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SELECTIVE RELEASE OF THE DISACCHARIDE 2-ACETAMIDO-2-DEOXY-3-*O*-(β-D-GALACTOPYRANOSYL)-D-GALACTOSE FROM EPIGLYCANIN BY ENDO-*N*-ACETYL-α-D-GALACTOSAMINIDASE

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ABSTRACT

Epiglycanin, the major glycoprotein of TA3-Ha mammary carcinoma ascites cells, was radiolabeled with tritium in the terminal D-galactose and 2-acetamido-2-deoxy-D-galactose residues. Alkaline-borohydride treatment, reported to release five *O*-glycosyl-linked chain types from epiglycanin, resulted in the cleavage of 98–99% of the radioactivity from the protein. Of this, 63% of the radioactivity from epiglycanin and 70% from asialoepiglycanin co-migrated with an authentic sample of 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactitol on a column of Bio-Gel P-6. Incubation of [^{3}H]galactose-epiglycanin with endo-*N*-acetyl -α-D-galactosaminidase *(Diplococcus pneumoniae)*, and fractionation of the mixture on a column of Bio-Gel P-4, gave only one oligosaccharide peak containing 62 and 70%, respectively, of the radioactivity of epiglycanin and asialoepiglycanin. This oligosaccharide comigrated with authentic 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactose (**1**) on columns of Bio-Gel P-2 and P-4 and on paper chromatograms. Results of experiments in which unlabeled epiglycanin was treated with enzyme and the products analyzed, by three different methods, suggested that 78–85% of **1** had been cleaved. Another enzyme, *N*-acetyl-α-D-galactosaminyl-oligosaccharidase from *Clostridium perfringens*, exhibited similar specificity and cleaved 65% of the radioactivity from ([^{3}H]galactose)asialoepiglycanin, which was eluted from a Bio-Gel P-2 column as the disaccharide **1**.

INTRODUCTION

Epiglycanin, a glycoprotein of ~500,000 molecular weight, is the major cell-surface component of the mouse TA3-Ha mammary carcinoma ascites cell[1,2]. Immunochemical and chemical evidence has shown that ~60% of the carbohydrate in epiglycanin is present as β-D-Gal (1→3)-D-GalNAc bound by an *O*-glycosyl linkage

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to serine and threonine[3]. The anomeric nature of the glycopeptide linkage has not been reported, even though, based on analogy to other glycoproteins containing similar structures[4–6], an α-anomeric linkage was considered probable. In order to obtain unequivocal proof for the configuration of this linkage, we examined the effect of endo-*N*-acetyl-α-D-galactosaminidase on epiglycanin. In addition, we were interested in partial enzymic deglycosylation of epiglycanin in order to investigate the specificity of the anti-epiglycanin antibody[7]. The results show that the disaccharide β-D-Gal(1→3)-D-GalNAc is α-linked to serine or threonine, and that the disaccharide may be removed virtually quantitatively from intact epiglycanin by the enzyme.

EXPERIMENTAL

Materials. — Epiglycanin was purified from TA3-Ha ascites cells as described previously[1,3]. Endo-*N*-acetyl-α-D-galactosaminidase and neuraminidase were purified from culture filtrates of *Diplococcus pneumoniae*[8,9]. These preparations were free from contaminating glycosidases and proteases[8,9]. *Vibrio cholerae* neuraminidase was purchased from Calbiochem, La Jolla, CA. Jack-bean exo-β-D-galactosidase[10] and *Escherichia freundii* endo-β-D-galactosidase[11] were gifts from Prof. Y.-T. Li, Tulane University, New Orleans, LA. *N*-Acetyl-α-D-galactosaminyl-oligosaccharidase *(Clostridium perfringens)* was purchased from Bethesda Laboratories, Rockville, MD. D-Galactose oxidase and horse-radish peroxidase were bought from Worthington Biochem. Corp., Freehold, N.J. NaB^3H_4 (340 mCi/mmol) was purchased from New England Nuclear, Boston, MA.

Asialoepiglycanin was prepared by treament of epiglycanin for 24 h at 37° with *Vibrio cholerae* neuraminidase (1 unit) in 200 μL of 0.1M sodium acetate buffer, pH 5.6, containing mM Ca^{2+}.

[^{3}H]Galactose-labeled epiglycanin and asialoepiglycanin were prepared by treatment with D-galactose oxidase followed by NaB^3H_4 according to the method of Morell and Ashwell[12]. After heat inactivation of the enzymes, the labeled samples were recovered by exhaustive dialysis against water followed by lyophilization.

Digestion with enzymes. — Treatment with endo-*N*-acetyl-α-D-galactosaminidase from *D. pneumoniae* was performed in 50mM Tris–maleate buffer, pH 7.0, with 2 mU of enzyme for 24 h at 37° in a volume of 50 μL. In some experiments, the treatment was continued for 72 h, with addition of enzyme (2 mU) every 24 h. Treatment with *E. freundii* endo-β-D-galactosidase was in 0.05M sodium acetate buffer, pH 5.8, for 24 h at 37° (ref. 11). Incubation with *C. perfringens N*-acetyl-α-D-galactosaminyl-oligosaccharase was in 50mM potassium phosphate buffer, pH 6.5, with 10 mU enzyme for 72 h at 37° (ref. 13). All digestions were terminated by heating for 2–5 min at 100°.

Gel filtration. — Columns of Bio-Gel P-2 (minus 400 mesh), P-4, P-6 (200–400 mesh), and P-100 (100–200 mesh) were equilibrated and eluted with 0.1M pyridine–acetic acid, pH 5.0.

RESULTS AND DISCUSSION

Characterization of [^{3}H]galactose-labeled epiglycanin. — The specific activities of [^{3}H]epiglycanin and [^{3}H]asialoepiglycanin prepared by treament with D-galactose oxidase followed by sodium borotritide were 13×10^4 and 20×10^4 d.p.m. per μg, respectively. On acid hydrolysis of the labeled asialoepiglycanin (M HCl, 5 h, 100°) and examination of the products by paper chromatography with 6:4:3 (v/v) 1-butanol–pyridine–water as solvent, it was found that 91% of the tritium was associated with galactose and 9% with 2-amino-2-deoxygalactose. Previous results have shown that ~19% of the 2-acetamido-2-deoxy-D-galactose residues in epiglycanin are present as single, non-reducing units[3]. The lower than expected level of labeling of GalNAc may be due to the unavailability of these residues for the D-galactose oxidase because of steric reasons[14] and to the observation that non-reducing terminals of GalNAc are poorer substrates than Gal for the enzyme (Bhavanandan, unpublished results). Bonnet-monkey cervical mucin[15] labeled similarly yielded, on hydrolysis, [^{3}H]galactose (64%) and 2-amino-2-deoxy-D-[^{3}H]galactose (36%).

Alkaline-borohydride treatment[16] of the [^{3}H]labeled epiglycanin and asialoepiglycanin, followed by chromatography on a calibrated column of Bio-Gel P-6, showed that 98 and 99%, respectively, of the labeled material was released by β-elimination. Of the eliminated oligosaccharides, 63 and 70%, respectively, co-migrated with authentic 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactitol isolated from fetuin[5,17].

*Action of endo-*N*-acetyl-α-*D*-galactosaminidase.* — The result of a typical experiment in which an epiglycanin sample was treated with the endo-*N*-acetyl-α-D-galactosaminidase and the digest examined by gel filtration is illustrated in Fig. 1. After 24 h of incubation, 43–50% of the label was released as a disaccharide that co-migrated with authentic 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactose on columns of Bio-Gel P-4 (Fig. 1) and P-2 (not illustrated). Incubation for longer periods (up to 72 h) or isolation of the material excluded from the column of Bio-Gel P-2 or P-4 (see Fig. 1) and retreatment with the enzyme resulted in the release of additional quantities of the disaccharide. A maximum of 62 and 70% of the label in epiglycanin and asialoepiglycanin, respectively, could be released as the disaccharide.

The product released from [^{3}H]asialoepiglycanin was recovered by combining fractions 78–86 (Fig. 1) and lyophilizing to remove pyridine and acetic acid. On paper chromatography, the recovered material comigrated with β-D-[^{3}H]Gal-(1→3)-D-GalNAc isolated from asialoglycophorin A and with β-D-Gal(1→3)-D-GalNAc isolated from fetuin (Fig. 2). Another portion of the material was dissolved in 50 μL of 0.05M citrate–phosphate buffer, pH 4.0, and incubated with 2 units of jack bean β-galactosidase for 29 h at 37°. Carrier galactose (1 mg) was added to the digest and the mixture chromatographed on a column of Bio-Gel P-2. Of the radioactivity, 37% was coeluted with galactose (Fig. 3), confirming that the

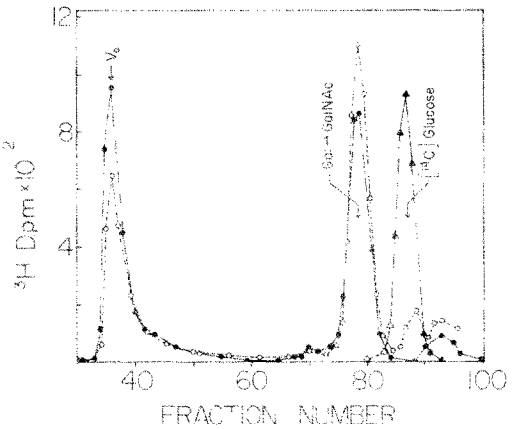

Fig. 1. Elution profiles on Bio-Gel P-4 of [^{3}H]epiglycanin (●——●) and [^{3}H]asialoglycophorin (○------○) after treatment with endo-α-*N*-acetylgalactosaminidase. The treated samples were mixed with D-[^{14}C]glucose (▲——▲) and chromatographed on a column (0.9 × 108 cm) of Bio-Gel P-4. The column was eluted with 0.1M pyridine–acetic acid, and 1-mL fractions collected and analyzed for radioactivity by liquid-scintillation counting. The void volume (V_o) and elution position of authentic β-D-Gal-(1→3)-GalNAc are indicated.

disaccharide consists of terminal [^{3}H]galactose linked in the β-D-configuration[18]. The partial cleavage of the disaccharide is because this enzyme is known to act very poorly on D-galactose β-(1→3)-linked to the aglycon[10]. In fact, under the same incubation conditions as before, this enzyme failed to release [^{3}H]galactose from [^{3}H]asialoepiglycanin or from the reduced disaccharide, β-D-[^{3}H]Gal-(1→3)-GalNAcol, isolated from asialoepiglycanin by alkaline borohydride treatment. Thus, it appears that the nature of the aglycon influences the activity of jack-bean β-galactosidase on β-D-(1→3)-linked galactose.

To determine on a mass basis the percent of the disaccharide, 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactose, released from epiglycanin, we performed the following experiments. Epiglycanin was incubated with endo-*N*-acetyl-α-D-galactosaminidase alone or in the presence of *D. pneumoniae* neuraminidase, for 48 h at 37°. The disaccharide released was quantitatively determined by the modified Morgan–Elson assay[19], with GalNAc as standard. In calculating the amount of the disaccharide released, the report that GalNAc substituted at O-3 gives 60% more color than unsubstituted GalNAc[20] was taken into consideration. It was found that 15 μg of epiglycanin yielded 17.9 and 21.4 nmol of the disaccharide when treated with the endo-enzyme alone or with the endo-enzyme and neuraminidase, respectively. This result corresponds to the release of 60 and 73%, respectively, of total GalNAc as the disaccharide.

In other experiments, 66 μg aliquots of epiglycanin were incubated with buffer alone or with the endo-enzyme (6 mU) and neuraminidase (42 mU). The digests were fractionated on pre-calibrated Bio-Gel P-2 columns to yield undigested material and enzyme (void-volume peak) and the released disaccharide (included

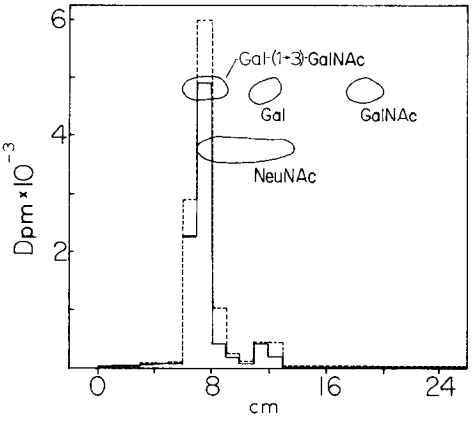

Fig. 2. Paper chromatography of the endo-α-*N*-acetylgalactosaminidase-released products from [^{3}H]asialoepiglycanin (——) and [^{3}H]asialoglycophorin (------). The [^{3}H]labeled products recovered from the Bio-Gel P-4 fractions 78–86 (Fig. 1) were chromatographed by using butyl acetate–acetic acid–water (3:2:1, v/v) as solvent. Paper strips containing the labeled samples were cut into pieces 1 cm wide, extracted with 1 mL of water, and the extracts analyzed for radioactivity. The migration positions of standard sugars were detected by the silver nitrate staining-procedure.

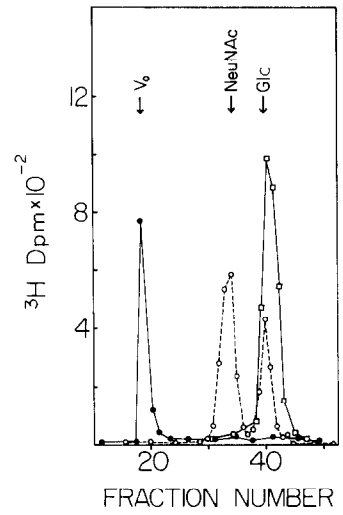

Fig. 3. Elution profiles on a column of Bio-Gel P-2 (0.9 × 70 cm) of β-D-[^{3}H]Gal-(1→3)-GalNAc (○------○) and [^{3}H]asialoepiglycanin (●——●) after treatment with jack bean β-galactosidase as described in the text. The column was eluted with 0.1M pyridine–acetic acid, 1-mL fractions were collected, and aliquots were analyzed for radioactivity and galactose (by the phenol–sulfuric acid reaction). The elution pattern of [^{14}C]glucose (□——□) and the peak elution positions of Blue Dextran (V_0) and NeuNAc are indicated.

TABLE I

HEXOSAMINE AND AMINO ACID COMPOSITION OF EPIGLYCAN BEFORE AND AFTER TREATMENT WITH *D. pneumoniae* endo-α-*N*-ACETYLGALACTOSAMINIDASE AND NEURAMINIDASE AND FRACTIONATION ON A COLUMN OF BIO-GEL P-4

Amino acids	*Untreated*	*Incubated with buffer*		*Incubated with enzymes*	
		Excluded material	*Included material*	*Excluded material*	*Included material*
	nmol per 33 μg of epiglycanin				
Aspartic acid	0.28	1.12	0	0.24[a]	0[a]
Threonine	13.10	9.92	0.12	11.14	0.02
Serine	8.79	9.38	0	8.54	0.04
Glutamic acid	0.44	1.44	0	0.58	0.12
Glycine	2.33	2.56	0	2.48	0
Alanine	3.38	2.40	0	2.79	0
Valine	0.43	0.40	0	0.31	0
Isoleucine	0.18	0.23	0	0.12	0
Leucine	1.06	0.86	0	0.89	0.01
Phenylalanine	0.05	0.16	0	0.02	0
2-Amino-2-deoxyglucose	0.81	1.07	0.15	1.11	0.18
2-Amino-2-deoxygalactose	10.01	9.88	0.12	3.88	6.03

[a]The values in these columns are corrected for contributions from the enzymes

peak). An enzyme control was fractionated similarly. Each of the fractions was hydrolyzed (6M HCl, 24 h, 110°, *in vacuo*) and the products analyzed on a Dionex amino acid analyzer equipped with a fluorescence detector. The results are summarized in Table I. It is clear that ~63% of the total GalNAc was released from epiglycanin that had been treated with both the endo-enzyme and neuraminidase.

In further experiments, 200 μg of the glycoprotein was treated with the endo-enzyme and neuraminidase, and the digest was fractionated on a column of Bio-Gel P-100. The undigested material, as well as the released oligosaccharides, were recovered, subjected to methanolysis, and the glycosides analyzed as their per(trimethylsilyl)ated methyl glycosides by gas–liquid chromatography[21]. The composition of the high-molecular-weight (epiglycanin) peak suggested that 70% of the Gal and 62% of the GalNAc, but no GlcNAc, had been cleaved. These results suggest that as much as 78–85% of the disaccharide had been released from the epiglycanin.

Our results clearly establish that the major carbohydrate component of epiglycanin is β-D-Gal(1→3)-D-GalNAc (partially substituted by NeuNAc), and that this disaccharide is linked to the protein by an α-anomeric linkage. Further, we have shown that up to ~80% of the disaccharide may be released from asialoepiglycanin by endo-*N*-acetyl-α-galactosaminidase.

Action of N-*acetyl-α-*D-*galactosaminyl oligosaccharidase.* — A minor portion of the carbohydrate in epiglycanin is present as a hexasaccharide and as a tetrasac-

charide[3]. As the *N*-acetyl-α-D-galactosaminyl-oligosaccharidase of *Clostridium perfringens* has been reported to cleave oligosaccharides larger than disaccharides bound by *O*-glycosyl linkages to protein[13], we tested the action of this enzyme on [^{3}H]asialoepiglycanin. The results obtained upon gel filtration of the incubation mixture through a column of Bio-Gel P-4 were identical to those obtained with *D. pneumoniae* endo-*N*-acetyl-α-D-galactosaminidase. Of the cleaved material, ~65% comigrated with β-D-Gal(1→3)-GalNAc, and no labeled components correspnding to higher oligosaccharides were detected; the tetrasaccharide β-D-[^{3}H]Gal-(1→4)-β-D-GlcNAc-(1→3)-D-Gal-(1→3)-β-D-GalNAc was expected. Retreatment of the material eluted at the void volume of the Bio-Gel P-4 column with the same enzyme also failed to release any tetrasaccharide.

Action of E. freundii *endo-β-D-galactosidase.* — When the excluded asialomaterial from the Bio-Gel P-4 column (Fig. 1) was treated with *E. freundii* endo-β-D-galactosidase, and the digest chromatographed on a column of Bio-Gel P-4, the results illustrated in Fig. 4 were obtained. Of the radioactivity, ~14% eluted in the same region as maltotriose. Insufficient material prevented further examination of the saccharide released. Tentatively, it appears that this endo-β-galactosidase may have released the trisaccharide, β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal, from the foregoing tetrasaccharide.

The specificity of endo-*N*-acetyl-α-D-galactosaminidase, as well as *N*-acetyl-α-D-galactosaminyl-oligosaccharidase, for cleaving the disaccharide in epiglycanin

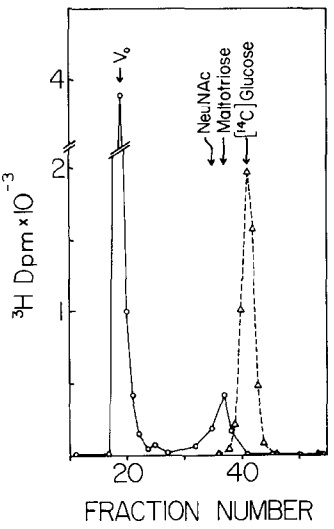

Fig. 4. Elution profile on Bio-Gel P-2 of endo-α-*N*-acetylgalactosaminidase-resistant [^{3}H]asialoepiglycanin after treatment with *E. freundii* endo-β-galactosidase. The enzyme digest was mixed with [^{14}C]glucose (△——△) and chromatographed on a column (0.9 × 68 cm) of Bio-Gel P-2. The column was eluted with 0.1M pyridine–acetic acid, and 1-mL fractions were collected and analyzed for radioactivity. The peak-elution positions of blue dextran (V_o), NeuNAc, and maltotriose are indicated.

suggests a unique role for this carbohydrate chain. Although the disaccharide structure itself has been identified in a variety of mammalian tissues and cells[3], it represents not only the preponderant carbohydrate structure in epiglycanin, but, as part of a glycopeptide structure, it serves uniquely as a receptor for either the anti-epiglycanin antibody[7] and the lectin from *Vicia graminea* beans [22]. Whether or not this chain is involved in the suggested antigen-masking function[23] of epiglycanin has not yet been determined.

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