

# 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  $\beta$ -aspartylglycosylamine linkage in glycopeptides with oligopeptide moieties.

The primary structures of the two oligosaccharide components,  $(\text{Man})_3(\text{Xyl})_1(\text{Fuc})_1(\text{GlcNAc})_2$  and  $(\text{Man})_2(\text{Xyl})_1(\text{Fuc})_1(\text{GlcNAc})_2$  were elucidated as  $\text{Man}\alpha 1 \rightarrow 6[\text{Xyl}\beta 1 \rightarrow 2]\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4[\text{Fuc}\alpha 1 \rightarrow 3]\text{GlcNAc}$  and  $\text{Man}\alpha 1 \rightarrow 6[\text{Xyl}\beta 1 \rightarrow 2]\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4[\text{Fuc}\alpha 1 \rightarrow 3]\text{GlcNAc}$ , respectively.

Stem bromelain is a thiol protease isolated from pineapple stem and contains one asparagine-linked oligosaccharide chain per molecule (1). The oligosaccharides<sup>1</sup> are composed of mannose, fucose, xylose, and *N*-acetylglucosamine (GlcNAc).<sup>2</sup> 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  $\beta$ -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. Methylation of the purified oligosaccharides established the structural relationship of the two components. Thus, the present studies demonstrate the usefulness of the almond glycopeptidase in the structural analysis of asparagine-linked oligosaccharides.

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<sup>1</sup> All sugars mentioned in this paper have the D-pyranose configuration except fucose which has the L configuration.

<sup>2</sup> The abbreviation used is: GlcNAc, *N*-acetylglucosamine.

## EXPERIMENTAL PROCEDURES<sup>3</sup>

### 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  $\text{NaB}^3\text{H}_4$ -reduced oligosaccharide alcohols were obtained as described under "Experimental Procedures." Fig. 3 shows that the [ $^3\text{H}$ ]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 0.8:1 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  $\mu\text{g}$  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 mannitols 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 mannitols (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 inner 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,

<sup>3</sup> The "Experimental Procedures," Figs. 1 to 3, Tables I to III, and references are presented in a miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-928, cite author(s), and include a check or money order for \$1.95 per set of photocopies.



and R' is Xyl $\beta$  or Fuca. The attachment of fucose at C-3 of asparagine-linked *N*-acetylglucosamine has already been suggested by Lee and Scocca (5). Xyl $\beta$ 1  $\rightarrow$  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  $\alpha$ 1  $\rightarrow$  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  $\alpha$ 1  $\rightarrow$  6. Second, the oligosaccharides contain a xylose residue linked to a  $\beta$ -mannosyl residue by  $\beta$ 1  $\rightarrow$  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.<sup>4</sup> Third, the oligosaccharides are shown to have the Man $\alpha$ 1  $\rightarrow$  6Man $\beta$ 1  $\rightarrow$  4GlcNAc $\beta$ 1  $\rightarrow$  4GlcNAc  $\rightarrow$  Asn sequence, which is common in a number of asparagine-linked oligosaccharides, while the oligosaccharides lacked another mannosyl residue linked to the  $\beta$ -mannosyl residue by  $\alpha$ 1  $\rightarrow$  3 linkage, which is also a common residue in asparagine-linked oligosaccharides (12, 22-28).

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#### REFERENCES

References are found on pp. 10718 and 10719.

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<sup>4</sup> M. Sasaki and N. Takahashi, unpublished experiments.

## SUPPLEMENTARY MATERIAL

to

Complete Structure of the Carbohydrate Moiety of Stem Bromelain: An Application of the Almond Glycopeptidase for Structural Studies of Glycopeptides

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## EXPERIMENTAL PROCEDURE

**Methylated Sugars**-----Methyl  $\alpha$ -fucoside and methyl  $\alpha$ -xyloside were synthesized from fucose and xylose respectively, according to James and Smith (29), and Percival and Zobrist (30). Methyl  $\alpha$ -mannoside was purchased from Nakarai Chemical Ltd., Tokyo. 2,3,4-Tri-O-methylfucose, 2,3,4-tri-O-methylxylose and 2,3,4,6-tetra-O-methylmannose were prepared by methylation of methyl  $\alpha$ -fucoside, methyl  $\alpha$ -xyloside and methyl  $\alpha$ -mannoside respectively, by the Hakomori's procedure (31), followed by hydrolysis with 1 N HCl at 100°C for 2 h. Benzyl N-acetyl- $\alpha$ -glucosaminide was synthesized according to Gross and Jeanloz (32). 2-(N-Methyl)-acetamido-2-deoxy-3,4,6-tri-O-methylglucose was prepared by methylation of benzyl N-acetyl- $\alpha$ -glucosaminide, followed by catalytic hydrogenation (33). Di-N-acetylchitobiose was prepared by partial hydrolysis of chitin with 11 N HCl at 40°C for 2.5 h and purification with a charcoal-Celite column (34). A mixture of 2-(N-methyl)-acetamido-2-deoxy-3,4,6-tri-O-methylglucose and 2-(N-methyl)-acetamido-2-deoxy-1,3,5,6-tetra-O-methylglucose was prepared from di-N-acetylchitobiose by reduction with NaBH<sub>4</sub>, permethylation and hydrolysis with 2 N HCl at 100°C for 16 h. 2-(N-Methyl)-amino-2-deoxy-3-O-methylglucose, 2-(N-methyl)-amino-2-deoxy-6-O-methylglucose and 2-(N-methyl)-amino-2-deoxy-3,6-di-O-methylglucose were synthesized from glucosamine hydrochloride according to Gorin and Finlayson (33). These partially methylated sugars were converted to their alditol acetates by reduction with NaBH<sub>4</sub> and acetylation with a mixture of pyridine and acetic anhydride (1:1) at 100°C for 3 h.

**Glycopeptidase from Almond Emulsin**-----The almond glycopeptidase hydrolyzing B-aspartylglucosylamine linkages in glycopeptides (7) was prepared from almond emulsin (B-glucosidase) as described previously (8).

**$\alpha$ -Mannosidase from *Turbo cornutus***----- $\alpha$ -Mannosidase from the liver of *Turbo cornutus* was prepared by the method of Muramatsu and Egami (35). Activity of the glycosidase was determined with p-nitrophenyl  $\alpha$ -mannoside as described previously (35).

**Glycopeptides from Stem Bromelain**-----The glycopeptides were prepared from purified stem bromelain by proteolytic digestion with pronase as previously described (6). The material obtained was shown to be a mixture of four kinds of glycopeptides that differed from one another in the peptide part (6) (Fig. 1). The unfractionated mixture was used in the present experiment in which the major interest was directed to its carbohydrate moiety. This is justifiable in view of the fact that the four component glycopeptides were all derived from a single portion of the parent glycoprotein and the overall amino acid composition of the mixture was known to be nearly constant from one preparation to another. The amount of the glycopeptide used was calculated from its content of glutamic acid as determined by amino acid analysis. The oligosaccharide in the glycopeptide was composed of the following sugars (numbers in parentheses expressed in relation to glutamic acid taken as 1.0): mannose (2.48), fucose (0.95), xylose (1.07), and N-acetylglucosamine (2.16).

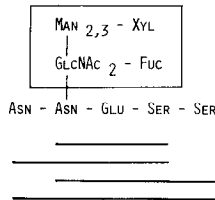


Fig. 1 Amino acid sequences of the glycopeptides from stem bromelain by proteolytic digestion with pronase. The horizontal bars indicate four kinds of glycopeptides.

**Glycopeptidase Digestion of Stem Bromelain Glycopeptides**-----Stem bromelain glycopeptide (22.5 mg) was digested with 99 millunits glycopeptidase from almond emulsin (7,8) in 3 ml of 0.2 M citrate-phosphate buffer, pH 5.2, at 37°C for 5 h. The reaction mixture was applied to two sheets of Whatman 3 MM filter paper (60 x 60 cm) and paper electrophoresis was carried out at pH 6.5 at a potential of 50 V per cm for 1 h. The released oligosaccharide migrated to the neutral position on electrophoretogram (7) (Fig. 2). The oligosaccharide (designated as "Z" in Fig. 2), located with peroxidase benzidine (36), was extracted twice with water from the paper. The amount of oligosaccharide was determined by orcinol-H<sub>2</sub>SO<sub>4</sub> reaction (37). The yield was 10 mg.

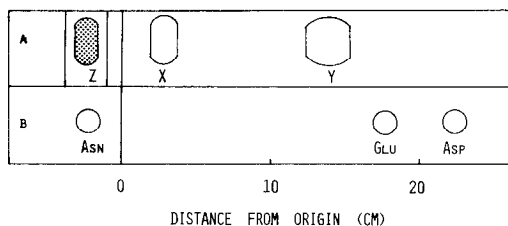


Fig. 2 Electrophoretogram of the glycopeptidase hydrolysis of stem bromelain glycopeptides. Samples were separated by paper electrophoresis at pH 6.5, at 50 V/cm for 1 h. Materials were stained with the following reagents: dotted, peroxidase-benzidine (32); white, ninhydrin. A, Stem bromelain glycopeptides incubated with glycopeptidase under the standard conditions (see the text). B, Reference amino acid. O, Start line on electrophoresis. X, Original glycopeptides. Y, Peptide product. Z, Oligosaccharides.

**NaB<sub>4</sub>H<sub>4</sub> Reduced Oligosaccharides**-----The NaB<sub>4</sub>H<sub>4</sub>-reduced oligosaccharides were prepared and purified by the method of Takasaki and Kobata (38). Thus, 10  $\mu$ mol of peroxide-free oligosaccharides were reduced with 3  $\mu$ mol of sodium borohydride (422 mCi/mmol, The Radiochemical Centre, England) and 27  $\mu$ mol of cold NaBH<sub>4</sub> in 2 ml of 0.05 M borate buffer, pH 8.0 at room temperature for 4 h. The reaction mixture was passed through a small column (0.5 x 3 cm) of Amberlite AG-50 (H<sup>+</sup>) and the effluent was evaporated to dryness. Borate was removed by evaporation with 0.5 ml of methanol (five times). The residue was dissolved in small amount of water, applied on Toyo No. 51 paper, and was subjected to paper chromatography for 4 days at 20-21°C (Fig. 3). [<sup>3</sup>H]oligosaccharide alcohols were recovered from paper by elution with water.

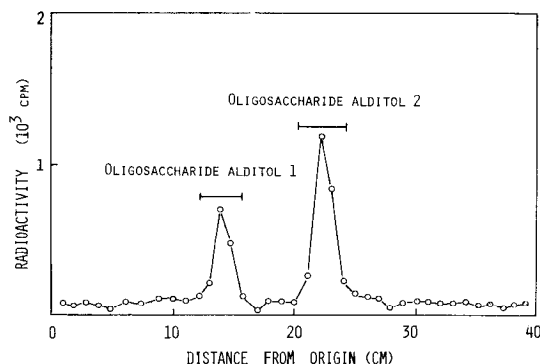


Fig. 3 Paper chromatogram of the radioactive oligosaccharide alditols obtained from bromelain glycopeptides by the glycopeptidase digestion, followed by NaB<sub>4</sub>H<sub>4</sub> reduction.

**Preparation of Fucose-free Glycopeptide**-----Fucose-free glycopeptide was prepared as described previously (3). To 15 mg of the glycopeptides in 250  $\mu$ l of water was added 2.5 ml of 13 N trifluoroacetic acid and the mixture was allowed to stand at 20°C for 3 days. The glycopeptide obtained upon purification by paper electrophoresis was virtually devoid of fucose. (cf. Table III).

**Preparation of  $\alpha$ -Mannose-free Glycopeptide**-----Twelve mg of the glycopeptide was incubated with 13 units of the  $\alpha$ -mannosidase from *Turbo cornutus* in 0.5 ml of 0.1 M sodium citrate-phosphate buffer pH 4.0. The incubation was continued at 37°C for 5 days with a few drops of toluene. At 2-day intervals 10 units each of the enzyme were added. The glycopeptide obtained by gel filtration on Sephadex G-25 was nearly devoid of mannose (cf. Table III).

**Analytical Procedures**-----Total neutral sugars were determined with orcinol-H<sub>2</sub>SO<sub>4</sub> method (37). Glucosamine was determined by the Elson-Morgan reaction (41) and by means of the amino acid analyzer (3). Other sugars were determined by either specific colorimetry as described previously (3) or with gas-liquid chromatography (39). Gas-liquid chromatography was performed with a Shimadzu gas chromatograph, Model GC-4BFT, equipped with a hydrogen flame ionization detector, by use of glass columns (2 m x 4 mm) packed with 2 % GE XF-1105 on Gas-Chrom P, 60 to 80 mesh, for Column A and 2 % OV-17 on Gas-Chrom Q, 80 to 100 mesh, for Column B. Neutral sugars were analyzed as their corresponding alditol trifluoroacetates on Column A and partially methylated sugars as their alditol acetates on Column B. Amino acid analysis of peptides was carried out with a Hitachi K1A-3B amino acid analyzer after hydrolysis of peptides in 6 N HCl at 100°C for 20 h. Radioactivity on paper strips were determined with a Packard Tri-Carb liquid scintillation spectrometer by use of a toluene system scintillation fluid.

**Paper Chromatography and Paper Electrophoresis**-----Toyo No. 51 paper was used for analytical purposes and Whatman 3 MM was used for preparative experiments. Descending paper chromatography was performed with the following solvent system, ethylacetate/pyridine/water (12:5:4). Paper electrophoresis was carried out at a potential of 50 V per cm for 1 h with Toyo HPE-V apparatus. The buffer was pyridine/acetic acid/water (5:0.2:95), pH 6.5.

**Methylation Study**-----Bromelain glycopeptides or [<sup>3</sup>H]oligosaccharide alcohols (2-5 mg) were dissolved in 0.5 ml of dimethylsulfoxide and methylated by the method of Hakomori (31). The permethylated product was applied on small column of Sephadex LH-20 equilibrated with chloroform/methanol (2:1) and eluted with the same solution instead of the reported chloroform (40). The fractions containing sugars were combined, and evaporated to dryness. The residue was dissolved in 5 ml of chloroform and washed twice with distilled water. The chloroform extract was evaporated to dryness and was hydrolyzed in a sealed tube with 2 N HCl at 100°C for 15 h for amino sugars or 1 N HCl at 100°C for 3 h for neutral sugars. After removal of HCl by repeated co-distillation with methanol, the residue was reduced to alditol and acetylated as described previously (40). The alditols were acetylated with 1 ml of pyridine/acetic anhydride (1:1) at 100°C for 3 h instead of the reported acetic anhydride at 100°C for 2 h. Separations and quantitative determinations of the alditol acetate derivatives of partially methylated sugars were performed with gas-liquid chromatography (21) on Column B. The column temperature was programmed from 130°C to 240°C at a rate of 3°C/min, the flow rate of nitrogen gas was 70 ml/min. The injection temperature was 260°C, and the detector temperature was 280°C. Identification of each peak was performed on a Hitachi M-52 gas chromatograph-mass spectrometer with a glass column (1.5 m x 3 mm) of 2 % OV-17 on Gas-Chrom Q, 80 to 100 mesh. Conditions for the mass spectrometry were: ionization potential, 20 eV; ion source temperature, 170°C; and separator temperature 160°C.

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Table I

Neutral carbohydrate composition of Oligosaccharide alditols 1 and 2

|                           | Mannose | Fucose | Xylose |
|---------------------------|---------|--------|--------|
| Oligosaccharide alditol 1 | 2.0     | 1.0    | 0.96   |
| Oligosaccharide alditol 2 | 1.9     | 1.0    | 0.98   |

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

Table II

Methylation analysis of Oligosaccharide alditols 1 and 2

| Methylated sugars                       | Molar ratio <sup>a</sup>  |                           |
|-----------------------------------------|---------------------------|---------------------------|
|                                         | Oligosaccharide alditol 1 | Oligosaccharide alditol 2 |
| Mannitols                               |                           |                           |
| 2,3,4,6-tetra-O-methyl-                 | 1.0                       | 1.0                       |
| 2,3,4-tri-O-methyl-                     | 0.9                       | -                         |
| 3,4-di-O-methyl-                        | 0.8                       | 0.9                       |
| Xylitol                                 |                           |                           |
| 2,3,4-tri-O-methyl-                     | 0.6                       | 0.7                       |
| Fucitol                                 |                           |                           |
| 2,3,4-tri-O-methyl-                     | 0.7                       | 0.8                       |
| 2-(N-Methyl)-acetamido-2-deoxyglucitols |                           |                           |
| 1,5,6-tri-O-methyl-                     | 0.9                       | 0.9                       |
| 3,6-di-O-methyl-                        | 1.0                       | 1.0                       |

<sup>a</sup>The amount of each sugar was determined by gas-liquid chromatography (21) and numbers were calculated by making the value of 2,3,4,6-tetra-O-methylmannitol as 1.0 for neutral sugars, and 2-(N-methyl)-acetamido-2-deoxy-3,6-di-O-methylglucitol as 1.0 for amino sugars. The ratio between the methylated amino sugars and the methylated neutral sugars was not determined.

Table III

Methylation analysis of glycopeptides and oligosaccharide from stem bromelain

| No. | Methylated sugar                        | Molar ratio <sup>a</sup>  |             |                |                                        |
|-----|-----------------------------------------|---------------------------|-------------|----------------|----------------------------------------|
|     |                                         | glycopeptide <sup>b</sup> |             |                | Oligosaccharide alditol 1 <sup>b</sup> |
|     |                                         | Intact                    | Fucose-free | α-Mannose-free |                                        |
|     | 2-(N-methyl)-acetamido-2-deoxyglucitols |                           |             |                |                                        |
| 1)  | 1,5,6-tri-O-methyl-                     | -                         | -           | -              | 1.2                                    |
| 2)  | 3,6-di-O-methyl-                        | 1.0                       | 2.0         | 1.0            | 1.0                                    |
| 3)  | 6-mono-O-methyl-                        | 0.9                       | -           | 0.9            | -                                      |
|     | Mannitols                               |                           |             |                |                                        |
| 4)  | 2,3,4,6-tetra-O-methyl-                 | 1.0                       | 1.0         | 0.2            | 1.0                                    |
| 5)  | 2,3,4-tri-O-methyl-                     | 0.6                       | 0.6         | 0.1            | 0.9                                    |
| 6)  | 3,4-di-O-methyl-                        | 0.8                       | 0.9         | 0.2            | 0.0                                    |
| 7)  | 3,4,6-tri-O-methyl-                     | 0.1                       | 0.1         | 0.7            | 0.1                                    |
|     | Xylitol                                 |                           |             |                |                                        |
| 8)  | 2,3,4-tri-O-methyl-                     | 0.5                       | 0.6         | 0.5            | 0.5                                    |
|     | Fucitol                                 |                           |             |                |                                        |
| 9)  | 2,3,4-tri-O-methyl-                     | 0.8                       | -           | 0.8            | 0.7                                    |

<sup>a</sup> The amount of each sugar was determined by gas-liquid chromatography (21) and number were calculated by taking the value of 2,3,4,6-tetra-O-methylmannitol as 1.0 for neutral sugars except in the case of α-mannose-free glycopeptide which was calculated by taking the value of 2,3,4-tri-O-methylfucitol as 0.8, and 2-(N-methyl)-acetamido-2-deoxy-3,6-di-O-methylglucitol as integers for amino sugar. The ratio between the methylated amino sugars and the methylated neutral sugars was not determined.

<sup>b</sup> Sources are given under "EXPERIMENTAL PROCEDURES".