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Conversion of Dextran to Cycloisomaltooligosaccharides Using an Enzyme-Immobilized Porous Hollow-Fiber Membrane

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This paper describes a cycloisomaltooligosaccharide glucanotransferase (CITase)-multilayer-immobilized porous hollow-fiber membrane used as an enzyme bioreactor. Dextran, a substrate with an average molecular mass of 43000, is converted into seven- to nine-glucose-membered cycloisomaltooligosaccharides, effective as a preventive for dental caries, aided by convective transport of the substrate to the vicinity of the enzyme through the pores. Epoxy-group-containing graft chains were uniformly appended onto the surface of pores throughout a porous hollow-fiber membrane by radiation-induced graft polymerization. Subsequently, a diethylamino group was introduced, as an anion-exchange moiety, to the graft chains, which caused the chains to expand toward the interior of the pores due to mutual electrostatic repulsion. The expanding graft chain provided multilayer binding sites for CITase. Fifty-five milligrams of adsorbed CITase per gram of membrane is equivalent to the degree of multilayer binding of 5. Finally, 80% of the multilayer-adsorbed CITase was immobilized via enzymatic cross-linking with transglutaminase to prevent the leakage of enzymes. CITase, with a degree of multilayer immobilization of 4, produced the target cycloisomaltooligosaccharides at a conversion yield of 55% in weight at 310 K during permeation by the dextran solution at a space velocity defined as the permeation rate divided by membrane volume of 6 per hour.

KEYWORDS: Radiation-induced graft polymerization; multilayer immobilization; cycloisomaltooligosaccharide glucanotransferase; cycloisomaltooligosaccharides; porous hollow-fiber membrane

INTRODUCTION

Cycloisomaltooligosaccharides, especially seven- to nine-glucose-membered cyclic oligosaccharides, have been reported to be preventives for dental caries (*1*). The cycloisomaltooligosaccharides are potentially convenient as a food additive because they are flavorless (*2*). The cycloisomaltooligosaccharides are produced via hydrolysis and cyclization of dextran in the presence of an enzyme or cycloisomaltooligosaccharide glucanotransferase (CITase). CITase, which has a molecular mass of 98000, was found and isolated by Oguma and co-workers (*3*, *4*).

For economical production of cycloisomaltooligosaccharides, enzyme immobilization is effective because there is no need to recover the enzyme from the products and remaining substrate. Conventional methods of enzyme immobilization include entrapment of the enzymes in gel matrices such as carrageenan and agarose (*5*) and immobilizaton of the enzymes on membranes (*6*, *7*). However, the use of substrates with high molecular mass induces a high diffusional mass-transfer resistance of the substrate into the interior of the matrices (*8*).

We suggested the use of a porous hollow-fiber membrane as a matrix to immobilize the enzyme (*9*). The permeation of the substrate solution driven by transmembrane pressure across the porous membrane minimizes the diffusional mass-transfer resistance of the substrate to the immobilized enzyme and hence enhances the overall enzymatic reaction, as illustrated in **Figure 1**. The ion-exchange polymer chains grafted onto a porous membrane extend themselves from the pore surface toward the pore interior, due to their mutual repulsion, and capture the enzyme by means of electrostatic interaction in a three-dimensional fashion, that is, in multilayers (*10*, *11*). Cross-linking of the enzymes is required to prevent the leakage of the enzymes from the polymer chains during enzymatic reaction.

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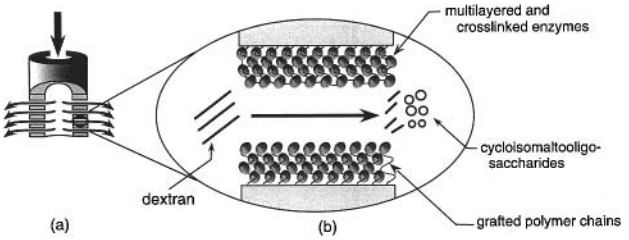

Figure 1. Schematic illustration of enzyme immobilized onto hollow-fiber membrane: (a) porous hollow-fiber membrane; (b) enzyme immobilized onto polymer chains grafted on pore surface.

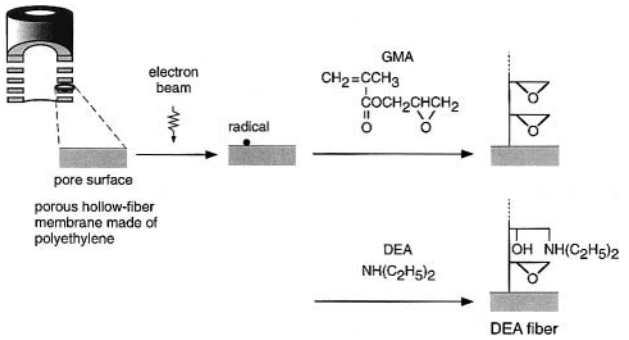

Figure 2. Introduction of diethylamino group into polymer chain. GMA = polyglycidyl methacrylate; DEA = diethylamine.

Conventional chemical reagents for the cross-linking of enzymes are glutaraldehyde (GA) (*12*) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (*13*). Here, we select the enzyme transglutaminase (TGase) as a cross-linker because of its safety in foods and the mildness of the reaction condition. TGase enables lysine and glutamic residues of a protein to form a ϵ-(γ-glutamyl) lysine peptide bond (*14*). This enzymatic cross-linking has been applied to the improvement of the foaming and emulsifying properties of proteins in various foods (*15*).

The objective of this study was twofold: (1) to immobilize CITase in multilayers onto a porous hollow-fiber membrane and (2) to efficiently convert dextran with an average molecular mass of 43000 to cycloisomaltooligosaccharide during the permeation of a dextran solution through a CITase-immobilized porous hollow-fiber membrane.

MATERIALS AND METHODS

Materials. A commercially available porous hollow-fiber membrane made of polyethylene was used as a trunk polymer for grafting. This hollow fiber had inner and outer diameters of 1.8 and 3.1 mm, respectively, with a pore size of 0.4 μm and a porosity of 70%. Glycidyl methacrylate (GMA, $CH_2{=}CCH_3COOCH_2CHOCH_2$) was purchased from Tokyo Kasei Co. and used without further purification. Cycloisomaltooligosaccharide glucanotransferase (CITase, M_r 98000) was purified according to the methods of Oguma et al. (*4*). Transglutaminase (TGase) was supplied by Ajinomoto Co., Ltd. Dextran with an average molecular mass of 43000 was purchased from Meito Sangyo Co., Ltd. Other reagents were of analytical grade or higher.

Preparation of Anion-Exchange Porous Hollow-Fiber Membrane. A porous hollow-fiber membrane containing a diethylamino (DEA) group [$-N(C_2H_5)_2$] as an anion-exchange group was prepared via the following three steps (**Figure 2**): (i) A porous hollow-fiber membrane 10 cm long was irradiated with an electron beam in nitrogen atmosphere at ambient temperature using a cascade-type accelerator (Dynamitron model, IEA 3000-25-2, Radiation Dynamics Inc., New York). The dose was set at 200 kGy. (ii) The irradiated membrane was immersed in a 10 (v/v)% GMA/methanol solution for 12 min at 313 K. The monomer solution was deaerated with nitrogen to purge oxygen dissolved in the monomer solution. After the prescribed time, the porous hollow-fiber membrane was washed thoroughly with dimethylformamide to remove residual GMA and poly-GMA homopolymer. The porous hollow-fiber membrane was then rinsed with methanol, dried under reduced pressure, and weighed. The degree of GMA grafting, defined as the percentage of weight increase (*16*), was 160%. (iii) The epoxy group of the polymer chain grafted onto the porous hollow-fiber membrane was reacted with a 50 (v/v)% diethylamine/water solution for 1 h at 303 K. The molar conversion of the epoxy group to the diethylamino group was evaluated as 60% from the weight increase (*9*). The resultant porous hollow-fiber membrane was referred to as a DEA fiber.

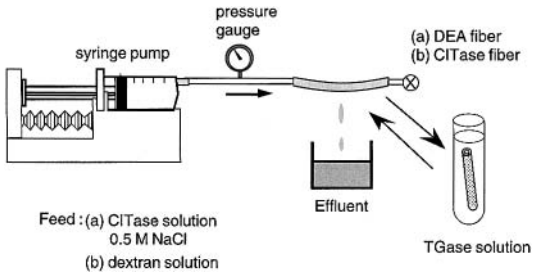

Figure 3. Experimental apparatus: (a) immobilization of CITase during the permeation of CITase solution through the DEA fiber; (b) enzymatic reaction during the permeation of a dextran solution through the CITase fiber.

Immobilization of Enzyme onto the Membrane by Cross-Linking. The CITase was immobilized via binding to the graft chain and subsequent cross-linking of the enzymes. First, the DEA fiber with a 2.0 cm effective length was positioned in an I-configuration (**Figure 3**). A 0.44 mg/mL CITase solution, buffered with 0.01 M phosphate buffer (pH 8.0), was forced to permeate outward from the inside surface of the DEA fiber through the pores. The permeation rate was maintained at 60 mL/h using a syringe pump (ATOM 1235N) at 310 K. The equilibrium binding capacity (EBC) of the enzyme to the anion-exchange polymer chain grafted onto the porous hollow-fiber membrane was evaluated as

$$\text{EBC (mg/g)} = \int_0^{V_e} (C_0 - C)\, dV/W \qquad (1)$$

where C_0 and C are the enzyme concentrations of the feed flowing in the inside surface of the DEA fiber and the effluent penetrating the outside surface, respectively. V, V_e, and W are the effluent volume, the effluent volume when C reaches C_0, and the weight of the DEA fiber, respectively. Second, the CITase bound to the graft chain was cross-linked by immersing the fiber in a 4.0 (w/w)% TGase solution buffered at pH 8.0 for 2 h at 300 K. Third, a buffer containing 0.5 M NaCl was permeated through the pores of the CITase-bound fiber to elute the un-cross-linked enzyme after the cross-linking treatment with TGase. Finally, the buffer was further permeated to wash the pores. The resultant porous hollow-fiber membrane that immobilizes the CITase via binding and subsequent cross-linking was referred to as a CITase fiber.

Conversion of Dextran to Cycloisomaltooligosaccharides during Permeation of Dextran. Dextran (2.0 (w/w)%) dissolved in a 0.1 M acetate buffer containing 10 mM $CaCl_2$ (pH 5.5) (*17*) was permeated across the 2.0-cm-long CITase fiber using an experimental apparatus similar to that shown in **Figure 3**. The permeation rate of the substrate solution ranged from 1.2 to 30 mL/h. This enzymatic reaction was performed at 310 K. The space velocity (SV) was defined as

$$\text{SV (h}^{-1}\text{)} = \text{(permeation rate)/(membrane volume excluding the lumen)} \qquad (2)$$

The effluent penetrating the outside surface of the CITase fiber was continuously sampled, and the contents of the cycloisomaltooligosaccharides consisting of seven to nine glucose members (CI-7, -8, and -9) were analyzed by high-performance liquid chromatography (Hitachi, L-7000). The chromatograph consisted of an L-7100 pump (Hitachi), a Rheodyne 7125 injection valve with a 20 μL of sample loop, and an

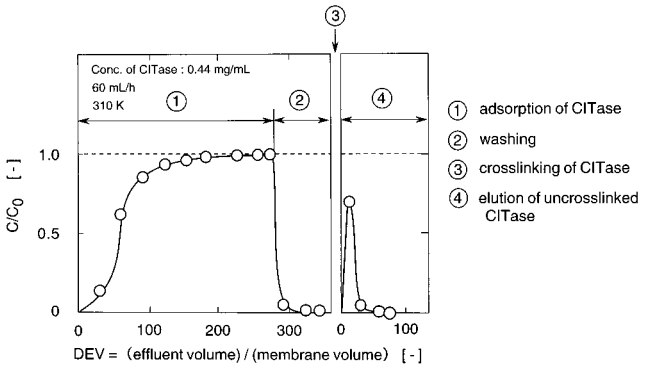

Figure 4. Change in CITase concentration of effluent during adsorption, washing, and elution.

RI-1530 detector (JASCO Corp.). The TSKgel Amide-80 column (Tosoh) was thermostated at 308 K. The mobile phase was prepared as acetonitrile/water (60:40, v/v). The conversion of the reaction was defined by the following:

$$\text{conversion (\%)} = 100\ (\text{total mass of CI-7, -8, and -9 in the effluent})/(\text{mass of dextran in the feed}) \quad (3)$$

To examine the stability of the immobilized enzyme over a long period, the concentration of CIs was determined continually monitored over a reaction duration of 220 h at a space velocity of 6 h^{-1} at 310 K.

RESULTS AND DISCUSSION

Properties of Anion-Exchange Porous Hollow-Fiber Membrane. The density of the DEA group of the porous hollow-fiber membrane, prepared at a degree of GMA grafting of 160% and 60% molar conversion, was 2.1 mol/kg of the DEA fiber. GMA grafting onto the porous hollow-fiber membrane and subsequent modification induced volume swelling of the membrane: the inner and outer diameters of the DEA fiber were 2.3 and 3.8 mm, respectively, compared to the corresponding diameters of 1.8 and 3.1 mm of the trunk hollow-fiber membrane.

Immobilization of CITase onto the Membrane. The change in the concentration of CITase in the effluent during the permeation of the CITase solution across the DEA fiber is shown in **Figure 4**. The abscissa is a dimensionless effluent volume defined as the ratio of effluent volume to membrane volume excluding the lumen. The ordinate is the CITase concentration of the effluent relative to that of the feed. According to eq 1, the EBC of CITase for the DEA fiber was evaluated as 55 mg/g, which is equivalent to the degree of enzyme multilayer binding of 5.0 defined as

$$\text{degree of enzyme multilayer binding } (-) = (\text{EBC})/(\text{theoretical monolayer binding capacity}) \quad (4)$$

$$\text{theoretical monolayer binding capacity (mg/g)} = (a_v M_r)/(aN_A) \quad (5)$$

where a_v and a are the specific surface area of the DEA fiber (5.5 m^2) and the area occupied by a CITase molecule (7.4×10^{-17} m^2), respectively. N_A and M_r are Avogadro's number and the molecular mass of CITase (98000), respectively. This multilayering of enzyme in the graft chain can be explained as the extension of the graft chain toward the pore interior due to mutual repulsion among diethylamino groups of the graft chain (*9*).

After cross-linking of CITase with TGase by permeating 0.5 M NaCl, 20% of the EBC was eluted from the fiber (**Figure 4**). The resultant degree of cross-linking of 80% with TGase was comparable with that with glutaraldehyde (GA); Nakamura et al. (*18*) reported that 90% of bovine serum albumin bound to the polymer chains grafted onto the porous hollow-fiber membrane was cross-linked with 0.025 (w/w)% GA under the reaction conditions of 303 K and 5 h. TGase is preferred over GA because its milder cross-linking conditions can prevent the deterioration of the enzymes.

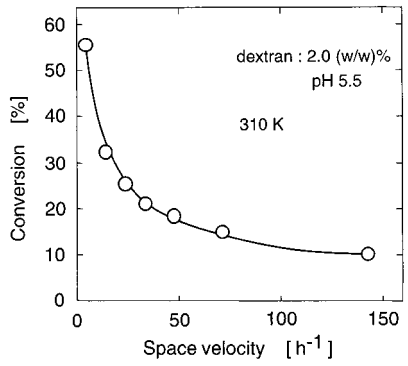

Figure 5. Conversion of dextran to cycloisomaltooligosaccharides during the permeation of a dextran solution through the CITase fiber.

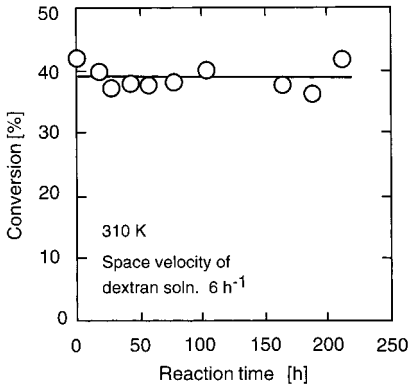

Figure 6. Stability of the CITase fiber.

Conversion of Dextran to Cycloisomaltooligosaccharides Using CITase-Immoblized Membrane. The CIs were produced during the permeation of dextran solution as a substrate across the CITase fiber. The conversion defined by eq 3 is shown in **Figure 5** as a function of space velocity (SV) of the substrate solution. The SV ranging from 6.0 to 140 h^{-1} corresponds to a residence time from 600 to 25 s. At an SV of 6 h^{-1}, the CITase fiber exhibited a conversion of 55%. The conversion decreased exponentially with increasing SV. High stability of the immobilized CITase was demonstrated over a duration of 220 h, as shown in **Figure 6**.

The CIs are produced via three steps: (i) convective transport of dextran through the pore of the CITase-immobilized membrane, (ii) diffusional mass transfer of dextran into the graft chain multilayering the cross-linked CITases, and (iii) intrinsic enzymatic conversion of dextran into the CIs at the active site on the CITase. Dependence of the conversion on the SV indicates that the latter two steps, that is, diffusion of the substrate with a high molecular mass and subsequent reaction, govern the overall enzymatic reaction rate, although the contribution of each process to the conversion is unclear at present.

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