ENDOGLYCOSIDASES ACTING ON CARBOHYDRATE MOIETIES OF GLYCOPROTEINS: DEMONSTRATION IN MAMMALIAN TISSUE

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SUMMARY Enzyme preparations from rat and porcine organs released [^{14}C]-acetyl-AsnGlcNAc from [^{14}C]-acetyl-Asn(GlcNAc)$_2$(Man)$_6$ without intermediate products, indicating the presence of an endoglycosidase acting on the substrate. The enzyme from porcine liver was separated from α-mannosidase by Sephadex G-200 column chromatography. The substrate specificity of the purified enzyme appears to be unique, since it hydrolyzed only ovalbumin glycopeptides among several glycopeptides and their derivatives.

Extensive studies have been carried out on the mechanism of glycoprotein catabolism in mammalian tissue (1,2,3). Evidence so far accumulated has established that exoglycosidases play important roles in the process. For example, Aronson and De Duve found that lysosomal extract progressively released monosaccharides from α_1-acid glycoprotein (3). Furthermore, various exoglycosidases purified from mammalian sources acted on glycoproteins or glycopeptides (1,4). In microorganisms, however, endoglycosidases acting on carbohydrate moieties of glycoproteins have recently been found and characterized (5,6,7,8,9).

In this communication, we will present evidence for the occurrence of an endoglycosidase in mammalian tissues. The significance of the endoglycosidases in glycoprotein catabolism in mammalian tissue will also be discussed.

MATERIALS AND METHODS

AsnGlcNAc[1] and purified ovalbumin glycopeptides with the composition of Asn(GlcNAc)$_2$(Man)$_5$, Asn(GlcNAc)$_2$(Man)$_6$ and Asn(GlcNAc)$_4$(Man)$_6$ (10) were kindly donated by Drs. S. Iwashita and Y. Inoue, Tokyo University. Calf thyroglobulin glycopeptides Unit A (11) and porcine thyroglobulin glycopeptides Unit B (12) were kindly donated by Dr. R. G. Spiro, Harvard Medical School and Drs. M. Fukuda and T. Osawa, Tokyo University, respectively. A bovine IgG glycopeptide was obtained by Pronase digestion of bovine IgG fraction (6). Glycopeptides and AsnGlcNAc were N-acetylated with [^{14}C]-acetic anhydride (19 mCi/mmole; the Radiochemical Centre, England) as described elsewhere (6). Defucosyl, degalactosyl and side-chain-free[2] IgG glycopeptide and side-chain-free Unit B glycopeptides of thyroglobulin were prepared by digestion of the [^{14}C]-acetylated intact glycopeptides with the corresponding exoglycosidases (6). (Man)$_6$[^{3}H]-N-acetylglucosaminitol was prepared from Asn(GlcNAc)$_2$(Man)$_6$ by digestion with endo-β-N-acetylglucosaminidase H followed by NaB^{3}H$_4$ reduction (9).

The male Sprague Dowley rats maintained at the condition free from specific pathogens were kindly provided by Takeda Pharmaceutical Co. The porcine organs were obtained from a local slaughter house. Rat or porcine organs were homogenized with Na-phosphate buffer, pH 7.0 (3 volumes per weight of organs) in a Waring blendor der for 15 min, and were centrifuged at 88,000 x g for 120 min. The supernatant was used as a crude enzyme solution.

Protein content was determined by the method of Lowry et al. (13) using bovine serum albumin as a standard.

1) Abbreviation used is: AsnGlcNAc, 2-acetamido-N-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine.

2) The term of side chain indicates oligosaccharide chains composed of galactose, N-acetylglucosamine and (sialic acid in some chains), which are attached to core structure composed of mannose and N-acetylglucosamine.

RESULTS AND DISCUSSION

[^{14}C]-Acetyl-Asn(GlcNAc)$_2$(Man)$_6$ was incubated with the crude extract of rat liver at pH 7.0 for 1 hour. When the reaction mixture was analyzed by paper electrophoresis at pH 5.4, release of [^{14}C]-acetyl-AsnGlcNAc was observed (Fig. 1). The identical ac-

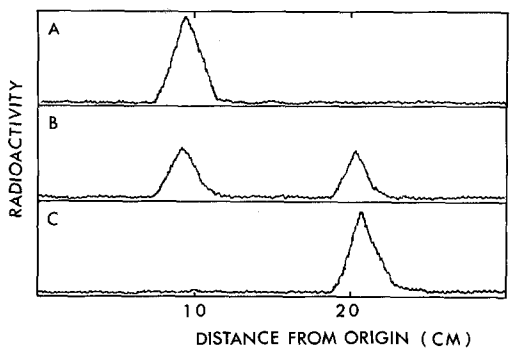

Fig. 1. Hydrolysis of [^{14}C]-acetyl-Asn(GlcNAc)$_2$(Man)$_6$ by the rat liver enzyme.

The crude extract of rat liver (50 µl, 2.9 mg of protein) in 0.05 M Na-phosphate buffer, pH 7.0 was incubated with 10 µl of substrate solution containing 0.3 nmoles of [^{14}C]-acetyl-Asn(GlcNAc)$_2$(Man)$_6$ at 37°C for 1 hour. The reaction was terminated by the addition of 0.1 ml of ethanol. After centrifugation the supernatant was analyzed by paper electrophoresis which was carried out at a potential of 73 volt per cm for 1 hour using pyridine-acetic acid-water (3:1:387), pH 5.4 as a solvent. Radioactivity was monitored by a Packard radiochromatogram scanner model 7201.

A: [^{14}C]-Acetyl-Asn(GlcNAc)$_2$(Man)$_6$
B: [^{14}C]-Acetyl-Asn(GlcNAc)$_2$(Man)$_6$ treated with the enzyme
C: [^{14}C]-Acetyl-AsnGlcNAc

At the above condition, [^{14}C]-acetyl-Asn and [^{14}C]-acetyl-Asn(GlcNAc)$_2$ migrated 31 cm and 17 cm, respectively.

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tivity was also found in the crude extracts of rat spleen, rat kidney, porcine liver and porcine kidney (Table I). The velocity of hydrolysis was practically proportional to reaction time up to at least 30% hydrolysis. In all cases, no intermediate products were found even after changing the reaction time and enzyme concentration. Therefore, an endoglycosidase appeared to catalyze the reaction as follows:

Table I. *Distribution of the Endoglycosidase in Mammalian Organs.*

The reaction was carried out as described in Fig. 1 except that incubation for 3 hours was employed. Paper electrophoresis of the reaction mixture performed as described in Fig. 1 revealed, in all cases, only one product with the mobility of [^{14}C]-acetyl-AsnGlcNAc. The radioactivity of the product and of the unhydrolyzed substrate was determined by liquid scintillation counting performed with a Packard liquid scintillation counter model 3320.

Organs		Degree of hydrolysis (%)
rat	liver	63.5
	kidney	58.1
	spleen	33.2
porcine	liver	24.2
	spleen	13.3

$$[^{14}C]\text{-Acetyl-Asn(GlcNAc)}_2\text{(Man)}_6 \longrightarrow [^{14}C]\text{-Acetyl-AsnGlcNAc} + \text{GlcNAc(Man)}_6$$

The enzymic activity in porcine liver was studied in detail as follows. The optimum pH of the enzyme was around pH 7.0, and no activity was observed below pH 5.0. After ammonium sulfate precipitation (0 - 40%), the enzyme was further purified by Sephadex G-200 column chromatography. Most of α-mannosidase, which was measured by using p-nitrophenyl-α-D-mannoside as a substrate, was removed by this treatment (Fig. 2). The purified enzyme was also free from any α-mannosidases acting on (Man)$_6$[^{3}H]-N-acetylglucosaminitol as is clearly shown in the following experiment. (Man)$_6$[^{3}H]-N-Acetylglucosaminitol (0.3 nmole) was incubated with 20 μl of the purified enzyme in 0.05 M Na-phosphate buffer, pH 7.0 at 37°C for 15 hours with a small amount of toluene. When the reaction mixture was analyzed by paper chromatography in ethylacetate-pyridine-water (12:5:4), none of oligosaccharide alcohols with

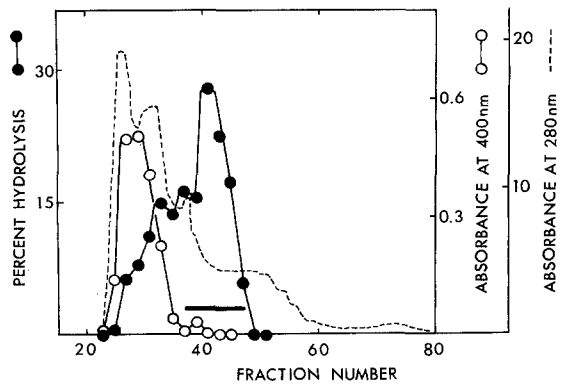

Fig. 2. Sephadex G-200 column chromatography of the endoglycosidase from porcine liver.

To 100 ml of the crude extract of porcine liver (50 g), ammonium sulfate was added up to 40% saturation. The precipitate was dissolved in 5 ml of 0.05 M sodium phosphate buffer, pH 7.0, dialyzed against the same buffer, and was applied to a column of Sephadex G-200 (2.6 x 95 cm), which was equilibrated with the same buffer. Elution was performed also with the same buffer, and fractions of 7 ml were collected.

The endoglycosidase activity (●—●) in the eluate was determined by incubating 20 µl aliquot of each fraction with 10 µl of substrate solution containing 0.3 nmoles of [^{14}C]-acetyl-Asn $(GlcNAc)_2(Man)_6$, at 37°C for 15 hours with a small amount of toluene. After incubation, the substrate and the product were separated by paper electrophoresis as described in the legend of Fig. 1. The result was expressed by per cent of the substrate hydrolyzed.

For the determination of α-mannosidase activity (○—○), 0.1 ml aliquot of each fraction was incubated with 0.1 ml of substrate solution containing 0.1 mg of p-nitrophenyl α-D-mannoside (Sigma Chemical Co.) at 37°C for 15 min. The reaction was terminated with 2.5 ml of 0.2 M Na_2CO_3 and the optical density at 400 nm was measured.

The fractions indicated by bar were pooled, concentrated by dialysis against polyethylene glycol 20,000 and were used as the purified enzyme. The protein content of the purified enzyme solution was 41 mg/ml.

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shorter carbohydrate chains was detected. At the identical enzyme and substrate concentration, 64.6% of [^{14}C]-acetyl-Asn$(GlcNAc)_2$ $(Man)_6$ was converted into [^{14}C]-acetyl-AsnGlcNAc. Therefore the enzymic activity was undoubtedly ascribed to an endoglycosidase. The mode of action of the enzyme seems to be similar to microbial endo-β-N-acetylglucosaminidases (5,6,7,8,9). Characterization of the oligosaccharide released from the substrate was unsuccesfull

Table II. *Comparison of the Substrate Sepcificity of the Mammalian Enzyme to those of Microbial Enzymes.*

[^{14}C]-Acetylated glycopeptides	Degree of hydrolysis		
	by the enzyme from porcine liver (%)	by Endo-β-N-acetylglucosaminidase D	H
Ovalbumin glycopeptides			
Asn(GlcNAc)$_2$(Man)$_5$	38.6	+	+*
Asn(GlcNAc)$_2$(Man)$_6$	64.6	-*	+
Asn(GlcNAc)$_4$(Man)$_6$	44.0	-*	+*
IgG glycopeptides			
intact	<1.0	-	-
defucosyl	<1.0	-	-
degalactosyl	<1.0	-	-
side-chain-free	<1.0	+	-
Thyroglobulin glycopeptides			
Unit A	<1.0	-	+
side-chain-free Unit B	<1.0	+	-

The purified enzyme from porcine liver in 20 μl of 0.05 M Na-phosphate buffer, pH 7.0 was incubated with 10 μl of substrate solution containing 0.3 nmoles of [^{14}C]-acetylated glycopeptides at 37°C for 15 hours with a small amount of toluene. The degree of hydrolysis was determined as described in Fig. 1 and Table I.

The specificities of microbial enzymes, *i.e.* endo-β-N-acetylglucosaminidase D from *D. pneumoniae* and endo-β-N-acetylglucosaminidase H from *S. griseus* have been described in previous papers (6,9).

+, hydrolyzed; -, not hydrolyzed; *, cited from an unpublished work done in colaboration with Drs. S. Iwashita and Y. Inoue.

at the present stage, for the enzyme preparation still contained large amounts of endogeneous glycoprotein substrates.

Among several glycopeptides and their derivatives, the enzyme hydrolyzed only ovalbumin glycopeptides (Table II). The substrate specificity is similar to that of endo-β-N-acetylglucosaminidase H, but is distinct from it with respect to its behaviour toward Unit A glycopeptides of thyroglobulin. Endo-β-N-acetylglucosaminidase H hydrolyzed [^{14}C]-acetylated Unit A glycopeptides at

about 30% of the velocity toward [^{14}C]-acetyl-Asn(GlcNAc)$_2$(Man)$_6$. Thus, the mammalian enzyme appears to have a different specificity.

Using an ovalbumin glycopeptide, we established the presence of at least one endoglycosidase in mammalian tissue. With other glycopeptides, the endoglycosidases with different substrate specificities may also be detected. Studies along this line are now in progress in our laboratory.

We propose that carbohydrate moieties of glycoproteins are degraded in mammalian tissue by a concerted action of exoglycosidases and endoglycosidases. Endoglycosidases may act on intact carbohydrate chains and in some cases on carbohydrate chains partially degraded by exoglycosidases. The released oligosaccharides may further be hydrolyzed by exoglycosidases.

Several oligosaccharides with the structure of (Manα1→2Man)$_n$·α1→3Manβ1→4GlcNAc were found to be excreted in the urine of patients suffered from mannosidosis (14,15). The oligosaccharides may be produced by the action of an endoglycosidase, since the sequence of Manβ1→4GlcNAcβ1→4GlcNAc→Asn is widely distributed in glycoproteins (16).

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