The Substrate Specificities of Endo-β-N-acetylglucosaminidases C_{II} and H

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SUMMARY A minor glycopeptide was newly isolated from the exhaustive pronase digest of crystalline ovalbumin by Dowex-50w column chromatography, and its structure was determined as Manα1→3Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc→Asn. This glycopeptide (GP-VI) has the smallest carbohydrate unit among the ovalbumin glycopeptides so far reported, and is also the smallest glycopeptide of all which are susceptible to endo-β-N-acetylglucosaminidases C_{II} and H. This finding, together with the already reported data of the action of both enzymes to glycopeptides of known structures, elucidates that the structural requirement of C_{II} enzyme for its substrate is R→2Manα1→3(R→6)Manα1→6(R→2Manα1→3)(R→4)Manβ1→4GlcNAcβ1→4GlcNAc→Asn, in which R represents either hydrogen or sugars, and that of H enzyme is R→2Manα1→3(R→6)Manα1→6(R→4)Manβ1→4GlcNAcβ1→4GlcNAc→Asn.

Endo-β-N-acetylglucosaminidase C_{II} (C_{II} enzyme) which was purified from the culture medium of *Clostridium perfringens* does not act on Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc→Asn ($Man_3GlcNAc_2Asn$), but acts on Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc→Asn (GP-V) (1). In this respect, the specificity of this enzyme is quite different from that of endo-β-N-acetylglucosaminidase D (2) but is the same to that of endo-β-N-acetylglucosaminidase H (H enzyme) (3,4). Since GP-V has the same inner core structure as $Man_3GlcNAc_2Asn$, it has become an essential experiment from the point of the enzyme specificity to study if Manα1→6Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc→Asn or Manα1→3Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc→Asn is susceptible to both enzymes. Fortunately, the latter glycopeptide has recently

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been found as GP-VI in our laboratory among ovalbumin glycopeptides. This paper deals with the isolation and the structural studies of this glycopeptide and the substrate specificities of endo-β-N-acetylglucosaminidases C_{II} and H.

MATERIALS AND METHODS

$Man_5GlcNAc_{OT}$ and $Man_6GlcNAc_{OT}$ (for structures, see Table I) were prepared from ovalbumin glycopeptides V and IV, respectively, as previously described (5). Manα1→3Manβ1→4$GlcNAc_{OT}$ and Manα1→2Manα1→3Manβ1→4$GlcNAc_{OT}$ were isolated from the acetolysates of $Man_5GlcNAc_{OT}$ and $Man_6GlcNAc_{OT}$, respectively, as reported previously (5). Manα1→2Manα1→2Manα1→3Manβ1→4$GlcNAc_{OT}$ was isolated from the acetolysate of calf thyroglobulin unit A (6). Manβ1→4$GlcNAc_{OT}$ was prepared by exhaustive α-mannosidase digestion of $Man_5GlcNAc_{OT}$. GlcNAc→N-[^{14}C]acetyl Asn was prepared as previously reported (5).

Pronase (45,000 p.u.k/g) was purchased from Kaken Chemical Co., LTD, Tokyo. α-Mannosidase was purified from jack bean by the method of Li and Li (7). β-Mannosidase purified from snail (8) and endo-β-N-acetylglucosaminidase H from *Streptomyces griseus* (4) were kindly supplied by Tokyo Seikagaku Kogyo, LTD.

Endo-β-N-acetylglucosaminidase C_{II} from *Clostridium perfringens* was prepared as reported previously (1). N-[^{14}C]acetylation of glycopeptides was performed by using [^{14}C]acetic anhydride (31 mCi/mmole, The Radiochemical Center, England) as reported previously (5).

Sugar composition was determined by using the radioelectrophoretic method devised by Takasaki and Kobata (9). Amino acid and glucosamine contents were determined by JEOL amino acid analyzer model JLC-5AH after hydrolysis in 6 N HCl at 110° for 20 hours and in 4 N HCl at 100° for 4 hours, respectively.

Descending paper chromatography was performed with the following solvents: I, ethylacetate-pyridine-water (12:5:4) and II, ethylacetate-pyridine-acetic acid-water (5:5:1:3). Paper electrophoresis was performed using pyridine-acetate buffer, pH 5.4 (pyridine-acetic acid-water, 3:1:387) at the potential of 73 volts/cm for 1.5 hours. Radioactivity on paper was determined after incubation of the paper pieces with 1 ml of water in the counting vials and addition of 7 ml of the scintillation fluid. Radiochromatogram scanning was performed with a Packard Radiochromatogram Scanner model 7201. Methylation analysis was performed as previously reported (10). The detail of the Bio-Gel P-2 permeation procedure for the molecular weight determination of $(Man)_nGlcNAc_{OT}$ was described in the previous paper (12).

Acetolysis of tritium labeled oligosaccharide was performed as described previously (5).

RESULTS AND DISCUSSION

As shown in Fig. 1, the exhaustive pronase digest of crystalline ovalbumin was separated into eight glycopeptide fractions by Dowex-50 (H^+) column chromatography. The small peak of GP-VI, which holds 0.54% dry wt of the total glycopeptide fraction was

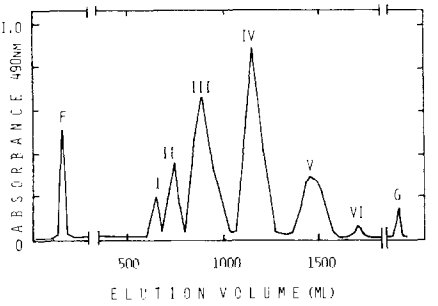


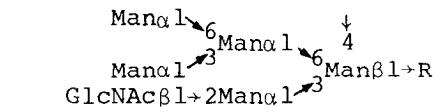
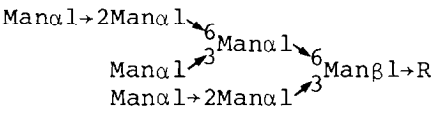
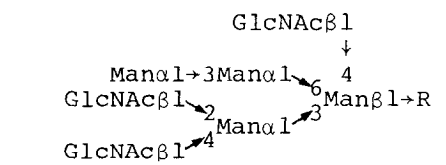
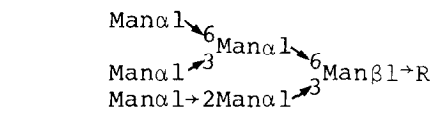
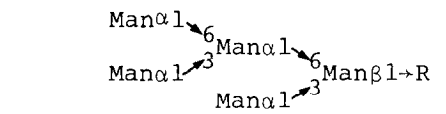
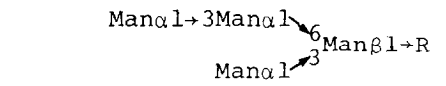
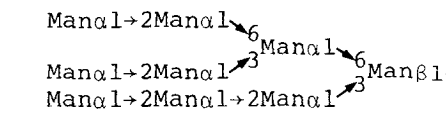
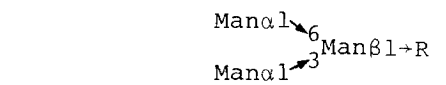
Fig. 1: Chromatography on Dowex 50W-X2 of the glycopeptide fraction from crystalline ovalbumin. Ovalbumin glycopeptide fraction was obtained by exhaustive pronase digestion of crystalline ovalbumin by the method of Huang et al. (14). The crystalline ovalbumin, which was prepared from eggs of White Leghorn strain by the method of Kekwick and Cannan (15), gave a single band with molecular weight of 45,000 in SDS gel electrophoresis. The glycopeptide fraction of ovalbumin was subjected to ion exchange chromatography using Dowex 50W-X2 column. The chromatographic condition was essentially the same as reported by Huang et al. (14). The chromatogram was obtained by monitoring the content of neutral sugars by phenol-sulfuric acid reagent (16). Peak F was the mixture of glycopeptides which were not retained by the column, and peak G was an incompletely digested glycopeptide.

collected. Compositional analysis of this glycopeptide gave 4.2 moles of mannose and 2.1 moles of N-acetylglucosamine per mole of aspartic acid. Other sugars and amino acids were not detected. Methylation analysis of GP-VI gave 2 moles of 2,3,4,6-tetra-O-methyl mannitol acetate, 1 mole of 2,4,6-tri-O-methyl mannitol acetate and 1.7 moles of 3,6-di-O-methyl-2-N-methylacetamido-2-deoxyglucitol acetate per mole of 2,4-di-O-methyl mannitol acetate.

The α-amino group of asparagine moiety of GP-VI was acetylated with [^{14}C]acetic anhydride by the method previously reported (5). When this radioactive glycopeptide (0.15 nmole, 4,000 cpm) was incubated with 0.4 munit of either C_{II} enzyme or H enzyme at 37° for 16 hours, it was completely converted to GlcNAc-N-[^{14}C]acetyl Asn. In order to study the structure of the oligosaccharide liberated from GP-VI by C_{II} enzyme, 1 mg of GP-VI was incubated with

7.5 munits of C_{II} enzyme in 0.1 ml of 0.05 M citrate phosphate buffer, pH 7.0 at 37° for 16 hours. The pH of the reaction mixture was then adjusted to 10.0 by adding 0.1 N NaOH, and reduced with NaB^3H_4 (specific activity, 316 mCi/mmole) according to the method of Takasaki and Kobata (9). The reaction mixture was passed through a small column of AG-50 (H^+) and the effluent was evaporated to dryness. Borate was removed from the residue by repeated evaporation with methanol. The residue was dissolved in 50 µl of water, spotted on Whatman No 1 paper and subjected to paper electrophoresis using pyridin-acetate buffer, pH 5.4. The radioactive oligosaccharide (sugar VI) which showed the same mobility as lactitol was recovered from paper by elution with water. That the radioactive sugar VI was a single component was confirmed by paper chromatography using solvent I and II. Aliquot of the tritium labeled sugar VI (2×10^4 cpm) was then hydrolyzed in 1 N HCl at 100° for 4 hours, freed from acid by repeated evaporation with water, and examined by paper electrophoresis with borate buffer (9). A single radioactive peak of glucosaminitol was detected, indicating that the reducing terminal of the liberated oligosaccharide was N-acetylglucosamine. When tritium labeled sugar VI (1.0 nmole, 3.2×10^4 cpm) was incubated with 0.2 unit of α-mannosidase in 10 µl of 0.05 M sodium acetate buffer, pH 4.0 at 37° for 15 hours, it was completely converted to a radioactive diitol with the same chromatographic mobility as authentic Manβ1→4GlcNAc$_{OT}$. This diitol could completely be converted to radioactive N-acetylglucosaminitol by β-mannosidase digestion. These results indicated that the structure of sugar VI is (Manα)$_3$Manβ1→4GlcNAc$_{OT}$. Molecular weight estimation of sugar VI by Bio-Gel P-2 permeation chromatography using a series of authentic (Manα)$_{0\sim5}$Manβ1→4GlcNAc$_{OT}$ gave a value of 940, which is consistent with the structure des-

Table I. The specificities difference of endo-β-N-acetyl-glucosaminidase C_{II} and H.

Glycopeptides		Endo-β-N-acetyl-glucosaminidase		Ref.
name	structure*	C_{II}	H	
ovalbumin				
GP-IIIA		+	+	12
GP-IIIB		+	+	12
GP-IIIC		-	+	12
GP-IV		+	+	5,12
GP-V		+	+	5, 12
GP-VI		+	+	
calf thyroglobulin				
unit A		+	+	6
bovine IgG				
IgG core		-	-**	13

* R = 4GlcNAcβ1→4GlcNAc→Asn

** H enzyme does act on this glycopeptide, however the rate of hydrolysis was around 1/10000 of GP-V (13).

cribed above. Since the methylation analysis of GP-VI indicated the presence of one 3,6-disubstituted mannose residue in its sugar chain, acetolysis was performed to the tritium labeled sugar VI to cleave the Manα1→6Man linkage specifically (11). One major peak with the same paper chromatographic mobility as authentic Manα1→3Manβ1→4GlcNAc$_{OT}$ was detected. Sequential α-mannosidase and β-mannosidase digestion further confirmed the structure of the radioactive acetolysis fragment as Manα1→3Manβ1→4GlcNAc$_{OT}$. Summarizing all the data so far described, the complete structure of GP-VI was proposed as shown in Table I.

Table I summarizes the available data of the action of endo-β N-acetylglucosaminidases C$_{II}$ and H on glycopeptides with different sugar chains. Comparison of the data of IgG core with that of GP-VI indicated that the additional Manα1→3 residue in the latter is essential for its susceptibility to both C$_{II}$ and H enzymes. The data with unit A and GP-IIIB indicated that this essential α-mannosyl residue can be substituted at the C-2 position by other sugar without losing its role. Comparison of the data of GP-IIIA, with that of GP-IIIC indicated that the α-mannosyl residue located at the C-3 position of the branching β-mannose is also recognized by C$_{II}$ enzyme. Glycopeptides with this α-mannosyl residue either in free state (GP-V and GP-VI) or substituted only at the C-2 position with other sugars (GP-IIIA, GP-IIIB, GP-IV and unit A) are all cleaved by C$_{II}$ enzyme. However, glycopeptide with this α-mannosyl residue substituted at C-4 position (GP-IIIC) can no more be a substrate. In contrast to C$_{II}$ enzyme, H enzyme seems to have no strict requirement on this residue, because this enzyme acts on GP-IIIC equally well as on GP-IIIA. Therefore, we can presume that the glycopeptides with the structure as shown in

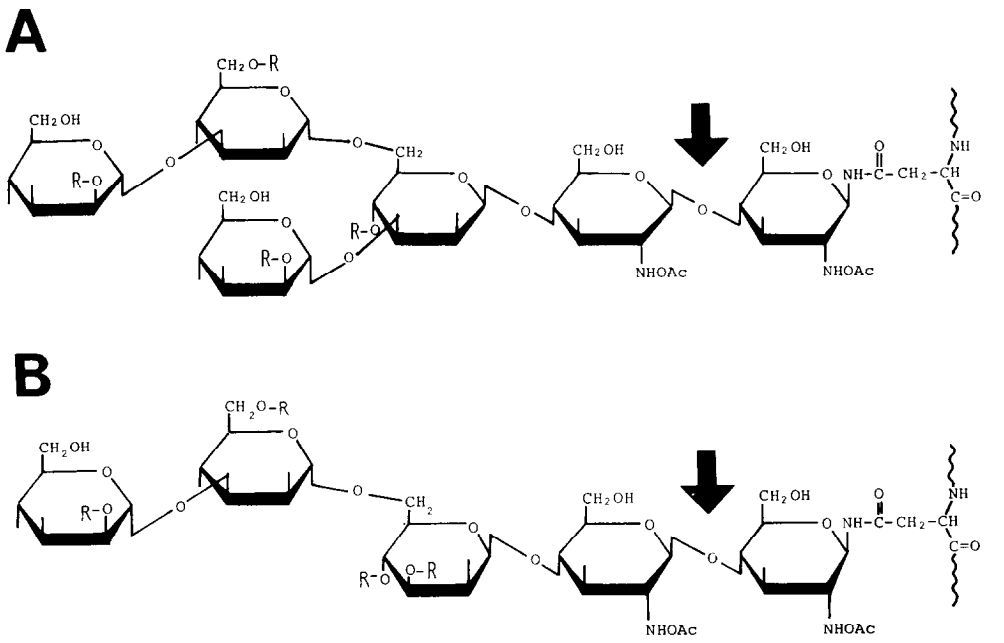


Fig. 2: General structures of the susceptible substrates for C_{II} enzyme (A) and H enzyme (B). Arrows indicate the positions of hydrolysis. R represent either hydrogen or sugars.

Fig. 2A are susceptible to endo-β-N-acetylglucosaminidase C_{II} and with the structure in Fig. 2B are susceptible to endo-β-N-acetylglucosaminidase H.

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