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 oC (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 oC, 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 oC. 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 Mn²⁺ ions for its action. The Mg²⁺ and Cu²⁺ ions showed about half of the galactosyltransferase activities with the Mn²⁺ ion. In the presence of the Fe²⁺ ion, partial activation was observed with the β-1,4-galactosyltransferase from N. meningitidis (64% of the enzyme activity with the Mn²⁺ ion), but not from N. gonorrhoeae. On the other hand, the Ni²⁺, Zn²⁺, and Cu²⁺ ions could not activate the β-1,4-galactosyltransferase activity. The inhibited enzyme activity with the Ni²⁺ ion was partially recovered with the Mn²⁺ ion, but in the presence of the Fe²⁺, Zn²⁺, and Cu²⁺ ions, the Mn²⁺ 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-galactosyltransferase (EC 2.4.1.22), is the transfer of galactose from UDP-β-D-galactose to terminal N-acetylgalcosamine 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;
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 somnus (GenBank accession no. AF096997), and Pateurella haemolytica (Potter and Lo, 1995). All of these bacterial enzymes are involved in the biosynthesis of lipooligosaccharide.

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, F, H, I, K, L, W135, X, Y, and Z (Frasch, 1987). Epidemic meningococcal disease is caused by serogroups A, B, and C. The serogroups of N. meningitidis 406Y (serogroup L) have not so far been associated with outbreaks (Frasch, 1987). The antigen property of N. meningitidis ATCC 13102 is classified into serogroup L, which is known to cause epidemic meningococcal disease and is different from the N. meningitidis 406Y (serogroup L) strain previously reported (Wakarchuk et al., 1998). Therefore, it would be interesting to clone the lgtB gene from the virulent N. meningitidis strain. A his tag 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% CO2. The lgtB genes were isolated from the template of genomic DNA samples by the PCR method using Pfu DNA polymerase. The forward and reverse deoxynucleotide primers of 5’CG GGAATTCATGCAAAAACACGTTATACG’ and 5’GCGGTACC GCAATACGATGCATCT’ 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 BamHI and KpnI and inserted into the BamHI/KpnI 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 BamHI-XhoI 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 OD600 = 0.5, the T7 promoter was induced with 0.5 mM IPTG. After an 8-h growth, the cells were centrifuged and resuspended in a 30 ml lysis buffer. After the cells were sonicated using a microtip with 50% power for ten 1-min 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).

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Galactosyltransferase assay with the purified β-1,4-galactosyltransferase The used galactosyltransferase assay was...
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$^5$ cpm [3H]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 lgtB 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 lgtB 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-harborin 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-harborin cells that were grown at 25°C. The expressed protein shows a major intact
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 ompT protease cleavage sites (Wakarchuk et al., 1998). Identification of the sites of proteolytic cleavage in the galactosyltransferase suggested that ompT was responsible for the observed degradation. To solve the degradation problem, E. coli BL21(DE3), one of the ompT 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 ompT deficient strains.

The reaction product (Galβ1-4GlcNAc) of the recombinant β1,4-galactosyltransferase 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 β1,4-galactosyltrasferrases showed a rather broad pH range within which it was active, pH 6.5-7.0

![Fig. 3. Purification of recombinant β-1,4-galactosyltransferase from N. meningitidis. The galactosyltransferase gene was induced at 25°C for 8 h in ompT 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.](image)

![Fig. 4. Identification of disaccharide (Galβ1-4GlcNAc) that was synthesized by β1,4-galactosyltransferase. GlcNAc was galactosylated by the recombinant β1,4-galactosyltransferase using [3H]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.](image)

![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.](image)
The optimum pH of the β-1,4-galactosyltransferase from a rat's brain is approximately 7.2, comparable to that of the recombinant β-1,4-galactosyltransferase (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 reaction. 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 rat's 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-galactosyltransferase 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 galactosyltransferases 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...
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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 ompT minus strain of *E. coli* BL21 (DE3) at 25°C. 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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