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Efficient Synthetic Method of Obtaining Oligosaccharide Units and Derivatives Utilizing Endoglycosidases

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Abstract Our purpose is to develop an efficient synthetic method of obtaining oligosaccharide unit in sufficient amounts to study functions of glycans. Many exoglycosidases have been used as tools for the oligosaccharide synthesis. In contrast, a limited number of reports are available on the utilization of endoglycosidases. We describe herewith the efficient synthetic method of useful oligosaccharides and derivatives as biomaterials utilizing lysozyme, cellulase, and lacto-*N*-biosidase-mediated transglycosylations.

Keywords: lysozyme; cellulase; lacto-*N*-biosidase; transglycosylation

INTRODUCTION

Oligosaccharide units play many important roles in biological processes such as cell-cell recognition, growth, differentiation, pathogenic infection, and cancer [1]. Oligosaccharide units are recognized as particularly challenging targets for regioselective glycosylation by either chemical or enzymatic synthesis to elucidate further biological function. Chemical methods for obtaining oligosaccharide units have been exclusively developed [2], but they involve various elaborate procedures for protection, glycosylation and deprotection. The enzymatic approach for oligosaccharide synthesis has been done in part with glycosyltransferase and glycosidase [3-6]. The use of enzymatic transglycosylation is synthetically useful, since no protection is required and the configuration at the newly formed anomeric center is determined by the specificity of the enzyme. The former enzymes generally catalyze the formation of the glycosidic linkages regio- and stereo-selectively with sugar nucleotides as donor substrate. However, it is not facilitated for practical synthesis of oligosaccharide units, because of great difficulty for obtaining sufficient amount of enzymes and their donor substrates. On the other hand, the latter enzymes usually not only hydrolyze glycosidic bonds, but also catalyze the newly formation of glycosides. From practical viewpoint, the use of glycosidase-mediated transglycosylation is attractive for oligosaccharide synthesis, because glycosidases are generally more available, and less expensive than the glycosyltransferases, and do not require expensive sugar nucleotide donors [5]. Therefore, many exoglycosidases have been used as tools for the oligosaccharide synthesis. In contrast, a limited number of reports are available on the utilization of endoglycosidases [7-11]. Here we describe the recent development of endoglycosidases-catalyzed oligosaccharide synthesis.

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LYSOZYME-MEDIATED TRANSGLYCOSYLATION

Hexa-*N*-acetylchitohexaose and Hepta -*N*-acetyl-chitoheptaose

Lysozyme, which is essentially hydrolase, was useful for the preparative scale synthesis of hexa-*N*-acetylchitohexaose $(GlcNAc)_6$ and hexa-*N*-acetylchitoheptaose $(GlcNAc)_7$, by utilizing the transglycosylation activity under controlled conditions [12]. An efficient synthesis of $(GlcNAc)_6$ and $(GlcNAc)_7$ was carried out by utilizing commercially available hen egg-white lysozyme from di-*N*-acetylchitobiose $(GlcNAc)_2$ as an initial substrate. Two hundred mg of $(GlcNAc)_2$ was dissolved in 2 mL of 0.1 M acetate buffer containing 30% ammonium sulfate. To this solution was added 20 mg of lysozyme and the mixture was kept for 8 h at 70°C. As time progressed, the medium became turbid and eventually formed a precipitate like gel. After the precipitate was dissolved in water, soluble portion was desalted and lyophilized to afford 42 mg containing mostly $(GlcNAc)_6$ and $(GlcNAc)_7$ in a molar ratio of 3 : 2. In this case, the addition of ammonium sulfate to the reaction system resulted in a remarkable increase of the hexamer and heptamer productions, which are desirable as biologically active oligosaccharides.

Chromogenic Chitooligoside

p-Nitorophenyl penta-*N*-acetyl-β-chitopentaoside (*p*NP-β-$(GlcNAc)_5$), which is available as a substrate for lysozyme assay, was efficiently synthesized utilizing

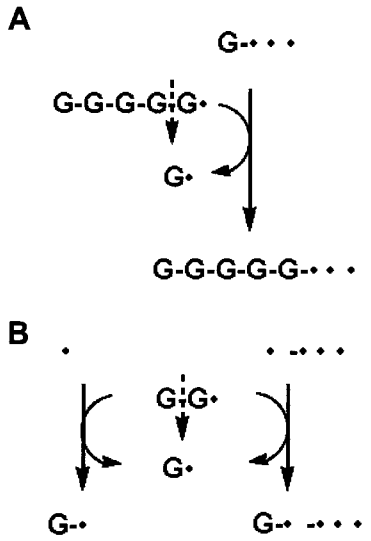

Fig. 1. Proposed mechanism of lysozyme-mediated transglycosylation. (A), Preparation of *p*NP-β-$(GlcNAc)_5$ from $(GlcNAc)_5$ and *p*NP-β-$(GlcNAc)_5$. (B), Preparation of GlcNAcβ1-4Man and GlcNAcβ1-4Manβ-*p*NP from $(GlcNAc)_2$ and Man or *p*NP-β-Man. G, *N*-acetyl-D-glucosamine. G*, reducing end residue. M, D-Mannose. *p*NP, *p*-nitrophenyl.

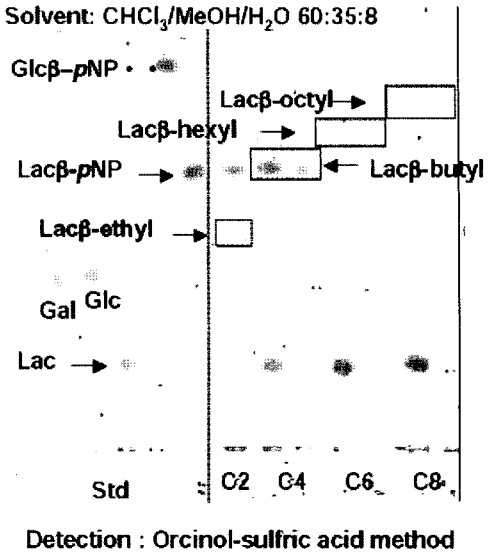

Fig. 2. TLC analysis of transglycosylation products to various 1-alkanols. Aliquot (2 μL) of each reaction mixture was analyzed on TLC plate by orcinol-sulfuric acid method. Acceptors: ethanol (C2), 1-butanol (C4), 1-hexanol (C6), and 1-octanol (C8).

transglycosylation from penta-*N*-acetylchitopentaose $(GlcNAc)_5$ to *p*-nitrophenyl β-*N*-acetylglucosaminide (*p*NP-β-GlcNAc) (Fig. 1A) [13,14]. To a solution of $(GlcNAc)_5$ (1 g, 1 mmol) and *p*NP-β-GlcNAc (3.97 g, 1.2 mmol) in 200 mM of acetate buffer containing 50% dimethylsulfoxide (Me_2SO) was added 1.5% hen egg-white lysozyme and the reaction mixture was left for 150 h at 50°C. As time progressed, *p*NP-β-$(GlcNAc)_5$ was precipitated from the reaction mixture. The precipitate was obtained as *p*NP-$(GlcNAc)_5$ by washing sufficiently with 50% methanol without using any column chromatographic separation in a high state of purity (98.5% on HPLC). The yield was 520 mg in a yield of 46% based on the $(GlcNAc)_5$ added. The reaction indicates that a disproportion of $(GlcNAc)_5$ as the donor splits predominantly tetrasaccharide unit to produce desired *p*NP-β-$(GlcNAc)_5$. Thus, $(GlcNAc)_5$ serves as a donor to transfer regioselectively tetra-*N*-acetylchitotetraosyl residue to *p*NP-β-GlcNAc acceptor. As a result, the addition of Me_2SO to the reaction system not only ensured the solubility of *p*NP-β-GlcNAc, but also resulted in the high yield of pNP-β-$(GlcNAc)_5$. The synthetic compound was very useful as a novel substrate for a colorimetric assay of hen egg white and human urinary lysozymes through a conjugated reaction involving β-*N*-acetylhexiosaminidase [15].

4-*O*-β-*N*-acetylglucosaminyl-mannose

Hen egg-white lysozyme also had GlcNAc transferase activity and was utilized to synthesize 4-*O*-β-*N*-acetylglucosaminyl-mannnose (GlcNAcβ1-4Man) sequence present in *N*-linked glycan. The enzyme produced GlcNAcβ1-4Man through a regioselective transglycosylation from *N,N'*-diacetylchitobiose $(GlcNAc)_2$ and Man in a yield of 20.9% based on the donor added (Fig. 1B) [16]. It was capable of transferring an *N*-acetylglucosaminyl group from the donor exclusively to the OH-4 of the Man moiety. When the donor was $(GlcNAc)_4$ instead of $(GlcNAc)_2$, the rate of formation of the desired GlcNAcβ1-4Man was 6-7 fold faster. This is also the case for the synthesis of GlcNAcβ1-4Manβ-$OC_6H_4NO_2$-*p* with *p*-nitrophenyl β-D-mannopyranoside as an acceptor. It was obtained in a yield of 10.5% based on the donor added. The addition of Me_2SO to the reaction enhanced the solubility of *p*-nitrophenyl β-D-mannopyranoside to result in a higher yield of the desired compound. The maximum production of the desired compound at 50% Me_2SO was about 3-fold higher than that obtained in a reaction done in the medium in its absence. These enzyme reactions were efficient enough to allow the one-pot preparation of the desired compounds.

CELLULASE-MEDIATED TRANSGLYCOSYLATION

Aliphatic β-lactoside

Our interest was directed to an enzymatic approach involved in lactosyl unit β-glycosidically linked to aliphatic groups as mimic units of glycosphingolipids. Endo-β-glycosidase activity, which cleaves the endo-

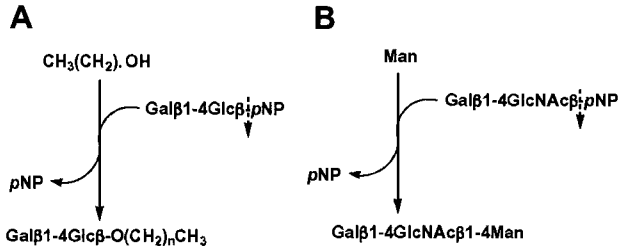

Fig. 3. Proposed mechanism of *Trichoderma reesei* cellulase-mediated transglycosylation. (A), Preparation of aliphatic β-lactosides from *p*-nitrophenyl β-lactoside and 1-alkanols. n= 7 and 11. (B), Preparation Galβ1-4GlcNAcβ1-4Man from Galβ1-4GlcNAcβ-*p*NP and D-mannose.

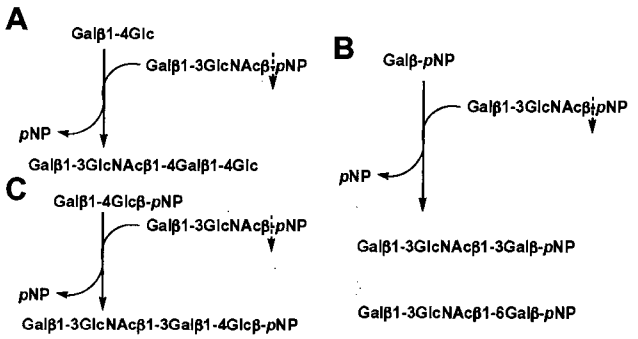

Fig. 4. Proposed mechanism of *Aureobacterium* sp. L-101 lacto-*N*-biosidase-mediated transglycosylation. (A), Preparation of LNT from lactose and Galβ1-3GlcNAcβ-*p*NP. (B), Preparation of LNTβ-*p*NP from *p*-nitrophenyl β-lactoseide and Galβ1-3GlcNAcβ-*p*NP. (C), Preparation of Galβ1-3GlcNAcβ-3Galβ-*p*NP and Galβ1-3GlcNAcβ-6Galβ-*p*NP from Galβ-*p*NP and Galβ1-3GlcNAcβ-*p*NP.

glycosidic linkage from synthetic *p*-nitrophenyl β-lactoside (Lacβ-*p*NP) and *p*-nitrophenyl β-*N*-acetyllactosaminide (LacNAcβ-*p*NP) substrates into, respectively, lactose and *N*-acetyllactosamine and *p*-nitrophenol, was searched from various kinds of commercially available glycosidase preparations. We found such hydrolytic activities in the cellulase preparation from *Trichoderma reesei* produced various types of β-1,4-glucanases. Since the crude enzyme also catalyzed translactosylation and trans-*N*-acetyllactosaminylation with Lacβ-*p*NP and LacNAcβ-*p*NP, respectively, it was directly used for the synthesis of oligosaccharide glycosides. Aliphatic β-lactosides were conveniently synthesized by β-lactosyl transfer reaction from Lacβ-*p*NP donor to various 1-alkanols (n = 2-12) acceptors, utilizing cellulase from *T. reesei* (Fig. 2). With ethanol acceptor, the enzyme induced ethyl β-lactoside in 18% yield based on the donor added in aqueous system. However, the yield decreased as the alkyl chain of 1-alkanol acceptors increased from C2 to C8. With 1-octanol acceptor, octyl β-lactoside was observed in a low yield (<1%). This problem was partially solved by using sodium cholate as the detergent. When 1-octanol and 1-dodecanol acceptors were incubated in the presence of sodium cholate, the transfer products octyl β-lactoside and dodecyl β-lactoside were obtained in 13 and 5% yields based on the donor added, respectively (Fig. 3A). The addition of sodium cholate to the reaction system not only guaranteed solubility of the substrate, but also resulted in a remarkable increase in the yield. These enzyme reactions were efficient enough to allow the one-pot preparation of the desired lactoside derivatives [17].

Galβ1-4GlcNAcβ1-4Man

The enzyme also had LacNAc transferring activity and was utilized to synthesize a part of antennary structure of *N*-linked sugar chain. Some monosaccharides were used to examine acceptor specificity on the enzyme-mediated transglycosylation. With Glc, Man, and GlcNAc as acceptors, only a single transfer product was observed in HPLC analysis. Gal and GalNAc did not serve as acceptors. These findings show that the configuration of OH-4 position in the glycosyl acceptors had a pronounced effect on the transglycosylation. Preparative scale syntheses were done utilizing Man and Glc as acceptors with a LacNAcβ-*p*NP donor. As a result, the enzyme transferred an *N*-acetyllactosamine unit from the donor exclusively to the OH-4 of Man and Glc moieties (Fig. 3B). The compounds Galβ1-4GlcNAcβ1-4Man and Galβ1-4GlcNAcβ1-4Glc were obtained in 13 and 9% yields based on the donor added, respectively. These products were conveniently isolated by one-step chromatographic separation on charcoal-Celite columns [17].

DIRECT CONVERSION OF LACTOSE INTO LNT

Human milk oligosaccharides, the third most abundant solid constituent in human milk, have several biological activities such as protective activity against pathogens and growth factor for bifidobacterium [18]. Therefore, there is a high current interest in synthesizing human milk oligosaccharides. In this section, we report the direct conversion of lactose into lacto-*N*-tetraose (Galβ-3GlcNAcβ1-3Galβ1-4Glc, LNT), which is one of core tetrasaccharide unit of human milk oligosaccharide, by lacto-*N*-biosidase-mediated transglycosylation. Lacto-*N*-biosidase as an endoglycosidase specifically hydrolyzes lacto-*N*-biosidic linkages in internal oligosaccharide chains [19]. It also acts on synthetic Galβ1-3GlcNAcβ-*p*NP to hydrolyze Galβ1-3GlcNAc and *p*-nitrophenol, that is, it can bypass a block of the disaccharide. When Galβ1-3GlcNAcβ-*p*NP was used as a donor and lactose was used as an acceptor, the enzyme from *Aureobacterium* sp. L-101 catalyzed the transfer of a β-lacto-*N*-biosyl unit to the OH-3' position of lactose (Fig. 4A). The resulting LNT was roughly separated by successive chromatography on ODS Chromatorex DM1020T and charcoal-Celite columns. The complete separation was finally effected by HPAEC-PAD in 0.6%

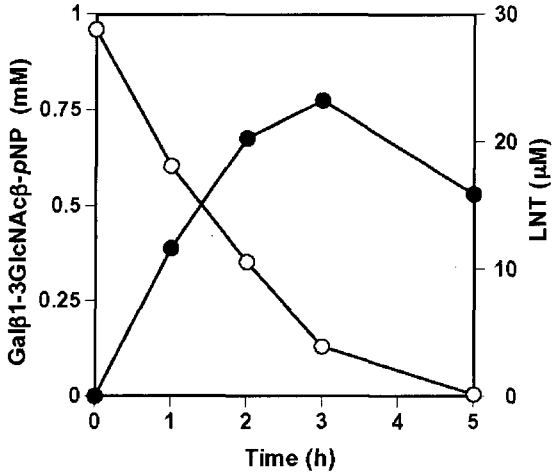

Fig. 5. Time course of the formation of LNT and the degradation of Galβ1-3GlcNAcβ-*p*NP. Four milligrams of Galβ1-4GlcNAcβ-*p*NP and 27.4 mg of lactose were incubated at 40°C in 300 mL of 40 mM Na-Ac. buf. (pH 5.5) with 200 mU of lacto-*N*-biosidase. ●, concentration of LNT; ○, concentration of Galβ1-4GlcNAcβ-*p*NP.

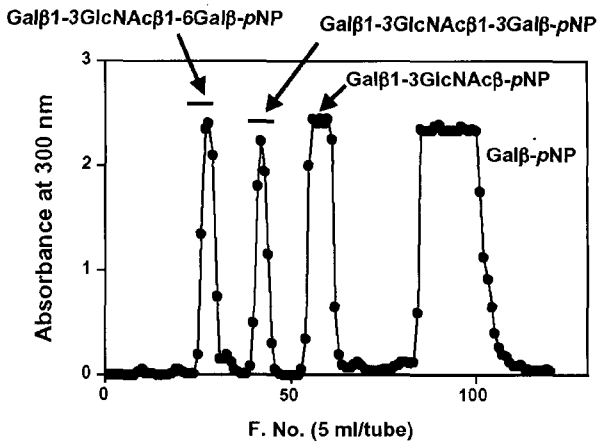

Fig. 6. Toyopearl HW-40S chromatograpy of transglycosylation products formed from Galβ1-3GlcNAcβ-*p*NP and Galβ-*p*NP by lacto-*N*-biosidase. The reaction was performed in the mixture of 100 mg of Galβ1-3GlcNAcβ-*p*NP, 480 mg of Galβ-*p*NP, 10.4 U of lacto-N-bisidase and 18 mL of 40 mM sodium phosphate buffer (pH 5.5) for 100 min at 40°C. The reaction mixture was applied onto a Toyopearl HW-40S column (ϕ 1.5 × 90 cm) equilibrated with 25% methanol. Flow rate, 5 mL/min.

yield based on the donor substrate [20]. The time course of LNT production and the degradation of donor substrate could be observed by HPAEC-PAD, as shown in Fig. 5. The maximum LNT production was reached at 3 h and its concentration rapidly decreased during the subsequent reaction. The yield of LNT at 3 h was observed by analysis to be 3.7% based on the donor. The large difference in the yields between the actual and analytical data should be accompanied by a loss of LNT through the process of three chromatographic separation procedures. Similarly, when Galβ-*p*NP and Lacβ-*p*NP were used as acceptors, the enzyme induced the formation of Galβ1-3GlcNAcβ1-3Galβ-*p*NP, Galβ1-3GlcNAcβ1-6Galβ-*p*NP, and Galβ1-3GlcNAcβ1-3Galβ-4GlcNAcβ-*p*NP, respectively, in 1.9, 1.8, and 0.6% based on the donor (Fig. 4B, C). These products were easily separated by Toyopearl HW-40S chromatography (Fig. 6). This is the first report that lacto-*N*-biosidase-mediated transglycosylation enabled the direct synthesis of LNT from lactose as an initial substrate, although it was rather inefficient.

CONCLUSION

Biologically important oligosaccharides and derivatives have been prepared through endoglycosidase-mediated transglycosylation. The enzymes catalyzed the transfer of oligosaccharide units (DP = 2 to 4) from donor substrates to not only sugar acceptors but also 1-alkanols. The yields were considered to be sufficiently high for the practical method except for lacto-*N*-biosidase, because of the simplicity of enzymatic synthesis. Such enzymes are readily available in large amounts. Excess of unreacted substrates are recovered by straightforward column chromatography and reutilized for the synthesis. We are presently engaged in expanding the method to other endoglycosidases for obtaining important oligosaccharides.

REFERENCES

[1] Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* 3: 97-130.
[2] Kanemitsu, T. and O. Kanie (1999) Carbohydrate-related libraries. *Trends Glycosci. Glycotechnol.* 11: 267-276.
[3] Murata, T. and T. Usui (2000) Enzymatic synthesis of important oligosaccharide units involved in *N*- and *O*-linked glycans. *Trends Glycosci. Glycotechnol.* 12: 161-174.
[4] Koeller, K. M. and C. H. Wong (2000) Emerging themes in medicinal glycoscience. *Nat. Biotechnol.* 18: 835-841.
[5] Murata, T. and T. Usui (1997) Preparation of oligosaccharide units library and its utilization. *Biosci. Biotech. Biochem.* 61: 1059-1055.
[6] Ichikawa, Y., G. C. Look, and C.-H. Wong (1992) Enzyme-catalyzed oligosaccharide synthesis. *Anal. Biochem.* 20: 215-238.
[7] Yamamoto, K., S. Kadowaki, J. Watanabe, and H. Kumagai (1994) Transglycosylation activity of Mucor hiemalis endo-β-*N*-acetylglucosaminidase which transfers complex oligosaccharides to the *N*-acetylglucosamine moieties of peptides. *Biochem. Biophys. Res. Commun.* 203: 244-252.
[8] Saitoh, H., K. Takagaki, M. Majima, T. Nakamura, A. Matsuki, M. Kasai, H. Narita, and M. Endo (1995) Enzymic reconstruction of glycosaminoglycan oligosaccharide chains using the transglycosylation reaction of bovine testicular hyaluronidase. *J. Biol. Chem.* 270: 3741-

3747.
[9] Shoda, S., M. Fujita, and S. Kobayashi (1998) Glycanase-catalyzed synthesis of non-natural oligosaccharides. *Trends Glycosci. Glycotechnol.* 10: 279-289.
[10] Takegawa, K., K. Fujita, J. Q. Fan, M. Tabuchi, N. Tanaka, A. Kondo, H. Iwamoto, I. Kato, Y. C. Lee, and S. Iwahara (1998) Enzymatic synthesis of a neoglycoconjugate by transglycosylation with *Arthrobacter* endo-β-*N*-acetylglucosaminidase: A substrate for colorimetric detection of endo-β-*N*-acetylglucosaminidase activity. *Anal. Biochem.* 257: 218-223.
[11] Ashida, H., K. Anderson, J. Nakayama, K. Maskos, C.-W. Chou, R. B. Cole, S.-C. Li, and Y.-T. Li (2001) A novel endo-β-galactosidase from *Clostridium perfringens* that liberates the disaccharide GlcNAcα1-4Gal from glycans specifically expressed in the gastric gland mucous cell-type mucin. *J. Biol. Chem.* 276: 28226-28232.
[12] Usui, T., H. Matsui, and K. Isobe (1990) Enzymic synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate. *Carbohydr. Res.* 203: 65-77.
[13] Usui, T., Y. Hayashi, F. Nanjo, and Y. Ishido (1988) Enzymatic synthesis of *p*-nitrophenyl *N,N',N'',N''',N''''*-penta-acetyl-β-chitopentaoside in water-methanol system; significance as a substrate for lysozyme assay. *Biochim. Biophys. Acta* 953: 179-184.
[14] Usui, T. and H. Matsui (1989) Lysozyme-mediated *p*-nitrophenyl penta-*N*-acetyl-β-chitopentaoside production in aqueous-dimethyl sulfoxide solvent system, as a substrate for a lysozyme assay. *Agric. Biol. Chem.* 53: 383-388.
[15] Nanjo, F., K. Sakai, and T. Usui (1988) *p*-Nitrophenyl penta-*N*-acetyl-β-chitopentaoside as a novel synthetic substrate for the colorimetric assay of lysozyme. *J. Biochem.* 104: 255-258.
[16] Matahira, Y., K. Ohno, M. Kawaguchi, H. Kawagishi, and T. Usui (1995) *N*-Acetylglucosaminyl disaccharide and trisaccharide formation through lysozyme-catalyzed transfer reaction. *J. Carbohydr. Chem.* 14: 213-225.
[17] Totani, K., N. Yasutake, H. Ohi, T. Murata, and T. Usui (2001) Enzymatic synthesis of aliphatic. β-lactosides as mimic units of glycosphingolipids by use of *Trichoderma reesei* cellulase. *Arch. Biochem. Biophys.* 385: 70-77.
[18] Newburg, D. S. (1997) Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria? *J. Nutr.* 127: 980S-984S.
[19] Sano, M., K. Hayakawa, and I. Kato (1993) Purification and characterization of an enzym releasing lacto-*N*-biose from oligosaccharides with type 1 chain. *J. Biol. Chem.* 268: 18560-18566.
[20] Murata, T., T. Inukai, M. Suzuki, M. Yamagishi, and T. Usui (1999) Facile enzymatic conversion of lactose into lacto-*N*-tetraose and lacto-*N*-neotetraose. *Glycoconj. J.* 16: 189-195.

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