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Development of a microbioreactor for glycoconjugate synthesis

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ABSTRACT

A microbioreactor immobilized with a synthase-type mutant enzyme, Endo-M-N175Q (glycosynthase) of endo-β-*N*-acetylglucosaminidase derived from *Mucor hiemalis* (Endo-M), was constructed and used for glycoconjugate synthesis. The transglycosylation was performed with a reaction mixture containing an oxazoline derivative of sialo complex-type glycoside (SG), which was prepared from a sialo complex-type glycopeptide SGP derived from hen egg yolk, as a glycosyl donor and *N*-Fmoc-*N*-acetylglucosaminyl-L-asparagine [Fmoc-Asn(GlcNAc)-OH] as an acceptor. The reaction mixture was injected into a glycosynthase microbioreactor at a constant flow rate. Highly efficient and nearly stoichiometric transglycosylation occurred in the microbioreactor, and the transglycosylation product was eluted from the other end of the reactor. The glycosynthase microbioreactor was stable and could be used repeatedly for a long time.

1. Introduction

Endo-β-*N*-acetylglucosaminidase derived from *Mucor hiemalis* (Endo-M) shows transglycosylation activity.[1] Using the transglycosylation activity of Endo-M, a "chemoenzymatic method" for the synthesis of glycoconjugate from a natural glycosyl donor and synthetic acceptor substrates was developed[2] and used for the synthesis of various complex glycoconjugates.[3]

As the Endo-M enzyme is originally a hydrolytic enzyme, it hydrolyzes both the glycosyl donor substrate and the transglycosylation product alongside the transglycosylation reaction; thus, the transglycosylation yield was moderate. The catalytic activity of the Endo-M enzyme is exhibited via an oxazolinium ion intermediate of *N*-acetylglucosamine (GlcNAc) as an active complex.[4] Recently, a synthase-type mutant enzyme, Endo-M-N175Q (glycosynthase), was developed.[5] This enzyme exhibits high transglycosylation activity using the oxazoline derivative of the reducing terminal GlcNAc of a glycosyl donor, but it scarcely shows hydrolytic activity. Therefore, highly efficient transglycosylation is possible using this enzyme.

We intended to develop a bioprocess for glycoconjugate synthesis using a glycosynthase-immobilized microbioreactor. Immobilized glycosidases had been used for the synthesis of various oligosaccharides.[6] Immobilized endoglycosidases were solely used for the de-glycosylation of glycoproteins and/or for the analysis of oligosaccharides in glycoconjugates.[7] Glycoconjugate synthesis using immobilized endoglycosidase, however, had scarcely been tried. Our concept of glycoconjugate synthesis using a glycosynthase microbioreactor is shown in Scheme 1. A complex glycoconjugate as the transglycosylation product is synthesized with a glycosynthase-immobilized microbioreactor using an *N*-glycan oxazoline derivative as a glycosyl donor and an appropriate acceptor substrate.

In this paper, we will describe the construction of a glycosynthase-immobilized microbioreactor, and its application to a transglycosylation reaction in a dynamic flow system.

2. Materials and methods

2.1. Materials

Sialo glycopeptide (SGP)[8] was prepared from hen egg yolk. Sialo complex-type glycoside (SG) was prepared from SGP using Endo-M enzyme. The oxazoline derivative of SG (SG-oxazoline) was prepared from SG using CDMBI[9] (2-chloro-1,3-dimethyl-1*H*-benzimidazol-3-ium chloride, Fushimi Pharmaceutical Co., Ltd.) as an oxazolination reagent. *N*-Fmoc (9-fluorenylmethyloxycarbonyl)-*N*-acetylglucosaminyl-l-asparagine (Fmoc-Asn(GlcNAc)-OH) was synthesized as reported previously[10] and prepared as a sodium salt. A carrier resin for enzyme immobilization, NHS-activated Sepharose 4 Fast Flow, was purchased from GE Healthcare Inc. Endo-M and glycosynthase (Endo-M-N175Q) were supplied by Tokyo Chemical Industry Co., Ltd.

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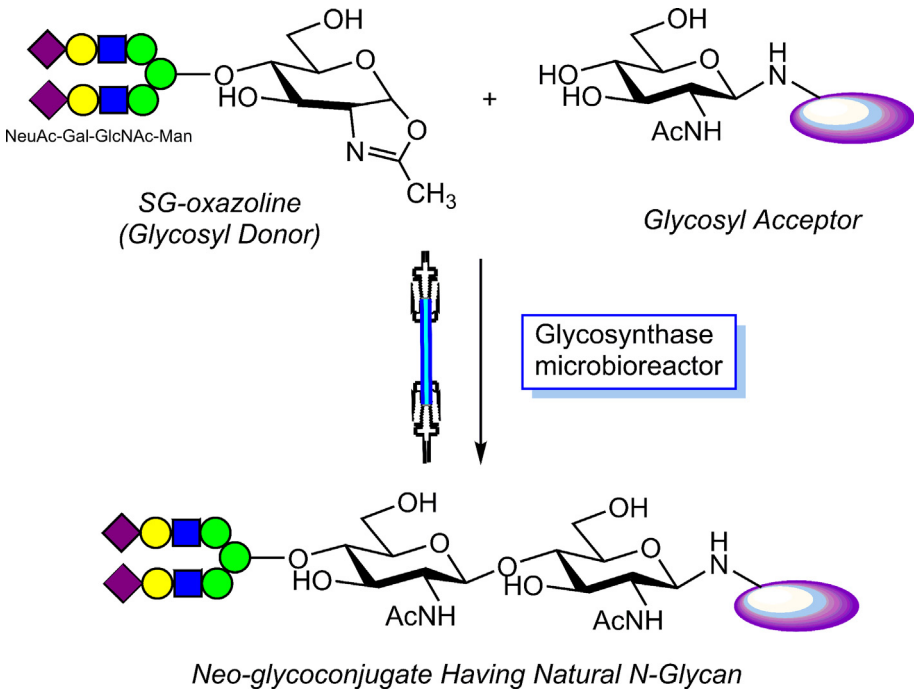

Scheme 1. A process for neoglycoconjugate synthesis using glycosynthase microbioreactor.

2.2. Methods

2.2.1. Preparation of SG-oxazoline

A sialo-complex-type glycoside SG was prepared by hydrolysis of SGP,[8] a sialo complex-type glycosyl peptide derived from hen egg yolk, using Endo-M-immobilized bioreactor. The oxazoline derivative of SG (SG-oxazoline) was prepared using 2-chloro-1,3-dimethyl-1*H*-benzimidazol-3-ium chloride (CDMBI) as reported by Noguchi et al.[9] The oxazolination rates of the SG-oxazoline derivatives prepared were around 60% to 70% as analyzed by ^{1}H NMR.

2.2.2. Preparation of glycosynthase microbioreactor

Glycosynthase microbioreactor M135: *N*-Hydroxysuccinimide-activated Sepharose resin (NHS-activated Sepharose 4 Fast Flow, GE Healthcare, 17–0906-01) was used for enzyme immobilization. Resin (150 μL, 50% suspension in 2-propanol) was washed with cold 1 mM HCl (1 M = 1 mol·dm^{-3}). Then, the resin slurry (60 μL) was immediately packed into a Teflon tube (ϕ1.6 mm × 4 cm) whose ends were plugged with 5 mm-length cotton filters. A solution of glycosynthase (20 μL, 20 mU) was mixed with 20 μL of a coupling buffer CB (0.1 M, pH 7.0 phosphate buffer (PB) and 0.5 M NaCl), and then this solution was immediately injected into a Teflon tube packed with the resin. After standing for 1.5 h at room temperature, the tube was washed with CB, and this was exchanged with 100 μL of a blocking buffer BB (0.2 M 2-aminoethanol, 0.08 M, pH 7.0 PB, and 0.4 M NaCl). After standing for 0.5 h at room temperature, the tube was washed with CB, and this was exchanged with a neutral buffer NB (0.1 M, pH 7.0 PB). Then, the glycosynthase microbioreactor named M135 (Glycosynthase 20 mU, resin 60 μL) was prepared. Both ends were sealed with union seals, and it was stored in a refrigerator after exchanging with the same buffer containing 0.1% NaN_3.

Glycosynthase microbioreactor M161: NHS-activated Sepharose 4 Fast Flow resin 50% solution (100 μL in 2-propanol) was placed in a 0.5 mL centrifuge filter tube (SpinTrap, GE Healthcare), then washed and activated with cold 1 mM HCl. Glycosynthase solution (40 μL, 40 mU) mixed with 20 μL of double concentration coupling buffer 2× CB (0.2 M, pH 7.0 PB, and 1 M NaCl) was placed in the SpinTrap tube packed with the resin. This resin slurry containing the enzyme was agitated by rolling for 1.5 h at room temperature. The resin was then washed with CB, exchanged with blocking buffer BB, and stood for 0.5 h. The resin was then washed with CB and neutral buffer NB. Then, this resin slurry (60 μL) immobilized with glycosynthase was packed into a Teflon tube (ϕ1.6 mm × 4 cm). Both ends of the tube were plugged with cotton filter (5 mm-length each) and sealed with union seals. The glycosynthase microbioreactor named M161 (glycosynthase 40 mU, resin 60 μL) was prepared, as shown in Fig. 1.

2.2.3. Transglycosylation reaction using a glycosynthase microbioreactor in a dynamic flow system

Glycosynthase microbioreactor M135 or M161 was used for the transglycosylation reaction. Before use, it was initialized by injecting a 60 mM, pH 7.0 PB solution. A reaction mixture containing 2.45 mg (1.2 μmol as weight basis) of SG-oxazoline as a glycosyl donor, 8 μL of 50 mM Fmoc-Asn(GlcNAc)-OH (400 nmol) in DMSO as an acceptor, 12 μL of 0.2 M, pH 7.0 PB, and 20 μL of distilled water in a total volume of 40 μL was injected into the glycosynthase microbioreactor using a microsyringe and a syringe pump with a constant flow rate at room temperature (25 °C). The flow rate was set at 1 μL/min in microbioreactor M135 immobilized with 20 mU glycosynthase, or 2 μL/min in microbioreactor M161 immobilized with 40 mU glycosynthase. After injecting the reaction mixture, 60 mM, pH 7.0 PB solution (220 μL) was successively injected at the same flow rate. The effluents eluted from the other end of the microbioreactor were collected every 20 μL, and the transglycosylation product and the remaining acceptor in each effluent fraction were analyzed by HPLC. A typical reaction system is shown in Fig. 2.

2.2.4. HPLC analysis of the transglycosylation product

Ten μL of the reaction mixture, which was diluted 20 times with distilled water, was applied to analytical HPLC using a