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The Composition and Structure of Carbohydrate Moiety of Stem Bromelain*

Yoko Yasuda, Noriko Takahashi, and Takashi Murachi

ABSTRACT: The composition and structure of the carbohydrate prosthetic group of stem bromelain have been investigated by using the glycopeptide preparation obtained from the Pronase digest of the parent protein. The composition of the carbohydrate unit, as expressed in nearest integers, is 3 moles of mannose, 1 of fucose, 1 of xylose, and 2 of *N*-acetylglucosamine per mole; none of the other sugars are present. There is a possibility of partial deletion or addition of some terminal residues since the actual data of analysis do not give strictly integral numbers, particularly for mannose and fucose. Two of the three mannoses, one fucose, and one xylose are specifically liberated by the action of α-D-mannosidase from the liver of *Turbo cornutus* and pig kidney, α-L-fucosidase from *T. cornutus*, and β-D-xylosidase from the liver of *Charonia lampas*, respectively. β-D-*N*-Acetylglucosaminidase from *T. cornutus* releases one *N*-acetylglucosamine from the neutral sugar-free glycopeptide. Partial acid hydrolysis of the glycopeptide yields, in addition to free monosaccharides, mannobiose, mannotriose, and mannosyl-*N*-acetylglucosamine. The glycopeptide consumes approximately 8 moles of periodate to produce more than 2 moles of formic acid, and, upon reduction and hydrolysis, close to 2 moles of glycerol, 1 mole of propylene glycol, and none of erythritol. In sequential Smith degradation, neutral sugar residues are first oxidized and removed, and two *N*-acetylglucosamine residues are then oxidized in stepwise fashion. From these and other pieces of evidence, the following structure is proposed for the carbohydrate moiety of stem bromelain:

α-D-Man(1→2)-α-D-Man-(1→2 or 6)-[α-L-Fuc-(1→6 or 2)]-α-D-Man-(β-D-Xyl)-β-D-GlcNAc-(1→3 or 4)-β-D-GlcNAc-(1→β-NH_2-*N* of Asn)-peptide

Stem bromelain is a proteolytic enzyme found in tissues of pineapple stem. It is a thiol protease and in this respect it resembles papain and ficin. However, unlike papain and ficin, stem bromelain was found to be a glycoprotein (Murachi *et al.*, 1964) which contains carbohydrate prosthetic group as a distinct single moiety (Murachi *et al.*, 1967). From the digest of stem bromelain by Pronase, glycopeptides were isolated and analyzed for their amino acid sequences around the carbohydrate moiety. The one with the longest sequence was shown to be Asn-Asn(sugar)-Glu-Ser-Ser (Takahashi *et al.*, 1969). The sugar moiety contained mannose, xylose, fucose, and *N*-acetylglucosamine.

Using the glycopeptide obtained from the Pronase digest, we have studied the composition and structure of the carbohydrate moiety. Various methods were employed for quantitative determination of component sugars in order to minimize fluctuation of data that would have arisen from different degrees of destruction of sugars depending upon the methods of assay. The sequence of the heterooligosaccharide moiety was studied by using several different kinds of glycosidases with different specificities, partial acid hydrolysis, and periodate oxidation. The latter two methods were rather conventional, while most of the specific glycosidases used in the present experiments were the enzymes only recently

* From the Department of Biochemistry, Nagoya City University School of Medicine, Nagoya, Japan. *Received September 3, 1969.* This work was supported in part by a grant from the Ministry of Education, Japan, and by a U. S. Public Health Service research grant (GM08714) from the National Institute of General Medical Sciences.

made available (Muramatsu, 1968; Fukuda *et al.*, 1969; Okumura and Yamashina, 1970). These enzymes were found to be extremely useful for determination of the nonreducing end groups and the nature of glycoside linkages.

The present paper describes details of these experiments and the results obtained by different methods of approach which have led us to propose the most probable structure of the carbohydrate moiety of the glycopeptide, and, hence, of stem bromelain.

Materials and Methods

Glycopeptide of Stem Bromelain. The glycopeptide was prepared from stem bromelain[1] by proteolytic digestion with Pronase as previously described (Murachi *et al.*, 1967). The material obtained appeared at first to be a homogeneous octaglycopeptide, but was later shown to be a mixture of four kinds of glycopeptides that differed from one another only in the peptide part (Takahashi *et al.*, 1969). The unfractionated mixture, designated as the "original glycopeptide" in the latter communication, was used as such in the present experiment where major interests were directed to its carbohydrate moiety. This has to be justified 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 almost constant from one preparation to the other (Takahashi *et al.*, 1969). The amount of the glycopeptide used was calculated from its content of glutamic acid as determined by amino acid analysis. The average molecular weight was assumed to be 1.5×10^3 (Takahashi *et al.*, 1969).

Glycosidases. α-D-Mannosidase, β-D-mannosidase, α-L-fucosidase, and β-D-*N*-acetylglucosaminidase prepared from the liver of *Turbo cornutus* (Muramatsu and Egami, 1967; Muramatsu, 1968) and β-D-xylosidase from the liver of *Charonia lampas* (Fukuda *et al.*, 1969) were gifts of Dr. Muramatsu and Dr. Fukuda. α-D-Mannosidase of pig kidney (Okumura and Yamashina, 1970) was kindly provided by Dr. Yamashina.

Quantitative Analysis. The total neutral sugar was determined by the orcinol–H_2SO_4 method (Winzler, 1955). When mannose was used as the standard, molar color values of 100% and 80% were obtained with xylose and fucose, respectively (Murachi *et al.*, 1967). For determination of the component sugars, the glycopeptide was hydrolyzed in 1 N HCl at 100° for 3 hr and the hydrolysate was subjected to paper chromatography. Individual sugars located on the paper were eluted and determined by specific colorimetric methods (Ashwell, 1957): for mannose, cysteine–H_2SO_4 reaction for hexoses; for xylose, orcinol–HCl reaction; for fucose, cysteine–H_2SO_4 reaction for methylpentoses. The phenol–H_2SO_4 method of Dubois *et al.* (1956) was also employed for determining isolated component sugars. The ferricyanide method of Park–Johnson (Ashwell, 1957) was used for reducing sugars. Glucosamine was determined by the Morgan–Elson reaction (Reissig *et al.*, 1955) and by means of the amino acid analyzer. Amino acids were analyzed, with isoleucine as an internal standard, on a Technicon Auto Analyzer by the method of Hamilton (1963) or on a Hitachi Model KLA-3B amino acid analyzer by the method of Spackman *et al.* (1953). Recovery of glucosamine after the hydrolysis in 6 N HCl at 100° for 20 hr was assumed to be 70% (Murachi *et al.*, 1967). *N*-Acetyl groups were determined by the method of Ludowieg and Dorfman (1960).

Paper Chromatography and Paper Electrophoresis. Toyo No. 51 filter paper was used. For descending paper chromatography two kinds of solvent systems were used: solvent I, acetic acid–ethyl acetate–water (1:3:3, upper phase), and solvent II, 1-butanol–ethanol–water (4:1:5, upper phase). The paper was developed for 16–72 hr depending upon the nature of the materials to be separated. Buffer solutions used for the paper electrophoresis at 2000–3000 V for 1–2 hr were: pyridine–acetic acid–water (5:0.2:95) at pH 6.5, and pyridine–acetic acid–water (1:10:89) at pH 3.5. The reducing sugars and most of their oxidation products on paper were detected with alkaline silver nitrate reagent. The ninhydrin reagent (0.2% in water-saturated 1-butanol) was used to detect amino acids and glucosamine. *t*-Butyl hypochlorite reagent (Greig and Leaback, 1960; Mazur *et al.*, 1962) was sprayed for peptide detection.

Gas–Liquid Partition Chromatography. Gas chromatography of neutral sugars was kindly performed by Dr. R. J. Winzler, State University of New York, Buffalo, and by Dr. Z. Tamura, University of Tokyo, Tokyo. The alditol acetate method (Lehnhardt and Winzler, 1968) and the trifluoroacetate method (Tamura *et al.*, 1968), respectively, were employed. The analysis by Dr. Tamura included also the determination of glucosamine.

Periodate Oxidation. To 1.5 mg of the glycopeptide were added 0.7 ml of 0.3 M sodium acetate buffer, pH 4.5, and 0.1 ml of 0.2 M sodium metaperiodate. The reaction was carried out at 5° in the dark. The consumption of the oxidant was estimated on 60-μl aliquots at appropriate time intervals by the microtitrimetric procedure based on the method of Fleury and Lange (1933). For the determination of *formic acid* formed, buffer solution was omitted. To 0.8 ml of the reaction mixture was added 0.1 ml of 50% ethylene glycol to terminate the oxidation, and after 30 min at 25° the mixture was titrated with 0.01 N NaOH in a Radiometer Model SBR2/SBU1/TTT1 pH-stat. *Glycerol, erythritol, and propylene glycol,* which could be found in the hydrolysate of the periodate-oxidized and borohydride-reduced glycopeptide (see below), were separated by paper chromatography with solvent II, and the eluted alcohols were further oxidized by periodate. The formaldehyde thus produced was determined by using chromotropic acid (Burton, 1957), and from the results obtained the amount of each alcohol was calculated.

Sequential Periodate Oxidation (Smith Degradation). After the oxidation with periodate, 0.8 ml of 0.3 M sodium borate buffer (pH 8.0) was added to 0.8 ml of the reaction mixture. Sodium borohydride (10 mg) was added and the reduction was continued at 25° for 7 hr. The solution was then treated with Amberlite IR-120 (H^+ form) and evaporated *in vacuo* with 5 ml of methanol to remove boric acid as volatile trimethyl borate. The products that remained were hydrolyzed in 0.1 N HCl at 25° for 16 hr. The hydrolysate was chromatographed on paper as described above. The glycopeptide material that remained at the origin of the chromatogram was eluted from the paper with water and the eluate was

[1] We are indebted to Dr. Ralph M. Heinicke, Dole Co., Honolulu, Hawaii, for a generous supply of the crude bromelain (lot UX11-1).

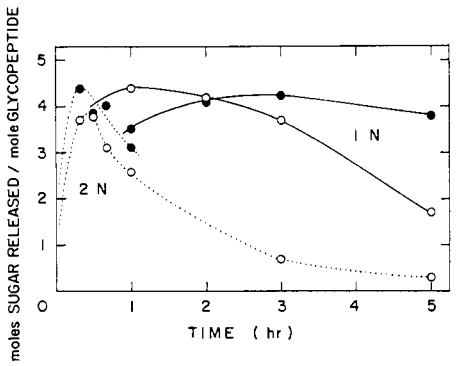

FIGURE 1: Release of neutral sugars from the glycopeptide of stem bromelain by acid hydrolysis at 100° in 1 N HCl (solid line) and 2 N HCl (dotted line). The liberated sugars were separated by paper electrophoresis at pH 3.5 and, after elution from the paper, determined by the orcinol-H_2SO_4 method (●) and Park-Johnson method (○).

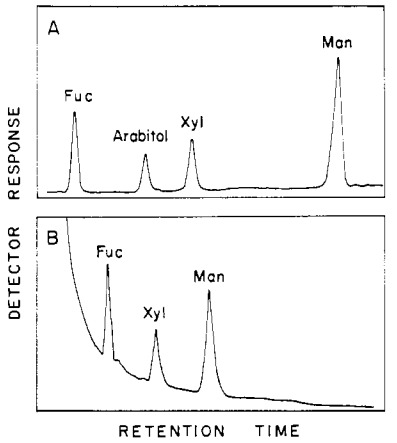

FIGURE 2: Gas-liquid partition chromatograms for neutral sugars of the glycopeptide of stem bromelain. The glycopeptide (1 mg) was analyzed by (A) alditol acetate method of Lehnhardt and Winzler (1968) and (B) trifluoroacetate method by Tamura *et al.* (1968). Arabitol was used as the internal standard in (A). In A, 14.4 min from fucose to mannose; in B, 5.5 min from fucose to mannose.

concentrated to dryness, thus completing the first cycle of Smith degradation (Goldstein *et al.*, 1965). The second and third cycles were started by periodate oxidation of the product in the preceding step of the cycle. With each product, *i.e.*, partially degraded glycopeptide, analyses were made for carbohydrates and amino acids in the same ways as those described for the original glycopeptide.

Preparation of Fucose-free Glycopeptide. Based on our observation that fucose residue was most labile in acids, we could choose ideal conditions under which only fucose was removed from the glycopeptide while all other sugar residues remained intact. Thus, to 1.5 mg of the glycopeptide in 10 μl of water was added 1 ml of trifluoroacetic acid and the mixture was allowed to stand at 20° for 20 hr. The glycopeptide obtained upon purification by paper chromatography with solvent I was practically devoid of fucose, and used as a substrate for α-D-mannosidase.

Hydrolysis of the Glycopeptide by Glycosidases from Turbo cornutus and Charonia lampas. The solutions of various enzymes were made so that each contained 3 enzyme units/ml. The substrate solution was prepared by dissolving 0.75 mg of the glycopeptide in 0.1 ml of 0.1 M sodium citrate–phosphate buffer at pH 4.0 containing 1 M NaCl. The reaction was started by mixing 0.2 ml of the enzyme solution with 0.1 ml of the substrate solution. The incubation was continued at 37° for several days with a few drops of toluene. Aliquots were withdrawn from the incubation mixture at appropriate time intervals and passed successively through columns of 2 ml each of Dowex-1 (carbonate form) and Amberlite IR-120 (H^+ form). The effluent was dried over phosphorus pentoxide and the residue was chromatographed on paper using solvent I. The monosaccharide thus separated was identified, and the quantitative analyses for neutral sugars were performed both of the liberated monosaccharide and of the glycopeptide that remained at the origin on the chromatogram. For the colorimetric determination of *N*-acetylglucosamine liberated by the enzyme, aliquots of the incubation mixture were used without further treatment.

Hydrolysis of the Glycopeptide by α-D-Mannosidase from Pig Kidney. The glycopeptide (4.5 mg) was incubated with 2 units of the enzyme dissolved in 1 ml of 0.1 M sodium citrate–phosphate buffer at pH 4.6 containing 0.005 M zinc acetate. The incubation was continued at 37° for 16 days with a few drops of toluene. At 4-day intervals 0.5 unit each of the enzyme was added. Further analyses were performed in the same way as that described above.

Results

Contents of Neutral Sugars of the Glycopeptide. It was previously reported that the glycopeptide contained 4.8 moles of neutral sugars/mole as determined by the orcinol-H_2SO_4 method and expressed in terms of mannose equivalent (Murachi *et al.*, 1967). More recent and repeated analyses gave an average value of 4.58 moles/mole. This was further confirmed by measuring the amounts of neutral sugars liberated by hydrolysis with HCl. Thus, 0.15 mg of the glycopeptide was hydrolyzed in 0.5 ml of 1 N and 2 N HCl at 100° for several hours. At appropriate time intervals the samples were subjected to paper electrophoresis at pH 3.5. The neutral sugars that slightly migrated were quantitatively eluted from the paper and the eluate was assayed for total sugar content (Winzler, 1955) and reducing power (Ashwell, 1957). The results obtained are shown in Figure 1. The maximal value for the liberation of total sugar is 4.4 moles, as mannose equivalent, per mole of glycopeptide. An almost identical value is attained also in terms of reducing power that appears during hydrolysis.

Carbohydrate Composition of the Glycopeptide. The previous report from this laboratory described that the glycopeptide contained mannose (3), xylose (1), and fucose (1), where the figures in parentheses were nearest integers that fit the data of colorimetric analyses (Murachi *et al.*, 1967). Gas chromatographic analysis was performed to reexamine the previous results. In Figure 2 are shown tracings of the chromatograms for component sugars. Both chromatograms clearly indicate the absence of neutral sugars other than mannose, fucose, and xylose. Numerical data for these three kinds of sugars are shown in Table I. The results of two independent analyses on the same glycopeptide satisfactorily agree with

TABLE I: Carbohydrate Composition of Glycopeptide of Stem Bromelain.

Method	Hydrolysis	moles/mole of Glycopeptide			
		Man	Fuc	Xyl	GlcN
Alditol acetate[a]	With Dowex 50 × 2 in 0.01 N HCl	2.48[b]	1.23[b]	1.00[b]	
Alditol trifluoroacetate[c]	In 2.5 N Trifluoroacetic acid at 100°				
	for 4 hr	2.02	1.00	1.09	
	8 hr	2.63	1.44	1.08	2.16
Specific colorimetry	In 1 N HCl at 100° for 3 hr	2.25	0.95	1.07	
Morgan–Elson	In 2 N HCl at 100° for 8 hr				1.96
	12 hr				1.99
	16 hr				1.87
	20 hr				1.81

[a] Lehnhardt and Winzler, 1968. [b] Molar ratio. [c] Tamura *et al.*, 1968.

each other and also with the results of colorimetric analyses. Xylose is always found to be close to 1 mole/mole of the glycopeptide, while the content of mannose is, in all cases, definitely less than 3 moles/mole. The fractional number for mannose residues could be explained by assuming microheterogeneity of the glycopeptide preparation with respect to one mannose residue. The latter possibility is likely, because, as will be described below, a mannosyl–mannosyl sequence is present in the branched oligosaccharide moiety and the terminal mannose residue could be a subject of deletion. The fact that the release of mannose from the glycopeptide by the action of specific mannosidases never exceeds 1.7 moles/mole (see Table II, below) is again consistent with the postulation of a partial deletion of the terminal mannose residue.

The content of fucose as determined by gas chromatography is higher than 1 mole/mole, while that by colorimetry is very close to 1 (Table I). By acid hydrolysis, again very close to 1 mole of fucose is liberated from 1 mole of the glycopeptide (see Figure 4, below). It is thus uncertain yet whether the number of fucose residue is indeed integral or fractional.

Table I shows that the glycopeptide contains 2 moles of glucosamine/mole. These two glucosamine residues are present in *N*-acetylated state, since *N*-acetyl groups of the glycopeptide, as determined by the method of Ludowieg and Dorfman (1960), were 2.0 moles/mole. An earlier report from this laboratory described that stem bromelain contained 4 glucosamine residues/mole of protein (Murachi, 1964). That seems to be inconsistent with the present data, if one assumes the occurrence of a single site of attachment of the sugar moiety to the protein molecule as was suggested previously by Murachi *et al.* (1967). To solve the problem, reexamination of glucosamine content of stem bromelain was carried out. Stem bromelain was hydrolyzed in 4 N HCl at 100° for 20 hr and the samples at 3- to 5-hr intervals were assayed for glucosamine by the method of Morgan–Elson. Extrapolation of the results obtained to 0 hr of hydrolysis yielded a value of 1.88 moles/mole, which implies the presence of 2, instead of 4, glucosamine residues/mole of the parent protein. The content of glucosamine of stem bromelain was also determined by gas–liquid chromatography. The alditol trifluoroacetate method, which involved hydrolysis of the protein in 2.5 N trifluoroacetic acid at 100° for 24 hr, gave a value of 1.85 moles of glucosamine/mole of protein.[2]

Enzymatic Hydrolysis of the Glycopeptide and Its Derivatives. It was expected that specific exo glycosidases could release monosaccharides one after another from the nonreducing end or ends of the oligosaccharide moiety and the sugars set free should reflect the specificity of the enzyme used. This was actually proved to be the case, when the glycopeptide was incubated with a variety of glycosidases from different origins as are shown in Table II. For example, β-D-xylosidase from the liver of *Charonia lampas* released 0.9 mole of xylose from 1 mole of glycopeptide without liberation of any other sugars and amino acids, indicating the occurrence of a β-D-xylosyl residue at one nonreducing end of the molecule.[3] Likewise, a value of 0.6 mole of fucose released after 181-hr incubation with α-L-fucosidase suggested that another nonreducing end had been occupied by an α-L-fucosyl residue. Still another nonreducing end of α-D-mannosyl residue should have been present, because α-D-mannosidase, not β-D-mannosidase, could release 1.6–1.7 moles of mannose from 1 mole of glycopeptide. The latter finding was consistent with the isolation of mannobiose and mannotriose by partial acid hydrolysis of the glycopeptide (see below). The addition of α-L-fucosidase at 133-hr incubation with α-D-mannosidase gave rise to release of 0.6 mole of fucose together with an increment of free mannose of 0.5 mole/mole of substrate. This could be explained by assuming that fucose was attached to the innermost mannosyl residue which became accessible only after removal of two outer mannosyl residues. The validity of this assumption was more obvious by comparing the results obtained with pig kidney α-D-mannosidase. When the glycopeptide, as such, was used as the substrate, the release of mannose was 1.6 moles/mole of substrate after 186 hr with no more release even upon longer incubation, while up to 2.4 moles of mannose could be released after 254 hr from the glycopeptide specimen that had previously been freed from fucose. A similar situation was found to

[2] Z. Tamura, personal communication.

[3] A preliminary report has been published (Fukuda *et al.*, 1968).

TABLE II: Enzymatic Hydrolysis of the Glycopeptide of Stem Bromelain with Glycosidases.

Glycosidase[a]	Moles of Monosaccharide Liberated per Mole of Glycopeptide[b]
β-D-Xylosidase (CL)	Xyl 0.9 (65)
α-L-Fucosidase (TC)	Fuc 0.6 (181)
α-D-Mannosidase (TC)	Man 1.6 (65), 1.7 (133)
α-D-Mannosidase (TC) with 133-hr addition of α-L-fucosidase (TC)	Man 2.2 (181); Fuc 0.6 (181)
α-D-Mannosidase (PK)	Man 1.4 (95), 1.6 (137), 1.6 (186), 1.4 (261)
α-D-Mannosidase (PK) on fucose-free glycopeptide	Man 0.8 (68), 2.1 (185), 2.4 (254), 2.2 (288)
β-D-Mannosidase (TC)	None (70)
β-D-*N*-Acetylglucosaminidase (TC)	None (41)
β-D-*N*-Acetylglucosaminidase (TC) on periodate-oxidized glycopeptide[c]	GlcNAc 1.0 (20), 1.1 (44), 1.1 (68)

[a] Sources of enzymes in parentheses are: CL, *Charonia lampas;* TC, *Turbo cornutus;* PK, pig kidney. [b] Figures in parentheses are periods of incubation in hours. [c] The substrate was $(GlcNAc)_2$-peptide obtained from the original glycopeptide as the product after the 1st step of Smith degradation (see below, Table V). *N*-Acetylglucosamine was determined by the Morgan–Elson reaction.

exist concerning the action of β-D-*N*-acetylglucosaminidase on the glycopeptide. No release of the sugar was encountered by incubating the substrate as such, but approximately 1 mole of *N*-acetylglucosamine was liberated when all the neutral sugar residues had previously been removed by the first step procedure of Smith degradation with periodate. The results indicated that two *N*-acetylglucosamine residues were located in positions closest to the peptide chain with a β-glucosaminide bond between these two residues.

Periodate Consumption. The consumption of periodate during the course of oxidation at three different concentrations of the oxidant is shown in Figure 3: A for the glycopeptide, and B for sucrose used as the reference compound to be oxidized. It is apparent from the figure that the periodate consumption occurs more rapidly and more extensively as the concentration of periodate increases. A theoretical value of 3 moles of periodate consumed per mole of sucrose was attained by 24-hr oxidation at 5° with 0.025 M periodate. By analogy, the value obtained under the latter conditions with the glycopeptide was taken as representing the result of an adequate oxidation. A value of 7.8 moles/mole was obtained after 24 hr. It follows that the number of bonds susceptible to periodate oxidation in the glycopeptide must have been 8 or slightly less than 8/mole.

Formation of Formic Acid, Erythritol, Glycerol, and Propylene Glycol. Determination of the reaction products were carried out after the glycopeptide had been oxidized with 0.025 M periodate for 20 hr at 5°. The results obtained are shown in Table III and the data may be interpreted as follows. One mole of glycopeptide consumes close to 8 moles of periodate and produces, after reduction and acid hydrolysis, approximately 1 mole of propylene glycol, approximately 2 moles of glycerol, more than 2 moles of formic acid, and practically no erythritol.

Carbohydrate Analysis of Glycopeptides Obtained by Smith Degradation. In Table IV are summarized the contents of carbohydrates in various samples of glycopeptides obtained by periodate oxidation that was performed under the conditions as specified in the table. After the first step of Smith

TABLE III: Products of the Periodate Oxidation[a] of the Glycopeptide of Stem Bromelain.

Product	moles/mole of Glycopeptide
$NaIO_4$ (Consumed)	7.8
HCOOH	2.2
Erythritol	0.2
Glycerol	1.6
Propylene glycol	0.7

[a] At 5° for 20 hr in the dark in 0.025 M $NaIO_4$, 20 moles/mole of the glycopeptide.

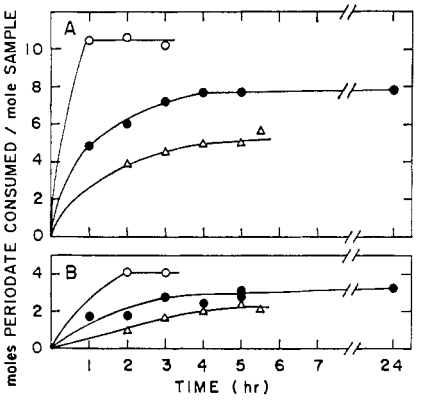

FIGURE 3: Periodate consumption of the glycopeptide of stem bromelain (A) and sucrose (B) at 5° in the dark. Concentrations of $NaIO_4$ are: 0.005 M (△); 0.025 M (●); 0.1 M (○).

TABLE IV: Carbohydrate Analysis of Glycopeptides Obtained by Smith Degradation.[a]

Step of Smith Degradation	Periodate Concentration (M)	Periodate moles/mole of Glycopeptide	moles/mole of Glycopeptide Man	Fuc	Xyl	GlcN[b]
Before Oxidation	0	0	3[c]	1[c]	1[c]	2[c]
1st	0.005	9	0.57	0.14	0.27	2.12
1st	0.005	9	0.20	0.08	0.82	1.99
1st	0.025	20	0	0	0	2.10
1st	0.1	50	0	0	0	1.86
2nd	0.1	50	0	0	0	1.15
3rd	0.1	50	0	0	0	0

[a] Periodate oxidation at 5° for 20 hr in the dark. [b] Determined on amino acid analyzer. [c] Nearest integral number of residues (see Table I).

degradation procedure with 0.005 M periodate, the destruction of neutral sugars is only incomplete so that variable portions of mannose, xylose, and, in one case, fucose residues remain unoxidized. When higher concentrations of the oxidant are used, complete destruction of neutral sugars occurs after the first step, but glucosamine residues are left almost intact even with 0.1 M periodate. The second step oxidation now eliminates one of the two glucosamine residues and the third step results in complete removal of glucosamine.

Release of Neutral Sugars by Acid Hydrolysis. When 0.45 mg of the glycopeptide was hydrolyzed in 1.0 ml of 0.05–1.0 N HCl at 100° for 1 hr, release of component sugars occurred to various extents depending upon the concentration of HCl. The amount of neutral sugars liberated was determined after separation on paper chromatography. As shown in Figure 4, xylose and fucose are hydrolyzed much more readily than mannose. The release of mannose does not seem to be completed even in 1 N HCl.

Identification of Oligosaccharides Released by Acid Hydrolysis. In addition to free neutral sugars, some oligosaccharides were also released by hydrolysis in 0.2 N HCl at 100° for 1 hr. The hydrolysate from 7.5 mg of the glycopeptide was successively subjected to paper electrophoresis runs at pH 6.5 and 3.5, paper chromatography with solvent I, and rechromatography on paper. The procedure is diagrammatically presented in Figure 5. As shown in section C of Figure 5, separation of three oligosaccharides, X, Y, and Z, was finally achieved. These oligosaccharides were eluted from the paper and analyzed for component sugars before and after reduction by 0.05 M sodium borohydride at 25° for 16 hr. For the analyses was employed hydrolysis in 1 N HCl at 100° for 3 hr. The results obtained are summarized in Table V. Oligosaccharides X and Y have different $R_{mannose}$ values, but both are composed of only mannose. The borohydride reduction causes decreases of mannose by 50% in X and 37%

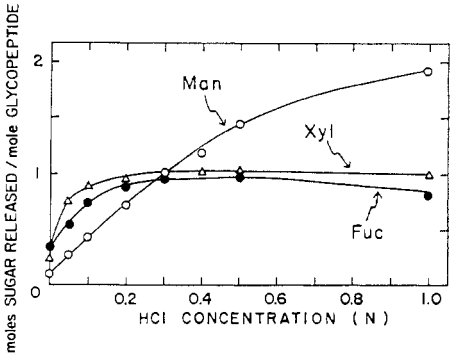

FIGURE 4: Hydrolysis of the glycopeptide of stem bromelain in varying concentrations of HCl at 100° for 1 hr. The data at zero concentration of HCl were obtained by heating the aqueous solution of the glycopeptide at 100° for 1 hr. The liberated sugars were separated by paper chromatography with solvent I and, after elution from the paper, determined by colorimetry.

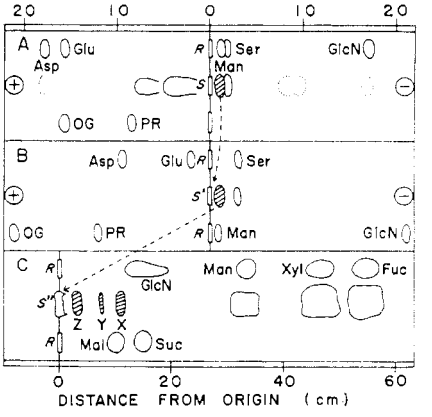

FIGURE 5: Separation of the hydrolysis products obtained from the glycopeptide of stem bromelain. The hydrolysis was carried out in 0.2 N HCl at 100° for 1 hr. (A) Paper electrophoresis at pH 6.5, 3000 V for 1 hr; (B) electrophoresis at pH 3.5, 3000 V for 2 hr; (C) chromatography with solvent I for 72 hr. Arrows with broken lines indicate the way of processing the oligosaccharide materials (shown with hatches). Authentic reference compounds and the sample were applied at R and S (S′ or S″), respectively. Detection of carbohydrates was made by staining with alkaline silver nitrate and that of amino acids and peptides by ninhydrin and *t*-butyl hypochlorite reactions. PR and OG are reference dyes, phenol red and orange G.

TABLE V: Oligosaccharides Released by Partial Acid Hydrolysis[a] of the Glycopeptide of Stem Bromelain.

Oligosaccharide	$R_{mannose}$[b]	Component Monosaccharide	μmole Found: Before $NaBH_4$ Reduction	μmole Found: After $NaBH_4$ Reduction	Proposed Structure
X	0.33	Man	0.77	0.39	Man-Man
Y	0.23	Man	0.15	0.10	Man-Man-Man
Z	0.10	Man	0.20	0.19	Man-GlcNAc
		Fuc	0.01	0.01	
		Xyl	0.03	0.02	
		GlcN[c]	0.18	0.02	
Maltose[d]	0.31	Glc	0.80	0.40	
Sucrose[d]	0.45				

[a] In 0.2 N HCl at 100° for 1 hr. [b] On the chromatogram (solvent I) shown in Figure 5C. [c] Determined by the Morgan–Elson reaction. [d] Reference disaccharides.

in Y, indicating mannobiose for X and mannotriose for Y. Oligosaccharide Z is composed of mannose and glucosamine and the borohydride reduction indicates that a glucosamine residue must have occupied the reducing end. In view of the neutral behavior in electrophoresis runs, the glucosamine in Z must have been *N*-acetylated.

Discussion

The experimental results described above have led us to propose a structure of carbohydrate moiety of stem bromelain as shown in Figure 6. The undecapeptide that carries the sugar moiety has recently been isolated from the pepsin digest of stem bromelain (Kito and Murachi, 1969). The glycopeptide preparation employed for the present experiments was obtained from the Pronase digest of stem bromelain. It corresponds to fragments ranging from Asn^6 to Ser^{10} of the pepsin peptide (Figure 6). The amino acid sequence of the Pronase peptides and the mode of attachment of the sugar moiety to Asn^7 were described in a previous communication (Takahashi *et al.*, 1969).

Although the proposed structure is shown as if it always contains three mannose residues, the terminal one may be subject to deletion in some of the preparations, since only less than three mannose residues per mole are known to exist (Table I). The deletion cannot be an artifact but must have occurred naturally in the parent protein molecule, since Pronase preparation used in the present experiment did not show any trace of glycosidatic activity toward *p*-nitrophenyl α-mannoside.

Three different nonreducing end groups shown in Figure 6 are deduced mainly from the results of enzymatic hydrolysis (Table II). Susceptibility to the specific glycosidase conclusively indicates only one possible configuration for the individual glycosyl group (Table II). Partial acid hydrolysis that leads to the isolation of mannobiose, mannotriose, and Man-GlcNAc, and the Smith degradation yielding $(GlcNAc)_2$-peptide, facilitate the reconstruction of the main chain (Tables IV and V). The fact that the removal of fucose, not xylose, enhances the enzymatic release of mannose suggests that the fucose residue is the one that may link to the innermost mannose residue (Table II). Branching of xylose from the outer rather than inner *N*-acetylglucosamine residue is reasonable, because an oligosaccharide preparation composed of xylose (1), fucose (0.5), mannose (2), and glucosamine (1) was obtained in a separate experiment where hydrazinolysis with 80% hydrazine hydrate was carried out. Mutual positioning of the residues can be further refined on the basis of the results of periodate oxidation (Tables III and IV). In Figure 6, the arrows indicate the theoretical sites of oxidation to occur. In theory, 8 moles of periodate are consumed to produce 3 moles of formic acid, and, upon reduction and acid hydrolysis, 3 moles of glycerol, 3 of glycolaldehyde, 2 of glyceraldehyde, 1 of ethylene glycol, and 1 of propylene glycol. The data actually obtained (Table III) are not incompatible with what one expects from the theory, although the agreement is not sufficiently quantitative. Man-(1→3)-Man linkage can be ruled out, because this would have left at least 1 mole of mannose intact even after the oxidation at high concentrations of periodate (Table IV). Man-(1→4)-

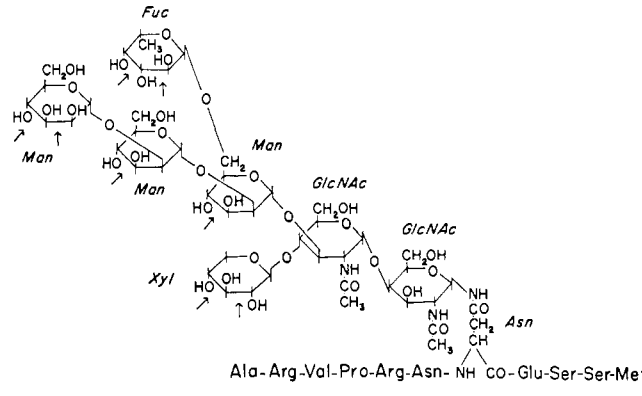

FIGURE 6: Proposed structure of the carbohydrate moiety of stem bromelain. Arrows show the possible sites of attack by periodate. Alternate structures Man-(1→6)-[Fuc-(1→2)]-Man and GlcNAc-(1→3)-GlcNAc are also possible. For the positions of Man and Xyl on the outer GlcNAc, six different possibilities of combination may exist and the one shown in the figure has been arbitrarily chosen (see text).

Man linkage is also unlikely, since this would have led to the production of at least 1 mole of erythritol instead of glycerol (Table III). The linkage between two *N*-acetylglucosamine residues cannot be 1→6 because of the resistance of these residues to periodate oxidation (Table IV). The β configuration of the innermost *N*-acetylglucosamine residue is assigned only by analogy to the cases of ovalbumin (Tsukamoto *et al.*, 1964), α_1-acid glycoprotein (Eylar, 1962), etc.

There must be a few alternative ways of reconstructing the possible structure. In Figure 6, the third, *i.e.*, the innermost, mannose residue links the second mannose at C-2 and fucose at C-6. Linking of the second mannose at C-6 and fucose at C-2 might be possible as well. The GlcNAc–GlcNAc linkage could be also 1→3 instead of 1→4. These alternatives do not conflict with the experimental data obtained. The Man-(Xyl)-GlcNAc structure could have six different possibilities with respect to which two of the three positions, C-3, C-4, and C-6, of the *N*-acetylglucosamine residue are occupied by mannose and xylose residues. The positions C-3 for mannose and C-6 for xylose shown in Figure 6 only represent an arbitrary choice. Further experimentation, including methylation studies, will be needed to determine which one of these possible structures is in fact correct.

Added in Proof

After the submission of this manuscript, a paper covering the work on the carbohydrate moiety of stem bromelain appeared (Scocca and Lee, 1969). Both papers are in general agreement.

Acknowledgments

We wish to express our appreciation to Dr. Ikuo Yamashina, Dr. Takashi Muramatsu, and Dr. Minoru Fukuda for the gifts of purified glycosidases. We are greatly indebted to Dr. Richard J. Winzler, State University of New York, and Dr. Zenzo Tamura, University of Tokyo, for performing the gas–liquid partition chromatographic analyses. The technical assistance of Miss Yoko Kasamatsu is gratefully acknowledged.

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