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Syntheses of mucin-type *O*-glycopeptides and oligosaccharides using transglycosylation and reverse-hydrolysis activities of *Bifidobacterium* endo-α-*N*-acetylgalactosaminidase

Hisashi Ashida・Hayato Ozawa・Kiyotaka Fujita・Shunichi Suzuki・Kenji Yamamoto

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H. Ashida*・H. Ozawa・K. Yamamoto

Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan

* E-mail: ashida@lif.kyoto-u.ac.jp; Tel: +81-75-753-4298; Fax: +81-75-753-9228.

K. Fujita

Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan

S. Suzuki

Ajinomoto Co., Inc., Kawasaki, Kanagawa 210-8681, Japan

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Abstract

Endo-α-*N*-acetylgalactosaminidase catalyzes the release of Galβ1-3GalNAc from the core 1-type *O*-glycan (Galβ1-3GalNAcα1-Ser/Thr) of mucin glycoproteins and synthetic *p*-nitrophenyl (*p*NP) α-linked substrates. Here, we report the enzymatic syntheses of core 1 disaccharide-containing glycopeptides using the transglycosylation activity of endo-α-*N*-acetylgalactosaminidase (EngBF) from *Bifidobacterium longum*. The enzyme directly transferred Galβ1-3GalNAc to serine or threonine residues of bioactive peptides such as PAMP-12, bradykinin, peptide-T and MUC1a when Galβ1-3GalNAcα1-*p*NP was used as a donor substrate. The enzyme was also found to catalyze the reverse-hydrolysis reaction. EngBF synthesized the core 1 disaccharide-containing oligosaccharides when the enzyme was incubated with either glucose or lactose and Galβ1-3GalNAc prepared from porcine gastric mucin using bifidobacterial cells expressing endo-α-*N*-acetylgalactosaminidase. Synthesized oligosaccharides are promising prebiotics for bifidobacteria.

Introduction

Glycosidases are divided into two types based on their catalytic mechanisms: retaining and inverting glycosidases. Most of the retaining glycosidases show transglycosylation activity in addition to hydrolysis activity. The transglycosylation reaction is recognized as a part of the hydrolysis reaction in which the water molecule is replaced with a compound containing a hydroxyl-group. In contrast to many examples of transglycosylation by exoglycosidases, there is a paucity of information describing transglycosylation by endoglycosidases acting on complex carbohydrates. Transglycosylation activities of endoglycosidases acting on complex carbohydrates are potentially very powerful tools for the syntheses of a wide variety of glycosyl compounds. This is because endoglycosidases are able to conjugate oligosaccharides *en bloc* to acceptors. Moreover enzymatic remodeling of *O*- and *N*-glycans in glycoproteins using endoglycosidases is one of the most promising approaches in the production of homogeneous glycoproteins [1]. We have reported that several microbial endoglycosidases showed strong transglycosylation activities and some of these enzymes could be applicable to the syntheses of bioactive glycoconjugates [2-7].

Recently, we identified the gene *engBF* encoding an endo-α-*N*-acetylgalactosaminidase from *Bifidobacterium longum* JCM1217, which belongs to a novel glycoside hydrolase family GH101 [8]. EngBF preferably released Galβ1-3GalNAc from the core 1-type *O*-glycan, also referred to as the Thomsen-Friedenreich antigen (T-antigen), of mucin glycoproteins. EngBF also showed transglycosylation activity of the released disaccharide to other mono- and disaccharides.

In the present paper, we report syntheses of the core 1 disaccharide-containing glycopeptides using the transglycosylation activity of EngBF. Furthermore, EngBF was found to catalyze the reverse-hydrolysis reaction, and using this activity we synthesized oligosaccharides without a synthetic donor substrate. Such an approach may be useful in the production of bifidus factors, the prebiotics for bifidobacteria.

Materials and methods

Peptides

PAMP-12, bradykinin and peptide-T were purchased from the Peptide Institute Inc., Japan. MUC1a and PAMP-12(S11T) were synthesized by Sigma Genosys (Sigma-Aldrich Japan). The peptide sequences are shown in Table 1.

Enzyme preparation

The six histidine-tagged EngBF was expressed in *E. coli* BL21(λDE3) and purified by affinity chromatography and gel filtration as previously described [8]. The purified recombinant EngBF showed no protease and exoglycosidase activities.

Transglycosylation and reverse-hydrolysis reaction of EngBF

For transglycosylation to various monosaccharides or amino acids as acceptors, 5 mM Galβ1-3GalNAcα1-*p*NP (Toronto Research Chemicals, Canada) and 500 mM of the acceptor substrate were incubated with 80 milliunits of EngBF in 50 mM sodium acetate buffer (pH 6.0) at 37 °C for an appropriate period. For transglycosylation to peptides, 5 mM Galβ1-3GalNAcα1-*p*NP and 15 mM peptide were incubated with 100 milliunits of EngBF in 50 mM sodium acetate buffer (pH 6.0) under the same conditions. For the reverse-hydrolysis reaction, 1 M glucose and 100 mM Galβ1-3GalNAc were incubated with 0.8 units of EngBF in 50mM sodium acetate buffer (pH 6.0) at 37 °C for an appropriate period.

High-performance Liquid Chromatography

Normal phase high-performance liquid chromatography (HPLC) was performed using a TSK-Gel Amide-80 column (4.6 × 250 mm, Tosoh, Japan). Elution was carried out with acetonitrile:water (3:1, by volume) as the solvent at a flow rate of 1.0 ml/min at 40 °C and detection

of GlcNAc-containing sugars and *p*-nitrophenol was monitored by the absorbance at 214 nm. Reversed phase HPLC was performed using a Cosmosil 5C18-AR-II column (4.6 × 250 mm Nacalai Tesque, Japan). Elution was carried out with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min at 40 °C and detection of compounds was monitored by absorbance at 214 or 280 nm.

Mass spectrometry

For glycopeptides, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analyses were carried out using a Bruker Daltonics Autoflex-G system (MA, USA) with a 337-nm nitrogen laser, using the positive-ion mode, the reflectron mode and external calibration with PEG. The sample was dissolved in ethanol at a concentration of 1 mg/ml. An α-cyano-4-hydroxycinnamic acid solution (25 mg/ml in acetonitrile:0.1% TFA = 3:1, by volume) was used as the matrix. Half-a-microliter of the mixture of these solutions (matrix:sample = 4:1, by volume) was placed on the AnchorChip target (Bruker, Germany). During the measurement, 30 laser shots were used and the data of the mass spectra were collected at different positions of the crystallized sample spot. For oligosaccharides, MALDI-TOF-MS analyses were carried out using a Voyager-DE STR (Applied Biosystems). Samples were analyzed in the positive ion mode using α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid or 2',4',6'-trihydroxyacetophenonen-hydorate as the matrix.

Thin layer chromatography

Thin layer chromatography (TLC) was performed using silica-gel TLC plates (Merck 5553) with 1-butanol:acetic acid:water = 2:1:1 (by volume) as the developing solvent. For separation of Galβ1-3GalNAc and Galβ1-3GlcNAc, 1-propanol:0.2% $CaCl_2$ = 11:2 (by volume) was used. The diphenylamine aniline reagent was used to visualize the sugars [9].

Preparation of Galβ1-3GalNAc from porcine gastric mucin

Bifidobacterium bifidum ATCC29521 was cultured in GAM broth (Nissui Pharmaceutical, Japan) containing 0.5% porcine gastric mucin (Sigma-Aldrich) for 16 h at 37 °C under anaerobic conditions using Anaeropack (Mitsubishi Chemical, Japan). For preparation of Galβ1-3GalNAc, 5 g porcine gastric mucin was incubated with a cell suspension of bifidobacteria in 50 mM sodium acetate buffer (pH 6.0) for 16 h at 37 °C. The supernatant was precipitated twice with 90% ethanol to remove proteins, concentrated and subsequently applied on to a Sephadex G-15 gel filtration column (2 × 50 cm, GE Healthcare). The disaccharide-containing fraction was concentrated and subjected to normal phase HPLC with acetonitrile:water = 4:1 (by volume) as a solvent to separate Galβ1-3GalNAc and Galβ1-3GlcNAc. Sugar compositions were confirmed after the treatment with β1,3-specific galactosidase from *Xanthomonas manihotis* (Calbiochem, CA).

Results

Transglycosylation of Galβ1-3GalNAc to serine and threonine

We previously demonstrated that EngBF could transfer the disaccharide Galβ1-3GalNAc from a donor substrate Galβ1-3GalNAcα1-*p*NP to acceptors such as 1-alkanols and mono- and disaccharides [8]. To apply this enzyme for syntheses of *O*-glycan-containing glycopeptides, we first examined whether EngBF could transfer the disaccharide to a hydroxyl group of a free threonine or serine. Galβ1-3GalNAcα1-*p*NP and threonine were incubated with the recombinant EngBF and the reaction mixture was analyzed by normal phase HPLC. The HPLC results showed a new peak appearing at the retention time (RT) of 32.25 min. This peak was considered to be a transglycosylation product (Fig. 1, middle panel). A new peak with a RT of 37.09 min was detected when serine was used as an acceptor (bottom panel). No new peaks were observed when no acceptor was present in the reaction mixture (upper panel). These two products were isolated and analyzed by

ESI-MS to determine their molecular masses. On the former product, a major mass ion peak was detected at *m/z* 507.75, which corresponded to the sodium adduct of Galβ1-3GalNAcα1-Thr (theoretical mass, 484.20). Similarly, on the latter product, a peak at *m/z* 493.72 was detected for the sodium adduct of Galβ1-3GalNAcα1-Ser (theoretical mass, 470.17). These isolated products were completely re-hydrolyzed by EngBF, indicating that these compounds were the transglycosylation products (data not shown).

Enzymatic syntheses of mucin-type glycopeptides by EngBF

We subsequently examined to determine whether EngBF could transfer the disaccharide to Ser or Thr residues in peptides. We selected PAMP-12 (FRKKWNKWALSR) as an acceptor, which is a potent hypotensive peptide processed from the adrenomedullin precursor and possess one Ser residue at the penultimate position in the sequence [10]. Incubation of PAMP-12 with EngBF in the presence of the donor substrate Galβ1-3GalNAcα1-*p*NP gave rise to two new peaks in the reversed phase HPLC chromatogram (Fig. 2A, bottom chart). The RT of the two new peaks were 52.2 and 61.6 min. The new peak eluting at a later RT value corresponded to *p*-nitrophenol released from the donor substrate. Consequently, the peak that eluted at an earlier RT value was assumed to represent the transglycosylation peptide product because such a product was not detected in the reaction mixture using an inactivated enzyme control (Fig. 2A, top chart). We analyzed this peak by MALDI-TOF-MS. The singly protonated mass ion peak at *m/z* 1984.68 corresponded to Galβ1-3GalNAc-attached PAMP-12 (Fig. 2B), indicating that it was the transglycosylation product formed by EngBF. The product was completely re-hydrolyzed by EngBF (data not shown), confirming that the disaccharide from the donor substrate was transferred to the hydroxyl group of the Ser residue in PAMP-12 via an α-linkage.

Furthermore, to examine whether EngBF could transfer the disaccharide to a Thr residue in peptides, we used a mutant PAMP-12 as an acceptor, in which the Ser residue was changed to a Thr

(PAMP-12(S11T)). Similarly in the case of PAMP-12, a new peak was observed in the reversed phase HPLC chromatogram. This peak was identified to be a transglycosylation product by MALDI-TOF-MS analysis (*m/z* 1999.08 for (M+H)+) (Fig. 2C). The amount of the transglycosylation product was about one-third when compared to the amount produced using PAMP-12 as the acceptor.

We further attempted to transfer Galβ1-3GalNAc to various Ser/Thr-containing peptides. The results of these analyses are summarized in Table 1. Bradykinin (RPPGFSPFR) is a bioactive peptide inhibitor for the angiotensin-converting enzyme, and therefore a potent hypotensive drug [11]. This peptide has a single Ser residue located four residues from the C-terminus. A transglycosylation product toward bradykinin was found by analysis using reversed phase HPLC, but the efficiency of the transglycosylation was about one-tenth of the observed transglycosylation of PAMP-12. MUC1a (AHGVTSAPDTR) is a part of the repetitive icosapeptides (AHGVTSAPDTRPAPGSTAPP) of a mucin MUC1 that is expressed by the normal mammary gland, stomach and pancreas, and is also highly expressed by cancer cells [12]. MUC1a contains one Ser and two Thr residues: i.e., three transglycosylation sites are available. However, only a single major peak in the HPLC chromatogram was observed and assumed to be a transglycosylation product of MUC1a with a single Galβ1-3GalNAc. Peptide T (ASTTTNYT) is a potent peptide for anti-HIV infection, having one Ser and four Thr residues [13]. The analysis of the transglycosylation products showed that a single major and two minor peaks were observed in the HPLC chromatogram. These products were isolated and analyzed by MALDI-TOF-MS and were found to be modified by one disaccharide.

Preparation of Galβ1-3GalNAc from porcine gastric mucin using bifidobacterial cells

To examine the reverse-hydrolysis reaction of EngBF, we first attempted to prepare the free disaccharide Galβ1-3GalNAc from porcine gastric mucin using the recombinant EngBF. However,

only a slight amount of released Galβ1-3GalNAc was observed even in the presence of sialidase and α-*N*-acetylglucosaminidase [14]. This observation may be due to the modification of the core 1-type *O*-glycan (Galβ1-3GalNAcα1-) by several other sugars such as α-Fuc, β-Gal and β-GlcNAc. We then used bifidobacterial cells to prepare the disaccharide because bifidobacteria possess many types of glycosidases. Among the various bifidobacteria, we found that *B. bifidum* ATCC29521 could effectively release the disaccharide from porcine gastric mucin as well as a few monosaccharides such as Gal and sialic acid (Fig. 3). We purified the released disaccharide and confirmed that this disaccharide was Galβ1-3GalNAc by treatment with β1,3-specific galactosidase from *X. manihotis* and MALDI-TOF-MS (*m/z* 407.45 for $(M+Na)^+$). Finally, approximately 120 mg of the pure Galβ1-3GalNAc was obtained from 5 g of porcine gastric mucin.

Reverse-hydrolysis reaction of EngBF

To assess the reverse-hydrolysis reaction of EngBF, 100 mM Galβ1-3GalNAc and 1 M glucose were incubated with active or heat-inactivated EngBF. After incubation for 3 h at 37 °C, the reaction mixtures were analyzed by normal phase HPLC. In the presence of the active enzyme, a broad new peak was found at a RT of 28 min (Fig. 4A, top chart), which was not observed in the chromatogram of the reaction mixture containing heat-inactivated enzyme (data not shown). The reaction was subsequently lengthened to 120 h. The peak of the new product reached a maximum after 72 h incubation, and then slightly decreased until 120 h (Fig. 4A). We collected the fractions corresponding to the new peak from the 72-h reaction mixture and analyzed this species by TLC (Fig. 4B, lane 4). The analysis showed that this compound had the same mobility as the authentic Galβ1-3GalNAcα1-Glc prepared by the transglycosylation reaction using Galβ1-3GalNAcα1-*p*NP and Glc (lane 3) [8]. The product was completely hydrolyzed to Galβ1-3GalNAc and Glc after incubating with EngBF (lane 5). MALDI-TOF-MS analysis of this new product detected mass ion peaks at *m/z* 569.18 for $(M+Na)^+$ and *m/z* 585.13 for $(M+K)^+$, confirming that the product was the

trisaccharide Galβ1-3GalNAcα1-Glc (theoretical mass, 545.49) (Fig. 4B). The yield of the trisaccharide at 72 h incubation based on the amount of the substrate Galβ1-3GalNAc was estimated to be 10.7 % by the peak areas of HPLC chromatogram. Furthermore, when 100 mM Galβ1-3GalNAc and 0.5 M lactose were incubated with EngBF, the peak representing the reverse-hydrolysis product was found at a RT of 47 min using normal phase HPLC. MALDI-TOF-MS analysis gave a mass ion peak at *m/z* 730.99 $(M+Na)^+$ and the product was assumed to be Galβ1-3GalNAcα1-lactose (theoretical mass, 707.63). The yield at 72 h incubation was approximately 2 %.

Discussion

In mammalian cells, the biosyntheses of mucin-type oligosaccharides are initiated by attachment of α-GalNAc to Ser/Thr residues of polypeptides by polypeptide:*N*-acetylgalactosaminyltransferases located on the luminal side of the Golgi apparatus. Following this initial attachment, other glycosyltransferases are used for elongation of the sugar chains. In lower eukaryotes and prokaryotes, this kind of protein modification is missing. Recently, genetically engineered yeast *Saccharomyces cerevisiae* capable of producing core 1 *O*-glycan-containing peptides was developed [15]. In this strain, human polypeptide:*N*-acetylgalactosaminyltransferase-1, *Drosophila melanogaster* core 1 β1,3-galactosyltransferase and several other genes were introduced. However, this approach may be complicated because there are twenty polypeptide:*N*-acetylgalactosaminyltransferases with different acceptor specificities to be selected [16]. Chemical synthesis of these glycopeptides *in vitro* is not easy, because this synthesis needs a multistep process. In this paper, we employed the transglycosylation activity of EngBF from *B. longum* to achieve a one-step synthesis of the core 1 disaccharide-containing glycopeptides. Transglycosylation of Galβ1-3GalNAc to a Ser-containing hexapeptide has already been reported using a similar enzyme, endo-α-*N*-acetylgalactosaminidase S

partially purified from the culture supernatant of *Streptomyces* sp. OH-11242 [17], though transglycosylation to Thr residues was not reported. In this study, EngBF was shown to have the ability to transfer Galβ1-3GalNAc to both Ser and Thr residues using the bioactive peptide PAMP-12 and its derivative. This enzyme seems to prefer Ser residue rather than Thr residue as an acceptor, though further experiments are necessary to conclude. We also showed that other three bioactive peptides could be acceptors for this enzyme. Therefore, a simple method to synthesize core 1 *O*-glycan-containing peptides using EngBF with broad acceptor specificity has been presented. The addition of sugar chain(s) to bioactive peptides may furnish some positive effects, such as increasing resistance to proteases, increasing stability and extending the half-life in the bloodstream. In the case of the mucin repetitive peptide MUC1a, sugar-conjugate could be a promising anti-tumor vaccine for blocking metastasis [18]. Enzymatic transglycosylation require donor substrates such as synthetic substrates which are usually expensive. Recently, an efficient chemical synthesis of α-GalNAc glycosides using the di-tert-butylsylene-directed method was developed [19-21] and this method is expected to lower the cost of production of the donor substrate. Although this enzymatic approach present in this study provides a simple and powerful tool for syntheses of *O*-glycopeptides, it is currently not easy to control the number and position of the disaccharide modification on the peptides with multiple Ser/Thr residues. Incomplete glycosylation may be due to the low efficiency of the transglycosylation by EngBF. The improvement of the transglycosylation efficiency of EngBF by mutagenesis and/or the use of higher amount of the donor substrate may overcome the weakness of this approach.

We also achieved the syntheses of oligosaccharides by the reverse-hydrolysis reaction of EngBF, which does not require donor substrates such as Galβ1-3GalNAcα1-*p*NP. Since the synthesized oligosaccharides are degraded by endo-α-*N*-acetylgalactosaminidases that are widely distributed in the *Bifidobacterium* species, these oligosaccharides could be assimilated by those bacteria and may function as a bifidus factor. We have previously described that bifidobacteria are

able to incorporate the Galβ1-3GalNAc into the cells through an ABC-type transporter specific for Galβ1-3GalNAc and Galβ1-3GlcNAc [22, 23]. The disaccharide is phosphorolyzed by intracellular Galβ1-3GalNAc/Galβ1-3GlcNAc phosphorylase to further degradation [24-26]. Since the β1,3-linked Gal is hardly hydrolyzed by enterobacterial β-galactosidases, Galβ1-3GalNAc may be specifically utilized by bifidobacteria.

The analysis of the crystal of EngBF has been completed [27]. Using the structural information will facilitate our focus on improving the efficiency of the transglycosylation and reverse-hydrolysis reactions.

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Figure legends

Fig. 1 Transglycosylation of Galβ1-3GalNAc to free threonine and serine by EngBF. The donor substrate Galβ1-3GalNAcα1-*p*NP was incubated with EngBF in the presence or absence of threonine or serine and analyzed by normal phase HPLC. Upper panel, no amino acid was added; middle panel, threonine was added as an acceptor; lower panel, serine was added as an acceptor. New peaks appeared at 32.25 min in the middle panel and at 37.09 min in the bottom panel. S, Galβ1-3GalNAcα1-*p*NP; NP, *p*-nitrophenol; DS, Galβ1-3GalNAc.

Fig. 2 Transglycosylation of Galβ1-3GalNAc to PAMP-12 by EngBF. (A) Reversed phase HPLC chromatograms of the transglycosylation reaction mixtures containing Galβ1-3GalNAcα1-*p*NP and PAMP-12 with heat-inactivated (upper chart) or active (bottom chart) EngBF. S, Galβ1-3GalNAcα1-*p*NP; NP, *p*-nitrophenol; TG, possible transglycosylation product. (B) MALDI-TOF-MS analysis of the PAMP-12 transglycosylation product. The theoretical mass of Galβ1-3GalNAc-attached PAMP-12 is 1984.24. (C) MALDI-TOF-MS analysis of the PAMP-12(S11T) transglycosylation product. The theoretical mass of Galβ1-3GalNAc-attached PAMP-12(S11T) is 1998.26.

Fig. 3 Production of Galβ1-3GalNAc from porcine gastric mucin using the cells of *Bifidobacterium bifidum* ATCC29521. The cells grown in the GAM medium were washed with phosphate buffered saline and incubated with porcine gastric mucin. The supernatant of the reaction mixture was precipitated by ethanol to remove proteins and then analyzed by TLC. Lane 1, bifidobacterial cells only; lane 2, mucin incubated with bifidobacterial cells; lane 3, mucin only; lane 4, standard Gal; lane 5, standard Galβ1-3GalNAc.

Fig. 4 Reverse-hydrolysis reaction of EngBF. (A) Normal phase HPLC chromatogram of the reverse-hydrolysis reaction product. Galβ1-3GalNAc and glucose were incubated with EngBF for indicated periods and then analyzed by HPLC. A new product (P) appeared with a RT of 28 min. (B) TLC analysis of the reverse-hydrolysis reaction product. Lane 1, standard Galβ1-3GalNAc; lane 2, standard Glc; lane 3, Galβ1-3GalNAcα1-Glc produced by transglycosylation using EngBF; lane 4, purified reverse-hydrolysis reaction product of EngBF; lane 5, purified reverse-hydrolysis reaction product incubated with EngBF. (C) MALDI-TOF-MS analysis of the reverse-hydrolysis reaction product. The theoretical mass of Galβ1-3GalNAcα1-Glc is 545.49.

Table 1 Transglycosylation of Galβ1-3GalNAc to various peptides by EngBF

Peptide	Sequence [a]	Acceptor peptide		Transglycosylated peptide			
		RT [b]	Mass	RT [b]	Mass [c]	Observed mass [d]	Yield [e]
PAMP-12	FRKKWNKWALSR-NH_2	54.8	1618.91	52.2	1984.24	1984.68 $(M+H)^+$	4.4
PAMP-12(S11T)	FRKKWNKWALTR-NH_2	55.1	1632.93	52.5	1998.26	1999.08 $(M+H)^+$	1.5
Bradykinin	RPPGFSPFR	58.0	1060.20	52.8	1425.53	1425.79 $(M+H)^+$	0.47
MUC1a	AHGVTSAPDTR	31.1	1111.17	28.5	1476.50	1476.57 $(M+H)^+$	2.4
Peptide T	ASTTTNYT	31.0	857.87	28.5-29.8 [f]	1223.20	1245.54 $(M+Na)^+$, 1261.62 $(M+K)^+$	9.7

a, Possible residues (Ser and Thr) for acceptor are underlined.

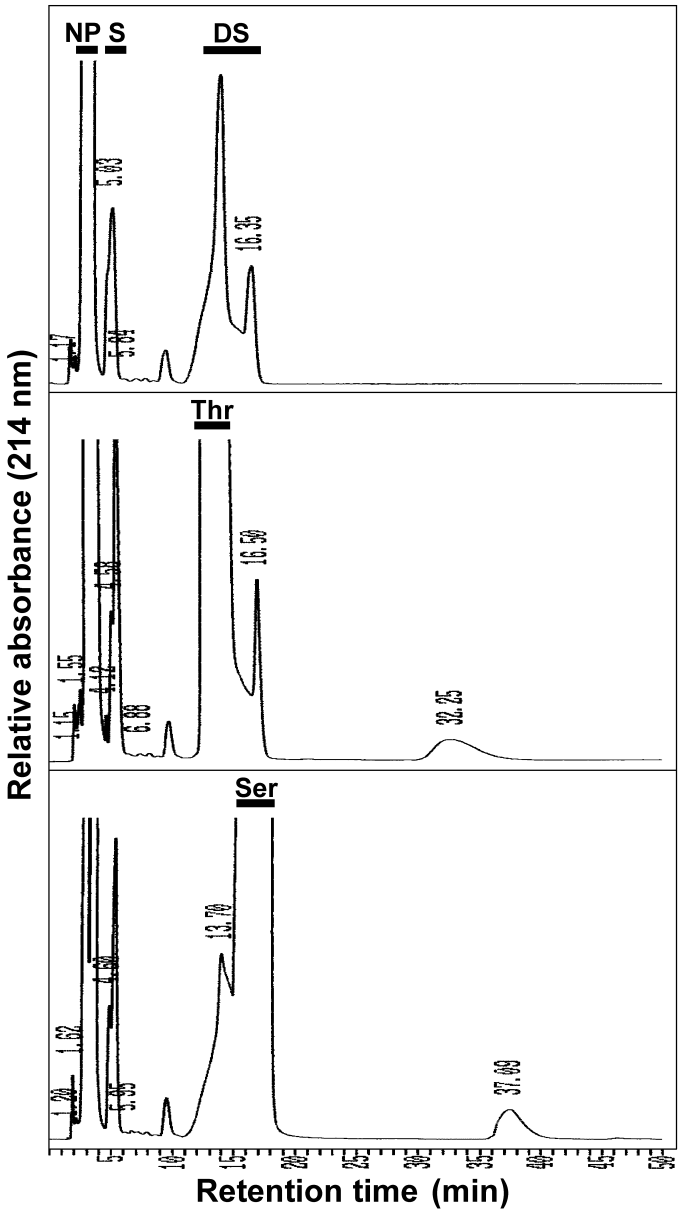
b, Retention times (min) of reversed phase HPLC under the condition described in Materials and method are shown.

c, Theoretical masses of glycopeptides attached with single disaccharide are shown.

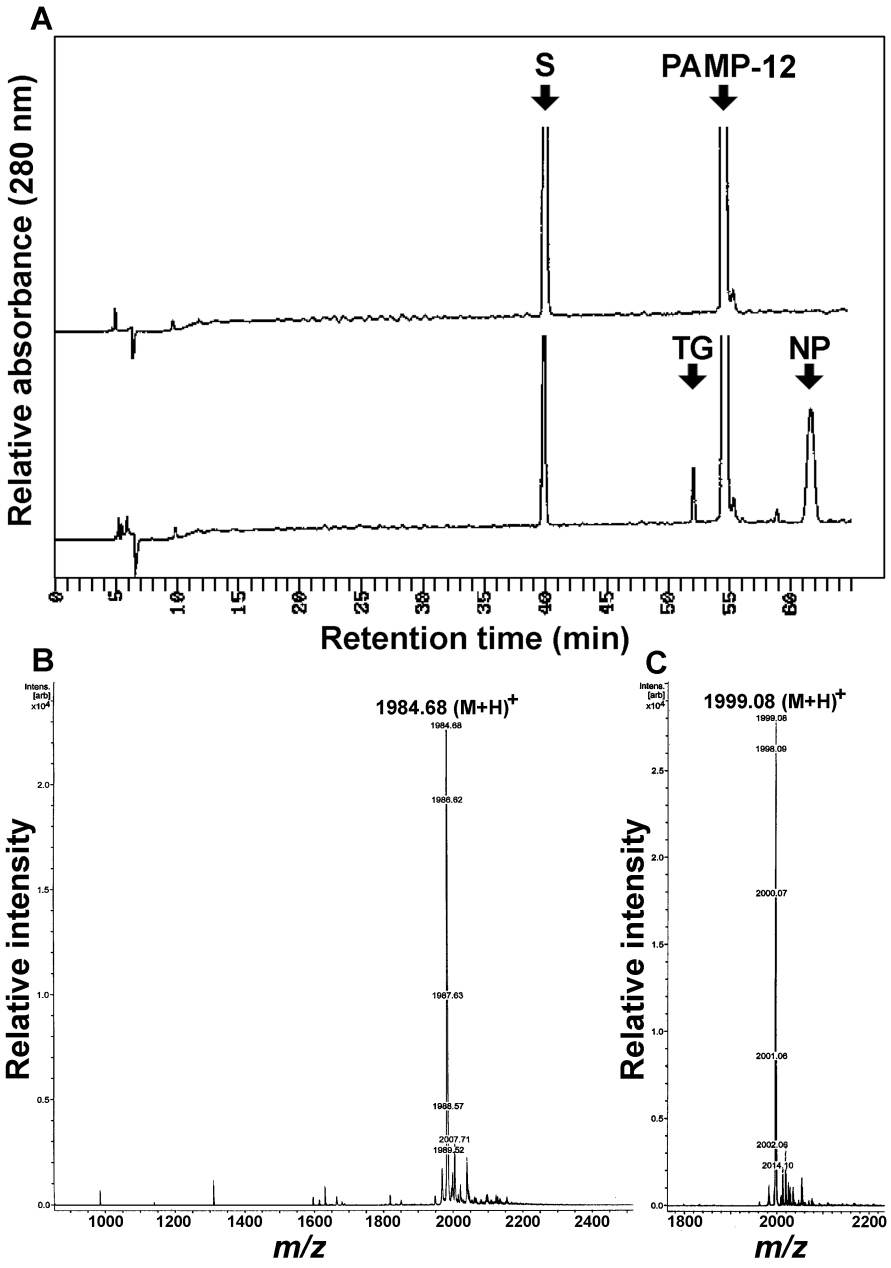
d, Observed masses in MALDI-TOF-MS analyses are shown.

e, Yields (%) were calculated from the following formula: yield = peak area of transglycosylation product × 100 / peak areas of acceptor and transglycosylation product.

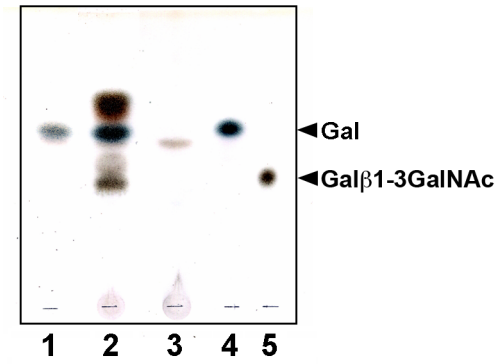
f, A single major peak and two minor peaks were observed.



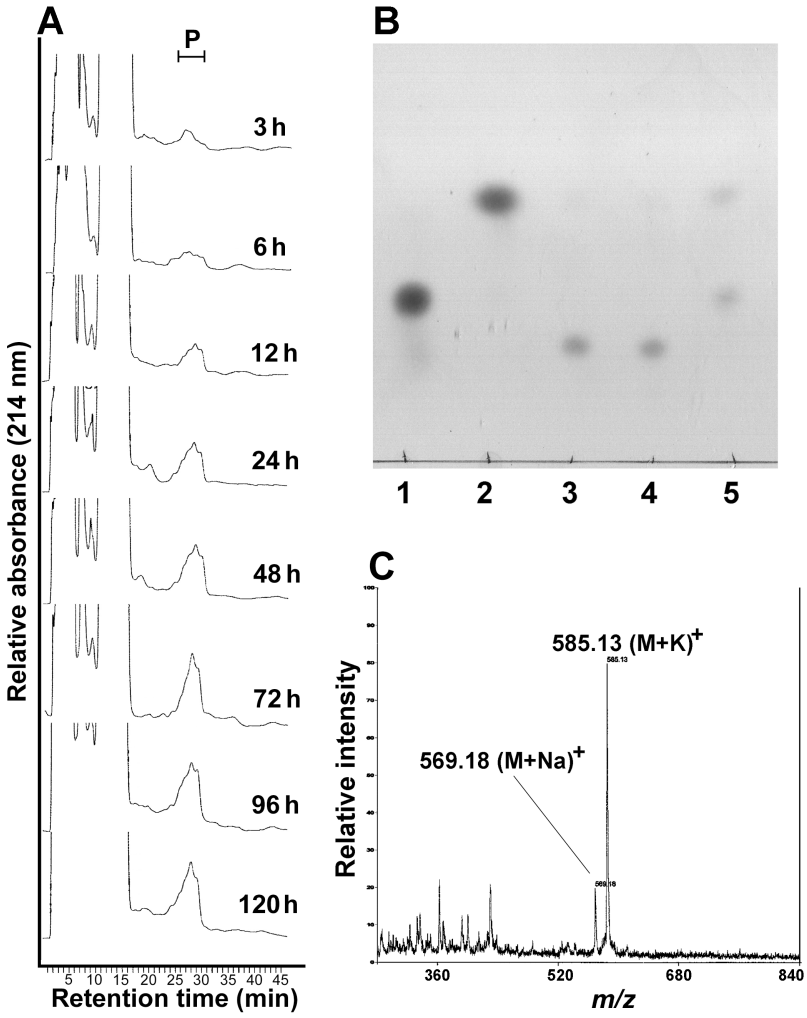
Ashida et al. Fig. 1



Ashida et al. Fig. 2



Ashida et al. Fig. 3



Ashida et al. Fig. 4