Communication

Activity of partially purified UDP-N-acetyl-α-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase with different peptide acceptors

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As part of investigations on the role of the UDP-GalNAc-ribosome complex in the initial O-glycosylation of proteins, we have isolated from porcine gastric mucosa GalNAc-transferase, mucin and apomucin, and its three fractions containing carbohydrate in the amounts: I - 1.6%, II - 0.63% and III - 0.00% (wt/wt) of apomucin mass.

Amino acid analysis showed that fractions I and II contained slightly higher amounts of serine and threonine as compared to native mucin and apomucin.

The short peptide Pro-Thr-Ser-Ser-Pro-Ile-Ser-Thr was the most effectively glycosylated. Our apomucin preparations are also good acceptors of GalNAc and can be used for testing of O-glycosylation in vitro.

Mucin-type O-glycosylation is initiated by a family of UDP-N-acetylgalactosamine:polypeptide transferases (GalNAc-transferase). In this reaction GalNAc is transferred to serine and threonine residues of the mucin polypeptide backbone. The animal GalNAc-transferase family has been reported to contain four members [1–5]. The specific activity of GalNAc-transferases from different sources toward various peptide substrates varies, and it has been clearly demonstrated that the flanking sequences of serine and threonine amino-acid residues significantly affect the rate of peptide glycosylation [6–8].

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Abbreviations: GalNAc, N-acetyl-α-D-galactosamine; TFMSA, trifluoromethane sulfonic acid; GalNAc-transferase, UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase; PTSP[ST], Pro-Thr-Ser-Ser-Pro-Ile-Ser-Thr synthetic peptide.
Mucins are the most intensely O-glycosylated glycoproteins and their deglycosylated protein backbones (apomucins) can serve as valuable acceptors of N-acetylgalactosamine, for in vitro studies of initial O-glycosylation. In this report we characterize the specificity of partially purified GalNAc-transferase from porcine gastric mucosa toward different carbohydrate acceptors: synthetic peptide and apomucin polypeptide obtained by chemical deglycosylation of gastric mucin. Furthermore, we have also examined the inhibition of glycosylation of peptide substrates by nucleotide sugar derivatives.

MATERIALS AND METHODS

All experiments were performed on porcine stomachs. GalNAc-transferase isolation from pig stomach mucosa was an adaptation of methods used for other tissues [9, 10]. Mucin was isolated from the surface of gastric mucosa and purified using a procedure elaborated in our laboratory [11].

Purification of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase. All operations during enzyme purification were carried out at 4°C. Scissors minced gastric mucosal tissue was homogenized in 25 mM imidazole/HCl buffer, pH 7.2 (buffer A), centrifuged at 13,000 × g for 1 h, and the pellet was rehomogenized in the same buffer containing 20 mM MnCl₂, and centrifuged as before. The enzyme was solubilized by suspending the pellet in the same buffer containing 1.5% Triton X-100, and stirring for 30 min. This step was repeated with 0.5% Triton X-100, in buffer A. The supernatant was passed through a DEAE-Sephadex A-50 column [9]. Effluent from the column containing GalNAc-transferase activity was applied to hydroxylapatite (Bio-Gel HT) column, as described by Bendiak & Schachter [10]. The fractions were eluted with 0.2 M phosphate buffer, pH 7.5, dialyzed against buffer A, concentrated in dialyzing tube against solid polyethylene glycol, dialyzed in buffer A containing 0.1 M NaCl and 0.1% Triton X-100, and then applied on Sephadex G-100 column [10].

During purification, enzymatic activity was tested in the mixture containing: the synthetic peptide Pro-Thr-Ser-Ser-Pro-Ile-Ser-Thr or an adequate quantity of apomucin as N-acetylgalactosamine acceptor, 0.4 nmol UDP-[1-¹⁴C]GalNAc, 100 mM imidazole buffer, pH 7.2, 10 mM MnCl₂, 4 mM dithiothreitol, 0.5% Triton X-100, and the enzyme preparation (0.1–0.5 mg) in a total volume of 100 µl [12]. Control test was performed using bovine serum albumin, a known poor substrate for glycosyltransferases [13], instead of polypeptide acceptor. Samples were incubated at 37°C for 30–60 min and the reaction was stopped by the addition of 100 µl 25 mM EDTA. The reaction mixture was applied to a Dowex 1×8 acetate form column (2 ml) and eluted with 10 ml of 20 mM sodium acetate. Aliquots from 1 ml fractions were submitted for radioactivity counting.

Separation and purification of mucus glycoproteins. Gastric mucus was obtained using a procedure elaborated in our laboratory [11]. Mucin extract (in 6 M urea) was purified (by gel exclusion chromatography) twice on Sepharose CL-2B (2.5 cm × 138 cm) and on Sephacryl S-500 (2.4 cm × 56 cm) columns. Both columns were equilibrated and eluted with 6 M urea solution in borate buffer (pH 7.0). Mucin containing fractions were identified by the phenol-sulfuric acid method [14], pooled, dialyzed against water and tested for purity by SDS/PAGE.

Deglycosylation of purified mucin. Purified mucin was reduced and carboxyalkylated according to Thornton et al. [15], then digested with pronase for 72 h at 37°C, at the initial enzyme/substrate ratio of 1:40 (wt/wt), in 0.1 M Tris buffer, pH 8.0, containing 5 mM CaCl₂ and 0.02% NaN₃. During the digestion the mixture was supplemented with additional portions of the enzyme after 24 and 48 h. The reaction was terminated by immersion of the sample tubes in boiling water, for
RESULTS AND DISCUSSION

Purification and properties of partially purified GalNAc-transferase

During the first step of purification, the GalNAc-transferase emerged from the DEAE-Sephadex A-50 column in the void volume of the column, with 70% recovery of the crude extract activity. After the two next steps of purification using hydroxylapatite adsorption and Sephadex C-100 column chromatography, 47% and 42%, respectively, of the initial activity was recovered. On SDS/PAGE this preparation showed the presence of four distinct protein bands (Fig. 1). The enzymatic activity corresponded to the band appearing at 70 kDa as confirmed by gel filtration on a Sephadex G-100 column.

The pH optimum of the enzyme activity was examined using four buffers at 0.1 M concentration: acetate, imidazole/HCl, Tris/HCl and borate over the pH range 4.5–6.0, 6.5–7.5, 8.0–8.5 and 9.0–9.5, respectively. The enzyme was active in a broad pH range, from 6.5 to 7.5, with maximal activity at pH 7.2. The enzyme required Mn²⁺ for maximal activ-

![Image of SDS/polyacrylamide gel electrophoresis](image-url)
ity at a concentration of 10 mM; however, Mg\(^{2+}\) and Ca\(^{2+}\) ions were also effective. EDTA at 10 mM concentration abolished the activity completely.

**Acceptor-substrate specificity of GalNAc-transferase**

Our procedure for deglycosylation of porcine gastric mucin gave three apomucin fractions (see Methods), two of them contained carbohydrate: Ip - 1.6%, IIP - 0.65% of the apomucin mass (wt/wt), respectively. Fraction IIP contained no carbohydrate. Amino acid composition of fractions I and II showed, NAc acceptors, was tested in O-glycosylation reaction with GalNAc-transferase.

We have also investigated the behaviour of some polypeptide as GalNAc acceptors, using native mucin, apomucin and its fragments. Apomucin preparations, Ip and IIP, which still contained 1.65% and 0.65% of carbohydrate residues, were better substrates for glycosylation (Table 1), whereas the completely deglycosylated IIP polypeptide chain showed considerably less activity (not shown).

The apomucin preparations were also compared with the synthetic peptide Pro-Thr-Ser-Ser-Pro-Ile-Ser-Thr as the acceptor for GalNAc-transferase, at two sugar/protein molar ratios (1:1 and 1:10). The results showed that this short peptide was the most effectively glycosylated (Table 2), demonstrating the highest accessibility of hydroxyamino acids for GalNAc transfer (0.62–0.82 mol sugar for 1 mol peptide). The apomucin fragments Ip and

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<th>Table 1. Chemical composition of apomucin preparations and their acceptor properties in the GalNAc-transferase reaction</th>
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<td>Threonine*</td>
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<td>GalNAc bound** (%)</td>
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*Expressed as mol/100 mol of whole amino acid content; **% of radioactivity initially present in reaction mixture and transferred from UDP-[^14]C|GalNAc to protein acceptor. The initial mole ratio of UDP[^14]C|GalNAc to protein was 1:2.

| Table 2. The dependence of UDP[^14]C|GalNAc to protein ratio and the amount of transferred carbohydrate |
|---------------------------------------------------------------|
| | Sugar/peptide ratio | 1:1 | 1:10 |
| | GalNAc bound: | PTSSPIST | Ip | IIP | PTSSPIST | Ip | IIP |
| d.p.m. (× 10\(^{-5}\)) | 3.7 | 1.9 | 1.3 | 4.9 | 2.7 | 2.0 |
| nmol sugar/mol acceptor* | 0.62 | 0.32 | 0.22 | 0.32 | 0.45 | 0.34 |
| % GalNAc** | 62 | 32 | 22 | 82 | 45 | 34 |

*Molecular mass of apomucin was assumed to be 100 kDa, as determined by gel electrophoresis; **% of radioactivity initially present in the reaction mixture and transferred from UDP[^14]C|GalNAc to peptide acceptor.

in comparison to our native mucin preparation, a higher amount of serine, threonine and proline (Table 1), with diminution of cysteine, an amino acid typically located in the non O-glycosylated area and of phenylalanine. The quality of apomucin preparations as Gal-
IIP were worse acceptors for GalNAc-transferase, with 0.32–0.45 mol sugar being bound to 1 mol of Ip and 0.22–0.34 mol sugar to 1 mol of IIP apomucin. A number of studies have shown that removal of the majority of the oligosaccharide chains from the polypeptide backbone of mucin glycoproteins dramatically alters their properties [21]. The change of the extended filamentous rod-like structure to a globular conformation causes that part of free threonine or serine are not available to O-glycosylation in vitro, as we have confirmed in our investigation.

The inhibitory effect of different nucleotides on O-glycosylation of polypeptide substrates

The GalNAc-transferase was very specific for UDP-GalNAc as a monosaccharide donor. The commonly applied incubation with two nucleotide substrates (UDP-GlcNAc and UDP-GalNAc or UDP-Gal and UDP-GalNAc), with a tenfold excess of UDP-GlcNAc or UDP-Gal over UDP-[14C]GalNAc, affected inhibition of the incorporated radioactivity into all of the peptide acceptors tested. However, greater inhibition was observed with the synthetic peptide. UDP-GlcNAc diminished radioactivity incorporation by about 50% with regard to the control value for apomucin preparations, and by about 40% for the synthetic peptide. UDP-Gal inhibited [14C]GalNAc incorporation to apomucin preparations by about 40% and 60% with respect to that of the synthetic peptide (Fig. 2).

α-Benzyl-GalNAc, the inhibitor of O-glycosylation in vivo did not inhibit the [14C]GalNAc incorporation into the different peptide substrates studied.

To sum up, we have isolated from porcine stomach mucosa by an original procedure GalNAc-transferase and practically carbohydrate-free apomucin preparations, suitable for use as acceptor in O-glycosylation by UDP-GalNAc-transferase in vitro.

REFERENCES


