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Expression and Characterization of β-1,4-Galactosyltransferase from *Neisseria meningitidis* and *Neisseria gonorrhoeae*

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The *lgtB* genes that encode β-1,4-galactosyltransferases from *Neisseria meningitidis* ATCC 13102 and *gonorrhoeae* ATCC 31151 were isolated by a polymerase chain reaction using the *pfu* DNA polymerase. They were expressed under the control of *lac* and T7 promoters in *Escherichia coli* M15 and BL21 (DE3). Although the genes were efficiently expressed in *E. coli* M15 at 37°C (33 kDa), most of the β-1,4-galactosyltransferases that were produced were insoluble and proteolysed into enzymatically inactive polypeptides that lacked C-terminal residues (29.5 kDa and 28 kDa) during the purification steps. When the temperature of the cell growth was lowered to 25°C, however, the solubility of the β-1,4-galactosyltransferases increased substantially. A stable N-terminal his-tagged recombinant enzyme preparation could be achieved with *E. coli* BL21 (DE3) that expressed *lgtB*. Therefore, the cloned β-1,4-galactosyltransferases were expressed under the control of the T7 promoter in *E. coli* BL21 (DE3), mostly to the soluble form at 25°C. The proteins were easily purified to homogeneity by column chromatography using Ni-NTA resin, and were found to be active. The galactosyltransferases exhibited pH optimum at 6.5-7.0, and had an essential requirement for the Mn^{+2} ions for its action. The Mg^{+2} and Ca^{+2} ions showed about half of the galactosyltransferase activities with the Mn^{+2} ion. In the presence of the Fe^{+2} ion, partial activation was observed with the β-1,4-galactosyltransferase from *N. meningitidis* (64% of the enzyme activity with the Mn^{+2} ion), but not from *N. gonorrhoeae*. On the other hand, the Ni^{+2}, Zn^{+2}, and Cu^{+2} ions could not activate the β-1,4-galactosyltransferase activity. The inhibited enzyme activity with the Ni^{+2} ion was partially recovered with the Mn^{+2} ion, but in the presence of the Fe^{+2}, Zn^{+2}, and Cu^{+2} ions, the Mn^{+2} ion could not activate the enzyme activities. Also, the β-1,4-galactosyltransferase activity was 1.5-fold stimulated with the non-ionic detergent Triton X-100 (0.1-5%).

Keywords: Galactosyltransferase, *Neisseria meningitidis*, *Neisseria gonorrhoeae*

Introduction

Carbohydrates play important cellular functions, including recognition, cell adhesion, proliferation, and differentiation (Varki, 1993). Chemical synthesis of carbohydrates is an inefficient and labor-intensive process (Ichikawa *et al*., 1992). Therefore, these enzymes may be unique and powerful tools as catalysts for regio- and stereospecific synthesis of oligosaccharides (Ichikawa *et al*., 1992; Gijsen *et al*., 1996). Glycosyltransferases, especially their large-scale expression, have become one of the prime targets in the pharmaceutical industry (Karlsson, 1991).

The reaction, catalyzed by β-1,4-galactosyltransferase (EC 2.4.1.22), is the transfer of galactose from UDP-α-D-galactose to terminal N-acetylglucosamine in glycoproteins and glycolipids with β-linkage (Ram and Munjal, 1985). The cDNAs that encode β-1,4-galactosyltransferase have been isolated from human (Appert *et al*., 1986; Masri *et al*., 1988), bovine (Narimatsu *et al*., 1986; D'Agostaro *et al*. 1989), and murine sources (Nakazawa *et al*., 1988; Shaper *et al*., 1988). Also, these mammalian galactosyltransferases have been expressed in higher eukaryotic cells (Nguyen *et al*., 1994; Borsig *et al*., 1997), yeast (Kleene *et al*., 1994; Borsig *et al*., 1997), and *E. coli* (Chatterjee, 1991; Nakazawa *et al*., 1993). However, when expressed in *E. coli* and yeast, the protein level of recombinant mammalian galactosyltransferase was very low or inactive (Chatterjee, 1991; Nakazawa *et al*., 1993;

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Borsig *et al*., 1997), the same as other recombinant eukaryotic proteins (Duggleby *et al*., 2000; Roytrakul *et al*., 2001). Also, in higher eukaryotic cells, it is impractical because of the high cost of the culturing process (Nguyen *et al*., 1994; Borsig *et al*., 1997). To overcome these problems and obtain a large amount of the recombinant β-1,4-galactosyltransferase, bacterial genes for β-1,4-galactosyltransferase from *Neisseria meningitidis* and *Neisseria gonorrhoeae* were cloned and expressed in *E. coli*.

The genes, which encode the glycosyltransferases that are involved in lipooligosaccharide (LOS) biosynthesis, were reported in *N. meningitidis* 406Y and belong to serogroup L (Jennings *et al*., 1995). In *N. meningitidis*, a locus that consisted of three genes (*lgtA*, *lgtB*, and *lgtE*) encoded the glycosyltransferase enzymes that are required for the addition of least three sugars in the lacto-N-neotetraose chain. Among these genes, the *lgtB* gene from the bacterial pathogen *N. meningitidis* 406Y is known to encode a β-1,4-galactosyltransferase enzyme (Wakarchuk *et al*., 1998). The amino acid sequence of β-1,4-galactosyltransferase of *N. meningitidis* showed homology to that of bacterial galactosyltransferases from *Haemophilus influenza* (High *et al*., 1993), *Haemophilus ducreyi* (Sun *et al*., 2000), *Haemophilus sommnus* (GenBank accession no. AF096997), and *Pateurella haemolytica* (Potter and Lo, 1995). All of these bacterial enzymes are involved in the biosynthesis of lipooligolysaccharide.

In the present study, the *lgtB* genes for a β-1,4-galactosyltransferase were cloned from *N. meningitidis* ATCC 13102 and *N. gonorrhoeae* ATCC 31151. Their primary structures and deduced amino acid sequences were analyzed. The serogroups of *N. meningitidis* are distinguished by the structure of the capsular polysaccharides. At least 13 main serogroups of *N. meningitidis* have been identified: A, B, C, D, E_{29}, H, I, K, L, W_{135}, X, Y, and Z (Frasch, 1987). Epidemic meningococcal disease is caused by serogroups A, B, and C. Other serogroups have so far not been associated with outbreaks (Frasch, 1987). The antigen property of *N. meningitidis* ATCC 13102 is classified into serogroup C, which is known to cause epidemic meningococcal disease and is different from of the *N. meningitidis* 406Y (serogroup L) strain previously reported. (Wakarchuk *et al*., 1998). Therefore, it would be interesting to clone the *lgt B* gene from the virulent N. meningitidis strain. A his_6tag was introduced to the N-terminus of the β-1,4-galactosyltransferase, and the fusion protein was properly expressed and purified to homogeneity by column chromatography. Also, the characteristics of the recombinant enzymes are presented.

Materials and Methods

Cloning of *lgtB* genes The genomic DNA samples for the cloning of the *lgtB* genes were prepared from the cells of *N. meningitidis* ATCC 13102 and *N. gonorrhoeae* ATCC 31151 that were grown for 18 h on Columbia blood agar plates at 37°C in 5% CO_2. The *lgt*B genes were isolated from the template of genomic DNA samples by the PCR method using *Pfu* DNA polymerase. The forward and reverse deoxyoligonucleotide primers of 5'CG GGATCCATGCAAACCACGTTATCAGC3' and 5'GCGGTACC GCAAATACGATGTCCATCT3' were used for amplifying both the *N. meningitidis* and *N. gonorrhoeae lgtB* genes. The amplified gene products were designed to anneal to upstream and downstream flanking sequences just outside the *lgtB* gene, and to contain the restriction sequences (underlined). A 0.73 kb PCR product was digested with *Bam*HI and *Kpn*I and inserted into the *Bam*HI/*Kpn*I site of the plasmid pQE30 that was purchased from QIAGEN (Valencia, USA), resulting in pQgal4M (*lgtB* gene from *N. meningitidis*) and pQgal4G. (*lgtB* gene from *N. gonorrhoeae*). The cloned genes were then transferred to the expression vector pET28a that was purchased from Novagen (Darmstadt, Germany). The β-1,4-galactosyltransferase gene, containing *Bam*HI-*Sal*I fragments of the above pQgal4M and pQgal4G, were then transferred into the same sites of the pET28a. The resulting recombinant was named pETgal4M (*lgtB* gene from *N. meningitidis*) and pETgal4G (*lgtB* gene from *N. gonorrhoeae*). Purification of the plasmids, agarose gel electrophoresis, and transformation of cells were performed according to the procedures previously described by Maniatis *et al*. (1982).

Expression and purification of the recombinant enzyme After several hours of inductions (4 h at 37°C and 8 h at 25°C) with isopropyl-D-thiogalactopyranoside (IPTG), the *E. coli* BL21(DE3) cells that harbor the pETgal4 series were harvested by centrifugation and resuspended in the lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM PMSF). After the cells were sonicated using a microtip with 50% power for five 20-s intervals on ice, the soluble and insoluble fractions were separated by centrifugation as needed. Whole cell lysates and the soluble and insoluble fractions from the 0.1-ml cultures were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The bands were visualized by Coomassie blue staining by the previously described standard procedures (Maniatis *et al*., 1982).

The recombinant proteins were purified by column chromatography using Ni-NTA resin (Koh *et al*., 2001). A 100 ml of BL21(DE3)/pET-gal4 series were grown in a LB medium that contained 50 μg/ml kanamycin at 25°C in a shaking flask. When the culture reached A_{600} = 0.5, the T7 promoter was induced with 0.5 mM IPTG. After an 8-h growth, the cells were harvested by centrifugation and resuspended in a 30 ml lysis buffer. After the cells were sonicated using a multitip with 50% power for ten 1-min intervals on ice, the lysates were centrifuged at $75{,}000 \times g$ for 90 min. The pellets were then extracted with another 10 ml of the same buffer. The combined supernatants were loaded on a 5-ml Ni-NTA column. After the column was washed with a lysis buffer, the proteins were eluted with an elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole, 1 mM PMSF). The elution fractions were dialyzed against a storage buffer (50 mM Tris-HCl pH 8.0, 20 mM NaCl, 20% glycerol). The column fractions were identified in each purification step by 12% SDS-PAGE.

Galactosyltransferase assay with the purified β-1,4-galactosyltransferase The used galactosyltransferase assay was

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N. go : 1    MQNHVISLASAAERRAHIADTFGSRGIPFQFFDALMPSERLEPAMAELVPGLSAHPYLSG
             MQNHVISLASAAERRAHIA TFG+RGIPFQFFDALMPSERLE AMAELVPGLSAHPYLSG
N. me : 1    MQNHVISLASAAERRAHIAATFGARGIPFQFFDALMPSERLEQAMAELVPGLSAHPYLSG

N. go : 61   VEKACFMSHAVLWKQALDEGVPYVAVFEDDVLFGKDAEKFLAEDTWLQERFDPDSAFVVR
             VEKACFMSHAVLWKQALDEG+PY+AVFEDDVL G+ AEKFLAED WL+ERFDPDSAF+VR
N. me : 61   VEKACFMSHAVLWKQALDEGLPYIAVFEDDVLLGEGAEKFLAEDAWLKERFDPDSAFIVR

N. go : 121  LETMFMHVLTSPSGLADYGGRAFPLLESEHCGTAGYIISRKAMRFFLDRFAVLPPERLHP
             LETMFMHVLTSPSG+ADY GRAFPLLESEH GTAGYIIS+KA+RFFL+ F +L PE++ P
N. me : 121  LETMFMHVLTSPSGVADYCGRAFPLLESEHWGTAGYIISKKAIRFFLEHFVMLQPEQIKP

N. go : 181  VDLMMFGNPDDREGMPVCQLNPALCAQELHYAKFHDQNSALGSLIEHDRRLNRKQQRRDS
             +DLMMF +  D+EGMPV Q++PALCAQELHYAKFHDQNSALGSLIEHDR LNRKQQRRDS
N. me : 181  IDLMMFSDFFDKEGMPVYQVSPALCAQELHYAKFHDQNSALGSLIEHDRLLNRKQQRRDS

N. go : 241  PANTFKHRLIRALTKIGREREKRRKRREQTIGKIIVPFQ    275
             PANTFK RLIRALTKIGREREKRR RREQ     IVPFQ
N. me : 241  PANTFKRRLIRALTKISREREKRRQRREQ----FIVPFQ    275
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Fig. 1. Amino acid sequence alignment of β-1,4-galactosyltransferases from *N. meningitidis* ATCC 13102 and *N. gonorrhoeae* ATCC 31151. An alignment of the amino acid sequences using the BLAST program is shown.

the modified method from a previous report (Kim *et al*., 1997). The enzyme activity of the purified protein was measured *in vitro* by 30-μl reactions that contained 20 mM MOPS (pH 7.85), 10 mM $MnCl_2$, 10 mM ATP, 20 mM N-acetylglucosamine (GlcNAc), and previously dried 5 × 10 cpm [^{3}H]UDP-α-D-galactose (UDP-Gal) in an Eppendorf tube. After incubation at 37°C for 1 h, adding 1 ml of distilled water terminated the reaction. A reaction mixture was loaded on a 1-ml Dowex (AG1-X8) pipette column that was pre-equilibrated with 5% sodium borate. After the column was washed 5 times with 1 ml of the same solution, the amount of tritium-labeled galactose β1-4 N-acetylglucosamine (Galβ1-4GlcNAc) in each fraction was quantified using a liquid scintillation counter. In order to identify the reaction product disaccharide (Galβ1-4GlcNAc), the reaction mixture was loaded on a Bio-Gel P-4 column (1.5 × 100 cm) that was pre-equilibrated with a 5% sodium borate solution. The incorporated tritium-labeled galactose in the disaccharide was counted using a Beckman liquid scintillation counter LS6500.

Results and Discussion

The *lgtB* genes that encoded β-1,4-galactosyltransferases were amplified from the bacterial pathogen *N. meningitidis* ATCC 13102 and *N. gonorrhoeae* ATCC 31151 genomic DNAs by the PCR method, which was based on the DNA sequence of the *lgt B* gene from the NCBI DNA database, and cloned into the pQE30 plasmid. DNA sequences of the cloned *lgtB* genes were fully determined and almost matched the DNA sequence of the *lgt* B gene (92% identity) that was previously reported (Wakarchuk *et al*., 1998). Also, a high similarity was found in the predicted amino acid sequences between the β-1,4-galactosyltransferases from *N. meningitidis* (279 amino acids) and *N. gonorrhoeae* (275 amino acids), shown in Fig. 1 (84.9% identity).

E. coli M15 cells that harbor pQgal4M were cultured at 37°C in the presence and absence of an inducer IPTG. The whole cell lysates were analyzed by 12% SDS-PAGE. One major band appeared approximately at the 33 kDa position in the case of IPTG induction, which was the expected position of the β-1,4-galactosyltransferase. When the cell growth temperature was decreased to 25°C, the galactosyltransferase was still induced by IPTG. However, the amount of total protein was reduced as the cell growth temperature was lowered from 37°C to 25°C (data not shown). When the *lgtB* gene-harboring cells that were grown with the IPTG induction at 37°C were harvested and lysed by sonication, most of the galactosyltransferase was precipitated, and only a small amount was found in the soluble (Fig. 2). On the other hand, the galactosyltransferase was only partially soluble within the cells that were grown at 25°C (Fig. 2). Therefore, the galactosyltransferase was purified using Ni-NTA affinity chromatography from the *lgtB* gene-harboring cells that were grown at 25°C. The expressed protein shows a major intact

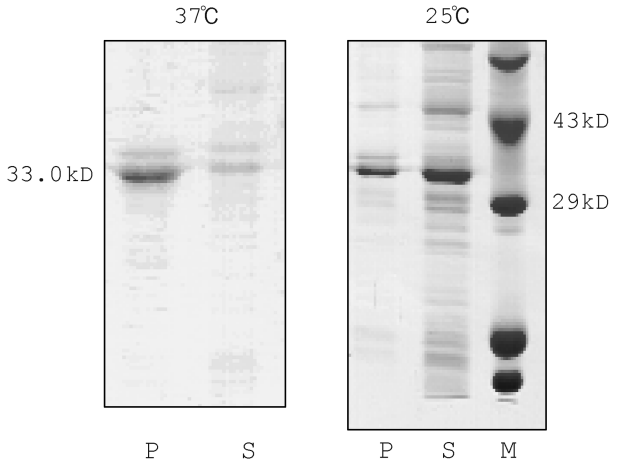

Fig. 2. Solubility of the recombinant β-1,4-galactosyltransferases from *N. meningitidis* that is expressed in the *E. coli* strain M15 that harbors pQgal4M at various temperatures, 37°C and 25°C. The total cell extracts were separated by centrifugation into a soluble supernatant (S) and insoluble pellet (P) portions, described in "Materials and Methods". Lane M shows the protein size standards.

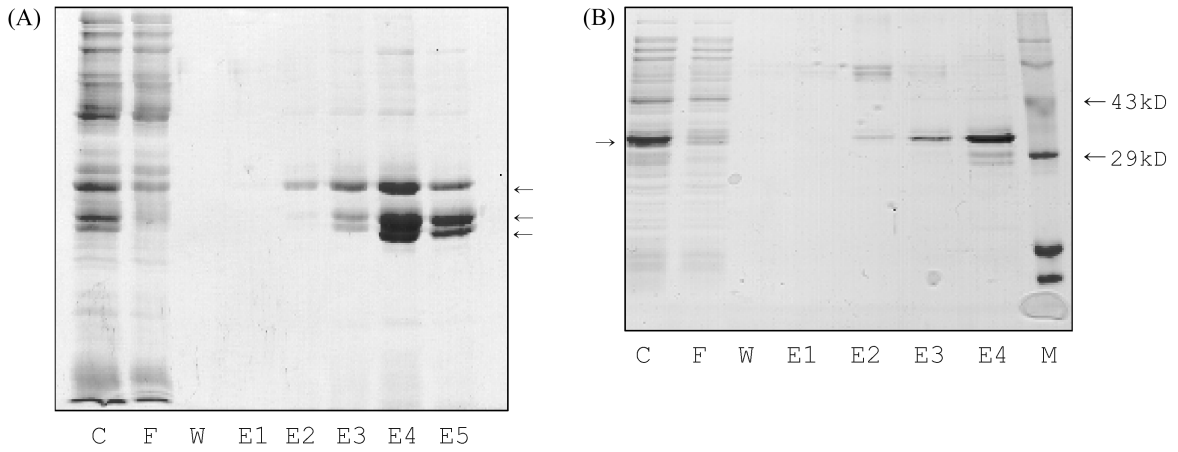

Fig. 3. Purification of recombinant β-1,4-galactosyltransferase from *N. meningitidis*. The galactosyltransferase gene was induced at 25°C for 8 h in *omp*T protease that is present the *E. coli* strain M15 that harbors pQgal4M, or deficient *E. coli* strain BL21 (DE3) that harbors pETgal4M. (A) The cell lysates from M15 were loaded onto a Ni-NTA column, and 250 mM imidazole elution fractions that contained mostly β-1,4-galactosyltransferase. (B) The same purification procedure was performed with the cell lysates from BL21 (DE3). C, cell lysate; F, flow-through; W, washing fraction; E1-E4, elution fractions; M, size standards.

galactosyltransferase (Fig. 2). However, the intact galactosyltransferase was cleaved into two major products (29.5 and 28 kDa) during purification steps of the galactosyltransferase from the *E. coli* M15 strain (Fig. 3A). It was previously reported that a clustering of pairs of basic amino acid residues in the C-terminal sequence of the galactosyltransferase produced *omp*T protease cleavage sites (Wakarchuk *et al*., 1998). Identification of the sites of proteolytic cleavage in the galactosyltransferase suggested that *omp*T was responsible for the observed degradation. To solve the degradation problem, *E. coli* BL21(DE3), one of the *omp* T deficient strains, was chosen as a host cell to express the *lgtB* gene. For this purpose, the cloned *lgtB* genes were transferred to an expression vector pET28a that contained the T7 bacteriophage promoter. The resulting recombinants were named pETgal4M and pETgal4G. As shown in Fig. 3B, no cleaved forms of the galactosyltransferase was observed in the protein purification steps with the *E. coli* BL21(DE3) cell that harbored the pETgal4M. It was demonstrated that proteolysis of the galactosyltransferase could be prevented by preparing the proteins that are expressed in the *E. coli omp*T deficient strains.

The reaction product (Galβ1-4GlcNAc) of the recombinant β-1,4-galatosyltransferase from *N. meningitidis* was identified with a Bio-gel P4 gel permeation column chromatography. As shown in Figure 4, the reaction product disaccharide (fractions 85-90) was separated from free galactose (fractions 95-100).

The recombinant β-1,4-galatosyltransferases showed a rather broad pH range within which it was active, pH 6.5-7.0

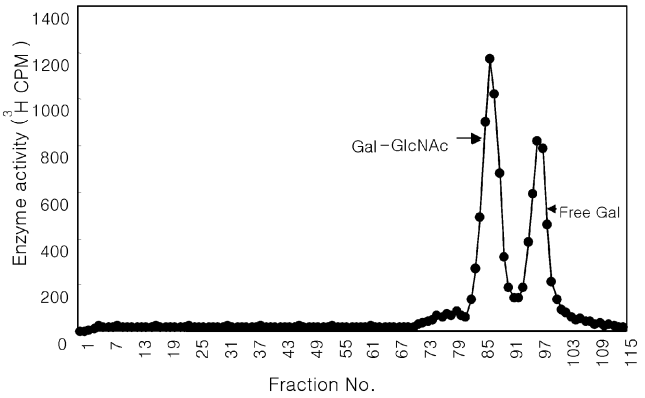

Fig. 4. Identification of disaccharide (Galβ1-4GlcNAc) that was synthesized by β1,4-galactosyltransferase. GlcNAc was galactosylated by the recombinant β1,4-galactosyltransferase using [^{3}H]UDP-Gal as substrate. The product was subjected to a Bio-Gel P-4 column chromatography. The migration positions of the authentic standards, disaccharide (Galβ1-4GlcNAc) and Galactose are indicated with arrows 1 and 2.

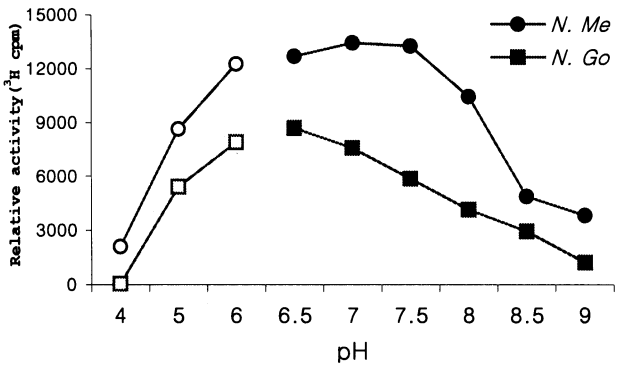

Fig. 5. Effect of pH on the activity of each recombinant β-1,4-galactosyltransferase from *N. meningitidis* and *N. gonorrhoeae*. The enzyme activities were assayed using 20 mM GlnNAc as an acceptor, except for the variable pH. Acetic acid buffer (pH 4-6) and MOPS buffer (pH 6.5-9) were used in the experiments.

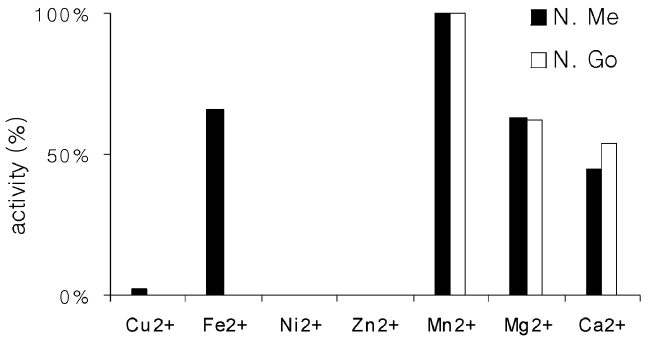

Fig. 6. Effect of divalent cations on the activity of recombinant β-1,4-galactosyltransferase from *N. meningitidis* and *N. gonorrhoeae*. The enzyme activities were assayed using 20 mM GlnNAc as an acceptor, except for the variable divalent cations. The concentration of cations were 10 mM.

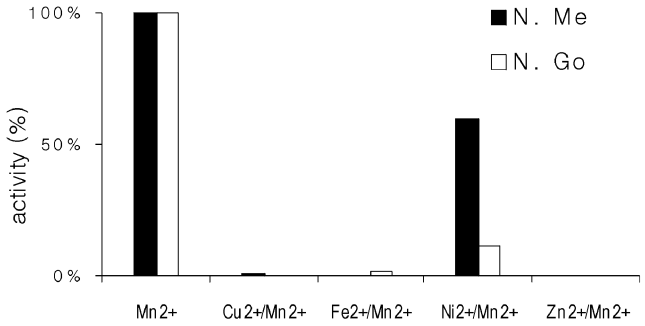

Fig. 7. Effect of divalent cations with Mn^{+2} on the activity of each recombinant β-1,4-galactosyltransferase from *N. meningitidis* and *N. gonorrhoeae*. The enzyme activities were assayed using 20 mM GlnNAc as an acceptor, except for the variable divalent cations. The concentration of Mn^{+2} and other cations were 10 mM.

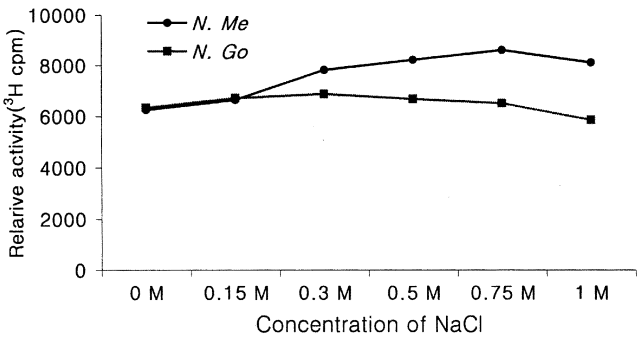

Fig. 8. Effect of NaCl salt concentration (0-1,000 mM) on the activity of each recombinant β-1,4-galactosyltransferase from *N. meningitidis* and *N. gonorrhoeae*.

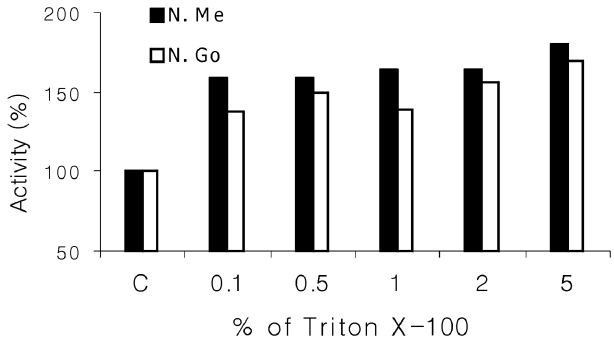

Fig. 9. Effect of Triton X-100 concentration (0-5%) on the activity of each recombinant β-1,4-galactosyltransferase from *N. meningitidis* and *N. gonorrhoeae.*

being optimal (Fig. 5). The optimum pH of the β-1,4-galatosyltransferase from a rats brain is approximately 7.2, comparable to that of the recombinant β-1,4-galatosyltransferase (Nomura *et al*., 1998). The effect of the divalent metal cation is shown in Fig. 6. Both of the β-1,4-galactosyltransferases require Mn^{+2} as a cofactor. To a lesser extent, the Mg^{+2} and Ca^{+2} ions could activate the enzyme reactions. The Fe^{+2} ion could be replaced with Mn^{+2} ion as a cofactor in the reaction with the β-1,4-galactosyltransferase from *N. meningitidis*, but not from *N. gonorrhoeae*. On the other hand, the Ni^{+2}, Zn^{+2}, and Cu^{+2} ions could not activate the β-1,4-galactosyltransferase activity. In the case of the β-1,4-galactosyltransferase from a rats brain, Mn^{+2} is essential for its activity. Also, Mg^{+2}, and Ca^{+2} partially activate the galactosyltransferase. However, Fe^{+2} and Ni^{+2} could not activate the galactosyltransferase (Nomura *et al*., 1998). Also, the *lgtA* gene that encodes the β-1,3-galatosyltransferase from *N. meningitidis* had an absolute requirement for the Mn^{+2} ion; whereas, the Mg^{+2}, Fe^{+2}, and Ca^{+2} ions were less effective (Blixt *et al*., 1999). These results show that both the prokaryotic and eukaryotic β-1,4-galactosyltransferases require the Mn^{+2} cation as a cofactor, and are partially activated by the Mg^{+2} and Ca^{+2} ions. We also observed the effect of divalent cations in the presence of the Mn^{+2} ion on the galactosyltransferase reaction. As shown in Fig. 7, the Mn^{+2} ion partially activated the enzymatic activities of galactosyltransferases from both *N. meningitidis* and *N. gonorrhoeae* in the presence of the Ni^{+2} ion. On the other hand, the Cu^{+2} and Zn^{+2} ions completely inhibited the activities of galactosyltransferases in the presence of the Mn^{+2} ion.

In variable conditions of the NaCl concentrations (0-1,000 mM), the activities of galactosyltranasferases from *N. meningitidis* and *N. gonorrhoeae* were unchanged (Fig. 8). It seems that the galactosyltransferase activity was unaffected by the NaCl salt concentration. As shown in Fig. 8, the non-ionic detergent (Triton X-100) was effective with about 1.5-fold stimulating of the galactosyltransferase activities from both microorganisms (0.1-5%). In general, most of galactosyltransferases from eukaryotes and prokaryotes are membrane-bound and purified with detergent-solubilization. Solubilization of the enzyme from membrane vesicles stimulates activity, apparently by increasing the accessibility of the substrate and acceptor. It was reported that the soluble-type mammalian β1-4 galactosyltransferase is activated with

nonionic detergent, such as Triton X-100 (Krezdorn *et al*., 1993; Oubihi *et al*., 2000). Evidently, nonionic detergent, such as Triton X-100, stabilizes active protein conformation, or increases the interaction between enzyme and substrate.

In this paper, β-1,4-galactosyltransferase genes from *N. meningitidis* and *N. gonorrhoeae* are cloned and expressed in an active form at high levels in the *omp*T minus strain of *E. coli* BL21 (DE3) at 25ºC. Also, the recombinant enzymes were easily purified to homogeneity by column chromatography using Ni-NTA resin and characterized. A large production of the β-1,4-galactosyltransferase would be helpful in the process of the enzymatic synthesis of oligosaccharides.

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