybee, hymenoptera, hyaluronidase, recombinant

Honeybee venom hyaluronidase (Api m 2) is one of the major allergens of bee venom allergy and anaphylaxis.¹ In previous work, we showed that hyaluronidase expressed in baculovirus-infected insect cells has enzymic activity and specific IgE binding that was comparable with that of native hyaluronidase, and hyaluronidase expressed in *Escherichia coli* has markedly depressed activity and binding.² When recombinant proteins expressed in prokaryotic cells fail to function as expected, the expression of these proteins in eukaryotic cells is desirable because such proteins are similar to native proteins in carbohydrate composition and protein conformation; this would be especially true for the expression of hymenoptera proteins in insect cells. The carbohydrate content and structure of the N-glycans of native Api m 2 has been determined^{3,4} (Table 1). In this article, we examine the car-

bohydrate chains of recombinant Api m 2 (rApi m 2) expressed in insect cells.

MATERIALS AND METHODS

Protein Expression and Purification

Expression of rApi m 2 in baculovirus-infected *Trichoplusia ni* (High-Five) BTI-TN-5B1-4 cells (Invitrogen, San Diego, CA), and the purification of the His-tagged protein by nickel-chelation chromatography, were performed as previously described.²

Carbohydrate Analysis by Electrophoresis

Fluorophore-assisted carbohydrate electrophoresis (FACE) for both monosaccharide content and N-linked oligosaccharide content⁵ were performed according to the manufacturer's directions (Glyco, Novato, CA). In the FACE technique, carbohydrates (oligosaccharides and monosaccharides) are released from the protein backbone under a variety of conditions and labeled with a suitable fluorophore. The carbohydrates are then separated by polyacrylamide gel electrophoresis and visualized by exposure to ultraviolet light. The rApi m 2 was subjected to three different acid hydrolysis protocols, to analyze its monosaccharide composition, and to enzymic treatment with PNGase F (peptide N-glycosidase F), to determine the sizes of the attached oligosaccharides.

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Table 1 Carbohydrate content of native bee venom hyaluronidase

Carbohydrate	Amount (nmol/mg)	Percent (w/w)
Fucose	107	1.75
Mannose	142	2.56
Galactose	12	0.22
Glucose	13	0.23
N-acetylglucosamine	95	2.10
N-acetylgalactosamine	17	0.38
Sialic acid	0	0
Glucuronic acid	0	0
Total		7.24

Source: Ref. 3 (reprinted with permission).

Carbohydrate Analysis by HPLC

For neutral and amino monosaccharides, 90 μ L of rApi m 2 (3 mg/mL) was hydrolyzed in 2N trifluoroacetic acid at 100°C for 6 hours. After removal of trifluoroacetic acid by lyophilization, the sample was dissolved in 300 μ L of water, passed through a 10,000 molecular weight cutoff membrane cartridge (Vivascience, Carlsbad, CA), and 10 μ L of filtrate was injected into a HPLC system using a CarboPak PA1 column (Dionex, Sunnyvale, CA).⁶ For sialic acid analysis, rApi m 2 was hydrolyzed in 0.1N trifluoroacetic acid at 80°C for 90 minutes, to avoid sialic acid destruction.⁷

Digestion of rApi m 2 with Endoproteinase Glu-C

Recombinant protein was prepared for proteolysis by denaturation in 8M urea and 0.1M ammonium bicarbonate, and reduction with 0.01M dithiothreitol in the presence of 0.04M methylamine. SDS (10%) was added to dissolve the small precipitate that formed during the reduction reaction. Endoproteinase Glu-C (Roche Diagnostics, Indianapolis, IN) was added in ammonium bicarbonate, such that the final digestion mixture contained rApi m 2 (2.1 mg/mL), endoproteinase Glu-C (0.1 mg/mL), 1M urea, 0.1M ammonium bicarbonate, 0.02M methylamine, 0.005M dithiothreitol, and 0.1% SDS. Digestion was allowed to proceed overnight at 25°C. The optimal amount of endoproteinase Glu-C was determined empirically.

Two-Dimensional Gel Electrophoresis, Transfer, and Staining of Peptide Fragments

Digested rApi m 2 was analyzed using the ZOOM IPGRunner System (Invitrogen). ZOOM Strips (pH 3–10NL) were rehydrated overnight in 8M urea, 2% 3-[3-(cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.5% ZOOM Carrier Ampholytes (pH 3–10), 0.002% bromphenol blue, and 20 mM dithiothre-

itol containing 52.2 μ g of digested rApi m 2 and were subjected to electrofocusing at 500 V for 4 hours. After electrofocusing, the peptides were reduced and alkylated according to the manufacturer's directions and separated electrophoretically on a NuPAGE Novex 4–12% Bis-Tris ZOOM Gel (Invitrogen) at 200 V for 30 minutes using the NuPAGE MES SDS running buffer. The peptides were transferred to a polyvinylidene fluoride membrane (Invitrogen) in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid buffer (pH 9.9) containing 10% methanol in an Invitrogen X Cell II Blot Module at 30 V for 2.5 hours. The transferred peptide fragments were then stained for protein using either the GelCode Blue Stain Reagent (Pierce, Rockford, IL) or for glycoprotein using the GelCode Glycoprotein Staining Kit (Pierce). Comparison of stained gels and membranes confirmed that all visible peptide bands transferred under these conditions.

N-Terminal Sequence Analysis

Bands of interest were excised and arranged in a single layer in the upper cartridge of Applied Biosystems model 494 protein sequenator (Applied Biosystems, Inc., Foster City, CA). Sequencing and analysis of phenylthiohydantoin derivatives were performed by online reverse-phase HPLC on Applied Biosystems model 140 Microgradient System followed by online detection on Applied Biosystems model 785A Programmable Absorbance Detector (Applied Biosystems, Inc.).⁸ In estimating the lengths of the detected glycopeptides, we determined the R_f of the peptide from the two-dimensional gel and subtracted the possible sizes of the oligosaccharide side chains. Based on the FACE analysis, the oligosaccharide sizes ranged from glucose-2 to glucose-7. The molecular masses of the glucose polymers may be calculated from the formula $(n - 1) * (180 - 16) + 180$ (16 is subtracted for water loss at each ketosidic bond). Thus, the molecular mass of glucose-2 is 344 Da, and molecular mass of glucose-7 is 1164 Da.

RESULTS

The monosaccharide content of rApi m 2 was evaluated by two methods. In the monosaccharide FACE technique, carbohydrates bound to rApi m 2 were hydrolyzed under various conditions and labeled with a fluorophore, 2-aminoacridon. The electrophoretic migration of the labeled monosaccharides was compared with the migration of a series of monosaccharide standards (Fig. 1). By this analysis, rApi m 2 contains mannose, fucose, N-acetylglucosamine, and trace amounts of glucose/galactose.

In a separate experiment using the same material, rApi m 2 was hydrolyzed and subjected to HPLC analysis for monosaccharides. In this method the only

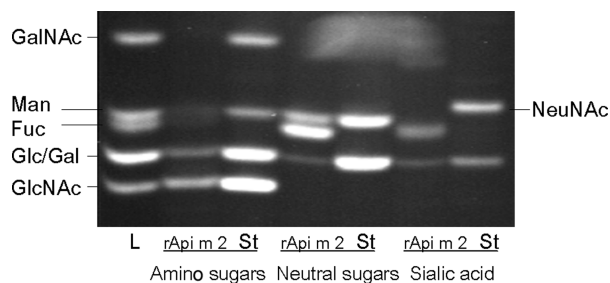


Figure 1. Monosaccharide composition of digested oligosaccharides from rApi m 2. Glycoprotein hydrolysis was performed under three different reaction conditions to enhance the greatest recovery of amine sugars (N-acetylglucosamine and N-acetylgalactosamine), neutral sugars (glucose, galactose, mannose, and fucose) and sialic acid. The released monosaccharides are fluorophore-labeled and separated by electrophoresis along with a glycoprotein standard (bovine fetuin) hydrolyzed under the three different conditions. The R_f for neutral and amine sugars and for N-acetylneuraminic acid are estimated from the standard ladder. L, monosaccharide ladder; St, glycoprotein standard (bovine fetuin).

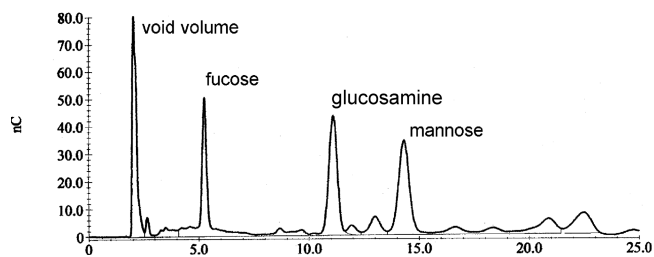


Figure 2. HPLC of released monosaccharides from rApi m 2. Peak identification is based on elution of monosaccharide standards. The glucosamine peak is N-acetylglucosamine from rApi m 2 that has been deacetylated during acid hydrolysis. The two small peaks between glucosamine and mannose may represent trace amounts of glucose and galactose.

monosaccharides detected were fucose, N-acetylglucosamine, and mannose; only a trace of glucose and galactose were observed (Fig. 2). In neither analysis was there any evidence of sialic acid or N-acetylgalactosamine (Fig. 1; HPLC sialic acid analysis not shown).

We used a modification of the FACE technique to examine the oligosaccharide content of rApi m 2. Oligosaccharides were released intact from the protein using PNGase F under denaturing conditions and labeled with a fluorophore, 8-aminonaphtalene-1,3,6-trisulfonate. PNGase F selectively cleaves the oligosaccharides from glycoproteins between the innermost N-acetylglucosamine of the oligosaccharide and the asparagine residue of the protein. The electrophoretic migration of the labeled oligosaccharides was compared with the migration of a series of glucose polymer standards. The N-linked oligosaccharides appeared to migrate as multiple carbohydrate chains consisting of two to seven monosaccharides (Fig. 3).

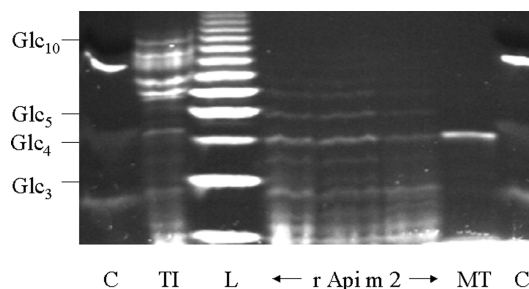


Figure 3. Oligosaccharide content of rApi m 2. Oligosaccharides were cleaved from the protein using PNGase F under denaturing conditions, labeled with a fluorophore and separated by electrophoresis along with cleaved trypsin inhibitor (TI), a glucose polymer ladder (L), maltotetraose (MT), and tracking dye (C).

Based on the consensus sequence of Asn-Xaa-Thr/Ser, Api m 2 contains four asparagine residues that are candidate N-glycosylation sites (at loci 37, 115, 223, and 263; see Fig. 5; see Swiss Protein Database accession number Q08169). To identify the specific glycosylation sites in the recombinant protein, rApi m 2 was exhaustively digested with the endoproteinase Glu-C under

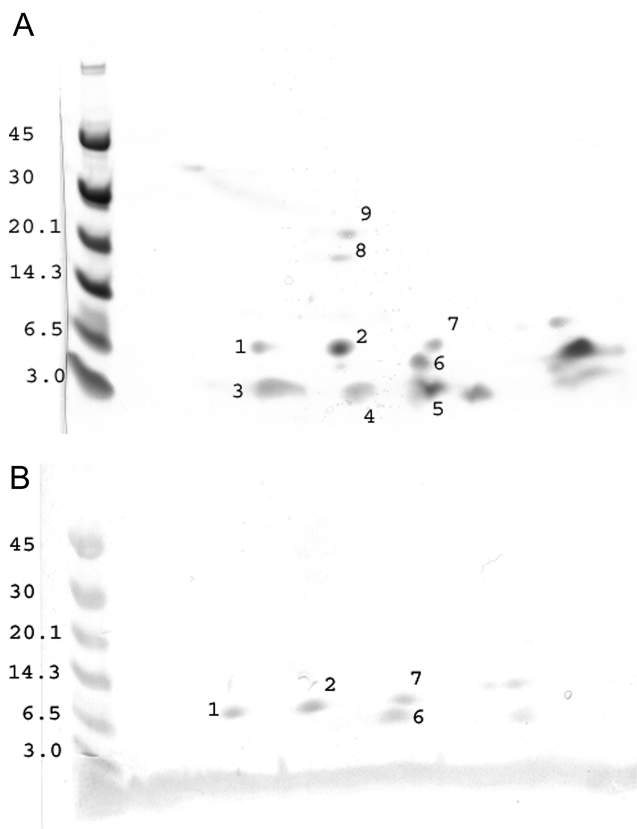


Figure 4. PVDF transfers of two-dimensional electrophoreses of digested rApi m 2. (A) Stained with GelCode Blue Stain Reagent (Pierce) and (B) stained with GelCode Glycoprotein Staining Kit (Pierce). The peptides selected for sequencing are numbered. Nominal molecular masses (kDa) of the molecular weight standards are designated on the left.

denaturing conditions and separated by two-dimensional electrophoresis. The separated peptides were transferred to a membrane support and stained on separate membranes for protein and carbohydrate content (Fig. 4, A and B). All of the four individual glycopeptides, and several peptides without evidence of carbohydrate side chains, were then subjected to N-terminal sequencing. The four hydrolysis fragments, which showed evidence of glycosylation (1, 2, 6, and 7), were estimated to contain two putative glycosylation sites (Asn 115 and Asn 263), based on their apparent molecular weights (Fig. 5).

DISCUSSION

Recombinant protein technology offers the opportunity to overcome several problems that have been associated with the use of allergens for diagnosis and immunotherapy, such as consistency, purity, and bioavailability. In addition, the use of modified recombinant allergens may permit allergists to administer effective immunotherapy with reduced risk of mast cell activation and anaphylaxis. However, recombinant allergens expressed in bacteria are not glycosylated. In some cases, allergens that are glycosylated in nature show no loss of potency when expressed without their native carbohydrate side chains.⁹⁻¹² This is not the case for Api m 2.² Whether this loss of potency is because fucose-containing oligosaccharides are essential com-

ponents of critical epitopes^{13,14} or because of a failure of aglycoproteins to fold properly¹⁵ is uncertain. One approach used to generate glycosylated allergens has been to express them in yeast.^{16,17} While allergens expressed in yeast are glycosylated, the carbohydrate structure is different from that of glycoproteins expressed in higher cell lines, and this may affect biological activity.

For allergens—such as the hymenoptera venoms proteins—that are naturally derived from insect tissues, expressing the recombinant products in insect cell lines would appear to be an ideal approach to the consistent generation of a product that closely approximates the native allergen in conformation and carbohydrate content.¹⁸ Our current data extend previous work, in which we showed that Api m 2 expressed in insect cells had antibody binding and enzymic activity at or near those of the native protein.²

This study confirms that, along with their functional similarities, recombinant and native Api m 2 appear to have comparable carbohydrate content. Although the only substantial difference—the presence of N-acetylgalactosamine in the native product—may reflect the insensitivity of our methods, it also may indicate a greater complexity of the oligosaccharides in the native protein. The N-linked oligosaccharides of rApi m 2 migrate electrophoreti-

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1   msrplviteg mmigvllmla pinalllgfv qstpdn[n]ktv refnvymvp tfmchkyglr

                                           115
61   feevsekygi lqnwmkfrg ee iaailydpg mfpallkdpn gmvarnggv pqlq[n]tkhl
      --1,2,7(est. MW = 5336 - 6156 Da)----->

121  qvfrdhlinq ipdksfpgvg vidfeswrpi frqnwaslqp ykklsvvevr rehpfwddqr
      ----->----->

                                           223
181  veqeakrrfe kygqlfmeet lkaakrmrpa anwggyaypy cy[n]tpnqps aqceattmqe

                                           263
241  ndkmswlifes edvllpsvyl r[n]ltsgerv glvggrvkea lriarqmtts rkkvlpwywy
      --6 (est. MW = 4136 - 4956 Da )----->----->

301  kyqdrddtdl sradleatlr kitdlgadgf iiwgssddin tkakclqfre ylnnelgpav

361  krialnnnan drltvdvsvd qv

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Figure 5. Primary amino acid sequence of Api m 2 (Swiss Protein Database accession number Q08169). The N-terminus of the mature protein is at Thr 33. Potential N-glycosylation sites at Asn 37, 115, 223, and 263 are indicated. The N-terminal sequences for the four glycopeptides (see Fig. 4) were IAILYDPG (for 1, 2, and 7) and SEDVLLPS (for 6). The lengths of the four glycopeptides were estimated from their R_f (Fig. 4) with 344 to 1164 subtracted for the possible oligosaccharide side chain sizes (see text); the ranges are indicated by the arrow marks within the dashed lines.

cally as multiple carbohydrate chains consisting of two to seven monosaccharides. In contrast, the most complex *N*-glycan identified in native Api m 2 contained 10 monosaccharides, and about 18 oligosaccharide chains of different size are present in the native protein.⁴ Recombinant proteins expressed in insect cells contain more heterogeneous, shorter and simpler, than native glycoproteins, consisting mostly of high-mannose type *N*-glycans.^{19–21} Our analysis of the *N*-linked oligosaccharide chains of rApi m 2 is consistent with these observations.

Quantitative amino sugar analysis of Api m 2 indicates the presence of four molecules of *N*-acetylglucosamine per protein molecule in the native³ and recombinant² proteins, suggesting the presence of one or two oligosaccharide chains. Prior data support the presence of *N*-linked glycans at residues 115 and 263. Kolarich subjected native Api m 2 from Bulgarian honeybees to tryptic digestion and identified two glycan species of different molecular mass by MALDI-TOF analysis at site 263.²² Markovic-Housley *et al.* performed an x-ray crystallographic analysis of the same rApi m 2 used in our studies. They identified an additional electron density at Asn 115, suggesting the presence of a sugar residue; they excluded Asn 223 as a possible glycosylation site because this residue is buried within the protein. On the other hand, Asn 263 is solvent exposed and can be a potential glycosylation site. No structural information is available for Asn 37 because the electron density is missing for the first 10 residues at the *N*-terminal end of the protein.²³ Our current data using rApi m 2 indicate that both sites Asn 115 and Asn 263 are glycosylated.

Api m 2 is an important hymenoptera allergen,¹ and the presence of enzymically active hyaluronidase in hymenoptera allergen extracts is required by the U.S. Food and Drug Administration.²⁴ This study confirms that rApi m 2 expressed in insect cells has similar carbohydrate composition to that reported for the native enzyme. This and the functional integrity that we previously reported² suggest that rApi m 2 from insect cells will be a useful immunotherapeutic reagent.

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