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Production of Sialyltrisaccharides Using β-Galactosidase and *trans*-Sialidase in One Pot

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Abstract Sialyltrisaccharides based on β-galactosyldisaccharides were synthesized using β-galactosidase and *trans*-sialidase in one pot. Using β-galactosidase from *Bacillus circulans* and *trans*-sialidase from *Trypanosoma cruzi* simultaneously, 6 mM sialyltrisaccharides composed of about 95% NeuAcα(2,3)Galβ(1,4)GlcNAc and 5% NeuAcα(2,3)Galβ(1,6)GlcNAc were produced from a reaction mixture containing 25 mM *o*-nitrophenyl-β-D-galactopyranoside, 100 mM N-acetylglucosamine and 10 mM p-nitrophenyl-α-D-N-acetylneuraminic acid. One beauty of this reaction was that a secondary hydrolysis of the disaccharide intermediate occurring between the activated galactopyranoside and N-acetylglucosamine was prevented. Using β-galactosidase from *Escherichia coli* and the same *trans*-sialidase, 15 mM sialyltrisaccharides composed of about 90% NeuAcα(2,3)Galβ(1,6)GlcNAc and 10% NeuAcα(2,3)Galβ(1,4)GlcNAc were produced from a reaction mixture containing 400 mM galactose, 800 mM N-acetylglucosamine and 20 mM *p*-nitrophenyl-α-D-N-acetylneuraminic acid. In this study, the reverse-galactosylation reaction between galactose and N-acetylglucosamine was dominant since the disaccharide intermediate mainly resulted in the sialylated product.

Keywords: *trans*-sialidase, β-galactosidase, *trans*-galactosylation, reverse-galactosylation

Sialyloligosaccharides are involved in a variety of important biological processes such as immune reaction [1], cell-surface communication [2], and microbial or viral infection [3]. Due to their high regioselectivity and stereoselectivity, enzymatic approaches using glycosyltransferases or glycosidases have been recognized to be useful for the production of sialyloligosaccharides [4]. Especially, a study on the production of sialyl-N-acetyllactosamine and its derivative sialyltrisaccharides was carried out using galactosyltransferase and sialyltransferase with *in situ* regeneration of sugar-nucleotides [5]. The sialylation of purified disaccharides produced by β-galactosidase catalyzed trans-galactosylation or reverse-galactosylation can also synthesize sialyltrisaccharides [6]. Although galactosylation using β-galactosidase does not require a high-cost sugar-nucleotide regeneration system, some problems in β-galactosidase catalyzed reactions limit wide use of β-galactosidase. Trans-galactosylation, *i.e.* a kinetically controlled β-galactosidase catalyzed reaction, makes the process control difficult due to the secondary hydrolysis of the product. Reverse-galactosylation, *i.e.* an equilibrium-controlled β-galactosidase catalyzed reaction, shows an extremely low yield of the product due to a thermodynamically unfavorable equilibrium for the production of disaccharide [6]. Thus, producing sialyltrisaccharides using β-galactosidase is eventually hindered in galactosylation step.

The main purpose of this communication was to show that the use of β-galactosidase with *trans*-sialidase in one-pot can change the reaction kinetics in a more favorable way, improving the yield in the production of sialyltrisaccharides. The problems of trans-galactosylation or reverse-galactosylation on the sialyltrisaccharide synthesis mentioned above mainly originate from the hydrolysis of the produced galactosyldisaccharides by β-galactosidase. Therefore, if newly synthesized galactosyldisaccharides were to be sialylated in one-pot, sialyltrisaccharides may be efficiently produced without the problems of hydrolysis by β-galactosidase since the sialylated galactosides would not be the substrates for β-galactosidase [7]. For the sialylation in one-pot reaction, recombinant *trans*-sialidase from *Trypanosoma cruzi* was examined. The *trans*-sialidase from *T. cruzi* is an enzyme that preferentially transfers sialic acid from donors to β-galactosides, subsequently yielding α(2,3)sialylgalactosides [8].

Expression of the cloned *trans*-sialidase from *T. cruzi* in *E. coli* strain carrying pTSCat was performed according to the method of Smith *et al.* [9], and its purification using His tag on the C terminus of the enzyme was carried out on a column of Ni-agarose (Qiagen, Santa Clarita, USA) (1 × 2 cm) according to Qiagen instructions. Purified *trans*-sialidase was assayed using ^{14}C-labeled lactose as a NeuAc acceptor [8]. One unit of

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trans-sialidase was defined as the amount of the enzyme catalyzing 1 μmol of sialic acid from sialyllactose incorporated into lactose per min. β-Galactosidase from *Bacillus circulans* was generously donated from Daiwa Kasei (Osaka, Japan), and all the other ingredients were purchased from Sigma. β-Glactosidases from *B. circulans* and *E. coli* were assayed using the o-nitrophenyl-β-D-galactopyranoside (ONPG) method [10] and one unit of β-galactosidase was defined as the amount of the enzyme catalyzing the hydrolysis of 1 μmol of ONPG per min. Reactions were monitored by HPLC (Model 660, Waters) equipped with a photodiode array detector using Aminex-87H column (Bio-Rad) at 200 nm. The column was eluted with isocratic 0.005 M sulfuric acid at a flow rate of 0.6 mL/min at 40°C. The amounts of galactosyldisaccharides (retention time (RT): 8.8 min) composed of Galβ(1,4)GlcNAc and Galβ(1,6)GlcNAc were determined using the calibration curves of original samples. Since Galβ(1,4)GlcNAc and Galβ(1,6)GlcNAc showed almost identical UV spectra, the amounts of sialyltrisaccharides (RT: 6.8 min) composed of NeuAcα(2,3)Galβ(1,4)GlcNAc and NeuAcα(2,3)Galβ(1,6)GlcNAc were determined using the calibration curve of the original NeuAcα(2,3)Galβ(1,4)GlcNAc based on the assumption that the unavailable NeuAcα(2,3)Galβ(1,6)GlcNAc would have the same extinction coefficient as that of NeuAcα(2,3)Galβ(1,4)GlcNAc. To monitor Galβ(1,4)GlcNAc (RT: 17.8 min) and Galβ(1,6)GlcNAc (RT: 22.1 min) separately, a polyamine II column (YMC Co. Kyoto, Japan) was eluted with isocratic solvent (acetonitrile:water = 75:25) at a flow rate of 1 mL/min [11].

Firstly, trans-galactosylation followed by *trans*-sialidase reaction was carried out in one pot (Fig. 1(a)). β-Galactosidase catalyzed disaccharide production was also executed as a control reaction. For the control reaction, ONPG, N-acetylglucosamine (GlcNAc), and β-galactosidase from *B. circulans* were used as the donor, acceptor and catalyst, respectively. The β-galactosidase from *B. circulans* is known to have β-1,4 linkage specificity and has been exploited in the synthesis of β-1,4-galactosyldisaccharides [10]. The closed circles in Fig. 1(b) plot the time course of the production of β-galactosyl N-acetylglucosamine in the control reaction. The maximum yield of the disaccharide based on the initial ONPG concentration was about 25%. Analysis by HPLC using a polyamine II column showed that the product consisted of 95% Galβ(1,4)GlcNAc and 5% Galβ(1,6)GlcNAc. This result was almost identical to that of a previous report [12]. As shown in Fig. 1(b), the secondary hydrolysis of the produced disaccharide occurred vigorously after a certain maximum yield. For one-pot reaction, *trans*-sialidase and p-nitrophenyl-α-D-N-acetylneuraminic acid (PNP-NeuAc) were additionally added to the above β-galactosidase catalyzed reaction. The open circles in Figure 1(b) plot the time course of the production of sialyltrisaccharide by one-pot reaction. The maximum yields of the trisaccharide based on the initial ONPG concentration and initial PNP-NeuAc concentration were about 24% and 60%, respectively. These values were comparable to the maximum yield of

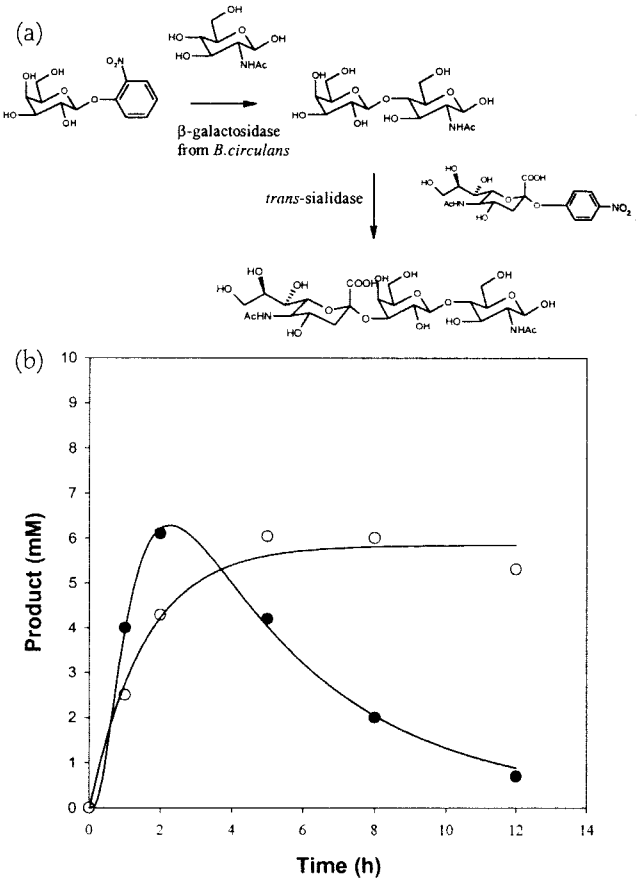

Fig. 1. (a) Schematic diagram of trans-galactosylation followed by *trans*-sialidase catalyzed reaction in one pot. (b) Time courses of galactosyldisaccharide production (●) using only β-galactosidase catalyzed trans-galactosylation and sialyltrisaccharide production (○) using one-pot reaction. For the trans-galactosylation, 50 μL reaction mixture containing 25 mM ONPG, 100 mM GlcNAc, and 0.5 unit/mL of β-galactosidase from *B. circulans* in 100 mM phosphate buffer (pH 7.0) was incubated for 12 h at 30°C, and the reaction was monitored with time. For one pot reaction, all reaction condition was same with that of the trans-galactosylation except that *trans*-sialidase and PNP-NeuAc were additionally added to result in the final concentrations of 0.5 unit/mL and 10 mM, respectively.

the disaccharide using only β-galactosidase. From the results obtained with the control reaction, we presumed that the produced sialyltrisaccharides were comprised of about 95% NeuAcα(2,3)Galβ(1,4)GlcNAc and 5% NeuAcα(2,3)Galβ(1,6)GlcNAc. The production profile showed that newly synthesized sialyltrisaccharide does not decrease with time, suggesting that secondary hydrolysis of the intermediate was somewhat suppressed.

A similar approach using the one-pot reaction mentioned previously was attempted by Herrman *et al.* [13] and Kren and Thiem [14]. However, their approach differs from ours in that they used sialyltransferase requiring a sugar-nucleotide (CMP-NeuAc) regeneration sys-

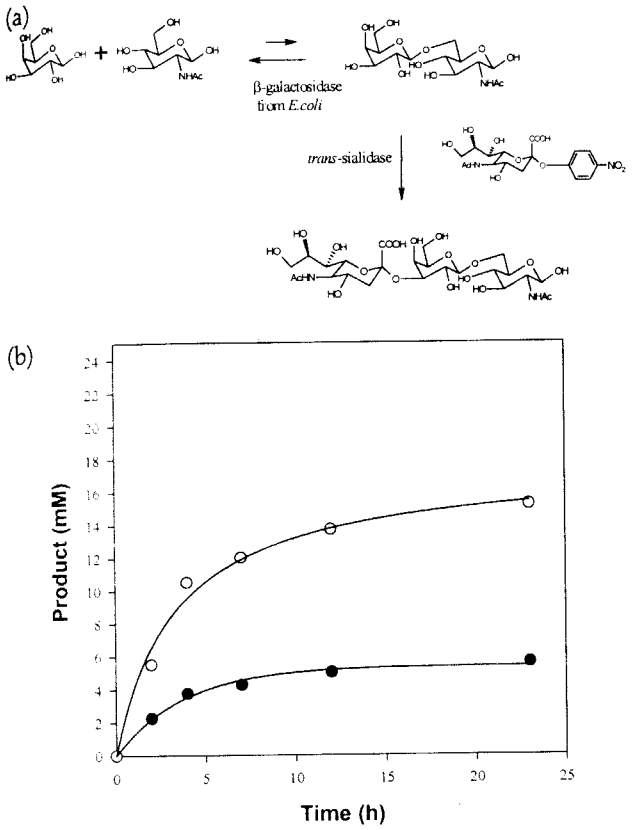

Fig. 2. (a) Schematic diagram of the reverse-galactosylation followed by *trans*-sialidase catalyzed reaction in one pot. (b) Time courses of disaccharide production (●) using β-galactosidase catalyzed reverse-galactosylation and sialyltrisaccharide production (○) using one-pot reaction. For reverse-galactosylation, 50 μL reaction mixture containing 400 mM galactose, 800 mM GlcNAc, and 20 unit/mL of β-galactosidase from *E. coli* in 100 mM phosphate buffer (pH 7.0) was incubated for 24 h at 30°C and the reaction was monitored with time. For one pot reaction, all reaction condition was same with that of the reverse-galactosylation except that *trans*-sialidase and PNP-NeuAc were additionally added to result in the final concentrations of 0.5 unit/mL and 20 mM, respectively.

tem for sialylation. As shown previously, we could overcome the limitation resulting from using sialyltransferase by using *trans*-sialidase from *T. cruzi*, the enzyme not requiring the high-cost sugar-nucleotide regeneration system. In addition, their approach had another problem in that different sialyltransferases must be used for the sialylation of different galactosyldisaccharides due to the strict acceptor specificities of sialyltransferases [15]. This limitation can also be overcome by using *trans*-sialidase due to the broad acceptor specificity of *trans*-sialidase [8].

Secondly, a reverse-galactosylation followed by *trans*-sialidase catalyzed reaction was executed in one pot (Fig. 2(a)). As a control reaction, disaccharide was produced by using β-galactosidase catalyzed reverse-galactosylation. Galactose (400 mM), GlcNAc (800 mM) and β-galactosidase from *E.coli* showing β-1,6 linkage specificity were used as the donor, acceptor and catalyst, respectively, for the control reaction. The closed circles in Fig. 2(b) plot the time course of the control reaction. The yield of disaccharide based on the initial galactose concentration was about 1.4% at equilibrium. Analysis by HPLC with polyamine II column showed that the produced disaccharide was composed of 90% Galβ(1,6)GlcNAc and 10% Galβ(1,4)GlcNAc, as reported by another study [16]. For one-pot reaction, *trans*-sialidase and PNP-NeuAc were additionally added to the β-galactosidase catalyzed reverse-galactosylation. The open circles in Fig. 2(b) plot the time course of sialyltrisaccharide production by one-pot reaction. The yields of trisaccharide based on the initial galactose concentration and initial PNP-NeuAc concentration were 3.8% and 76%, respectively. From the results obtained with the control reaction, we presumed that produced sialyltrisaccharides were comprised of about 90% NeuAcα(2,3)Galβ(1,6)GlcNAc and 10% NeuAcα(2,3)Galβ(1,4)GlcNAc. As shown in Fig. 2(b), the sialyltrisaccharide yield from one-pot reaction was higher than the disaccharide yield using only reverse-galactosylation. This result suggests that reverse-galactosylation in one-pot reaction can result in the product by following the sialylation. To our knowledge, this reaction mode is the first sialyltrisaccharide production method shifting the direction of reverse-galactosylation. Despite the use of very inexpensive donor (galactose), reverse-galactosylation has not been used widely compared to trans-galactosylation because of very low yields. If reverse-galactosylation were to be coupled with sialylation as shown above, reverse-galactosylation can be more widely used for the production of sialyltrisaccharides.

In this paper, we showed that one of the two reaction modes, either *trans*-galactosylation or reverse-galactosylation, followed by *trans*-sialidase catalyzed sialylation in one pot, produced sialyl-trisaccharides. The advantages of the suggested scheme are as follows: 1) The yield of sialyltrisaccharide can be increased by reducing the secondary hydrolysis reaction of β-galactosidase. 2) Laborious purification of the intermediates can be avoided since sialyltrisaccharides can be easily purified from the mixture using an anion exchange column. 3) By using both β-galactosidase and *trans*-sialidase, the sugar-nucleotide regeneration step is not required. 4) Although we showed only the synthesis of sialyl-N-acetyllactosamine sequences in this report, various sequences can be produced by varying one-pot reaction due to the broad acceptor specificities of β-galactosidases and *trans*-sialidase. The limitation of this scheme was that some by-product can be produced at the same time, since the regioselectivity of β-galactosidase is not strict compared with that of galactosyltransferase. However, this limitation can be overcome if β-galactosidases of high regioselectivity [11] or reaction conditions which bestow high regioselectivity of the enzymes [17] were to be used. In conclusion, although reaction conditions were not optimized in our experi-

ments, our results suggest that it is possible to produce various sialylgalactosides more efficiently by using our one-pot reaction scheme.

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