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Performance of Column Type Bioreactor Packed with Immobilized Cyclodextrin Glucanotransferase for Cyclodextrin Production

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Performance of column type bioreactor packed with immobilized cyclodextrin glucanotransferase (CGTase) on chitosan and Amberite IRA 900 was evaluated for cyclodextrin(CD) production. For CGTase immobilized on chitosan, the maximum CD conversion yield of 42% was achieved at the range of 88-168 units of immobilizied CGTase per gram of chitosan, retention time of 0.3 hr, and from 5.0% (w/v) of partially cyclized soluble starch. On the other hand, for CGTase immobilized on Amberite IRA 900, the maximum conversion yield of 40% was obtained at the range of 3.6-11.0 units of immobilized CGTase per gram of carrier and retention time of 1.2 hr from 5.0% of substrate. Above CD conversion yields are almost identical level with that can be obtained with soluble CGTase of 47%. The productivities of bioreactor packed with immobilized CGTase were 17.0 g of CD/*l*·hr for amberite IRA 900 and 15.5 g of CD/*l* · hr for chitosan. The partially cyclized starch with soluble CGTase were more suitable as substrate to achieve better CD conversion yield, and 5% (w/v) of partially cyclized soluble starch containing 10% (w/w) of CD was found to be most suitable to obtain maximum CD conversion yield.

Cyclodextrin(CD) production has been mainly carried out batchwisely using soluble CGTase (2-5, 7). However, a continuous production of CD using immobilized CGTase would have potential advantages of permitting reuse of expensive CGTase for an extended period of time, simplification of CD purification process, easy control of reaction, and scale-up availability.

CD production using immobilized CGTase has been rarely attempted except by only two research groups (6, 13). Production of CD derivative, α-glycosylated CD, was intensively investigated by Japan CD Reseach Associates (13) who utilized immobilized CGTase, amylase, glucoamylase, and yeast cell serially to obtain highly pure CD derivative. Hitashi *et al.* (6) undertook an experiment for continuous formation of CD by the column method using CGTase immobilized on ion exchange carrier, and found that CD yield was mainly affected by the relationship between the amount of immobilized CGTase and the flow rate of substrate.

In our pervious work (10), immobilization of CGTase on chitosan by covalent linking and on Amberite IRA 900 by adsorption were found to be the most suitable immobilization methods for CGTase considering the yield of activity and the stability of immobilized CGTase. The enzymatic properties of immobilized CGTase also investigated and compared with those of the soluble CGTase.

In this work, the optimum operational conditions for column type enzyme reactor packed with CGTase immobilized on chitosan and Amberite IRA 900 were investigated for CD production. This research will facilatate not only the development of effective immobilized enzyme bioreactor but also continuous process for industrial-scale production of CD.

MATERIALS AND METHODS

Preparation of Immobilized CGTase

CGTase of *B. macerans* partially purified (3,500 units/mg of protein, Amano Pharm. Co.) as described

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in our previous work (11) was immobilized on macroporous chitosan by covalent linking and on Amberite IRA 900 by adsorption. Chitosan was prepared from crab shell by the successive procedures of Stanley *et al.* (12) and Rigby (Rigby, G. W. 1934. U. S. Patent 2,040, 889); on the other hand, Amberite IRA 900 (Sigma Co.) was used without any modification. The immobilization procedures of CGTase on chitosan and Amberite IRA 900 are documented in our previous work (10).

Physical Properties and Environment of Immobilized CGTase

One gram of chitosan was swelled to 7 ml in buffer solution, and can immobilize 8-168 units of CGTase per gram of chitosan; in other word, 1 ml of bed volume of swollen chitosan can immobilize 1.1-24.0 units of CGTase. One gram of Amberite IRA occupies about 1.0 ml of volume, and can hold 0.8-16.8 units of CGTase.

The amorphous and highly macroporous chitosan could evenly fix CGTase on the internal-surface as well as outer-surface; on the other hand, bead-type and rather macroporous Amberite IRA 900 adsorbed CGTase mainly on the outer-surface as observed previously (10).

Measurement of CGTase Activity

5 ml 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 reactant 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 described by Kitahata *et al.* (8).

Preparation of Partially Cyclized Soluble Starch

To achieve high CD conversion yield in immobilized CGTase column type bioreactor, soluble starch (5%, w/w) was partially cyclized with soluble CGTase (10 units/g of soluble starch) at pH 6.0 and 60℃ for 2 hrs. The remained activity of CGTase was removed by heating for 10 min at 90℃ before feeding into column type bioreactor.

Operation of Column Type Bioreactor

Column type bioreactor system was consisted of water jacketted glass column (1.6×33.0 cm, bed volume 65 ml), water bath, and flow rate controller (Spectra/Chrom Flow Rate Controller, 5-300 ml/hr) as illustrated in Fig. 1.

A thermostatted glass column was filled with 50 ml of bed volume of immobilized CGTase to a column height of 25 cm. 5% (w/v) of partially cyclized soluble starch solution in 10 mM of maleic acid-Tris-NaOH buffer (pH 6.0) containing 10 mM of $CaCl_2$ was feeded into the bed of immobilized CGTase from the top by flow rate controller with the range of 15-60 ml/hr (retention time; 2.3-0.6 hr for Amberite IRA 900 and 3.3-0.8 hr for chitosan), and maintained at 50℃.

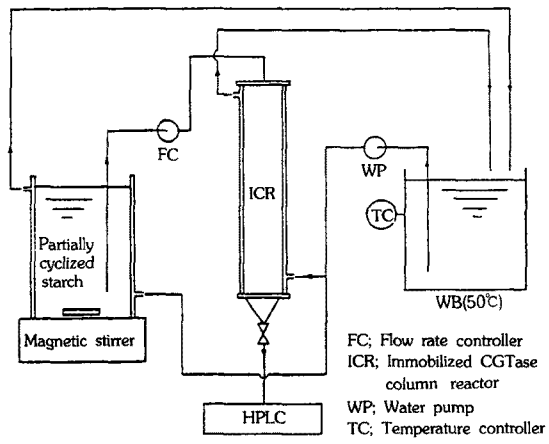

Fig. 1. Column type immobilized CGTase bioreactor system utilized for continuous formation of CD.

RESULTS AND DISCUSSION

Effect of Degree of Partial Cyclization on CD Conversion Yield in Column Bioreactor

In order to increase the CD conversion yield, partial cyclization of starch with soluble CGTase under mild conditions seems to be an essential prerequisite procedure before main cyclization reaction for CD formation in immobilized column bioreactor as reported in our previous work (10). Fig. 2 compares the effect of partially cyclized (10% CD) and non-treated soluble starches on conversion yield of CD in main immobilized column bioreactor that was packed with the range of 0.9-16.8 units of CGTase immobilized on one gram of Amberite IRA 900. Five percents of soluble starch solutions partially cyclized as described previously or non-treated were passed through the column reactor at the flow rate of 30 ml/hr (retention time, 1.2 hr).

CD conversion yields of partially cyclized starch were generally 5-18% higher than those of non-treated starch regardless of the amount of immobilized CGTase. The maximum conversion yield for partially cyclized substrate was 40% at the immobilized yield for partially cyclized substrate was 40% at the immobilized CGTase amount of 7.2 units per gram of carrier, whereas gradually decreased at the above level, which seems to be related with other side reactions, such as, coupling and disproportionation reaction, in addition to CD forming cyclization reaction under the excessive reaction of CGTase.

In the case of non-treated substrate, CD conversion yield was consecutively increased with respect to the

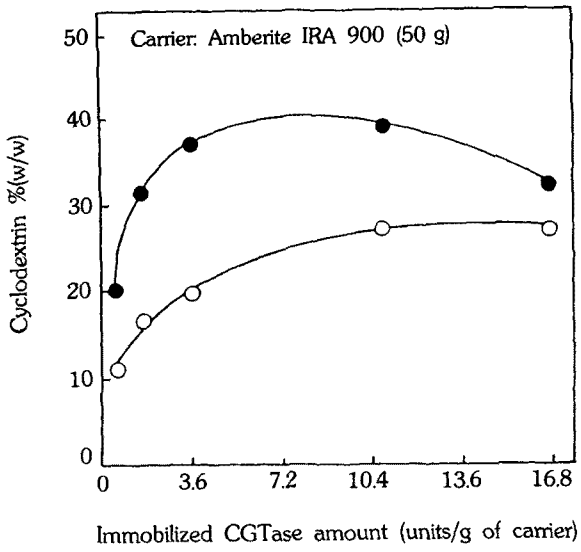

Fig. 2. Effect of partial cyclization of substrate on the formation of cyclodextrin in immobilized CGTase column reacter.

Partially cyclized starch (−●−) containing 10% (w/w) of CD, non-treated starch (−○−): 5% (w/v) of soluble starch solutions were passed through the immobilized CGTase column reactor at flow rate of 30 ml/hr, pH 6.0, and 50℃.

increasement of immobilized CGTase amount, which indicating that quite a large amount of CGTase is required for effective CD conversion of non-treated starch. The partially cyclized substrate was found to be more proper than non-treated one not only for obtaining better CD conversion yield but also for preventing excessive requirement of CGTase at immobilized CGTase column bioreactor.

Table 1 compares the effect of different degree of partial cyclization on CD conversion yield in immobilized CGTase and that obtained from free CGTase. Partially cyclized 5% (w/v) soluble starch (0-20%(w/w) of CD by soluble CGTase) were reacted with 6 units of immobilized CGTase on Amberite IRA 900 and the same amount of soluble CGTase in order to investigate CD conversion yield. Relatively high CD conversion yields (33-42%) were obtained at the broad range of degree of partial cyclization (0-15%) in accordance with the degree of partial cyclization, and the highest CD conversion yield (42%) was achieved at 10% of partial cyclization. In contrast, the reaction carried out using soluble CGTase, the CD conversion yields were around 5% higher compared with those of immobilized CGTase system, and the maximum achievable CD conversion yield was 47% for 5% (w/v) of soluble starch whose degree of partial cyclization is 10%.

The rapid decrease of CD conversion yield above 10% of partial cyclization seems to be related with the increased concentration of glucose which is gradually liberated during the partial cyclization of starch as shown in Table 1, which not only inhibiting the formation of CD by the coupling reaction of CGTase where the rings of CD are opened and transferred to co-substrates such as glucose to make various oligosaccharides (9), but also lowering yield of CD by the over-cutting of the soluble starch chain that are required for 6-8 units of glucose for effective cyclization.

Table 1. Effect of degree of partial cyclization on CD conversion yield

Partial cyclization time (min)	Degree of partial cyclization: Total CD (%, w/w)	Degree of partial cyclization: Glucose (%, w/w)	CD conversion yield at 2nd stage main reactor: Immobilized CGTase (%, w/w)	CD conversion yield at 2nd stage main reactor: Free CGTase (%, w/w)
0	0	0.01	37	43
17	5	0.15	38	43
24	7	0.16	40	45
39	10	0.16	42	47
67	15	0.18	37	43
100	20	0.25	32	38

To obtain desirable degree of partial cyclization, 10 ml of 5% (w/v) soluble starch solutions were mixed with 6 units of CGTase and reacted for various time interval between 0-100 min at pH 6.0 and 60℃. Those reactants were inactivated by heating at 90℃ for 5 min. CD and glucose concentrations were measured by HPLC.

Immobilized CGTase, 6 units of CGTase immobilized on 1 gram of Amberite IRA 900 was mixed with above partially cyclized starches and reacted for 200 min while stroking (120 strokes/min) at pH 6.0 and 50℃.

Soluble CGTase, fresh solutions of CGTase (6 units) were added into above partially cyclized starches and reacted at the same conditions.

Effect of Substrate Flow Rate on CD Conversion Yield

The effects of substrate flow rate and the amount of CGTase immobilized on Amberite IRA 900 on CD conversion yields was evaluated as shown in Fig. 3(A). The highest CD conversion yield of 40% was achieved at the broad ranges of immobilized CGTase amount of 3.6-11.0 units per gram of carrier and substrate flow rate of 30 ml per hour which is corresponding to retention time of 1.2 hr.

In the case of immobilized CGTase on chitosan (Fig. 3(B)), the highest CD conversion yield (42%) was obtained at the immobilized.

CGTase amount ranged from 88-168 units per gram of carrier and substrate flow rate of 15 ml per hour, which was a slightly higher than that obtained from Amberite IRA 900 (40%).

Above conversion yields represent the maximum ob-

tainable yield in immobilized bioreactor and they are comparable with that obtained by soluble CGTase. Both in the Amberite IRA 900 and chitosan, CD conversion yield increased as the amount of the immobilized CGTase increased up to a certain level, however slight decrease of CD conversion yield was provided when excessive amount of immobilized CGTase was applied. This decrease in CD conversion yield may be presumable due to side reactions of CGTase such as, coupling and disproportionation reactions other than the normal cyclization reaction (9). Consequently, to obtain the maximum CD conversion yield, the substrate flow rate must be controlled according to the amount of immobilized CGTase.

Effect of Substrate Flow Rate on the Ratio of α-, β-, γ-CD Formation

Fig. 4 shows the effect of substrate flow rate (or retention time) on the ratio of α-, β-, γ-CD formation. 5% (w/v) partially cyclized soluble strach was pumped through the immobilized CGTase bed (10.9 units/g of Amberite IRA 900, 104 units/g of chitoasn) at various flow rates (15-60 ml/hr, retention time; 2.33-0.58 hr for Amberite IRA 900, 3.3-0.8 hr for chitosan). For both immobilized CGTase on chitosan and Amberite IRA 900, the relative ratio of α-CD to β-CD was remained without any significant changes at the lower flow rate of 30 ml/hr, whereas α-CD were predominated over β-CD at higher flow rate. This phenomenon indicates that α-CD formation is more favorable than β-CD at the short reaction (or retention) time indicating *B. macerans* CGTase produces mainly α-CD at earlier stage of reaction as reported by Hitoshi *et al.* (4). As reaction time prolonged, the quantity of α-CD once produced decreased by coupling and disproportionation reaction of CGTase which has higher affinity for α-CD rather then β-CD, as a consequence β-CD's quantity increased.

Effect of Substrate Concentration on CD Conversion Yield

Fig. 5 illustrates the effects of substrate concentration and immobilized CGTase amount on the CD conversion yield. Soluble starch concentration of 5, 10 and 15% (w/v) partially cyclized were injected into the immobilized CGTase column bioreactor. The CD conversion yields for 5, 10 and 15% of substrate were 40, 26 and 12% for Amberite IRA 900 (Fig. 5(A)), whereas 42, 24 and 15% for chitosan (Fig. 5(B)).

As the concentration of substrate increased, the CD conversion yield decreased rapidly. This decrease of CD conversion yield seems to be mainly due to diffusional limitation of high molecular weight and viscous substrate into the immobilized CGTase on the outer surface as well as internal surface of matrix. This low CD conversion yield seems to be a major limitation for industrial-scale application of immobilized CGTase compared with that of soluble CGTase system which can obtain 40-50% of CD conversion yield from 15-25% (w/v) of starch.

Productivity of CD Formation using Immobilized CGTase in Column Bioreactor

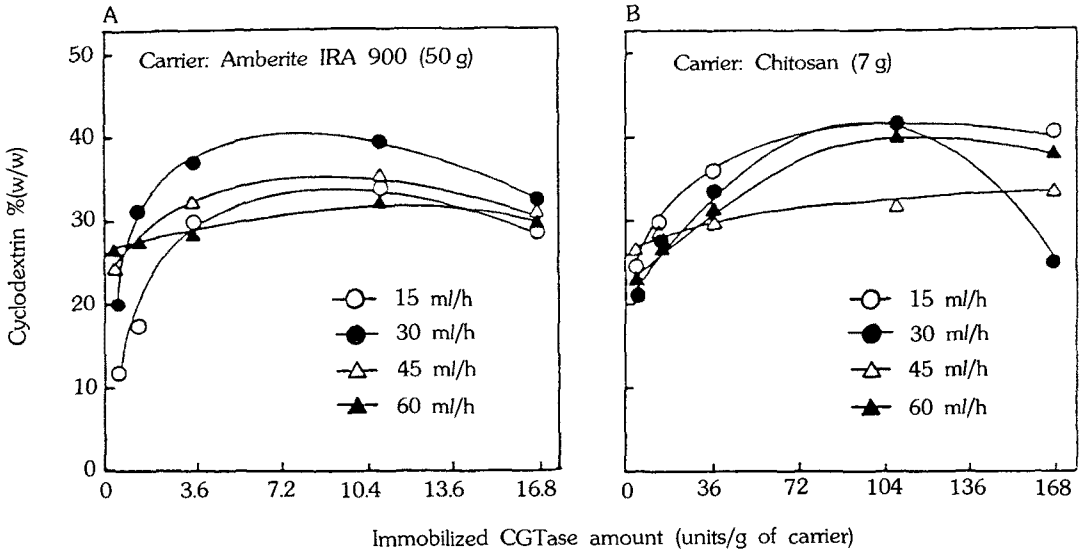

Fig. 3. Effect of immobilized CGTase amount and substrate flow rate on the formation of CD.

Partially cyclized soluble starch 5% (w/v), pH 6.0, 50℃, and column size (1.6×33.0 cm, 65 ml).

A) Amberite IRA 900; 1 gram of Amberite IRA 900 can immobilize 0.8-16.8 units of CGTase and occupy about 1.0 ml of volume.

B) Chitosan; 1 gram of chitosan is swelled to 7 ml and can immobilize 8-168 units of CGTase per gram of chitosan, therefore, 1 ml of bed volume can immobilize 1.1-16.8 units of CGTase.

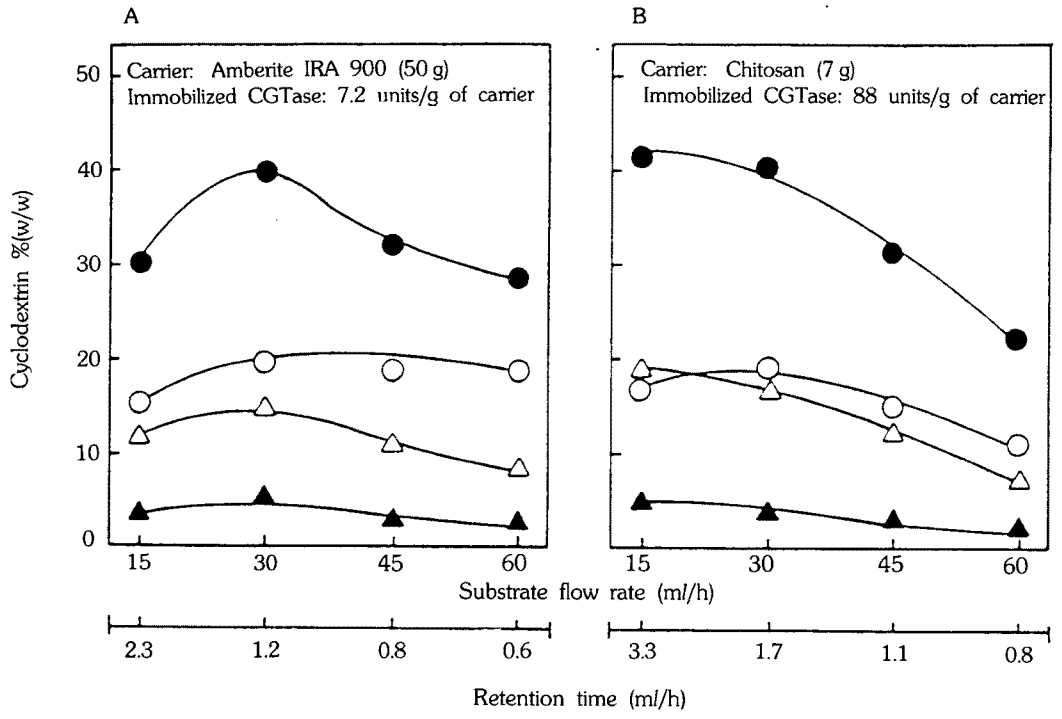

Fig. 4. Effect of substrate flow rate on the ratio of cyclodextrin formation.
Total-CD(−●−), α-CD(−○−), β-CD(−△−), γ-CD(−▲−): partially cyclized soluble starch 5%(w/v), pH 6.0, thermostatted column (1.6×33.0 cm), and 50℃.

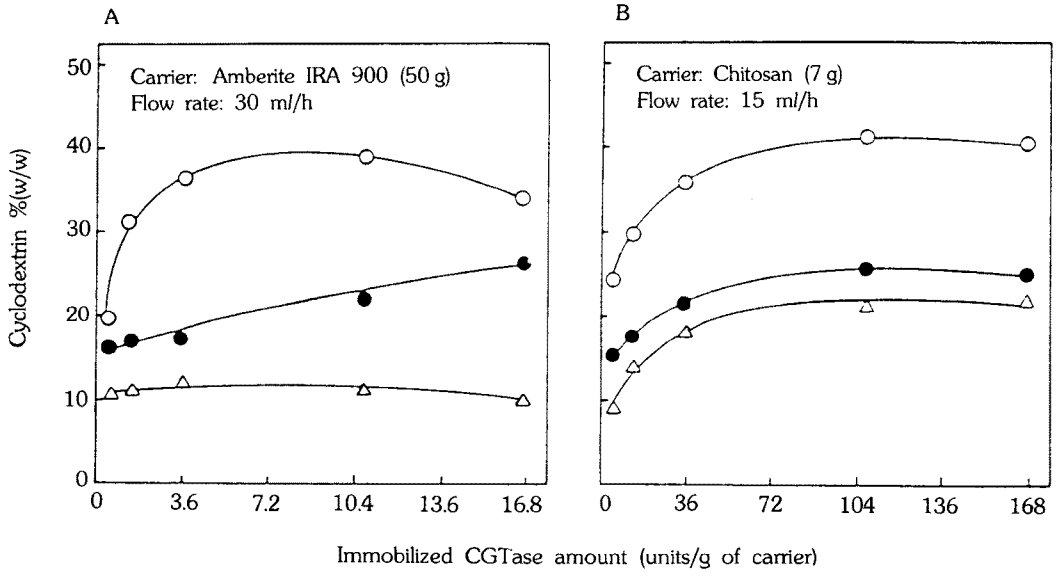

Fig. 5. Effect of substrate concentration and immobilized CGTase amount on the formation of cyclodextrin.
Partially cyclized soluble starch, 5(−○−), 10(−●−), 15(−△−) %(w/v): thermostatted column, 1.6×33.0 cm, 50℃.

Retention time of substrate: Fig. 6 illustrates the effect of retention time of substrate on CD conversion yield and productivity of column type bioreactor for both packed with CGTase immobilized on chitosan and Amberite IRA 900. For both chitosan and Amberite IRA 900 were higher than those of chitosan at short retention time between 0.5-1.7 hr, which indicate that the reaction rate of CGTase immobilized on Amberite IRA 900 is higher than that of chitosan. On the other hand, productivities of Amberite IRA 900, the conversion yield rea-

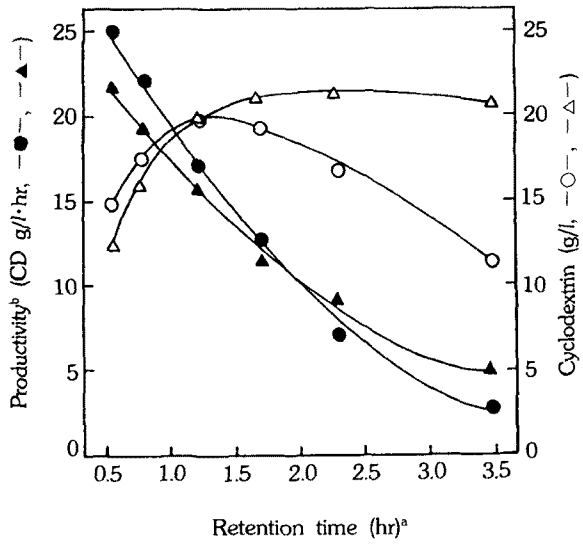

Fig. 6. Effect of substrate retention time on the formation of CD and productivity of bioreactor packed with CGTase immobilized on chitosan(—△—, —▲—) and Amberite IRA 900(—○—, —●—).
The amount of CGTase immobilized were 520 units on chitosan and 616 units on Amberite IRA 900.
a) Retention time (hr)=void volume (50 ml for chitosan and 35 ml for Amberite IRA 900)/flow rate
b) Productivity=g of CD/l · hr

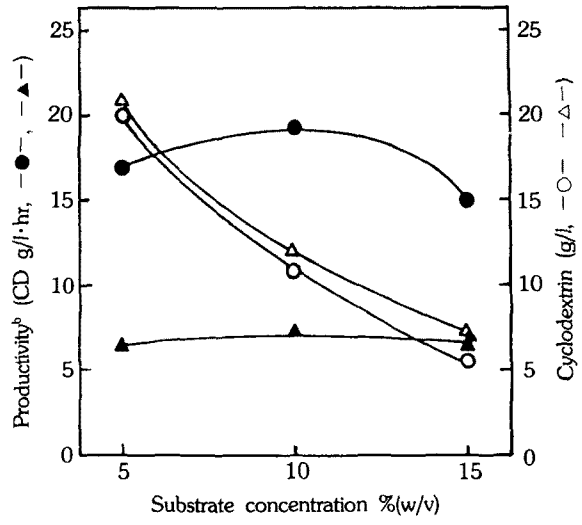

Fig. 7. Effect of substrate concentration on the formation of CD and productivity of bioreactor packed with CGTase immobilized on chitosan(—△—, —▲—) and Amberite IRA 900(—○—, —●—).
The amount of CGTase immobilized and retention time were 616 units, 1.2 hrs for Amberite IRA 900 and 520 units 3.3 hrs for chitosan.

Table 2. Comparison of immobilized CGTase on Amberite IRA 900 and chitosan

	Immobilized CGTase on Amberite IRA 900	Immobilized CGTase on Chitosan
Yield of[b]		
enzyme activity[a]	77	37
Half life(days)[b]	21	23
Max CD[c]	40	42
Productivity[d]	17.0	15.5
Preparation	simple	intermediate
Binding force	strong	strong
Regeneration	possible	difficult
High pressure drop	rare	serious
Cost of immobilization	intermediate	intermediate

[a]Yield of enzyme activity: (initial activity-effluent activity)×100/initial activity
[b]Time for 50% reduction of initial immobilized CGTase activity
[c]Maximum CD conversion yield (%, w/w)
[d]Productivity: g of CD produced/l·hr at the reaction time when CD conversion yields reached up to the maximum

ched maximum at retension time of 1.5 hrs, however, the productivities were constantly decreased in accordance with the increase of retention time. The productivities of Amberite IRA 900 were lower than those of chitosan above 1.7 hr of retention time. This phenomena can be explained as gradual decrease of CD conversion yield due to excessive reaction of CGTase under the influence of the structure of immobilized CGTase on Amberite IRA 900, whereas constant levels of CD conversion yield were maintained for chitosan at broad ranges of long retention time.

The optimal retention time should be determined by economic optimization in terms of CD conversion yield which is related with substrate cost and productivity of bioreactor which is related with operational cost.

Substrate concentration: Fig. 7 illustrates the effects of substrate concentration on CD conversion yield and productivity of column type bioreactor packed with CGTase immobilized on chitosan and Amberite IRA 900. For substrate concentration of 5, 10, 15% (w/v), the productivity for Amberite IRA 900 were 16.7, 19.2, 15.0 g/l·hr, which were much higher than those of chitosan, 6.4, 7.3, 6.8 g/l·hr, respectively. These differences of productivity can be mainly explained by the physical environments of immobilized CGTase on Amberite IRA 900 and chitosan, *i.e.* most of the CGTase immobilized on Amberite IRA 900 are located on the outer-surface of carrier, and thus the reaction rate is faster than that of CGTase on chitosan where most of enzyme is immobilized on inner-surface rather than outer-surface as described in our previous work (10).

For both chitosan and Amberite IRA 900, the conversion yields of CD were rapidly decreased in accordance

with increase of substrate concentration. Especially, CD conversion yield for Amberite IRA 900 were somewhat rapidly decreased comparing with those of chitosan with increasing of substrate concentration, which indicate that diffusional limitation of high concentration of substrate is more seriously occurred in Amberite IRA 900 than in chitosan.

Comparison of Chitosan and Amberite IRA 900 as Carrier for CGTase Immobilization

Table 2 summarizes characteristics and applicability of immobilized CGTase on chitosan especially for Amberite IRA 900. Good yield of enzyme activity and stability were obtained for both CGTase immobilized on Amberite IRA 900 and chitosan. They also showed almost same maximum achievable conversion yield of CD (40-42%) comparable with that of soluble enzyme (47%). However, for the industrial production of CD by immobilized CGTase bioreactor, Amberite IRA 900 seems to be more proper carrier than chitosan from the viewpoints of the high productivity, easy of regeneration of spent carrier (1), and low pressure drop at scale-up size bioreactor.

Acknowledgement

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