RELEASE OF GALACTOSYL OLIGOSACCHARIDES BY ENDO-β-N-ACETYLGLUCOSAMINIDASE D

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SUMMARY Endo-β-N-acetylglucosaminidase D from *Diplococcus pneumoniae* released galactosyl oligosaccharides from IgG glycopeptides treated with β-N-acetylglucosaminidase. The structure of the major oligosaccharide was proposed to be as follows.

$$\text{Gal}\beta\rightarrow\text{GlcNAc}\beta\rightarrow\text{Man}\alpha\begin{matrix}\nearrow\\\searrow\end{matrix}\begin{matrix}\text{Man}\beta\rightarrow\text{GlcNAc}\\\text{Man}\alpha\end{matrix}$$

Since α-mannosidase digestion of the β-N-acetylglucosaminidase-treated glycopeptides made them again resistant to the endoglycosidase, we concluded that an unsubstituted α-mannosyl residue was required for the enzymic action.

Endo-β-N-acetylglucosaminidase D, an endoglycosidase acting on carbohydrate moieties of glycoproteins has been purified from the culture fluid of *Diplococcus pneumoniae* (1). The enzyme hydrolyzed di-N-acetylchitobiose structure of glycopeptides and their derivatives, releasing oligosaccharides composed of mannose and N-acetylglucosamine (1). In this communication we report that the enzyme can also release large oligosaccharides containing galactose.

MATERIALS AND METHODS

Bovine IgG glycopeptides were prepared by pronase digestion of bovine IgG fraction and were [^{14}C]N-acetylated as described previously (1). Tritiation of galactose residues of the glycopeptides was performed by oxidation with galactose oxidase followed by NaB^3H_4 reduction as outlined by Morell and Ashwell (2). The [^{14}C]acetylated IgG glycopeptides treated with β-N-acetylglucosaminidase were prepared by digestion of 0.43 μmole of the [^{14}C]acetylated glycopeptides with 4.8 units of β-N-acetylglucosaminidase in 0.66 ml of 0.015 M citrate-phosphate buffer, pH 4.0 at 37° for 60 hours with a small amount of toluene. By the treatment, 0.39 mole of N-acetylglucosamine was released per

mole of the glycopeptides as measured by a modified Morgan-Elson reaction (3) and no further release was observed even after the addition of excess enzyme. In one experiment, [^{14}C]acetylated β-N-acetylglucosaminidase-treated glycopeptides (14 nmoles) were further digested with 1.33 units of α-mannosidase in 0.03 M citrate-phosphate buffer, pH 4.0 at 37° for 16 hours with a small amount of toluene. These glycosidase-treated glycopeptides were purified by paper electrophoresis at pH 5.4.

Endo-β-N-acetylglucosaminidase D was prepared by N. Koide in our laboratory as described previously (1). β-N-Acetylglucosaminidase (4), β-galactosidase (5) and α-mannosidase (4), all from jack bean meal, were prepared according to cited references. Milk oligosaccharides were prepared as described elsewhere (6). NaB^{3}H$_4$ reduction was carried out according to Takasaki and Kobata (7).

RESULTS AND DISCUSSION

The oligosaccharide so far released from bovine IgG glycopeptides by endo-β-N-acetylglucosaminidase D was $(Man)_3GlcNAc$, since the enzyme acted on bovine IgG glycopeptides treated with β-galactosidase and β-N-acetylglucosaminidase but did not act on the intact glycopeptides (1). However, we now report that a part of the glycopeptides treated with β-N-acetylglucosaminidase alone was also hydrolyzed by the endoglycosidase, releasing oligosaccharides with galactose residue. The β-N-acetylglucosaminidase-treated glycopeptides had been labeled in the peptide moiety by [^{14}C]N-acetylation and in the galactose residue by tritiation using the galactose oxidase method. When the reaction mixture was analyzed by paper electrophoresis at pH 5.4 (Fig. 1-B), we observed two products (I and II) in addition to undigested substrates which migrated 8.0 cm from the origin (Fig. 1-A). Product I was labeled with [^{3}H] and the product II with [^{14}C]. The latter product migrated identically with Fuc→GlcNAc→[^{14}C]N-acetylpeptide (Fig. 1-C). When the product I was recovered from the paper and analyzed by paper chromatography, about 70% of the label behaved as an oligosaccharide with the mobility of a hexasaccharide and the rest of the product migrated more slowly (Fig. 2-A). No free galactose was detected by shorter development.

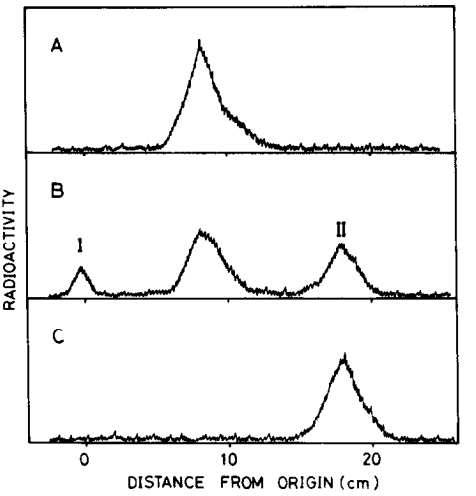

Fig. 1. Action of endo-β-N-acetylglucosaminidase D on IgG glycopeptides treated with β-N-acetylglucosaminidase.

Analysis was performed by paper electrophoresis at pH 5.4 (pyridine-acetic acid-water 3:1:387) at 73 volts per cm for 1.5 hours, followed by radiochromatogram scanning performed with Packard radiochromatogram scanner model 7201.

A: [^{14}C]Acetylated IgG glycopeptides treated with β-N-acetylglucosaminidase. The glycopeptide derivatives were also labeled with [^{3}H] in their galactose residues.

B: The above substrates (43 nmoles) were treated with 4 milliunits of endo-β-N-acetylglucosaminidase D in 0.1 ml of 0.025 M citrate phosphate buffer, pH 7.0 at 37° for 30 min.

C: [^{14}C]Acetylated IgG glycopeptides were treated with β-galactosidase and β-N-acetylglucosaminidase followed by endo-β-N-acetylglucosaminidase D as described previously (1). This product showed the properties of Fuc→GlcNAc→[^{14}C]N-acetyl-peptide.

The structure of the major component of the galactosyl oligosaccharides was studied as follows. Substantial amount of the oligosaccharide was isolated from bovine IgG glycopeptides by β-N-acetylglucosaminidase treatment followed by digestion with endo-β-N-acetylglucosaminidase D. The oligosaccharide was labeled with $NaB^{3}H_{4}$ and purified with paper chromatography to get the oligosaccharide alcohol. When the oligosaccharide alcohol was hydrolyzed by 1 N HCl at 100° for 4 hours and analyzed by paper electrophoresis at pH 5.4, glucosaminitol was detected as a sole radioactive spot, indicating that the reducing end of the oligo-

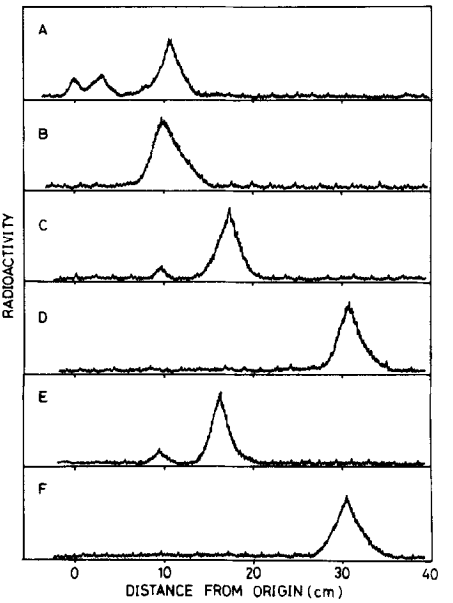

Fig. 2. Analysis of the galactosyl oligosaccharides by exo-glycosidase treatment.

Oligosaccharides and their derivatives were subjected to paper chromatography in ethylacetate-pyridine-water (12:5:4) for 6 days in a descending manner. Radioactivity on the chromatogram was monitored by radiochromatogram scanning. The standards migrated from the origin as follows: Lacto-N-tetraitol (25.4 cm), Lacto-N-fucopentaitol III (13.3 cm), Lacto-N-difucohexaitol II (8.1 cm).

A: [^{3}H]Galactose labeled oligosaccharides released by endo-β-N-acetylglucosaminidase D from β-N-acetylglucosaminidase-treated IgG glycopeptides. The product I in the experiment shown in Fig. 1-B was extracted from the chromatogram and was analyzed.

B: The major galactosyl oligosaccharide reduced with NaB^3H_4.

C: The oligosaccharide alcohol (0.36 nmole) shown in Fig. 2-B was hydrolyzed by 0.095 unit of β-galactosidase in 0.035 ml of 0.03 M citrate-phosphate buffer, pH 4.0 at 37° for 16 hours with a small amount of toluene.

D: The oligosaccharide alcohol (0.36 nmole) shown in Fig. 2-C was treated with 0.23 unit of β-N-acetylglucosaminidase at the same condition as that of Fig. 2-C.

E: The oligosaccharide alcohol (0.36 nmole, Fig. 2-B) was treated with 1.33 units of α-mannosidase at the same condition as that of Fig. 2-C.

F: Man_3-[^{3}H]N-acetylglucosaminitol. This material was obtained by NaB^3H_4 reduction of the oligosaccharide which was released by endo-β-N-acetylglucosaminidase D (1) from IgG glycopeptides treated with β-galactosidase and β-N-acetylglucosaminidase.

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saccharide was N-acetylglucosamine. After β-galactosidase treatment, the apparent hexasaccharide alcohol (Fig. 2-B) was convert-

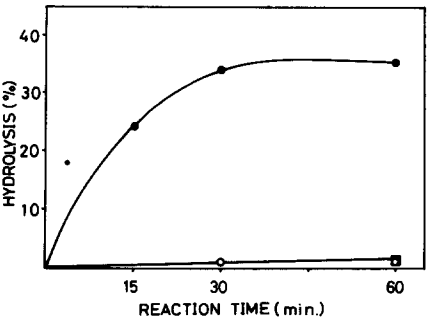

Fig. 3. Time course of the hydrolysis of derivatives of IgG glycopeptides by endo-β-N-acetylglucosaminidase D.

Fourteen nmoles of [^{14}C]acetylated IgG glycopeptides and their derivatives were incubated with 0.6 milliunit of endo-β-N-acetylglucosaminidase D in 0.04 ml of 0.025 M citrate-phosphate buffer, pH 7.0 at 37°. After the reaction, products were analyzed by paper electrophoresis at pH 5.4 as shown in Fig. 1. Radioactive material detected was either undigested substrates or the product with the mobility of Fuc→GlcNAc-[^{14}C]N-acetylpeptide. Results were expressed by per cent of the radioactivity in the product region to the total radioactivity recovered on the electrophoretogram. The radioactivity was determined by liquid scintillation counting as described previously (1).

o—o [^{14}C]acetylated IgG glycopeptides

●—● [^{14}C]acetylated IgG glycopeptides which had been treated with β-N-acetylglucosaminidase

■—■ [^{14}C]acetylated IgG glycopeptides which had been treated with β-N-acetylglucosaminidase and α-mannosidase

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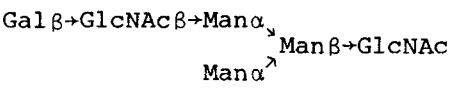
ed to a product with the mobility of a pentasaccharide alcohol (Fig. 2-C), which was further converted to a product (Fig. 2-D) with the same mobility as Man_3-N-acetylglucosaminitol (Fig. 2-F) by β-N-acetylglucosaminidase digestion. Successive treatment with α-mannosidase and β-mannosidase shows that among the three mannosyl residues of the oligosaccharide alcohol (Fig. 2-D and 2-F) two were α-linked and the innermost one was β-linked (1). The α-mannosyl residues were linked to the β-mannosyl residue as in the case of human myeloma IgG glycopeptides (8), based on the methylation analysis of the glycopeptides (Tai et al., unpublished results). The parental oligosaccharide alcohol (Fig. 2-B) was resistant to β-N-acetylglucosaminidase but was hydrolyzed by α-mannosidase and yielded a product with the mobility of a pentasaccharide alcohol (Fig. 2-E), indicating that one α-mannosyl

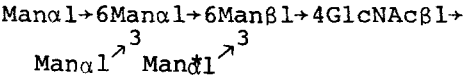
residue was also located at a non-reducing end.

Summarizing the results, the structure of the major galactosyl oligosaccharide was proposed to be as follows.



Among the β-N-acetylglucosaminidase-treated glycopeptides, about 40% of them were hydrolyzed by endo-β-N-acetylglucosaminidase D (Fig. 3). Since 0.39 mole of N-acetylglucosamine had been released per 1 mole of the glycopeptide mixture by β-N-acetylglucosaminidase digestion, it seemed likely that an α-mannosyl residue exposed by β-N-acetylglucosaminidase digestion was essential for the action of the endoglycosidase. This assumption was verified by the finding that α-mannosidase treatment after the β-N-acetylglucosaminidase digestion made the glycopeptides again resistant to the endoglycosidase (Fig. 3).

As above, we established that endo-β-N-acetylglucosaminidase D can release galactosyl oligosaccharides from IgG glycopeptides, and thus expanded the scope of the application of endo-β-N-acetylglucosaminidase D. The result is also interesting from the view point of the specificity of endo-β-N-acetylglucosaminidase D, since we can conclude that the α-mannosyl residue exposed by β-N-acetylglucosaminidase digestion is essential for the action of the endoglycosidase. The key role of an α-mannosyl residue for the action of the endoglycosidase has also been revealed by different approach. A purified ovalbumin glycopeptide with the composition of $Man_5GlcNAc_2Asn$ was susceptible to the enzyme and the major component of it had the following structure (9).



Whereas another glycopeptide with the composition of $Man_6GlcNAc_2$-

Asn was resistant to the enzyme and the major component had the structure identical with the above except that one more α-mannosyl residue was linked to the α-mannosyl residue marked with asterisk by an α1→2 linkage (9). Thus, the substrate specificity of the enzyme toward IgG glycopeptides and ovalbumin glycopeptides is based on the same requirement, i. e. the presence of an unsubstituted α-mannosyl residue linked to the β-mannosyl residue.

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