Carbohydrate Research, 162 (1987) 257–269
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF THE CARBOHYDRATE PORTION OF THE SIALOGLYCOPEPTIDE ISOLATED FROM THE SKIN OF THE FISH *Mastacembalus armatus*

RINA GHOSH, SANTOSH KUMAR SIKDER*, AND AMALENDU DAS**
Department of Chemistry, Jadavpur University, Calcutta-700 032 (India)
(Received April 11th, 1986; accepted for publication, November 18th, 1986)

ABSTRACT

The sialoglycopeptide (PSGP-A), isolated from the degelled skin of the fish *Mastacembalus armatus*, contained sialic acids (NANA and NGNA, 13.5%), GlcNAc (19.3%), Gal (14.7%), Man (12.6%), Fuc (2.5%), and amino acids of which Asp, Ser, Thr, and Pro preponderated. Affinity chromatography showed that the two sialic acids were integral parts of the same glycopeptide molecule. The carbohydrate portion was linked through GlcNAc to Asn of the peptide core. Methylation analysis of PSGP-A and its degradation products and periodate-oxidation studies indicated a structure for the carbohydrate portion of PSGP-A which resembled those of other sialoglycopeptides having similar carbohydrate compositions.

INTRODUCTION

Sikder and Das[1] reported the isolation of a sialoglycopeptide from the skin of the fish *Mastacembalus armatus*, but did not carry out any structural investigation. We now report further purification of the sialoglycopeptide fraction isolated from the degelled skin from the same source, and a probable structure for the carbohydrate portion.

RESULTS AND DISCUSSION

The defatted dry skin of the fish *M. armatus* was extracted with hot water to remove the glycosaminoglycan portion[1]. The residual skin was digested with pronase for 2 h and the released sialoglycopeptide (SPG) was purified on CM-Sephadex and DEAE-cellulose. The resulting sialoglycopeptide (SGP_1) had a protein-to-sialic acid (SA) ratio of 2.5 and contained Gal, Glc, Man, Fuc, GlcNAc,

*Present address: Department of Microbiology, Columbia University, College of Physicians & Surgeons, New York, N.Y. 10032, U.S.A.
**Author for correspondence.

0008-6215/87/$ 03.50

TABLE I

COMPOSITION OF THE SIALOGLYCOPEPTIDES PSGP-A AND PSGP (IN PARENTHESIS)

Components		*Percent*	*Molar ratio*[a]
SA	NANA 9% NGNA[b] 4.5%	13.5[c]	3.2 (3.5)
GlcNAc		19.3[d]	6.2 (5.8)
Gal		14.7	5.8 (5.6)
Man		12.6	5
Fuc		2.5	1.1 (1.2)
Total hexose		33.8[e] (27.2)	
Protein		12.4[f] (14.3)	

[a]Relative to Man (5 molar proportions). [b]Glycolic acid estimation[5]. [c]Thiobarbituric acid method[6]. [d]Amino acid analyser. [e]Direct estimation (phenol–sulfuric acid method[7]). [f]Lowry method[8].

NANA, and NGNA, together with some protein (Lowry) and nucleic acid (absorbance[2], 260 nm). SGP_1 gave a single broad band in gel electrophoresis (PAS stain[3]), which indicated some polydispersity and/or microheterogeneity at least in the peptide portion of the glycopeptide. Therefore, in order to obtain a purer sialoglycopeptide and also to overcome the problem of solubility, SGP_1 was digested with pronase for 72 h and the resulting sialoglycopeptide was fractionated on Sephadex G-25. Fractions containing both protein (absorbance, 280 nm) and SA (resorcinol method[4]) were combined and lyophilised. The product was recycled through the same column to yield the purified sialoglycopeptide (PSGP), which had a protein-to-SA ratio of 1.1 and contained SA, GlcNAc, Gal, Man, and Fuc in

TABLE II

AMINO ACID COMPOSITION OF PSGP AND PSGP-A

Amino acids	*PSGP (mmol/100 g)*	*PSGP-A (mmol/100 g)*
Aspartic acid	31.3	36.2
Threonine	18.8	24.2
Serine	10.6	10.9
Glutamic acid	17.5	19.0
Proline	26.3	25.3
Glycine	29.4	22.3
Alanine	8.1	6.9
Cysteine	0.2	trace
Isoleucine	0.3	3.2
Leucine	0.5	3.4
Phenylalanine	trace	1.9
Lysine	0.2	2.4
Valine	0.4	8.1
Arginine	trace	trace

the molar ratios 3.5:5.8:5.6:5:1.2, together with protein (14.3%, Table I). Electrophoresis of PSGP on Whatman No. 1 paper or cellulose acetate furnished a single but slightly elongated spot, showing the preparation to be reasonably homogeneous.

Since SA sometimes interferes with enzyme activity, an attempt was made to shorten the peptide chain by treatment of the asialoglycopeptide with pronase, but no substantial change of the protein content or amino acid composition could be effected.

When PSGP was subjected to the alkaline β-elimination reaction[9], no oligosaccharides were released (data not shown). This finding and the absence of GalNAc established the absence of any *O*-glycosylic[10] linkage in PSGP, in spite of the significant contents of Ser and Thr (Table II).

Hydrazinolysis[11] of PSGP released no oligosaccharides (data not shown), but they were formed on drastic treatment[12] with base–borohydride. The oligosaccharides were isolated by chromatography on Sephadex G-15 and hydrolysed, and the hexosamine and neutral components were separated. Acetylation of the hexosamine component and g.l.c. of the products revealed 2-amino-2-deoxyglucitol. This result established the presence of a GlcNAc-(1→peptide (Asn) linkage. However, no attempt was made to isolate the oligosaccharide because of the poor yield and the peeling reaction[12,13] as evidenced by p.c. of the products obtained on treatment with base–borohydride. Hydrazinolysis of the GlcNAc–Asn linkages is difficult[14] where the innermost GlcNAc residue (linked to Asn) is 3-substituted. Also, hydrazinolysis usually yields lower oligosaccharides[15] and hence gives poor yields of the desired product.

In order to verify whether both the sialic acids were integral parts of the same glycopeptide molecule, PSGP was subjected to affinity chromatography on Sepharose 4B–serotonin (which binds[16] NANA but not NGNA). The results are shown in Fig. 1. The fractions (16–18) which were eluted with 0–100mM phosphate buffer and contained carbohydrate and protein were further fractionated on Sephadex G-25 (Fig. 2) to yield PSGP-A. This contained NANA (9%) and NGNA[5] (4.5%) in almost the same proportions as in PSGP, and furnished a single spot in electrophoresis; there was only a slight enrichment in the carbohydrate portion (protein:SA 0.9). This finding clearly established that the sialic acids were integral parts of the same glycopeptide molecule. The carbohydrate composition in PSGP-A (SA:GlcNAc:Gal:Man:Fuc 3.2:6.2:5.8:5:1.1, Table I) was almost unchanged and the amino acid composition (Table II) showed only slight differences compared to that of PSGP, suggesting that PSGP-A was essentially homogeneous. However, quantitative analysis of the amino acid data (Table II) indicated that there could be some microheterogeneity and/or polydispersity in the large peptide portion of PSGP-A, but this did not interfere with the structural investigation of the glycan moiety (*cf.* refs. 17 and 18).

Mild hydrolysis of PSGP-A with acid effected complete desialation, but the relative molar proportions of Gal, Man, Fuc, and GlcNAc (Table III) in the

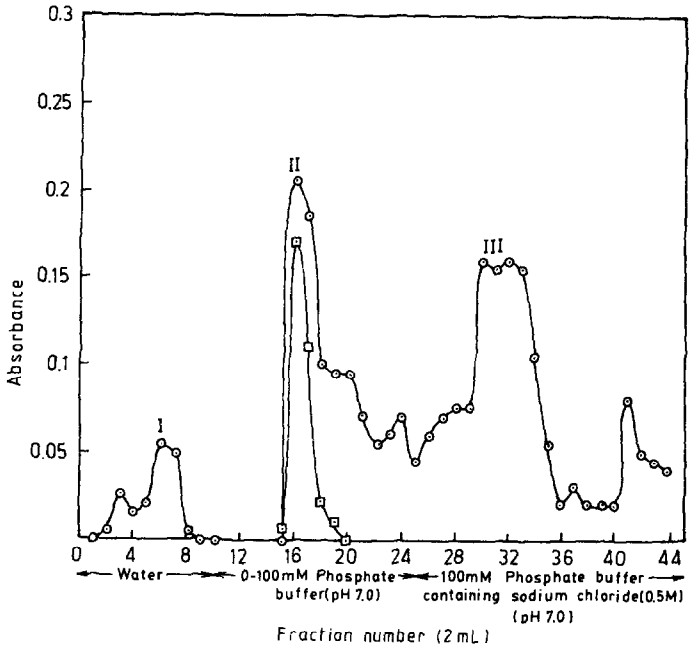


Fig. 1. Affinity chromatography of PSGP on Sepharose 4B-serotonin: —○—, protein (280 nm); —□—, hexose (phenol–sulfuric acid, 490 nm).

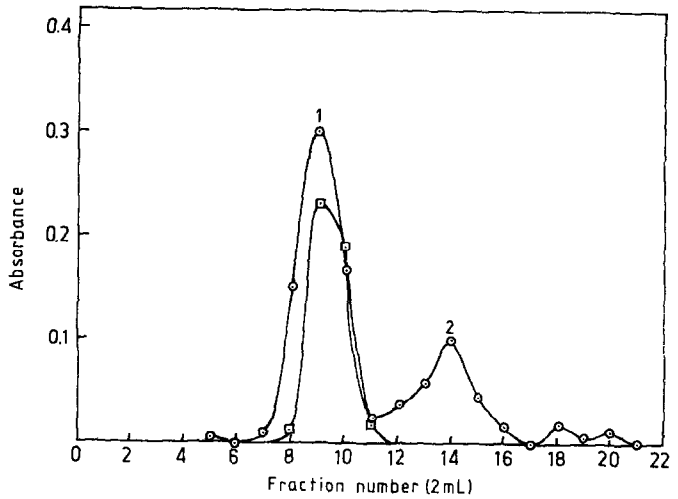


Fig. 2. Fractionation of fractions 16–18 in Fig. 1 on Sephadex G-25: —○—, protein; —□—, hexose.

TABLE III

CARBOHYDRATE COMPOSITION OF THE SIALOGLYCOPEPTIDE (PSGP-A), ASIALOGLYCOPEPTIDE (asGP), AND FRACTION I[a]

Components	*Molar ratio*[b]		
	PSGP-A	*asGP*	*Fraction I*
SA	3.2	—[c]	—[c]
GlcNAc	6.2	6.0	5.7
Gal	5.8	5.9	4.1
Man	5	5	5
Fuc	1.1	0.8	0.2

[a]Obtained by removing both SA and Fuc from PSGP-A. [b]Relative to Man (5). [c]—, Absent.

resulting asialoglycopeptide (asGP) were unchanged. This finding showed that all the SA residues occupied terminal positions and that the glycopeptide had a multi-antennary structure.

Treatment of asGP with β-D-galactosidase[19] released only Gal. This finding and the methylation analysis data (see later) suggested that, at least in some antennae, the SA residues were linked to Gal residues. No GlcNAc was released when the galactosidase-treated product (containing ~2 mol of Gal) was treated separately with exo-*N*-acetyl-β-D-glucosaminidases[19] A and B. This result must be viewed with caution since the lack of proper substrate precluded a control experiment. The presence of a Man→GlcNAc unit in the antennae was ruled out by the fact that no Man was released when the hexosaminidase-treated product was digested with α-D-mannosidase[19]. This finding was consistent with the assumption that PSGP-A contained a conventional oligomannosyl core with the GlcNAc residue blocking the action of the mannosidase.

Fuc may also occupy terminal positions, and both Fuc and SA residues may occupy such positions[20,21] in various chains of the same glycoprotein.

In order to prepare a Fuc-free glycopeptide, PSGP-A was subjected to mild hydrolysis (0.5M sulfuric acid, 1 h, 80°), bearing in mind that the removal of Fuc is always accompanied by partial release of some of the other peripheral sugar residues[22]. Fractionation of the product, after removal of SA and inorganic acids, on Sephadex G-25 gave peak I which contained the degraded glycopeptide, and peak II which contained the released Fuc and Gal (80 and 30%, respectively, of that in PSGP-A). The carbohydrate composition of the material (fraction I) in peak I is given in Table III.

Methylation analysis of PSGP-A, asGP, and fraction I gave the results in Table IV. The molar responses of methylated sugars (especially of mono- and di-*O*-methyl derivatives) are not usually quantitative[23a,b] and it is advisable to quantitate the different monosaccharides on the basis of the total number of sugar residues (as

TABLE IV

METHYLATION ANALYSIS (G.L.C. AND G.L.C.–M.S.) OF PSGP-A, asGP, AND FRACTION I

Monosaccharide unit	O-*Methyl sugars*	*PSGP-A (mol)*	*asGP (mol)*	*Fraction I (mol)*	*Major mass fragments*	*Nature of linkage*
Fuc	2,3,4-	0.7(1)	0.4(1)	—	43, 57, 71, 89, 101, 117, 131, 161, 175	Fuc*p*-(1→
Man	3,4,6-	3	3	3	43, 45, 71, 87, 99, 129, 145, 161, 189	→2)-Man*p*-(1→
	2,4-	1.5(2)	1.8(2)	1.9(2)	43, 87, 101, 117, 129, 189, 201, 233	→3,6)-Man*p*-(1→
Gal	2,3,4,6-	0.7(1)	2.6(3)	3.3(4)	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	Gal*p*-(1→
	2,4,6-	4.3(4)	1.5(2)	0.9(1)	43, 45, 71, 87, 101, 117, 129, 161, 203, 233	→3)-Gal*p*-(1→
	3,6-	0.7(1)	0.7(1)	0.8(1)	43, 45, 55, 87, 99, 113, 129, 173, 189, 233	→2,4)-Gal*p*-(1→
GlcNAc	3,4,6-	0.6(1)	0.7(1)	0.5(1)	43, 45, 74, 87, 116, 129, 142, 158, 202	GlcNAc-(1→
	3,6-	3	4	4	43, 45, 74, 99, 116, 142, 158, 233	→4)-GlcNAc-(1→
	6-	0.5(1)	0.9(1)	0.8(1)	43, 45, 74, 87, 99, 116, 129, 158	→3,4)-GlcNAc-(1→
	3-	0.5(1)	—	—	43, 74, 116, 142, 158, 261	→4,6)-GlcNAc-(1→

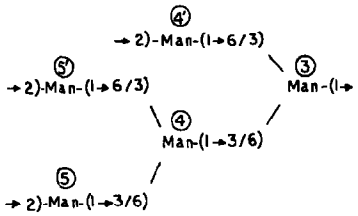


Fig. 3. Partial structure of the oligomannosidic portion of the glycopeptide.

determined by sugar analysis) present in the glycopeptide. On this basis, the methylation analysis data were normalised, as indicated in parentheses in Table IV.

PSGP-A, asGP, and fraction I each gave 3 mol of 3,4,6-tri- and 2 mol of 2,4-di-*O*-methylmannose (Table IV). These data coupled with the fact that graded hydrolysis of PSGP-A did not release Man suggests that the mannosyl residues constituted the core structure (Fig. 3).

The Fuc (1 mol) in PSGP-A and asGP furnished only 2,3,4-tri-*O*-methylfucose (1 mol); fraction I was practically devoid of Fuc (Table IV). This finding suggested that the fucosyl residues in PSGP-A or asGP occupied only the terminal positions of antennae (like SA).

PSGP-A, asGP, and fraction I contained (Table III) 5.8 (6), 5.9 (6), and 4.1 mol, respectively, of Gal. The lower value for fraction I reflects some loss (~30%) of terminal Gal during the mild acid treatment of PSGP-A. The corresponding *O*-methyl derivatives of Gal, obtained on methylation analysis, are given in Table IV.

The above results show that, of the six Gal residues present in PSGP-A, one (⑦ in Fig. 4) must be a non-reducing terminal (→2,3,4,6-tetra-*O*-methylgalactose). The formation of 4 mol of 2,4,6-tri-*O*-methylgalactose requires 4 Gal units (⑥, ⑦, ⑧, and ⑩) to be linked through O-3 and the remaining 1 mol of Gal (⑦) to be linked through O-2 and O-4 (→3,6-di-*O*-methylgalactose).

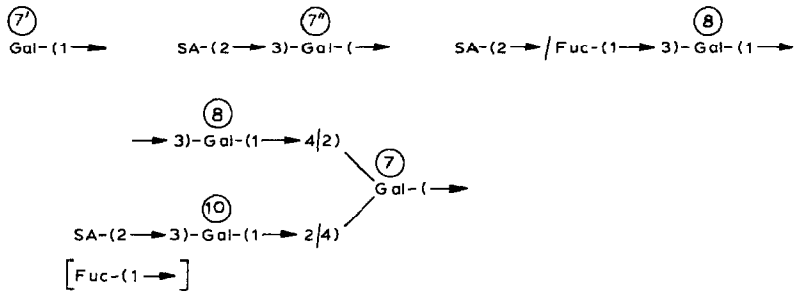
Methylation analysis of asGP gave 2,3,4,6-tetra-, 2,4,6-tri-, and 3,6-di-*O*-methylgalactose in the molar proportions 2.6(3):1.5(2):0.7(1). Comparison of these data with those for PSGP-A (Table IV) showed an increase of 2 mol of tetra-*O*-methylgalactose and a decrease of 2 mol of 2,4,6-tri-*O*-methylgalactose, indicating that two SA residues were joined to two Gal residues at O-3 and that these Gal residues were exposed on desialation. This finding substantiates the proposed sequence for two of the antennae.

The above *O*-methyl derivatives of Gal were also formed from fraction I, but in the molar ratios 3.3(4):0.9(1):0.8(1) for the tetra-, tri- and di-*O*-methyl sugars. The slightly lower proportion of methylated galactoses, as opposed to those obtained from methylated PSGP-A or asGP, is to be expected because, during the preparation of fraction I, 2 mol of Gal was lost. There was also an increase of the tetra-*O*-methylgalactose with a consequent decrease (1 mol) of the 2,4,6-tri-*O*-

methylgalactose, which established that the terminal fucosyl residue was attached to O-3 of a Gal residue.

Again, the formation of 1 mol of 3,6-di-*O*-methylgalactose on methylation of each of the three products (Table IV) clearly indicated that one Gal (⑦ in Fig. 4) was branched through O-2 or O-4. Further, in order to account for the gradual increase in the proportion of tetra-*O*-methylgalactose in the products from PSGP-A, asGP, and fraction I, it was also necessary to assume that the branched unit (⑦) carried a Gal unit which, in turn, was linked to an SA/Fuc residue through O-3.

On the basis of the above results, the following partial structures may be suggested for the peripheral portions of the glycan part of PSGP-A in which the numbers in circles relate to Fig. 4.



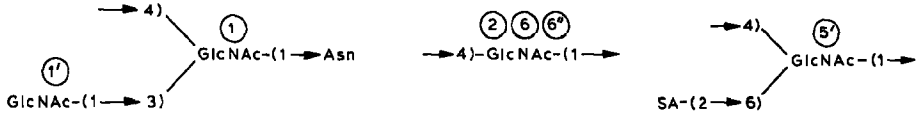
Methylation analysis of PSGP-A furnished 3,4,6-tri-, 3,6-di-, and 6- and 3-*O*-methyl derivatives of GlcNAc in the molar proportions 0.6(1):3:0.5(1):0.5(1) (Table IV).

Characterisation of 3,4,6-tri-*O*-methylGlcNAc (1 mol) revealed that, of the six GlcNAc residues in PSGP-A, one constituted a non-reducing end. The formation of 3 mol of 3,6-di-*O*-methylGlcNAc showed that these residues were linked through O-4 (②, ⑥, and ⑥' in Fig. 4) as commonly found in glycopeptides containing lactosaminyl and/or chitobiosyl residues. The formation of the 3- and 6-*O*-methyl derivatives revealed that two of the GlcNAc residues had branching at O-6 (⑤') and O-3 (①). The two remaining non-reducing ends (one giving the above 3,4,6-tri-*O*-methylGlcNAc and the other an SA residue) were attached to positions 3(6) and 6(3) of the GlcNAc residues which gave the mono-*O*-methylGlcNAc derivatives. Therefore, if the position of one could be found, that of the other would follow.

Further information in this respect came from methylation analysis of the asialoglycopeptide (Table IV), which furnished 3,4,6-tri-, 3,6-di-, and 6-*O*-methylGlcNAc in the molar proportions 0.7(1):4:0.9(1). The disappearance of the 3-*O*-methyl derivative and increase by 1 mol of 3,6-di-*O*-methylGlcNAc revealed that the SA residue was joined to a GlcNAc residue through O-6. Therefore, the remaining GlcNAc residue (①' in Fig. 4) must have occupied O-3 of a GlcNAc residue (①).

No further change in the molar ratios [0.5(1):4:0.8(1), Table IV] of the 3,4,6-tri-, 3,6-di-, and 6-*O*-methylGlcNAc found on methylation analysis of fraction I confirmed that Fuc was not linked to GlcNAc but only to Gal.

As noted above, the innermost GlcNAc residue was linked to Asn. This finding together with the above methylation analysis data suggest the following linkages for the GlcNAc residues in the glycopeptide.



Thus, a probable structure for the carbohydrate portion of the glycopeptide (PSGP-A) is that shown in Fig. 4; although no direct evidence for the location of the GlcNAc residue ①' could be obtained, the structure explains the resistance of the glycopeptide to hydrazinolysis[14].

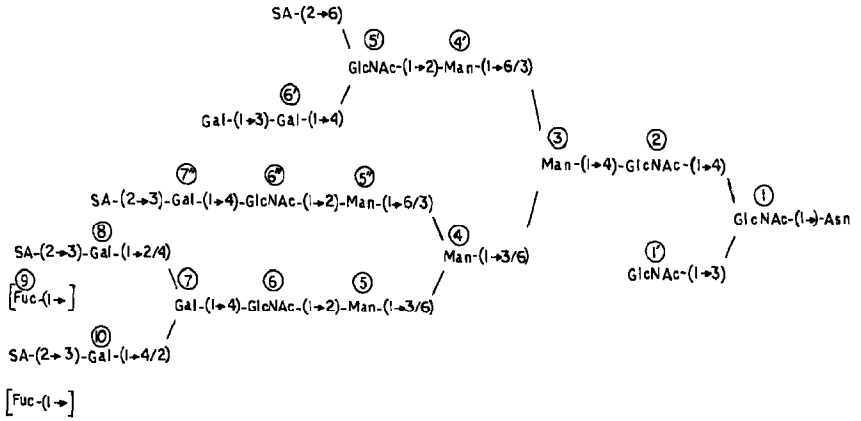


Fig. 4. Proposed structure of the carbohydrate portion of PSGP-A.

Smith degradation (mild hydrolysis) of PSGP-A furnished only glycerol, thereby showing that none of the surviving monosaccharide residues was flanked by oxidisable sugar residues. Vigorous hydrolysis of the Smith-degradation product furnished Gal and Man in the molar ratio 2:1 (*cf.* 2.5:1 required by the structure in Fig. 4). This discrepancy can be rationalised by assuming that, besides ⑦', some of the Gal residues ⑦", ⑧, or ⑩ became exposed to periodate due to desialation during the prolonged oxidation reaction; the other oxidisable residues were the Man residues ④, ⑤, and ⑤" and the fucosyl residue ⑨. Therefore, this finding is also consistent with the proposed structure (Fig. 4).

The possibility that multiple oligosaccharide chains are attached to the same glycopeptide molecule could not be ruled out completely by the available data.

EXPERIMENTAL

The materials and analytical methods were similar to those described earlier[1,24]. Neutral sugars were determined after hydrolysis of the glycopeptide sample with 2M hydrochloric acid for 3 h at 100°. CNBr-activated Sepharose 4B, serotonin hydrochloride, β-D-galactosidase (from *Aspergillus niger*), β-*N*-acetylglucosaminidases A and B (from human placenta), and α-D-mannosidase (from Jack bean) were purchased from Sigma. Solvents for p.c. were *A*, 8:2:1 ethyl acetate–pyridine–water: *B*, 5:5:1:3 ethyl acetate–pyridine–acetic acid–water; and *C*, 3:2:1 butyl acetate–acetic acid–water; and detection reagents were (*a*) alkaline silver nitrate, (*b*) benzidine–periodate, and (*c*) 0.5% ninhydrin in acetone. G.l.c. was performed with glass columns (6 mm × 1.83 m) containing *A*, 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of neutral and methylated amino sugars) or 170° (for alditol acetates of partially methylated neutral sugars); *B*, 3% of OV-225 on SIL RUB (80–100 mesh) at 170° (for alditol acetates of partially methylated neutral sugars); and *C*, 3% of Poly A-103 on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of hexosamines), using the earlier instrument[24]. G.l.c.–m.s. was performed as described earlier[25].

Electrophoresis of the glycopeptide samples. — The sample was subjected to high-voltage electrophoresis[26] (3000 V, 1 h) on Whatman No. 1 paper, using *A*, pyridine–acetic acid–water (200:8:1800) buffer (pH 6.55); or *B*, acetic acid–aqueous 98% formic acid–water (58:104:1838) (pH 1.85); and detection with ninhydrin and/or benzidine–periodate.

Low-voltage electrophoresis[27] (300 V, 45 min) was performed on cellulose acetate strips in 0.05M lithium chloride–0.1M hydrochloric acid (pH 2.0) with detection by Alcian Blue[28].

Polyacrylamide gel electrophoresis was conducted according to the method of Davis[29].

Isolation and purification of the sialoglycopeptide. — The sialoglycoprotein was isolated[1] from eel fishes, and the corresponding sialoglycopeptide (SGP) was released by digestion with pronase[1] (2 h). The SGP was purified by chromatography on CM-Sephadex and DEAE-cellulose to yield SGP_1 which had a protein-to-sialic acid (SA) ratio of 2.5. A solution of SGP_1 (300 mg) in 0.2M sodium phosphate buffer (15 mL, pH 7.8) containing 1.5mM calcium chloride and pronase (15 mg) was kept at 37° under a drop of toluene for 72 h. The enzyme was deactivated (80°, 10 min), the suspension was centrifuged (6000 r.p.m., 30 min, 4°), the supernatant solution was lyophilised, and a solution of the residue in pyridine acetate buffer (0.1M, 3 mL, pH 5.0) was added to a column (70 × 2 cm) of Sephadex G-25 and eluted with the same buffer at 15 mL/h. Fractions (5 mL) responding positively to both protein (absorbance, 280 nm) and SA (resorcinol) were combined and lyophilised to give the purified sialoglycopeptide (PSGP, 70 mg).

Preparation of the asialoglycopeptide (asGP). — PSGP (or PSGP-A, 2.5 mg) was treated with 25mM sulfuric acid (1 mL) for 1 h at 80° in a sealed tube. The

hydrolysate was diluted with water (10 mL), adsorbed onto a column (10 × 0.8 cm) of Dowex 1-X4 ($HCOO^-$) resin, and eluted with water. The combined effluent and washings (100 mL) were lyophilised to yield the asialoglycopeptide (asGP, 1.9 mg).

On high-voltage electrophoresis in solvent *B*, asGP gave a single spot (mobility, 1.2 cm towards the cathode).

Treatment of PSGP with borohydride. — PSGP (5 mg) was treated with M NaOH–M $NaBH_4$ (1 mL) for 5 h at 100° in a sealed tube under nitrogen. Excess of borohydride was decomposed by cold 6M acetic acid, and the mixture was eluted from a column (70 × 2 cm) of Sephadex G-15 with water at 20 mL/h (5-mL fractions). Carbohydrate-containing fractions were combined and lyophilised, and the residue was hydrolysed with 4M hydrochloric acid (1 mL) for 8 h at 100°. The hexosamines were separated from the neutral part, using Dowex 50W-X8 (H^+) resin, and acetylated[30], and the products were subjected to g.l.c. (column *C*, 190°). The control experiment involved 2 mg of sample in 1 mL of water.

Fractionation of the sialoglycopeptide (PSGP). — The bed of Sepharose 4B–serotonin was prepared according to the manufacturer's instructions[32]. The material (2 g) was packed in a column (20 × 0.8 cm) and washed with water (20 mL). PSGP (3 mg) was applied and the column was washed with water until the u.v. absorption (280 nm) of the effluent became zero (Fig. 1), and then with a linear gradient of phosphate buffer (0→100mM, 30 mL, pH 7.0) followed by a phosphate buffer (100mM, pH 7.0) containing sodium chloride (500mM). Fractions (2 mL) were collected at 8 mL/h. Fractions (16–18) which responded positively to phenol–sulfuric acid and had absorption at 280 nm were combined and lyophilised to give PSGP-A. The column was regenerated by washing with acetate buffer (100mM, 400 mL, pH 3.0) followed by water, and two more portions (3 mg) of PSGP were processed in the same way. The carbohydrate-positive fractions (see above) from these three lots were combined and lyophilised to give PSGP-A.

The combined PSGP-A was charged onto a column (64 × 0.8 cm) of Sephadex G-25 and eluted with water at 10 mL/h (2-mL fractions). Fractions (8–11, peak 1, Fig. 2) which responded positively to phenol–sulfuric acid and absorbed at 280 nm were combined and lyophilised to give the sialoglycopeptide (PSGP-A).

The small amount of material obtained by lyophilisation of the fractions associated with peak 2 (Fig. 2) were ninhydrin-negative and were rejected.

PSGP-A, when recycled over Sephadex G-25, furnished a single peak corresponding to peak 1 of Fig. 2. The fractions comprising this peak were combined and lyophilised to give the purified sialoglycopeptide (PSGP-A, 5 mg).

Treatment of the asialoglycopeptide with enzymes. — The glycopeptides were digested at 37° with the appropriate glycosidase (0.1–0.5 U) in 50mM sodium acetate buffer (0.5 mL, pH 4) under a layer of toluene for 18–50 h. The enzyme was deactivated by heating at 80° for 10 min, and each mixture was passed through Dowex 50W-X8 (H^+) and 1-X4 ($HCOO^-$) resins, and then lyophilised. Each residue was taken up in water (2 mL), an aliquot (0.2 mL) was concentrated, and the residue was converted into the alditol acetates. Another aliquot (0.3 mL) was

hydrolysed and the products were converted into alditol acetates. The alditol acetates were analysed by g.l.c. The remaining portion, obtained after deactivation of the enzyme and lyophilisation, was treated with the next glycosidase.

Removal of both SA and Fuc from PSGP-A. — PSGP-A (4.7 mg) was hydrolysed with 0.5M sulfuric acid (0.7 mL) for 1 h at 80° in a sealed tube. The hydrolysate was diluted (25-fold) with water and passed through a column (20 × 1.2 cm) of Dowex 1-X4 ($HCOO^-$) resin. The effluent and water washings (200 mL) were combined and concentrated to ~0.5 mL by lyophilisation. The resulting solution was fractionated (in 3-mL portions) through a previously calibrated column (36 × 1.2 cm) of Sephadex G-25 to give fractions I (7–9, 1.9 mg) and II (11–12, containing only Fuc and Gal).

Methylation analysis of PSGP-A, asGP, and fraction I. — The glycopeptide (2–3 mg) methylated first by the Kuhn method[33] (thrice) still had some i.r. absorption for hydroxyl, but was soluble in methyl sulfoxide and was further methylated by the Hakomori[34] method (once). The resulting permethylated product was subjected to acetolysis–hydrolysis[35] (0.25M sulfuric acid in aqueous 95% acetic acid, 14 h, 100°). The hydrolysate was neutralised using Dowex 1-X4 ($HCOO^-$) resin, and the neutral and the amino sugar components were separated using Dowex 50W-X8 (H^+) resin, converted into the alditol acetates, and analysed by g.l.c. and g.l.c.–m.s.

Smith degradation of PSGP-A. — PSGP-A (5.29 mg) was oxidised with 0.05M sodium metaperiodate in 0.05M sodium acetate buffer (2.5 mL, pH 4.25) for 112 h in the dark at 4°. The reaction was terminated by adding ethylene glycol (50 μL). After 1 h at room temperature, *myo*-inositol (240 μg) and 0.1M sodium borate (2 mL, pH 8.0) containing 0.1M sodium borohydride were added, and the mixture was stored overnight at 4°. After the usual processing, the product was hydrolysed (0.1M hydrochloric acid, 2 mL, 1.5 h, 80°). An aliquot (0.6 mL) of the hydrolysate was worked-up and the product was acetylated. G.l.c. (programming[25], column *A*) furnished glycerol triacetate (retention time, 12.5 min), but no derivative of Gal or Man could be detected (190°, column *A*).

The remainder of the oxidised–reduced product (in 1.4 mL) was further hydrolysed (2M hydrochloric acid, 1 mL, 3 h, 100°), and the products were converted into alditol acetates and subjected to g.l.c. (column *A*, 190°) which furnished peaks corresponding to derivatives of Gal and Man (ratio, 2:1) with an unidentified peak of retention time 90 min.

ACKNOWLEDGMENTS

The authors thank Professor M. Futai (Department of Microbiology, Okayama University, Japan) and Dr. T. Samanta (Bose Institute, Calcutta) for amino acid analyses, Professor H. Mayer and Dr. S. Basu (Max-Planck Institute, Freiburg, F.R.G.) for the g.l.c.–m.s. analyses, and the C.S.I.R. (New Delhi) for a fellowship to R.G.

REFERENCES

1 S. K. SIKDER AND A. DAS, *Indian J. Chem.*, 19B (1980) 389–392.
2 J. D. HIGGINBOTHAM, A. DAS, AND M. HEIDELBERGER, *Biochem. J.*, 126 (1972) 225–231.
3 R. A. KAPITANY AND E. J. ZEBROWSKI, *Anal. Biochem.*, 56 (1973) 361–369.
4 L. SVENNERHOLM, *Biochim. Biophys. Acta*, 24 (1957) 604–611.
5 E. MÅRTENSSON, A. RAAL, AND L. SVENNERHOLM, *Biochim. Biophys. Acta*, 30 (1958) 124–129.
6 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971–1975.
7 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
8 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
9 M. BERTOLINI AND W. PIGMAN, *J. Biol. Chem.*, 242 (1967) 3776–3781.
10 A. NEUBERGER, A. GOTTSCHALK, R. D. MARSHALL, AND R. G. SPIRO, in A. GOTTSCHALK (Ed.), *Glycoproteins*, Vol. 5A, Elsevier, Amsterdam, 1972, pp. 450–485.
11 M. FUKUDA, T. KONDO, AND T. OSAWA, *J. Biochem. (Tokyo)*, 80 (1976) 1223–1232.
12 Y.-C. LEE AND J. R. SCOCCA, *J. Biol. Chem.*, 247 (1972) 5753–5758.
13 J. BAENZIGER, S. KORNFELD, AND S. KOCHWA, *J. Biol. Chem.*, 249 (1974) 1897–1903.
14 Y. INOUE AND K. KITAJIMA, *Carbohydr. Res.*, 135 (1985) 342–347.
15 J. C. MICHALSKI, J. P. KATALINIC, H. EGGE, J. P. PARENTE, J. MONTREUIL, AND G. STRECKER, *Carbohydr. Res.*, 134 (1984) 177–189.
16 R. J. STURGEON AND C. M. STURGEON, *Carbohydr. Res.*, 103 (1982) 213–219.
17 J. CONCHIE, A. J. HAY, AND J. A. LOMAX, *Carbohydr. Res.*, 112 (1983) 281–295.
18 G. V. AVVAKUMOV AND O. STREL'CHYONOK, *Carbohydr. Res.*, 138 (1985) 91–98.
19 K. YAMAMOTO, T. TSUJI, T. IRIMURA, AND T. OSAWA, *Biochem. J.*, 195 (1981) 701–713.
20 Z. DISCHE, C. PALLAVICINI, H. KAVASAKI, J. SMIRNOW, I. CIZEK, AND S. CHEN, *Arch. Biochem. Biophys.*, 97 (1962) 459–469.
21 R. C. CALDWELL AND W. PIGMAN, *Arch. Oral Biol.*, 11 (1966) 437–449.
22 S. K. SIKDER AND A. DAS, *Carbohydr. Res.*, 95 (1981) 249–262.
23 (*a*) S. N. BHATTACHARYA AND W. S. LYNN, *J. Biol. Chem.*, 252 (1977) 1172–1180. (*b*) K. FUKUDA, M. TOMITA, AND A. HAMADA, *Biochim. Biophys. Acta*, 677 (1981) 462–470.
24 S. K. SIKDER AND A. DAS, *Carbohydr. Res.*, 71 (1979) 273–285.
25 R. GHOSH AND A. DAS, *Carbohydr. Res.*, 126 (1984) 287–296.
26 M. I. HOROWITZ AND A. DAS, *Immunochemistry*, 4 (1967) 303–313.
27 M. BREEN, H. G. WEINSTEIN, M. ANDERSON, AND A. VEIS, *Anal. Biochem.*, 35 (1970) 146–159.
28 J. K. HERD, *Anal. Biochem.*, 23 (1968) 117–121.
29 B. J. DAVIS, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404–427.
30 W. NEIDERMEIER AND M. TOMANA, *Anal. Biochem.*, 57 (1974) 363–368.
31 R. G. SPIRO, *Methods Enzymol.*, 28 (1972) 3–43.
32 *Affinity Chromatography Manual, Principles and Methods*, Pharmacia, 1977, p. 8.
33 R. KUHN, H. TRISCHMANN, AND I. LÖW, *Angew. Chem.*, 67 (1955) 32.
34 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
35 K. STELLNER, H. SAITO, AND S. HAKOMORI, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.