

Studies on carbohydrate moieties of *Aspergillus niger* glucoamylase II: Isolation, purification and characterization of glycopeptides

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Abstract. Six glycopeptide fractions namely GP-C₁, GP-C₂, GP-C_{3a}, GP-C_{3b}, GP-D₁, and GP-D₂ were isolated after exhaustive digestion of glucoamylase II (Glucozyme) from *Aspergillus niger* with pronase. They were purified using gel-filtration, high-voltage paper electrophoresis and ion-exchange chromatography on Dowex-50 and Dowex-1. They appeared homogeneous on electrophoresis under different conditions of pHs. The molecular weights ranged from 1600 and 4000 for these glycopeptides. All of them contained serine at the N-terminal end. Serine and threonine were the major amino acids with glycine, alanine, proline and tryptophan present as minor constituents. Carbohydrate analysis revealed the presence of different sugars. Based on this, the glycopeptides were grouped into three types: (1) GP-C₁ and GP-C₂ containing mannose, glucose and galactose; (2) GP-C_{3a} and GP-C_{3b} containing mannose, glucose and glucosamine; and (3) GP-D₁ and GP-D₂, containing mannose, glucose, galactose and xylose. Most sugar constituents in each glycopeptide occurred in non-integral ratios implying a microheterogeneity of the carbohydrate moiety in *Aspergillus niger* glucoamylase II.

Keywords. Glucoamylase; *Aspergillus niger*; glycopeptides.

Introduction

Glucoamylase (α -1, 4-glucan glucohydrolase EC. 3.2.1.3) from fungal sources is a microbial glycoprotein that has received considerable attention particularly because it is used in the commercial production of dextrose. It has been isolated from fungal sources and studied by several investigators. In most instances two forms have been reported (glucoamylase I and glucoamylase II). The gross carbohydrate composition of these glycoproteins from *Aspergillus* species has been investigated (Pazur, *et al.*, 1971; Lineback *et al.*, 1972; Venkataramu *et al.*, 1975; Manjunath and Raghavendra Rao, 1979). Lineback *et al.*, (1972) have

Abbreviations used: FDNB, 1-Fluoro-2, 4-dinitrobenzene; DNP-amino acids, dinitrophenyl amino acids; TNBS, trinitrobenzene sulphonic acid; Gly, glycine; Val, valine; Arg, arginine.

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provided some information on the structure of the carbohydrate moieties. However, further studies such as the isolation of glycopeptides, the sequence and distribution of the carbohydrate moieties were not conducted. Only one such study seems to have been made on the glucoamylase of *Rhizopus javanicus* (Watanabe and Fukimbara, 1974a, 1974b, 1975; Watanabe, 1976).

In the present investigation, an attempt has been made to prepare glycopeptides from *A. niger* glucoamylase II in order to obtain information on the size, number, composition and structure of the carbohydrate units and their distribution in the enzyme molecules, and the nature of the bonds linking them to polypeptide.

Materials and methods

Dowex-1×2 (200-400 mesh), Dowex-50 ×2 (200-400 mesh) and 1-fluoro-2,4-dinitrobenzene (FDNB) were purchased from the Sigma Chemical Company, St. Louis, Missouri, USA. p-Dimethylaminobenzaldehyde (recrystallised by us according to Boas, 1953) and dinitrophenyl amino acids (DNP-amino acids) were from the Council of Scientific and Industrial Research Centre for Biochemicals, V.P. Chest Institute, Delhi. Pronase (1,170,800 tyrosine units/g) was from Kaken Chemicals Co. Ltd., Tokyo, Japan. Biogels were obtained from Bio Rad, Granada Hills, USA. Trinitrobenzene sulphonic acid (TNBS) was purchased from Eastman Chemicals, Rochester, New York, USA. Glucose, galactose, maltose, pyridine (distilled over KOH and ninhydrin before use), phenol (distilled over Zn turnings before use) and benzidine were from the British Drug House, Bombay. All other reagents and chemicals used were of analytical grade unless otherwise described.

Glucozyme', a crude preparation of glucoamylase prepared from *A. niger* was obtained from Anil Starch Products, Ahmedabad. Glucoamylase II from this source was purified to homogeneity as described earlier (Manjunath and Raghavendra Rao, 1979).

Total carbohydrate by phenol-H₂SO₄ reaction, neutral sugars by gas liquid chromatography, amino sugars by the Elson Morgan reaction were determined as described earlier (Manjunath and Raghavendra Rao, 1979).

For amino acid analysis, glycopeptides were hydrolysed for 24 h at 110°C with constant boiling HCl (5.8 N) in evacuated tubes. Amino acids were determined in a Beckman Model 120 B amino acid analyser (Kedenburg, 1971). Free amino acids were determined using trinitrobenzenesulphonic acid as described by McKelvey and Lee (1969). The N-terminal amino acid was determined by the procedure of Sanger (Schroeder, 1967).

Paper electrophoresis of peptides was carried out on Whatman No. 3 MM paper (27×60cm) at various pH (Ambler, 1963): (a) pH 1.8, acetic acid-formic acid-water, 10:3:107 (v/v) (b) pH 3.4, pyridine-acetic acid-water 1:8:9 (v/v) and (c) pH 6.4, pyridine-acetic acid-water, 225:7.5:2018 (v/v). Electrophoresis was usually carried out for a period of 1 h at 3000 V, 90-110 mA in a Savant high-voltage electrophoresis apparatus. Peptides and amino acids were detected by staining with ninhydrin-cadmium acetate reagent (Heilmann, *et al.*, 1957). Carbohydrate was detected using the periodate-benzidine reagent (Gorden, *et al.*, 1956).

Molecular weights of glycopeptides were determined by Biogel filtration (Spiro, *et al.*, 1976). Biogel P-10 equilibrated with 0.1 M pyridine-acetate buffer pH 5.0 was packed into a column (0.9× 126cm). The glycopeptides (0.1 mg) were dissolved in 200 µl of 0.1 M pyridine-acetate buffer and applied to the column. They were eluted with the same buffer and the fractions containing carbohydrates were detected by the phenol-H₂SO₄ reaction. Blue Dextran was used to determine the void volume. Glucose, raffinose, Gly-Val-Arg, insulin, glycopeptide (unit A and B) prepared from the Pronase digest of thyroglobulin and glycopeptide prepared from the pronase digest of ovalbumin were used as standards.

Molecular weights of some glycopeptides were also determined by analytical ultracentrifugation (Klainer and Kegeles, 1955). For centrifugation analysis, the glycopeptide (1%) was dissolved in 0.01M potassium phosphate buffer (pH 7.0) containing 0.09 M KCl and the Archibald runs were made at 21740 rpm (Jamieson, 1965).

Results

Preparation of glycopeptides from glucoamylase II

Treatment with pronase: Initially attempts were made to prepare glycopeptides from native glucoamylase II. The native enzyme was treated with pronase (ratio of pronase: glucoamylase II, 1:50) (pH 7.5-8.0) for 20 h and the digest was then passed through a calibrated Sephadex G-200 column. The first fraction eluted had a molecular weight of 65,000 (while the native enzyme had a molecular weight of 80,000), contained most of the carbohydrate and had more than 60% the original glucoamylase, 1:200). The reaction mixture was incubated for 36 h at 37°C in the amino acids but was not digested any further and was too large for structural studies.

Therefore glucoamylase II was denatured before proteolysis (Anai *et al.*, 1966) with the following modification. A solution of 1.4 g of GA II in 56ml water was brought to pH 1.9 by carefully adding 6 N HCl at 25°C. The mixture was then kept at 60°C for 1 h, cooled to room temperature and the pH rapidly adjusted to 8.0 with 2 N NaOH. Calcium acetate was added to the cloudy suspension to a concentration of 0.01 M and proteolysis was started by adding 7.0 mg of pronase (pronase: glucoamylase, 1:200). The reaction mixture was incubated for 36 h at 37°C in the presence of toluene to prevent microbial growth. The pH of the solution was maintained at 8.0 by the addition of 2 N NaOH. The progress of the digestion was followed by TNBS analysis of the increase in amino groups and it levelled off after 33 to 36 h. The digest was then concentrated by lyophilization.

Biogel filtration: The lyophilized pronase digest was subjected to gel filtration on a column of Biogel P-30 as described in figure 1a. The recovery of the applied sample was 87% with respect to neutral sugars. The major carbohydrate peak (fractions 28-55, pooled and lyophilized), designated GP-A, contained 85% carbohydrate.

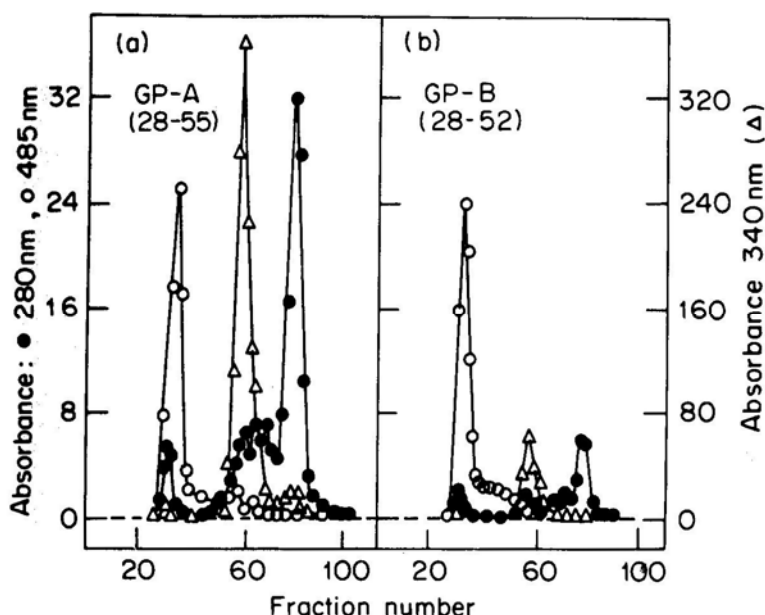


Figure 1. Gel filtration patterns of first (a) and second pronase(b) digest of glucoamylase II.

A column of Biogel P-30 (2×120cm) equilibrated with 0.01 N acetic acid was used. Fractions of 5ml were collected: Carbohydrate (O) was monitored by the phenol-H₂SO₄ reaction; free amino groups (Δ) detected by TNBS method. Absorbance (●) at 280nm.

Pronase P used in the present investigation was free of carbohydrases and contained approximately 2.5% carbohydrate (phenol-H₂SO₄ reaction). During gel filtration on P-30, pronase elutes with the carbohydrate-rich fraction (GP-A). However, the amount of pronase used was only 1% of glucoamylase II protein. This contributed only a negligible amount (approximately 0.35 mg) of carbohydrate compared to the glucoamylase carbohydrate (250 mg).

Second pronase treatment and Biogel filtration: Fraction GP-A was digested a second time with pronase as described above. The digest was chromatographed again on a Biogel P-30 column. The elution profile is shown in figure 1b. The carbohydrate containing fractions (designated GP-B) were pooled and concentrated by lyophilization. The recovery of neutral sugars was almost quantitative.

Preparative paper electrophoresis: GP-B was further purified by preparative paper electrophoresis by streaking 1 mg of material (as glucose)/cm width on Whatman No. 3 MM paper and electrophoresing at pH 1.8 (43 volts/cm for 60 min). The glycopeptide zones were located on test strips with periodate-benzidine and ninhydrin reagents. Two distinct glycopeptide bands were observed: GP-C, 10cm and GP-D, 4.5cm from the cathode. The areas of the electropherogram showing a positive reaction to both the ninhydrin and periodate reagents were cut out separately, eluted with water and freeze-dried. Several other ninhydrin-positive

bands on the same electropherogram gave negative tests with the periodate reagent. The total recovery of glycopeptides was about 88% based on the carbohydrate content.

Chromatography on Dowex-50 \times 2: The glycopeptide GP-C was chromatographed as shown in figure 2a. Three carbohydrate peaks were observed in the effluent. The distribution of carbohydrate as percentage of the total recovered neutral sugar is as follows: peak 1 (GP-C₁), 33%; peak 2 (GP-C₂), 20%; and peak 3 (GP-C₃), 38%. The fractions constituting these peaks were separately pooled, concentrated and further purified on a column of Dowex-1 \times 2.

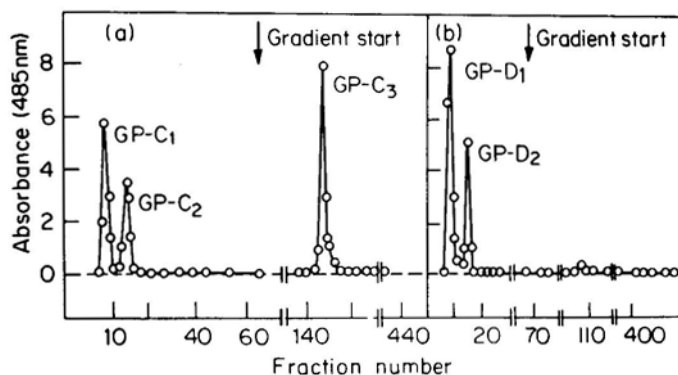


Figure 2. Dowex-50 \times 2 chromatographic patterns of glycopeptides GP-C(a) and GP-D(b).

The cation exchange resin Dowex-50W \times 2 was prepared as described by Schroeder (1967). The resin was equilibrated in 2mM pyridine-acetate buffer pH 3.1 and packed into a 0.9 \times 110 cm column. The sample of glycopeptide was applied with the above buffer and the column was washed with same buffer. Around fraction 70, a linear gradient of 500ml each of 2mM pyridine-acetate pH 3.1 (in the mixing chamber) and 700mM pyridine-acetate pH 5.8 (in the reservoir) was started, resulting in both salt and pH gradients. Fractions of 3ml were collected at a flow rate of 45ml/h. Glycopeptides were detected by the phenol-sulphuric acid reaction.

GP-D was also similarly chromatographed. The chromatographic pattern obtained is shown in figure 2b. This material which was homogeneous by electrophoresis, gave rise to two carbohydrate-positive peaks. The distribution of carbohydrate as percentage of the total recovered neutral sugar is: peak 1 (GP-D₁), 80% and peak 2 (GP-D₂), 20%. The fractions were separately pooled and concentrated, and further purified on a column of Dowex-1 \times 2.

Dowex-1 \times 2 chromatography: The glycopeptides GP-C₁, GP-C₂, GP-C₃, GP-D and GP-D₂ were chromatographed separately on Dowex-1 \times 2. The elution pattern was similar to that described by Hilschman and Craig (1965) and is shown in figure 3. Glycopeptides GP-C₁, GP-C₂, GP-D₁ and GP-D₂ all eluted as one major and several minor peaks. The major fractions were pooled separately and freeze-dried. The minor glycopeptide fractions amounted to about 3% of the total glycopeptides and were not therefore pursued any further. Glycopeptide GP-C₃ was eluted in two carbohydrate-containing fractions (Figure 3c). The distribution of

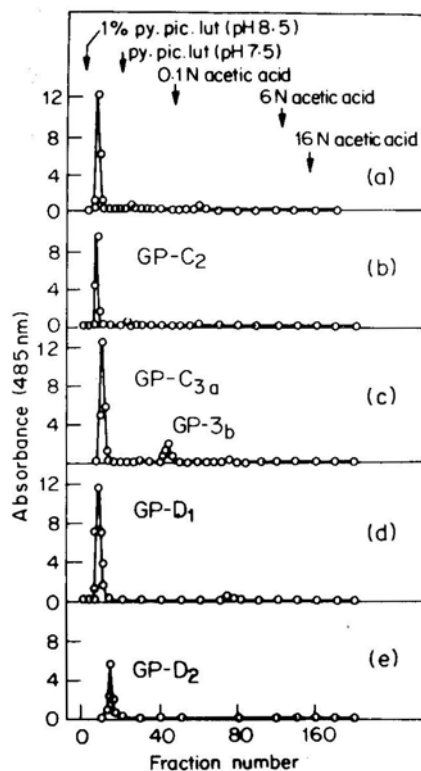


Figure 3. Dowex-1×2 Chromatographic separation of glycopeptides. a, GP-C; b, GP-C₂; c, GP-C₃; d, GP-D, and e, GP-D₂.

The anion exchange resin Dowex-1×2 was prepared as described by Schroeder (1967). The resin was equilibrated in aqueous solution containing 1% pyridine-picoline-lutidine, and sufficient acetic acid to reach a pH 8.5 and packed into a 0.9×60 cm column. The glycopeptide was applied in the above buffer and elution was carried out using different buffers as indicated by arrows. Fractions of 2.1ml were collected at a flow rate of 45ml/h. Glycopeptides were detected by the phenol-H₂SO₄ acid reaction.

the recovered carbohydrate was: peak 1 (GP-C_{3a}), 83% and peak 2 (GP-C_{3b}), 17%. Table 1 gives the yield and total recovery of glycopeptides during each step of purification.

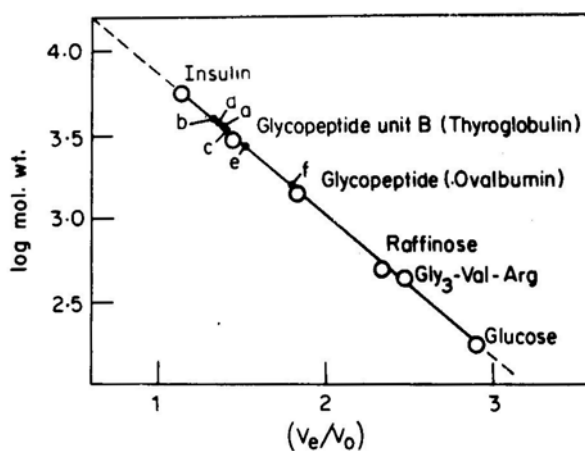
Properties of glycopeptides

Homogeneity: When subjected to electrophoresis at pHs 1.8, 3.4 and 6.4, the purified glycopeptides moved as single bands.

Molecular weight: A plot of log molecular weight against V_e/V_o of the standards is shown in figure 4. From the graph, the molecular weights calculated for glycopeptides GP-C₁, GP-C₂, GP-C_{3a}, GP-C_{3b}, GP-D₁ and GP-D₂ were 3600, 3900, 3500, 3700, 2750 and 1600 respectively.

Table 1. Yield and total recovery of glycopeptides.

Fraction	Yield (mg)	Overall recovery ^a %
Pronase digest (from 1.4 g of glucoamylase II)	250	100
GP-A (Biogel P-30)	212	85
GP-B (Biogel P-30)	200	80
High voltage electrophoresis		
GP-C	145	70
GP-D	30	
Dowex-50×2 chromatography		
GP-C ₁	35	
GP-C ₂	30	52
GP-C ₃	40	
GP-D ₁	20	
GP-D ₂	5	
Dowex-1×2 chromatography		
GP-C ₁	30	
GP-C ₂	25	
GP-C _{3a}	25	42
GP-C _{3b}	5	
GP-D ₁	16	
GP-D ₂	4	

^a As glucose equivalent.**Figure 4.** Molecular weight determination of glycopeptides on Biogel P-10

Experimental details are given in Materials and Methods. V_e , elution volume; V_o , void volume
a, GP-C; b, GP-C₂; c, GP-C_{3a}; d, GP-C_{3b}; e, GP-D₁; f, GP-D₂.

The molecular weight of two glycopeptides namely GP-C₂ and GP-C_{3a}, was also determined by the Archibald method. Values of 4000 (± 300) and 3800 (± 200) were obtained respectively. A partial specific volume of 0.664 ml/g was employed in the molecular weight calculations.

Amino-terminal end group: Serine was found to be the N-terminal amino acid in all the glycopeptides. Traces of glycine and alanine were also seen on the chromatograms.

Amino acid composition: The amino acid composition of purified glycopeptides is given in Table 2. The amino acids serine and threonine were the major components and proline, glycine and alanine were the minor ones in all of the isolated glycopeptides. Small amounts of tyrosine were also present in GP-D glycopeptides. The ratio of threonine to serine was 2.3 to 2.4 in GP-C glycopeptides, but was unity in GP-D glycopeptides. The number of residues of different amino acids present in each glycopeptides based on molecular weights determined by gel filtration are also given in Table 2. Most of the amino acids, including minor ones, occurred in non-integral molar ratios, perhaps due to destruction during hydrolysis; in some instance, less than one residue was obtained. Treatment of each glycopeptide with pronase caused no change in the amino acid composition. It is likely therefore that pronase attacks different peptide bonds at different rates, producing a mixture of glycopeptides, each of which is no longer susceptible to pronase action.

Table 2. Amino acid and carbohydrate composition of the glycopeptides.

Components	GP-C ₁ mols/ mol	GP-C ₂ mols/ mol	GP-C _{3a} mols/ mol	GP-C _{3b} mols/ mol	GP-D ₁ mols/ mol	GP-D ₂ mols/ mol
<i>Amino acids</i>						
Thr ^a	6.68(7)*	6.63(7)	5.77(6)	5.6(6)	3.98(4)	3.68(4)
Ser ^a	2.88(3)	2.73(3)	2.55(3)	2.62(3)	3.70(4)	3.52(4)
Pro	Traces	0.50(1)	0.50(1)	0.59(1)	0.68(1)	0.61(1)
Gly	0.54(1)	0.82(1)	0.42	0.66(1)	Traces	0.52(1)
Ala	Traces	0.74(1)	0.32	0.70(1)	Traces	0.19
Tyr					0.19	0.19
<i>Sugars</i>						
Man ^b	11.5(12)	14.5(15)	10.5(11)	10.36(10)	7.98(8)	3.68(4)
Glc ^b	1.18(1)	0.7(1)	0.7(1)	1.1(1)	0.96(1)	
Gal ^b	1.18(1)	0.66(1)			0.82(1)	0.51(1)
Xyl ^b					0.96(1)	0.31
GlcN ^c			2.5(3)	2.6(3)		

^a Uncorrected for destruction

^b By gas liquid chromatography as alditol acetates

^c By the Elson-Morgan reaction

* Figures in parentheses have been rounded off to the nearest integer.

Carbohydrate composition: The sugar composition determined for these glycopeptides is also given in table 2. On this basis glycopeptides appeared to belong to three distinct types: Type 1 glycopeptides, GP-C₁ and GP-C₂, contained mannose, glucose and galactose in the molar proportion 12-15:1:1 but not glucosamine or xylose. Type 2 glycopeptides, GP-C_{3a} and GP-C_{3b}, contained mannose, glucose and glucosamine in the molar proportion 10-11:1:2-3, but not galactose or xylose. Type 3 glycopeptides, GP-D₁ and GP-D₂, contained mannose, glucose, galactose and xylose in the molar proportion 3.7-8:0-1:0.5-0.8:0.3-1 but not glucosamine. In most of the isolated glycopeptides, each constituent sugar was present in a non-integral ratio.

Discussion

The glucoamylase II of *A. niger* contained 18% carbohydrate and its molecular weight was 72,000 (Manjunath and Raghavendra Rao, 1979). It contained, per mole, 45 residues of mannose, 3 of galactose, 4 of glucose, 2 of xylose and 3 of glucosamine. This is one of the many instances of glycoproteins containing so many different monosaccharides.

In order to investigate the carbohydrate moiety in the glycoprotein molecule, a convenient approach is to proteolyse the glycoprotein to a number of smaller fragments. The extent of proteolysis is likely to depend upon the size and the number of carbohydrate moieties connected to the peptide backbone, and also upon the protease used. In the present investigation, native glucoamylase II was not amenable to extensive pronase digestion: about 60% activity remained after two prolonged digestions, and the residual enzyme had a molecular weight of around 60,000. However, the denatured enzyme is susceptible to pronase action and yielded, after exhaustive digestion, four major glycopeptides that were purified to homogeneity by a combination of separation methods. The yield of the homogeneous glycopeptides was 42% of the original carbohydrate in the glycoprotein, which is fairly satisfactory. In the case of glucoamylase from *R. javanicus*, Watanabe and Fukimbara, (1973) obtained a 65% yield of glycopeptide. In the case of ovalbumin (Marshall and Neuberger, 1972) and α -amylase of *A. oryzae* (McKelvy and Lee, 1969) the yields of glycopeptides were reported to be around 80-90%.

The glycopeptides varied in their molecular weights from 1600 to 4000 and in the carbohydrate content from 50 to 70%. In *R. javanicus*, two glycopeptides had asparagine and either threonine or glycine as the amino acid components, whereas the third one had four amino acids (threonine, serine, alanine and proline) in addition to 60% carbohydrate (Watanabe and Fukimbara, 1974a. and 1974b). Such patterns of amino acid composition have been attributed to the use of a protease such as pronase with broad specificity (Watanabe and Fukimbara, 1973; Yamauchi, *et al.*, 1968; Spiro, 1962). Apparently pronase digestion proceeds to a different degree and rate in each case: perhaps the carbohydrate moieties and/or the amino acid sequence affect the extent of digestion.

The carbohydrate compositions of the glycopeptides generally differed from one another and also from that of the glycoprotein itself. All the six glycopeptides

contained mannose and glucose and were grouped into three types on the basis of the monosaccharide present: one containing galactose; the second containing glucosamine instead of galactose; and the third with galactose and xylose. Further, in most of these glyco-peptides, the constituent sugars occurred in non-integral ratios. For example, the glycopeptide GP-D₂ contained 0.33 mole of xylose and 0.51 mole of galactose per mole. Each glycopeptide fraction therefore presumably represented a family of extremely similar molecules all of broadly the same chemical composition and differing only slightly in carbohydrate composition. Since the compositional variations within each glycopeptide fraction were apparently rather small, the fraction, though not entirely homogeneous, behaved as a single compound during column chromatography and electrophoresis. Such "microheterogeneity" was reported to be a common feature of glycoproteins (Spiro, 1970; Sharon, 1974) particularly ovalbumin and α -amylase of *A. oryzae*.

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References

- Ambler, R. P. (1963) *Biochem. J.*, **89**, 349.
 Anai, M., Tkenaka, T. and Matsushima, Y. (1966) *J. Biochem. (Tokyo)*, **59**, 57.
 Boas, N. F. (1953) *J. Biol. Chem.*, **204**, 553.
 Gordon, H. T., Thornburg, W. and Werum, L. N. (1956) *Anal. Chem.*, **28**, 849.
 Heilmann, J., Barollier, J. and Watzke, E. (1957) Hoppe-Seyler's *Z. Physiol. Chem.*, **309**, 219
 Hilschmann, N. and Craig, L. C. (1965) *Proc. Natl. Acad. Sci. USA.*, **53**, 1403.
 Jamieson, G. A. (1965) *J. Biol. Chem.*, **240**, 2019.
 Kedenburg, C. P. (1971) *Anal. Biochem.*, **40**, 35.
 Klainer, S. M. and Kegeies, G. (1955) *Z. Physiol. Chem.*, **59**, 952.
 Lineback, D. R., Aira, L. A. and Harner, R. L. (1972) *Cereal Chem.*, **49**, 283.
 Manjunath, P. and Raghavendra Rao, M. R. (1979) *J. Biocsci.*, **1**, 409.
 Marshall, R. D. and Neuberger, A. (1972) in *Glycoproteins, their composition, structure and function* (ed. A. Gottschalk), (Amsterdam: Elsevier Publishing Company) Vol. 5B, p. 732.
 McKelvy, J. F. and Lee, Y. C. (1969) *Arch. Biochem. Biophys.*, **132**, 99.
 Pazur, J. N., Cepure, A., Knull, H. R. (1971) *Carbohydr. Res.*, **20**, 83.
 Sharon, N. (1974) *Scientific American*, **230**, 78.
 Schroeder, W. A. (1967) *Methods Enzymol.*, **11**, 351. 361
 Spiro, R. G. (1962) *J. Biol. Chem.*, **237**, 382.
 Spiro, R. G. (1970) *Ann. Rev. Biochem.*, **39**, 599.
 Spiro, M. J., Spiro, R. G. and Bhoryroo, V. C. (1976) *J. Biol. Chem.*, **251**, 6400.
 Venkataramu, K., Manjunath, P. and Raghavendra Rao, M. R. (1975) *Ind. J. Biochem. Biophys.*, **12**, 107.
 Watanabe, K. (1976) *J. Biochem. (Tokyo)*, **80**, 379.
 Watanabe, K. and Fukimbara, T. (1973) *Agr. Biol. Chem.*, **37**, 2755.
 Watanabe, K. and Fukimbara, T. (1974a) *Agr. Biol. Chem.*, **38**, 1643.
 Watanabe, K. and Fukimbara, T. (1974b) *Agr. Biol. Chem.*, **38**, 1973.
 Watanabe, K. and Fukimbara, T. (1975) *Agr. Biol. Chem.*, **39**, 1711.
 Yamauchi, T., Makino, M. and Yamashina, I. (1968) *Biochem. (Tokyo)*, **64**, 683.