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Studies on carbohydrate moieties of the glycoprotein, glucoamylase II of *A spergillus niger:* Nature of carbohydrate-peptide linkage and structure of oligosaccharides

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Abstract. Electrophoretically homogeneous type 1 (GP-C_1, and GP-C_2), type 2 (GP-C_{3a} and GP-C_{3b},) and type 3 (GP-D_1, and GP-D_2) glycopeptides from *Aspergillus niger* glucoamylase II (Manjunath and Raghavendra Rao, preceding paper) were separately treated with alkaline borohydride. The (*ß*-eliminated oligosaccharides were subjected to single and sequential digestion with specific glycosidases and the products analysed by gas liquid chromatography. The studies revealed that carbohydrate moieties were present as mannose, Man-Man-, and trisaccharide structures, namely, (a) GIc-Man-Man-, (b) Gal-Man-Man, (c) Man-Man-Man-, (d) GlcNAc-Man-Man-, and (e) Xyl-Man-Man. None of the glycopeptides contained all the trisaccharide structures (a) to (e). Type 1 glycopeptide contained structures (a), (b) and (c); type 2, (a) and (d)and type 3, (a), (b)and (e). The number of carbohydrate units (mono-, di-and trisaccharides) present in the major glycopeptides was determined and tentative structures for the glycopeptides proposed. Carbohydrate units appeared to occur in clusters of 4 to 7 in each glycopeptide, a structure unique to the carbohydrate moiety in *Aspergillus ni*ger glucoamylase. Based on carbohydrate analysis and yields of glycopeptide, the number of units of each type of glycopeptide present in glucoamylase II was tentatively calculated to give two of type Man:Glc:Gal = 12-15:l:l, one of type Man:Glc:GlcN = 10-l 1:1:2 and one of type Man :GIc :Gal :Xyl =4-8:0.1:0.5-0.8:0.3-1 glycopeptides.

Keywords. Glucoamylase; *Aspergillus niger;* glycopeptide; carbohydrate-peptide linkage; central heterogeneity.

Introduction

The isolation and purification of six glycopeptides viz., GP-C , GP-C_2, GP-C_{3a}, GP-C_{3b} GP-D and GP-D_2 have been described and their molecular weight and composition has been indicated (Manjunath and Raghavendra Rao preceding paper). All of them contained mannose and glucose besides other monosaccharides. Based on their content of other monosaccharides, the glycopeptides were grouped into three types: GP-C_1 and GP-C_2 forming one type, contained galactose, GP-$C_{3a}$and GP-C_{3b} forming another type, contained galactosamine, and GP-D_1 and GP-D_2 forming the third, contained galactose and xylose.

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The present investigation describes the results of experiments conducted to determine the structure of the carbohydrate moieties, the nature of the bonds linking them to peptide and the probable distribution of these units in glycopeptides.

Materials and methods

D-Galactose oxidase (EC 1.1.3.9),peroxidase (EC 1.11.1.7), jack-bean meal and *o*-dianisidine were purchased from Sigma Chemical Company, St. Louis, Missouri, USA, *β*-Galactosidase (EC 3.2.1.23) (*Escherichia coli*) was from Worthington Biochemical Corporation, Freehold, New Jersey, USA. Sources of the chemicals have been described previously (Manjunath and Raghavendra Rao, previous paper).

Glycopeptides GP-C_1, GP-C_2, GP-C_{3a}, GP-C_{3b}, GP-D_1, and GP-D_2 were purified from the pronase digest of glucoamylase II of *A. niger as* described (Manjunath and Raghavendra Rao, previous paper).

Glycosidase preparation and activity

β-Galactosidase was purchased from the Worthington Biochemicals Corporation, Freehold, New Jersey, USA. α-Mannosidase (EC 3.2.1.24) and *β*-N-acetyl glucosaminidase (EC 3.2.1.30) were purified from Jack bean meal (from Sigma Chemical Co., St. Louis, Missouri, USA) according to the procedure of Li (1966 and 1967). α-Mannosidase and *β*-N-acetyl glucosaminidase thus prepared were free from each other and also from *β*-galactosidase and *β*-xylosidase. An aqueous extract of Jack bean meal was found to contain ß-xylosidase (EC 3.2.1.37) and was used as such. Amyloglucosidase (glucoamylase) was purified as described earlier (Manjunath and Raghavendra Rao, 1979). The assay conditions, substrates and specific activities for these enzymes used in the present investigation are indicated in table 1.

Table 1. Specific activities of glycosidases used*

Enzyme	Substrate	Specific activity
α-Mannosidase[a] (JBM)†	methyl-α-D-gluco-pyranoside	5.0
β-N-Acetyl glucosaminidase[b] (JBM)	ovalbumin glyco-peptides	0.2
β-Galactosidase[c] (*E. coli*)	p-nitrophenyl-β-D-galactopyranoside	330.0
β-Xylosidase[d] (JBM)	*o*-nitrophenyl-β-D-xyloside	0.01
Glucoamylase[e] (*A. niger*)	Soluble starch	90.0

* All specific activity units are expressed as μ mols of product liberated/mg protein/min at 37°C in 0.5 M acetate buffer pH 4.6. Activity measured on the basis of the release of

[a] mannose [c] and [d] p-nitrophenol † JBM—Jack Bean Meal

[b] N-acetylglucosamine [e] Glucose

Preparation of reduced oligosaccharides

Glycopeptides were treated with alkaline borohydride according to Carlson (Carlson, 1966) with the following modification. Freeze-dried glycopeptides (2-3 umol) were treated with 10 ml of 0.1 M NaOH containing 1 M $NaBH_4$ at 37°C. After 50 h, the excess of borohydride was destroyed with dilute acetic acid (to pH 5) and concentrated. Borate was removed by repeated evaporations with methanol. The peptide-free material, obtained by passage through a column of Dowex-50×2 (H+), was subjected to preparative paper chromatography (n-butanol:ethanol: water=10:l:2) and the various mono-, di and trisaccharides were isolated.

The peptide portion was eluted from the Dowex column with 1 M pyridine acetate (pH 5.0), evaporated to dryness and subjected to amino acid analysis as described earlier (Manjunath and Raghavendra Rao, previous paper).

Determination of terminal non-reducing monosaccharide residue using glycosidases.

The following procedure was used to detect terminal non-reducing sugars. To 1 to 2 umol of oligosaccharide in 0.05 M acetate buffer pH 4.6 was added *ß*-galactosidase (3 units), ß-N-acetyl glucosamidase (8 units), α-mannosidase (15 units), glucoamylase (10 units) or ß-xylosidase (0.1 unit). The reaction mixture (0.5 ml) was incubated for 30 to 48 h at 37°C with a few drops of toluene, then heated in a boiling water-bath for 3 min and centrifuged to remove the precipitated enzyme protein. The supernatant was then applied to a column (0.5×5 cm) of Dowex-50× 2(H^+) and washed with water. The washings were concentrated and examined by paper chromatography. The oligosaccharides eluted from the paper were evaporated to dryness, redissolved in 0.05 M acetate buffer pH 4.6 and incubated with another glycosidase. Controls consisting only of substrate or the enzyme accompanied each experiment. The released sugars were determined by gas liquid chromatography as described earlier (Manjunath and Raghavendra Rao, 1979). Glucose was also determined using tris glucose oxidase reagent. N-Acetyl glucosamine was determined by the method of Reissig *et al.* (1955), following the modification of Marshall and Neurber (1972). The presence of non-reducing (i.e., terminal) residues of D-galactose in the oligosaccharides was also detected using D-galactose oxidase following the procedure of Dawson and Clamp (1968).

Results

Structure of type 1 glycopeptides (*GP-C_1 and GP-C_2*)

The glycopeptides GP-C_1, and GP-C_2 were shown to contain threonine, serine, glycine, mannose, glucose and galactose in a molar ratio of approximately 7:3:1:12:1:1 (Manjunath and Raghavendra Rao, previous paper). In addition, GP-C_2 also contained one residue each of alanine and proline and 3 more residues of mannose. Fractional amounts of other amino acids were also present. The calculated molecular weights were 3320 and 3900 for GP-C_1, and GP-C_2 respectively. These values are in reasonable agreement with a value of 3600 and 3950 obtained by gel filtration.

Structure of glycopeptide GP-C_1: The glycopeptide GP-C_1 (10 mg=3 µmols) was subjected to alkaline borohydride treatment and mono-, di- and trisaccharides were isolated by paper chromatography as described in "Materials and methods".

The sugar components were examined by paper chromatography and quantitated by gas liquid chromatography. The results obtained are shown in table 2. High recoveries of glucose and galactose were obtained following alkaline borohydride treatment, Dowex-50 (H^+) column chromatography and paper chromatography and hydrolysis. The recovery of mannose was only 40% but most of the undetected mannose was recovered as mannitol. Mannitol was formed in the system from mannose units linked by alkali-sensitive glycopeptide bonds. On a molar basis approximately 7 mol of mannitol were formed per mol of glycopeptide. On the basis of yield of saccharides and their reduced products, there were approximately 2 monosaccharide units, 3 disaccharide units and 2 trisaccharide units per mole of glycopeptide.

Table 2. Molar ratios of sugar recoveries from alkaline borohydride treated glycopeptide GP-C_1.

Fraction	Mannose[a]	Mannitol[b]	Glucose[a]	Galactose[a]
1. GP-C_1	12	—	1	1
2. Dowex-50 eluate	5	7	1	1
3. Paper Chromatography				
Monosaccharide	—	2	—	—
Disaccharide	3	3	—	—
Trisaccharide	2	2	1	1

[a] Analyzed by gas liquid chromatography as alditol acetates

[b] Determined by gas liquid chromatography after separation from mannose by paper chromatography using solvent 1-butanol-pyridine H_2O, 6:4:3(v/v).

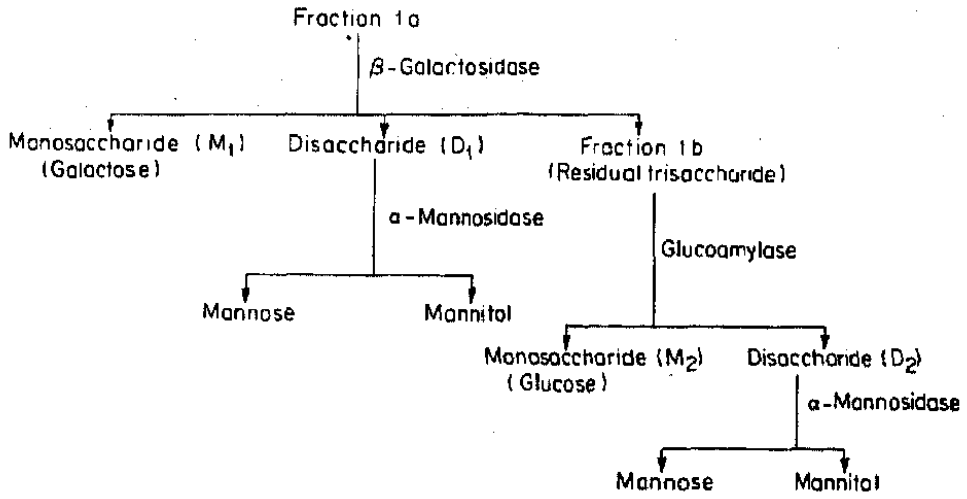
— means not present.

Structure of disaccharides: Upon treatment with α-mannosidase the disaccharide was cleaved into mannose and mannitol. Therefore the disaccharide was α-Man-Man-OH.

Structure of trisaccharides: Sugar composition of the trisaccharide fraction designated as fraction la indicated the presence of mannitol, mannose, glucose and galactose in the molar ratios 2:2:1:1. Hence fraction 1a should be a mixture of trisaccharides of differing composition.

The structure of the trisaccharides in fraction la was determined as follows: Fraction la was treated successively with various glycosidases as shown in figure 1

Figure 1. Scheme for the action of glycosidases on fraction la derived from GP-C_1.



and the released sugars were quantitatively determined at each step. The results obtained in such studies are shown in table 3. From these results it was deduced that two trisaccharides with the possible structure Glc-Man-Man-OH and Gal-Man-Man-OH were present.

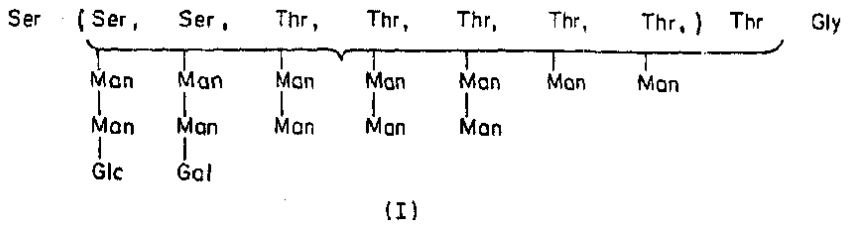
Table 3. Sugar composition of fraction la and its derivatives.

Fraction	Mannose	Mannitol	Glucose	Galactose
				(mols./mol glycopeptide)
Fraction 1a (original)	1.8	1.78	0.86	0.87
Fraction M_1	—	—	—	0.82
Fraction D_1	0.85	0.85	—	—
Fraction 1b	0.8	0.82	0.8	—
Fraction M_2	—	—	0.8	—
Fraction D_2	0.78	0.78	—	—

Monosaccharides were quantitated by gas liquid chromatography as alditol acetates. M_1 and other fractions were obtained from la by the procedure in figure 1. — = not present.

Amino acid composition: The amino acid composition both before and after treatment with alkaline borohydride was determined (table 4). Approximately 5 residues of Thr and 2 residues of Ser were lost per mole of glycopeptide. The combined loss of threonine and serine was found to be equivalent to the mannitol formed in the system. These results therefore suggested that there are seven mannose residues in O-glycosidic linkages with 5 residues of Thr and 2 of Ser.

From the data obtained, it may be inferred that GP-C_1 contained (per mole) 2 units of Thr/Ser-Man, 3 units of Thr/Ser-Man-Man, 1 unit of Thr/Ser-Man-Man-Gal and 1 unit of Thr/Ser-Man-Man-Glc. The sequence of amino acids in glycopeptide was not determined. A tentative structure (I) is proposed for GP-C_1, on the basis of the facts now known:



(I)

The saccharides could be present in a different order from that shown.

Structure of glycopeptide GP-C_2: This glycopeptide (10 mg) when subjected to alkaline borohydride treatment yielded di- and trisaccharides. These saccharides were isolated and their composition determined as described above for GP-C_1.

Table 4. Effect of alkaline borohydride treatment on amino acid composition of GP-C_1.

Component	Original	After alkaline borohydride treatment
Threonine[a]	7	2
Serine[a]	3	1
Glycine	1	1
Alanine	—	2
α-amino-butyrate	—	1

[a] Values not corrected for any destruction due to acid hydrolysis.

The results obtained are shown in table 5. Nearly 7 moles of mannose of a total of 15 disappeared and most of it was recovered as mannitol. There were approximately 4 units of disaccharides and 3 units of trisaccharides.

Structure of disaccharide: The disaccharide was cleaved by α-mannosidase to give mannose and mannitol in the ratio 1:1. Hence the disaccharide has a Man-Man-OH structure.

Structure of trisaccharides: The trisaccharide fraction designated fraction 2a was found to contain Man-OH, Man, Glc and Gal in the ratio 4.7:7.1:1:1 (table 5). Therefore fraction 2a is clearly a heterogeneous mixture of trisaccharides. The trisaccharide structures in fraction 2a were determined as follows:

Table 5. Molar ratios of sugar recoveries from alkaline-borohydride treated glycopeptide GP-C_2

Fraction	Mannose	Mannitol	Glucose	Galactose
1. GP-C_2	15	—	1	1
2. Dowex-50	8	7	1	1
3. Paper chromatography				
Disaccharide	4	4	—	—
Trisaccharide	4	3	1	1

Experimental conditions as in Table 2. — = not present.

Fraction 2a was treated with various glycosidases as shown in figure 2 and the released sugars were quantitatively determined at each step. The results obtained by such studies are shown in table 6. From these results it is possible to deduce the structures of three trisaccharides, namely Man-Man-Man-OH, Gal-Man-Man-OH and Glc-Man-Man-OH.

Figure 2. Scheme for the action of glycosidases on fraction 2a derived from GP-C_2

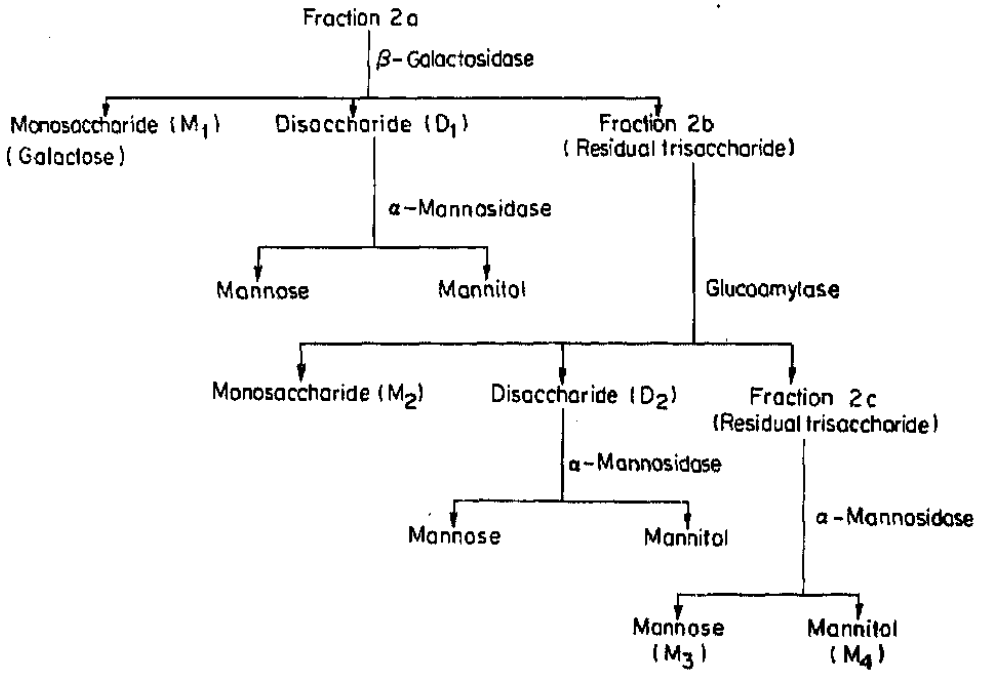


Table 6. Sugar composition of fraction 2a and its derivatives.

Fraction	Mannose	Mannitol	Glucose	Galactose
	(mol./mol. of glycopeptide)			
2a	4.00	2.64	0.60	0.56
M_1	—	—	—	0.50
D_1	0.50	0.50	—	—
2b	3.38	2.00	0.56	—
M_2	—	—	0.52	—
D_2	0.50	0.50	—	—
2c	2.78	1.40	—	—
M_3	2.70	—	—	—
M_4	—	1.30	—	—

Fractions mentioned were obtained from 2a by the procedure in figure 2. — = not present.

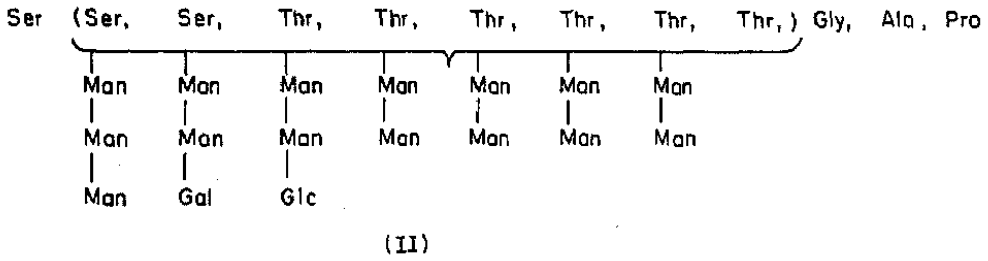
Amino acid composition: Amino acid composition of glycopeptide GP-C_2 before and after alkaline borohydride treatment is given in table 7. Nearly 5 mol of threonine and 2 mol of serine were destroyed and this was almost equal to the mannitol formed in the system. Therefore 7 residues of mannitol are involved in 0-glycosidic linkage with 5 residues of threonine and 2 residues of serine.

The data for GP-C_2 show that it contained per mol, 4 units of Thr/Ser-Man-Man, 1 unit of Thr/Ser-Man-Man-Man, 1 unit of Thr/Ser-Man-Man-Glc and 1 unit of Thr/Ser-Man-Man-Gal. Based on these data, a tentative structure (II) is proposed forGP-C_2.

Table 7. Effect of alkaline borohydride treatment on amino acid composition of GP-C_2.

Component	Original (mol./mol.)	After alkaline borohydride treatment
Threonine[a]	7	2
Serine[a]	3	1
Proline	1	1
Glycine	1	1
Alanine	1	2
α-amino-butyrate	—	1

[a] Values not corrected for any destruction by acid hydrolysis. — = not present.



(II)

Structure of type 2 glycopeptides (GP-C_{3a} and GP-C_{3b})

Glycopeptide GP-C_{3a} has been shown to contain threonine, serine, proline, mannose, glucose and glucosamine in the molar ratio 6:3:1:11:1:3 (Manjunath and Raghavendra Rao, previous paper). Glycopeptide GP-C_{3b} had the same composition but one residue less of mannose. In addition, GP-C_{3b} also contained one mole each of glycine and alanine. The calculated molecular weights for GP-C_{3a} and GP-C_{3b} were 3500 and 3480, which were in good agreement with the values of 3500 and 3700 obtained by gel filtration.

Structure of glycopeptide GP-C_{3a}: This glycopeptide yielded mono-, di- and trisaccharides on treatment with alkaline borohydride. The saccharides were separated and their composition determined as before. The results are shown in table 8. Approximately 6 mol of mannitol were formed in the system indicating mannose to be linked with the peptide chain.

Structure of disaccharide: The disaccharide had a Man-Man-OH structure since it was cleaved by α-mannosidase.

Structure of trisaccharides: The trisaccharide fraction designated as fraction 3a contained Man, Glc, GlcN and Man-OH, and hence was heterogeneous. The structure of trisaccharides in fraction 3a was determined following the scheme

shown in figure 3. The results obtained are indicated in table 9. From these results two trisaccharides were deduced to occur, namely Glc-Man-Man-OH and GlcNAc-Man-Man-OH.

Table 8. Molar ratios of sugar recoveries from alkaline-borohydride treated glycopeptide GP-C_{3a}.

Fraction	Mannose	Mannitol	Glucose (mols/mol)	Glucosamine[a]
1. GP-C_{3a}	11	—	1	3
2. Dowex-50 eluate	5	5	1	3
3. Paper Chromatography				
Monosaccharide	—	1	—	—
Disaccharide	1	1	—	—
Trisaccharide	3	3	1	2-3

Experimental conditions as in table 2.

[a] Determined as N-acetyle glucosamine by Reissig *et al,* (1955).

Figure 3. Scheme for the action of glycosidases on fraction 3a and its derivatives.

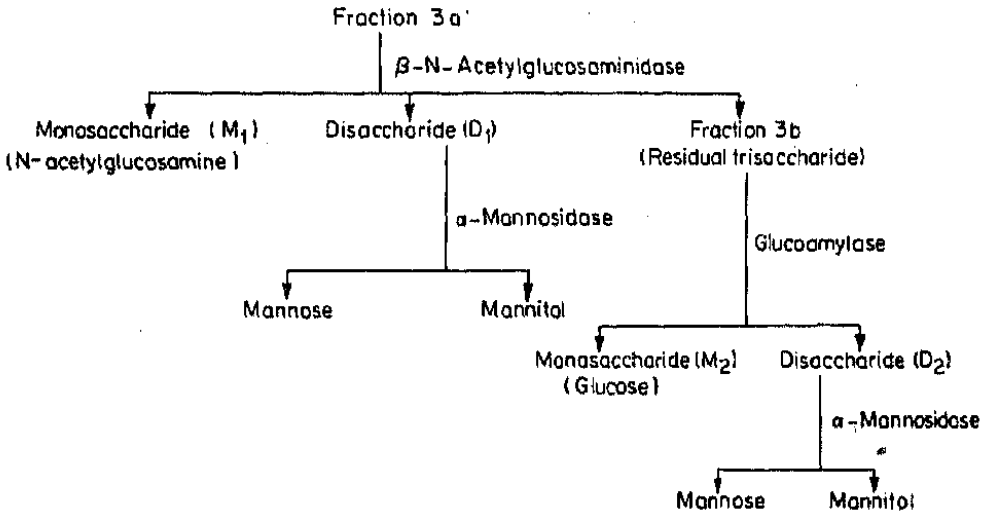


Table 9. The composition of fraction 3a and its derivatives.

Fraction	Mannose	Mannitol	Glucose	N-acetyl-Glucosamine
			(mol./mol. of glycopeptide)	
3a	3.48	3.48	0.72	2.72
M_1	—	—	—	2.60
D_1	2.63	2.63	—	—
3b	0.65	0.65	0.65	—
M_2	—	—	0.60	—
D_2	0.60	0.60	—	—

The fractions were obtained from 3a by the procedure in figure 3. GlcNAc was determined according to Reissig *et al.* (1955).

Amino acid composition: Glycopeptide GP-C_{3a} after treatment with alkaline borohydride resulted in the loss of approximately 4 residues of threonine and 2 residues of serine (table 10). These are therefore involved in the linkage.

Data obtained for GP-C_{3a} suggest that it contained approximately 1 unit of Thr/Ser-Man, 1 unit of Thr/Ser-Man-Man, 1 unit of Thr/Ser-Man-Man-Glc and 3 units of Thr/Ser-Man-Man-GlcNAC. Based on these results, a tentative structure (III) is proposed for this glycopeptide.

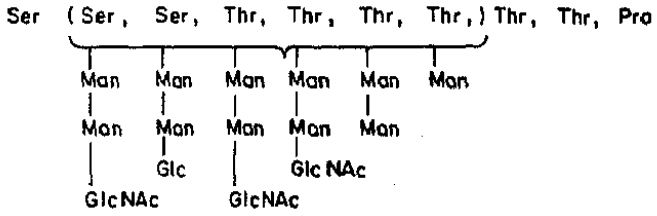


Table 10. Effect of alkaline borohydride treatment on amino acid composition of GP-C_{3a}.

Component	Original	After alkaline borohydride treatment
	(mols/mol)	
Threonine[a]	6	2
Serine[a]	3	1
Proline	1	1
Alanine	—	2
Glycine	—	—
α-amino-butyrate	—	1

[a] Values not corrected for any destruction by acid hydrolysis.

Structure of glycopeptide GP-C_{3b}: Because of low yields of glycopeptide GP-C_{3b}, no quantitative data could be obtained on this glycopeptide. However, some qualitative results were obtained.

Treatment of GP-C_{3b} with alkaline boroKydride eliminated all the carbohydrate from the glycopeptide. This also resulted in the loss of nearly 60-70% of both serine and threonine. Mannitol was detected in the system. These results therefore indicated the occurence of O-glycosidic linkages involving threonine and serine with mannose. From the ß-eliminated carbohydrate fraction mono- and trisaccharide fractions were isolated. The trisaccharide fraction, on treatment with ß-N-acetylglucosaminidase, released N-acetyl-glucosamine. Therefore trisaccharide structures similar to those present in GP-C_{3a} seem to be present in glycopeptide GP-C_{3b} also. It appears reasonable to conclude that the glycopeptide GP-C_{3b} has a structure similar to GP-C_{3a}, but differs from it since it contained an additional residue each of alahine and glycine.

Structure of type 3 glycopeptides (GP-D_1 and GP-D_2)

Glycopeptide GP-D_1: Glycopeptide GP-D_1 was shown to contain threonine, serine, proline, mannose, glucose, galactose and xylose in the molar ratio approximately 4:4:1:8:1:1:1 (Manjunath and Raghavendra Rao, previous paper).

Fractional amounts of other amino acids were also present. This glycopeptide yielded only di- and trisaccharides on treatment with alkaline borohydride. The compositions of these fractions are shown in the table 11. Approximately 4 mol of mannitol were formed in the system indicating mannose to be involved with the peptide chain.

Structure of disaccharide: As before, the disaccharide was identified as Man-Man-OH.

Table 11. Molar ratios of sugar recoveries from alkaline borohydride treated glycopeptide GP-D_1

Fraction	Mannose	Mannitol	Glucose	Galactose	Xylose
GP-D_1	8	—	1	1	1
Dowex-50	4	4	1	1	1
Paper Chromatography Disaccharide	1	1	—	—	—
Trisaccharide	3	3	1	1	1

Experimental conditions as in Table 2. Monosaccharides and derivatives were determined as alditol acetates.

Structure of trisaccharide: The trisaccharide fraction (designated fraction 4a) consisted of mannose, mannitol, glucose, galactose and xylose and was hence heterogeneous. The structures of the trisaccharides in fraction 4a were established following the scheme shown in figure 4 and the results obtained are shown in table 12. From these results, structures of three trisaccharides were deduced as Gal-Man-Man-OH, Glc-Man-Man-OH and Xyl-Man-Man-OH.

Figure 4. Scheme for the action of glycosidases on fraction 4a and its derivatives.

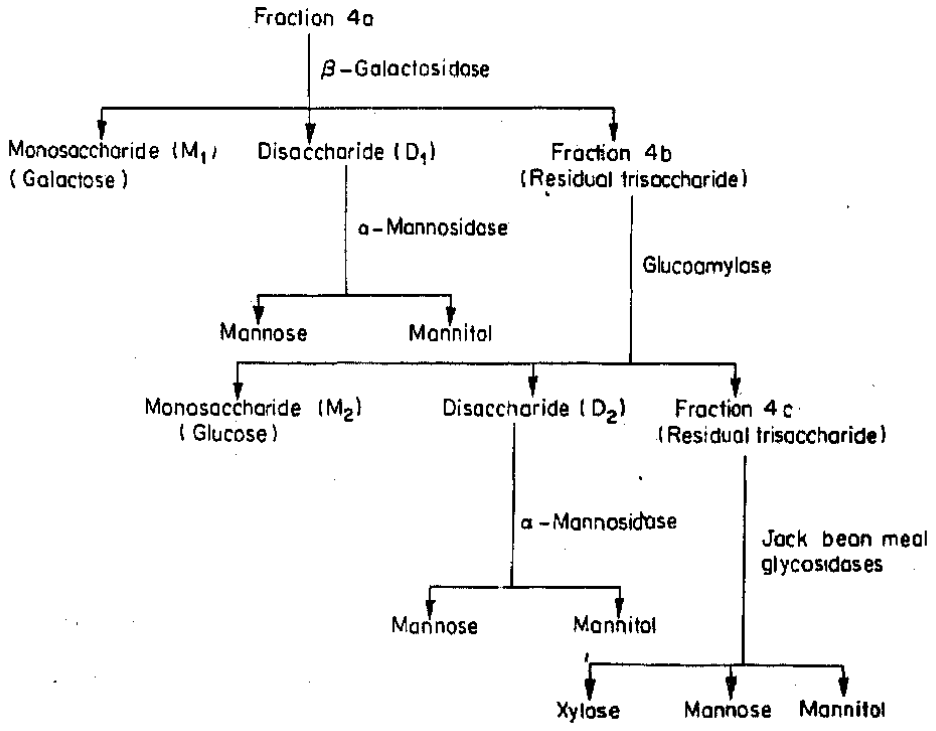


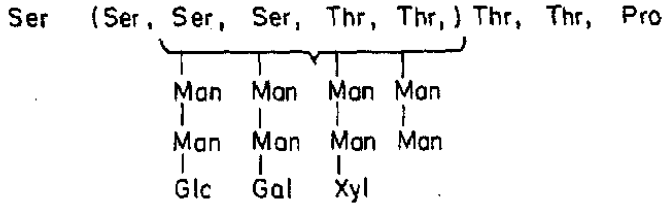
Table 12. Sugar composition of fraction 4a and its derivatives.

Fraction	Mannose	Mannitol	Glucose	Galactose	Xylose
			(mol./mol. of glycopeptide)		
4a	3.64	3.64	1.3	1.08	1.28
M_1	—	—	—	0.98	—
D_1	0.98	0.98	—	—	—
4b	2.50	2.48	1.2	—	1.20
M_2	—	—	1.2	—	—
D_2	1.20	1.20	—	—	—
4c	1.10	1.10	—	—	1.10
M_3	—	—	—	—	1.00
D_3	1.02	1.02	—	—	—

The fractions were obtained from 4a by the procedure in figure 4.

Amino acid composition: Amino acid composition after treatment with alkaline borohydride is shown in Table 13. Approximately 2 residues each of threonine and serine disappeared during the treatment and these are therefore involved in the O-glycosidic linkage. Therefore there is one unit each of Thr/Ser-Man-Man, Thr/Ser-Man-Man-Glc, Thr/Ser-Man-Man-Gal and Thr/Ser-Man-Man-Xyl.

On the basis of these results'a tentative structure (IV) is proposed for GP-D_1



(IV)

Table 13. Effect of alkaline borohydride treatment on amino acid composition of GP-D_1.

Component	Original	After alkaline borohydride treatment
	(mol./mol.)	
Threonine[a]	4	2
Serine[a]	4	2
Proline	1	1
Glycine	—	—
Alanine	—	1
α-amino-butyrate	—	1
Tyrosine	—	—

[a] Values uncorrected for any destruction by acid hydrolysis.

Glycopeptide GP-D_2: The very limited amount of GP-D_2 available did not permit detailed investigations on this glycopeptide. On chemical analysis, this glycopeptide showed mannitol, galactose, threonine, serine, proline, glycine and xylose in the molar ratio 4:1:4:4:1:1:0.3. Treatment of GP-D_2 with alkaline borohydride eliminated all the carbohydrate,, besides resulting in the loss of nearly 45-50% of both threonine and serine. Mannitol was detected in the system. These results again indicated an O-glycosidic linkage similar to GP-D_1. The ß-eliminated product when chromatographed on paper gave spots corresponding to mono-, di- and trisaccharides. The monosaccharide was identified as mannitol and the disaccharide as Man-Man-OH. The trisaccharide fraction contained mannitol, mannose, galactose, traces of glucose and xylose indicating heterogeneity of the trisaccharide. On the basis of these data it may be inferred that GP-D_2 has a structure similar to GP-D_1.

Discussion

The present study was directed at examining the nature of linkage between carbohydrate and peptide moieties, and the structure of the oligosaccharide, following which an average structure for each glycopeptide has been proposed. Four of the six glycopeptides isolated, namely GP-C_1, GP-C_2, GP-C_{3a} and GP-D_1, were obtained in substantial quantity. Only these glycopeptides were investigated in detail. Although no extensive studies were made on GP-C_{3b} and GP-D_2, the available data indicate that GP-C_{3b} has a structure similar to GP-C_{3a} and GP-D_2 was similar to GP-D_1. No work on the sequence of amino acids in the glycopeptides except the determination of the N-terminal amino acids has been conducted.

Nature of linkage

When the glycopeptides were treated with alkaline borohydride, a decrease of serine and threonine with a simultaneous increase in alanine and *de novo* appearance of α-amino butyric acid and mannitol was noted with all the glycopeptides. Besides, mannitol was the only sugar alcohol obtained. Clearly the carbohydrate moieties in all the glycopeptides are O-glycosidically linked through mannose to the hydroxyls of serine and threonine. However, the number of such linkages differed for each type of glycopeptide. Thus type 1, type 2 and type 3 glycopeptides contained 7, 6 and 4 O-glycosidic linkages, respectively. In all of them, two linkages involved serine and mannose and the rest involved threonine and mannose.

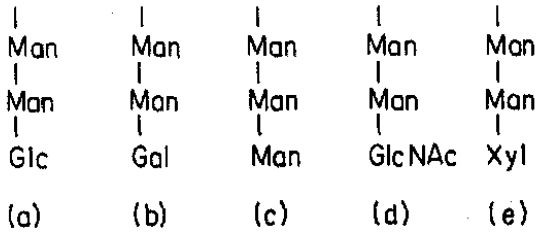
The presence of N-acetylglucosamine might indicate a linkage with asparagine. However there was no trace of aspartic acid in the acid hydrolyzate of the glycopeptides. Besides, the GlcNAc-Asn linkage is resistant to alkaline borohydride (Spiro, 1966). Secondly, the trisaccharides obtained by ß-elimination did not contain N-acetyl glucosaminitol but only N-acetylglucosamine which could be released from the trisaccharides by N-acetylglucosaminidase. These facts ruled out any direct linkage of N-acetylglucosamine with the peptide backbone in the present instances. Therefore the linkage of the carbohydrate to the peptide chain seemed to be exclusively through the O-glycosidic bonds with the aliphatic hydroxy amino acids. These results in general are in conformity with those of Lineback *et al.* (1972) and Pazur *et al.* (1971) on the glucoamylases of *A. niger* and

A. foetidus respectively. However their results were based on alkaline borohydride treatment of glucoamylase itself and not the isolated glycopeptides.

The glycopeptides of glucoamylase of *R. javanicus* (Watanabe and Fukimbara, 1974a, b, and 1975; Watanabe, 1976) seem to be quite distinct from the ones described here. Two of them had GlcNAc-Asn bonds whereas the third glycopeptide had O-glycosidic bonds between aliphatic hydroxy amino acids and mannose. If this proves typical of the *Rhizopus* glucoamylases, then these differ very markedly from the enzyme from black *Aspergilli* in the linkage of the carbohydrates to the peptide backbone.

Structure of the Oligosaccharides and heterogeneity

Sequential digestion of the trisaccharides with specific glycosidases revealed at least 5 different trisaccharide structures:



(V)

None of the glycopeptides investigated here contained all the five trisaccharide structures (a) to (e). Type 1 glycopeptides contained structures (a), (b) and (c); type 2 glycopeptides contained structures (a) and (d), and type 3 glycopeptides contained structures (a), (b) and (e). Thus, the glycopeptides differ from one another in their trisaccharide structures. This type of heterogeneity has been described as 'central heterogeneity' (Clamp, *et al.*, 1968). In addition to trisaccharides, all the glycopeptides in the present investigation contained either mannose or Man-Man- or both in varying amounts. These are probably the products of incomplete glycosylation. Though a molar monosaccharide composition in each glycopeptide has been assigned, in most of the glycopeptides each constituent monosaccharide occurred in a non-integral (fractional) ratio. This has been termed "microheterogeneity" and has been discussed already (Manjunath and Raghavendra Rao, previous paper). Even the very simple Glc-Gal-disaccharide unit of collagens manifests "microheterogeneity" since units consisting only of the internal galactose occur in addition to the disaccharide (Spiro, 1969). The microheterogeneity observed in the carbohydrate structures of glycopeptides is consistent with the present concept of glycoprotein biosynthesis in which it is proposed that each sugar residue is added stepwise to a growing chain (Spiro, 1970 and 1973; Sharon, 1974). Thus the microheterogeneity found in so many glycoproteins might represent oligosaccharide chains in varying stages of completion, or resulting from transglycosylations that do not have an absolute specificity (Spiro, 1970 and 1973; Montgomery, 1972). With its large number of units, heterogeneity in glucoamylase may be even more apparent than in proteins with fewer units, for, the presence of multiple attachment sites along the peptide chain may lead to a greater number of sterically unfavourable conditions in which glycosylation may be impeded.

Two instances which have provided information on the structure of carbohydrate moieties of glycoamylases are due to Lineback *et al.,* (1972) and Watanabe and Fukimbara (1974a, b, 1974; Watanabe, 1976). Lineback *et al.,* (1972) isolated carbohydrate units after treating *A. foetidus* (Diazyme) glycoamylase I and glucoamylase II with alkaline borohydride and obtained trisaccharides and also a small fraction of mono- and disaccharides. They interpreted their results to mean that both glucoamylase I and glucoamylase II contained only trisaccharide structures of the types Man-Man-Man- and Man-Glc-Man- since they found that the trisaccharide structures were hydrolyzed partially to give mono- and disaccharide units. This apparently was due to the acid condition they used (0.1 N acetic acid) during the isolation of the oligosaccharides. In our experiments, it is quite unlikely that there is any hydrolysis of the trisaccharides because incubating isolated oligosaccharides or glycopeptides in 0.1 N acetic acid did not release any monosaccharides and therefore there was no cleavage of glycosidic linkage. Other trisaccharide structures involving galactose were also indicated by Lineback *et al.,* (1972) in their experiment and the trisaccharide units must therefore be heterogeneous in nature. One difference lies in the sequence of monosaccharides in the glucose containing trisaccharide which in the present studies is clearly shown to be Glc-Man-Man-. Moreover, the Diazyme glucoamylase did not contain trisaccharides containing Xyl and GlcNAc, since the native protein itself did not carry these sugars.

One of the three glycopeptides (GP-II) from *R. javanicus* glucoamylase contained six disaccharide units linked O-glycosidically to 5 threonine and 1 serine residues (Watanabe and Fukimbara, 1974a, 1974b and 1975). These glycopeptides bear an obvious resemblance to the ones described here. *R. javanicus* glycopeptide GP-II contained only Man-Man-disaccharides, whereas the present *A. niger* glucoamylase glycopeptides contained structures varying from monosaccharides to trisaccharides. Further, the trisaccharides in *A. niger* glucoamylase were heterogeneous.

On the other hand, the carbohydrate moiety of two other glycopeptides (GP-Ia and GP-Ib) of *R. javanicus* glucoamylase have a complex structure (Watanabe, and Fukimbara, 1974a, 1974b and 1975; Watanabe, 1976). In glycopeptides GP-Ia and GP-Ib the carbohydrate was shown to be present in the form of large and branched heterosaccharides. Carbohydrate-peptide link was through an N-glucosidic linkage between N-acetylglucosamine and asparate. The carbohydrate unit in this instance resembled that of ovalbumin (Montgomery, 1972; Marshall and Neuberger, 1970) or of α-amylase (Marshall and Neuberger, 1970). However such heteropolysaccharide structures were totally absent from *A. niger* glucoamylase.

Glycopeptide types in glucoamylase II

Although glucoamylase II contained a large number of glycopeptides which differed in the monosaccharide composition, an attempt was made to allocate carbohydrate units in terms of the relative abundance of each type of glycopeptide isolated. This might give us a picture of the number of mol of each glycopeptide per mole of glucoamylase II (table 14). Thus one mol of glucoamylase II appeared to contain an average two mols of type 1, one of type 2 and one of type 3 glyco-

Table 14. Glycopeptide units of glucoamylase II.

	Monosaccharide residues				
	Extrapolated from experimentally obtained data on resolved glycopeptides[d]				Present in original glycoprotein[e]
	GP-Type 1 2 units[a]	GP-Type 2 1 unit[b]	GP-Type 3 1 unit[c]	Total	Total
Mannose	26	11	8	45	45
Glucose	2	1	1	4	4
Galactose	2	0	1	3	3
Xylose	0	0	1	1	2
Glucosamine	0	3	0	3	3

The results are expressed as residues of monosaccharides experimentally found in each glycopeptide corrected to the nearest whole number, from which the sum of the residues in the postulated glycopeptide units has been totalled. This figure is compared with the number of residues experimentally found in the original glycoprotein.

GP, Glycopeptide

[a] Equal quantities of GP-C_1, and GP-C_2, hence total values employed.
[b] Almost 80-90% GP-C_{3a}, hence this value employed.
[c] Almost 80-90% GP-D_1, hence this value employed.
[d] Manjunath and Raghavendra Rao, previous paper.
[e] Manjunath and Raghavendra Rao, (1979).

peptide units. The number of mols of monosaccharide per mol of the glucoamylase calculated on the basis of the above allocation of glycopeptide units agreed very well with the analytical values for the same. The minor glycopeptides may perhaps replace the major units in a smaller number of protein molecules, and may represent incomplete or aberrant units.

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