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Carbohydrate Research 330 (2001) 487–493

CARBOHYDRATE RESEARCH

Enzymatic syntheses of T antigen-containing glycolipid mimicry using the transglycosylation activity of *endo*-α-*N*-acetylgalactosaminidase

Hisashi Ashida,* Kenji Yamamoto, Hidehiko Kumagai

Laboratory of Molecular Biology of Bioresponse, Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan

Received 2 August 2000; accepted 21 December 2000

Abstract

Thomsen–Friedenreich antigen (T antigen) disaccharide, β-D-galactose-(1→3)-α-*N*-acetyl-D-galactosamine (β-D-Gal-(1→3)-α-D-GalNAc), containing glycolipid mimicry was synthesized using the transglycosylation activity of *endo*-α-*N*-acetylgalactosaminidase from *Bacillus* sp. This enzyme could transfer the disaccharide from a *p*-nitrophenyl substrate to water-soluble 1-alkanols and other alcohols at a transfer ratio of 70% or more. Although the transfer ratios were lower for water-insoluble than water-soluble alcohols, they were shown to increase by adding sodium cholate to the reaction mixtures. The enzyme also transferred the disaccharide directly from asialofetuin to 1-alkanols. The anomeric bond between the disaccharide and 1-alkanols of the transglycosylation product is in the α configuration as determined by sequential digestion of jack bean β-galactosidase and *Acremonium* α-*N*-acetylgalactosaminidase. Since the transglycosylation product, β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl, efficiently inhibits the binding of anti-T antigen monoclonal antibody to asialofetuin, it has potential as an agent for blocking T antigen-mediated cancer metastasis.

Keywords: *endo*-α-*N*-Acetylgalactosaminidase; T antigen; Glycolipid mimicry; Transglycosylation; Disaccharide

1. Introduction

Many glycosyl hydrolases (glycosidases) have been reported to have transglycosylation and/or reversed hydrolysis activity in addition to hydrolysis activity. Some endo-type glycosidases, as well as exo-type ones have these activities. We have studied the transglycosylation activity of endo-type glycosidases such as endoglycoceramidase,[1] *endo*-β-*N*-acetylglucosaminidase,[2–4] and *endo*-α-*N*-acetylgalactosaminidase (*endo*-α-GalNAc-ase),[5] which act on sugar chains of complex carbohydrates. Transglycosylation activities of these *endo*-glycosidases are a powerful tool for glycotechnology because an intact oligosaccharide can be transferred to a suitable acceptor. Previously, we reported the purification and characterization of *endo*-α-GalNAc-ase (EC 3.2.1.97) from *Bacillus* sp. The enzyme hydrolyzes the *O*-glycosidic α linkage between β-D-galactose-(1→3)-α-*N*-acetyl-D-galactosamine (β-D-Gal-(1→3)-α-D-GalNAc) and a serine or threonine residue in mucin-type glycoproteins.[5] In that paper we also reported that the enzyme could transfer the disaccharide, β-D-Gal-(1→3)-α-D-GalNAc, to mono- or disaccharides, resulting in the syntheses of oligosaccharides.

* Corresponding author. Fax: +81-75-7536275.
E-mail address: byd01621@nifty.ne.jp (H. Ashida).

0008-6215/01/$ - see front matter
PII: S0008-6215(01)00008-8

The *O*-glycosidic disaccharide, β-D-Gal-(1→3)-α-D-GalNAc, is the common core structure of the mucin-type sugar chain and is known as a carcinoma antigen, also termed the Thomsen–Friedenreich antigen (T antigen). T antigen on the normal cell surface is usually masked by sialic acids or neutral oligosaccharides attached at the nonreducing terminus; however, it is exposed on most carcinoma cells of various human tissues such as those of the intestines, breasts, ovaries and bladder.[6] Furthermore, T antigen has been thought to mediate adhesion of cancer cells, resulting in tumor metastases or infiltrations.[7] Thus, it is speculated that T antigen-containing glycolipid mimetics could inhibit the cell adhesion of carcinoma mediated by T antigen, and such compounds are considered as potential antimetastatic drugs for cancer. In addition, as the tumor-associated oligosaccharide antigens are important targets of cancer vaccines, T antigen-containing glycolipid may be used as a vaccine for T antigen-expressing cancer. For these reasons, we attempted to synthesize T antigen-containing glycolipid mimetics using the transglycosylation activity of *Bacillus endo*-α-GalNAc-ase as described by the following reaction:

β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitro phenyl (disaccharide donor)

+ 1-alkanol (acceptor) → β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-alkyl + *p*-nitrophenol

2. Experimental

Materials.—β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl was a gift from Dr Usui of Shizuoka University. Asialofetuin was prepared from bovine serum fetuin (Sigma Chemical Co.) by neuraminidase digestion. Various alcohols and detergents were purchased from Nacalai Tesque, Japan. 5-Benzyloxy-1-pentanol and 2-benzyloxyethanol were from Aldrich Chemical Co. Mouse anti-T antigen monoclonal antibody (DAKO-HB-T1) was from DAKO. Peroxidase (POD)-conjugated goat anti-mouse IgM antibody was from Sigma. Block Ace (blocking reagent) was from Dainippon Pharmaceutical, Japan. 3,3′,5,5′-Tetramethylbenzidine (TMBZ) was from Dojindo Lab., Japan.

Enzymes.—*endo*-α-GalNAc-ase was purified from the culture supernatant of *Bacillus* sp. A198 as described previously.[5] β-Galactosidase and β-*N*-acetylhexosaminidase from jack beans were purchased from Seikagaku Co., Japan. α-*N*-Acetylgalactosaminidase was purified from *Acremonium* sp. as described previously.[8,9] Neuraminidase from *Arthrobacter ureafaciens* was purchased from Nacalai Tesque, Japan.

Enzyme reaction.—The reaction mixture, composed of 60 μg of β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl (approx 2.0 mM, final concentration) and 6 mU of *endo*-α-GalNAc-ase in 50 mM potassium phosphate buffer (pH 7.0), was incubated with various acceptors (15%, v/v) in a total volume of 60 μL at 37 °C for an appropriate period. When asialofetuin was used as a disaccharide donor, 1.2 mg (20 mg/mL, final concentration) was added to the reaction mixture instead of above β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl.

Thin-layer chromatography.—The enzyme reaction mixture was analyzed on a TLC plate (no. 5547, E. Merck) using 65:35:8 chloroform–methanol–water as the developing solvent. Carbohydrate-containing compounds were visualized by spraying with orcinol–H_2SO_4 reagent and heating at 120 °C for 5 min. For quantitative determination, the TLC plate was scanned using an HP DeckScan II (Hewlett–Packard), and the color intensity of the spots was quantified with imaging software, NIH IMAGE 1.54. The transfer ratio was calculated with the following equation.

$$\text{Transfer ratio (\%)} = \frac{\text{color intensity (CI) of transglycosylation product}}{\text{CI of transglycosylation product} + \text{CI of hydrolysis product}} \times 100$$

HPLC analysis.—HPLC analysis was performed with a Hitachi L-6200 chromatograph and a L-4200 UV–Vis detector. The transglycosylation products were purified using a reversed-phase column (4.6 × 150 mm, Cosmocil $5C_{18}$-AR, Nacalai Tesque, Japan). Elution was carried out with a solvent of 10%

MeCN in water at a flow rate of 0.8 mL/min for 5 min at 40 °C, then with a linear gradient of 10–50% MeCN for 10 min. Elution was monitored by absorbance at 214 nm for β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl and at 280 nm for β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-2-benzyloxyethyl.

TOF–MS analysis.—Time of flight (TOF)–mass spectrometry was performed in the positive-ion mode on a Voyager Biospectrometry Workstation (PerSeptive Biosystems, USA) using α-cyano-4-hydroxycinnamic acid as the matrix.

ELISA.—Asialofetuin (50 μL of a solution of 500 μg/mL in PBS) was put on a 96-well plate and stored at 4 °C, overnight. After the asialofetuin solution in each well was discarded, and the plate was washed with PBS. Then 200 μL of Block Ace (diluted four-times with PBS) was added, and the mixture was incubated for 2 h at rt. Blocking solutions were discarded, and the plate was washed with PBS containing 0.1% Tween 20. Then 50 μL of anti-T antigen monoclonal antibody solution (DAKO-HB-T1, 15 μg/mL in PBS), and various amounts of T antigen-containing glycolipid mimicry were added and incubated at rt for 3 h. Peroxidase (POD)–conjugated goat anti-mouse IgM (diluted 10,000-fold with PBS) was used as the secondary antibody, and 3,3′,5,5′-tetramethylbenzidine (TMBZ) and H_2O_2 were used as substrate for POD. The enzyme reaction was carried out in 100 mM AcONa buffer (pH 5.5) containing 0.1% Tween 20 at 30 °C for 30 min, and then absorbance was measured at 450 nm.

3. Results

Transglycosylation of disaccharide from p-nitrophenyl substrate to 1-hexanol.—To examine the transglycosylation activity of *Bacillus endo*-α-GalNAc-ase to 1-alkanols, 2 mM β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl and 100 mU/mL of the enzyme were incubated with 15% (v/v) 1-hexanol as the acceptor in the absence or presence of 0.2% (w/v) sodium cholate. After incubation at 37 °C for 3 h, the reaction mixtures were analyzed by TLC using 65:35:8 chloroform–methanol–water as the developing solvent. The transglycosylation product could be hardly detected by TLC when detergent was absent from the reaction mixture. However, a new product was clearly found (R_f 0.43) when sodium cholate was added to the reaction mixture. To isolate the product, the part of the TLC plate corresponding to the mobility of the product was removed and extracted with 2:1 chloroform–methanol, and the extract was applied to the reversed-phase column on HPLC. Only one sugar-containing peak was observed, and the appropriate fractions were collected and lyophilized. The purified product was subjected to TOF–MS analysis in the positive-ion mode. The charged mass ions $[M+Na]^+$ and $[M+K]^+$ were found at m/z 490.4 and 506.4, respectively (data not shown). The molecular mass of 467.4 calculated from these values coincided with the theoretical value of β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl. These results support the hypothesis that the disaccharide liberated from β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl was transferred to 1-hexanol by *Bacillus endo*-α-GalNAc-ase.

Effects of detergents on transglycosylation.—The effects of various detergents on the transglycosylation of disaccharide to 1-hexanol by *endo*-α-GalNAc-ase were investigated. The detergents tested were Triton X-100, Tween 20, Tween 80, sodium cholate, sodium deoxycholate, and sodium dodecyl sulfate (SDS). Each was added to the reaction mixture at a concentration of 0.2% (w/v). Among the detergents tested, sodium cholate gave the highest yield of transglycosylation product (60% in transfer ratio). Sodium deoxycholate and nonionic detergents (Triton X-100, Tween 20 and Tween 80) were slighty effective (20–30%). SDS strongly inhibited the enzyme activity; thus, neither a hydrolysis nor transglycosylation product was detected.

Acceptor specificity for transglycosylation.—Acceptor specificity was examined using various 1-alkanols as the acceptor with or without sodium cholate. As shown in Fig. 1(A and B), the corresponding transglycosylation products from the various 1-alkanols were found on TLC. The color intensity of each spot was quantified with an image scanner, and the results are shown in Table 1. When sodium

cholate was not added to the reaction mixture, about 70% of the disaccharide from β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl was transferred to methanol, ethanol, 1-propanol and 1-butanol. However, when the carbon number of 1-alkanol was more than five, the yield of transglycosylation product decreased. When sodium cholate was added, the degree of transglycosylation of disaccharide to short-chain alkanols was the same as that without sodium cholate, but the transglycosylation to long-chain alkanols (C_5–C_8) was more effective than that without sodium cholate. The transfer ratios for various 1-alkanols were also determined by HPLC using reversed-phase column. The results were almost identical with those obtained by the TLC-scanning method.

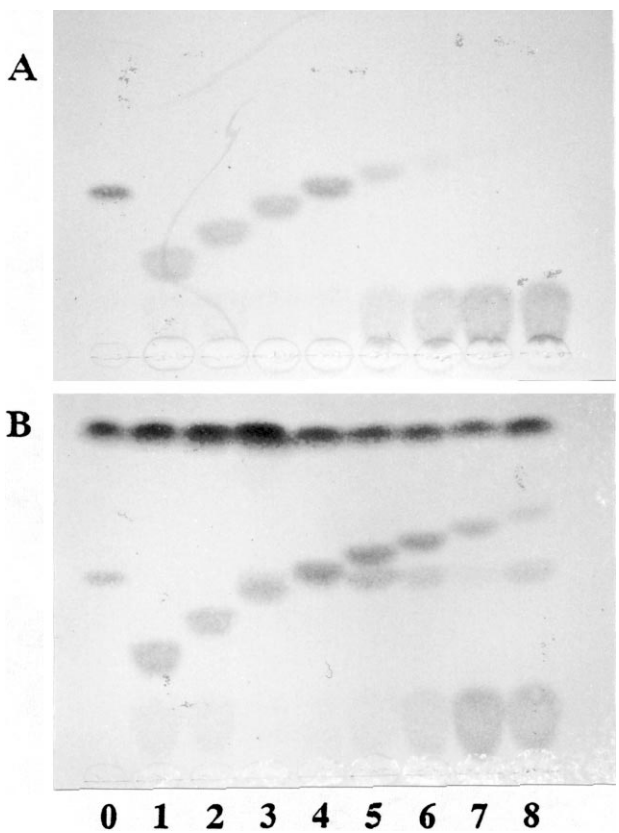

Fig. 1. Transglycosylation of disaccharide from β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl to various 1-alkanols by *endo*-α-GalNAc-ase. (A) β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl + 1-alkanol + enzyme. (B) β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl + 1-alkanol + sodium cholate + enzyme. Lane 0, donor only; lane 1, add methanol as acceptor; lane 2, ethanol; lane 3, 1-propanol; lane 4, 1-butanol; lane 5, 1-pentanol; lane 6, 1-hexanol; 7, 1-heptanol; 8, 1-octanol.

In addition to 1-alkanols, other alcohols were also tested for the transglycosylation of the disaccharide. The results are shown in Table 1. Allyl alcohol, ethylene glycol monoethyl ether and diethylene glycol monoethyl ether, which are soluble in water, were good acceptors, and their disaccharide transfer ratios were $>70\%$. 2-Benzyloxyethanol and 5-benzyloxy-1-pentanol were also accepted, but the transfer ratio of the latter was not so high.

Estimation of the anomeric bond of disaccharide to aglycone.—To confirm the anomeric configuration of the disaccharide in the transglycosylation product, 2-benzyloxyethanol was selected as the acceptor, because it is easy to identify by UV detection. The transglycosylation product was purified by HPLC as in β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl and analyzed by TOF–MS. The mass ion at 540.5 is from $[M + Na]^+$. The molecular mass of 517.5 calculated from this value coincided with the theoretical value of the transglycosylation product (data not shown). Then, the product was analyzed by sequential digestion of *exo*-glycosidases, namely β-galactosidase from jack bean and α-*N*-acetylgalactosaminidase from *Acremonium* sp. Each reaction mixture was subjected to HPLC with a reversed-phase column. As shown in Fig. 2, the transglycosylation product was completely hydrolyzed on addition of these *exo*-glycosidases. On the other hand, the product hydrolyzed with β-galactosidase was not degraded by β-*N*-acetylhexosaminidase from jack bean (data not shown). These results indicate that the anomeric bond of the transglycosylation product is of the α-D configuration.

Transglycosylation of disaccharide from native glycoprotein.—To examine the transferring activity of *endo*-α-GalNAc-ase using native glycoprotein as a donor of disaccharide, neuraminidase-treated bovine fetuin (asialofetuin) was used. Asialofetuin (20 mg/mL, final concentration) and *endo*-α-GalNAc-ase 100 (mU/mL, final concentration) were incubated with various 1-alkanols (15%, v/v) as the acceptor in the presence of 0.2% sodium cholate. After incubation at 37 °C for 6 h, the reaction mixtures were evaporated and extracted with 2:1 chloroform–methanol, and then assayed by TLC. As shown in Fig. 3, the

Table 1
Acceptor specificity for transglycosylation of disaccharide

Acceptor	Donor	Transfer ratio [a] (%)		
	Sodium cholate	β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-*p*-nitrophenyl		Asialofetuin
		−	+	+
Methanol (C_1)		71.3	70.8	56.0
Ethanol (C_2)		65.8	65.0	50.1
1-Propanol (C_3)		69.8	76.8	63.3
1-Butanol (C_4)		76.8	85.6	48.4
1-Pentanol (C_5)		28.2	73.3	18.9
1-Hexanol (C_6)		7.5	58.8	3.8
1-Heptanol (C_7)		3.1	22.7	trace
1-Octanol (C_8)		trace	14.9	trace
Allyl alcohol		85.1	86.8	60.2
Ethylene glycol monoethyl ether		79.1	79.4	59.5
Diethylene glycol monoethyl ether		77.1	78.6	58.2
2-Benzyloxyethanol		79.7	80.8	56.4
5-Benzyloxy-1-pentanol		22.5	56.3	13.7

[a] transfer ratio = color intensity (CI) of transglycosylation product/(CI of transglycosylation product + CI of hydrolysis product) × 100.

disaccharide was readily transferred to methanol, ethanol, 1-propanol and 1-butanol (transfer ratio 50–65%), and also to 1-pentanol (20%). However, little transfer occurred with 1-hexanol, and none was observed for 1-heptanol or 1-octanol. In spite of the presence of sodium cholate, the transfer ratios from asialofetuin to 1-alkanols were lower than those from *p*-nitrophenyl substrate to 1-aklanols even without sodium cholate.

Inhibition of binding of anti-T antigen antibody to asialofetuin by glycolipid mimicry.— The inhibitory activity of T antigen-containing glycolipids mimicry for binding of anti-T antigen monoclonal antibody to asialofetuin was investigated. To evaluate the activity, ELISA was carried out. Mouse anti-T (DAKO-HB-T1) and various amounts of β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl, β-D-Gal-(1→3)-D-GalNAc (free T disaccharide), or β-D-GlcNAc-(1→O)-hexyl were added to a 96-well plate previously coated with asialofetuin. As shown in Fig. 4, the control compound, β-D-GlcNAc-(1→O)-hexyl, did not inhibit the binding at all, while free T disaccharide inhibited it to a certain degree at the concentration of 250 μmol/L. However, the inhibition of enzymatically synthesized β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl was observed even at 0.25 μmol/L, and its inhibition at 2.5 μmol/L was almost the same degree as that of free T disaccharide at 250 μmol/L. These results seemed to indicate the inhibitory activity of β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl is 100-times stronger than that of free T disaccharide.

4. Discussion

In this paper, we described the syntheses of T antigen-containing glycolipid mimicry using the transglycosylation activity of *endo*-α-GalNAc-ase from *Bacillus* sp. This is the first report of enzymatic synthesis of neo-glycolipid having a mucin-type oligosaccharide. In addition to the *Bacillus* enzyme used in this study, there are two commercially available *endo*-α-GalNAc-ases from microbes, *Diplococcus pneumoniae*[10,11] and *Alcaligenes* sp.[12] These three enzymes have the same specificity for glycone, which is β-D-Gal-(1→3)-α-D-GalNAc, but differ slightly in their specificities for aglycone, viz., *Bacillus* enzyme has broader aglycone specificity than the others.[5] It was reported that *Diplococcus* enzyme catalyzed

the reversed-hydrolysis reaction as well as the transglycosylation. Although the syntheses of oligosaccharides and aminodisaccharides were achieved using the reversed-hydrolysis reaction of *Diplococcus* enzyme, the synthesis of the alkyldisaccharide has not been reported.[13] So far as we have examined, *Diplococcus* enzyme could not transfer the disaccharide from the *p*-nitrophenyl substrate to 1-alkanols in the presence of sodium cholate (data not shown). In contrast, since the *Bacillus* enzyme has relatively broad specificity for aglycone and greater stability in organic solvents such as 15% (v/v) of methanol, ethanol, acetone and dimethyl sulfoxide (data not shown), the enzyme seems to be suitable for the synthesis of neo-glycolipid. Indeed, the *Bacillus* enzyme could transfer the disaccharide from *p*-nitro phenyl substrate to various water-soluble alcohols at a transfer ratio of 70% or more. Although the transfer ratios were lower for water-insoluble than water-soluble alcohols, the transfer ratios efficiently increased when sodium cholate was added to the reaction mixtures. Furthermore, the *Bacillus* enzyme could transfer the disaccharide directly from

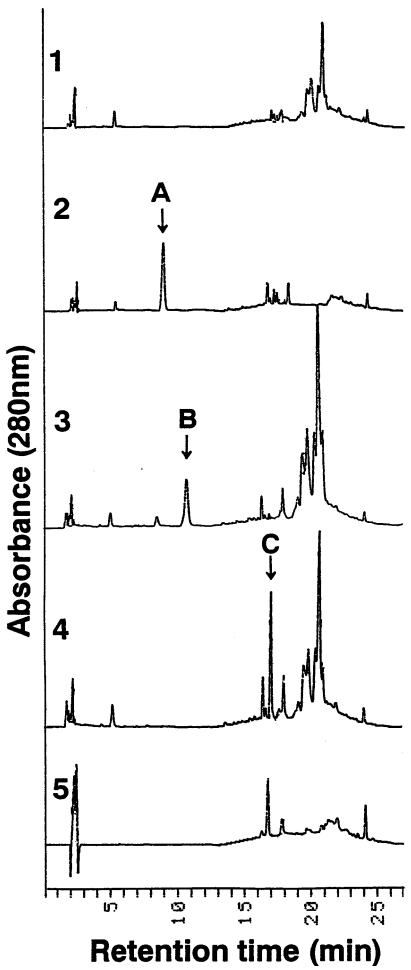

Fig. 2. Exoglycosidase digestion of β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-2-benzyloxyethyl. The reaction mixtures of exoglycosidase digestion were analyzed by HPLC on a reversed-phase column. (1) β-Galactosidase and α-*N*-acetylgalactosaminidase; (2) purified β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-2-benzyloxyethyl; (3) β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-2-benzyloxyethyl + β-galactosidase; (4) β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-2-benzyloxyethyl + β-galactosidase + α-*N*-acetylgalactosaminidase; (5) 2-benzyloxyethanol. The arrows indicate as follows: (A) β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-2-benzyloxyethyl; (B) α-D-GalNAc-(1→O)-2-benzyloxyethyl; (C) 2-benzyloxyethanol.

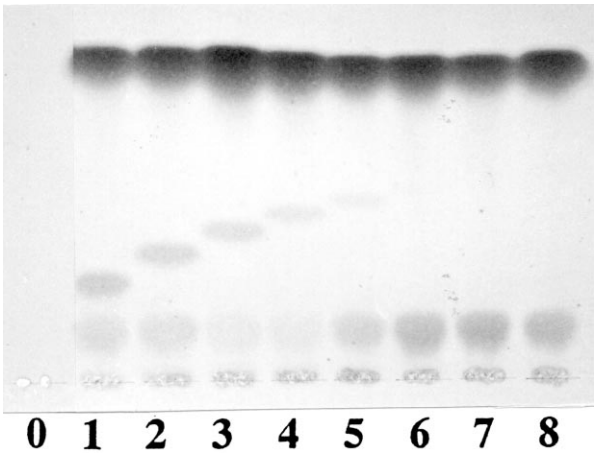

Fig. 3. Transglycosylation of disaccharide from asialofetuin to various 1-alkanols by *endo*-α-GalNAc-ase. The reaction mixture consisted of asialofetuin, 1-alkanol, sodium cholate and enzyme. Lane 0, donor only; lane 1, add methanol as acceptor; lane 2, ethanol; lane 3, 1-propanol; lane 4, 1-butanol; lane 5, 1-pentanol; lane 6, 1-hexanol; 7, 1-heptanol; 8, 1-octanol.

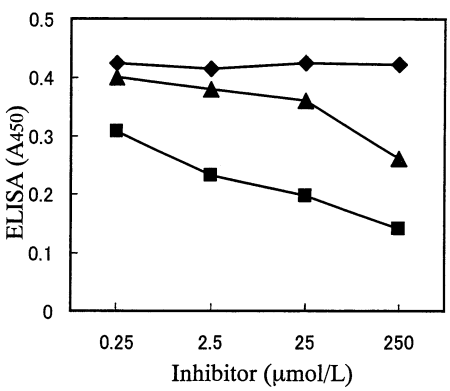

Fig. 4. Inhibition of binding of anti-T antigen antibody to asialofetuin by β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl. Anti-T antigen monoclonal antibody and various concentrations of inhibitor were incubated in wells previously coated with asialofetuin. Each point represents the mean of three determinations. Symbols: ■, β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl; ▲, β-D-Gal-(1→3)-α-D-GalNAc; ◆, β-D-GlcNAc-(1→O)-hexyl.

native glycoprotein, asialofetuin, to various alcohols. Synthesis using *p*-nitrophenyl disaccharide as the glycosyl donor is impractical because of the cost; however, the method using glycoprotein seems to be valuable. As the enzyme is a retaining type, the disaccharide was selectively transferred to the acceptor in the α configuration. It is difficult to introduce GalNAc to an alcoholic hydroxyl group in the α configuration by organic chemical methods because of interference by the *N*-acetyl group at the GalNAc C-2 position. Therefore enzymatic synthesis using *Bacillus* enzyme is an alternative to chemical synthesis. The enzymatically synthesized β-D-Gal-(1→3)-α-D-GalNAc-(1→O)-hexyl efficiently inhibited the binding of anti-T antigen antibody to asialofetuin. The binding of T-antigen to a cell receptor must play a significant role in cancer metastases or infiltrations.[7] Although our inhibition test is a model experiment, the synthetic T antigen-containing glycolipid is expected as an agent for blocking T antigen-mediated cancer metastasis.

Acknowledgements

We thank Dr K. Haneda of the Noguchi Institute, Tokyo, Japan, for help with mass analyses. We also thank Drs T. Murata and T. Usui of Shizuoka University, Shizuoka, Japan, for generously donating *p*-nitrophenyl substrate.

References

1. Ashida, H.; Tsuji, Y.; Yamamoto, K.; Kumagai, H.; Tochikura, T. *Arch. Biochem. Biophys.* **1993**, *305*, 559–562.
2. Yamamoto, K.; Kadowaki, S.; Watanabe, J.; Kumagai, H. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 244–252.
3. Haneda, K.; Inazu, T.; Yamamoto, K.; Kumagai, H.; Nakahara, Y.; Kobata, A. *Carbohydr. Res.* **1996**, *292*, 61–70.
4. Yamamoto, K.; Takegawa, K. *Trends Glycosci. Glycotechnol.* **1997**, *9*, 339–354.
5. Ashida, H.; Yamamoto, K.; Murata, T.; Usui, T.; Kumagai, H. *Arch. Biochem. Biophys.* **2000**, *373*, 394–400.
6. Brockhausen, I. *Biochim. Biophys. Acta* **1999**, *1473*, 67–95.
7. Springer, G. F. *Science* **1984**, *224*, 1198–1206.
8. Kadowaki, S.; Ueda, T.; Yamamoto, K.; Kumagai, H.; Tochikura, T. *Agric. Biol. Chem.* **1989**, *53*, 111–120.
9. Ashida, H.; Tamaki, H.; Fujimoto, T.; Yamamoto, K.; Kugamai, H. *Arch. Biochem. Biophys.* **2000**, *384*, 305–310.
10. Endo, Y.; Kobata, A. *J. Biochem. (Tokyo)* **1976**, *80*, 1–8.
11. Bhavanandan, V. P.; Umemoto, J.; Davidson, E. A. *Biochem. Biophys. Res. Commun.* **1976**, *70*, 738–745.
12. Yamamoto, K.; Fan, J.-Q.; Kadowaki, S.; Kumagai, H.; Tochikura, T. *Agric. Biol. Chem.* **1987**, *51*, 3169–3171.
13. Bardales, R. M.; Bhavanandan, V. P. *J. Biol. Chem.* **1989**, *264*, 19893–19897.