SPECIFIC INHIBITION OF ENDO-β-N-ACETYLGLUCOSAMINIDASE D BY MANNOSE AND SIMPLE MANNOSIDES

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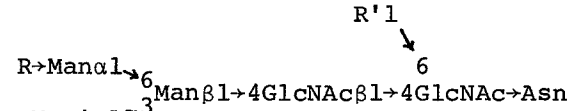
SUMMARY p-Nitrophenyl α-mannopyranoside competitively inhibited endo-β-N-acetylglucosaminidase D. Mannose and methyl α-mannoside were also inhibitory, while glucose, galactose, N-acetylglucosamine and p-nitrophenyl α-glucopyranoside showed no significant effect. The result indicated that the specific α-mannosyl residue in the substrates played a key role in the binding of the substrates to the enzyme. In contrast, endo-β-N-acetylglucosaminidase H was not affected by mannose and the simple mannosides, but was inhibited by yeast mannan.

Endo-β-N-acetylglucosaminidase D is a unique endoglycosidase isolated from the culture fluid of *Diplococcus pneumoniae* (1) and cleaves di-N-acetylchitobiose structure in various glycoproteins and glycopeptides (1-4). An important property of the enzyme is the strict specificity with respect to oligomannosyl cores in the substrates (1, 4-6). Structural studies of the susceptible glycopeptides and the resistant glycopeptides enabled us to postulate that the following sugar sequence was required for the enzymatic action (5, 6).



R = H, monosaccharides or oligosaccharides

R'= H or Fuc

All sugars mentioned in this paper have a D configuration except fucose which has an L configuration.

The α-mannosyl residue marked with an asterisk was concluded to be essential for the enzymatic action, since glycopeptides in which the α-mannosyl residue was either substituted or removed were resistant to the endoglycosidase (4-6). In order to further clarify the role of the α-mannosyl residue in the interaction with the endoglycosidase, we examined effects of mannose and simple mannosides on the enzymatic activity.

MATERIALS AND METHODS

Purified ovalbumin glycopeptides, $Man_5GlcNAc_2Asn$ and $Man_6GlcNAc_2Asn$ were prepared by M. Nishigaki in our laboratory according to Huang and Montgomery (7). p-Nitrophenyl α-mannopyranoside and methyl α-mannoside were purchased from Sigma Chemical Co. Yeast mannan was prepared from baker's yeast according to Haworth et al. (8).

Endo-β-N-acetylglucosaminidase D (1) and endo-β-N-acetylglucosaminidase H (9, 10) were prepared from the culture fluid of *Diplococcus pneumoniae* and *Streptomyces griseus*, respectively as described before. Assays of the enzymes were performed as described previously (1, 10). Shortly, 0.15 nmoles of [^{14}C]-acetylated ovalbumin glycopeptides ($Man_5GlcNAc_2Asn$ for endo-β-N-acetylglucosaminidase D and $Man_6GlcNAc_2Asn$ for endo-β-N-acetylglucosaminidase H) were incubated with the enzymes in 0.03 ml of reaction mixture at 37° for 15 min. The buffers were 0.05 M citrate-phosphate buffer, pH 6.5 for endo-β-N-acetylglucosaminidase D and 0.05 M of the same buffer, pH 5.0 for endo-β-N-acetylglucosaminidase H. The reaction was terminated by addition of 0.1 ml of ethanol. The reaction mixture was subjected to paper electrophoresis at pH 5.4, and the amount of released GlcNAc-Asn-[^{14}C]acetyl was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Endo-β-N-acetylglucosaminidase D was significantly inhibited by 0.05 M of mannose (Table I). In contrast, glucose, galactose and N-acetylglucosamine showed little effect even at the concentration of 0.5 M. Methyl α-mannoside and p-nitrophenyl α-mannopyranoside were also inhibitory, whereas p-nitrophenyl α-glucopyranoside was not. Yeast mannan was not inhibitory at the concentration of 15 mg/ml.

Among mannose, methyl α-mannoside and p-nitrophenyl α-mannopyranoside, the last compound was the most potent inhibitor.

Table I

Effects of mannose and related compounds on the activity of endo-β-N-acetylglucosaminidase D

Compounds	Concentration	Relative activities
none	-	100
mannose	0.5 M	6.1
	0.05	31.6
	0.01	69.8
glucose	0.5	82.8
galactose	0.5	76.9
N-acetylglucosamine	0.5	108
methyl α-mannoside	0.05	46.5
p-nitrophenyl α-mannopyranoside	0.01	30.0
p-nitrophenyl α-glucopyranoside	0.01	97.1
yeast mannan	15 mg/ml	106

Therefore, we carried out a kinetical experiment using p-nitrophenyl α-mannopyranoside in order to know the type of inhibition. As shown in Fig. 1, p-nitrophenyl α-mannopyranoside was revealed to be a competitive inhibitor. The Ki value was estimated to be 2.2 mM. Thus, p-nitrophenyl α-mannopyranoside was concluded to bound to the vicinity of the catalitic site of the enzyme. The most probable binding site of the mannoside is the substrate binding site.

As summerized in INTRODUCTION, the specific α-mannosyl residue in the substrates was essential for the action of the endoglycosidase. The above result gave a sufficient evidence to

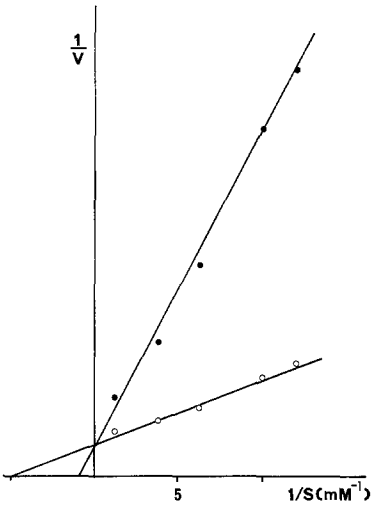


Fig. 1. Inhibition of endo-β-N-acetylglucosaminidase D by p-nitrophenyl α-mannopyranoside. o - o: Without inhibitor. ● - ●: With 0.01 M p-nitrophenyl α-mannopyranoside. The Km value of the enzyme was reported to be 0.25 mM (1), however, from the figure, the Km value was estimated to be 0.20 mM. The difference was due to the fact that the Km value decreased at higher substrate concentration. For the calculation of Ki value, the latter Km value was used.

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the conclusion that the specific α-mannosyl residue in the substrates played a direct role in the interaction with the enzyme, and excluded the possibility that the α-mannosyl residue played only secondary roles such as determination of the configuration of the glycopeptides. Furthermore, it is possible that only after binding to the α-mannosyl residue, the enzyme is allowed to interact with di-N-acetylchitobiose structure at a definite distance from the α-mannosyl residue. Although analysis by using various model compounds and employment of physicochemical methods are certainly required to clarify the sequence of the recognition of glycopeptide structures by the enzyme, the endoglycosidase appears to be a suitable system to study protein-carbohydrate interaction of a relatively complex nature.

There exists another type of endo-β-N-acetylglucosaminidase

acting on di-N-acetylchitobiose structure of glycoproteins. This enzyme, endo-β-N-acetylglucosaminidase H (9, 10) was different from endo-β-N-acetylglucosaminidase D with respect to the specificity toward oligomannosyl cores in the substrates (10). Therefore we examined effects of mannose and their derivatives on endo-β-N-acetylglucosaminidase H. The enzyme was not significantly inhibited by 0.5 M of mannose, 0.5 M of methyl α-mannoside and 0.01 M of p-nitrophenyl α-mannopyranoside (Table II). On the other hand, yeast mannan was inhibitory at the concentration of 15 mg/ml.

The above result was consistent with the specificity of the endoglycosidase. Although both endo-β-N-acetylglucosaminidase D and H required α-mannosyl residues for their action (1, 4-6, 10), oligomannosyl cores recognized by the two enzymes were different. The D enzyme recognized relatively simple cores as illustrated in INTRODUCTION. On the other hand, the H enzyme

Table II

Effects of mannose and related compounds on the activity of endo-β-N-acetylglucosaminidase H

Compounds	Concentration	Relative activities
none	-	100
mannose	0.5 M	80.1
N-acetylglucosamine	0.5	79.3
methyl α-mannoside	0.5	89.7
p-nitrophenyl α-mannopyranoside	0.01	105
yeast mannan	15 mg/ml	38.0

hydrolyzed glycopeptides with relatively complex cores such as Unit A glycopeptides of thyroglobulin, but did not act on those with simple cores such as IgG glycopeptides (10). Therefore, the required structure for the H-enzyme was certainly more complex than that for the D-enzyme, and appeared to include the sequence of Manα1→XManα1→XManβ1→4GlcNAcβ1→4GlcNAc. Thus, mannose and their simple derivatives such as p-nitrophenyl α-mannopyranoside were probably too simple to be able to interact with endo-β-N-acetylglucosaminidase H. The inhibitory effect of yeast mannan toward endo-β-N-acetylglucosaminidase H could be explained by the occurrence of oligomannosyl sequence complex enough to be recognized by the H enzyme.

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REFERENCES

1. Koide, N., and Muramatsu, T. (1974) J. Biol. Chem., 249, 4897-4904
2. Muramatsu, T. (1971) J. Biol. Chem., 246, 5535-5537
3. Muramatsu, T., Atkinson, P. H., Nathenson, S. G., and Ceccarini, C. (1973) J. Mol. Biol., 80, 781-799
4. Ito, S., Muramatsu, T., and Kobata, A. (1975) Biochem. Biophys. Res. Commun., 63, 938-944
5. Tai, T., Ito, S., Yamashita, K., Muramatsu, T., and Kobata, A. (1975) Biochem. Biophys. Res. Commun., in press
6. Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., and Kobata, A. (1975) submitted for publication
7. Huang, C. C., Mayer, H. E., Jr., and Montgomery, R. (1970) Carbohyd. Res., 13, 127-137
8. Haworth, W. N., Hirst, E. L., and Isherwood, F. A. (1937) J. Chem. Soc., 784-791
9. Tarentino, A. L., and Maley, F. (1974) J. Biol. Chem., 249, 811-817
10. Arakawa, M., and Muramatsu, T. (1974) J. Biochem. (Tokyo), 76, 307-317