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Evaluation of Immobilization Methods for Cyclodextrin Glucanotransferase and Characterization of its Enzymatic Properties

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Cyclodextrin glucanotransferase(CGTase) derived from *Bacillus macerans* was immobilized by (1) covalent linkage on chitosan and chitin with glutaraldehyde, (2) adsorption on DEAE-cellulose and Amberite IRA 900 after succinylation, and (3) entrapment on alginate and polyacrylamide by cross linking. Adsorption on Amberite IRA 900 and covalent linking on chitosan were identified to be the most suitable immobilization methods considering the yield of activity and stability of immobilized CGTase. The enzymatic properties of immobilized CGTase were investigated and compared with those of the soluble CGTase. Thermal stability of CGTase immobilized on chitosan was increased from 50 to 55°C, and the optimum temperature of CGTase immobilized on Amberite IRA 900 was shifted from 55 to 50°C. The effect of molecular size of soluble starch (substrate) on immobilized CGTase investigated using partially liquefied substrates with different dextrose equivalent(DE). Cyclodextrin(CD) conversion yield augmented according to the increase of DE level for immobilized CGTase on Amberite IRA 900. CD conversion yield of partially cyclized starch with soluble CGTase was higher compared with liquefied one with α-amylase.

Cyclodextrin glucanotransferase(CGTase) is an enzyme that catalyzes the formation of cyclodextrin (CD) from starch and related carbohydrates. The CD which is a ring structure molecule built up of 6, 7 or 8 glucopyranose unites (α-, β-, γ-CD) can entrap other molecules at inside of hydrophobic site; therefore, it can be widely utilized in pharmaceutical, cosmetic, chemical, and food industries (17). This CGTase can be produced by several microorganisms, *i. e. Bacillus macerans, B. circulans, B. stearothermophilus, B. megaterium , Klebshiella pneumoniae,* and alkalophilic *Bacillus* sp. (6). In our previous work (14), we have newly isolated alkalophilic *Bacillus circulans,* and then purified and characterized the excreted cyclodextrin glucanotransferase.

For the industrial-scale production of cyclodextrin, conventional batch production methods which utilized directly the soluble state CGTase have been mainly adopted; however, continuous production of CD using immobilized CGTase would have great advantages of permitting reuse of expensive CGTase for an extended period of time, obtaining homogeneous CD product, simplifying CD purification process, and availability for scale-up.

Nakamura and Horikoshi (12) immobilized CGTase by ionic binding on a vinylpyridine copolymer anion-exchange resin, but continuous leakage of immobilized enzyme was occurred. Katalin *et al.* (4) reported that the leakage of enzyme might be overcome by formation of covalent bonds between the enzyme and the Akrilex C, a polyacrylamide-type bead polymer activated by carbodiimide. Adsorptions of CGTase on styrene-divinyl benzene copolymer were attempted with (Yoneyama, M. and Souga, 1982, U.S. Patent 4,338,398) or without (5) cross-linking of enzyme with carbodiimide, and good recovery yield of enzyme (70-80%) were obtained as well as the long operational stability. Boross *et al.* (Boross *et al.*, 1986, U.S. Patent 4,593,004) attempted covalent binding of CGTase on acrylamide copolymer showing dependency of enzyme recovery yield on pH. Hitoshi *et al.*(3) carried out immobilization of CGTase on several

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ion exchange resins for continuous production of CD.

In this work, CGTase was immobilized using a variety of immobilization methods including covalent linkage on chitosan and chitin with glutaraldehyde, adsorption on DEAE-cellulose and Amberite IRA 900 after succinylation, and entrapment on alginate and polyacrylamide by cross linking. The various immobilization techniques were compared to identify the most suitable immobilization method. The enzymatic properties of immobilized CGTase was also investigated , and compared with those of soluble CGTase.

MATERIALS AND METHODS

CGTase used

Crude CGTase (280 units/mg of protein, Amano Pharma. Co.) of *B. macerans* was partially purified by successive DEAE-Sephadex A-50 and Sephadex G-100 chromatography as described in our previous work (14). The partially purified CGTase had specific activity of 3,500 units/mg of protein with about 13 folds of increase in specific activity.

The optimal pH and temperature of soluble CGTase, that were determined measuring after 2 hr reaction, were 6.0 and 55℃; and the pH and thermal stability were 5.0-11.0 and 50℃, respectively. The ratio of α-, β-, γ-CD formation after 24 hrs of reaction was measured to be 6:4:1.

Assay of CGTase Activity

5 m*l* of 5% soluble starch in 10 mM maleic acid-Tris-NaOH buffer (pH 6.0) containing 10 mM $CaCl_2$ was incubated with CGTase at 50℃ for 60 min. The content of α-CD in the product solution was measured by HPLC, and one unit of the CGTase was defined as the amount of enzyme which produces 1 mg of α-CD per hour.

Determination of Cyclodextrins

The profile and content of CDs were determined by HPLC (7) under the following condition; HPLC (Model-441 Waters Co. U.S.A.), carbohydrate analysis column, acetonitrile/water (65/35), 2.0 m*l*/min., 10 μ*l* sample size, and RI detector.

Immobilization of CGTase on Chitin and Chitosan by Covalent Linking

Chitin was purified by the procedure of Stanley *et al.*(15), and chitosan was prepared by method of Rigby (Rigby, 1934, U.S. Patent 2,040,880). 10 mM buffer solution was added to carriers at room temperature for 30 min to prepare moist chitin and chitosan, with the ratio of 10 m*l* of buffer to 1 g of carrier. Glutaraldehyde concentration of 0.1% (w/v) was added, thoroughly mixed for 30 min, and then desired amout of CGTase was added; after standing 20 min, the carrier were washed several times with 10 mM buffer (pH 6.0) to remove unadsorbed CGTase.

Immobilization of CGTase on Amberite IRA 900 and DEAE-Cellulose

CGTase was mixed with 20 m*l* of 0.1% (w/v) succinic anhydride for succinylation and stirred at 0℃ for 30 min while maintaining pH 7.0 by adding 1.0 M of sodium bicarbonate in order to increase the negative charge of enzyme (8). The remained reagents were dialyzed with 10 mM of maleic acid-Tris-NaOH buffer (pH 6.0), and then adsorbed on Amberite IRA 900 and DEAE-cellulose (Sigma Co.) directly without any modification.

Immobilization of CGTase on Alginate and Polyacrylaminde Gel

Alginate gel entrapment was prepared by the procedure of Shigeaki *et al.* (13). The alginate solution containing CGTase treated with cross-linking reagents was added dropwisely into 200 m*l* of 1% $CaCl_2$ and stand for 1 hr to fix bead formation (diameter 3-5 mm). Polyacrylamide gel entrapment was prepared by the procedures of Makkar (10) and Wheatly *et al.* (16).

Determination of Enzymatic Properties of Immobilized CGTase

Thermal stability of immobilized CGTase was measured by preincubating the immobilized CGTase at the temperature range of 55-70℃ for 60 min in the absence of calcium ion. pH stability was investigated by treatment of immobilized CGTase with 20 mM of maleic buffer (pH 5.0-9.0) at 50℃ for 30 min and by measuring the residual activity of immobilized CGTase.

Operational Stability of Immobilized CGTase

To examine the operational stability of immobilized CGTase, a thermostatted (50℃) glass column (1.6×33.0 cm) was filled with immobilized CGTase (Amberite IRA 900 and chitosan as carrier) and 1% of soluble starch solution (pH 6.0) containing 10 mM of $CaCl_2$ was passed through the column with a constant flow rate of 30 m*l*/hr. The residual activity was expressed as the percentage of remained immobilized CGTase to initial immobilized CGTase activity.

Preparation of Partially Liquefied and Cyclized Soluble Starch

5% (w/v) of soluble starch solutions were liquefied with 2-12 units of α-amylase (Fungamyl, Novo) per g of starch for 10 min at pH 6.0 and 50℃ to obtain dextrose equivalent level of 2.5-30.0. DE is defined as the percentage of reducing sugar to maximum convertible sugar. 5% of soluble starch solution was also partially cyclized with 10 units of soluble CGTase per g of starch for 2 hr at pH 6.0 and 60℃.

RESULTS AND DISCUSSION

Immobilization of CGTase on Chitosan by Covalent Linking

The effect of various immobilization conditions on yield of activity of CGTase immobilized were summarized in Table 1. For immobilization of CGTase on chitosan, glutaraldehyde was utilized as cross-linking reagent. The effect of glutaraldehyde treatment pH was studied under the pH range of 5.0-10.0. It was found that the CGTase was immobilized effectively at the weakly acidic pH range of 5.0-7.0 with activity yields of 34-40%, and the highest activity yield (40%) was obtained at pH 5.5. The effect of pH on immobilization yield seems to be related with the physical property of chitosan that swells significantly at the weakly acidic pH region (9), as a result, quite a large amount of CGTase could bind effectively on surface area of swollen chitosan compared to alkaline pH region. The optimum concentration of glutaraldehyde treatment was found to be 0.1% (w/v), and the proper reaction time for glutaraldehyde treatment was identified to be 30 min, on the other hand, optimum enzyme coupling time was determined to be 20 min.

Table 2 show the effect of the amount of enzyme on immobilization of CGTase on chitosan. The activity yield of the CGTase increased with increasing the amount of the enzyme, but the activity yield reached to the maxium of 42% when 15 units of soluble CGTase is added. Although total activity of immobilized CGTase was increased with according to the increase of added soluble CGTase activity, suitable amout of CGTase have to be determined considering not only the activity yield of immobilized CGTase but also the efficiency of immobilized CGTase at enzyme reactor for economic production of CD.

Immobilization of CGTase on Amberite IRA 900 and DEAE-Cellulose by Adsorption

To increase the adsorptivity of CGTase on anion-exchange resin (Amberite IRA 900, DEAE-Cellulose), it is neccessary to enhance the negative charge of enzyme in order to compensate the weak binding force. However,excessive modification of enzyme have to be avoided in order to prevent enzyme inactivation, and therefore, suitable treatment concentration of modifying reagent have to be determined. CGTase was treated with 0.1-0.5% (w/v) of succinic anhydride as modifying reagent, and the residual activity of CGTase was depicted in Fig. 1. The residual activity of CGTase after succinylation remained 95% of initial activity up to 0.2% of succinic anhydride, whereas rapidly decreased above 0.2% of succinic anhydride concentration. The optimum succinic anhydride concentration for succinylation of CGTase was identified to be as 0.2% for strong adsorption on the

Table 2. Effect of the amount of CGTase on covalent linkage on chitosan

Soluble CGTase activity (units) Added	Immobilized CGTase	
	Immobilized activity (units/g of chitosan)	Yield of activity (%)
5	1.8	35
10	4.0	40
15	6.3	42
20	7.5	38
25	8.3	33
30	8.8	29

CGTase (5-30 units) was coupled with 1g of chitosan treated with 0.1% of glutaraldehyde for 20 min at room temperature.

Table 1. The effect of experimental conditions on immobilization yields of CGTase on chitosan

GA activation pH[a]		GA concentration[b]		GA activation time[c]		CGTase coupling time[d]	
pH	YOA	% (w/v)	YOA	Time (min)	YOA	Time (min)	YOA
5.0	34	0.05	33	10	37	10	39
5.5	40	0.1	41	20	39	20	41
6.0	39	0.2	30	30	41	30	39
7.0	37	0.3	27	60	40	60	38
8.0	31	0.5	24	120	39	120	37

GA (glutaraldehyde)

$$\text{YOA (yield of activity, \%)} = \frac{\text{initial activity-unbound activity}}{\text{initial activity}} \times 100$$

[a]Glutaraldehyde concentration, 0.1% (w/v)
[b]Glutaraldehyde activation pH 5.5
[a,b]Time for GA activation and CGTase coupling, 30 min
[c]CGTase coupling time, 20 min
[d]GA activation time, 30 min
[c,d]GA concentration, 0.1% (w/v): GA activation pH 5.5

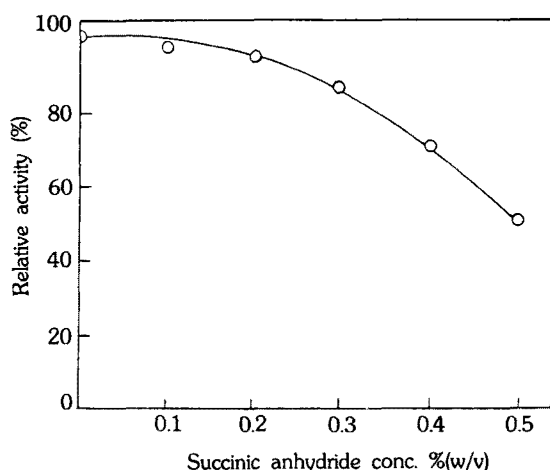

Fig. 1. Effect of succinic anhydride concentration on the activity of CGTase.

positively charged groups of Amberite IRA 900 and DEAE-Cellulose. Thus continuous desorption of enzyme due to its weak binding force (2, 11) could be overcome prevented by succinylation of CGTase during continuous operation in column reactor.

Comparison of Various Immobilization Methods for CGTase

The yield of activity and stability of immobilized CGTase on various carriers including not mentioned at previous sections are compared in Table 3. The highest recovery yield of CGTase activity with 77% was obtained in the case of adsorption on Amberite IRA 900, compared with those of DEAE-cellulose (67%), chitosan (37 %), chitin 32%), alginate (13%), and polyacrylamide (11 %).

Table 4. Comparison of physical properties of various carriers used for CGTase immobilization

Carrier Properties	Chitosan	Amberite IRA 900	DEAE-Cellulose	Alginate[a]
Particle size (mm)	0.1-0.3	0.16-0.4	0.1-0.2	3.0-5.0
Porosity	HMP[a]	MP[b]	HMP	HMP
Rigidity	medium	hard	medium	soft
Water regain (ml/g)	10.0	2.3	7.5	60.0
Morphology	amorphous	bead	bead	bead
Functional group	amine	quaternary amonium	diethyl amino ethyl	–
Cost ($/kg)	20	33	147	61
Origin	natural	synthetic	synthetic	natural

HMP (highly macroporous), MP (macroporous)
[a] manufactured gel (1.6 %, w/v)

Immobilized CGTase on Amberite IRA 900 also showed good stability (64%) after 2 weeks of continuous operation in column reactor compared with that of DEAE-cellulose (34%). As shown in Table 3, the succinylation of CGTase is essential to prevent the leakage of absorbed CGTase during the continuous operation in enzyme reactor. Both immobilized CGTase on chitosan and chitin treated with glutaraldehyde were also found to have good operational stability of 52 and 62%, compared with the non-treated ones of 47, 50%, respectively. On the other hand, the entrapment methods showed poor yield of activity and stability, indicating incompatiable as the CGTase immobilization methods.

In order to select suitable carriers for immobilization,

Table 3. The effect of experimental conditions on immobilization yield of CGTase

Carrier	Treatment	Immobilized enzyme activity (units)	Yield of activity(%)[a]	Stability (%)[b]
Chitin	Glutaraldehyde	4.8	32	52
Chitin	None	5.0	33	47
Chitosan	Glutaraldehyde	6.3	42	62
Chitosan	None	5.4	36	50
DEAE-cellulose	Succinylation	10.1	67	34
DEAE-cellulose	None	10.5	70	20
Amberite IRA 900	Succinylation	11.6	77	64
Amberite IRA 900	None	11.7	78	46
Alginate	Glutaraldehyde	2.0	13	14
Polyacrylamide	Glutaraldehyde	1.7	11	24

15 units of soluble CGTase was added into each carrier.

$$^{a}\text{YOA (yield of activity, \%)} = \frac{\text{initial activity-unbound activity}}{\text{initial activity}} \times 100$$

[b]Stability was determined by measuring residual CGTase activity after 2 weeks of continuous operation at column reactor.

the following additional factors have to be considered such as porosity and particle size of carrier, rigidity with respect to the reactor environment, regeneration of carrier, resistance to microbial contamination, and most of carrier. Table 4 compares the various physical properties of the selected carriers used for CGTase immobilization. Especially, the porosity of carrier seems to be the most critical factor, because the high molecular size of substrates *i.e.*, starch or related carbohydrates may, either not penetrate the pores, or access only to the larger pores, therefore, major quantaties of the immobilized CGTase may never be utilized. Also the rapid diffusion of large cyclodextrin molecules from immobilized carrier is required for effective CD formation in immobilized enzyme reactor.

Recently chitin and its derivative (chitosan, colloidal chitin) were utilized extensively as carriers for immobilization of several other enzymes (1, 9), but not for CGTase. The feature of chitin and chitosan justifying this upsurge of interest are the abundance of chitin as a by-product of the fishing (crab, shrimp and prawn) and fermentation industries along with relatively low cost compared to other carriers, lack of toxicity, and its chemical reactivity allowing easy fixation of enzymes using glutaraldehyde as cross-linking reagent. Chitosan seems to be an ideal carrier for immobilization of CGTase whose main product (CD) is used mainly in food or pharmaceutical purpose.

Considering the immobilization yield and stability along with several factors mentioned above, both chitosan and Amberite IRA 900 are seems to be most suitable carriers for immobilization of CGTase.

Physical Features of Chitosan and Amberite IRA 900 as Carriers

Fig. 2 shows the scanning electron micrographs of chitosan and Amberite IRA 900. The amorphous and highly macroporous chitosan can fix CGTase on the inner-space as well as outer-surface, on the other hand, bead-type and rather macroporous Amberite IRA 900 seems to absorb most CGTase on the outer-surface. Chitosan is highly macroporous, and thus able to minimize the diffusional limitation through the internal space of the carrier. On the other hand, Amberite IRA 900 seems to have the advantage of hard rigidity which make it

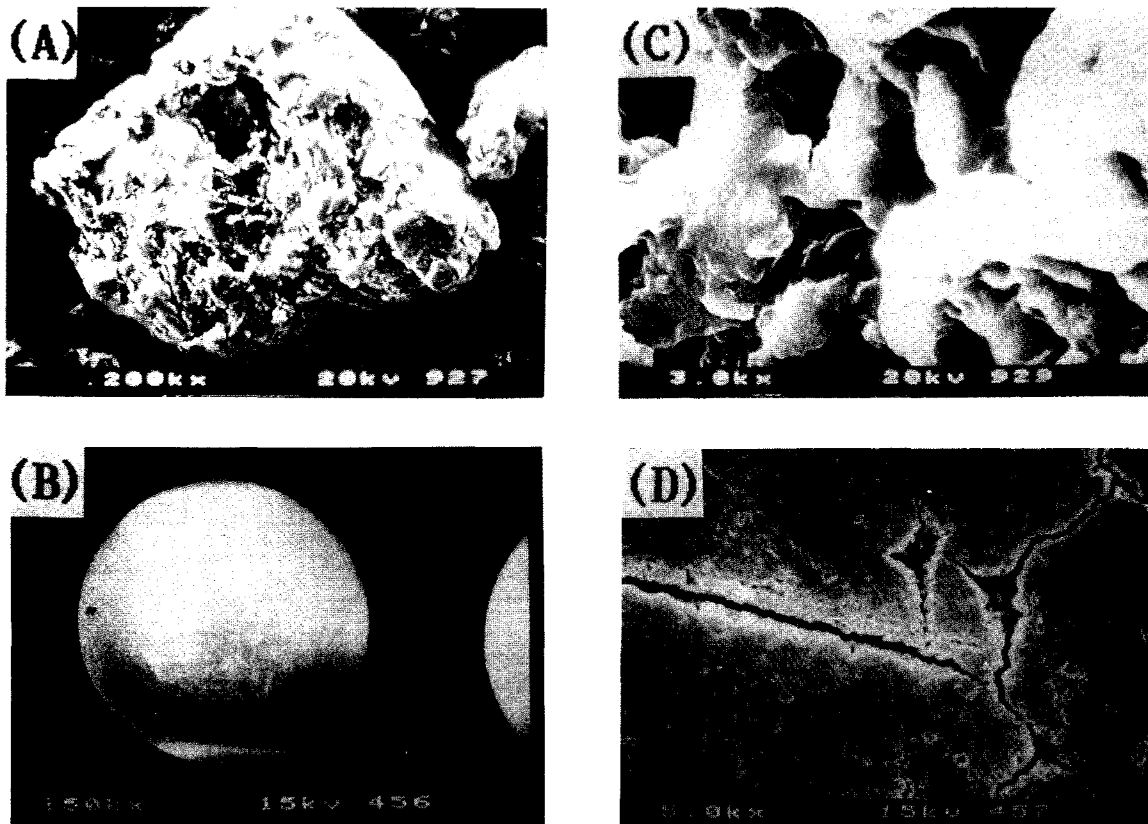

Fig. 2. Scanning electron micrograph of chitosan and Amberite IRA 900 (A) chitosan particle (×200), (B) porous structure of chitosan inner space (×3,000), (C) Amberite IRA 900 (×150), and (D) outer surface structure of Amberite IRA 900 (×5,000).

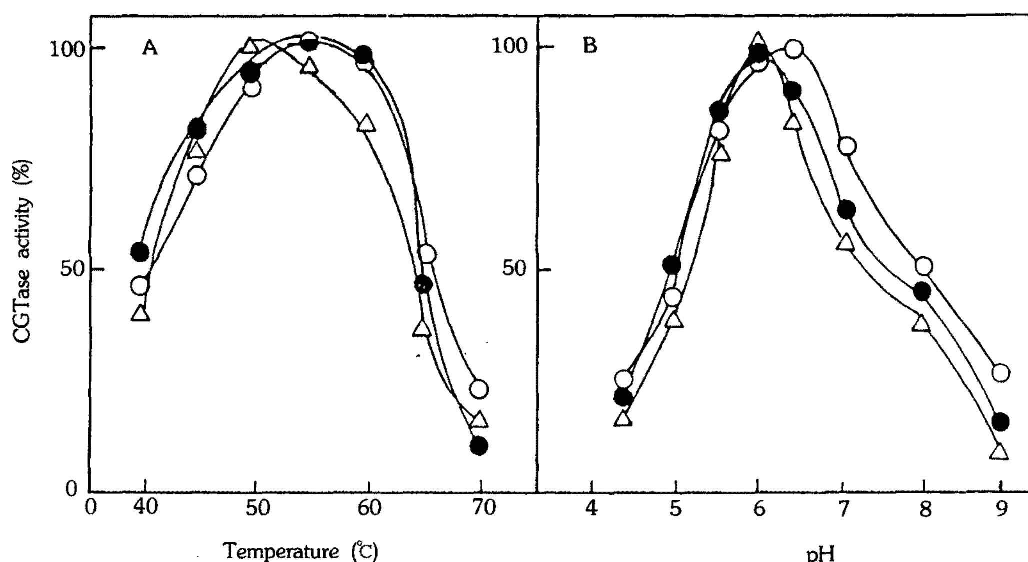

Fig. 3. Effect of temperature (A) and pH (B) on CGTase activity of both soluble and immobilized enzyme (Soluble —●—; Chitosan —○—; Amberite IRA 900 —△—).

possible to be easily applied into packed bed bioreactor without imposing serious pressure drop, and it is also cheap and regenerable carrier.

Enzymatic Properties of CGTase Immobilized on Chitosan and Amberite IRA 900

The enzymatic properties of immobilized CGTase on chitosan and Amberite IRA 900 were compared with those of soluble CGTase. The immobilized CGTase on Amberite IRA 900 showed optimum temperature shift from 55 to 50℃ as well as optimum temperature range narrowing between 50 and 55℃ compared with that of soluble CGTase (50-60℃), whereas no change of optimum temperature was occurred for the immobilized CGTase on chitosan as shown in Fig. 3(A). And the immobilized CGTase on chitosan was found to have an optimum pH shift from 6.0 to 6.5 but the optimum pH of the immobilized CGTase on Amberite IRA 900 didn't shift as shown in Fig. 3(B).

The immobilized CGTase on chitosan was found to have an increased thermal stability, maintaining its activity above 75% compared with that of soluble CGTase (below 50%) even after heat treatment for 1 hr at 60℃ as shown in Fig. 4. This apparent increase of heat stability is very desirable property because of the long duration of immobilized CGTase for continuous CD production. Optimum temperature shift of immobilized CGTase on Amberits IRA 900 (55→50℃) also makes it possible to maintain the enzyme activity for long time operation, whilst the danger of microbial contamination is more probable.

As shown in Fig. 5, pH stability of immobilized CGTase showed little change except small increase of immobilized CGTase on chitosan at the weakly acidic pH range (pH 5.0-6.0) compared to that of soluble enzyme.

Half Life of Immobilized CGTase during Column Operation

Fig. 6 illustrates the operational stability of the immobilized CGTase during prolonged column operation. Covalently bound CGTase on chitosan with glutaraldehyde maintained around 53% of its initial activity after 3 weeks of continuous reaction with half life of 23 days, whereas for the simply absorbed CGTase on chitosan, only 37% of its initial activity remained with half life of 16 days. Both immobilized CGTase on Amberite IRA 900 with and without succinylation (half life, 21 and 15 days, respectively) showed almost the same degree of decrease until 4 days of continuous reaction, whereas rapid decrease of activity for immobilized CGTase without succinylation occured over 4 days operation compared with succinylated one. These results of long operational stability for both immobilized CGTase on chitosan and Amberite IRA 900 may indicate higher applicability of immobilized CGTase for continuous production of CD.

Effect of Dextrose Equivalent (DE) of Soluble Starch on Immobillized CGTase

To achieve high CD conversion yield, the starch is partially liquified with α-amylase or partially cyclized with CGTase before feeding into the main enzyme reactor for industrial productions of CD (2). Fig. 7 shows the effect of DE (dextrose equivalent) levels of substrate treated by α-amylase on the formation of CD by both immobilized and soluble CGTases.

For soluble CGTase, the CD conversion yield increased up to DE level of 10 with maximum yield of 46%,

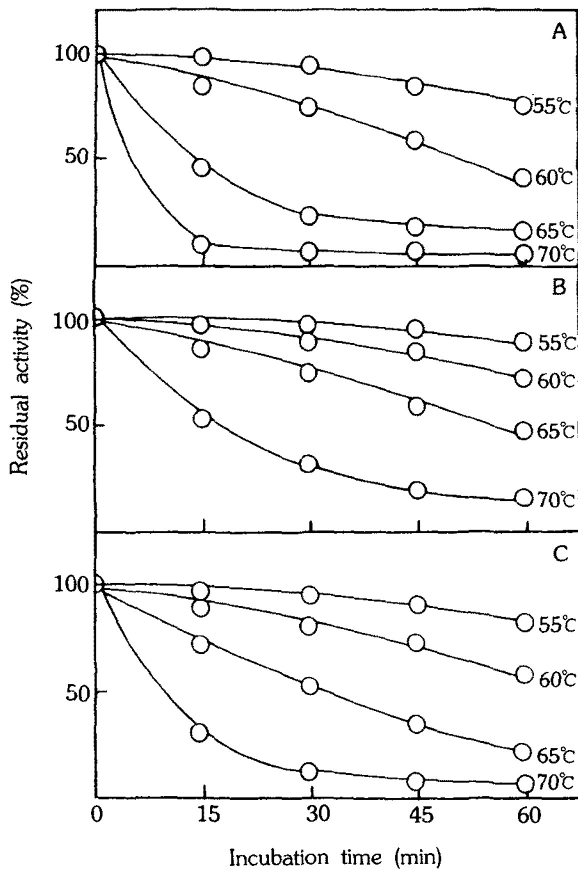

Fig. 4. Thermal stability of soluble and immobilized CGTase.
All the enzyme preparation were preincubated at indicated temperatures in the absence of calcium ion and residual activities were determined. (A) soluble CGTase, (B) immobilized CGTase on chitosan, and (C) immobilized CGTase on Amberite IRA 900.

whereas rapidly decreased abve that level and immobilized CGTase on chitosan also showed the same propencity with maximum yield of 40%. Those phenomena of the decrease indicated that suitable DE level of substrate is needed for efficient CD conversion yield. But CGTase immobilized on Amberite IRA 900 showed gradual increase of CD conversion yield with according to increase of DE level, however, the overall CD conversion yield was lower than both immobilized CGTase on chitosan and soluble enzymes. This result may be explained by diffusional limitation of macromolecular substrate through the internal space of immobilized CGTase on Amberite IRA 900 or changes of substrate specificity and catalytic action of immobilized CGTase on Amberite IRA 900.

Comparison of CD Conversion Yield Between Partially Liquefied and Partially Cyclized Starch

Table 5 compares the maximum CD conversion yield

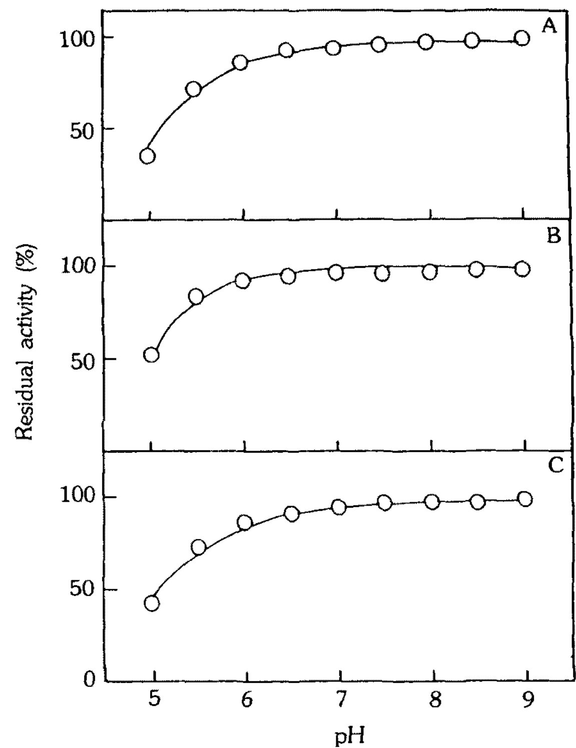

Fig. 5. pH stability of soluble and immobilized CGTase.
All the enzyme preparations were preincubated at different pH values (2 mM Maleic buffer) at 50℃ for 60 min and residual activities were determined. (A) soluble CGTase, (B) immobilized CGTase on chitosan, and (C) immobilized CGTase on Amberite IRA 900.

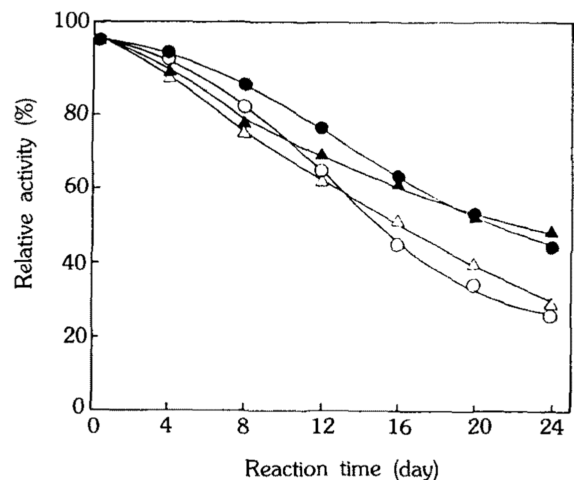

Fig. 6. Operational stability of immobilized CGTase on chitosan with (–▲–) or without (–△–) glutaraldehyde and on Amberite IRA 900 with (–●–) or without (–○–) succinylation of enzyme.
Soluble starch solutions (1%, containing 10 mM $CaCl_2$, pH 6.0) were passed through the thermostatted column (1.6× 33.0 cm, 50℃) at a constant flow rate of 30 ml/hr.

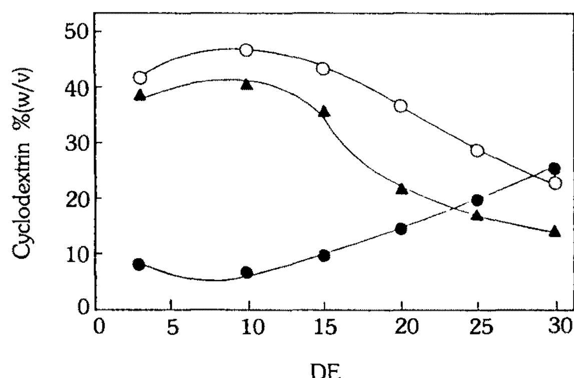

Fig. 7. Effect of DE (dextrose equivalent) levels of substrate on the formation of CD by the same amount of soluble CGTase (—○—), immobilized CGTase on chitosan (—▲—) and Amberite IRA 900 (—●—). 5% (w/v) of soluble starch solutions were liquefied with α-amylase (Fungamyl, Novo, 2-12 units/g of starch) for 10 min at pH 6.0, 50℃ (DE is defined as the percentage of reducing sugar to maximum conversible reducing sugar).

Table 5. Comparison of maximum CD conversion yields between partially liquefied starch with α-amylase and partially cyclized starch with CGTase

Substrate / CGTase	Partially liquefied starch[a]	Partially cyclized starch[b]
Soluble	46	48
Immobilized on chitosan	40	42
Immobilized on Amberite	25	40

[a] 5% (w/v) of soluble starch solution was liquefied with α-amylase (Fungamyl, Novo, 4 unit/g of starch) for 10 min at pH 6.0 and 50℃.

[b] 5% (w/v) of soluble starch solution was cyclized with CGTase (10 units/g of starch) for 2 hr at pH 6.0 and 60℃.

between liquefied starch with α-amylase and partially cyclized starch with CGTase. Regardless of soluble or immobilized CGTase, partially cyclized starch showed superior CD conversion yield compared with liquefied starch. This fact indicates that partially cyclized starch may have more suitable molecular length which can be easily diffused and transformed to CD by CGTase than that of liquefied starch. The partial cyclization of starch seems to be an essential procedure to achieve the high degree of conversion of CD by immobilized CGTase.

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