Immobilization of a Cyclodextrin Glucanotransferase (CGTase) onto Polyethylene Film with a Carboxylic Acid Group and Production of Cyclodextrins from Corn Starch using CGTase-Immobilized PE Film

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ABSTRACT: Carboxylic acid groups were introduced onto polyethylene (PE) film by radiation-induced graft copolymerization. Subsequently, the clodextrin glucanotransferase (CGTase) was immobilized on the PE film with a carboxylic acid group. The activity of the immobilized CGTase on PE film was in the range of 0.40–1.04 U/cm^2 per min. The production of cyclodextrins (CDs) from corn starch was examined using the CGTase-immobilized PE film. The production ratios of CDs using CGTase-immobilized PE film was in the following order: α–CD > β–CD > γ–CD. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 85: 2451–2457, 2002

Key words: immobilization; cyclodextrin glucanotransferase (CGTase); radiation-induced grafting copolymerization; polyethylene film; acrylic acid; cyclodextrins (CDs)

INTRODUCTION

Radiation-induced graft copolymerization (RIGP) is a well-known method used to introduce functional groups into different polymer materials using specially selected monomers. There have been several reports on the use of the radiation graft copolymerization of polar monomers onto polymer film to obtain hydrophilic properties for versatile applications.[1–5] For example, an ion-exchange membrane can be used in water desalination,[6] as a carrier for the immobilization of medical products,[7] as a separator in alkaline batteries,[8] and so forth.[9]

Cyclodextrin glucanotransferanse (CGTase; 1,4-α−D-glucan: 1,4-α−D-glucopyrannosyltransferase, cyclizing, EC 2.4.1.19) is a unique enzyme capable of converting starch and related substrates into cyclodextrins (CDs) possessing a hydrophilic outside and hydrophobic central cavity.[10] CDs are homogeneous cyclic nonreducing oligosaccharides in which from six to 12 glucose units are joined by means of α-1,4-glucosidic bonds.[11] Most of the bacterial CGTase produce mainly α-CD, β-CD, and a trace amount of γ-CD consisting of six, seven, or eight glucose units, respectively. Recently, these cyclic CDs have been widely used in food, pharmaceutical, chemical, cosmetic, and agricultural industries because of their ability to form inclusion complexes with a wide variety of chemicals by partially encapsulating them in their cavity, thereby altering the physical and chemical properties of these compounds.

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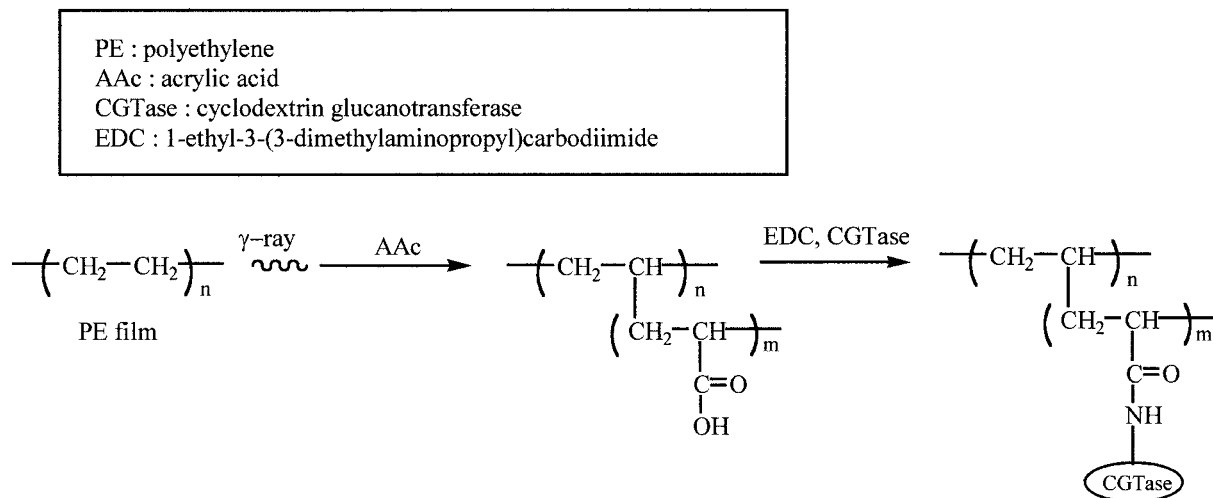

Scheme 1 Immobilization of the CGTase onto PE film with carboxylic acid group.

In a previous work,[12] the complexation of the nonsteroidal anti-inflammatory drug loxoprofen with modified and unmodified β-CD was examined. It was found that loxoprofen is soluble in the following order: glycerol ether β-CD > sulfated β-CD > β-CD. However, the inclusion study of the styrene derivative and phenol derivatives was also performed by Fourier transform–Raman spectroscopy.[13,14]

The adsorption behavior of urokinase by polypropylene film with various functional groups—amine, hydroxylamine, polyol, amino acid, carboxylic acid, phosphoric acid, and sulfonic acid—was examined under various condition.[15–17] It was found that the adsorption behavior of the PP films with various functional groups at pH 7.4 were higher than at pH 9.0. In trimethylamine, diethanolamine, and tri(hydroxymethyl) aminomethane groups, the adsorption of urokinase without salts also was higher than with salts.

In this study, polyethylene (PE) films with carboxylic acid were prepared by RIGP and by subsequent immobilization of the CGTase. The immobilization degree of the CGTase was determined by Bradford method. The activity of the CGTase-immobilized PE film was determined. Furthermore, the production of the cyclodextrins using the immobilized CGTase PE film was also examined.

EXPERIMENTAL

Materials

A PE film of thickness 0.05 mm (Tae-Syeng Chemistry Co., Korea) was washed with methanol and dried in a vacuum oven at 50°C for 8 hours. Reagent-grade acrylic acid (AAc; Junsei, 99%) and the CGTase from *Bacillus macerans* (Amano Pharmaceutical Co., Ltd., Babsuaerd, Denmark) were used as received. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Sigma Co. (USA). All the other chemicals were also reagent grade and used without further purification.

Grafting Procedure

Scheme I shows the immobilization of the CGTase on PE film with the carboxylic acid group. The PE film was used as the base polymer for grafting polymerization. The PE film of the area 4 × 5 cm was irradiated by γ-ray of Co-60 under atmospheric pressure and ambient temperatures and then immediately reacted with monomer. The monomer and homopolymers after grafting of AAc were removed with hot water. The grafted PE film was dried in a vacuum oven at 50°C for 14 hours. For the studies of the effects of various parameters on the grafting polymerization, the degree of grafting can be defined as

$$\text{Degree of grafting (\%)} = [(\text{Wg} - \text{Wo})/\text{Wo}] \times 100 \quad (1)$$

where W_g and W_o denote the weights of the grafted and the ungrafted PE film, respectively.

The content of the carboxylic acid group was determined from the measurement of total carboxylic acid group capacity by back titration method. PE film of the COOH– form was im-

mersed in 0.05 N NaOH aqueous solution at room temperature for 12 hours, and then the hydroxyl ion remaining in the solution was titrated with 0.025 N HCl aqueous solution.

Purification of the CGTase

The CGTase (activity = 85,936 U/mL × min, content of protein = 1427 mg/mL) was treated with 80% $(NH_4)_2SO_4$ solution and then centrifuged (RPM=15,000, for 15 min). The soluble part was separated; the activity of enzyme and the content of protein was determined. The activity of the CGTase, measured by the phenolphthalein method, and protein content, measured by the Bradford method, were 75,057 U/mL × min and 904 mg/mL, respectively. To obtain fine CGTase, the concentration of the CGTase using dialysis with Dialysis Tubing (Sigma) was established, and then the activity of the CGTase and protein content was determined. The activity of the CGTase, base CGTase, and protein content was 47,433 U/mL × min and 681 mg/ mL, respectively. The fine CGTase with the activity of 27,591 U/mL × min and 538 mg/mL was used in immobilization experiments.

Determination of the Activity of the CGTase

The β-cyclodextrin (CD) solution was prepared with the 0.4-mL Tris-maleic NaOH buffer solution (pH = 6.0) and 0.5-mL soluble starch. The solution was incubated at 50°C for 1 hour and then added to 0.5 mL phenolphthalein solution and 3.5 mL 1.0% Na_2CO_3 solution. The absorbance was recorded by an ultraviolet spectrophotometer at 550 nm. From the absorbance results, the unit equation was obtained as follows:

$$Y = 0.06029X - 0.1522$$

where X and Y are the absorbance and the amount of β-CD, respectively.

Determination of the Protein Amounts

The CGTase-immobilized PE film (2 × 2 cm) was hydrolized in 6.0 N HCl (0.5 mL) at 110°C for 24 hours. The solution was used with a 6.0 N NaOH (0.5 mL) solution. The samples were prepared by mixing the obtained solution (0.5 mL) and Bradford solution and then maintained at room temperature for 30 min.

Immobilization of the CGTase onto PE Film with Carboxylic Acid as the Covalent Bond

The PE film (2.0 × 2.0 cm) with carboxylic acid groups was immersed in base CGTase solution (10 mL), EDC (50.0 mg) added at 4.0°C for 20 hours in a shaking incubator, rinsed in 1.0 M glycine/20 mM $CaCl_2$ for 4 hours, and subsequently washed in 1.0 M NaCl/20 mM $CaCl_2$ solution. The activity and protein amounts of the immobilized CGTase were also determined as described earlier.

Characterizations

Infrared (IR) spectra were recorded using a Nicolet Model 205 FTIR (Fourier transform IR) spectrometer (Texas, USA). For scanning electron microscopy (SEM), a sample 0.5 × 0.5 cm^2 in size that was coated with a gold–palladium alloy before the measurement. The sputtered sample was then scanned by the electron beam in a scanning electron microscope (JSM-840A; JEOL Co., Tokyo, Japan).

The X-ray photoelectron spectra of the samples were obtained using an ESCALab 220i (VG Scientific, West Sussex, England) equipped with a full 180° hemispherical electrostatic analyzer to examine the chemical state of the constituent elements. As a phonon source, Al K_α radiation (1486.6 eV) was used. The half-width at half-maximum of the 4f7/2 line in the XPS (X-ray photoelectron spectroscopy) spectrum of gold obtained in our XPS spectrometer was smaller than 1.0 eV. The energy scale of the spectrometer was calibrated using the lowest BE component of the C 1s peak (285.0 eV).

RESULTS AND DISCUSSION

In previous papers,[2,4,8] the carboxylic acid group was introduced onto polypropylene nonwoven fabric (PNF), polyethylene film, and polyolefin PNF, wherein the PNF comprises at least about 60% of polyethylene that has a melting temperature at ~132°C and no more than about 40% of a second polypropylene having a lower melting temperature at ~162°C, for a battery separator. The carboxylic acid group was a good functional group to introduce enzymes into different polymer materials using specially selected enzymes. Cho et al.[15] reported on the immobilization of Trypsin onto the carboxylic acid group–introduced glass bead

Table I Effects of the Acrylic Acid (AAc) Concentration on the Grafting of AAc onto Polyethylene Film in the Presence of Salt and Acid[a]

No.	AAc Concentration (M)	Degree of Grafting (%)		Content of Carboxylic Acid (mmol/g)[b]	
		0.03 mm	0.05 mm	0.03 mm	0.05 mm
1	neat	Gelation	—	—	—
2	12.0	120.0	97.0	2.06	1.68
3	10.0	78.0	72.5	1.20	1.10
4	8.0	61.2	58.0	0.96	0.92
5	6.0	46.5	40.0	0.84	0.78
6	4.0	20.0	22.3	0.22	0.25

[a] Irradiation dose: 30 kGy; reaction time: 240 min at 70°C; salt = $FeSO_4$ $7H_2O$ (2.5×10^{-3} M); Acid = H_2SO_4, in water.
[b] Back titration method.

for Caseinnophosphopetide. In contrast, Mohamade[16] immobilized the *Paenibacillus macerans* NRRL B-3186 cyclodextrin glucosyltransferase onto aminated polyvinylchloride (PVC) by covalent binding with a bifunctional agent (glutaraldehyde). However, the immobilization of the CGTase onto a PE film carboxylic acid group prepared by radiation-induced graft polymerization has not been previously reported to our knowledge.

Preparation and Characterization of PE Film with Carboxylic Acid Group

Ferrous sulphate ($FeSO_4$) and Mohr's salt [$(NH_4)_2SO_4 \cdot FeSO_4$] are two of the most commonly used compounds for inhibiting the homopolymerization of vinyl monomers during grafting polymerization.[8] Table I shows the effects of AAc concentration on the grafting of AAc in the presence of $FeSO_4$ and sulfuric acid. The grafting yield and carboxylic acid content increased in accordance with the AAc concentration. When PE film is irradiated by ionizing radiation in air, trapped radicals and peroxy radicals are generally formed. Irradiation in the air leads to the formation of hydroxyperoxide species, which may result in undesirable homopolymerization, initiated by the mobile OH radical formed in the thermal decomposition radical. It is possible to solve this problem by using a reducing agent, for example, a metallic salt, to decompose the peroxy species.

Figure 1 shows IR spectra of the original PE film (0.05 mm) [Fig. 1(a)], the 97% AAc-grafted PE [Fig. 1(b)], the 72.5% AAc-grafted PE [Fig. 1(c)], and the 58 % AAc-grafted PE [Fig. 1(d)]. The IR spectra of the original PE film and an AAc-grafted PE film with a carboxylic acid group were compared. In the IR spectrum of the AAc-grafted PE film, a carbonyl peak of poly(AAc) was observed at 1730 cm^{-1}. No such peak was observed in IR spectrum of original PE film. In addition, broad –OH stretching and C–O stretching of the carboxylic acid appear around 3000 and 1235 cm^{-1}, respectively. The $–CH_2–$ stretching appeared at 1163 cm^{-1}.

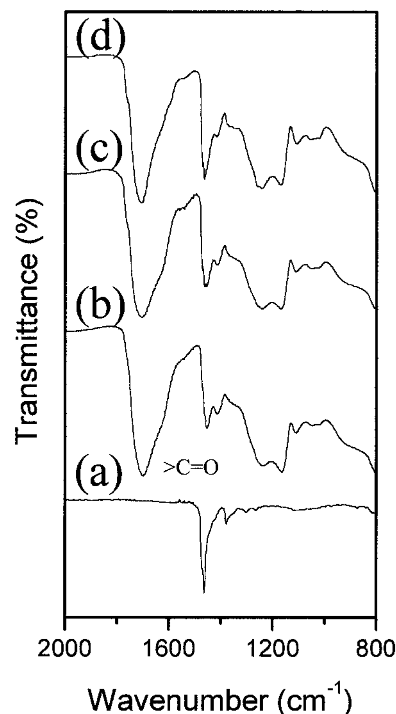

Figure 1 IR spectra of original PE film with thickness of 0.05 mm and AAc-grafted PE film. (a) original PE film, (b) No. 2, (c) No. 3, and (d) No. 4 in Table I.

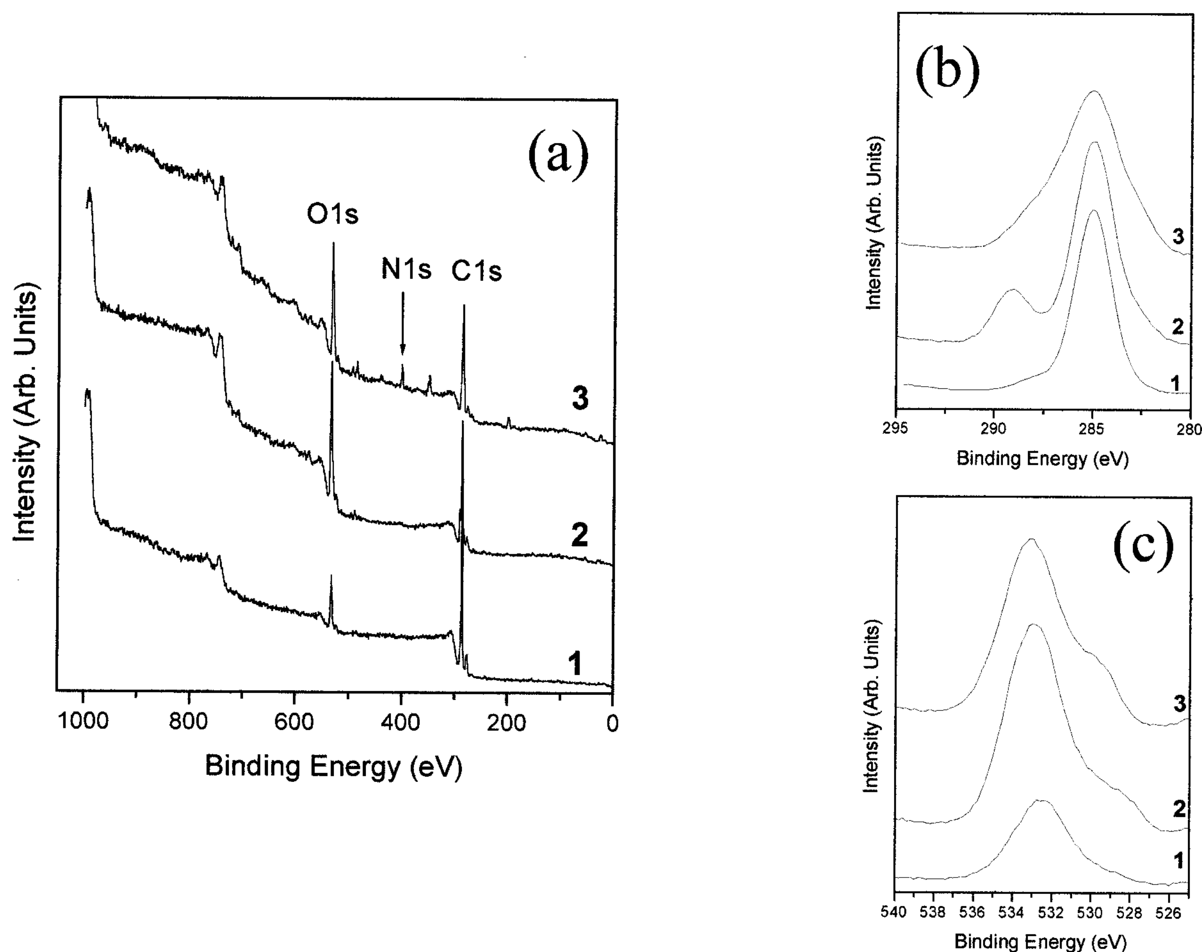

Figure 2 XPS survey scan spectra (a), C1s (b), and Ols (c) of (1) original PE film, (2) 58% AAc-grafted PE film, and (3) CGTase-immobilized PE film.

Figure 2 shows the XPS survey scan spectra of PE film [Fig. 2(a)], the C 1s [Fig. 2(b)], and the O 1s [Fig. 2(c)] of the original PE film (1), 58% AAc-grafted PE film (2), and CGTase-immobilized PE film (3) (dg = 58%). In Figure 2(a), the PE film showed two peaks corresponding to C 1s (binding energy, 285 eV) and O 1s (binding energy, 532 eV), whereas the CGTase-immobilized PE film showed one additional peak corresponding to N 1s (binding energy, 400 eV). The chemical compositions of the PE film, AAc-grafted PE film, and CGTase-immobilized PE film were calculated from the XPS survey scan spectra. The atomic percent (%) of the PE films was 81.9% of C and 18.1% of O, whereas the atomic percent (%) of the AAc-grafted PE film was 68.9% of C and 31.1% of O. The oxygen content (%) of the AAc-grafted PE film increased compared with the original PE film. However, the atomic percents (%) of the CGTase-immobilized PE film were 60.2% of C, 38.4% of O, and 1.4% of N. From these results, the CGTase was successfully immobilized onto the surface of AAc-grafted PE film. In Figure 2(b), the core-level binding energy of the PE film is calculated to be 285 eV. As the PE film was grafted with acrylic acid, an additional peak was observed at 290–292 eV because of the carboxylic group at the polymer chains. The original PE film has no carbonyl group peal. However, in the CGTase-immobilized PE film (3), the broad C 1s peak was observed between 282 and 292 eV. This seems to be attributable to the amide bond of the CGTase immobilized onto PE film. In Figure 2(c), the core-level binding energy of PE film is calculated to be 533 eV. In the AAc-grafted PE film, the additional peaks were observed around 529 eV because of the O of the carbonyl group. However, the 529 eV peak intensity of the CGTase-immobilized PE film was greater than in the AAc-grafted PE film. From these results, the CGTase success-

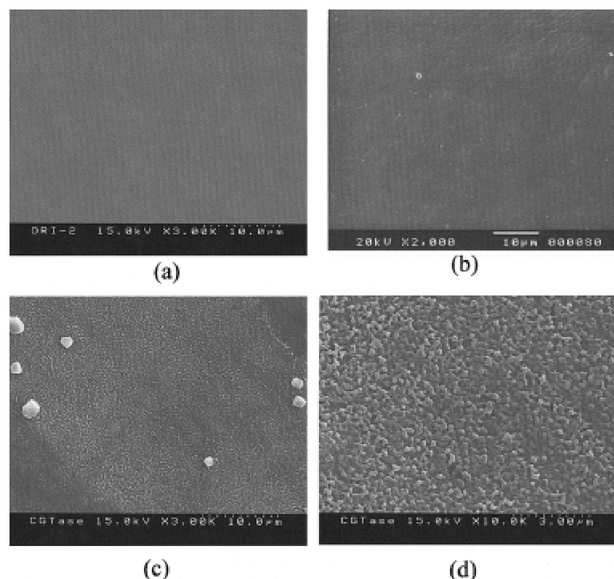

Figure 3 Scanning electron phothomicrographs of (a) original PE film, (b) 58% AAc-grafted PE, (c) CGTase-immobilized PE film, and (d) 30 × (c).

fully immobilized PE film with a carboxylic acid group.

Figure 3 shows the surface morphology of [Fig. 3(a)] original PE and [Fig. 3(b)] 58% AAc-grafted PE film, [Fig. 3(c)] CGTase-immobilized PE film, and [Fig. 3(d)] (c) ×30. The grafted poly(AAc) on the PE film is shown as bright hills in Figure 3(b), in contrast to the uniform texture of the original PE film in Figure 3(a). In Figure 3(c and d), the photographs showed the high-immobilization CGTase on PE film with the carboxylic acid group. From the results, the CGTase was immobilized on PE film with a carboxylic acid group.

Immobilization of the CGTase onto PE Film with Carboxylic Acid Group and Production of Cyclodextrins Using the CGTase-Immobilized PE Film

Figure 4 shows the activity of the immobilized CGTase onto PE film with carboxylic acid for 24 hours as the function of EDC concentration. The activity of the immobilized CGTase increased to EDC concentration of 15 mg/mL, while the activity of the immobilized CGTase decreased above EDC concentration of ~20 mL/min. The maximum activity of the CGTase-immobilized PE film was ~20 mg/mL EDC concentration.

Figure 5 shows the activity of the immobilized CGTase onto PE film with carboxylic acid (0.92 mmol/g), using EDC of 15 mg/mL as the function

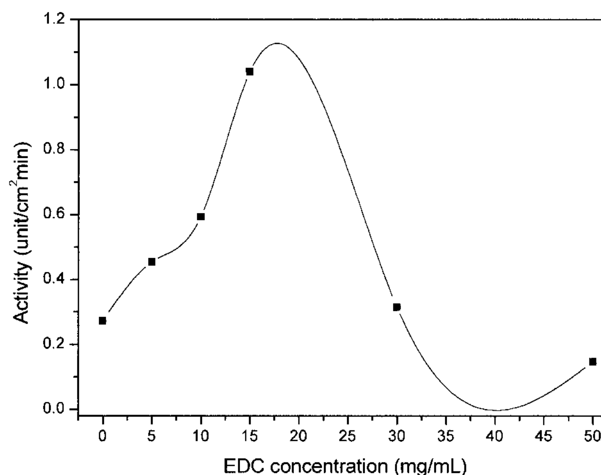

Figure 4 Activity of the immobilized CGTase onto PE film with carboxylic acid of 0.92 mmol/g for 24 hrs as the function of EDC concentration.

of immobilization time. The maximum activity of the immobilized CGTase was observed around 24 hours.

The ratios of the producing cyclodextrins were a very important factor because the isolated cyclodextrin was very expensive. Therefore, many workers reported the ratios of producing cyclodextrins using various CGTases.[17–19] Figure 6 shows the production of the cyclodextrins (CDs) using CGTase-immobilized PE film (activity, 1.04 unit/cm^2 min) as a function of reaction time: [Fig. 6(a)] total CD, [Fig. 6(b)] α-CD, [Fig. 6(c)] β-CD, and [Fig. 6(d)] γ-CD. The production amounts of the CDs were determined by HPLC method[20] and

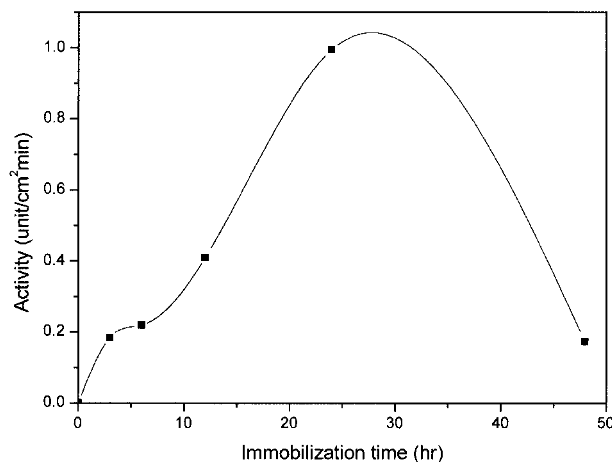

Figure 5 Activity of the immobilized CGTase onto PE film with carboxylic acid of 0.92 mmol/g using EDC 15 mg/mL as the function of the immobilization time.

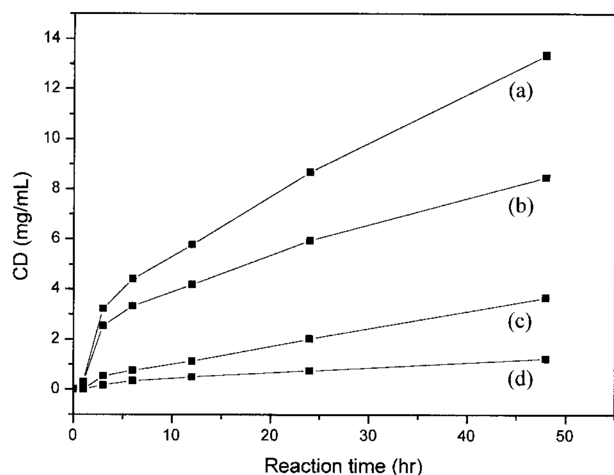

Figure 6 Production of the cyclodextrins (CDs) using CGTase-immobilized PE film (activity, 1.04 unit/cm^2 min) as the function of reaction time. (a) total CD, (b) α-CD, (c) β-CD, and (d) γ-CD.

increased in accordance with reaction time in the following order: α-CD > β-CD > γ-CD. Blackwood et al.[21] reported on the production ratios of α-CD, β-CD, and γ-CD, using *B. circulans* strain 251 CGTase in various solvents. He found that production ratios in the following order: β-CD > γ-CD > α-CD. Kim et al.[22] also reported on the production of cyclodextrin using raw corn starch at high temperature. The production amounts of cyclodextrin were highest around 65°C. However, the production ratio of α-CD, β-CD, and γ-CD was not examined.

CONCLUSIONS

The CGTase was immobilized onto the PE film with carboxylic acid group using EDC. The production of the cyclodextrins from soluble starch using CGTase-immobilized PE film was examined. From the results, the conclusion was as follows: First, the content of the introduced carboxylic acid group onto PE film was in the range of 0.22 to 2.06 mmol/g.

Second, the activity of the immobilized CGTase onto PE film was 0.27 U/cm^2 $\times$ min without EDC, whereas the activity of the immobilized CGTase onto PE film with EDC was in the range of 0.40 to 1.04 U/cm^2 $\times$ min. Third, the production amounts of α-CD, β-CD, and γ-CD using the CGTase-immobilized PE film increased with increasing the reaction time. Finally, the production ratio of CDs using CGTase-immobilized PE film was in the following order: α-CD > β-CD > γ-CD.

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