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THE CHEMICAL STRUCTURE OF A TETRASACCHARIDE CONTAINING N-ACETYLGLUCOSAMINE OBTAINED FROM BOVINE COLOSTRUM κ-CASEIN

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Summary

A tetrasaccharide consisting of D-galactose, *N*-acetylneuraminic acid, *N*-acetylglucosamine and *N*-acetylgalactosaminitol (1 : 1 : 1 : 1) was obtained from bovine colostrum κ-casein. It is the main carbohydrate portion of a caseinoglycopeptide formed by alkaline borohydride treatment. The carbohydrate sequence was analyzed by enzymatic and chemical methods and by mass spectrometry, and was shown to be *N*-acetylglucosaminyl-β-1-3-galactosyl-β-1-3-[*N*-acetylneuraminyl-α-2-6-]-*N*-acetylgalactosaminitol.

Introduction

The stability of the casein micelle system in bovine milk is dependent on the stabilizing ability of κ-casein. κ-Casein is the only casein fraction which has a carbohydrate moiety in its structure and in which only three different sugars, *N*-acetylneuraminic acid, D-galactose and *N*-acetylgalactosamine have been identified [1]. However, the identification of their sequence was not easy because of the observed microheterogeneity of the carbohydrates [2]. We have reported a structural analysis of a trisaccharide isolated from bovine normal casein [3] and this structure is identical to that of a trisaccharide found by Fournet et al. [4].

Colostrum casein contains more κ-casein than does normal casein, but in colostrum casein micelles were not observed until 2 days after calving [5]. Colostrum κ-casein also contains about twice as much carbohydrate as does κ-casein from normal milk, and also an additional sugar, *N*-acetylglucosamine

[6]. Moreover, 3 days after calving, the sugar content of colostrum caseinoglycopeptide decreased to the normal level and this was also accompanied by the disappearance of *N*-acetylglucosamine [6]. Fournet et al. [2] isolated four different oligosaccharides from colostrum κ-casein and analyzed their sugar composition. The results obtained were much more complex in the case of oligosaccharides from colostrum κ-casein than those from normal κ-casein. No reports have yet been published on the chemical structure of the sugar moiety in colostrum κ-casein.

The present paper describes the fractionation of an oligosaccharide, containing *N*-acetylglucosamine, from bovine colostrum κ-casein and the determination of its chemical structure.

Materials and Methods

Preparation of colostrum caseinoglycopeptide

Colostrum (collected 12 h after parturition) was obtained from a cow. Whole casein from colostrum was prepapred according to the method of Alais and Jollès [7]. Colostrum κ-casein was obtained from purified whole colostrum casein by gel filtration on Sephadex G-150 (Pharmacia, Sweden) according to Yaguchi et al. [8]. Caseinoglycopeptide was prepared by chymosin digestion [3] of the purified colostrum κ-casein. Chymosin (EC 3.4.23.4) was purified from rennet powder (Chr. Hansen Lab., Denmark), the method has been described earlier [9].

Alkaline β-elimination of caseinoglycopeptide and preparation of oligosaccharides

Alkaline β-elimination of caseinoglycopeptide was carried out by incubation of a sample in 0.5 M NaOH at 45°C for 100 min. The qauntities of unsaturated amino acids and chromogen formed were measured spectrophotometrically at 241 nm [10] and 540 nm [11], respectively.

For the preparation of oligosaccharides from caseinoglycopeptide by alkaline borohydride treatment, caseinoglycopeptide was incubated in 0.05 M NaOH containing 1.0 M $NaBH_4$ at 50°C for 24 h [12]. The sugar moieties were purified, by gel filtration on a Sephadex G-25 column, from the reaction mixture and were desalted by ion-exchange chromatography (AG50W X8, H^+ form, Bio-Rad Lab., U.S.A.), and then fractionated by ion-exchange chromatography and gel filtration by the method of Farrar and Harrison [13].

Carbohydrate analyses

The identification and analysis of the sugar components in caseinoglycopeptide and oligosaccharides were performed by gas-liquid chromatography (GLC) of the trimethylsilyl derivatives prepared by the method of Clamp et al. [14] after methanolysis in 0.65 M HCl-CH_3OH at 90°C for 24 h. The conditions for GLC were as follows: JEOL Model JGC-20 KFP (Japan Electron Optics Lab., Japan) gas chromatograph equipped with a glass column packed with 1.5% Silicone SE-52 (Gasukuro Kogyo, Japan) was operated with a temperature gradient of 3 K/min from 100—200°C. The total amount of neutral sugars was determined by the phenol-H_2SO_4 reaction [15]. Sialic acids were determined by the method of Warren [16].

Paper and thin-layer chromatography

Paper chromatography of sugars was carried out on Toyo No. 514 paper by the ascending technique using butan-1-ol/pyridine/water (6 : 4 : 3, v/v) [17] and the papers were developed with alkaline $AgNO_3$ reagent [18]. Thin-layer chomatography (TLC) was developed with propan-2-ol/butan-1-ol/water (5 : 3 : 2, v/v) [13]. The positions of sugars were detected by spraying with 50% H_2SO_4 and heating at 110°C for 10 min.

Methylation analysis of the sugar chain

The oligosaccharide was permethylated according to the method of Hakomori [19,20]. The oligosaccharide was also treated in the same way (by heating 0.1 M H_2SO_4 at 80°C for 1 h). The permethylated products were purified through a silicagel column according to the method of Yamashita et al. [21], and then hydrolyzed, reduced and acetylated as described by Bouveng et al. [22] (for neutral sugars) and by Stellner et al. [23] (for amino sugars). Analysis of the methylated product was performed with a gas-liquid chromatograph column packed with 3% ECNSS-M and was operated at 190°C isothermally. The mass spectrometer (Model JEOL-JMS-06) was used with an ion source pressure of $6 \cdot 10^{-7}$ mmHg with an ionizing current of 300 μA and an electron energy of 75 eV.

Anomer analysis by several glycosidases

Oligosaccharide was successively digested with the following enzymes: α-neuraminidase (EC 3.2.1.18) in 0.1 M sodium acetate buffer containing 10^{-2} M $CaCl_2$ (pH 6.5) at 37°C for 48 h; β-*N*-acetylglucosaminidase (EC 3.2.1.30) from jackbean (Seikagaku Kogyo, Japan) in 0.05 M sodium citrate buffer (pH 4.0) at 37°C for 24 h; β-galactosidase (EC 3.2.1.23) from *Escherichia coli* (Seikagaku Kogyo) in 0.1 M sodium phosphate buffer (pH 7.0) at 37°C for 48 h. Toluene was added as an antiseptic in the reaction mixtures and each step of the reactions was stopped by heating at 80°C for 10 min.

Results

Carbohydrate composition of colostrum caseinoglycopeptide

Four different carbohydrates, D-galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and *N*-acetylneuraminic acid, were detected by GLC in caseinoglycopeptide from colostrum κ-casein, although only three carbohydrates, D-galactose, *N*-acetylgalactosamine and *N*-acetylneuraminic acid, were observed in caseinoglycopeptide from normal milk, confirming the data of Guérin et al. [6].

Alkaline β-elimination reaction of colostrum caseinoglycopeptide

The formation of chromogen and unsaturated amino acids slowly increased. The absorbance at 241 nm by unsaturated amino acids reached a steady value after approx. 80 min. The absorbance at 540 nm by chromogens increased essentially parallel to the formation of the unsaturated amino acids, and reached a maximum after about 70 min. No *N*-acetylgalactosamine was detected in the reaction mixture by GLC analysis after alkali treatment. These results confirm

the observations of Gottschalk [10] and Kochetkov et al. [11] that all *N*-acetylgalactosamine in the carbohydrate unit of the colostrum caseinoglycopeptide is linked *O*-glycosidically to threonine or serine.

Preparation of the oligosaccharides from colostrum caseinoglycopeptide and their carbohydrate composition

Colostrum caseinoglycopeptide was treated by alkaline borohydride, and the sugar-containing fractions from the Sephadex G-25 column were combined, lyophilized, and then fractionated by DEAE-Sephadex A-25 ion-exchange chromatography.

Elution was monitored by hexose and sialic acid analysis. Five well-separated peaks were obtained (Fig. 1). Fractions corresponding to each peak (I—V) were separately combined and rechromatographed on a Sephadex G-25 column. Fractions II—V gave a major peak containing hexose and sialic acid, accompanied by a few small peaks. The major peak from fraction I contained hexose but no sialic acid.

The sugar compositions of the five purified oligosaccharides analyzed by GLC are summarized in Table I. Amino acids were detected in the acid hydrolysate (after treatment with 6 M HCl at 100°C for 20 h) of fractions I and II by paper chromatography. This indicates that β-elimination by alkaline borohydride is not complete under these conditions. No amino acids were detected in oligosaccharides III, IV and V. The amount of oligosaccharide III with a low D-galactose content was too small to conduct further structural analysis. Oligosaccharide V contained no *N*-acetylglucosamine and was considered to be the same trisaccharide as the main sugar moiety of normal caseinoglycopeptide. Oligosaccharide IV had a mobility, R_{Gal}, of 0.37 on TLC, and was composed of D-galactose, *N*-acetylgalactosaminitol, *N*-acetylglucosamine and *N*-acetylneuraminic acid in a molar ratio of 1 : 1 : 1 : 1. The results of GLC of the trimethylsilyl derivatives are shown in Fig. 2. Oligosaccharide IV was, therefore, considered to be a tetrasaccharide and was subjected to further structural analysis.

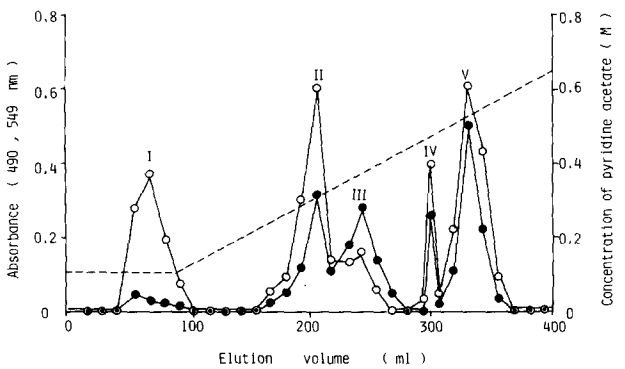


Fig. 1. Ion-exchange chromatography of carbohydrate-containing fractions from colostrum caseinoglycopeptide. Column: 2.5 X 20 cm, DEAE-Sephadex A-25. ------, linear gradient of 0.05—0.65 M pyridine acetate buffer, pH 5.0. o———o, absorbance at 490 nm using the phenol-H_2SO_4 reaction for hexose. o———o, absorbance at 549 nm using the method of Warren [16] for sialic acid.

TABLE I

CARBOHYDRATE COMPOSITION OF OLIGOSACCHARIDES (I, II, III, IV AND V) OBTAINED FROM COLOSTRUM CASEINOGLYCOPEPTIDE BY ALKALINE BOROHYDRIDE TREATMENT

I, II: relative molar ratio estimated by setting *N*-acetylgalactosamine to 1.00. III, IV, V: relative molar ratio estimated by setting *N*-acetylgalactosaminitol to 1.00.

Sugars	Oligosaccharide				
	I	II	III	IV	V
Amino acids	+	+	—	—	—
D-Galactose	1.02	2.32	0.19	1.22	0.87
N-Acetylgalactosamine	1.00	1.00	—	—	—
N-Acetylgalactosaminitol	—	—	1.00	1.00	1.00
N-Acetylglucosamine	—	—	1.02	0.85	—
N-Acetylneuraminic acid	—	1.05	0.82	1.04	0.94

Identification of sugar linkages, and their anomer types, of oligosaccharide IV

One neutral and one basic fraction were obtained by hydrolysis of permethylated oligosaccharide IV and subsequent ion-exchange chromatography. The fractions were converted into their alditol acetate derivatives and then were analyzed by GLC and mass spectrometry. The identification data of both fractions by GLC and mass spectrometry are summarized in Table II.

Only one peak was observed on GLC of the neutral fraction, which was identified by mass spectrometry as 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol (*m/e* values of 45, 117, 161 and 233). Two peaks were detected on GLC of the basic fraction, they were identified by mass spectrometry as 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol and 1,4,5-tri-*O*-methyl-3,6-di-*O*-acetyl-2-deoxy-2-*N*-methylacetaminidogalactitol. The *N*-acetylglucosamine was, thus, not substituted, the *N*-acetylgalactosaminitol was di-substituted at positions 3 and 6.

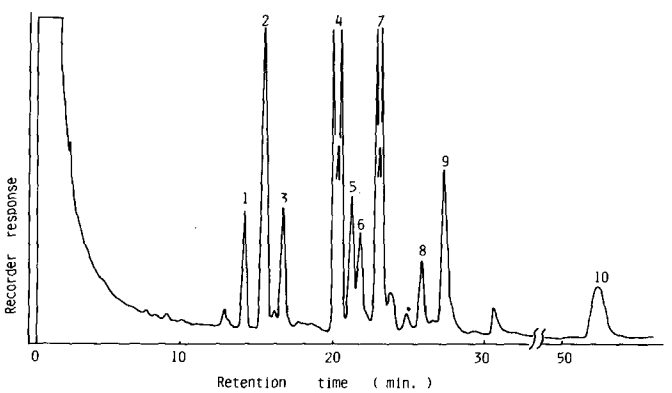


Fig. 2. Gas-liquid chromatography of trimethylsilyl ether derivatives of *O*-methylglycosides obtained by methanolysis of oligosaccharide IV. GLC conditions: column: 0.3 × 300 cm, glass, 1.5% SE-52 on chromosorb W AWDMCS (100/120). column temperature: 100—200°C (3 K/min). Identification of peaks: 1,2,3: D-galactose; 4: D-mannitol (internal standard); 7: *N*-acetylgalactosaminitol; 5,6,8,9: *N*-acetylglucosamine; 10: *N*-acetylneuraminic acid.

TABLE II

IDENTIFICATION BY GAS-LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY OF ALDITOL ACETATE DERIVATIVES OBTAINED FROM PERMETHYLATED OLIGOSACCHARIDE IV

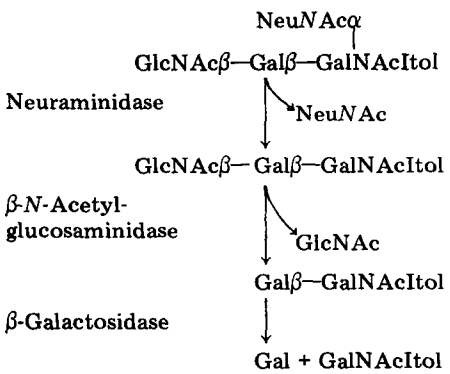
(1) intact oligosaccharide IV, (2) oligosaccharide IV after disialylation.

Methylated sugars	Oligosaccharide IV		Major peaks: *m/e*
	(1)	(2)	
Galactitol			
2,4,6-Tri-*O*-methyl (1,3,5-Tri-*O*-acetyl)	+	+	45, 117, 161, 233
2-Deoxy-2-*N*-methylacetamidoglucitol			
3,4,6-Tri-*O*-methyl (1,5-Di-*O*-acetyl)	+	+	45, 116, 129, 142, 145, 158, 161, 202, 205
2-Deoxy-2-*N*-methylacetamidogalactitol			
1,4,5-Tri-*O*-methyl (3,6-Di-*O*-acetyl)	+	—	43, 88, 101, 117, 130, 142, 161
1,4,5,6-Tetra-*O*-methyl (3-Mono-*O*-acetyl)	—	+	43, 45, 88, 101, 130, 133, 142, 216

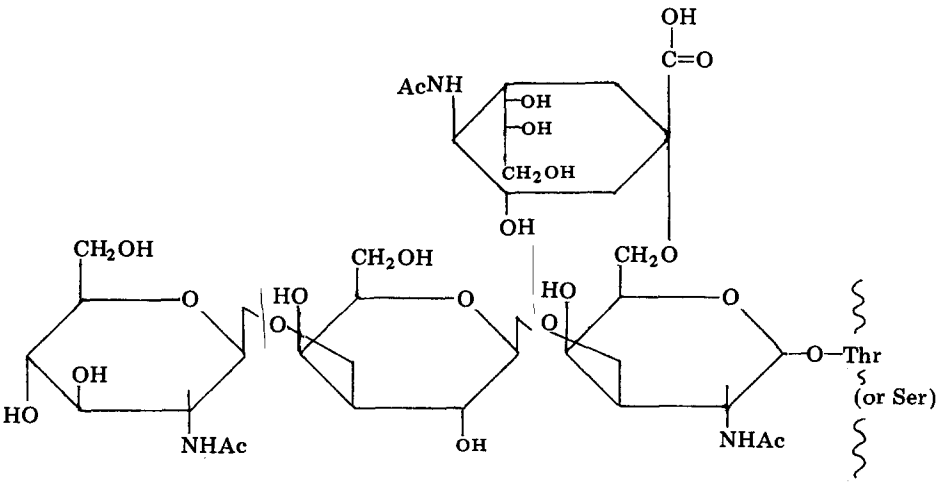
The oligosaccharide IV was desialylated, permethylated and analyzed as above. 2,4,6-Tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol and 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol and a new galactosaminitol derivative, 1,4,5,6-tetra-*O*-methyl-3-mono-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol were identified by GLC and mass spectrometry analysis. This indicates that the *N*-acetylneuraminic acid is linked to the *N*-acetylgalactosaminitol by a 2 → 6 bond.

The pattern of successive digestion of the oligosaccharide IV by several glycosidases is shown in Scheme I. The identification of carbohydrates liberated was carried out by paper chromatography.

In conclusion, the chemical structure shown in Scheme II is proposed as the sugar sequence of oligosaccharide IV isolated from bovine colostrum κ-casein.



Scheme I. Enzymatic digestion with several glycosidases of oligosaccharide IV isolated from colostrum κ-casein.



Scheme II. Sugar sequence of the oligosaccharide obtained from colostrum κ-casein. (*N*-acetylglucosaminyl-β-1-3-galactosyl-β-1-3-[*N*-acetylneuraminyl-α-2-6-]-*N*-acetylgalactosaminyl-X (threonine or serine)).

Discussion

The sugar composition of bovine colostrum κ-casein was characterized by the presence of *N*-acetylglucosamine together with *N*-acetylgalactosamine [1], but, unexpectedly, the former had disappeared 66 h after parturition [6]. Further reports illustrating such phenomena from a biochemical standpoint have not yet been presented.

The tetrasaccharide obtained in this work from colostrum κ-casein was a characteristic oligosaccharide, having GlCNAc at a non-reducing end, which was not observed in normal κ-casein. The same sugar structure is rarely found in glycoproteins other than blood group substances. A similar tetrasaccharide moiety, Gal-β-1-3(or 4)-GlcNAc-β-1-3-Gal-β-1-3-GalNAc, was presented as a commonly occurring sugar chain in blood groups A, B, H and Le^a [24]. In this case, the different types of blood group determinant can be formed by the enzymatic binding of additional carbohydrates to D-galactose at the non-reducing end. A biosynthetic pathway for the incorporation of carbohydrates into the carbohydrate side-chain of glycoproteins was presented by Schachter et al. [25].

The fact that kinds of oligosaccharides differing were from those of normal κ-casein liberated from colostrum κ-casein by alkaline borohydride treatment [2] suggests that the sugar moiety in κ-casein is variably modified by enzymatic action in the early colostrum period. The reason for the colostrum κ-casein oligosaccharide found in the present work being different from that found by Fournet et al. [2] may be due to some factor that alters during the early stages of lactation.

Further investigations are necessary for elucidation of the compositional and structural alterations in the sugar moiety of κ-casein following parturition.

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