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Kinetic Studies on Endo-β-galactosidase by a Novel Colorimetric Assay and Synthesis of Poly-*N*-acetyllactosamines Using Its Transglycosylation Activity

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Abstract: Novel chromogenic substrates for endo-β-galactosidase were designed on the basis of the structural features of keratan sulfate. Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP (2), which consists of two repeating units of *N*-acetyllactosamine, was enzymatically synthesized by consecutive additions of GlcNAc and Gal residues to *p*-nitrophenyl β-*N*-acetyllactosaminide. In a similar manner, GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP (1), GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (3), Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (4), Galβ1,3GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (5), and Galβ1,6GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (6) were synthesized as analogs of 2. Endo-β-galactosidases released GlcNAcβ-*p*NP or Glcβ-*p*NP in an endo-manner from each substrate. A colorimetric assay for endo-β-galactosidase was developed using the synthetic substrates on the basis of the determination of *p*-nitrophenol liberated from GlcNAcβ-*p*NP or Glcβ-*p*NP formed by the enzyme through a coupled reaction involving β-*N*-acetylglucosaminidase or β-D-glucosidase. Kinetic analysis by this method showed that the value of V_{max}/K_m of 2 for *Escherichia freundii* endo-β-galactosidase was almost equal to that for keratan sulfate, indicating that 2 is very suitable as a sensitive substrate for analytical use in an endo-β-galactosidase assay. In addition, the hydrolytic action of the enzyme toward 2 has shown to be remarkably promoted by the presence of 2-acetamide group adjacent to *p*-nitrophenyl group in comparison with 4. In addition, enzymatic synthesis of GlcNAc-terminated poly-*N*-acetyllactosamine β-glycosides GlcNAcβ1,3 (Galβ1,4GlcNAcβ1,3)$_n$ Galβ1,3GlcNAcβ-*p*NP (n = 1–5) has been demonstrated using a transglycosylation reaction of *E. freundii* endo-β-galactosidase. The enzyme catalyzed a transglycosylation reaction on 1, which served both as a donor and an acceptor, and converted 1 into *p*-nitrophenyl β-glycosides GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_n$Galβ1,4GlcNAcβ-*p*NP (9, n = 1; 10, n = 2; 11, n = 3; 12, n = 4; 13, n = 5). The efficiency of production of poly-*N*-acetyllactosamines by *E. freundii* endo-β-galactosidase was significantly enhanced by the addition of BSA and by a low temperature condition.

Key words: endo-β-galactosidase, enzyme assay, poly-*N*-acetyllactosamine, kinetics, transglycosylation

Endo-β-galactosidases (EC 3.2.1.103) were discovered as keratan sulfate-degrading enzymes, so-called keratanases, in culture filtrates of *E. freundii*,[1] *Coccobacillus* sp.,[2] *Pseudomonas* sp.,[3] *Flavobacterium keratolyticus*[4,5] and *Bacteroides fragilis*.[6] *E. freundii* keratanase was found to have hydrolyzing activity for a wide range of nonsulfated oligosaccharides isolated from human milk and carbohydrate moieties of glycoproteins and glycolipids.[7−10] The use of endo-β-galactosidase has been expanded to detection of poly-*N*-acetyllactosamine chains in a variety of complex glycoconjugates in addition to keratan sulfate. *Bacteroides fragilis* endo-β-galactosidase has properties similar to those of *E. freundii* endo-β-galactosidase.[11−13] Therefore, the endo-β-galactosidases from *E. freundii* and *B. fragilis* have been widely used as tools for structural and functional analyses of glycans involved in glycoconjugates. An assay using keratan sulfate as a substrate has been widely used for estimation of endo-β-galactosidase activity. However, this method is not always reproducible because of lack of uniformity of the polymer. Methods using low-molecular-weight substrates defined their structures have been preferred and recommended for accurate determination of endoglycosidases such as α-amylase,[14] lysozyme,[15] and endo-β-*N*-acetylglucosaminidase,[16] because the purity of the substrate and the reaction pattern can be determined exactly. Therefore, a series of chromogenic substances having a partial substituted unit of poly-*N*-acetyllactosamine were designed as substrate analogs for the enzyme.

Poly-*N*-acetyllactosamine has been shown to be present on membrane glycoconjugates,[17,18] and has been identified as a precursor of Lewis X, sialyl Lewis X, and blood group antigens.[19] The amounts of poly-*N*-acetyllactosamine chains have been shown to be changed during cellular differentiation and malignant transformation of cells.[20,21] Furthermore, B16 melanoma cells that expressed sialyl Lewis X on long poly-*N*-acetyllactosamine were highly metastatic, while cells expressing even more sialyl Lewis X on short poly-*N*-acetyllactosamine were not metastatic.[22] In addition, poly-*N*-acetyllactosamines have been shown to be directly recognized with high affinity by galectins,[23] and to be involved in apoptosis.[24] These findings suggest

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Abbreviations: β3GnT, β-1,3-*N*-acetylglucosaminyltransferase (EC. 2.4.1.149); β-NAHase, β-*N*-acetylhexosaminidase (EC 3.2.1.52); BSA, bovine serum albumin; ESI-MS, electrospray ionization mass spectrometry; β4GalT, β-1,4-galactosyltransferase (EC 2.4.1.22); HPLC, high-performance liquid chromatography; *p*NP, *p*-nitrophenyl; NMR, nuclear magnetic resonace.

that poly-*N*-acetyllactosamines play important roles in biological events. Therefore, it is important to generate a series of pure and well-characterized poly-*N*-acetyllactosamines. The chemical syntheses of poly-*N*-acetyllactosamines have been reported.[25–28] These methods have contributed to the elucidation of functions of their glycans. In addition, methods using β-1,3-*N*-acetylglucosaminyltransferases (β3GnT) and β-1,4-galactosyltransferases (β4GalT) have been developed for the synthesis of poly-*N*-acetyllactosamines and are excellent in terms of stereo- and regioselectivities of products.[29,30] On the other hand, there has been no report of enzymatic synthesis of poly-*N*-acetyllactosamines using glycosidase-catalyzed transglycosylation. Glycosidases have often been used for synthesis of glycans utilizing transglycosylation reaction.[31–33] Therefore, we tried to synthesize a series of GlcNAc-terminated poly-*N*-acetyllactosamines using the enzyme with **1** as an initial substrate.

In this review, we describe the enzymatic synthesis of a novel substrate **2** and its analogs for a colorimetric assay of endo-β-galactosidase activity and the usefulness of the resulting chromogenic substrates for kinetic studies on the enzyme. In the latter part of this review, synthesis of poly-*N*-acetyllactosamine β-glycoside utilizing endo-β-galactosidase-mediated transglycosylation is described.

Synthesis of colorimetric substrates.

A series of chromogenic substances were designed as substrates of endo-β-galactosidase based on the structural features of keratan sulfate, which is an alternating polymer of *N*-acetyllactosamine units joined to each other by β-(1-3)-linkage.[34] Tetrasaccharide **2** containing two *N*-acetyllactosamine repeats and its analogs were synthesized by the alternative addition of β-(1-3)-linked GlcNAc and β-(1-4)-linked Gal to respective Galβ1-4GlcNAcβ-*p*NP and Galβ1-4Glcβ-*p*NP utilizing two kinds of glycosyltransferases. Thus compounds **1** and **3** were first prepared by the regioselective transfer of GlcNAc residue from UDP-GlcNAc to Galβ1-4GlcNAcβ-*p*NP and Galβ1-4Glcβ-*p*NP by β3GnT from bovine serum. They were further converted into **2** and **4**, utilizing β4GalT from bovine milk (Figs. 1A, B). The enzyme efficiently catalyzed the transfer of a Gal moiety to the OH-4″ position of the acceptors in high yields (82 and 71%) based on the acceptors. The positional isomers **5** and **6** were prepared simultaneously by Gal transfer from Galβ-*o*NP to the OH-3″ and OH-6″ positions of **3** using *B. circulans* β-D-galactosidase-mediated transglycosylation (Fig. 1B). The resulting products were obtained in a molar ratio of 1 : 1.3 and in a 12% overall yield based on the acceptor added. Compound **7** and its isomer **8** were prepared from Galβ-*p*NP using β-*N*-acetylhexosaminidase (β-NAHase)-mediated transglycosylation (Fig. 1C).

Hydrolytic actions of endo-β-galactosidases.

The hydrolytic actions of endo-β-galactosidases on synthetic chromogenic substances were investigated by using enzyme preparations from *E. freundii* and *B. fragilis* (Table 1).[34] Each enzyme splits compounds **1**–**6** into the corresponding reducing oligosaccharides and chromogenic

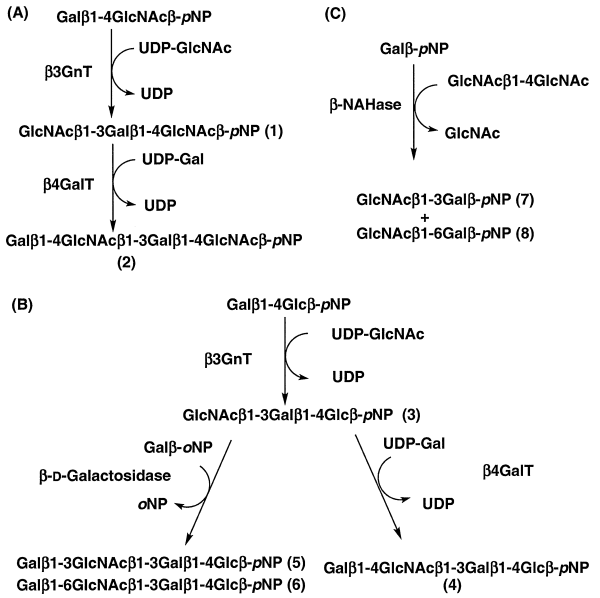

Fig. 1. Summary of enzymatic synthesis of *p*-nitrophenyl oligosaccharide β-glycosides used in this work.

(A) Consecutive additions of GlcNAc and Gal to Galβ1-4GlcNAcβ-*p*NP by β3GnT and β4GalT. (B) Consecutive additions of GlcNAc and Gal to Galβ1-4Glcβ-*p*NP by β3GnT and β-D-galactosidase or β4GalT. (C) *N*-acetylglucosaminylation of Galβ-*p*NP by β-NAHase-mediated transglycosylation.

Table 1. Relative hydrolytic rates of endo-β-galactosidases on *p*-nitrophenyl oligosaccharide β-glycosides.

Substrates	Relative hydrolytic rates (%)[a]	
	E. freundii	*B. fragilis*
GlcNAcβ1-3Galβ1-4GlcNAcβ-*p*NP (**1**)	47	54
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-*p*NP (**2**)	100[b]	100
GlcNAcβ1-3Galβ1-4Glcβ-*p*NP (**3**)	<1[c]	<1
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-*p*NP (**4**)	10	11
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-*p*NP (**5**)	<1	<1
Galβ1-6GlcNAcβ1-3Galβ1-4Glcβ-*p*NP (**6**)	<1	<1
GlcNAcβ1-3Galβ1-4GlcNAc ↓ Galβ1-4GlcNAcβ-*p*NP	<1	<1
	—[d]	—
GlcNAcβ1-3Galβ-*p*NP (**7**)	—	—
GlcNAcβ1-6Galβ-*p*NP (**8**)	—	—

The hydrolytic actions of endo-β-galactosidase on different substrates were investigated. The vertical arrow indicates the point of cleavage. [a]The conditions for hydrolytic reaction were described under MATERIALS AND METHODS. [b]Relative hydrolytic rate of compound **2** was arbitrarily set at 100. [c]<1, hydrolyzed only in the presence of 10 mU of the enzyme. [d]—, not hydrolyzed even in the presence of 10 mU of the enzyme.

substances, GlcNAcβ-*p*NP/Glcβ-*p*NP. For example, compound **2** was completely hydrolyzed in an endo-manner into Galβ1-4GlcNAcβ1-3Galβ and GlcNAcβ-*p*NP. The relative rates of **1** and **4** compared with **2** (set at 100) were 47 and 10, namely, 2- and 10-fold differences. Compounds **3**, **5** and **6** acted slightly as substrates. Furthermore, the hydrolytic rate of reducing trisaccharide GlcNAcβ1-3Galβ1-4GlcNAc was compared with that of its glycoside **1** in order to examine how the *p*-nitrophenyl group participates in the hydrolytic action. Hydrolysis of the reducing trisaccharide preceded little under the experimental condition, although the reducing trisaccharide was hydrolyzed very slowly to form GlcNAcβ1-3Gal and GlcNAc when a tenfold amount of enzyme was added. *p*-Nitrophenyl disaccharide β-glycosides **7**, **8**, Galβ1-4GlcNAcβ-*p*NP and GlcNAcβ1-6Galβ1-4GlcNAcβ-*p*NP and Glc NAcβ1-6Galβ1-4Glcβ-*p*NP did not act as substrates toward the enzyme. Based on the hydrolytic action toward the enzyme, **2** was the best substrate among the synthetic substances.

Colorimetric assay of endo-β-galactosidase activity.

As shown in Fig. 2A, a novel method for endo-β-galactosidase assay using **2** was designed on the basis of the above-described results.[34] This assay involves the colorimetric determination of *p*-nitrophenol liberated from the substrate by the action of the enzyme through a coupled reaction involving β-NAHase. Thus, the enzyme produces exclusively GlcNAcβ-*p*NP from **2** and then β-NAHase hydrolyzes GlcNAcβ-*p*NP to free *p*-nitrophenol. Compound **2** was incubated with *E. freundii* endo-β-galactosidase in the presence and absence of the β-NAHase. The increase of absorption at 405 nm was observed only in the presence of the enzyme. The rate of hydrolysis was first-order with respect to endo-β-galactosidase throughout the course of the determination. The reaction proceeded linearly for at least 20 min under the conditions. In this case, only 10 ng of the endo-β-galactosidase could be determined by this assay method. The dose-response plot of the coupled enzyme *vs*. the color intensity of the enzyme was shown to be a plateau in the range of 20–40 mU for 15 min. The addition of 20 mU of coupled enzyme to the assay system was sufficient to obtain the maximal activity of endo-β-galactosidase. In a similar manner, compound **4** was applied to determination of endo-β-galactosidase activity with β-D-glucosidase as a coupled enzyme (Fig. 2B). When **4** was used as a substrate, 100 ng of the enzyme was required for the determination of the activity by this assay method. The addition of 50 mU of coupled enzyme to the assay system was sufficient to obtain the maximal activity of the endo-β-galactosidase. The present assay system has better reproducibility and is simpler than the method of Park and Johnson. From a practical viewpoint, **2**, a well-defined substrate, was shown to be very useful for routine submicrogram assay of endo-β-galactosidase in biological materials.

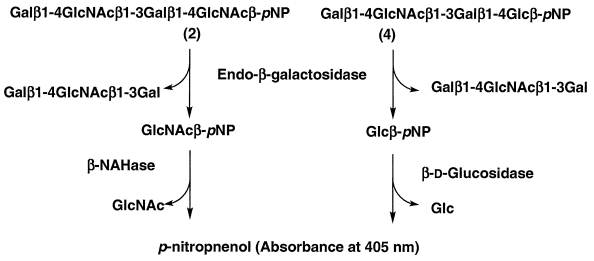

Fig. 2. Principle of colorimetric assay method for endo-β-galactosidase using compounds **2** and **4** through a coupled reaction involving β-NAHase or β-D-glucosidase.

Substrate specificity of endo-β-galactosidase.

In order to elucidate in more detail the substrate specificity of endo-β-galactosidase, parameters of Michaeris-Menten-type kinetics for **1**–**5** were evaluated by $1/v - 1/[S]$ plot.[34] Compound **2** was assayed with *Amycolatopsis orientalis* β-NAHase as a coupled enzyme and **3**–**5** with almond β-D-glucosidase, using the newly developed colorimetric assay as described above. Compound **1** was assayed by HPLC. The kinetic parameters are summarized in Table 2. The catalytic efficiencies of **2** for both enzymes were the highest among the synthetic substrates. The value of V_{max}/K_m of **2** for *E. freundii* enzyme was almost equal to that for keratan sulfate, indicating that it is very useful as a substrate instead of keratan sulfate for analytical use in the endo-β-galactosidase assay. In addition, this similarity in the values of V_{max}/K_m suggests that the sulfate group on the 6-position on GlcNAc of keratan sulfate is not always essential for the hydrolytic action of the enzyme. Replacement of Glc by internal GlcNAc of **2** resulted in a remarkable reduction in the catalytic effi-

Table 2. Kinetic parameters of endo-β-galactosidases from *Escherichia freundii* and *Bacteroides fragilis*.

Substrates	*E. freundii*					*B. fragilis*			
	K_m[a]	V_{max}	k_{cat}	V_{max}/K_m	k_{cat}/K_m	K_m	V_{max}	k_{cat}	k_{cat}/K_m
GlcNAcβ1-3Galβ1-4GlcNAcβ-*p*NP (**1**)[b]	0.07	42.1	19.7	601	281	0.46	12.2	6.10	13.3
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-*p*NP (**2**)	0.10	129	60.2	1290	602	0.13	11.1	5.59	43
GlcNAcβ1-3Galβ1-4Glcβ-*p*NP (**3**)	1.01	1.66	0.76	1.6	0.75	2.16	0.43	0.21	0.10
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-*p*NP (**4**)	1.20	43.7	20.4	36.4	17.0	2.75	2.17	2.93	1.07
Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-*p*NP (**5**)	1.42	3.52	1.64	2.5	1.15	—[d]	—	—	—
Keratan sulfate[c]	0.35	408	—	1166	—	—	—	—	—
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer[c]	0.30	39	—	130	—	—	—	—	—

The parameters of Michaelis-Menten-type kinetics were evaluated by $1/v - 1/[S]$ plots and the least-squares method. This summary is compiled from results reported here and from data in the literature. [a]K_m, mM; V_{max}, μmol·min^{-1}·mg^{-1}; k_{cat}, s^{-1}; k_{cat}/K_m, s^{-1}·mM^{-1}. [b]Kinetic parameters were determined by HPLC method. [c]These data were reported by M.N. Fukuda.[10] [d]not determined.

ciency on **4**. This was also the same tendency for the comparison of **1** and **3**. The increase in catalytic efficiency was clearly due to the *N*-acetyl group on C-2 of GlcNAc linked to the aglycon moiety. However, compound **4** still acts as a fairly good substrate: the V_{max}/K_m value of **4** is 28% of that of Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer. It may be substitute for the detection of glycosphingolipid-degrading endo-β-galactosidase. This concept for the enzyme assay could be applied to other types of endo-β-galactosidases from *Diplococcus pneumonia*,[35] *Clostridium perfringens*[36,37] and mollusk *Painopecten* sp.,[38] which hydrolyze internal β-galactosidic linkages of blood group A and B antigens, Galα1-4Galβ1-4GlcNAc, GlcNAcα1-4Galβ1-4GalNAc and GlcAβ1-3Galβ1-3Gal structures, respectively.

The structure of the site of cleavage by the enzyme, which was deduced from results of kinetic studies using well-defined synthetic oligosaccharides, is shown in Fig. 3A. We propose a binding structure so that Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-*p*NP has a matching shape, which can accommodate a chain of five residues (A, B, C, D and E) to fit into the active site. Synthetic tri- and tetrasaccharide glycosides had only one cleavage site for the endo-β-galactosidase, which splits the glycoside bond between C and D. On the other hand, Fukuda and Matsumura reported that the endo-β-galactosidases from *E. freundii* hydrolyzed corneal keratan sulfate, releasing GlcNAc6SO_3^- β1-3Gal and GlcNAc6SO_3^- β1-3Gal6SO_3^- β1-4GlcNAc6SO_3^- β1-3Gal as major products.[8] This result suggests that the enzyme tolerates C-6 sulfation of the sugar residues A and B, which have to be partially *O*-sulfated in keratan sulfate, but not C-6 sulfation of the sugar residue C. The hydrolyzates GlcNAc6SO_3^- β1-3Gal and GlcNAc6SO_3^- β1-3Gal6SO_3^- β1-4GlcNAc6SO_3^- β1-3Gal may occupy corresponding sites B-C and -A-B-C, respectively, as shown in Fig. 3B. The sugar residue D at the cleavage site influences the sensitivity of oligosaccharides to the enzyme, because hydrolytic action was promoted by the presence of an *N*-acetyl group on C-2 of GlcNAc corresponding to sugar residue D. Disaccharide

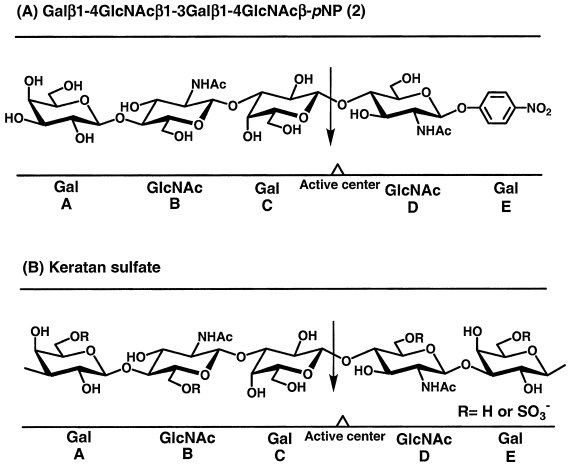

Fig. 3. Proposed structure of the cleavage site of endo-β-galactosidase.

The enzyme hydrolyzes both *N*-acetyllactosamine-repeating tetrasaccharide β-glycoside (A) and keratan sulfate (B). Arrows show the cleavage site of the glycosidic linkages of each substrate.

β-glycoside **7** did not act as a substrate. Reduction of reducing-end residue of Galβ1-4GlcNAcβ1-3Galβ1-4Glc prohibited the action of endo-β-galactosidases from *E. freundii*.[7,39] These results indicate that a sugar pyranose structure such as Glc*p*NAc or Glc*p* on cleaving site D is required for the hydrolytic action of endo-β-galactosidases. Furthermore, the mode of linkage of sugar residues A to B was strict for the binding locus in the active site, because conversion of the (1-4) into (1-3)-linkages of terminus Gal to GlcNAc residues remarkably decreased the enzyme action (Tables 1, 2). These observations indicate that a tetrasaccharide sequence consisting of two LacNAc repeating units such as **2** is accommodated to the binding locus in the active site. A series of chromogenic substrates were shown to be advantageous as probes for substrate recognition at the active site in the enzyme.

Enzymatic synthesis of poly-N-acetyllactosamines.

In order to examine the transglycosylation activity of endo-β-galactosidase, commercially available *E. freundii* endo-β-galactosidase was directly used for the synthesis of poly-*N*-acetyllactosamines.[40] When the enzyme was incubated with a high concentration of **1** (10.5 mM) at a low temperature (1°C) in the presence of BSA, a series of transfer products were formed during the incubation. In this process, the conditions for obtaining GlcNAc-terminated poly-*N*-acetyllactosamines were optimized by controlling the temperature or by adding an activator mentioned in the next section. The reaction mixture was readily separated by using a column of Toyopearl HW-50S as shown in Fig. 4A. The purity of each fraction was verified by HPLC analysis as described above. The purities of Fr. B (**11**), C (**10**), D (**9**) and E (**1**) were $>$ 98% based on their peak area obtained by HPLC analysis. Fr. A (**12**), however, was contaminated with a large amount of Fr. B (**11**), which amount was 4-times higher than that of Fr. A (**12**). The structures of transglycosylation products were characterized by using NMR and ESI-MS analyses. ^{1}H- and ^{13}C-NMR data for Fr. D matched those of GlcNAcβ1,3 (Galβ1,4 GlcNAcβ1,3)$_1$Galβ1,4 GlcNAcβ-*p*NP (**9**). The structure of Fr. C was characterized by the appearance of new proton and carbon signals at δ4.68 and δ4.37 corresponding to GlcNAc H-1 and Gal H-1 and at δ56.4, δ61.0, δ70.5, δ74.4 and δ76.8 corresponding to GlcNAc C-2, C-6, C-4, C-3 and C-5, respectively, of the additional terminal GlcNAcβ1,3Gal unit added to **9**. The β1,4-linkage between the terminal GlcNAcβ1,3Gal and GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP was confirmed by a downfield shift of the signal for terminal GlcNAc C-4, which newly linked with Gal residue, from δ70.5 to δ81.3. The relative numbers of Gal and GlcNAc residues were estimated to be 3 : 4 by integration of anomeric proton signals. ESI-MS analysis of Fr. C shows molecular ions at m/z 719, 730 and 741, presumably arising from $[M+2H]^{2+}$, $[M+H+Na]^{2+}$ and $[M+2Na]^{2+}$, respectively. From these data, Fr. C was characterized as GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_2$Galβ1,4GlcNAcβ-*p*NP (**10**). ^{1}H-NMR spectra of Fr. B, showed the characteristic anomeric proton signals of β-1,4-linked Gal residue at δ4.47–4.51 and the expected ratio between the anomeric protons of Gal and methyl protons of

GlcNAc residues. The relative integrated values for Gal H-1s and GlcNAc CH3s of Fr. B is 4 : 5, indicating that Fr. B consists of four Gal and five GlcNAc residues. ESI-MS of Fr. B shows molecular ions at *m/z* 902 ($[M+2H]^{2+}$), 913 ($[M+H+Na]^{2+}$) and 924 ($[M+2Na]^{2+}$), which coincide with calculated values of $GlcNAc_5Gal_4$ *p*NP. These data revealed that Fr. B is GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_3$Galβ1,4GlcNAcβ-*p*NP (**11**). Fr. A was also subjected to ESI-MS analysis without further purification. Three molecular ions $[M+2H]^{2+}$, ($[M+H+Na]^{2+}$) and ($[M+2Na]^{2+}$) were detected at *m/z* 1084, 1095 and 1106, which correspond with the theoretical values of $GlcNAc_6Gal_5$*p*NP, with the molecular ions of **11**.

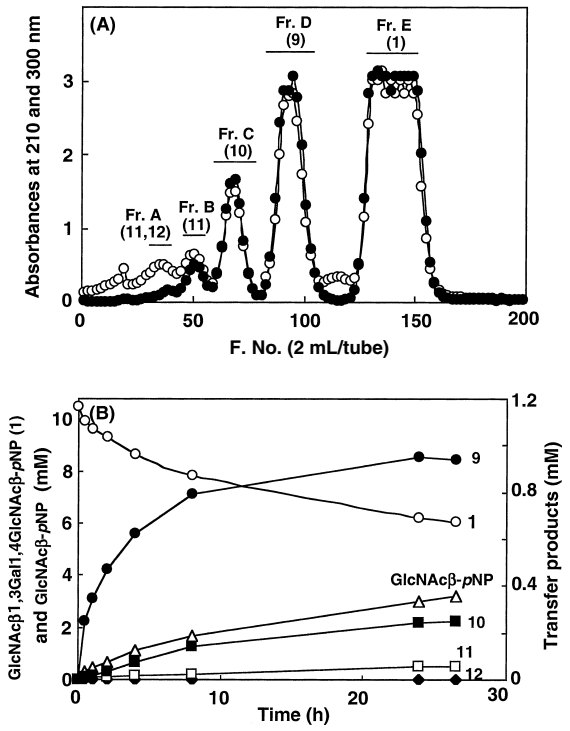

Fig. 4. Enzymatic synthesis of poly-*N*-acetyllactosamines from compound **1**.

(A) Toyopearl HW-50S chromatography of transglycosylation products formed by *E. freundii* endo-β-galactosidase. Absorbances at 210 (○) and 300 nm (●). (B) Time courses of the formation of transfer products and degradation of the initial substrate. ○, compound **1**; ●, compound **9**; ■, compound **10**; □, compound **11**; ◆, compound **12**.

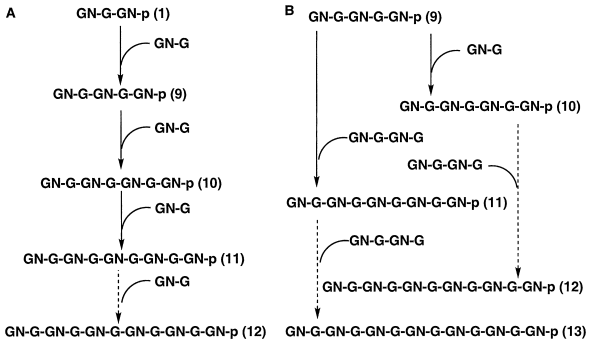

Fig. 5. Proposed mechanism of *E. freundii* endo-β-galactosidase-mediated transglycosylation reaction.

(A) Enzymatic synthesis of poly-*N*-acetyllactosamines from compound **1** as an initial substrate. (B) Enzymatic synthesis of poly-*N*-acetyllactosamines from compound **9** as an initial substrate. **GN**, GlcNAc; **G**, Gal; **p**, *p*NP.

This result indicate that Fr. A contains both **11** and GlcNAcβ1,3 (Galβ1,4 GlcNAcβ1,3)$_4$Galβ1,4 GlcNAcβ-*p*NP (**12**). These results indicated that the enzyme catalyzed the regioselective transfer of GlcNAcβ1,3Galβ from **1** onto the OH-4 position of the non-reducing end GlcNAc residue of acceptors. In this synthesis, the enzyme would first transfer the disaccharide unit GlcNAcβ1,3Gal from **1** to the non-reducing end GlcNAc residue of **1** to form **9**. Then **10**–**12** would be synthesized through sequential transfer reaction of the disaccharide unit GlcNAc β1,3Gal from **1** to the non-reducing end GlcNAc residue of acceptor substrates as shown in Fig. 5A. In this reaction, the resulting **9** may also act as a donor substrate of subsequent sugar-elongation reactions in some degree. However, its frequency would be low because the concentration of **9** was much lower than that of **1** in the reaction system. Compounds **9**–**12** were obtained in a total yield of 11.1% based on the amount of **1** added. Time courses of transfer products and GlcNAcβ-*p*NP are shown in Fig. 4B. **9** was the main product during the entire course of the reaction along with **10**–**12** as minor products. The amount of **9** was increased nearly linearly up to 8 h, and the time required for maximum production was ~24 h.

On the other hand, when **9** was used as a substrate instead of **1**, transfer products **10**–**13** was observed after 24-h incubation. The time for maximum production of **11** was ~24 h, but the amount decreased remarkably during the next 20 h. **10** formation was much slower and the time for maximum production was ~50 h. Thus, much more of **11** than **10** were formed in the initial stage of the reaction, but the relation between their yields were reversed in the latter stage of the reaction. Trace amounts of undecasaccharide **12** and **13**, which was presumed to be tridecasaccharide GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_5$Galβ1,4GlcNAcβ-*p*NP, were also detected by HPLC analysis after 24-h incubation. This result indicates that the enzyme transferred not only disaccharide GlcNAcβ1,3Gal but also tetrasaccharide GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Gal to form heptasaccharide **10** and nonasaccharide **11**. Then the resulting **10** and **11** would be further converted into **12** or **13** (Fig. 5B).

Effects of reaction conditions on the transglycosylation reaction.

Nakagawa *et al*. reported that an enzyme was activated by addition of BSA to the enzyme solution.[39] The effect of BSA on *E. freundii* endo-β-galactosidase-catalyzed transglycosylation was examined with **1**. Figure 6 shows time courses for the production of the transfer products in the presence (A) and absence (B) of 0.2% BSA at 37°C. In the former case, transfer products **9**–**11** were detected during 4–8 h of incubation (Fig. 6A), while only **9** was detected in the latter case (Fig. 6B). The maximum production of **9** was obtained in the presence of BSA, about 4-fold higher than that in its absence, and the total yield of transfer products (**9**–**11**) was also 6-fold higher in the presence of BSA. This result indicates that the addition of BSA to the reaction system significantly improves the efficiency of production of poly-*N*-acetyllactosamines.

The use of low temperature for transglycosylation has recently been reported to increase of its yield.[41,42] To in-

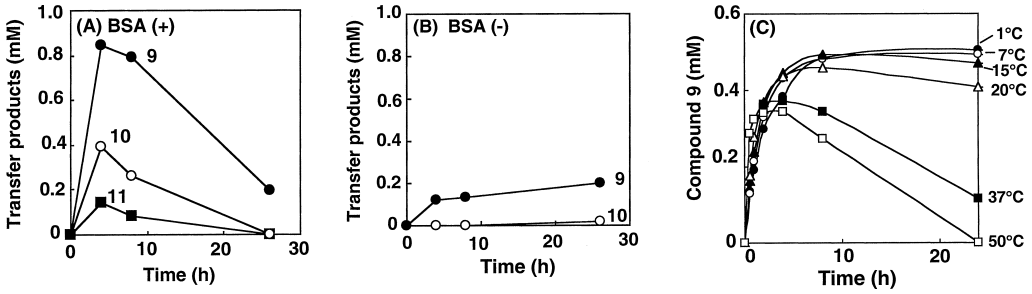

Fig. 6. Effects of reaction conditions on the transglycosylation reaction.

Compound **1** was incubated at 37°C in the presence (A) and absence (B) of 0.2% BSA. ●, compound **9**; ○, compound **10**; ■, compound **11**. (C) Effect of reaction temperature on the formation of **9**. The reactions were performed with **1** in the presence of 0.2% BSA. ●, 1°C; ○, 7°C; ▲, 15°C; △, 20°C; ■, 37°C; □, 50°C.

vestigate the effects of temperature on *E. freundii* endo-β-galactosidase-catalyzed transglycosylation, we focused on the formation of **9** at different temperatures in the presence of 0.2% BSA (Fig. 6C). The maximum production of **9** gradually increased with a decrease in the reaction temperature. The production of **9** at 1°C was the highest among the reaction temperatures tested. The maximum production of **9** at 1°C was about 1.4-fold higher than that at 37°C, which is optimum for the hydrolytic activity. The use of low temperature is considered to be effective for suppressing competitive hydrolytic reaction of **9** once it has been formed. To prove this hypothesis, the effect of temperature on hydrolytic action of the enzyme on **1** and **9** was examined. The relative hydrolytic rate of **9** toward **1** at 1°C apparently decreased to about half its value at 37°C. This result suggests that undesired product hydrolysis upon transglycosylation would be diminished by the low temperature condition. In this way, the efficiency of production of poly-*N*-acetyllactosamines has been improved by nearly 12-fold by the addition of BSA and by a low temperature condition.

Conclusion and perspectives.

A highly sensitive colorimetric assay for determining endo-β-galactosidase activity has been developed using Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP (**2**), which consists of two repeating units of *N*-acetyllactosamine. This method would be useful for the detection of endo-β-galactosidase activity in various biological sources.

Poly-*N*-acetyllactosamine is one of most attractive structures in the field of chemical and enzymatic synthesis of glycans due to its numerous biological functions.[43] In this study, we demonstrated one-pot synthesis of GlcNAc-terminated poly-*N*-acetyllactosamine β-glycosides GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_n$Galβ1,4GlcNAcβ-*p*NP (n = 1−5) by *E. freundii* endo-β-galactosidase. The synthetic poly-*N*-acetyllactosamines were also shown to be useful for characterizing endo-β-galactosidases in hydrolytic and transglycosylation reactions. This enzyme would be useful as a tool for preparing a series of poly-*N*-acetyllactosamines that are valid for studying their counterparts such as natural receptors and glycosidases.

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エンド-β-ガラクトシダーゼの基質特異性解析と糖転移反応によるポリ-*N*-アセチルラクトサミンの合成

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エンド-β-ガラクトシダーゼは，複合糖質糖鎖に存在するポリ-*N*-アセチルラクトサミンやケラタン硫酸のβ(1-4)ガラクトシル結合をエンド的に分解する酵素である．本酵素はこれまで糖鎖の構造解析や機能解析などに広く用いられてきたが，加水分解反応における速度論的な基質特異性の解析や糖転移反応によるオリゴ糖合成ついての報告はなかった.そこで,ケラタン硫酸をモデルとし発色団として利用可能な *p*-ニトロフェニル基（*p*NP）を有する GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP (**1**), Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-*p*NP (**2**), GlcNAcβ1,3Galβ1,4Glcβ-*p*NP(**3**), Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ-*p*NP(**4**), Galβ1,3GlcNAcβ1,3Galβ1,4Glcβ-*p*NP (**5**) and Galβ1,6GlcNAcβ1,3Galβ1,4Glcβ-*p*NP(**6**)を系統的に酵素合成した．*Escherichia freundii* 由来のエンド-β-ガラクトシダーゼは，これらの基質をエンド的に加水分解しGlcβ-*p*NPあるいはGlcNAcβ-*p*NPのみを特異的に遊離した．この水解特性を利用して，共役酵素としてβ-グルコシダーゼあるいはβ-*N*-アセチルヘキソサミニダーゼを添加することにより生成した *p*-ニトロフェノールを定量し，本酵素の簡便かつ分析適応性に優れた測定法を確立した．この活性測定法に基づき **2** の本酵素に対する動力学的パラメータの測定を行ったところ，V_{max}/K_m はケラタン硫酸の V_{max}/K_m とほぼ同等であった．このように，**2** は本酵素に対する高感度基質として利用可能であることが明らかとなった．**2** に対する高い基質特異性から，基質の *p*NP に隣接した糖残基の *N*-アセチル基が本酵素の基質認識に重要であることが示唆された．さらに，本酵素の糖転移反応により，GlcNAcを末端にもつポリ-*N*-アセチルラクトサミン GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_n$Galβ1,4GlcNAcβ*p*NPの合成を行った．高濃度の **2** に対して *E. freundii* 由来のエンド-β-ガラクトシダーゼを作用させたところ，本酵素は，**2** からGlcNAcβ1-3Gal二糖単位を高位置選択的に受容体基質の非還元末端GlcNAc残基の4位OH基に転移する反応を触媒することが明らかとなった．その結果，GlcNAcβ1,3(Galβ1,4GlcNAcβ1,3)$_n$Galβ1,4GlcNAcβ*p*NP (**9**, $n=1$; **10**, $n=2$; **11**, $n=3$; **12**, $n=4$; **13**, $n=5$)の合成が可能となった．この糖転移反応は，ウシ血清アルブミンの添加および低温での反応により著しく転移効率が増加した．

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〔質　問〕　食総研　北岡

Bovine serumを用いた反応系にATPを加えている理由は．

〔答〕

血清中には糖ヌクレオチドを加水分解する酵素が存在するので，その阻害剤として反応系にATPを添加しています．

〔質　問〕　横浜市大院　梶原

エンド-β-ガラクトシダーゼによる加水分解反応の際，還元末端をGlcβ-*p*NPからGlcNAcβ-*p*NPに代えると酵素活性が増大しました．酵素触媒部位においてGlcNAcβ-*p*NPに対して強い親和性をもつ結合サイトがあるような気がしますが，生成物等を用いた阻害実験等で調べた知見はないでしょうか．

〔答〕

種々の合成基質を用いた動力学的な解析から考えると，ご指摘の通り触媒部位にGlcNAcの*N*-アセチル基を強く認識する部位の存在が推測されます．しかしながら，生成物を用いた阻害試験はこれまでに行っていません．