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THE STRUCTURE OF THE GLYCAN MOIETY OF TORA-BEAN (*Phaseolus vulgaris*) LECTIN

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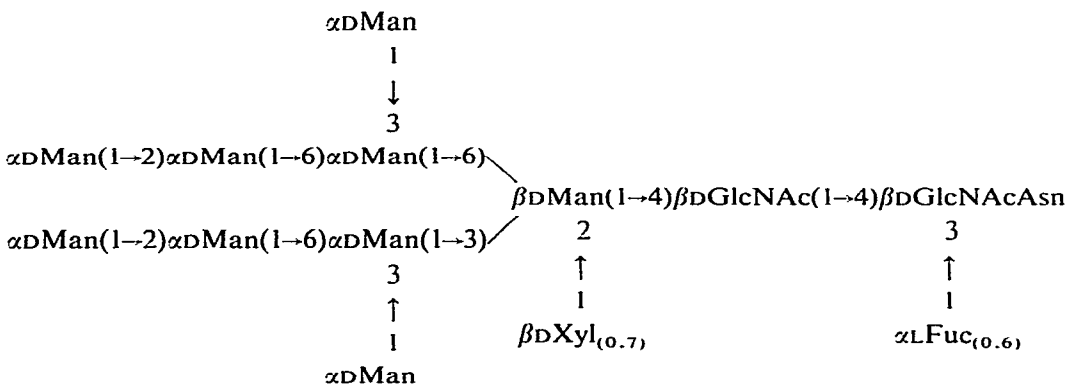
ABSTRACT

The structure of the carbohydrate unit of Tora-bean lectin, which contains 7.8% of neutral carbohydrate, was elucidated by isolation of the glycopeptide from the digest by the action of proteolytic enzymes. The purified glycopeptide (mol. wt. 2,700) comprises 9.2 moles of D-mannose, 2.0 moles of 2-acetamido-2-deoxy-D-glucose, 0.6 mole of L-fucose, and 0.7 mole of D-xylose per mole of asparagine.

Methylation analysis, Smith degradation and enzymic-degradation studies permitted formulation of a possible structure of the glycan of the glycopeptide, as follows.



INTRODUCTION

In recent years, various types of plant lectins, isolated from legumes, have been studied in view of their important, biological properties. However, information on the structure of their carbohydrate chains has been limited to a few lectins, *e.g.*, lima-bean (*Phaseolus lunatus*)[1] and soybean (*Glycine max*)[2] lectins.

In previous work[3], the lectin of Tora bean, a variant of *Phaseolus vulgaris* (and one of the edible beans common in Japan), was purified principally by affinity chromatography using concanavalin A. This lectin reacts with erythrocytes and mammalian glycoproteins, such as fetuin and human parotid glycoprotein. Like

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other types of phytohemagglutinins of *Phaseolus vulgaris*, this lectin was shown to be specific to the particular, internal-carbohydrate sequence, *i.e.*, β-D-Gal*p*-β-D-Glc*p*NAc-D-Man*p* of the mammalian glycoproteins.

As Tora-bean lectin contains D-mannose, 2-acetamido-2-deoxy-D-glucose, L-fucose, and D-xylose in the molar ratios of 9.2:2.0:0.6:0.7, it was of interest to establish the structure of the carbohydrate moiety, in comparison with that of other phytohemagglutinins.

We here describe the isolation of the glycopeptide from Tora-bean lectin, and the elucidation of the chemical structure of the carbohydrate unit-chain, as revealed mainly by methylation, Smith degradation, and enzymic-degradation studies.

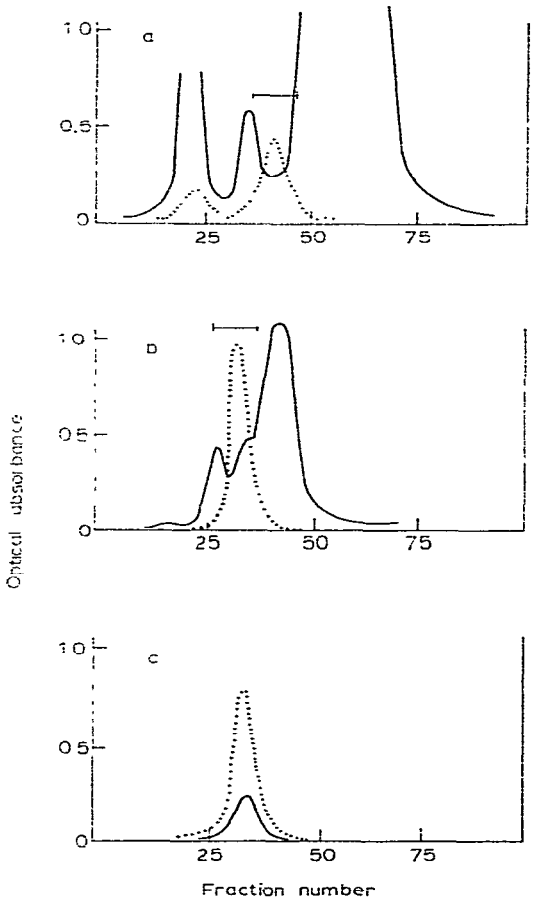


Fig. 1. Profiles of the column-chromatographic purification of glycopeptide from Tora-bean lectin. [Lectin (192.5 mg) was digested with pronase, followed by insoluble trypsin, and the product purified by gel filtration. (———), absorbance at 280 nm; (.....), absorbance at 490 nm, for sugar analysis by the phenol–sulfuric acid method. Each 1-mL fraction was collected. a, Sephadex G-50 column chromatography after pronase digestion; b, Sephadex G-50 column chromatography after insoluble trypsin digestion; c, rechromatography of the glycopeptide on Bio-Gel P-4 after elution from a Sephadex G-25 column.]

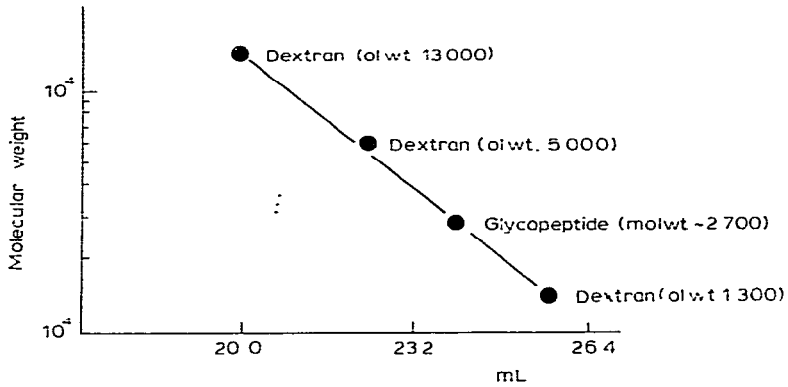


Fig. 2. Determination of the molecular weight of the glycopeptide by high-performance, liquid chromatography. [Elution was performed with 0.1M phosphate buffer, pH 7.8, at a flow rate of 0.8 mL per min.]

RESULTS AND DISCUSSION

Isolation and properties of the glycopeptide. — The purified, Tora-bean lectin was repeatedly digested with pronase, and then with insoluble trypsin, and the enzyme digest was subjected to column chromatography on Sephadex G-50, Sephadex G-25, and Bio-Gel P-4. The elution profile in each step is shown in Fig. 1. The purified glycopeptide, which corresponded to 10% of the lectin, showed a symmetrical peak on the Bio-Gel P-4 column. The molecular weight of the glycopeptide was 2,700, as estimated by h.p.l.c. (see Fig. 2). The g.l.c. analysis of the carbohydrate components showed that the glycopeptide comprises, per mole, 9.2 moles of D-mannose, 0.6 mole of L-fucose, 0.7 mole of D-xylose, and 2.0 moles of 2-acetamido-2-deoxy-D-glucose. Itoh *et al.*[4] reported the presence of D-glucose in Tora-bean lectin; however, the fact that the purified glycopeptide isolated in the present study contains no glucose confirmed our previous result[3]. Amino acid analysis of the glycopeptide showed that the molar ratio of 2-acetamido-2-deoxy-D-glucose to aspartic acid is 2.0:0.9. In addition to aspartic acid, small proportions of threonine and serine were detected in the acid hydrolyzate of the glycopeptide.

The mode of glycosidic linkages in the glycopeptide. — The glycopeptide was methylated by the method of Hakomori[5], and the product was hydrolyzed with acid. The identities and the molar ratios of the methylated sugars are listed in Table I. These methylated carbohydrates were identified by analysis by g.l.c.–m.s. The results of the methylation analysis indicated that the carbohydrate chain of the glycopeptide has a highly branched structure, consisting (per mole) of 0.6 mole of L-fucose, 0.7 mole of D-xylose, and 4 moles of D-mannose residues located at nonreducing terminals, 2 moles of (1→2)-linked D-mannose, 2 moles of D-mannose residues substituted at O-3 and O-6, 1 mole of D-mannose residue substituted at O-2, O-3, and O-6, and 2 moles of (1→4)-linked 2-acetamido-2-deoxy-D-glucose residues, one of which is substituted at O-3. In order to ascertain the location of the terminal L-fucose and D-xylose resi-

TABLE I

IDENTITIES AND MOLAR RATIOS OF METHYLATED SUGARS FROM THE ACID HYDROLYZATE OF THE METHYLATED GLYCOPEPTIDES

Methylated sugars	T[a]	*Intact*	*After hydrolysis with glycosidase*		*After Smith degradation*
			α-L-*fucosidase plus* β-D-*xylosidase*	α-D-*mannosidase*[b]	
2,3,4-Tri-*O*-methyl-D-xylose	0.54	0.5	0.2	0.5	—
2,3,4-Tri-*O*-methyl-L-fucose	0.58	0.6	0.2	0.5	—
2,3,4,6-Tetra-*O*-methyl-D-mannose	1.00	4.0	4.0	1.0	2.0
3,4,6-Tri-*O*-methyl-D-mannose	1.44	2.1	2.3	—	—
2,3,4-Tri-*O*-methyl-D-mannose	1.69	—	—	0.6	—
3,4-Di-*O*-methyl-D-mannose	2.15	—	—	0.5	—
2,4-Di-*O*-methyl-D-mannose	2.33	2.0	2.5	—	1.2
4-*O*-Methyl-D-mannose	3.04	1.2	0.5	—	—
2-Acetamido-2-deoxy-3,6-di-*O*-methyl-D-glucose	3.30	1.5	2.2	1.3	2.2
2-Acetamido-2-deoxy-6-*O*-methyl-D-glucose	3.90	0.6	±	0.5	—

[a]Retention time of the alditol acetate, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol. [b]Under the present conditions, 7 out of 9 moles of D-mannose residues were released (see text).

dues, both sugar residues were removed by the action of α-L-fucosidase (from *Charonia lampas*) and β-D-xylosidase (from *Charonia lampas*). Although D-xylose and L-fucose residues were not completely hydrolyzed under the present conditions, as shown in Table I, comparison of the methylation data for the glycosidase-treated glycopeptide with those for the intact glycopeptide showed that the proportions of the triply substituted D-mannose residue and the doubly substituted 2-acetamido-2-deoxy-D-glucose residue of the enzyme-treated glycopeptide were significantly decreased, suggesting that one of these sugar residues is attached to O-2 of the mannose residue, and the other, to O-3 of the 2-acetamido-2-deoxy-D-glucose residue.

The results of the methylation analyses were supported by the analyses of Smith-degradation products. By periodate oxidation and borohydride reduction of the intact glycopeptide, followed by complete hydrolysis, D-mannose and 2-acetamido-2-deoxy-D-glucose (molar ratio, 2.6:2.0), in addition to glycerol and 1,2-propanediol, were identified as the hydrolysis products. The glycerol must have arisen from nonreducing (terminal) D-mannosyl groups, whereas the D-mannose, which is resistant to periodate cleavage, must have originated from the branch points of the D-mannosyl residues substituted at O-3.

In order to obtain knowledge on the sequence of the sugar residues in the

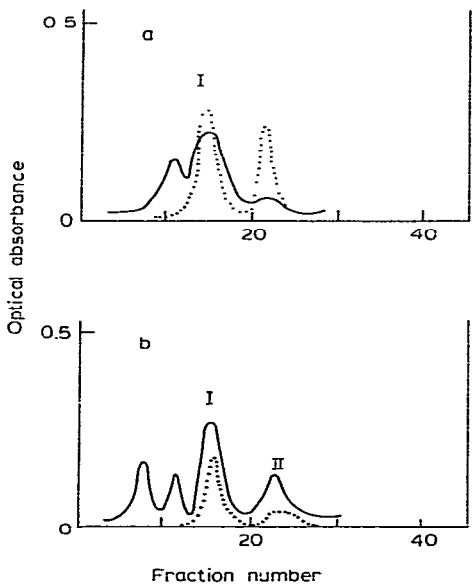
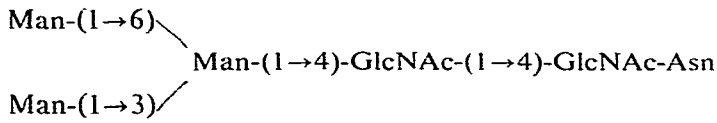


Fig. 3. Purification of the enzyme-degraded glycopeptide by chromatography on a column of Bio-gel P-4. [Key: (———), absorbance at 230 nm; (.....), absorbance at 490 nm, for sugar analysis by the phenol–sulfuric acid method. Each 1-mL fraction was collected. a, elution profile after α-D-mannosidase treatment; b, elution profile after treatment of α-D-mannosidase-degraded glycopeptide (peak I in a) with β-D-mannosidase and β-*N*-acetyl-D-glucosaminidase.]

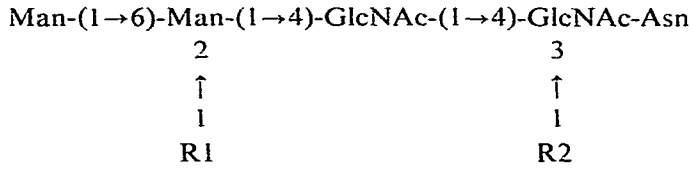
glycopeptide, an Asn-oligosaccharide* was isolated from the glycopeptide after mild, Smith degradation (hydrolysis with 0.1M sulfuric acid for 20 h at 20°) by chromatography on a column of Bio-Gel P-4. Complete hydrolysis of the Asn-oligosaccharide yielded D-mannose and 2-acetamido-2-deoxy-D-glucose in the molar ratio of 2.8 : 2.0, in addition to aspartic acid, indicating that this oligosaccharide fragment surviving the periodate cleavage corresponds to $(Man)_3$-GlcNAc-(1→4)-GlcNAc-Asn.

In order to obtain more information on the sequence of the carbohydrate chain, the glycopeptide was digested with α-D-mannosidase, whereby a rapid release of D-mannose was observed during 7 to 10 h, but, thereafter, essentially no increase in the release of reducing sugar was observed. Fig. 3a shows the fractionation profile of the α-D-mannosidase-degraded glycopeptide in chromatography on a column of Bio-Gel P-4. The purified Asn-oligosaccharide was shown to contain L-fucose, D-xylose, D-mannose, and 2-acetamido-2-deoxy-D-glucose in the molar ratios of 0.7 : 0.6 : 2.2 : 2.0, as the carbohydrate components, indicating that the α-D-mannosidase digestion might have been incomplete.

The results of methylation analyses of both oligosaccharide fragments (obtained by mild, Smith degradation and by α-D-mannosidase digestion) are listed in Table I. Thus, the mildly Smith-degraded oligosaccharide, which may represent the core moiety, possesses the following sequence.



In a similar way, the α-D-mannosidase-resistant oligosaccharide may have the following structural feature, although a micro-heterogeneity might exist with regard to the D-xylose and L-fucose residues.



where R1 and R2 represent a D-xylosyl or L-fucosyl group, respectively.

Comparison of the sequence of sugar residues in these two oligosaccharide fragments strongly suggests that the L-fucose and D-xylose residues must be attached to the 2-acetamido-2-deoxy-D-glucose residue adjacent to the asparagine, and that the D-mannose residue is joined by (1→4)-linkage to another 2-acetamido-2-deoxy-D-glucose residue. This conclusion was supported by the following, sequential, enzymic degradation of the glycopeptide.

When the aforementioned α-D-mannosidase-resistant glycopeptide was further

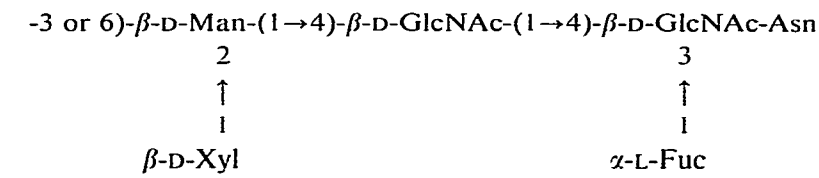
*Abbreviation: Asn-oligosaccharide, an oligosaccharide linked to asparagine through a glycosyl–N bond.

treated with α-D-mannosidase (0.2 unit) for 24 h, one more mole of D-mannose was released per mole, leaving an Asn-oligosaccharide which was isolated by chromatography on a column of Bio-Gel P-4. The oligosaccharide was shown to be comprised of L-fucose, D-xylose, D-mannose, and 2-acetamido-2-deoxy-D-glucose in the molar ratios of 0.7:0.6:1.1:2.0, indicating that 8 of the 9 moles of D-mannose residues of the intact glycopeptide have the α configuration. The glycopeptide finally obtained (freed of α-D-mannose) was treated with β-D-mannosidase (0.1 unit; from *Aspergillus niger*) for 24 h at pH 4.5, followed by Jack-bean β-*N*-acetyl-D-glucosaminidase (0.1 unit) for 24 h at pH 4.5, and the enzyme digest was fractionated on a column of Bio-Gel P-4, affording two carbohydrate fractions (peaks I and II), as shown in Fig. 3b. As already mentioned, if a D-xylose residue is attached to a β-linked D-mannose residue, the α-D-mannosidase-degraded glycopeptide should not be hydrolyzed by β-D-mannosidase, whereas, if the degraded glycopeptide is devoid of the xylose residue, the successive action of β-D-mannosidase and *N*-acetyl-D-glucosaminidase should result in release of the corresponding two sugars.

Paper-chromatographic and g.l.c. analyses of the foregoing degradation products revealed that the more-slowly emerging fraction (peak II) was a mixture of mannose, 2-amino-2-deoxyglucose, and an oligosaccharide. This oligosaccharide showed slower migration on a paper chromatogram, and, upon acid hydrolysis, gave fucose and 2-amino-2-deoxyglucose, in addition to aspartic acid, as detected by t.l.c. with 5:5:1:3 ethyl acetate–pyridine–acetic acid–water. These results indicated that the oligosaccharide showing slower migration on a paper chromatogram is an Asn-oligosaccharide having the sequence α-L-Fuc-β-D-GlcNAc-Asn.

The faster-emerging fraction (peak I), whose elution volume was the same as that of the original, α-D-mannosidase-degraded glycopeptide, was shown to be comprised of L-fucose, D-xylose, D-mannose, and 2-acetamido-2-deoxy-D-glucose (molar ratios 0.3:0.9:1.1:2.0), suggesting that it was a D-xylose-containing glycopeptide fragment, resistant to the action of β-D-mannosidase.

It may be concluded from these findings that the carbohydrate chain of Tora-bean lectin contains a core carbohydrate structure like those in glycoproteins already known, as follows.



However, there is the possibility of a micro-heterogeneity of the carbohydrate chain, at least, with regard to the D-xylosyl group. Thus, the oligosaccharide of higher molecular weight (peak I in Fig. 3b) may represent the D-xylose-bearing Asn-oligosaccharide that is resistant to β-D-mannosidase, and the fraction of lower molecular weight, which contains D-mannose, 2-acetamido-2-deoxy-D-glucose, and the degraded

TABLE II

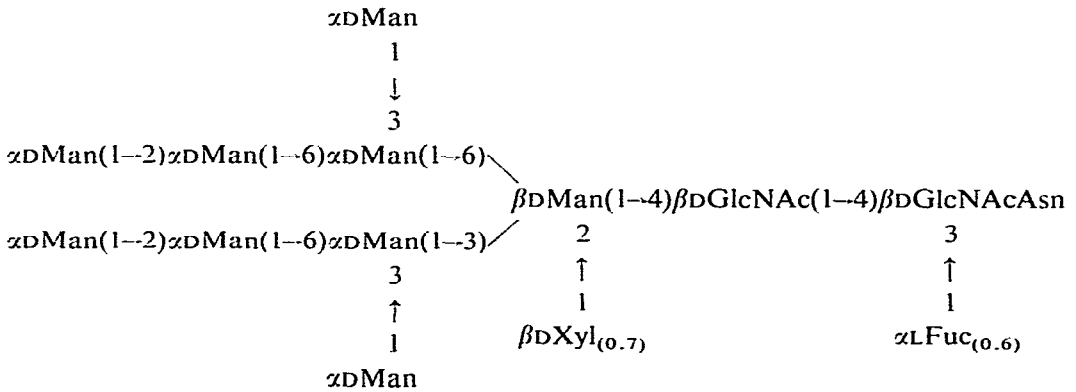
EFFECT OF VARIOUS GLYCOSIDASES ON GLYCOPEPTIDE

Substrate	*Glycosidase*	*Source*	*Unit*[a]	*pH*	*Carbohydrate composition*			
					Fuc	*Xyl*	*Man*	*GlcNAc*
Glycopeptide (Intact)					0.6	0.7	9.2	2.0
	(1→2)-α-D-mannosidase	*A. niger*	0.1	4.5	0.6	0.7	7.3	2.0
	α-D-mannosidase	Jack bean	0.3	4.5	0.7	0.6	1.1	2.0
	α-L-fucosidase	*Ch. lampas*	0.01	4.0	0.2	0.2	9.2	2.0
	β-D-xylosidase	*Ch. lampas*	0.05	3.0				

[a]One unit of the glycosidase hydrolyzes 1 μmole of the corresponding *p*-nitrophenyl glycoside per min at 37°.

Asn-oligosaccharide, in the sequence L-Fuc(1→3)-D-GlcNAc-Asn, could have originated from the D-xylose-deficient carbohydrate-chain.

There may be a possibility of α-D-(1→2)-linkages of (terminal) D-mannosyl groups in the carbohydrate-chain unit. When the intact glycopeptide was digested with the purified (1→2)-α-D-mannosidase of *Aspergillus niger* in 0.1M acetate buffer, pH 4.5, for 3 h at 37°, liberation of D-mannose, by separation of the released sugar from the residual glycopeptide, was observed by chromatography on a column (1.2 × 22 cm) of Bio-Gel P-2 (400 mesh), and also by g.l.c. analysis. From this fact, it was confirmed that the (nonreducing) terminal D-mannosyl groups are joined by an α-D-(1→2) bond to the neighboring D-mannosyl residues. The effects, on the glycopeptide, of various glycosidases are summarized in Table II. Thus, the most-probable, structural feature of the carbohydrate unit of Tora-bean lectin may be depicted as follows.



There may be a micro-heterogeneity, particularly with regard to the D-xylosyl group that is attached to the (1→4)-β-D-linked D-mannosyl residue. From the molar ratio of L-fucose residue (0.6 mole per 2.0 moles of 2-acetamido-2-deoxy-D-glucosyl residues), it is also possible that some carbohydrate units could be devoid of L-

fucosyl groups. However, such micro-heterogeneities of the glycan moiety in glycoproteins is not surprising, as they have been reported for α-lactalbumin[6] and IgG (ref. 7).

Most lectins, except for a very few (*e.g.* concanavalin A, wheat-germ agglutinin, and peanut agglutinin), are known to be glycoproteins; however, the structural features of their glycan moieties have not yet been fully elucidated. Recently, Misaki and Goldstein[1] reported the structure of the carbohydrate chain of lima-bean (*Phaseolus lunatus*) lectin. The glycopeptide isolated from the pronase digest has branched, α-D-linked, D-mannosyl residues which are joined to the core moiety: β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-Asn. More recently, Lis and Sharon[2] reported the structure of two branched, carbohydrate chains in soybean lectin. It is noteworthy that these plant lectins bear a similarity to each other in their core structure, *viz.*, β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-Asn, and also to those of glycoproteins of mammalian and microbial origins.

It is interesting that the attachment of the β-D-linked D-xylosyl group to the backbone of D-mannosyl residues in the present lectin is similar to that in pineapple bromelein, as reported by Ishihara *et al.*[8].

As regards their biological activities, the Tora-bean lectin having α-D-mannosyl residues partially removed (removal of 5 of the 9 moles of D-mannose residues per mole) still exhibited potent, hemagglutinin activity, as with the native lectin[3].

EXPERIMENTAL

Materials. — Tora-bean lectin was purified as already described[3]. Pronase (70,000 p.u.k./g) was purchased from Kaken Kogyo, Co., Tokyo. Jack-bean α-D-mannosidase and α-L-fucosidase of *Charonia lampas*[9] were purchased from Sigma. β-D-Xylosidase of *Charonia lampas* was purified according to the method of Fukuda *et al.*[10]. β-D-Mannosidase and (1→2)-α-D-mannosidase[11] of *Aspergillus niger* were kindly provided by Professor O. P. Bahl, Department of Biochemistry, State University of New York at Buffalo. Jack-bean β-*N*-acetyl-D-glucosaminidase was prepared according to the method of Li[12].

Preparation of the glycopeptide. — Tora-bean lectin (192 mg), purified as previously described[3], was twice digested with pronase (each, 8 mg) in 0.1M Tris-HCl buffer, pH 8.0, for 48 h at 37°. The digest was applied to a column (1.5 × 50 cm) of Sephadex G-50 and the carbohydrate fraction, monitored by the phenol–sulfuric acid method[13], was collected. This fraction was now treated with insoluble trypsin (1 mg, Sigma) in 0.1M Tris-HCl buffer, pH 8.0, for 24 h at 37°, and then applied successively to Sephadex G-50, G-25, and Bio-Gel P-4 columns (each, 1.5 × 50 cm), the purified glycopeptide fraction being collected, and evaporated to dryness.

Estimation of the molecular weight of the glycopeptide. — The molecular weight of the purified glycopeptide was determined by high-performance liquid chromatography (h.p.l.c.), with a Hitachi High-Performance Liquid Chromatograph, model 635, having a column (7.5 × 600 mm) of Toyo-Soda TSK-GEL G 3000SW (Toyo

Soda Co.), by using 0.1M phosphate buffer, pH 7.8, as the carrier at a flow rate of 0.8 mL per min. Purified dextrans having different molecular weights, supplied by Meito Sangyo Co., Nagoya, were used as standards.

General, analytical method. — Total carbohydrate content was determined colorimetrically by the phenol–sulfuric acid method[13]. Reducing sugar was measured by the Nelson–Somogyi method[14]. Complete hydrolysis of the glycopeptide was achieved by heating with 2M trifluoroacetic acid in a sealed tube for 6 h at 100°; the hydrolyzate was evaporated, and water was repeatedly added to, and evaporated from, the residue.

G.l.c. was performed with a Hitachi Gas Chromatograph, model 163, fitted with a flame-ionization detector. After reduction with sodium borohydride followed by conversion into the corresponding alditol acetates, the analyses of both the neutral and the amino sugars were performed in a column packed with 3% of OV-17 on Chromosorb W (80–100 mesh), programmed from 150 to 205° at 1° per min. As the hydrolyzate contained 2-amino-2-deoxy-D-glucose, acetylation of the mixture of alditols was achieved by heating with acetic anhydride in the presence of sodium acetate, after decomposition of the excess of sodium borohydride by acidifying with acetic acid and removing borate as methyl borate[15] (instead of the ordinary method, which involves decomposition of the excess of borohydride with a cation-exchange resin, and acetylation of the resulting alditols with pyridine and acetic anhydride). The mixture of acetyl derivatives was dissolved in chloroform, washed several times with water (to remove acetic anhydride) and applied to the g.l.c. column.

Methylation analysis. — A sample of glycopeptide (2 mg) was dissolved in dimethyl sulfoxide (2 mL) in a nitrogen atmosphere, and treated with methylsulfinyl carbanion (0.5 mL) and methyl iodide (1.5 mL) according to the method of Hakomori[5]. The methylated product was extracted into chloroform, and the extract was washed with water, and evaporated to dryness. The methylation procedure was repeated until no absorbance at 3600–3400 cm^{-1} (hydroxyl group) was observed in the infrared spectrum. The fully methylated product was purified by passing it through a column (1 × 20 cm) of Sephadex LH-20, the methylated glycopeptide being eluted with 2:1 (v/v) chloroform–methanol. The methylated glycopeptide was hydrolyzed with 4M trifluoroacetic acid for 6 h at 100°, the solution evaporated *in vacuo*, and the mixture of methylated sugars analyzed, as their alditol acetates, by g.l.c., using an OV-17 column programmed from 150 to 205° at 1° per min.

Periodate oxidation. — The glycopeptide (2 mg) was oxidized with 0.05M sodium periodate (0.5 mL) at 5°. After complete oxidation (5 days), the pH of the solution was adjusted to 8.0, and the product reduced with sodium borohydride (5 mg) overnight at room temperature. The excess of sodium borohydride was decomposed by careful addition of acetic acid, and the glycopeptide-polyalcohol was purified by passage through a column (1.5 × 40 cm) of Bio-Gel P-4. A portion of the resulting glycopeptide-polyalcohol was hydrolyzed completely with 0.5M sulfuric acid for 6 h at 100°, the acid neutralized, the product reduced with sodium borohydride, the alditol acetylated with acetic anhydride, and the acetate analyzed by

g.l.c. Another portion of glycopeptide-polyalcohol was moderately hydrolyzed with 0.1M sulfuric acid for 20 h at 20°, and the hydrolyzate was applied to a column of Bio-Gel P-4, to give a degraded glycopeptide that was resistant to periodate oxidation. The g.l.c. analysis of its carbohydrate components, and the methylation analysis, were performed as already described.

Enzymic degradation of the glycopeptide. — Treatment of the glycopeptide (3.8 mg, as neutral sugar) with α-D-mannosidase was conducted in 0.1M acetate buffer, pH 4.5, for 48 h at 37°, and the resulting digest was fractionated on a column of Sephadex G-50 and then on a column of Bio-Gel P-4, and the glycopeptide fraction was collected. It was hydrolyzed, and the products analyzed, as their corresponding alditol acetates, by g.l.c. The digestion of α-D-mannose-depleted glycopeptide (2.5 mg, as neutral sugar) with β-D-mannosidase of *Aspergillus niger* (0.1 unit) and Jack-bean β-*N*-acetyl-D-glucosaminidase (0.1 unit) was performed in 0.2M acetate buffer, pH 4.5, at 37°, and the resulting digest was fractionated on a column of Bio-Gel P-4. The digestion of the glycopeptide (~1 mg) with α-L-fucosidase of *Charonia lampas* (0.01 unit) was performed, at pH 4.0, in 0.1M acetate buffer, and the digestion with β-D-xylosidase of *Charonia lampas* (0.05 unit), at pH 3.0, in 0.1M acetate buffer. The treatment of the glycopeptide (700 μg, as neutral sugar) with (1→2)-α-D-mannosidase of *Aspergillus niger* (0.1 unit) was performed at pH 4.5, in 0.1M acetate buffer.

Amino acid analysis of the glycopeptide. — The glycopeptide (0.8 mg) was hydrolyzed for 24 h and 48 h with 6M hydrochloric acid at 110°. The amino acids were analyzed with an Amino Acid Analyzer, Japan Electronic Optical Laboratory, model JLC-5AM.

ACKNOWLEDGMENTS

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