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Characterization of a novel galactose β1,3-*N*-acetylglucosaminyltransferase (β3Gn-T8): the complex formation of β3Gn-T2 and β3Gn-T8 enhances enzymatic activity

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We characterized a novel member of the β1,3-*N*-acetylglucosaminyltransferase (β3Gn-T) gene family, β3Gn-T8. A recombinant soluble form of β3Gn-T8 was expressed in *Pichia pastoris* (*P. pastoris*), and its substrate specificity was compared with that of β3Gn-T2. The two enzymes had similar substrate specificities and recognized tetraantennary *N*-glycans and 2,6-branched triantennary glycans in preference to 2,4-branched triantennary glycans, biantennary glycans, and lacto-*N*-neotetraose (LNnT), indicating their specificity for 2,6-branched structures such as [Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manα1→6Man]. Interestingly, when soluble recombinant β3Gn-T2 and β3Gn-T8 were mixed, the Vmax/Km value of the mixture was 9.3- and 160-fold higher than those of individual β3Gn-T2 and -T8, respectively. Sephacryl S-300 gel filtration of the enzymes revealed that apparent molecular weights of each β3Gn-T2, β3Gn-T8, and the mixture were 90–160, 45–65, and 110–210 kDa, respectively, suggesting that β3Gn-T2 and -T8 can form a complex with enhanced enzymatic activity. This is the first report demonstrating that *in vitro* mixed glycosyltransferases show enhanced enzymatic activity through the formation of a heterocomplex. These results suggested that β3Gn-T8 and β3Gn-T2 are cooperatively involved in the elongation of specific branch structures of multiantennary *N*-glycans.

Key words: *N*-acetylglucosaminyltransferase/enzyme complex/galactose/*Pichia pastoris*/tetraantennary *N*-glycan

Introduction

β1,3-Linked GlcNAc residues are present in the backbone of various biologically important glycans. For example, *GlcNAcβ1→3*GalNAcα1→ is the core 3 structure found in many *O*-linked glycans expressed in the digestive organs (Podolsky, 1985; Vavasseur *et al*., 1995). Lacto- and neolacto-series glycolipids contain a *GlcNAcβ1→3*Galβ1→4Glcβ1→Cer sequence, which is the backbone for various tumor-specific glycolipid antigens (Hakomori, 1989). Keratan sulfate, a poly-*N*-acetyllactosamine structure (Galβ1→4*GlcNAcβ1→3*)$_n$ with high 6-*O*-sulfation, maintains appropriate hydration levels in the cornea and is believed to have a functional role in processes such as cell motility and embryo implantation (Funderburgh, 2000). Lymphocyte homing is initially mediated by L-selectin and its ligand glycans, one of which is a sulfated *N*-acetyllactosamine on a core 1 glycan (Yeh *et al*., 2001). Expression of oligo- or poly-*N*-acetyllactosamine moieties in multiantennary *N*-glycans has been correlated with baby hamster kidney cell transformation (Yamashita *et al*., 1984), hepatic carcinogenesis (Yamashita *et al*., 1989), and granulocyte differentiation (Lee *et al*., 1990). The variety of β1,3-linked GlcNAc residues in different types of glycoconjugates and cells at varying stages of differentiation suggests the presence of various β1,3-*N*-acetylglucosaminyltransferases (β3Gn-T) with different substrate specificities and expression profiles in human tissues.

In fact, eight *β3Gn-T* genes (*iGn-T*, *β3Gn-T2*, *-T3*, *-T4*, *-T5*, *-T6*, *-T7*, and *-T8*) have been identified thus far, and their activities have been characterized. Several of the enzymes, iGn-T, β3Gn-T2, -T3, -T4, and -T8, mediate poly-*N*-acetyllactosamine synthesis (Sasaki *et al*., 1997; Shiraishi *et al*., 2001; Ishida *et al*., 2005), and β3Gn-T3 is also able to synthesize *O*-linked core 1 structures (Yeh *et al*., 2001). β3Gn-T5, -T6, and -T7 are involved in the synthesis of lactotriose (Togayachi *et al*., 2001), *O*-linked core 3 glycans (Iwai *et al*., 2002), and the keratan sulfate backbone (Kataoka and Huh, 2002; Seko and Yamashita, 2004), respectively. Their substrate specificities can explain which enzymes are responsible for the addition of β1,3-linked GlcNAc to particular glycoconjugates, but which enzymes contribute to β1,3-GlcNAc elongation of oligo- or poly-*N*-acetyllactosamine in multiantennary *N*-glycans is not yet fully understood.

In this study, we extensively characterized β3Gn-T8. Although the same gene has been recently designated β1,3-galactosyltransferase-7 (β3GALT7) by Huang *et al*. (2004) without enzymatic characterization, we could not detect any β3GalT activity in it, and we also redesignated the gene as *β3Gn-T8*, like as shown by Ishida *et al*. (2005). The enzyme acts efficiently on tetraantennary and 2,6-branched triantennary *N*-glycans, with a specificity similar to that of β3Gn-T2. Additionally, the *in vitro* mixing of β3Gn-T8 and β3Gn-T2 forms a heterocomplex whose enzymatic activity is greatly enhanced, compared with the individual enzymes.

Results

Characterization of the enzymatic activity of β3Gn-T8

To analyze the β3Gn-T activity, we expressed the catalytic domain of the β3Gn-T8 protein with $(His)_6$ tag sequence by

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Pichia pastoris KM71 cells. Six transformant colonies were preliminary cultured in a small scale, and the media were assayed for β3Gn-T activity to select expression-positive clones. Three clones secreted similar levels of β3Gn-T activity, whereas the other three clones showed no β3Gn-T activity. In a large-scale preparation as materials and methods, the purified enzyme (T8 fraction) was used as an enzyme source. As for the time dependency, the linearity of the activity was maintained at least for 3 h (data not shown). We similarly prepared a soluble form of β3Gn-T2, too (T2 fraction). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of T8 and T2 fractions was shown in Figure 1. Both exhibited rather broad bands, and apparent molecular weights of β3Gn-T8 and -T2 were 50–70 and 80–200 kDa, respectively. After peptide:*N*-glycanase (PNGase) F digestion, these broad bands were concentrated to 44 kDa (β3Gn-T8) and 45 kDa (β3Gn-T2), indicating that large *N*-glycans were present in yeast-derived β3Gn-T8 and -T2. The putative molecular weights of the truncated β3Gn-T2 and -T8 were 45,338 and 42,360, respectively, and the possible N-glycosylation sites of the truncated β3Gn-T2 and -T8 were five and two, respectively. Large and broad molecular weight of β3Gn-T2 should be because of heterogeneity of large *N*-glycans. The GlcNAc-transferase activity of T8 and T2 fractions was 2.0 and 43 nmol/min/mg protein, respectively, using tetraGP as an acceptor.

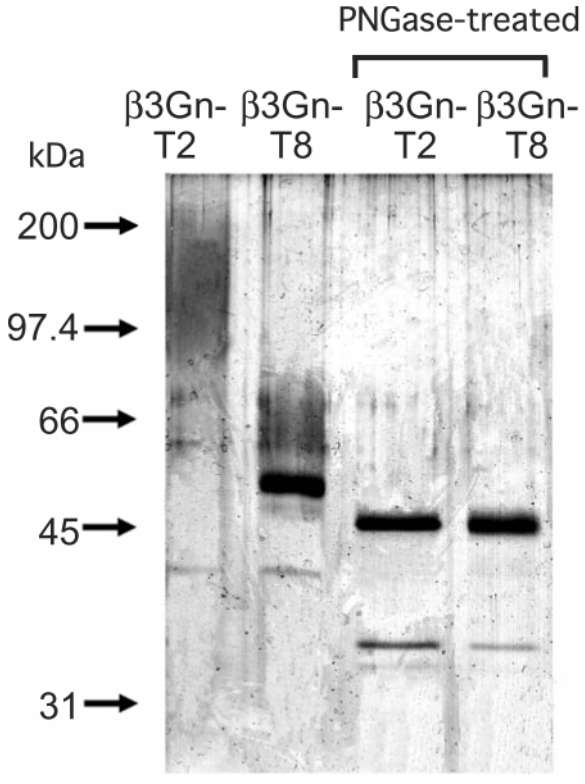

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of recombinant β3Gn-T2 (T2 fraction) and β3Gn-T8 (T8 fraction) produced by *Pichia pastoris* KM71 cells. The enzyme fractions (300 ng for intact proteins and 100 ng for peptide: *N*-glycanase [PNGase] F-digests) were subjected to SDS–PAGE (12% gel) under reducing conditions and visualized with SYPRO Orange.

The linkage position of GlcNAc was determined using tetraGP as an acceptor. The enzymatic reaction product, [^{3}H]GlcNAc→tetraGP, was purified by paper electrophoresis and paper chromatography. [^{3}H]GlcNAc was β-linked, because the [^{3}H]GlcNAc→tetraGP bound to a *Psathyrella velutina* lectin-Sepharose (PVL-Sepharose) column which binds to nonsubstituted β-GlcNAc residues (Kochibe and Matta, 1989; Endo *et al*., 1992). Next, the reaction product was β1,4-galactosylated by bovine milk β1,4-galactosyltransferase to cap the [^{3}H]GlcNAc residue. When the resulting Galβ1→4[^{3}H]GlcNAcβ1→tetraGP was digested with *Escherichia freundii* endo-β-galactosidase and applied to a Bio-Gel P-4 gel filtration, radioactivity eluted at 4.0 Glc units (two hexose and one HexNAc, data not shown) (Kobata *et al*., 1987). This indicates that the radioactive product was Galβ1→4[^{3}H]GlcNAcβ1→Gal, and that Galβ1→4[^{3}H]GlcNAcβ1→ could be linked to either of the four branching *N*-acetyllactosamine moieties of the tetraGP.

The [^{3}H]GlcNAc linkage should be at the C-3 position of Gal, based on the substrate specificity of the endo-β-galactosidase (Fukuda *et al*., 1984). To further confirm the linkage position, we subjected Galβ1→4[^{3}H]GlcNAcβ1→tetraGP to periodate oxidation and the Smith degradation. If the [^{3}H]GlcNAc was attached to the C-2, -4, or -6 position of the Gal residue, CHO-CH(*O*-β[^{3}H]GlcNAc)-CH_2OH, CH_2OH-CH(*O*-β[^{3}H]GlcNAc)-CHOH-CH_2OH, or CH2 (*O*-β[^{3}H]GlcNAc)-CHOH-CH_2OH, respectively, would be produced by the reactions. In contrast, if the [^{3}H]GlcNAc was attached to the C-3 position of Gal, the theoretical products would be 1-*O*-([^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→)-glycerol, when [^{3}H]GlcNAc is attached to the 6-branch of α1,6-linked Man, or 2-*O*-([^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→)-glyceraldehyde, when [^{3}H]GlcNAc is attached to the 2-branch of α1,6-linked Man, or [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3Manβ1→4XylNAcol, when the linkage is to the 4-branch of α1,3-linked Man, or [^{3}H]GlcNAcβ1→3Galβ1→ 4GlcNAcβ1→2(GlcNAcβ1→4)Manα1→3Manβ1→4XylNAcol, when the linkage is to the 2-branch of α1,3-linked Man.

The oxidized products were applied to a Bio-Gel P-4 column chromatography (Figure 2). The major ^{3}H-labeled compound (peak II, 61% of the total radioactivity) was eluted at the 6.3 Glc unit position, which corresponds to that of authentic [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→glycerol. This result indicates that the Smith degradation product is [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→glycerol or glyceraldehyde, and that [^{3}H]GlcNAc introduced by β3Gn-T8 is attached to the C-3 position of Gal. Accordingly, these data confirm that the enzyme is a β1,3-GlcNAc-transferase. Peak I (10.8 Glc unit, 8% of the total radioactivity) seemed to be a heptaose, corresponding to authentic [^{3}H]$GlcNAc_1Gal_1GlcNAc_2Man_2$XylNAcol, which should be produced when [^{3}H]GlcNAc is attached to either the 2- or the 4-branch of α1,3-linked Man. The result of the Smith degradation implies that β3Gn-T8 preferentially acts on the 2,6-branch of α1,6-linked Man of tetraGP.

To examine whether β3Gn-T8 has β1,3-galactosyltransferase (β3GalT) activity, we used UDP-[^{3}H]Gal as a donor

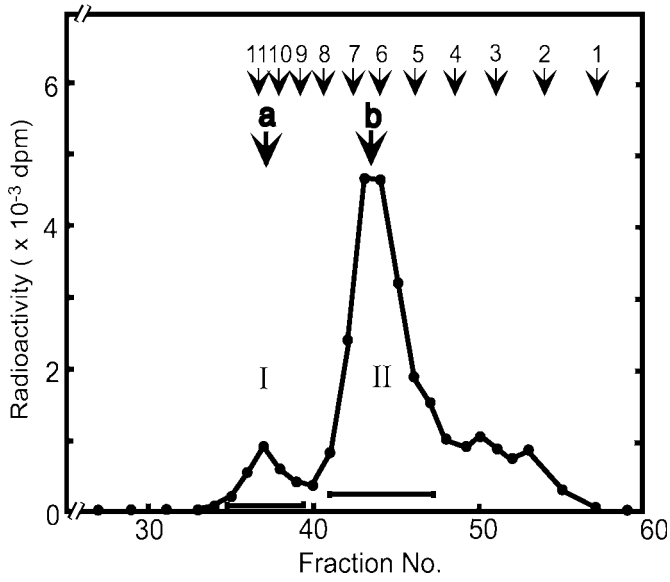

Fig. 2. Bio-Gel P-4 gel filtration of the periodate oxidation product of Galβ1→4GlcNAcβ1→tetraGP. The Bio-Gel P-4 (<45 μm) column (1.5 × 50 cm) was equilibrated and eluted with distilled water at 55°C, and 1.5 mL fractions were collected. Elution positions of standard Glc units were indicated by arrows. Authentic compounds, [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→2 or 4(GlcNAcβ1→4 or 2)Manα1→3Manβ1→4XylNAcol (a) and [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→glycerol (b), were produced by the periodate oxidation, Smith degradation, and $NaBH_4$ reduction from Galβ1→4[^{3}H]GlcNAcβ1→3(2,4-branched triGP) and Galβ1→4[^{3}H]GlcNAcβ1→3biGP, respectively, which were synthesized by β3Gn-T2 and bovine milk β1,4-GalT.

substrate and GlcNAcβ1→3Galβ1→4Glc, agalacto biGP, or GalNAcα-*O*-pNP as acceptor substrates. The reaction mixture was applied to paper electrophoresis and paper chromatography like as βGn-T assay. No GalT activity was detected for the three acceptor substrates (data not shown), indicating that β3Gn-T8 does not have β3-GalT activity.

The optimal pH of the β3Gn-T8 activity was 7.0–7.5. The addition of ethylenediaminetetraacetic acid (EDTA) (2 mM) instead of 10 mM $MnCl_2$ completely inhibited the enzymatic activity, and the addition of 10 mM $CaCl_2$ or $MgCl_2$ showed no activity, suggesting a requirement of Mn^{2+} for the enzymatic activity.

The substrate specificity of β3Gn-T8 is summarized in Table I. β3Gn-T8 can efficiently act on *N*-linked glycans (tetraGP and 2,6-branched triGP), and its relative activity is positively correlated with increasing numbers of branch chains. An *O*-linked-type glycan (Gal-core2-*O*-pNP) and a glycolipid-type glycan (lacto-*N*-neotetraose [LNnT]) were also acceptors, but lacto-*N*-tetraose (LNT) which contains the type 1 chain was a poor substrate. Lacto-*N*-fucopentaose-III (LNF-III), core 1-pNP, GalNAcα-*O*-pNP, lactosylceramide, galactosylceramide, and L2L2 were also poor substrates (data not shown). These results suggested that β3Gn-T8 is involved in the elongation of *N*-acetyllactosamine sequences in various type of glycans, especially in multibranched *N*-glycans. The transferred position of [^{3}H]GlcNAc to Gal-core2-*O*-pNP by β3Gn-T8 was determined by peanut agglutinin-agarose (PNA-agarose) affinity chromatography. PNA binds to the nonreducing terminal of the Galβ1→3(R→6)GalNAc structure, but not to the R→3Galβ1→3GalNAc (Lotan *et al.*, 1975). The [^{3}H]GlcNAcβ1→3(Gal-core2-*O*-pNP) completely bound to the PNA-agarose column and was eluted with 0.3 M lactose (data not shown), indicating that the ^{3}H-labeled compound was [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcα1-*O*-pNP.

Next, to determine to which branches of biGP [^{3}H]GlcNAc was transferred, we digested [^{3}H]GlcNAcβ1→3biGP with *Streptococcus* 6646K β-galactosidase and then was applied to an E_4-phytohemagglutinin-agarose (E_4-PHA-agarose) column. Galβ1→4GlcNAcβ1→2Man*α1*→*3*Manβ1→4GlcNAcβ1→ is slightly retarded on the lectin column at 2°C, whereas Galβ1→4GlcNAcβ1→2Man*α1*→*6*Manβ1→4GlcNAcβ1→ is more retarded (Kobata and Yamashita, 1989). Because the C-3 substitution at the Gal residue does not affect the elution profile, [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→2Man*α1*→*6*(GlcNAcβ1→2Manα1→3) Manβ1→4GlcNAc should be more retarded on the column than its counterpart having a Manα1→3. As shown in Figure 3, [^{3}H]GlcNAcβ1→3biGP was separated into fractions I and II by E_4-PHA-agarose chromatography, and fractions I and II should be oligosaccharides containing [^{3}H]GlcNAc on the α1→3 branch and on the α1→6 branch, respectively. The ratio of the radioactivities of fractions I and II was 59:41, indicating that β3Gn-T8 slightly preferred the α1→3-branching LacNAc to the α1→6-branching LacNAc as a substrate.

We were interested whether β3Gn-T2, which has a strong LacNAc-elongation activity (Shiraishi *et al.*, 2001), can act on multivalent *N*-linked glycans, similar to the activity of β3Gn-T8. We examined the substrate specificity of β3Gn-T2, which was also prepared by KM71 cells as a truncated soluble protein with $(His)_6$ tag sequence. As summarized in Table I, β3Gn-T2 has a similar substrate specificity to that of β3Gn-T8,

Table I. Substrate specificities of β3Gn-T8, β3Gn -T2, and the mixture of the two enzymes

	TetraGP[a]	2,6-Branched triGP	2,4-Branched triGP	BiGP	MonoGP	LNnT	LNT	Gal-core2-pNP
β3Gn-T8	*100*[b]	102[c]	69	39	23	17	3	34
β3Gn-T2	*100*	120	48	23	11	8	2	14
β3Gn-T8 + β3Gn-T2	*100*	106	62	37	18	11	2	35

LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose.
[a]The concentrations of the substrates were 0.5 mM.
[b]The enzymatic activities were the means of four independent experiments. The standard deviations were less than 5% (data not shown). Specific activities of β3Gn-T8 (T8 fraction), β3Gn-T2 (T2 fraction), and the mixture the two enzymes for tetraGP were 2.0, 43, and 270 nmol/min/mg protein, respectively.
[c]The values are normalized to tetraGP as 100.

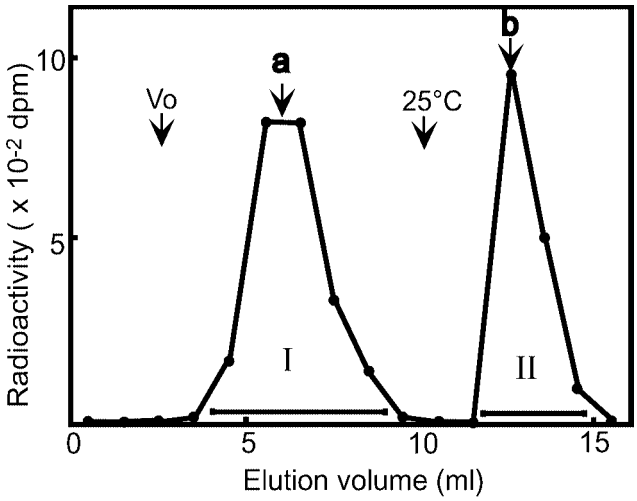

Fig. 3. E_4-phytohemagglutinin (E_4-PHA) affinity chromatography of β-galactosidase-treated [^{3}H]GlcNAcβ1→3biGP produced by β3Gn-T8. [^{3}H]GlcNAcβ1→3biGP was digested with *Streptococcus* 6646K β-galactosidase (3 mU, in 0.1 M sodium acetate buffer (pH 5.3), 10 mM $MnCl_2$ at 37°C for 16 h). The digests were heated at 100°C for 3 min and were applied on an E_4-phytohemagglutinin-agarose (E_4-PHA-agarose) column (0.7 × 5.2 cm) at 2°C, equilibrated and eluted with 10 mM Tris–HCl (pH 8.0), 0.15 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. The column was moved to 25°C at the position indicated by an arrow. Vo, void volume. Arrows a and b are the elution positions of authentic Galβ1→4GlcNAcβ1→2Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc-$_{OT}$ and Galβ1→4GlcNAcβ1→2Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc-$_{OT}$, respectively (Kobata and Yamashita, 1989).

although β3Gn-T2 had relatively low activities toward 2,4-branched triGP, biGP, monoGP, Gal-core2-pNP, and LNnT.

Both β3Gn-T8 and β3Gn-T2 had higher activities for 2,6-branched triGP than for 2,4-branched triGP (Table I). To further confirm this, we measured kinetic values of these enzymes for tetraGP, triGPs, and biGP (Table II). The Vmax/Km values of β3Gn-T8 for tetraGP and 2,6-branched triGP were higher than those for 2,4-branched triGP and biGP. Similar results were obtained for β3Gn-T2. These results indicate that a Galβ1→4GlcNAcβ1→ 6Manα1→6Man branch is important for good recognition by the both enzymes.

Mixing β3Gn-T8 with β3Gn-T2 increases enzymatic activity

We found that enzymatic activity in the mixture of β3Gn-T2 and -T8 was several times higher than the sum of the activities of each enzyme (Figure 4). By the addition of T8 fraction to the reaction mixture of T2 fraction, β3Gn-T activity was enhanced in comparison with the sum of the individual activities of the two fractions. At 2.2 ng protein of T8 fraction (the relative amount of T8 fraction/T2 fraction = 1.1:1), the activity was six times higher than the sum of the two. Further addition of T8 fraction did not enhance β3Gn-T activity, suggesting that β3Gn-T8 may interact with β3Gn-T2 and that β3Gn-T8 was saturated at the ratio of T8 fraction/T2 fraction (1.1:1). We thereafter studied character of the enhanced β3Gn-T activity in the mixture of the two fractions by this ratio. The kinetic values of β3Gn-T2, -T8, and the mixture of the two enzymes for tetraGP are shown in Table II. The Vmax value of the mixture (470 nmol/min/mg protein) was approximately four times higher than the sum of the each Vmax value (124 nmol/min/mg protein), and the Vmax/Km value of the mixture (1400 nmol/min/mg protein/mM) was approximately nine times higher than that for β3Gn-T2 (150 nmol/min/mg protein/mM). The substrate specificity of the mixture is summarized in Table I. The specificity of the mixture is similar to that of β3Gn-T2 and -T8, but is closer in substrate specificity to β3Gn-T8 than to β3Gn-T2.

This activity-enhancing effect was also observed using membrane-bound β3Gn-T2 and -T8. We prepared crude membrane fractions containing β3Gn-T2 or -T8 from these expression vector-transfected COS-7 cells and used the fractions as enzyme sources. The Km values for β3Gn-T2, -T8, and the mixture were almost the same as those for the soluble enzymes (data not shown). The Vmax/Km value for the mixture (480 pmol/min/mg protein/mM) was 4.8- and 15-times higher than those for β3Gn-T2 (100 pmol/min/mg protein/mM) and β3Gn-T8 (32 pmol/min/mg protein/mM), respectively. From these results, it was speculated that β3Gn-T8 binds to β3Gn-T2 and that the hetero-oligomer has much higher enzymatic activity than either of the individual enzymes.

Gel filtration of soluble β3Gn-T2, β3Gn-T8, and the mixture of β3Gn-T2 and -T8

To determine whether β3Gn-T2 interacts with β3Gn-T8, we applied each enzyme alone and the mixture to Sephacryl S-300 gel filtration columns and measured the enzymatic activities of the individual fractions. As shown in Figure 5, the apparent molecular weights of β3Gn-T2 (peak II) and -T8 (peak III) were estimated to be 90–160 and 45–65 kDa, respectively. In the case of the mixture (peak I), the apparent molecular weight shifted to 110–210 kDa. From the results of SDS–PAGE analysis in Figure 1, the soluble β3Gn-T2 and -T8 were likely present as a monomer. Similarly, based on the apparent molecular weight, the mixture

Table II. Kinetic analysis of β3Gn-T8, β3Gn-T2, and mixture of the two enzymes for several *N*-linked oligosaccharides

	β3Gn-T8				β3Gn-T2				β3Gn-T8 + β3Gn-T2
	TetraGP	2,6-Branched triGP	2,4-Branched triGP	BiGP	TetraGP	2,6-Branched triGP	2,4-Branched triGP	BiGP	TetraGP
Km (mM)	0.42	0.19	1.9	1.8	0.80	0.42	5.1	3.3	0.33
Vmax (nmol/min/mg protein)	3.6	2.9	3.4	3.4	120	150	150	93	470
Vmax/Km	8.6	15	1.8	1.9	150	360	29	28	1400

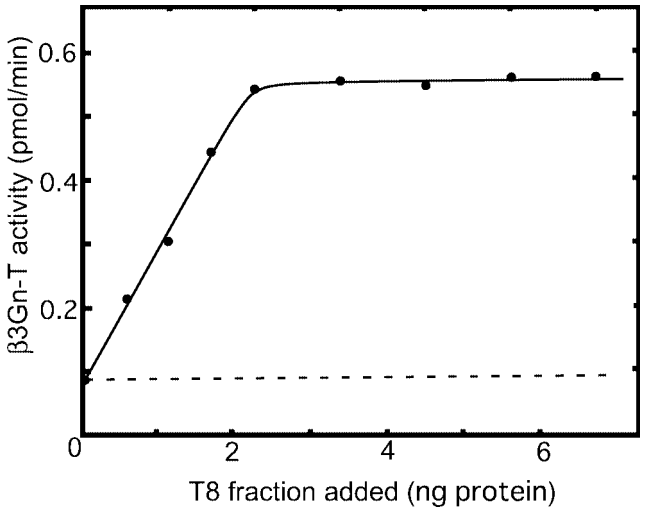

Fig. 4. The effect of the addition of T8 fraction on β3Gn-T2 (T2 fraction) activity, with tetraGP as the acceptor. Various amounts of T8 fraction were added to the reaction mixture containing 2 ng protein of T2 fraction, and β3Gn-T activity was assayed (•). Dotted line indicates the predicted sum of the individual activities of 2 ng protein of T2 fraction and indicated amounts of T8 fraction.

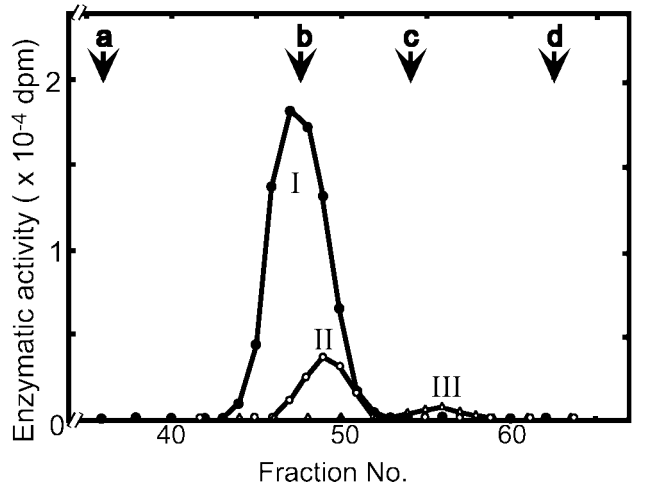

Fig. 5. Sephacryl S-300 HR gel filtration of the soluble forms of β3Gn-T2, -T8, and the mixture of the two enzymes. The column (1.4 × 72 cm) was equilibrated and eluted with 10 mM Tris–HCl (pH 8.0) and 0.15 M NaCl. The individual enzymes or the mixture of the two enzymes were applied to the column, and the enzymatic activities of β3Gn-T2 (○), β3Gn-T8 (Δ), and the mixture (•) were measured as in *Materials and methods*. Elution positions of standard proteins are indicated by arrows: a, porcine thyroglobulin (669 kDa); b, human IgG (150 kDa); c, bovine serum albumin (BSA) (67 kDa); and d, soy bean trypsin inhibitor (21.5 kDa). The fraction numbers of the void volume and total volume were 27 and 80, respectively.

of β3Gn-T2 and -T8 was likely to form a heterodimer consisting of one β3Gn-T2 unit and one β3Gn-T8 unit.

Discussion

We clearly demonstrated in this study that β3Gn-T8 has similar substrate specificity to that of β3Gn-T2. β3Gn-T8 belongs to a β1,3-GlcNAc-transferase family, based on its β3Gn-T activity. Huang *et al.* (2004) designated the same enzyme as β3GALT7 without the demonstration of the enzymatic activity. We showed that the enzyme does not have β3Gal-T activity and designated it as β3Gn-T8. Recently, Ishida *et al.* (2005) showed that β3Gn-T8 has a β1,3-GlcNAc-transferase activity toward tetraantennary *N*-glycans. In this study, β3Gn-T8 and -T2 act most efficiently on 2,6-branching *N*-acetyllactosamine moieties of tetraGP and 2,6-branched triGP. Soluble β3Gn-T8 and β3Gn-T2 formed a heterocomplex when the proteins were mixed in vitro, and the complex exhibits enhanced enzymatic activity in comparison with the individual enzymes.

We used *P. pastoris* cells for the preparation of soluble forms of β3Gn-T8 and -T2. This organism is advantageous to use because, unlike *Escherichia coli* (*E. coli*), it is able to add *N*-linked glycans, which are high mannose-type glycans, and, unlike *Saccharomyces* (*S. cerevisiae*), the glycans are relatively small. In fact, we were unable to prepare a soluble and enzymatically active form of β3Gn-T8 and -T2 in *E. coli*, although soluble, inactive proteins were obtained. Some *N*-linked glycans of β3Gn-T8 and -T2 may be essential for proper folding or maintenance of their functional structure. Grinna and Tschopp (1989) reported that *P. pastoris* synthesizes relatively small high mannose-type *N*-glycans (average 8–14 mannose residues) in comparison with *Saccharomyces cerevisiae* (*S. cerevisiae*). However, our SDS–PAGE data indicated that recombinant β3Gn-T2 and -T8 have much larger *N*-glycans than those they reported. The reason for this discrepancy remains unclear, but it is possible that tertiary structure of recombinant proteins may be related to the size of high mannose-type *N*-glycans.

From the results of the periodate oxidation and substrate specificities, β3Gn-T8 and -T2 preferentially act on 2,6-branching *N*-acetyllactosamine moieties. Van den Eijnden *et al.* (1988) showed that a β3Gn-T partially purified from Novikoff tumor cell ascites fluid strongly preferred the moieties in tri- and tetraantennary *N*-glycans. This partially purified enzyme is likely to be β3Gn-T2, -T8, or the complex of the two enzymes, because the substrate specificity of the enzyme is very similar to that of β3Gn-T2 and -T8. It has been shown that oligo-*N*-acetyllactosamine moieties in tri- and tetraantennary *N*-glycans are present in various glycoproteins including calf thymocyte plasma membrane glycoproteins (Yoshima *et al.*, 1980), plasma α_1-acid glycoproteins (Yoshima *et al.*, 1981), human erythropoietins produced in Chinese hamster ovary cells (Sasaki *et al.*, 1987; Takeuchi *et al.*, 1988; Hokke *et al.*, 1995), and lysosomal membrane glycoprotein (LAMP)-1 and LAMP-2 in dimethyl sulfoxide-treated HL-60 cells (Lee *et al.*, 1990). In these cases, *N*-acetyllactosamine residues elongate preferentially or exclusively at the 2,6-branching. Although it is unknown whether these cells or tissues express β3Gn-T2 and/or -T8, the glycan structures are consistent with the substrate specificities of β3Gn-T2 and -T8 found in this study.

The result of Sephacryl S-300 gel filtration indicates that β3Gn-T2 and -T8 can form a complex. The molecular weights of polypeptide moieties of recombinant soluble β3Gn-T2 and -T8 are 45,338 and 42,360, respectively, which include an EAEAHHHHHHGSDDDDKYVEF sequence derived from the pPIC9-His expression vector.

This is consistent with the results of SDS–PAGE analysis of PNGase F-treated proteins (Figure 1). On the other hand, intact recombinant β3Gn-T2 and -T8 proteins exhibit rather broad bands on SDS–PAGE with molecular weight 80–200 and 50–70 kDa, respectively (Figure 1). The apparent molecular weight (90–160 kDa) of β3Gn-T2 alone by gel filtration (Figure 5) should correspond to a monomer form, as that of β3Gn-T8 alone (45–65 kDa) can be considered to be a monomer. The mixture of the two proteins elutes at 110–210 kDa, suggesting a heterodimer of each β3Gn-T2 and β3Gn-T8.

The β3Gn-T2/T8 complex shows enhanced enzymatic activity in comparison with the individual enzymes, in either the soluble or membrane-bound forms. The substrate specificity of the complex is similar to that of each enzyme. The Vmax value of the complex was consistently much higher than either enzyme alone, although the Km value is comparable with that of β3Gn-T8. Recently, it has been reported that *in vivo* co-expression of two individually inactive enzyme proteins resulted in robust enzymatic activity in the cases of core1 β3GalT and Cosmc (Ju and Cummings, 2002), chondroitin synthase and chondroitin polymerizing factor (Kitagawa *et al.*, 2003), and protein *O*-mannosyltransferase 1 and 2 (Manya *et al.*, 2004). These enzymatic activities are very low or not detected when mixing individually prepared enzyme fractions *in vitro*. Ju and Cummings (2002) proposed that Cosmc functions as a molecular chaperone to support active core1 β3GalT. Our results are quite different from these cases with respect to the enhanced effect obtained by *in vitro* mixing and the occurrence of the enzymatic activities in individual β3Gn-T2 and -T8 preparations. Although at present it is unresolved which subunit(s) have enhanced activity, X-ray structural studies should be able to elucidate this issue in the future.

Materials and methods

Materials

UDP-[6-^{3}H]GlcNAc (2.21 TBq/mmol) and UDP-[6-^{3}H]Gal (659 GBq/mmol) were purchased from Perkin Elmer Biosciences (Boston, MA) and Amersham Biosciences (Buckinghamshire, UK), respectively. Fucα1→ 3(Galβ1→4) GlcNAcβ1→3Galβ1→4Glc (LNF-III), Galβ1→ 3GalNAcα1-*O*-pNP (core1-*O*-pNP), and Galβ1→ 3(GlcNAcβ1→6) GalNAcα1-*O*-pNP (core2-*O*-pNP) were purchased from Funakoshi (Tokyo, Japan). Bovine milk β1,4-galactosyltransferase, GalNAcα1-*O*-pNP, and UDP-GlcNAc were purchased from Sigma (St. Louis, MO). Galβ1→ 3GlcNAcβ1→3Galβ1→4Glc (LNT) and Galβ1→ 4GlcNAcβ1→3Galβ1→4Glc (LNnT) were prepared from human milk (Kobata, 1972). *Ricinus communis* agglutinin-I-agarose (RCA-I-agarose) (4 mg/mL gel) was obtained from Hohnen Oil (Tokyo, Japan). PVL was prepared according to the method described by Kochibe and Matta (1989), and the lectin was conjugated to CNBr-activated Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. PNA-agarose (4.5 mg/mL gel) was purchased from E-Y Laboratories (San Mateo, CA). Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4GlcNAc (monoGP), Galβ1→4GlcNAcβ1→2Manα1→3(Galβ1→ 4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAc (biGP), a mixture of Galβ1→4GlcNAcβ1→2 (Galβ1→4GlcNAcβ1→4) Manα1→3(Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→ 4GlcNAc (2,4-branched triGP) and Galβ1→4GlcNAcβ1→ 2Manα1→3[Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→ 6) Manα1→6]Manβ1→4GlcNAc (2,6-branched triGP), and Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→4) Manα1→ 3 [Galβ1→4GlcNAcβ1→2 (Galβ1→4GlcNAcβ1→ 6)Manα1→ 6]Manβ1→4GlcNAc (tetraGP) were obtained from the urine of G_{M1} gangliosidosis patients (Yamashita *et al.*, 1981). Galβ1→4(SO_3^-→6)GlcNAcβ1→3Galβ1→ 4(SO_3^- →6)GlcNAc (L2L2) was the kind gift of Seikagaku (Tokyo, Japan). *Streptococcus* 6646K β-galactosidase, *E. freundii* endo-β-galactosidase, and E_4-PHA-agarose (3.6 mg/mL gel) were obtained from Seikagaku.

cDNA cloning of β3Gn-T8

Based on the amino acid sequence of human *β3Gn-T2* (Shiraishi *et al.*, 2001), we found one sequence [GenBank, AY277592, submitted by Huang *et al.* (2004)] in the NCBI database (NIH, Bethesda, MD) with a high degree of similarity. The cDNA encoding the full open reading frame was amplified by polymerase chain reaction (PCR) from QUICK-Clone™c DNA for human colon adenocarcinoma (Clontech, Palo Alto, CA). The oligonucleotide primers used were 5′-tttaagcttATGCGCTGCCCCAAGTG-3′ (forward primer) and 5′-ttttctagaTCAGCACTGGAGCCTTG-3′ (reverse primer). The sequences in lowercase letters contain appropriate restriction sites. Amplified cDNA was digested with HindIII and XbaI and cloned into pcDNA3 (Invitrogen Life Technologies, Carlsbad, CA). The constructed plasmid was named pcDNA3-β3Gn-T8 and sequenced using a PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Similarly, the cDNA encoding β3Gn-T2 was amplified by PCR using the oligonucleotide primers 5′-tttaagcttGAGAAATGAGTGTTGGA-3′ (forward primer) and 5′-ttttctagaACACAACATGGGAAC-3′ (reverse primer) and was cloned into pcDNA3 between the same sites as β3Gn-T8 (pcDNA3-β3Gn-T2).

Expression of β3Gn-T8 and -T2 in COS-7 cells was performed, as described previously (Seko *et al.*, 2001). The crude membrane fraction was used for examining the enzymatic activity.

Expression of soluble forms of β3Gn-T2 and β3Gn-T8 in P. pastoris

The cDNA fragments of truncated forms of β3Gn-T2 and β3Gn-T8, lacking cytoplasmic and transmembrane domains, were amplified by PCR using with pcDNA3-β3Gn-T2 and -T8, respectively. The oligonucleotide primers used were 5′-tttgaattcTCCAAAAGCAGTAGCC-3′ (forward primer for β3Gn-T2), 5′-tttgcggccgcTAATGTGAGAACACAAC-3′ (reverse primer for β3Gn-T2), 5′-tttgaattcAGCAAGGCCTACCC-3′ (forward primer for β3Gn-T8), and 5′-tttgaattcAGCACTGGAGCCTTG-3′ (reverse primer for β3Gn-T8). An expression vector, pPIC9-His, was produced from pPIC9 (Invitrogen Life Technologies) by the insertion of 5′-CATCACCATCACCATCACGGATCCGATGACGATGACAAA-3′ sequence

at the upstream of SnaB1 site. The cDNAs were cloned into pPIC9-His between EcoRI and NotI for β3Gn-T2 and at the EcoRI site for β3Gn-T8. The resulting plasmids were sequenced with a Prism 310 Genetic Analyzer.

Production of the recombinant proteins in culture media of *P. pastoris* was performed using a Pichia Expression Kit, Version M (Invitrogen Life Technologies). Briefly, the plasmids were linearized with SalI and used for the transformation of *Pichia* KM71 cells. The transformants were inoculated in 300 mL of buffered glycerol-complex medium and cultured at 30°C at 230 rpm for 3 days. After centrifugation at 1500 *g* for 5 min, cell pellets were resuspended in 100 mL of buffered methanol-complex medium containing 1% casamino acids and cultured at 28°C at 230 rpm for 2 days. Methanol (0.5 mL each) was added every day to maintain induction. The cultures were centrifuged at 5000 rpm for 10 min at 4°C. The supernatants were collected, and final concentrations of 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 0.02% bovine serum albumin (BSA) were added. The solutions were dialyzed against 10 mM sodium phosphate and 0.15 M NaCl (pH 8.0) and then adjusted to final concentration of 5 mM imidazole (pH 8.0). The solutions were applied to nickel-nitrilotriacetic acid (Ni-NTA) agarose (0.7 × 1.3 cm; equilibrated with 20 mM sodium phosphate, 0.3 M NaCl, and 10 mM imidazole [pH 8.0] [buffer A]) (QIAGEN GmbH, Hilden, Germany). The columns were washed with buffer A, and the proteins were eluted with buffer A containing 0.25 M imidazole. The eluates were concentrated with Ultrafree-PFL (NMWL: 10,000) (Millipore, Yonezawa, Japan) and washed repeatedly with 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)-NaOH, 0.15 M NaCl, and 10% glycerol (pH 7.2). The concentrates (0.3 mL) for β3Gn-T2 and -T8 were named T2 fraction and T8 fraction, respectively, and were used for measurement of β3Gn-T activities. Purified enzymes were analyzed by SDS–PAGE followed by staining with SYPRO Orange (Molecular Probes, Eugene, OR) and detected with FLA-2000 (Fuji Photo Film, Tokyo, Japan). PNGase F (Takara Shuzo, Kyoto, Japan) digestion was performed with denatured condition according to the manufacturer's instructions, and the digests were analyzed by SDS–PAGE. Protein concentrations were estimated by SDS–PAGE and SYPRO Orange staining using BSA as a standard.

Assay of β3Gn-T activity

As for T2 and T8 fractions, a reaction mixture (20 μL) consisted of 50 mM HEPES-NaOH (pH 7.2), 10 mM $MnCl_2$, 0.1% (w/v) Triton X-100, 0.5 mM acceptor substrate, 2.5 μM UDP-[^{3}H]GlcNAc (6.7×10^6 dpm), 50 μg/mL protamine chloride, 0.5 mM spermine, and appropriately diluted enzyme fractions. When assaying the activities of mixed enzymes, enzyme mixtures without substrates were preincubated on ice for 20 min. As for COS-7-derived membrane fractions, a reaction mixture (20 μL) consisted of 50 mM HEPES-NaOH (pH 7.2), 10 mM $MnCl_2$, 0.5% (w/v) Triton X-100, 0.5 mM acceptor substrate, 2.5 μM UDP-[^{3}H]GlcNAc (6.7×10^6 dpm), 30 μM UDP-GlcNAc, 0.1 M GlcNAc, 1 mM adenosine 5′-phosphate, and appropriately diluted enzyme fractions. The reaction mixture was incubated at 37°C for 3 h. The ^{3}H-labeled products were purified by paper electrophoresis (pyridine : acetic acid : water, 3:1:387, pH 5.4) and then by paper chromatography (pyridine : ethyl acetate : acetic acid : water, 5:5:1:3). After drying, the paper was monitored for radioactivity with a radiochromatogram scanner, and the ^{3}H-labeled products were extracted with water and counted. When using glycolipids as acceptors, the reaction mixture was applied to Sep-Pak C18 cartridges (Waters, Milford, MA), and the ^{3}H-labeled products were eluted with methanol.

Characterization of the ^{3}H-labeled product

The enzymatic reaction of β3Gn-T8 was performed using the tetraGP as an acceptor substrate. The [^{3}H]GlcNAc→tetraGP was purified by paper electrophoresis and paper chromatography as above and then was galactosylated by bovine milk β1,4-galactosyltransferase (Sigma) in a 30 μL-reaction mixture containing 50 mM HEPES-NaOH (pH 7.2), 10 mM $MnCl_2$, 15 mU β1,4-galactosyltransferase, [^{3}H]GlcNAc→tetraGP, and 200 μM UDP-Gal at 37°C for 16 h. The galactose-capped product was purified by paper electrophoresis and paper chromatography and was subjected to periodate oxidation and the Smith degradation (Spiro, 1966; Seko *et al.*, 2001). Briefly, the radioactive products were dissolved in 30 μL of 75 mM sodium acetate (pH 5.3) and 75 mM sodium periodate and incubated at 4°C for 48 h in the dark. Three microliters of 20% ethyleneglycol was added and incubated at 25°C for 3 h. The mixture was added to 300 μL of 0.1 M sodium borate (pH 9.0), 0.1 M sodium borohydride and incubated at 25°C overnight. After acidification with 1 M acetic acid, the mixture was applied to a Bio-Rad AG50W-X8 (H^+ form) column (Bio Rad, Hercules, CA), and the flow through fraction was evaporated repeatedly with methanol. The residues were hydrolyzed in 100 μL of 0.05 N H_2SO_4 at 80°C for 1 h. After neutralizing with 1 N NaOH, the mixture was desalted with AG50W-X8 (H^+ form) and AG1-X8 (OH^- form) and applied to a Bio-gel P-4 (<45 μm) gel filtration column (1.5 × 50 cm), equilibrated and eluted with distilled water at 55°C.

Separation of 2,6-branched triGP from 2,4-branched triGP

A triGP mixture (500 μg) was applied to an L_4-phytohemagglutinin-agarose (L_4-PHA-agarose) column (4.1 mg/mL gel; 0.9 × 7.9 cm) (Seikagaku), which was equilibrated and eluted with 10 mM Tris–HCl (pH 8.0), 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (Buffer A). L_4-PHA has a weak affinity for 2,6-branched glycans such as [Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manα1→6Manβ1→4GlcNAc] (Cummings and Kornfeld, 1982; Kobata and Yamashita, 1993). The glycans were applied to the L_4-PHA column at 4°C, and after washing with 10 mL of Buffer A, the column was stored at room temperature and washed with 20 mL of Buffer A. The retarded fraction was eluted at room temperature and contained the 2,6-branched triGP. The flow through fraction, eluted at 4°C, was applied to a concanavalin A-Sepharose (Con A-Sepharose) column (10 mg/mL of gel; 0.9 × 7.9 cm) (Amersham Biosciences), which was equilibrated and eluted with Buffer A, to remove biantennary glycans possessing

three *N*-acetyllactosamine units (Yamashita *et al*., 1981). The flow through fraction contained the 2,4-branched triGP. The purified triGPs were desalted with Sephadex G-25 gel filtration columns (1.4 × 68 cm), which were equilibrated and eluted with 5% ethanol, and used for β3Gn-T substrates in enzymatic activity assays.

*Preparation of Gal-core2-*O*-pNP*

A reaction mixture (7 mL) containing 2 mM core2-*O*-pNP, 50 mM HEPES-NaOH (pH 7.2), 10 mM $MnCl_2$, 60 μg/mL bovine milk β1,4-galactosyltransferase, and 2.5 mM UDP-Gal was incubated at 37°C for 16 h. After heating at 100°C for 5 min, the reaction mixture was applied to an RCA-I-agarose column by divided eight times (2.3 × 6.5 cm; equilibrated with 10 mM Tris–HCl [pH 8.0], 0.15 M NaCl, and eluted with the same buffer containing 10 mM lactose). The bound fractions were applied to a Sep-Pak C18 column to remove salts and lactose. Finally, 7.3 μmol of Gal-core2-*O*-pNP was obtained.

Abbreviations

β3Gn-T, β1,3-*N*-acetylglucosaminyltransferase; β3Gal-T, β1,3-galactosyltransferase; E_4-PHA, E_4-phytohemagglutinin; HEPES-NaOH, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid-NaOH; L_4-PHA, L_4-phytohemagglutinin; LNnT, lacto-*N*-neotetraose; PCR, polymerase chain reaction; *P. pastoris, Pichia pastoris;* PNA, peanut agglutinin; PNGase, peptide:*N*-glycanase; pNP, *p*-nitrophenyl; PVL, *Psathyrella velutina* lectin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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