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STRUCTURE OF THE GLYCAN PORTION OF A PURE SIALOGLYCOPEPTIDE FRACTION ISOLATED FROM THE SKIN OF THE FISH *Labeo rohita**

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ABSTRACT

The residue left after hot-water extraction of the defatted skin of the fish *Labeo rohita* has been found to contain sialoglycoprotein(s). On treatment with Pronase, this residue released all of the sialoglycopeptide (SGP) in 2 h. The resulting SGP was subjected to exhaustive, proteolytic treatment and several purification steps, whereupon it finally yielded a pure sialoglycopeptide (PSGP) fraction containing NeuAc, GlcNAc, Gal, Man, and Fuc in the molar ratios of 3.0:2.9:3.2:2.3:1.0, together with protein (10%). The major amino acid components of PSGP were aspartic acid, threonine, phenylalanine, and proline. From the results of base–borohydride treatment, methylation analysis, and enzymic reactions, a probable structure has been assigned to the glycan portion, which is linked through GlcNAc to Asn of the peptide core in PSGP.

INTRODUCTION

In an earlier report[1], it was shown that hot-water extraction of the defatted skin of the fish *Labeo rohita* furnishes a soluble, gel-like material whose principal carbohydrate constituents are dermatan sulfate, chondroitin 4-sulfate, and hyaluronic acid.

The residue (degelled skin) after hot-water extraction has been found to contain sialic acid (*N*-acetylneuraminic acid, NeuAc), 2-acetamido-2-deoxyglucose (GlcNAc), 2-acetamido-2-deoxygalactose (GalNAc), Gal, Glc, Man, and Fuc, and protein. We now report the isolation of a pure, sialoglycopeptide fraction from the degelled skin, and a probable structure for the glycan portion thereof.

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*Part II; this work was presented by A. Das at the Xth International Symposium on Carbohydrate Chemistry, held in Sydney in 1980. For Part I, see ref. 1.
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RESULTS AND DISCUSSION

The native skin of the fish *Labeo rohita* was first defatted by extraction with several changes of cold acetone, to remove the fatty matter and the repulsive fishy odor. The resulting, dry skin retained all of its carbohydrate components (*viz.*, NeuAc, GlcNAc, GalNAc, Gal, Glc, Man, and Fuc) intact, but no protein or sialoglycopeptide (SGP) could be extracted from it by direct extraction with cold water[2], cold electrolyte solution[3], or aqueous phenol[4]. The defatted skin (36 g) was, therefore, extracted with water at 80°, to remove the soluble gel-material[1]. The residue (degelled skin, 7 g) was treated with Pronase in phosphate buffer, pH 7.8, at 37°, as described earlier[5]. A kinetic study[5] showed that all of the SGP could be released within 2 h, and the residual skin, which became very disintegrated, did not retain any sialic acid. The resulting SGP did not yield any precipitable complex with detergents[2,3,6], although it was nondialyzable. The ratio of protein to sialic acid (12.1 : 1) was quite high; hence, to remove the adhering protein or peptides, or both, this SGP was treated with CM-Sephadex, whereupon the ratio of protein to sialic acid changed to 2.5 : 1. The product retained all of the carbohydrate components mentioned earlier, and a repetition of the treatment with Sephadex caused very little change in the composition.

Further purification of the SGP was effected by chromatography on a column of DEAE-cellulose (acetate form), using a linear gradient of sodium chloride (0 to 1M); this resolved the SGP into two peaks, of which the fractions (eluted at ~0.5M

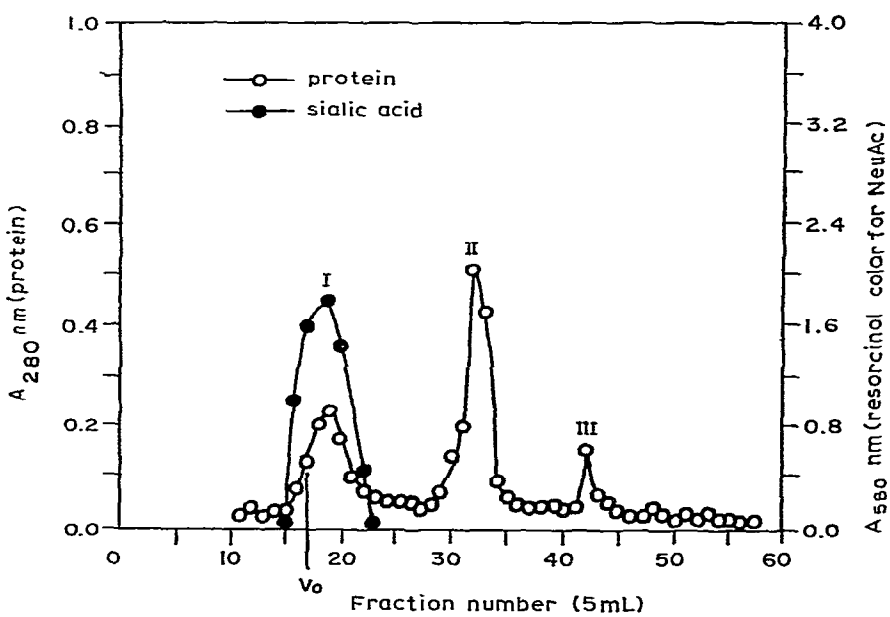


Fig. 1. Purification of the sialoglycopeptide fraction (SGP-1) by chromatography on a column of Sephadex G-25. Peak I afforded purified sialoglycopeptide (PSGP).

sodium chloride) constituting the sialic acid peak were pooled, dialyzed, and lyophilized, to yield SGP-1. This had a protein to sialic acid ratio of 1.1 : 1, and a content of nucleic acid of 4.8%, but its carbohydrate composition remained qualitatively unchanged. Ultracentrifugation of this preparation in phosphate buffer, pH 7, at 50,000 r.p.m. revealed the presence of two peaks. Also, electrophoresis of the SGP-1, on paper, at 3 kV, and on cellulose acetate strip, at 300 V, furnished long trailings towards the positive electrode. These findings indicated that the preparation still had some heterogeneity, or, at least, some polydispersity.

It should be mentioned that considerable difficulty was encountered in redissolving the lyophilized sialoglycopeptides obtained at each stage of the purification. Similar difficulties were experienced by earlier workers[7,8]. Hence, with a view to removing some of the protein, or to shortening the peptide chain (so that further fractionation leading to isolation of a pure sialoglycopeptide might be easier), and also to overcoming the problem with solubility, the SGP-1 preparation was redigested with Pronase (48 h). The digest was centrifuged, and the supernatant liquor was concentrated by lyophilization to ~2 mL. This concentrate was fractionated through a column of Sephadex G-25. Fractions constituting the sialic acid peak (which included some protein fractions; see peak I in Fig. 1) were pooled, and lyophilized, to give the purified sialoglycopeptide (PSGP, 52.5 mg). This was once again recycled through the Sephadex column, but the elution profile remained practically the same as before, and no further change in the composition of PSGP could be effected. (An attempt to isolate the PSGP by exhaustive, proteolytic treatment of the degelled skin presented considerable difficulties in fractionating the products. Hence, subsequent lots were isolated by this stepwise procedure.)

This preparation of PSGP could not be further examined by ultracentrifugation, probably because of its low molecular weight. However, electrophoresis of this PSGP on paper or cellulose acetate furnished a single spot, showing the preparation

TABLE I

COMPOSITIONS OF PURIFIED SIALOGLYCOPEPTIDE (PSGP), ASIALOGLYCOPEPTIDE (GP), AND FRACTION (F_1)

Components		*Molar ratios*		
		PSGP	*GP*	F_1[a]
NeuAc		3.0	—[b]	—
GlcNAc		2.9	3.2	3.0
Gal		3.2	3.1	2.5
Man		2.3	2.1	2.2
Fuc		1.0	1.0	0.2
Protein	10% (Lowry) 9.8% (Amino acid analyzer)			

[a]Obtained by removing both sialic acid and fucose (and part of the galactose) from PSGP. [b]Key: —, absent.

TABLE II

AMINO ACID COMPOSITION OF PSGP

Amino acids	*g/100 g*	*mmol/100 g*
Aspartic acid	3.30	24.9
Threonine	2.46	20.8
Serine	0.64	6.2
Glutamic acid	0.96	6.5
Proline	1.76	15.4
Glycine	0.43	5.8
Alanine	0.51	5.8
Cysteine	0.56	4.6
Isoleucine	0.40	3.0
Leucine	0.05	0.4
Phenylalanine	3.29	20.0
Lysine	trace	
Histidine	trace	
Arginine	trace	

to be homogeneous. It had the composition; NeuAc (24.8), GlcNAc (16.5), Gal (14.7), Man (10.5), and Fuc (4.2%), in the molar ratios of 3.0:2.9:3.2:2.3:1.0 (see Table I); it also contained Lowry protein (~10%). The preparation was free from nucleic acid. The small amount of GalNAc and Glc, detected in the earlier stages of purification, completely disappeared in the final, purified product (*i.e.*, in the PSGP). This indicated that these two sugars were contributed by some extraneous sources.

The amino acid composition of PSGP is given in Table II. An estimate of the protein content (from the amino acid composition of PSGP) amounted to 9.8%, which is very close to the value estimated by the Lowry method.

The presence of significant amounts of threonine and serine in PSGP indicated the possibility of *O*-glycosylic (mucin type[9,10]) linkages between the glycan portion and the polypeptide chain. PSGP was, therefore, subjected to the alkaline β-elimination reaction under the conditions of Carlson[11] and those of Bertolini and Pigman[12], separately, but neither method yielded any oligosaccharide. Also, similar treatment (Carlson) of the precursor of PSGP (*i.e.*, SGP-1, which was nondialyzable, and which contained some GalNAc) did not yield any dialyzable carbohydrate, and no hexosaminitol could be detected in the hydrolyzate of the material treated with alkali–borohydride. Hence, the possibility of any *O*-glycosylic linkage in PSGP seems remote; the absence of any GalNAc in PSGP is also consistent with this conclusion, because a mucin-type linkage occurs through O-1 of GalNAc.

On the other hand, because of the presence of an appreciable amount of aspartic acid in PSGP, the possibility of a 2-acetamido-2-deoxyglucosyl–asparagine linkage, found elsewhere[9,10], was to be expected. Furthermore, the 3:1 molar ratio

of GlcNAc to aspartic acid suggested that one of the GlcNAc residues could be linked to asparagine.

To explore this possibility, the PSGP was subjected to treatment with alkaline borohydride[13] for 5 h. The hexose-positive peak was isolated by chromatography on a column of Sephadex G-15, and hydrolyzed, and the hexosamines were separated from the neutral sugars by means of Dowex 50 (H^+) resin. Direct acetylation of the hexosamine portion, and g.l.c., revealed the presence of aminodeoxyglucitol. This finding clearly indicated that the glycan portion of PSGP was linked through the reducing group of a GlcNAc residue, possibly to the amide nitrogen atom of the asparagine of the peptide core. The neutral sugars were identified as Gal, Man, and Fuc, as expected, along with some unidentified products. Because of the uncontrollable, peeling effect[13,14], no attempt was made to obtain oligosaccharides by this method.

When PSGP was treated with neuraminidase in 0.1M acetate buffer, pH 5.0, at 37°, complete removal of sialic acid (as estimated by the thiobarbituric acid method[15]) was possible; this observation was rather unusual. Complete removal of NeuAc from PSGP was also possible by treatment with 25mM sulfuric acid for 1 h at 80°. In both cases, the resulting asialoglycopeptide (GP) was homogeneous (electrophoresis) and had the same carbohydrate composition (neutral sugars and hexosamines) as that of the original PSGP (see Table I). This proved that the PSGP itself must have been homogeneous, that the NeuAc residues in PSGP were all peripheral, and that the treatment with acid did not cause any undesirable degradation.

Usually, fucosyl residues also occupy terminal positions, and may have reciprocal arrangements[16–19] with sialic acids. Controlled, acid hydrolysis[20] of such glycopeptides releases all the sialic acids and most of such fucosyl residues. Although this kind of acid treatment has some disadvantages[20], because partial release of some of the other neutral, peripheral sugars may occur, the method nevertheless has some

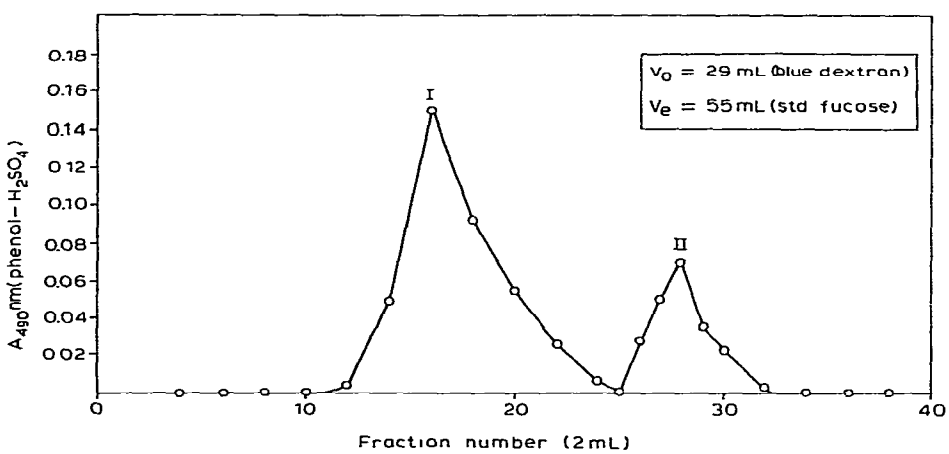


Fig. 2. Purification of material from treatment of PSGP with 0.5M H_2SO_4, for 1 h at 80°, by chromatography on Sephadex G-25. Peak I furnished fraction I (F_1).

utility, as it offers a convenient method for determining the peripheral composition and for obtaining partially degraded product(s) in one step. Methylation analysis of such products, and of their precursors, would be expected to furnish important, structural information.

With this objective, the PSGP was subjected to treatment with 0.5M sulfuric acid for 1 h at 80°. The hydrolyzate was passed through a column of Dowex-1 X4 (formate) resin, and the resulting material was fractionated by passage through a calibrated, Sephadex G-25 column, with monitoring with phenol–sulfuric acid[21], furnishing two peaks (I and II, see Fig. 2). Peak I was processed to give Fraction I (F_1), which was electrophoretically homogeneous in 0.05M borate buffer, pH 9.3 (3 kV, 45 min; migrated 4.8 cm towards the positive electrode), and had the composition: GlcNAc, Gal, Man, and Fuc in the molar ratios of 3.0:2.5:2.2:0.2 (see Table I). It was evident from these results that the foregoing acid treatment removed all of the sialic acids, and 80% of the fucosyl and 20% of the galactosyl residues of the original PSGP.

Peak II, which appeared at the elution volume of standard Fuc, contained mainly fucose, together with a small amount of galactose, as expected.

In order to determine the sequence of, and the nature of the linkage between, the different monosaccharide residues in the glycan portion, the three preparations [*viz.*, PSGP, GP, and F_1 (prepared from PSGP)] were separately subjected to methylation analysis; the results are given in Table III. According to previous

TABLE III

RESULTS OF METHYLATION ANALYSIS OF PSGP, GP, AND F_1

No.	*O-Methyl derivatives*[a]	*Molar ratios*		
		PSGP	*GP*	*F_1*
	Gal			
1	2,3,4,6-	1.0	1.5	2.2
2	2,4,6-	1.3	0.5	0.2
2a	2,3,6-	—[b]	0.8	0.5
3	2,6-	0.58 (~1)	—	—
	Man			
4	3,4,6-	1.0	1.0	1.0
5	2,4-	0.6 (~1)	0.63 (~1)	0.6 (~1)
	Fuc			
6	2,3,4-	1.0	0.97	0.22
	GlcN(Me)Ac			
7	3,4-	1.2	—	—
8	3,6-	1.0	1.0	1.0
9	6-	0.55 (~1)	0.45 (~1)	0.62 (~1)
10	3,4,6-	—	1.1	1.4

[a]Identified by g.l.c. of the alditol acetates, by comparing the retention times[30–32] relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]Key: —, absent.

reports[22,23], sugar positions linked to sialic acid residues remain blocked (and, hence, not methylated) during such permethylation, and this was found true in this case, also.

As, when estimated by g.l.c., di-*O*-methylhexoses and mono-*O*-methylhexosamines always give a low value (as has been found, using some authentic samples, in this work and elsewhere[24–26]), the molar ratios (0.55–0.62, see Table III) of these two kinds of sugar were taken to be equal to unity in computing the actual molar ratio.

In the foregoing experiment, permethylated PSGP (PM-PSGP) furnished 2,3,4,6-tetra- (**1**), 2,4,6-tri- (**2**), and 2,6-di-*O*-methylgalactose (**3**) in the molar ratios of 1.0:1.3:0.58 (~1). Therefore, of the three galactosyl residues present in PSGP, one constituted a nonreducing terminal unit (giving **1**); another, giving **3** (and, hence, being linked through O-3 and O-4, besides O-1), must have originated from a branch point; the unit giving **2** must have been linked through O-1 and O-3 in the glycan portion of PSGP.

The mannosyl residues of PM-PSGP were identified as 3,4,6-tri- (**4**, 1 mol) and 2,4-di-*O*-methylmannose (**5**, ~1 mol). Compound **4** was, therefore, derived from a unit linked through O-1 and O-2, and **5** must have originated from another branch point, where it was linked through O-1, O-3, and O-6.

The fucosyl residues of PM-PSGP furnished only 2,3,4-tri-*O*-methylfucose (**6**, 1 mol), showing that they were also situated at nonreducing ends.

The hexosamine portions of PM-PSGP were characterized as 2-deoxy-3,4-di-*O*-methyl-2-(*N*-methylacetamido)glucose (**7**, 1 mol), 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose (**8**, 1 mol), and 2-deoxy-6-*O*-methyl-2-(*N*-methylacetamido)glucose (**9**, ~1 mol). This shows that the precursor of **7** was linked through O-1 and O-6, that of **8** was linked through O-1 and O-4, and that of **9**, being linked through O-1, O-3, and O-4, constituted another branch point in PSGP.

In other words, there are three branch points in PSGP: one from a galactosyl residue; one from a mannosyl residue, and the third from a GlcNAc residue.

In permethylated asialoglycopeptide (PM-GP), the molar proportion of **1** (1 mol in PM-PSGP) increased to 1.5 mol (see Table III). At the same time, **2** decreased to almost half of its original value (1.3 mol in PM-PSGP). Therefore, it may be concluded that, because of asialation, one of the galactosyl residues of PSGP released a sialic acid residue from O-3, and thus created another nonreducing, terminal unit (in GP); this would account for the increment in **1**. One more important observation is that, whereas PM-PSGP could not provide any 2,3,6-tri-*O*-methylgalactose (**2a**), the latter made its appearance from PM-GP. Also, **3** (furnished by PM-PSGP), disappeared in the case of PM-GP. These two findings can be readily correlated by assigning one sialic acid residue to O-3 of another galactosyl residue, which afforded **3**. The O-3 atom of this residue became exposed due to asialation, and then appeared as **2a**. Thus, it may be concluded that, of the three sialic acid residues in PSGP, two are linked to O-3 atoms of two galactosyl residues. The other galactosyl unit had already been shown to constitute a nonreducing (terminal) group.

The methyl sugars from the mannosyl and fucosyl residues, and their molar

proportions in the case of PM-GP, remained the same as that of PM-PSGP. This corroborated the earlier contention regarding their linkage.

The hexosamine portion of PM-GP furnished 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)glucose (**10**, 1.1 mol), **8** (1 mol), and **9** (~1 mol). As **10** can originate only from a nonreducing terminal unit, and as **7** was absent in this case, it is apparent that asialation exposed the O-6 atom of a hexosamine residue (giving **7** in the case of PM-PSGP). In other words, the remaining sialic acid residue must have been linked to O-6 of a GlcNAc residue.

As mentioned earlier, mild treatment with acid removed all of the sialic acids, and ~80% of the fucosyl and ~20% of the galactosyl residues, to give F_1. Permethylated F_1 (PM-F_1) furnished **1** (2.2 mol), **2** (0.2 mol), and **2a** (0.5 mol). There was no di-*O*-methylGal (*viz.*, **3**) present. As it had been shown that two sialic acid residues were linked to two galactosyl residues, further increment in the content of **1** indicated that the fucosyl residue(s) are also linked to galactosyl residues. As expected, the molar proportion of the tri-*O*-methylfucosyl residues (**6**, 0.2 mol) decreased to one-fifth of its original value.

In this instance, also, the molar proportions of the methylated sugars (**4** and **5**) of the mannosyl residues remained intact. Thus, their location in the core of the proposed structure (**11**) is amply justified. It may be mentioned that it is considered that mannose is incorporated in the early stages of biosynthesis[27], and, therefore, its location near the GlcNAc–Asn linkage region, as has been suggested in the proposed structure, is justified.

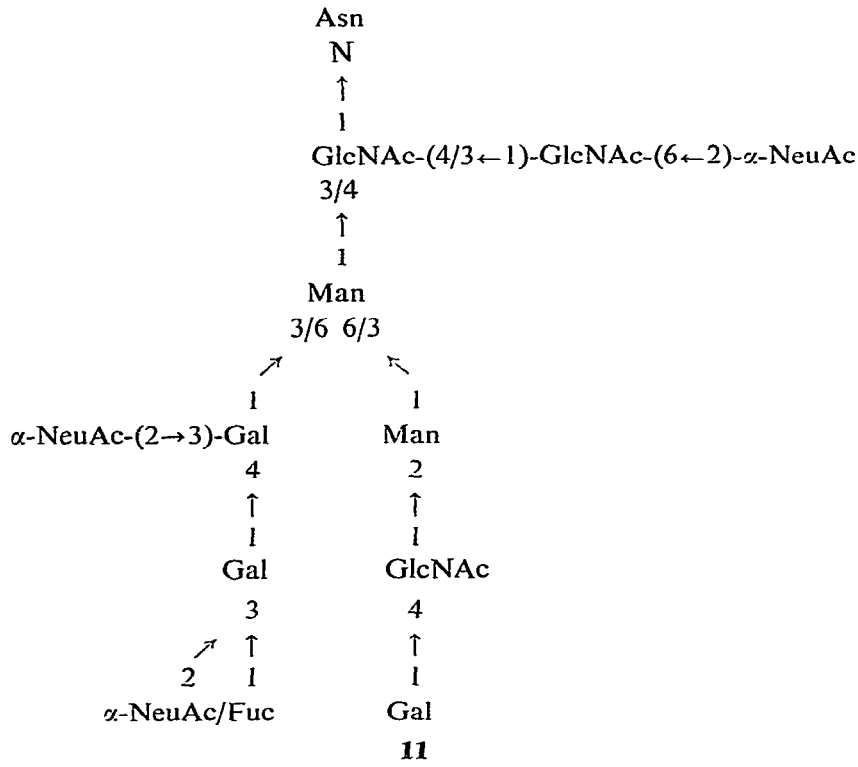
Again, to explain the further increment of the tri-*O*-methylhexosamine (**10**) in the case of PM-F_1, it is reasonable to conclude that the loss (~20%) of some terminal galactosyl residues, during acid treatment of PSGP, exposed some of its GlcNAc residues. This means that, next to a terminal galactosyl residue, there is a GlcNAc residue, as shown in the proposed structure.

Therefore, the presence of the following sequences in PSGP may be taken to be well established.

(*a*) Gal-(1→4)-GlcNAc-(1→
(*b*) α-NeuAc-(2→3)-Gal-(1→
(*c*) Fuc-(1→3)-Gal-(1→
(*d*) α-NeuAc-(2→6)-GlcNAc-(1→
(*e*) GlcNAc-(1→N)Asn

The α-anomeric nature of the linkage of sialic acid has been suggested on the basis of enzyme specificity.

In view of the foregoing results, structure **11** may be assigned to the glycan portion of PSGP.



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It should, however, be noted that, although the proposed structure bears similarities to those of a number of sialoglycopeptides[24,28,29] having a more or less similar carbohydrate composition, alternative possibilities cannot be completely excluded by the present data. Furthermore, as fucose has been shown to be an alternative to NeuAc, this would mean that, even though we could not detect any heterogeneity, the final PSGP might have some peripheral heterogeneity, as has been found elsewhere[16–19].

EXPERIMENTAL

Materials and methods. — The defatted, dry skins of the fish *Labeo rohita* used in this investigation were prepared as described earlier[1]. Materials, and the methods used for the identification of component sugars, the analytical procedures, and the solvent systems for paper partition-chromatography were the same as those reported earlier[5]. For determination of the amino acid composition, the sialoglycopeptide sample (2.6 mg) was hydrolyzed[20] with 6M HCl (2.5 mL) during 18 h at 110° under nitrogen, and the hydrolyzate evaporated *in vacuo*, and analyzed by means of a Beckman 120 C amino acid analyzer.

For g.l.c., glass columns (6 mm × 1.83 m) containing (*1*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 195° (for alditol acetates of neutral sugars),

at 170° (for alditol acetates of partially methylated, neutral sugars[30,31]), and at 190° (for alditol acetates of partially methylated hexosamines[32]); (*II*) 3% of POLY A-103 on Gas Chrom Q (100–120 mesh) at 200° (for alditol acetates of hexosamines); and (*III*) 5% of OV-225 on SIL. RUB. (80–100 mesh) at 170° (for alditol acetates of partially methylated, neutral sugars[30,31]) were used. For quantitative evaluation of gas–liquid chromatograms, a Hewlett–Packard 3370B integrator was used.

Isolation and purification of the sialoglycopeptide (SGP). — Defatted, dry skin (36 g) was extracted with hot water for 1 h at 80°, to separate the soluble, gel-like glycosaminoglycan fraction. The residue (degelled skin) was washed with cold water (4 × 25 mL), and then triturated with cold acetone, to yield dry material (7.5 g). This was treated with Pronase in 0.2M phosphate buffer, pH 7.8, containing 1.5mM calcium chloride, for 2 h at 37°, by which time, all of the sialoglycopeptides had been released. The mixture was centrifuged at 20,000 r.p.m. for 30 min at 4°, and the supernatant liquor (350 mL) was dialyzed for 72 h at 4°. A portion (~25 mL) of the dialyzed solution was lyophilized, and the resulting solid was used for component identification and analysis.

The rest of the solution (~400 mL) was stirred with CM-Sephadex (H^+) (12 g) for 4 h at 4°, filtered through glass wool, and the filtrate lyophilized (yield, ~300 mg). This was analyzed for protein and sialic acid.

The foregoing SGP preparation was further purified by chromatography on a column (40 × 2.5 cm) of DEAE-cellulose (acetate form), using a linear gradient of sodium chloride (0–1M) as described earlier[5]. Fractions responding positively to tests for both sialic acid and protein were pooled, and lyophilized, to yield SGP-1 (105 mg).

Electrophoresis of SGP-1. — The SGP-1 preparation was subjected to high-voltage electrophoresis[33] (3 kV, 1 h) on Whatman No. 1 paper in 25:1:225 (v/v) pyridine–acetic acid–water buffer, pH 6.5, and the paper was stained with periodate–benzidine reagent[34], whereupon it showed a long trailing towards the positive electrode.

The SGP-1 was also subjected to electrophoresis[35] on a cellulose acetate strip in 0.05M lithium chloride–0.01M hydrochloric acid, pH 2.0, at 300 V for 40 min. The strip was stained with Alcian Blue[36]; here, also, an elongated spot appeared.

Ultracentrifugation of SGP-1. — A solution (1%) of the sialoglycopeptide in 0.1M phosphate buffer, pH 7.0, containing sodium chloride (0.1M), was centrifuged (50,000 r.p.m., at 25°) and photographs were taken after 30 min.

Further pronase treatment of SGP-1, and fractionation of the resulting sialoglycopeptide through a column of Sephadex G-25. — The SGP-1 (80.2 mg) was subjected to exhaustive digestion (48 h) with Pronase under the conditions already described. The suspension was centrifuged, and the supernatant liquor was lyophilized. The resulting solid was dissolved in water (2 mL), and the solution was applied to a column (61 × 2 cm) of Sephadex G-25. The column was eluted with water, and fractions (5 mL) were collected at the rate of 15 mL/h. Each fraction was monitored for protein (optical absorbance at 280 nm) and sialic acid (resorcinol method). Fractions 15–22, constituting peak I (see Fig. 1), responded positively to tests for

both protein and sialic acid. These were pooled, and lyophilized, to give the purified sialoglycopeptide (PSGP); yield 52.5 mg.

The PSGP was subjected to electrophoresis on paper, and on cellulose acetate strips, as already described, and in both experiments, a single spot appeared. This material (PSGP) was used for subsequent investigations.

Treatment of PSGP with alkaline borohydride. — The PSGP was subjected to the alkaline β-elimination reaction under the conditions of Carlson[11] and of Bertolini and Pigman[12], in order to release the carbohydrate moiety from the protein core. In Carlson's method, the glycopeptide sample (5 mg) was dissolved in alkaline borohydride solution (5 mL of 0.05M potassium hydroxide, containing M sodium borohydride), and kept for 15 h at 45°. In the second method, the sample was treated with 0.1M sodium hydroxide–0.3M sodium borohydride solution for 10 h at 45°. In both cases, the excess of borohydride was decomposed with cold acetic acid (6M), and the solution was decationized with Dowex 50 (H^+) resin, and evaporated to dryness. Boric acid was removed as methyl borate, and the residue was hydrolyzed with 4M hydrochloric acid (1 mL) for 8 h at 100°. The hexosamines were separated from the neutral sugars by means of a small column of Dowex 50 (H^+) resin, and, after the usual processing, the residue was directly acetylated[37], and the acetates were subjected to g.l.c. (Col. *II*, 200°).

The PSGP was also subjected to drastic, alkali–borohydride treatment under the conditions of Lee and Scocca[13]. The sample (3 mg) was dissolved in alkali–borohydride solution (1 mL of M sodium hydroxide–M sodium borohydride solution), and heated, in a sealed tube under nitrogen, for 5 h in a boiling-water bath. The solution was cooled, the excess of the borohydride was decomposed by adding a few drops of cold acetic acid, and the solution was applied directly to a column (70 × 1.8 cm) of Sephadex G-15, and eluted with water at the rate of 20 mL/h (5-mL fractions). The eluate containing the carbohydrate peak (monitored by the phenol–sulfuric acid reaction[21]) was lyophilized, the residue hydrolyzed, and the hydrolyzate subjected to g.l.c. as before.

Asialation of (removal of sialic acid from) PSGP. — (*a*) *By means of neuraminidase*[20]. To a solution (1 mL) of PSGP (0.5 mg/mL) in acetate buffer (0.1M, pH 5.0) was added 5 μL (0.1 unit) of neuraminidase (from *Clostridium perfringens*, EC 3.2.1.18; Sigma), and incubated for 20 h at 37° under a drop of toluene. The sialic acid liberated was determined by the thiobarbituric acid method of Warren[15].

(*b*) *By acid*[20]. The PSGP (5.6 mg) was heated for 1 h at 80° with 25mM sulfuric acid (2 mL) in a sealed tube, and the sialic acid released was estimated as before. The hydrolyzate was diluted with water (10 mL), and passed through a column (5 × 0.8 cm) of Dowex-1 X4 (formate). The effluent and water washings (100 mL) were combined, and lyophilized, to yield the asialoglycopeptide (GP, 3.5 mg).

Removal of NeuAc and Fuc (and part of Gal) from PSGP by acid treatment[20], *to yield Fraction I* (F_1). — The PSGP (8.2 mg) was heated with 0.5M sulfuric acid (1 mL) for 1 h at 80°. Sulfate and NeuAc were removed from the hydrolyzate with Dowex-1 X4 (formate) resin. The solution was concentrated by lyophilization, and

the concentrate fractionated on a calibrated column (36 × 1.5 cm) of Sephadex G-25, to give Fraction I (F_1, 3.5 mg) and Fraction II (0.8 mg).

F_1 was subjected to high-voltage electrophoresis on paper (Whatman No. 1) in 0.05M borate buffer, pH 9.3, at 3 kV for 45 min, and then stained with the periodate–benzidine reagent[34]. A single spot appeared at 4.8 cm towards the positive electrode. Similar electrophoresis of Fraction II revealed the presence of Fuc, along with a trace of Gal.

Methylation analysis. — The PSGP, GP, and F_1 (2–3 mg) were separately methylated by the method of Hakomori[38] as described by Jansson *et al.*[30]. The sample was dissolved in dimethyl sulfoxide (2 mL; predried over molecular sieve 4A), 2M methylsulfinylmethylsodium (2 mL) was added, and the mixture was stirred overnight at room temperature under nitrogen. Methyl iodide (2 mL) was added dropwise, and the mixture was stirred for 3 h, and poured into water (20 mL); the aqueous solution was transferred to a column of 2:1 (w/w) charcoal (Norit A)–Celite 545, and the methylated compound was eluted as described by Hatcher *et al.*[20]. Completion of methylation was checked by the i.r. spectrum (no OH band).

The respective, permethylated product was hydrolyzed by heating with M sulfuric acid (1 mL) for 5 h on a boiling-water bath, according to Kornfeld *et al.*[24]. The methylated (neutral and amino) sugars were separated on a column (5 × 0.8 cm) of Dowex 50 (H^+) resin, and the hexosamines were eluted from the column with 2M hydrochloric acid (20 mL). Each fraction was reduced with potassium borohydride, and the products were acetylated for g.l.c. as described by Hatcher *et al.*[20].

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REFERENCES

1 S. K. Sikder and A. Das, *Carbohydr. Res.*, 71 (1979) 273–285.
2 R. L. Katzman and E. H. Eylar, *Arch. Biochem. Biophys.*, 117 (1966) 623–637.
3 G. Tettamanti and W. Pigman, *Arch. Biochem. Biophys.*, 124 (1968) 41–50.
4 O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
5 S. K. Sikder and A. Das, *Indian J. Chem.*, 19B (1980) 389–392.
6 J. Moschera and W. Pigman, *Carbohydr. Res.*, 40 (1975) 53–67.
7 Y. Hashimoto, S. Hashimoto, and W. Pigman, *Arch. Biochem. Biophys.*, 104 (1964) 282–291.
8 F. A. Bettelheim and S. K. Dey, *Arch. Biochem. Biophys.*, 109 (1965) 259–265.
9 R. G. Spiro, *Annu. Rev. Biochem.*, 39 (1970) 599–638.
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 D. M. Carlson, *J. Biol. Chem.*, 243 (1968) 616–626.
12 M. Bertolini and W. Pigman, *J. Biol. Chem.*, 242 (1967) 3776–3781.
13 Y. C. Lee and J. R. Scocca, *J. Biol. Chem.*, 247 (1972) 5753–5758.
14 J. Baenziger, S. Kornfeld, and S. Kochwa, *J. Biol. Chem.*, 249 (1974) 1897–1903.
15 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971–1975.
16 Z. Dische, C. Pallavicini, H. Kavasaki, J. Smirnow, I. Cizek, and S. Chen, *Arch. Biochem. Biophys.*, 97 (1962) 459–469.

17 Z. DISCHE, *Ann. N. Y. Acad. Sci.*, 106 (1963) 259–270.
18 R. C. CALDWELL AND W. PIGMAN, *Arch. Oral Biol.*, 11 (1966) 437–449.
19 R. J. WINZLER, E. A. JOHNSON, AND C. LOMBART, in *Membrane-Mediated Information*, Vol. 1, American Elsevier, New York, 1973, pp. 3–19.
20 V. B. HATCHER, G. O. H. SCHWARZMAN, R. W. JEANLOZ, AND J. W. MCARTHUR, *Biochemistry*, 16 (1977) 1518–1524.
21 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
22 H. U. CHOI AND R. CARUBELLI, *Biochemistry*, 7 (1968) 4423–4430.
23 M. LEMONNIER AND R. BOURRILLON, *Carbohydr. Res.*, 51 (1976) 99–106.
24 R. KORNFELD, J. KELLER, J. BAENZIGER, AND S. KORNFELD, *J. Biol. Chem.*, 246 (1971) 3259–3268.
25 B. NILSSON AND S. SVENSSON, *Carbohydr. Res.*, 72 (1979) 183–190.
26 NASIR-UD-DIN, R. W. JEANLOZ, V. N. REINHOLD, AND J. W. MCARTHUR, *Carbohydr. Res.*, 75 (1979) 349–356.
27 N. SHARON, in *Complex Carbohydrates: Their Chemistry, Biosynthesis, and Functions*, Addison–Wesley, Reading, MA, 1975, pp. 118–126.
28 R. G. SPIRO, *Adv. Protein Chem.*, 27 (1973) 349–455.
29 S. N. BHATTACHARYA AND W. S. LYNN, *J. Biol. Chem.*, 252 (1977) 1172–1180.
30 P.-E. JANSSON, L. KENNE, H. LIEDGREN, B. LINDBERG, AND J. LÖNNGREN, *Chem. Commun. (Univ. Stockholm)*, 8 (1976) 22–23.
31 J. LÖNNGREN AND Å. PILOTTI, *Acta Chem. Scand.*, 25 (1971) 1144–1145.
32 K. STELLNER, H. SAITO, AND S.-I. HAKOMORI, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
33 M. I. HOROWITZ AND A. DAS, *Immunochemistry*, 4 (1967) 303–313.
34 J. A. CIFONELLI AND F. SMITH, *Anal. Chem.*, 26 (1954) 1132–1134.
35 M. BREEN, H. G. WEINSTEIN, M. ANDERSON, AND A. VEIS, *Anal. Biochem.*, 35 (1970) 146–159.
36 J. K. HERD, *Anal. Biochem.*, 23 (1968) 117–121.
37 W. NEIDERMEIER AND M. TOMANA, *Anal. Biochem.*, 57 (1974) 363–368.
38 S.-I. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.