STUDIES ON THE CARBOHYDRATE STRUCTURE OF BOVINE MILK GALACTOSYLTRANSFERASE

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1. Introduction

The lactose synthase enzymic system of bovine milk consists of two protein components, a galactosyltransferase (EC 2.4.1.22) and the well-studied milk protein, α-lactalbumin [1]. The galactosyltransferase is a glycoprotein [2] which is reported to have about 12% by weight of carbohydrate [3]. The soluble enzyme can exist in two major multiple protein forms [4] which have similar activity and which contain carbohydrate [5]. The multiple forms are a result of proteolysis and no carbohydrate is lost during the proteolytic conversion of a higher mol. wt from (58 000) to lower mol. wt form (42 000) [4].

The purpose of this paper is to report on the detailed carbohydrate composition of the galactosyltransferase isolated from bovine milk and to present evidence for heterogeneity in the carbohydrate moiety and for the presence of *O*-glycosidic glycopeptide linkages in this protein.

2. Materials and methods

Galactosyltransferase was prepared from bovine milk as previously described [6] and shown to be pure by electrophoresis [4]. Neutral monosaccharides were determined on a Technicon chromatographic system [7] after hydrolysis with 2 N H_2SO_4 for 4 hr at 100°C and passage of hydrolysate through coupled columns of Dowex 50 and Dowex 1 resin [8]. Hexosamines were determined on the amino acid analyzer [9] after hydrolysis of the protein or glycopeptide with 4 N HCl at 100°C for 6 hr [8]. Amino acid analyses were performed on a Beckman 120 analyzer [9] after hydrolysis of samples at 110°C for 24 and 48 hr in 6 N HCl containing 4% thioglycolic acid [10] in evacuated sealed tubes. Sialic acid was determined by the thiobarbituric assay method [11] after hydrolysis of the sample with 0.05 M H_2SO_4 at 80°C for 1 hr. Sialic acids were identified after hydrolysis by thin-layer chromatography on cellulose sheets developed in *n*-butyl acetate–acetic acid–H_2O (3:2:1) for 18 hr and stained with thiobarbituric acid [12]. For the enzymatic release of sialic acid the glycoprotein was incubated with neuraminidase from *Cl. perfrigens* at 37°C in 0.1 M sodium acetate buffer, 0.001 M $CaCl_2$, pH 5.0 [13].

Galactosyltransferase (17.8 mg) was digested with pronase (Calbiochem) in 1 ml of 0.2 M Tris–HCl buffer, pH 7.8, containing 0.0015 M $CaCl_2$, at 37°C in the presence of a small amount of toluene. Initially pronase was added equal to 1% of the weight of galactosyltransferase, and 0.5% pronase (w/w) was added at 25 and 48 hr. Total digestion time was 96 hr after which the digestion was stopped by heating to 100°C for 10 min. After centrifugation the supernatant solution, which accounted for all the carbohydrate in the transferase, was removed and lyophilized. The lyophilized material was dissolved in 0.2 ml of 0.1 M pyridine acetate buffer, pH 5.0, and applied to a column of Bio-Gel P-10 (100–200 mesh) equilibrated with same buffer. The column was eluted with the pyridine acetate buffer at a flow rate of 3.0 ml/hr and fractions of 0.2 ml were collected. Aliquots of fractions were analyzed for neutral

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sugars, sialic acids, and amino acids and peptides by micro-modifications of the anthrone reaction [14], thiobarbituric method [11] and ninhydrin reaction [15], respectively. Selected fractions were pooled for further study.

Glycopeptide fractions, I-III from the Bio-Gel P-10 column were incubated with 0.5 M Na_2SO_3 in 0.1 M NaOH for 48 hr at 37°C [16]. At the end of the reaction time, the samples were acidified with glacial acetic acid, lyophilized, and analyzed.

3. Results and discussion

3.1. *Carbohydrate and amino acid composition*

The carbohydrate composition of the galactosyltransferase is presented in table 1. Sialic acids were identified as predominantly *N*-acetyl neuraminic acid and a small amount of *N*-glycolylneuraminic acid by thin-layer chromatography. The total amount of hexoses, hexosamines and sialic acids of 4.98%, 5.32%, and 3.02%, respectively, differs from the values of 8.1%, 2.2%, and 2.0% reported by Trayer and Hill [3]. They also reported the presence of glucose but this monosaccharide was absent in our preparations. Their higher hexose value may reflect the glucose content which may be a contaminant from a column chromatographic step. The amino acid composition of galactosyltransferase was similar to that reported by Trayer and Hill [3]. Complete removal of the sialic acid from the glycoprotein by treatment with neuraminidase over a three hour period had no effect on the specific activity of the enzyme.

Table 1
Carbohydrate composition of galactosyltransferase

Monosaccharide	Residue weight (g/100g of protein)	Residues/molecule[a]
Hexoses	4.98	15.7
Mannose	2.17	6.8
Galactose	2.81	8.9
Hexosamines[b]	5.32	13.3
Glucosamine	2.39	6.0
Galactosamine	2.93	7.3
Fucose	0.52	1.7
Sialic acid[b]	3.02	5.0

[a] Calculations based on equal amounts of 44 000 and 58 000 mol. forms as determined by SDS polyacrylamide gel electrophoresis.
[b] Expressed as *N*-acetyl derivative.

3.2. *Fractionation and composition of glycopeptides*

In order to investigate the structure of the carbo-

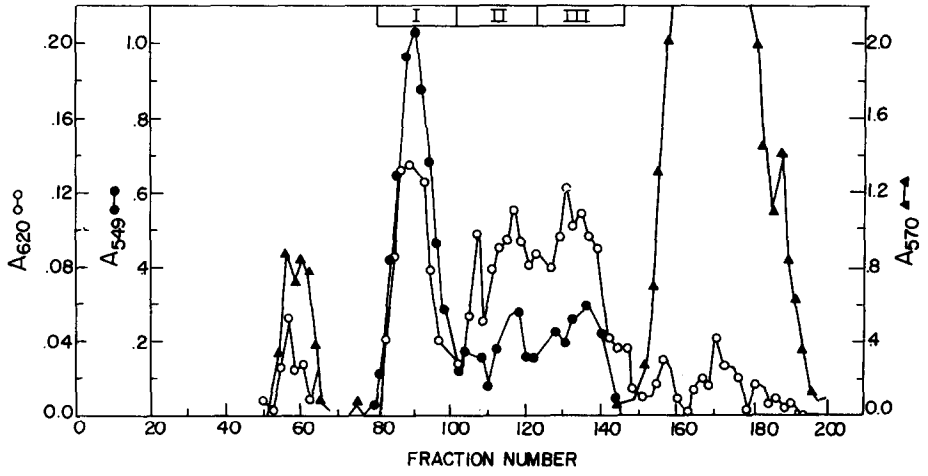

Fig.1. Gel filtration on Bio-Gel P-10 of a pronase digest of 17.8 mg of galactosyltransferase (see Materials and methods). Color formation with; anthrone (○), tiobarbituric (●) and ninhydrin (▲) were determined. Numbered areas (I–III) designate fractions pooled for further study.

hydrate moiety of galactosyltransferase, the enzyme was digested extensively with pronase and the resulting glycopeptides were fractionated by gel filtration chromatography (fig.1). A small amount of carbohydrate containing material was present in the void volume of the column which probably represents undigested protein. The glycopeptide region was heterogeneous but showed the presence of three major fractions (I, II, III) of glycopeptides differing in mol. wt. It is apparent that the relative amounts of sialic acid compared to hexoses were highest in the higher mol. wt fraction (I) and least in the lower mol. wt fractions (II and III).

The composition of these glycopeptide fractions is presented in table 2. Each fraction contains all the monosaccharides present in the intact enzyme but their relative proportions vary. The amount of sialic acid relative to the neutral monosaccharides was highest in fraction I and progressively decreased in fractions II and III, as shown by the ratios 0.79, 0.46, and 0.36, respectively. This result was also clearly evident from the elution profile in fig.1. Also, the ratio of mannose to galactosamine increased from 0.59 in fraction I to 1.43 and 1.29 in fractions II and III, respectively. These three major fractions of glycopeptides differ distinctly with regard to the size of their respective carbohydrate units as opposed to difference in the protein moiety, since the carbohydrate accounts for 71, 72 and 49% of the weight of fractions I, II, and III, respectively. This would argue that fractions II and III are not extensive proteolytic digestion products of fraction I. Therefore, the monosaccharide residues of galactosyltransferase appear to the distributed about carbohydrate units which are heterogeneous with respect to both composition and size.

Table 2
Composition of glycopeptide fractions from Bio-Gel P-10 chromatography of pronase digest of galactosyltransferase

Component	Bio-Gel Fraction		
	I	II	III
	μmol/μmol aspartic acid		
Mannose	2.4	3.0	1.2
Galactose	3.7	2.2	0.72
Fucose	0.93	0.03	0.22
Sialic acid	5.5	2.4	0.75
Glucosamine	2.7	2.8	1.1
Galactosamine	4.1	2.1	0.93
Aspartic acid	1.00	1.00	1.00
Threonine	2.8	1.4	1.1
Serine	3.0	1.8	0.9
Glutamic acid	0.35	0.74	1.3
Proline	0.35	1.6	2.2
Glycine	2.6	0.75	0.69
Alanine	0.99	0.51	0.50
Valine	0.82	0.46	0.38
Isoleucine	0.0	0.15	0.27
Leucine	0.96	0.27	0.39
Lysine	0.0	0.31	0.41
Histidine	0.56	0.39	0.21
Arginine	0.56	0.29	0.16
Percentage carbohydrate by weight	71	72	49

3.3. *Studies on the glycopeptide linkage in the Bio-Gel fractions*

The presence of galactosamine in each fraction suggests that each contains the *O*-glycosidic glycopeptide linkages involving galactosamine and serine or threonine. To determine the presence of these linkages, the three glycopeptide fractions (fig. 1), which do not contain half-cystine, were treated with alkaline sulfite. The results of this study, as presented in table 3, showed the destruction of threonine and serine in each fraction with a concomitant destruction of galactosamine. Only a small amount of glucosamine was destroyed. In addition, there was a concomitant increase in the amount of cysteic acid and/or 3-methyl cysteic acid produced from sulfite addition to the unsaturated amino acids formed during the β-elimination of serine and threonine. Therefore, these results strongly indicate the existence of *O*-glycosidic linkages in each of the fractions which involved galactosamine and threonine and serine.

The yields of the sulfonated amino acids as compared to the amount of serine and threonine destroyed (68%, 51%, and 26% for fractions, I, II, and III, respectively) are consistent with the yields of 25% [17], 30% [18] and 92% [16] reported by others. The lower yield in fraction II and III relative to I probably reflects the increased amount of threonine which is destroyed relative to serine because the conversion of threonine through α-aminocrotonic

Table 3
Effect of alkaline sulfite treatment on glycopeptides of galactosyltransferase

Component	Bio-Gel P-10 fraction[a]					
	I		II		III	
	Control	difference[b]	Control	difference[b]	Control	difference[b]
Cysteic acid + 3-methyl cysteic acid	0.0	+1.50	0.0	+0.76	0.0	+0.88
Threonine	9.20	−0.59	6.64	−0.66	10.3	−2.0
Serine	9.70	−1.62	8.83	−0.84	8.3	−1.35
Galactosamine	12.2	−1.80	9.20	−1.97	7.8	−1.75
Glucosamine	8.0	−0.14	12.4	−0.3	8.95	−0.45
△GalN/△Thr + △Ser		0.82		1.31		0.52
$\triangle CySO_3H/\triangle Thr + \triangle Ser$		0.68		0.51		0.26

[a] Glycopeptide fractions from Bio-Gel P-10 column shown in fig.1.
[b] Difference between control value and value after treatment with 0.1 N NaOH–0.5 M Na_2SO_3 for 48 hr at 37°C.

acid into 3-methylcysteic acid is more difficult [19].

The presence of mannose in each of the glycopeptide fractions suggests the presence of carbohydrate units with the glycosylamine type glycopeptide linkage involving glucosamine and asparagine. This postulation is based on the generalization made by Spiro [20] and Kramer [21] regarding the composition, structural features, and glycopeptide bonds of carbohydrate units which have been studied in detail for a variety of unrelated glycoproteins. That is, the glucosamine-asparagine linked unit lacks galactosamine and possesses a central core of alternating glucosamine and mannose residues from which peripheral branches extend, and the galactosamine-serine or -threonine linked unit lacks mannose and possesses a central core of alternating glucosamine and galactose residues from which peripheral residues are attached.

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