Complete Structure of the Carbohydrate Moiety of Stem Bromelain
AN APPLICATION OF THE ALMOND GLYCOPEPTIDASE FOR STRUCTURAL STUDIES OF GLYCOPEPTIDES

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Asparagine-linked oligosaccharides of stem bromelain glycopeptides were quantitatively released by digestion with the almond glycopeptidase which cleaves β-aspartylglycosylamine linkage in glycopeptides with oligosaccharide moieties.

The primary structures of the two oligosaccharide components, (Man)α(Xyl)1,(Fuc)1(GlcNAc)2 and (Man)α-(Xyl)1(=Fuc)1(GlcNAc)2 were elucidated as Manα1 → 6Manα1 → 6[Xylβ1 → 2]Manβ1 → 4GlcNAcβ1 → 4[Fucα1 → 3]GlcNAc and Manα1 → 6[Xylβ1 → 2]Manβ1 → 4GlcNAcβ1 → 4[Fucα1 → 3]GlcNAc, respectively.

Stem bromelain is a thiol protease isolated from pineapple stem and contains one asparagine-linked oligosaccharide chain per molecule (1). The oligosaccharides are composed of mannose, fucose, xylose, and N-acetylglucosamine (GlcNAc).2 Because of their unusual carbohydrate compositions, several studies have been performed on the asparagine-linked oligosaccharides and, as yet, revealed only their partial structures (2-5). In this communication, we present the complete primary structure of the oligosaccharides. To the best of our knowledge, this is the first report in which the primary structure of an asparagine-linked oligosaccharide is established in a higher plant.

Elucidation of the complete oligosaccharide structures was difficult because of their heterogeneity with respect to mannosyl residues (2, 3) and because of the large number of amino acids remaining in the glycopeptides even after extensive pronase digestion (6). These difficulties have been overcome by the use of the almond glycopeptidase which was found by one of the authors in 1977 (7, 8). The enzyme cleaves β-aspartylglycosylamine linkages in glycopeptides with multiple amino acid residues and quantitatively releases oligosaccharides from the bromelain glycopeptides. The released oligosaccharides were separated by paper chromatography into two components with an integral number of mannosyl residues (2,3) and because of the large number of amino acid residues in the glycopeptides, 2-dimensional analyses on the same oligosaccharide alditols 1 and 2 (Tables I and II) satisfactorily agreed with each other.

In the methylation analysis of oligosaccharide alditol 1, three kinds of methylated manninitols were produced: (Table II): 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol from the nonreducing terminal mannose; 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylmannitol from the second mannose (substituted in position 6); and 1,2,5,6-tetra-O-acetyl-3,4-di-O-methylmannitol from the innermost mannose (substituted in positions 2 and 6). The products from oligosaccharide alditol 2 were two kinds of methylated manninitols (Table II): 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol from the nonreducing terminal mannose and 1,2,5,6-tetra-O-acetyl-3,4-di-O-methylmannitol from the innermost mannose (substituted in positions 2 and 6). The results of two independent analyses on the same oligosaccharide alditols 1 and 2 (Tables I and II) satisfactorily agreed with each other. Thus, oligosaccharide alditol 1 was a heptasaccharide alcohol containing 3 residues of mannose and oligosaccharide alditol 2 a hexasaccharide alcohol containing 2 residues of mannose.

EXPERIMENTAL PROCEDURES

Results

Liberation and Isolation of Two Oligosaccharides from Bromelain Glycopeptides—Asparagine-linked oligosaccharides of stem bromelain glycopeptides were quantitatively released by digestion with the glycopeptidase. Product analysis by paper electrophoresis and paper chromatography detected no smaller oligosaccharides or monosaccharides as by-products (8). The NaB³H₄-reduced oligosaccharide alcohols obtained as described under “Experimental Procedures.” Fig. 3 shows that the [³H]oligosaccharide alcohols separated into two peaks (named Oligosaccharide alditol 1 and Oligosaccharide alditol 2). The yield ratio of oligosaccharide alditol 1 to oligosaccharide alditol 2 was 0.81 after the second chromatography. These two sugar alcohols were eluted from the paper and analyzed by gas liquid chromatography and methylation studies.

Carbohydrate Composition of Oligosaccharide Alditols 1 and 2—About 500 μg each of oligosaccharide alditols 1 and 2 was hydrolyzed in 1 ml of 2.5 n trifluoroacetic acid at 100°C for 4 h. The amounts of each neutral sugar in oligosaccharide alditols 1 and 2 were determined by gas-liquid chromatography on Column A. Table I shows that oligosaccharide alditol 1 contained 3 mol of mannose/mol of fucose, whereas the value for oligosaccharide alditol 2 was 2. In the methylation analysis of oligosaccharide alditol 1, three kinds of methylated manninitols were produced (Table II): 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol from the nonreducing terminal mannose; 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylmannitol from the second mannose (substituted in position 6); and 1,2,5,6-tetra-O-acetyl-3,4-di-O-methylmannitol from the innermost mannose (substituted in positions 2 and 6). The products from oligosaccharide alditol 2 were two kinds of methylated manninitols (Table II): 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylmannitol from the nonreducing terminal mannose and 1,2,5,6-tetra-O-acetyl-3,4-di-O-methylmannitol from the innermost mannose (substituted in positions 2 and 6). The results of two independent analyses on the same oligosaccharide alditols 1 and 2 (Tables I and II) satisfactorily agreed with each other. Thus, oligosaccharide alditol 1 was a heptasaccharide alcohol containing 3 residues of mannose and oligosaccharide alditol 2 a hexasaccharide alcohol containing 2 residues of mannose,

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† To whom correspondence should be addressed.
‡ All sugars mentioned in this paper have the D-pyranose configuration except fucose which has the L configuration.
§ The abbreviation used is: GlcNAc, N-acetylglucosamine.

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whereas all other glycosyl residues were the same in number and linkages. Therefore, oligosaccharide alditols 1 and 2 appear to have an identical structure except for the external mannosyl residue. Although the previous studies (2-5) were done on the mixture of oligosaccharides, they do show that the terminal \(\alpha\)-fucosyl, terminal \(\alpha\)-xylosyl, and terminal \(\alpha\)-mannosyl residues must be present on both oligosaccharides, and that the Man\(\beta\) \(\rightarrow 4\)GlcNAc\(\beta\) \(\rightarrow 4\)GlcNAc \(\rightarrow\) Asn sequence must also be present in both oligosaccharides. This inference should be correct because of completely overlapping structures of the two oligosaccharides except for the external mannosyl residue (Table II). 1. Configuration of fucose and \(\beta\) configuration of all other sugars were assigned by susceptibility toward specific glycosidases (3). The remaining question is, therefore, the mode of linkage of external fucosyl, xylosyl, and mannosyl residues to the core Man\(\beta\) \(\rightarrow 4\)GlcNAc\(\beta\) \(\rightarrow 4\)GlcNAc sequence.

Determination of Glycosyl Residue Linked to GlcNAc-GlcNAc Sequence—The \(N\)-acetylglucosamine residue at the reducing end must be substituted either by fucose, xylose, or mannose in both oligosaccharides 1 and 2. In the methylation analysis of the oligosaccharide alditol 1, 1 mol of 2-(\(N\)-methyl)acetamido-3,4-di-\(O\)-acetyl-2-deoxy-1,5,6-tri-\(O\)-methylglucitol and 1 mol of 2-(\(N\)-methyl)acetamido-1,4,5-tri-\(O\)-acetyl-2-deoxy-3,6-di-\(O\)-methylglucitol but none of 2-(\(N\)-methyl)acetamido-4-\(O\)-acetyl-2-deoxy-1,3,5,6-tetra-\(O\)-methylglucitol were found (Table III, 1 to 3). In order to determine the sugar residue substituting the reducing \(N\)-acetylglucosamine residue, the glycopeptides were partially hydrolyzed by trifluoroacetic acid. This treatment selectively removed fucose from the glycopeptides, but not the other sugars. We found that 2-(\(N\)-methyl)acetamido-1,3,4,5-tetra-\(O\)-acetyl-2-deoxy-6-\(O\) methylglucitol, which was detected in intact glycopeptides, disappeared completely after defucosylation, and 2-(\(N\)-methyl)acetamido-1,4,5-tri-\(O\)-acetyl-2-deoxy-3,6-di-\(O\)-methylglucitol increased concomitantly (Table III, 2 and 3). These results establish that a fucosyl residue is linked to position 3 of the reducing \(N\)-acetylglucosamine in both oligosaccharides 1 and 2.

Determination of Glycosyl Residue Linked to Innermost Mannose Residue—In the methylation analysis of the oligosaccharide alditols, approximately 1 mol of 1,2,5,6-tetra-\(O\)-acetyl-3,4-di-\(O\)-methylglucitol was detected per mol of each oligosaccharide, indicating that the innermost mannose residue in both of the oligosaccharides is substituted in positions 2 and 6 (Table II). Since the fucosyl residue has been shown to be linked to the di-\(N\)-acetylglucosamine residue, the sugar residues linked to the branching \(\beta\)-mannosyl residue are \(\alpha\)-mannosyl and \(\beta\)-xylosyl residues. The \(\text{Man} \rightarrow [\text{Xyl}] \rightarrow \text{Man}\beta\) structure could have two different possibilities with respect to the linkage of the \(\alpha\)-mannosyl and \(\beta\)-xylosyl residues to C-2 and C-6 of the \(\beta\)-mannosyl residue. The removal of the terminal \(\alpha\)-mannosyl units with \(\alpha\)-mannosidase were performed on the intact glycopeptide which contained both oligosaccharides. The terminal mannosyl residues were almost completely removed and thus must have been removed from both oligosaccharides. The 6-linked mannosyl residue was removed from the mixture of oligosaccharides and thus must have been removed from oligosaccharide alditol 1. That no other glycosyl residues were removed was confirmed by paper chromatography. Above two points indicates that terminal and 6-linked mannosyl residues in oligosaccharide alditol 1 must be connected to each other and both must be \(\alpha\)-linked. Since only 2-linked mannosyl residues were produced by enzyme treatment (Table III, 7), the terminal mannosyl residue in oligosaccharide alditol 2 must be attached to C-6 of the 2,6-linked mannosyl residue and the Man\(\alpha\) \(\rightarrow 6\)Man\(\alpha\) residue must be attached to C-6 of the 2,6-linked mannosyl residue in oligosaccharide alditol 1. Xylose, therefore, must be linked to position 2 of the innermost mannosyl residue.

The Proposed Structures of Oligosaccharides 1 and 2 from Bromelain Glycopeptides—Based on these data, the complete structures of oligosaccharides 1 and 2 are proposed as shown in Fig. 4.

**FIG. 4:** The proposed structures of oligosaccharides 1 and 2 from bromelain glycopeptides.

**DISCUSSION**

The isolation of peptide-free oligosaccharides from glycopeptides is desirable for the structural analysis of carbohydrate moieties, since precise fractionation of glycopeptides that comprise both heterogeneous peptide and carbohydrate moieties is practically impossible. In this respect, endo-\(\beta\)-\(N\)-acytlylgulosaminidases from *Diplococcus pneumoniae* (9) and *Streptomyces griseus* (10) are excellent tools to remove oligosaccharide moieties from intact glycopeptides. However, because of the strict specificity of the enzymes with respect to the oligomannosyl cores of glycopeptides, they cannot hydrolyze intact glycopeptides derived from stem bromelain. Although hydrazinolysis has recently been introduced as a means to prepare peptide-free oligosaccharides from glycoproteins (2, 11, 12), hydrazinolysis of stem bromelain glycopeptides was accompanied by degradation of about 40% reducing terminal \(N\)-acytlylgulcosamine residue (2). Furthermore, selection of reaction conditions in each different glycopeptide preparation will be needed to avoid undesirable degradations of released oligosaccharide chains by chemical cleavage such as hydrazinolysis. In this paper, we have demonstrated the use of a novel procedure for analysis of the oligosaccharide moiety of stem bromelain, namely, enzymatic liberation of intact oligosaccharides by the glycopeptidase. The method is expected to be valuable in the structural study of other glycopeptide preparations.

The present data give the complete structure of the oligosaccharides of stem bromelain. Fukuda et al. (2) recently proposed as Man\(\alpha\) \(\rightarrow 6\) or 2[R\(\beta\) \(\rightarrow 2\) or 6]Man\(\beta\) \(\rightarrow 4\)GlcNAc\(\beta\) \(\rightarrow 4\)GlcNAc, where R is Fuca or Xyl\(\beta\)
and R' is Xylβ or Fuca. The attachment of fucose at C-3 of asparagine-linked N-acetylglucosamine has already been suggested by Lee and Scocca (5). Xylβ1 → 2Man linkage was also suggested by Lee and Lee (13), although these proposals (5, 13) lacked evidence. The proposed complete structure includes, without contradiction, most of the partial structures already proposed (2-5).

The established structure of stem bromelain oligosaccharides have three interesting features, as follows. First, fucose is attached to the N-acetylglucosamine involved in protein-carbohydrate linkage by the α1 → 3 bond. Although attachment of fucose to the N-acetylglucosamine residue has been shown in a number of cases (11, 12, 14-20), the mode of linkage is always α1 → 6. Second, the oligosaccharides contain a xylose residue linked to a β-mannosyl residue by β1 → 2 linkage. Occurrence of xylose is very rare in asparagine-linked sugar chains, and this is the first case in which mode of linkage of a xylosyl residue is established for this class of sugar chains. Recently, we have found that stem bromelain glycopeptides conjugated to Freund’s incomplete adjuvant is antigenic in the rabbit, and the xylose residue appears to take part in the antigenic determinant. Third, the oligosaccharides are shown to have the Manα1 → 6Manβ1 → 4GlcNAcβ1 → 4GlcNAc → Asn sequence, which is common in a number of asparagine-linked oligosaccharides, while the oligosaccharides lacked another mannosyl residue linked to the β-mannosyl residue by α1 → 3 linkage, which is also a common residue in asparagine-linked oligosaccharides (12, 22-28).

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REFERENCES

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4 M. Sasaki and N. Takahashi, unpublished experiments.
Complete Structure of Bromelain Carbohydrate

Fig. 3. Paper chromatogram of the radioactive oligosaccharide aldolase, which was obtained after digestion of bromelain by the glycoseptase digestion, followed by HCl-Na reduction.

Preparation of HCl-Na-free Glycoside—HCl-Na-free glycoside was prepared as described previously.55 After the isolation of the oligosaccharide, 25 μl of the glycoside in 250 μl of water was added 2.5 ml of 10 N tris(hydroxymethyl)aminomethane (tris) and the mixture was heated on a steam bath at 100°C for 15 min. The mixture was cooled, then 0.8 N hydrochloric acid and 0.2 N sodium acetate buffer pH 7.4 were added, followed by paper chromatography and densitometry. The paper chromatogram was scanned at 254 nm.

Analytical Procedure—Total neutral sugars were determined by the method of Dubois et al.56 Neutral sugars were determined by gas-liquid chromatography using a gas chromatograph of a 10% Carbowax 20M/1.5% trimethylsilyl 10% Carbowax 20M column. The paper chromatogram was stained with 0.02% ninhydrin solution.

Paper Chromatography and Paper Electrophoresis—Toyo No. 51 paper was used for analytical purposes and Whatman No. 1 paper was used for preparative experiments. The paper chromatography was performed by eluting the paper in 95% ethanol and the paper electrophoresis was performed by the method of Yamaguchi et al.57

REFERENCES

Table III
Methylated sugars

<table>
<thead>
<tr>
<th>No.</th>
<th>Methylated sugar</th>
<th>Molar ratio a</th>
<th>Glycopeptide b</th>
<th>Oligosaccharide</th>
<th>Intact</th>
<th>Panose</th>
<th>α-Neomannose</th>
<th>α-Clue</th>
<th>α-alditol 1b</th>
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<tbody>
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<td>1</td>
<td>2,3,6-tetra-O-methyl-</td>
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<tr>
<td>2</td>
<td>3,6-di-O-methyl-</td>
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<tr>
<td>3</td>
<td>4-O-methyl-</td>
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<td>-</td>
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<tr>
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<tr>
<td>5</td>
<td>2,3,4-tri-O-methyl-</td>
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<td>0.6</td>
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<td>6</td>
<td>2,4,6-tri-O-methyl-</td>
<td>0.8</td>
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<td>0.2</td>
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<td>7</td>
<td>3,4,6-tri-O-methyl-</td>
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<td>8</td>
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<td>9</td>
<td>2,3,4-tetra-O-methyl-</td>
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<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The amount of each sugar was determined by gas-liquid chromatography (11) and numbers were calculated by taking the value of 2,3,4,6-tetra-O-methyl-1,2,3,4,6-penta-O-methyl-1-glucitol as 1.0 for neutral sugars except in the case of α-mannose-free glycopeptide, and 2-(α-methyl)-acetaenyl-2-deoxy-3,5-di-O-methyl-1-glucitol as an internal standard for major sugar. The ratio between the methylated α-mannose and the methylated neutral sugars was not determined.

b Intact and Panose are given under “glycopeptide” and “Oligosaccharide”.

Neutral carbohydrates were determined by gas-liquid chromatography, while glucosamine was not estimated. Values expressed in relation to fucose taken as 1.0.

Table II
Methylated sugars

<table>
<thead>
<tr>
<th>Methylated sugars</th>
<th>Molar ratio a</th>
<th>Oligosaccharide</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>1,2</td>
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<tr>
<td>Mannitols</td>
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<tr>
<td>2,3,4,6-tetra-O-methyl-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3,4,6-tri-O-methyl-</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>2,3,4-tri-O-methyl-</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>2,3,4,6-tetra-O-methyl-</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>2,3,4-tri-O-methyl-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The amount of each sugar was determined by gas-liquid chromatography (11) and numbers were calculated by taking the value of 2,3,4,6-tetra-O-methyl-1,2,3,4,6-penta-O-methyl-1-glucitol as 1.0 for neutral sugars except in the case of α-mannose-free glycopeptide, and 2-(α-methyl)-acetaenyl-2-deoxy-3,5-di-O-methyl-1-glucitol as an internal standard for major sugar. The ratio between the methylated α-mannose and the methylated neutral sugars was not determined.
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