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TRANSGLYCOSYLATION ACTIVITY OF *Mucor hiemalis* ENDO-β-*N*-ACETYL-GLUCOSAMINIDASE WHICH TRANSFERS COMPLEX OLIGOSACCHARIDES TO THE *N*-ACETYLGLUCOSAMINE MOIETIES OF PEPTIDES

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Summary: A novel endo-β-*N*-acetylglucosaminidase in the culture fluid of *Mucor hiemalis* isolated from soil was found to have transglycosylation activity. This endo-β-*N*-acetylglucosaminidase, Endo-M, could liberate the complex type of asparagine-linked oligosaccharides by hydrolysis of diacetylchitobiose linkage from glycoproteins. The treatment of Endo-M with *N*-acetylglucosamine and asialotransferrin glycopeptide having the complex type of oligosaccharides resulted in the transfer of the released oligosaccharide from the glycopeptide to *N*-acetylglucosamine. The structure of the product after transfer was deduced to be (GlcNAc)2-Man-(Gal-GlcNAc-Man)2 by a combination method of pyridylamination and high perfomance liquid chromatography, and mass-spectrometry. The enzyme could transfer the complex type of oligosaccharide from asialotransferrin glycopeptide to peptidyl-*N*-acetylglucosamine prepared from bovine ribonuclease with the high-mannose type of oligosaccharide. This will lead to the construction of neoglycoproteins containing different types of oligosaccharides.

Many exoglycosidases have been shown to have transglycosylation activities in addition to hydrolytic activity. The transglycosylation reaction is regarded as a special reaction of hydrolysis in which the monosaccharide moiety of the substrate is transferred to the hydroxyl groups of various compounds instead of water. This activity is very useful for the enzymatic synthesis of various oligosaccharides, since chemical synthesis is very complex and yields anomeric isomers (1). In contrast to the transglycosylation activities of various exoglycosidases, those of endoglycosidases acting on complex carbohydrates are less known and not well-studied. To date, there have been

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The abbreviation used are:
DNS, dansyl (5-dimethylaminonaphthalene-1-sulfonyl); Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; PA-, pyridylamino-.

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only a few reports regarding the transglycosylation activities of bacterial endo-β-*N*-acetylglucosaminidases (2,3), endo-α-*N*-acetylgalactosaminidase (4), and ceramide glycanase from leech (5). Recently, we also reported that of endoglycoceramidase from *Corynebacterium* sp. (6).

A few years ago, we found a novel endo-β-*N*-acetylglucosaminidase (endo-β-GlcNAc-ase) in the culture fluid of *Mucor hiemalis* isolated from soil. This endo-β-GlcNAc-ase, Endo-M, was found to cleave not only the high-mannose type and hybrid type of asparagine-linked oligosaccharides but also the complex type of biantennary oligosaccharides by hydrolysis of diacetylchitobiose core of the oligosaccharide chain in glycoproteins (7-9), unlike other well-characterized endo-β-GlcNAc-ases which can act on only high-mannose type and hybrid types. During studies on substrate specificity of Endo-M, we found that the enzyme could transfer the intact complex oligosaccharide from human asialotransferrin glycopeptide to suitable acceptors during hydrolysis of the glycopeptide. This oligosaccharide transferring reaction can be used for remodeling of sugar chains of glycoproteins/glycopeptides: high-mannose types of oligosaccharides in glycoproteins/glycopeptides can be exchanged to complex types. This finding will lead to synthesis of neoglycoproteins and neoglycopeptides. This is the first report that the complex type of oligosaccharide was added to peptide.

MATERIALS AND METHODS

Preparation of endo-β-N-acetylglucosaminidase from Mucor hiemalis: The endo-β-*N*-acetylglucosaminidase of *Mucor hiemalis*, that is Endo-M, was partially purified from the culture fluid of the fungus by CM-cellulose treatment, ammonium sulfate fractionation and column chromatography on DEAE-Sepharose CL-6B and hydroxylapatite, as described previously (7). The enzyme preparation was almost free of protease and other glycosidase activities.

Preparation of substrates: Asialotransferrin glycopeptide was prepared by repeated exhaustive Pronase digestion of human transferrin, followed by separation on a Sephadex G-25 gel column and reaction with neuraminidase before the last Pronase digestion, as described previously (7).

An asparaginyl oligosaccharide, Asn-(GlcNAc)$_2$-(Man)$_6$, was prepared from ovalbumin glycopeptides by repeated exhaustive Pronase digestion of ovalbumin followed by separation on a Sephadex G-25 gel column and then Dowex 50W-X2 column chromatography by the procedure of Huang *et al.* (10).

Peptidyl *N*-acetylglucosamine was prepared from bovine pancreas ribonuclease B with one high-mannose type sugar chain by enzymatic treatment with pepsin and endo-β-GlcNAc-ase. Ribonuclease was digested with pepsin, and peptides containing sugar chains were separated by Concanavalin A-Sepharose 4B column chromatography. The glycopeptides thus obtained were treated with *Flavobacterium* sp. endo-β-GlcNAc-ase (11), and peptidyl GlcNAc were isolated by high performance liquid chromatography (HPLC).

Enzyme assay: The enzyme activity was measured using dansyl-asialotransferrin glycopeptide as the substrate, as described previously (7). One unit was defined as the amount of enzyme that yields 1 μmol of dansyl-asparaginyl *N*-acetylglucosamine (DNS-Asn-GlcNAc) from DNS-asialotransferrin glycopeptide per min at 37℃.

Pyridylamination of oligosaccharides: The oligosaccharides were pyridylaminated using 2-aminopyridine and borane-dimethylamine complex by the method of Kondo *et al.* (12) except with the use of benzene to remove excess 2-aminopyridine.

High performance liquid chromatography (HPLC): High performance liquid chromatography of dansyl derivatives was performed using a reversed-phase column (4.6×250mm, Unicil QC-18: Gasukuro-Kogyo Inc., Japan) by the method of Iwase *et al.* (13). The reaction mixture was analyzed with a Hitachi L-6200 chromatograph with an F-1050 fluorescence spectrophotometer. Elution was carried out with 11% acetonitrile in 25 mM sodium borate buffer (pH 7.5) at 0.5 ml/min, and monitored with an excitation wavelength of 320 nm and emission wavelength of 540 nm.

The pyridylamino (PA) derivatives of oligosaccharides were separated on a reversed-phase column (4.6×250 mm, Cosmosil 5C18-AR: Nacalai Tesque Co., Japan) using 100 mM acetic acid-triethylamine (pH4.0) containing 0.25% n-butanol at 40℃ at a flow rate of 0.5 ml/min. For separation of PA-derivatives of high-mannose oligosaccharides, 125 mM sodium citrate-HCl buffer (pH4.0) containing 1% acetonitrile was used. PA-derivatives were also analyzed using a size-fractionation column (4.6×250 mm , TSK-gel Amide 80: Tosoh Co., Japan) with a solution of acetonitrile-3% acetic acid (70:30), adjusted to pH 7.3 with triethylamine, at 70℃ at a flow rate of 1.0 ml/min. For detection of PA-oligosaccharides, the above-mentioned fluorescence spectrophotometer at an excitation wavelength of 320 nm and emission wavelength of 400 nm was used.

Peptides were separated on a reversed-phase column (4.6×250 mm, Cosmosil 5C18-P: Nacalai Tesque Co., Japan) equilibrated with 0.05% trifluoroacetic acid and eluted with a linear acetonitrile gradient (0~35%) at 30℃ at flow rate of 1 ml/min. Ultraviolet spectrophotometer (Hitachi L-4200) at 220 nm was used for detection of absorbance of peptides.

Mass spectrometry: Ion spray liquid chromatography/mass spectrometry (ISLC/MS) was performed in the positive ion mode on a Perkin Elmer-Sciex API-III spectrometer. Liquid chromatography was performed using a normal-phase column (4.6×250 mm , PALPAK Type S: Takara Shuzo Co., Japan) with a solvent of acetonitrile-500 mM acetic acid (53:47), adjusted to pH 7.3 with triethylamine at a flow rate of 0.5 ml/min. Mass spectra were analyzed first in the full scan mode, and the fragment mass ions were further confirmed by a linked scan daughter ion analysis.

Materials: Human transferrin and bovine pancreas ribonuclease B were obtained from Sigma Chemical Co. Standard PA-oligosaccharides and PA-glucose oligomers were obtained from Takara Shuzo Co., Japan. All other chemicals used were obtained from commercial sources.

RESULTS

Transfer of biantennary complex oligosaccharides from asialotransferrin to N-acetylglucosamine by Endo-M. To examine whether Endo-M has transglycosylation activity in addition to hydrolytic activity, we added *N*-acetylglucosamine to the reaction mixture of enzymatic deglycosylation of asialotransferrin. The reaction mixture was composed of 2.5 mg of asialotransferrin glycopeptide and 32 munits of Endo-M in 66 mM potassium phosphate buffer (pH 6.0), and incubated in the absence or presence of *N*-acetylglucosamine (100 mM) in a total volume of 1.5 ml at 37℃. After incubation for 6 hr, each reaction mixture was boiled for 3 min and centrifuged. The supernatant was chromatographed on a Sephadex G-10 column (0.9×50 cm) to remove excess *N*-acetylglucosamine, and the fractions containing carbohydrates in the void volume position were collected. The solution was lyophilized and the oligosaccharides were pyridylaminated. The PA-oligosac-charides obtained after Sephadex G-10 column chromatography were analyzed by HPLC with a reversed-phase column. One major peak was obtained in the reaction without *N*-acetyl-

glucosamine (Fig.1A). On the other hand, two separate large peaks were found in the reaction mixture to which was added *N*-acetylglucosamine (Fig.1B). The front peak was eluted at the position of the peak in the reaction mixture without *N*-acetylglucosamine, and seemed to correspond to the hydrolytic product. The later peak was found at the elution position coinciding with PA-biantennary complex oligosaccharide (Fig.1C). The fractions of the peak were collected and concentrated, and then analyzed by HPLC with a size-fractionation column. One peak was obtained and identified as PA-biantennary complex oligosaccharide judged from its elution position to be exactly the same as that of the authentic PA-sugar chain. Next, this PA-oligosaccharide was subjected to ion spray liquid chromatography/mass spectrometry (ISLC/MS) analysis in the positive mode. The doubly charged mass ion $[M+2H]^{2+}$ was at m/z 860.5 (Fig.2). The molecular mass of 1719.6 calculated from this value coincided with the theoretical value of PA-(GlcNAc)$_2$-Man-(Man-GlcNAc-Gal)$_2$. These results showed that free *N*-acetylglucosamine was transferred to the biantennary complex oligosaccharide of asialotransferrin glycopeptide by Endo-M during hydrolysis of core diacetyl-chitobiose linkage.

Transfer of oligosaccharides from glycopeptides to diacetylchitobiose by Endo-M. We also found that diacetylchitobiose was an effective acceptor of the biantennary complex oligosaccharide released from asialotransferrin glycopeptide. Asialotransferrin glycopeptide (2.5 mg) was incubated with 32 munits of Endo-M in 66 mM potassium phosphate buffer (pH 6.0) in the presence of 50 mM diacetylchitobiose in a total volume of 1.5 ml. After incubation for 6 hr and heat inactiva-

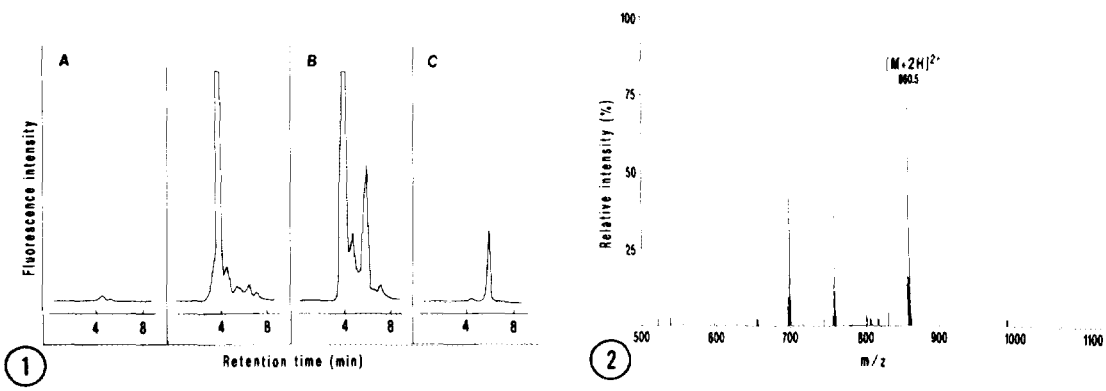

Fig. 1. HPLC profiles of PA-oligosaccharides derived from the reaction mixtures of hydrolysis of asialotransferrin glycopeptides by Endo-M. (A) Portions of the reaction mixture without GlcNAc were withdrawn at 0 hr (left) and 6 hr (right) and analyzed after pyridylamination. (B) A portion of the reaction mixture with GlcNAc was withdrawn at 6 hr and analyzed after pyridylamination. (C) Authentic PA-complex type biantennary oligosaccharide was analyzed. HPLC was carried out using a reversed-phase column.

Fig. 2. Mass spectrum of PA-oligosaccharide isolated from the reaction mixtures of hydrolysis of asialotransferrin glycopeptides in the presence of GlcNAc.

tion, the reaction mixture was treated in the same manner described above and the oligosaccharides were pyridylaminated. The PA-oligosaccharides were analyzed by HPLC, and the trans-glycosylated product was found (data not shown). This product was deduced to be PA-(GlcNAc)$_3$-Man-(Man-GlcNAc-Gal)$_2$ having triacetylchitotriose by the two-dimensional sugar map method of Tomiya *et al.* (14) and the means of ISLC/MS (the molecular mass of 1922.8). It is a novel oligosaccharide.

As diacetylchitobiose was an effective acceptor for transglycosylation reaction by Endo-M, we investigated the use of PA-diacetylchitobiose as an acceptor, because PA-oligosaccharide is very convenient for rapid assay by HPLC. Asialotransferrin glycopeptide (0.17 mg) was incubated with 2.2 mM PA-diacetylchitobiose and 2 munits of Endo-M in 75 mM potassium phosphate buffer (pH 6.0) in a total volume of 0.1 ml at 37℃. After incubation for 6 hr, portions of the reaction mixture were analyzed by HPLC (Fig. 3A). A new peak of the PA-compound was found and was certified to correspond to the product of transglycosylation reaction, PA-(GlcNAc)$_3$-Man-(Man-GlcNAc-Gal)$_2$, according to its two-dimensional sugar map. PA-cellobiose was not effective as the acceptor.

Besides complex oligosaccharides, Endo-M is also capable of transferring high-mannose oligosaccharides from ovalbumin to the acceptor. Asn-(GlcNAc)$_2$-(Man)$_6$ (0.2 mg) was incubated with 2.2 mM PA-diacetylchitobiose and 2 munits of Endo-M under the same reaction conditions as described above for 2 hr. A new PA-compound was found by HPLC analysis of a portion of the reaction mixture (Fig.3B). This compound was judged to be the product of transglycosylation reaction, PA-(GlcNAc)$_3$-(Man)$_6$, according to its two-dimensional sugar map.

Using PA-diacetylchitobiose as the acceptor, we examined the optimum reaction conditions of Endo-M for the transfer of oligosaccharide in asialotransferrin glycopeptide as the donor; the

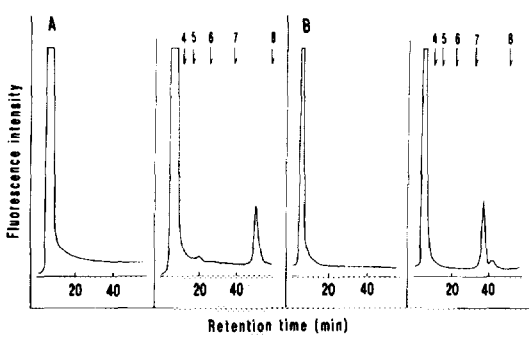

Fig. 3. HPLC profiles of the products formed by incubation of PA-diacetylchitobiose and asialotransferrin glycopeptide (A) or Asn-(GlcNAc)$_2$-(Man)$_6$ (B). A portion of each reaction mixture with enzyme (right) and inactivated enzyme (left) was analyzed using a normal-phase column. Elution positions of PA-glucose unit numbers are indicated by arrows. Numbers indicate numbers of sugars.

optimum pH was approximately 6.0 and optimum temperature was 40℃, which were same as those for hydrolytic activity of Endo-M.

Transfer of oligosaccharides from glycopeptides to asparaginyl N-acetylglucosamine by Endo-M. We examined the transfer of oligosaccharides from glycopeptides to asparaginyl *N*-acetyl-glucosamine by Endo-M. We used DNS-Asn-GlcNAc as the acceptor because this compound is fluorescent and thus the transglycosylation product can be easily analyzed by HPLC. DNS-Asn-GlcNAc 2 mM was incubated with 1 mg of asialotransferrin or Asn-(GlcNAc)$_2$-(Man)$_6$ in 75 mM potassium phosphate buffer (pH 6.0) using 1 munit of Endo-M in a total volume of 0.1 ml at 37℃. The reaction was carried out for 2 hr and terminated by heating, and a portion of the reaction mixture was analyzed by HPLC. In the reaction mixture containing Asn-(GlcNAc)$_2$-(Man)$_6$ as the donor, a large peak corresponding to DNS-Asn-(GlcNAc)$_2$-(Man)$_6$ was observed (Fig. 4). However, only a small peak corresponding to DNS-Asn-(GlcNAc)$_2$-Man-(Man-GlcNAc-Gal)$_2$ was found in the reaction mixture containing asialotransferrin glycopeptide as the donor.

Transfer of oligosaccharide from glycopeptides to peptidyl N-acetylglucosamine by Endo-M. Using Endo-M, we next attempted to transfer complex oligosaccharides from asialotransferrin glycopeptides to peptidyl GlcNAc which was prepared from bovine pancreas ribonuclease by pepsin and *Flavobacterium* sp. endo-β-GlcNAc-ase treatment. The ribonuclease has one sugar chain of the high-mannose type. Asialotransferrin glycopeptide (1 mg) was incubated with 0.1 mg of peptidyl GlcNAc using 0.5 munits of Endo-M in 75 mM potassium phosphate buffer (pH 6.0) in a total volume of 0.1 ml at 37℃. After incubation for 1 hr, portions of the reaction mixture were

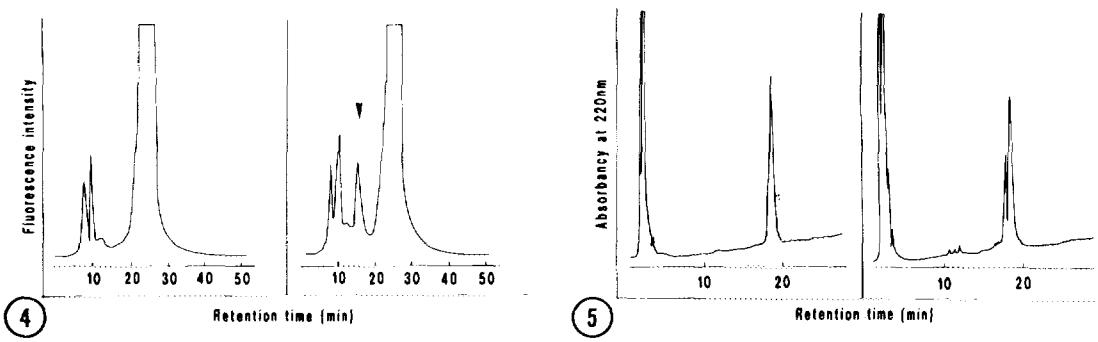

Fig. 4. HPLC profiles of the products formed by incubation of dansyl asparaginyl GlcNAc and Asn-(GlcNAc)$_2$-(Man)$_6$. A sample of the reaction mixture with enzyme (right) and inactivated enzyme (left) was analyzed using reversed-phase column. The arrow indicates the product. Large peak at retention time of 20-25 min is corresponding to DNS-Asn-GlcNAc as substrate.

Fig. 5. HPLC profiles of the products formed by incubation of peptidyl GlcNAc prepared from bovine ribonuclease, and asialotransferrin glycopeptide. Samples of the reaction mixture with enzyme (right) and inactivated enzyme (left) were analyzed using a reversed-phase column. Detail is described in text.

withdrawn and subjected to HPLC. Only one peak was found in the reaction mixture with inactivated enzyme, which corresponded to peptidyl GlcNAc. On the other hand, a new peak occurred in the enzyme reaction with an earlier retention time than that of peptidyl GlcNAc. This seemed to be the transglycosylation product and its elution position corresponded to that of ribonuclease glycopeptide (Fig. 5). This peak disappeared after Endo-M digestion, but not *Flavobacterium* sp. endo-β-GlcNAc-ase digestion (this enzyme has only reactivity for high-mannose type and hybrid type of oligosaccharide (11)). These results demonstrate that complex type oligosaccharide was transferred to peripheral *N*-acetylglucosamine of the peptide by Endo-M, and show the possibility of remodeling or exchanging oligosaccharides on glycopeptides or glycoproteins.

DISCUSSION

The transglycosylation activity of endoglycosidases acting on complex carbohydrates is less common than that of exoglycosidases. However, it has attracted attention in the fields of protein engineering and cell biology because there are very few methods of glycosylating proteins and lipids. Therefore, the enzymatic method using the transglycosylation activity of endoglycosidase seems promising.

The transglycosylation reaction by endoglycosidase was first found about the endo-β-GlcNAc-ase from *Flavobacterium meningosepticum* (Endo-F); Trimble *et al.* observed that a high-mannose oligosaccharide was transferred to glycerol during the cleavage of chitobiose linkage in Asn-$(GlcNAc)_2$-$(Man)_6$ (2). Recently, Takegawa et al. reported that endo-β-GlcNAc-ase from *Arthrobacter protophormiae* (Endo-A) could transfer released high-mannose types of oligosaccharide to mono- and disaccharides (3). However, there have been no reports of any enzymes capable of transferring complex types of oligosaccharide to acceptors such as sugars and peptidyl GlcNAc (Endo-A can not hydrolyze complex types of oligosaccharides). Moreover, no other endo-β-GlcNAc-ases were reported to have transglycosylation activity.

We found that Endo-M had transglycosylation activity, transferring complex types of oligosaccharide to suitable acceptors such as GlcNAc and peptidyl GlcNAc. The reaction scheme is depicted in Fig. 6. The optimum conditions for transglycosylation activity seemed to be almost the same as those of its hydrolytic activity. The results suggest that even though the oligosaccharide was once bound to the acceptor by transglycosylation reaction, it can also be easily hydrolyzed. This is one of the reasons why the yield of transglycosylation product is low. We should, therefore, consider the relative concentrations of donor and acceptor to maximize yield of the product.

However, using the transglycosylation activity of Endo-M, it may be possible that the high-mannose type of oligosaccharides in glycoproteins/glycopeptides which are produced by yeasts or

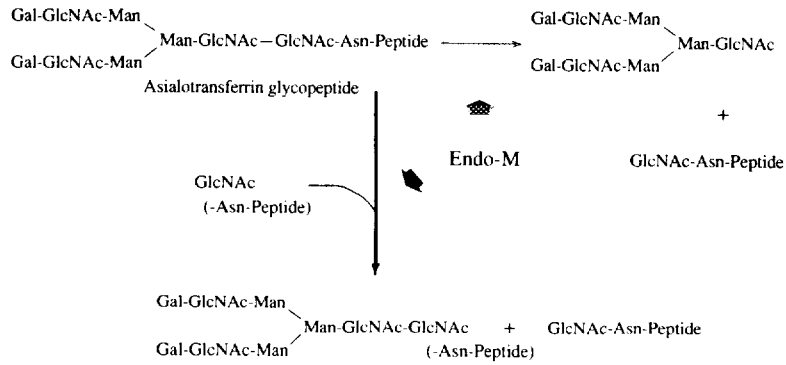

Fig. 6. Scheme of enzyme reactions of Endo-M.

molds could be exchanged or remodeled to the complex types which are not synthesized by these microorganisms. The transglycosylation reaction catalyzed by Endo-M should become useful for synthesizing neoglycoproteins and as a technique in protein engineering.

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