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SHORT COMMUNICATION

Isolation and characterization of a sialo-glycopeptide from buffalo colostrum

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A sialoglycopeptide was isolated from buffalo colostrum in pure form by chromatography on Sephadex G-25 and QAE-Sephadex A-25. This was found to be homogeneous by cellulose acetate membrane electrophoresis and reverse phase HPLC. It consisted of fucose, galactose, mannose, *N*-acetyl glucosamine and *N*-acetyl neuraminic acid in the ratio 1:2:3:4:1, and aspartic acid, serine, threonine, proline and glutamic acid were the major amino acids. Glycine was identified as the N-terminal amino acid residue. The structure elucidation of the carbohydrate moiety was carried out by methylation analysis, mass spectrometry, ^{1}H-NMR spectroscopy and the probable structure was revealed to be that of a complex biantennary type.

Keywords: buffalo, colostrum, glycopeptide

Introduction

Colostrum, the first milk, is an extremely complex biological fluid and helps in the development of immunity in the new born. This is mainly due to the presence of a complex mixture of oligosaccharides, glycopeptides, glycoproteins and glycolipids often resembling cell surface glycoconjugates, along with immunoglobulins and phagocytes [1, 2]. Absorption of many of these macromolecules takes place during early lactation [3] and is crucial for the growth and development of the newborn. Some of the glycopeptides isolated from human milk [4, 5] have attracted considerable attention for their bacteriostatic, bactericidal and antiviral activities [6–8]. In this communication, we report the structure of the glycan moiety of a sialoglycopeptide isolated from buffalo (the major milking mammal of Asia) colostrum.

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Materials and methods

Materials

Fresh buffalo colostrum from the first 2 days after delivery was collected locally and kept frozen until analysed.

Fractionation of glycopeptides

The colostrum was extracted with $CHCl_3$:MeOH mixture (2:1, v/v) [9]. The concentrated crude mixture was fractionated on Sephadex G-25 column (105 × 2.5 cm) using water as eluent. Fractions were assayed for neutral sugars [10] and proteins [11] and pooled. Peak I obtained from the above fractionation was subjected to chromatography on QAE-Sephadex A-25 column (40 × 2.5 cm) using H_2O and ammonium acetate buffer (pH 5.4, 0.1–0.5 M) as eluants and fractions were desalted on Biogel P-2 column (40 × 2.5 cm) and lyophilized.

Homogeneity of the glycopeptide

The homogeneity of the glycopeptide was tested by cellulose acetate membrane electrophoresis (Beckman Instruments, Switzerland) using acetate buffer (pH 5.0,

50 mM) and the electrophoretograms were stained with silver nitrate reagent prepared in ethanol instead of acetone [12]. Reverse phase HPLC was carried out on a C-18 column [13] using Shimadzu HIC-6A chromatograph. The mobile phase consisted of (A) 0.1% TFA (5 min) and a linear gradient (0–100%) of CH_3CN (B) containing 0.1% TFA (30 min) at a flow rate 1 ml min^{-1}. The glycopeptide was detected at 220 nm. SDS-PAGE (12%) of the lyophilized sample was carried out using Tris-glycine buffer (pH 8.3) and stained with Coomassie Brilliant Blue [14].

Chemical methods

The amino acid composition of the hydrolysed peptide (6 N HCl containing 1% liquid phenol, 110°, 24 h under vacuum) was determined as phenylthiohydantoin labelled amino acids [15] which were identified on a Waters PICO-TAG column (0.25 × 250 mm, Waters Assoc., USA) using HPLC system. N-terminal amino acid analysis was carried out by 4-dimethyl-amino-azobenzene-4′-isothiocyanate double coupling method [16].

Neutral sugars, amino sugars and sialic acids were estimated according to the methods of McKelvy *et al.* [10], Reissig *et al.* [17] and Aminoff [18]. Identification and quantitation of neutral and amino sugars were done by GLC as their alditol acetates using 3% OV-225 column (2.4 m × 3.8 cm, Pierce Chemicals Co., USA) at 190° and 230°, respectively [19, 20]. Permethylation of pure samples was performed as reported by Hakomori [21] and the partially methylated monosaccharides were analysed by GLC-MS using CP-Sil-5 capillary column (0.22 mm × 25 m, Chromatopak, Middelburg, Netherlands) [22].

NMR analysis

The 400 MHz^1 H-NMR spectra of glycopeptide in D_2O was recorded on a Bruker AMX-400 spectrometer at room temperature. Chemical shifts are reported as ppm on the delta scale and are relative to external standard tetramethyl silane.

Results and discussion

The chloroform-methanol extract of colostrum (yield was 3.3 g per 100 ml colostrum) fractionated on Sephadex G-25 gave three saccharide pools eluting prior to lactose. The first fraction (0.7% of the material loaded on the column) eluted in the void volume and was found to contain glycopeptides, whereas the second (1.2%) and third (2.8%) fractions contained oligosaccharides as reported earlier from bovine and buffalo milk [23, 24]. When the first fraction was chromatographed on QAE-Sephadex A-25 (Fig. 1), two unbound fractions were eluted with water, and the bound fractions were subsequently eluted with buffer into six separate pools in 0.1 M, seven in 0.25 M and two in 0.5 M buffer, respectively.

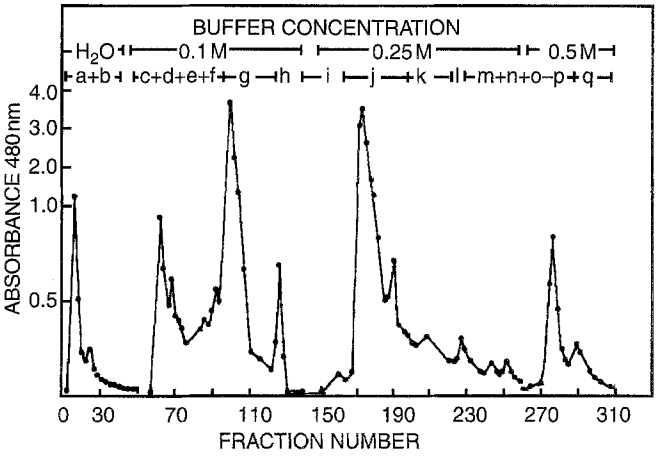

Figure 1. QAE-Sephadex column chromatographic (40 × 2.5 cm) analysis of the first fraction from Sephadex G-25. Elution buffer: water, ammonium acetate buffer (pH 5.4, 0.1–0.5 M). Flow rate 25 ml h^{-1}.

Electrophoresis of the isolated fractions on cellulose acetate membranes showed only fractions "c" and "d" to be homogeneous. They were designated as GP-1 and GP-2 and were obtained in an average yield of 25 mg and 11 mg l^{-1} colostrum, respectively. A detailed study was done only on GP-1. On reverse phase HPLC, GP-1 eluted as a single peak at retention time of 26.75 min. On SDS-PAGE, it gave a single band with a molecular weight of 17 kDa (Fig. 2).

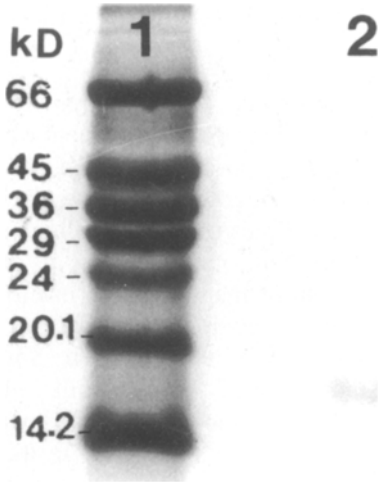

Figure 2. SDS-PAGE of GP-1 run on 12% slab gel along with low molecular weight standards. Gels were stained with Coomassie Brilliant Blue and destained in methanol: acetic acid: water, 25:15:60, v/v mixture.

The amino acid composition of GP-1 is presented in Table 1. Aspartic acid and glutamic acid were the major amino acids. Higher amounts of hydroxy amino acids, serine and threonine are noteworthy. In addition, a higher content of proline was also observed. Glycine was identified as an N-terminal amino acid.

The carbohydrate analysis of GP-1 indicated Fuc, Gal, Man, GlcNAc and NeuNAc in a molar ratio of 1:2:3:4:1.

Permethylation analysis of GP-1 indicated 2,3,4-Me_3-Fuc and 2,3,4,6-Me_4-Gal at terminal positions (Table 2). Man was involved in the formation of the biantennary structure (2,4-Me_2-Man) while the branched Man residues were linked to GlcNAc by a 1,2 linkage (3,4,6-Me_3-Man). Antennary GlcNAc was substituted in a 1,4 linkage (3,6-Me_2-GlcNAcNMe) and the presence of 3-Me-GlcNAcNMe is attributed to branching with Fuc. The presence of 2,4,6-Me_3-Gal is attributed to the substitution by NeuNAc in 2,3 linkage at the terminal position. Fragmentation pattern of NeuNAc could not be observed due to the hydrolytic condition employed [25]. Structural features of biantennary GP-1 were very similar to the glycopeptides isolated from human milk [5], serotransferrin [26], pancreatic ribonuclease [27], antichymotrypsin [28] and also to the complex oligosaccharides excreted in urine during sialidosis [29].

In order to further elucidate the primary structure of the glycan moiety, ^{1}H-NMR spectra of GP-1 was recorded and its characteristic chemical shifts are summarized in Table 3. The presence of mannotrioside branching core [26, 29, 30] was evidenced by the spectral integration of H-2 protons of Man-3, Man-4 and Man-4′ at 4.210 ppm, 4.190 ppm and 4.144 ppm respectively. Substitution of NeuNAc in α-linkage to Gal could be inferred from the set of chemical shifts of the NeuNAc H-3 atoms (H-3ax = 1.800 ppm; H-3eq = 2.758 ppm) as reported earlier [28, 29]. The substitution of α-(2 → 3) NeuNAc to Gal 6′ was evidenced by the shift from 4.0 ppm to 4.116 ppm as previously described in human serotransferrin [31].

Anomeric proton at 5.076 ppm is attributed to β-GlcNAc-1 linked to an Asn residue while the chemical shift at 4.690 ppm indicates the β-linkage between

Table 1. Amino acid composition of GP-1

Amino acids	*Number of residues*
Asx	19
Glx	15
Ser	21
Gly	10
His	04
Arg	05
Thr	20
Ala	13
Pro	17
Tyr	02
Val	04
Ileu	02
Leu	06
Phe	03
Lys	02

Table 2. Methylation analysis data of GP-1

Methyl ether	*GP-1*	*Major peaks* (m/z)
Fucitol		
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetate)	+	175, 161, 131, 117, 101, 89, 58, 43
Galactitol		
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetate)	+	205, 161, 145, 129, 117, 101, 87, 45, 43
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetate)	+	233, 161, 129, 117, 101, 87, 45, 43
Mannitol		
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetate)	+	189, 161, 129, 117, 101, 87, 45, 43
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetate)	+	189, 129, 117, 101, 87, 58, 45, 43
2-*N*-methyl acetamido-2-deoxyglucitol		
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetate)	+	233, 158, 142, 129, 116, 98, 74, 43
3-mono-*O*-methyl (1,4,5,6-tetra-*O*-acetate)	+	261, 158, 142, 116, 74, 43

Table 3. ^{1}H-NMR chemical shifts of structural reporter groups of constituent monosaccharides for the complex oligosaccharide of GP-1

Residue		*Reporter group*	*Chemical shifts (ppm)*
		Anomeric protons	
GlcNAc	1		5.076
	2		4.690
Man	3		4.799
	4		5.163
	4′		4.925
		H-2 protons	
Man	3		4.210
	4		4.190
	4′		4.144
		N-acetyl protons	
GlcNAc	1		2.014
	2		2.094
	5		2.055
	5′		2.063
NeuNAc	2 → 3		2.041
		H-3 protons	
Gal	6,6′		4.116
NeuNAc	H-3ax		1.800
	H-3eq		2.758
Fuc	CH_3		1.215

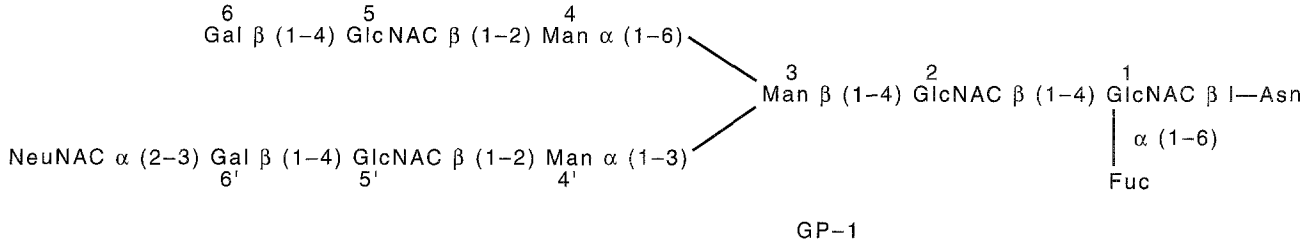

Figure 3. Probable structure of GP-1.

GlcNAc-1 and GlcNAc-2. Mannosyl residues 3,4 and 4′ had characteristic anomeric signals of 4.799 ppm, 5.163 ppm and 4.925 ppm indicating β-linkage of Man with GlcNAc-2 and Man-4, Man-4′ in α-linkage to Man-3, respectively. Values for other anomeric signals could not be determined as the representative signals were not clear due to interference from the peptide moiety. The *N*-acetyl protons of GlcNAc and NeuNAc residues were clear (Table 3). The presence of signals at 2.014 ppm and 2.094 ppm demonstrated the occurrence of an Asn linked di-*N*-acetyl chitobiose unit with fucose in α-(1 → 6) linkage to GlcNAc-1. Similarly, the presence of Fuc α-(1 → 6) GlcNAc-1 causing a shift from 2.076 ppm to 2.094 ppm for the *N*-acetyl protons of GlcNAc-2 was similar to the shift observed with the complex asparagine bound glycopeptide isolated from pancreatic ribonuclease [27]. The presence of a fucosyl residue was also supported by characteristic CH_3 signals at 1.215 ppm.

From the methylation analysis and ^{1}H-NMR data, the probable structure of the glycan moiety of biantennary GP-1 is presented in Fig. 3. The complex biantennary type of oligosaccharide unit reported here appears to be similar to many well characterized glycopeptides from various other sources [32] including human and bovine milk [5]. However the important feature in the proposed structure is the substitution of sialic acid at C-3 of galactose rather than at C-6 as observed in most other cases. This situation is similar to those glycopeptides isolated from fetuin [33], calf thymocyte membrane [34] and galactoprotein [35]. Secondly the presence of biantennary monosialylated oligosaccharide possessing β-galactose exclusively at the terminal position of α-(1 → 6) linked mannose antenna is similar to the situation observed in human fibronectin [36]. Further studies on the physiological significance of this glycopeptide in colostrum will be interesting.

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