Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRIN GLUCANOTRANSFERASE AND INDUSTRIAL PROCESSES FOR PRODUCTION OF CYCLODEXTRIN

Yong-Hyun Lee

Department of Genetic Engineering, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea, tel.: +82-53-950-5384, fax: +82-53-959-8314, e-mail: leeyh@knu.ac.kr

Various approaches for industrial production of CDs have been presented. In order to increase the bioavailable of the substrate, the raw starch was extruded instead of liquefaction. The processes of CDs production using raw starch as substrate, liquefied starch and swollen extrusion starch were compared. The highest yield of CDs (54 g/l) was obtained after 24 hours for extrusion starch, compared to 45 g/l of that of liquefied starch and only 6 g/l CDs from raw starch (starch concentration was 100 g/l).

Представлены различные подходы промышленного получения ЦД. Для увеличения биодоступности субстрата, сырой крахмал подвергали экструзии вместо разжижения. Сравнились процессы получения ЦД с использованием сырого, разжиженного и разбухшего экструзионного крахмала в качестве субстратов. Наибольшой выход ЦД (54 г/л) получен после 24ч в случае экструзионным крахмалом, по сравнению с 45 г/л из разжиженного крахмала и только 6 г/л из сырого крахмала (концентрация крахмала 100 г/л).

Ներկայացված են ՑԴների արդյունաբերական ստացման տարբեր մոտեցումներ: Սուբստրատի կենսահասանելիությունը բարձրացնելու նպատակով, մասնակի հիդրոլիզի փոխարեն, չմշակված օսլան ենթարկել են էկստրուզիայի: Համեմատվել են ՑԴների ստացման եղանակների արդյունավետությունը չմշակված, հիդրոլիզացված և էկստրուզիոն օսլայի դեպքում: ՑԴների ամենաբարձր քանակություն (54գ/լ) ստացվել է 24ժամ հետո էկստրուզիոն օսլայից, համեմատած 45գ/լ ջրիկացված օսլայի և միայն 6գ/լ չմշակված օսլայի դեպքերի հետ:

Introduction

Cyclodextrins (cyclomaltooligosaccharides, cyclic(1→4)-α-D-glucans, CDs) are produced by the action of cyclodexrin glucanotransferase(CGTase) from starch, amylose, amylopectin, and other long-chain maltooligosaccharides, and posses a hydrophilic outside and hydrophobic central cavity. The formation of inclusion complex between CDs and dye was discovered by Cramer *et al.* at the beginning of the 1950s, and a patent covering practical application of CD in drug formulations was filed by Freudenberg *et al.* Thereafter, the enzymatic production of CDs, fractionation of CDs, and characterization of their chemical and physical properties had been intensively studied, and the commercialization of CD was initiated in Japan at 1970s [1].

CDs have been widely utilized 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 into their cavity, thereby, altering the physical and chemical properties of these compounds. The utilization of CDs in Korea was stated at 1984 using the import β-CD, and their utilization has been gradually increased in pharmaceutical, food, and cosmetic industry. CD mixture comprising α-CD as a major CD as well as pure β-CD are manufactured by Sewon Inc. since the end of the 1980s [2].

Cyclodextrin Glucanotransferase (CGTase)

CGTase enzymes are able to produce CDs from starch via the cyclization reaction, which is the basis of their industrial application. Recent developments, however, also concentrate on the use of the CGTase catalyzed coupling and disproportionation reactions for synthesis of modified oligosaccharides by using alternative acceptor substrates.

(1) Reactions catalyzed by CGTase

The synthesis of CDs can easily be performed by enzymatic reaction of starch or other CGTase. CGTase are able to catalyze several reactions [3]. As shown in Figure 1, there is the unique cyclization reaction that is only efficient if long-chain substrates are used (dextrose equivalent (DE) < 20). According to Bender, optimal cyclization rate are achieved with 1,4-α-D-glycopyranosyl chins at a length of 16-80 glucose residues. Chain > G8 can be cyclized directly [6]. The cyclization reaction is initiated by an exo-attack of suitable substrates from their nonreducing chain ends.

CGTase can catalyze the reverse reaction of cyclization called "coupling". This reaction explains the time-dependent shift of CD-products during conversion of starch to CDs. For example, during the enzymatic reaction of a α-CGTase, a high initial peak of α-CD can be transformed to a significant extent to β-CD as shown in Figure 1 via the coupling-cyclization equilibrium. The reverse coupling reaction is favored at higher concentrations of maltooligosaccharieds of glucose [7].

The disproportionation reaction occurs mainly at the beginning of the CGTase reaction, if longer chain starch substrates are used. This is apparent from the rapid and drastic decrease of the viscosity of substrate. It is assumed that the disproportionation reaction does not influence the synthesis of CDs any further. The difference between the cyclization reaction and disproportionation is that cyclization is a one-substrate and disproportionation is a two-substrate reaction [7,8]

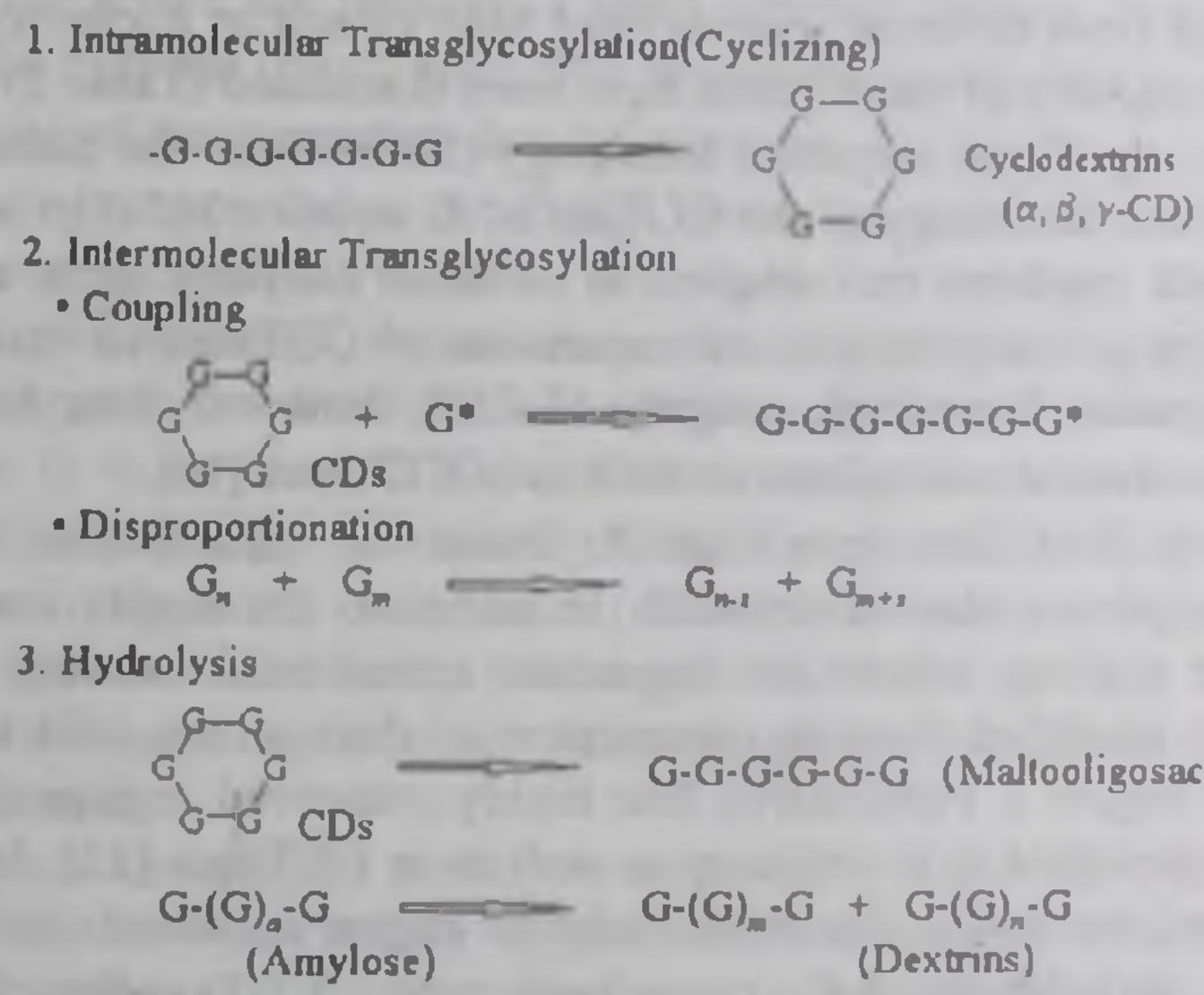

Figure 1. Various catalytic reactions of CGTase.

(2) Classification and source

CGTase are divided into three types (α, β, and γ) according to the type of CD formed initially. As the enzymatic reaction continues, the two other forms of CD are usually also

synthesized. When the reaction is allowed to reach equilibrium, β-CD is the main end-product; formation of β-CD is thermodynamically more favorable than that of α- and γ-CD [7].

Table 1 classifies microorganisms producing CGTase according to its main product from starch. Hence, α-CGTase produces (initially) α-CD. Typical representatives are the CGTase of *B. macerans*, *B. stearothermophilus*, *Thermoacaerobacter* sp., and *Klebsiella oxytoca* M5al. β–CGTase have been analyzed from *B. circulans* and a variety of alkalophilic bacteria. Examples of unique enzyme are the β-CGTase of the alkalophilic strains, *B. ohbensis*, *Bacillus* 1-1, and *B. firmus* var. *alkalophilus*. Both enzymes produce high amounts of β-CD, some γ-CD, but no α-CD under standard reaction conditions [4, 5, 7].

Table 1. Microorganisms producing cyclodextrin glucanotransferase (CGTase)

Main product from starch	Microorganisms
α-CD	*Bacillus macerans*, *B. stearothermophilus*, *Klebsiella pneumoniae*, *Thermoanaerobacter sp.*
β–CD	*B. megaterium*, *B. circulans*, *B. ohbensis*, Alkalophilic *Bacilli*, *B. firmus* var. *alkalophilus*
γ–CD	*Bacillus sp* AL6, *B. subtilis* No. 313, *Brevibacterium* sp. No. 9605

(3) Biochemical and genetic features

Typically, CGTase have a molecular weight of about 70-75 kDa, which is significantly higher than that of α-amylases (45-55 kDa). Most of them require Ca^{2+} for optimal activity and stability. They show activity over a broad pH-range, often from pH 5 to pH 10. The thermostability of the mesophilic enzymes is limited to about 60°C. An exception is the CGTase of *Theroanaerobacter* sp. which shows highest activity at 95°C [10].

At least 18 genes from different sources have been cloned in *Escherichia coli* and the DNA sequence of the majority of these genes have been elucidated (Table 2) [4]. Among the *Bacillus* sp. CGTase a significant sequence homology (50-70%) of the deduced amino acid sequence exists. Sequence homology of the CGTase of *K. oxytoca* M5al to other CGTases is only about 30%, which confirms the uniqueness of these enzymes from a gram-negative bacterium. Interestingly, primary sequence comparisons of CGTases to various α-amylases indicate moderate sequence homology, roughly 15-25%, however, four highly conserved regions have been identified in α-amylases as well as in CGTases [10].

In our laboratory, β-CGTase gene from *B. firmus* var. *alkalophilus* has been cloned and it s nucleotide sequence also determined. In addition, ten highly conserved regions including above four regions, which are important amino acid residues in catalysis of CGTase, were further identified through comparison of their amino acid sequence among CGTases as shown in Figure 2. Particularly, four highly conserved regions (region II, V, VI, and VIII) have been identified in α-amylases as well as in CGTases [12]. In addition, other enzymes were analyzed containing this conserved four-region sequence motif. The so called α-amylase family includes-besides α-amylase and CGTase-branching enzymes, neopullulanase, cyclodextrinase, and amylomaltase. β-Amylase and glucoamylase do not possess these sequence motifs and belong to another protein family [10]. Region IX and X are belonged to putative raw starch-binding motif, and important in CGTase catalysis.

Table 2. Bacterial sources and enzymatic properties of CGTases

Enzyme source	M.W.	CGTase type	Opt. PH	pH stability	T. S.	Gene cloned
Bacillus megatrium	75,000	β-type	5.2-6.2	7.0-10.0	<55°C	-
Bacillus circulans	-	β-type	5.2-5.7	7.0-9.0	<55°C	+
B. stearothermophilus	68,000	α-type	6.0	8.0-10.0	<50°C	+
Bacillus macerans	75,000	α-type	5.2-5.7	8.0-10.0	<55°C	+
Klebsiella pneumoniae	-	α-type	5.2	5.0-7.5	-	+
Alkalophilic *Bacillus* sp. 38-2	88,000	β-type	4.5-4.7	6.0-10.0	<65°C	+
B. firmus var. *alkalophilus*	75,000	β-type	6.0	5.5-10.0	<60°C	+
Bacillus ohbensis	35,000	β-type	5.5	6.5-9.5	<55°C	-
Bacillus sp. AL6	45,000	γ-type	7.0	6.0-10.7	<55°C	-
Bacillus subtilis	64,000	γ-type	8.0	6.0-8.0	<50°C	+
Thermoanaerobacter sp.	75,291	α-type	6.0	5.0-6.7	100°C	+
Brevibacterium sp	75,000	γ-type	10.0	6.0-8.0	<50°C	-

M.W.: molecular weight, T.S. : temperature stability

(4) Crystallography, active site, and site directed mutagenesis

Crystallographic structural data confirm that four highly conserved regions are involved in the active site (substrate - binding catalytic center) and the Ca^{2+}-ion binding. Despite the low sequence homology between α-amylase and CGTase they show a common overall folding pattern and active arrangements [9]

In both cases the amino-terminal domain (A) is a TIM barrel structure, consisting of eight inner, parallel β-sheets, surrounded by eight α-helixes (β/α) 8-barrel). This crystal structure is now known for 21 proteins. Characteristic for the α-amylase family is the arrangement of the active site between a small separate domain (B-domain) in between β-strand 3 and helix 3 of the (β/α)8 fold. The B-domain is supposed to be responsible for the functional diversity of the α-amylase family enzymes.

A C-domain is succeeded to the (β/α)8 barrel, approximately 100 amino acid residues ling and folds as eight parallel β-sheets in a Geek key topology. The function of the C-domain is unknown, although mutations in the C-domain of α-amylases inactivate the enzymes which suggests that the domain is necessary for enzyme activity.

In contrast to α-amylses, CGTase exhibit two further domains (D and E) which are anti-parallel β-Sheets at the C-terminal end of the protein and which cause the higher molecular weight compared to α-amylases (~25kDa) as shown in Figure 3. The D-domain following the C-domain is similar to immunoglobulin topology [11]. No function has been suggested for this domain up to now. Surprisingly, the α-CGTase of *K.oxytoca* M5al is missing this D-domain.

The E-domain found in the tertiary structure of CGTases has a raw starch binding site and shows high similarity to region of glucoamylase enzyme which are responsible for binding starch granules, and also contained two maltose-binding sites (MBS); one would seems to be involved in raw starch binding (MBS1) and the other guides the starch chain into the active site as well as playing an inhibitory role in CD production through accessibility control of substrates (MBS2) from X-ray crystallographic studies [9,11].

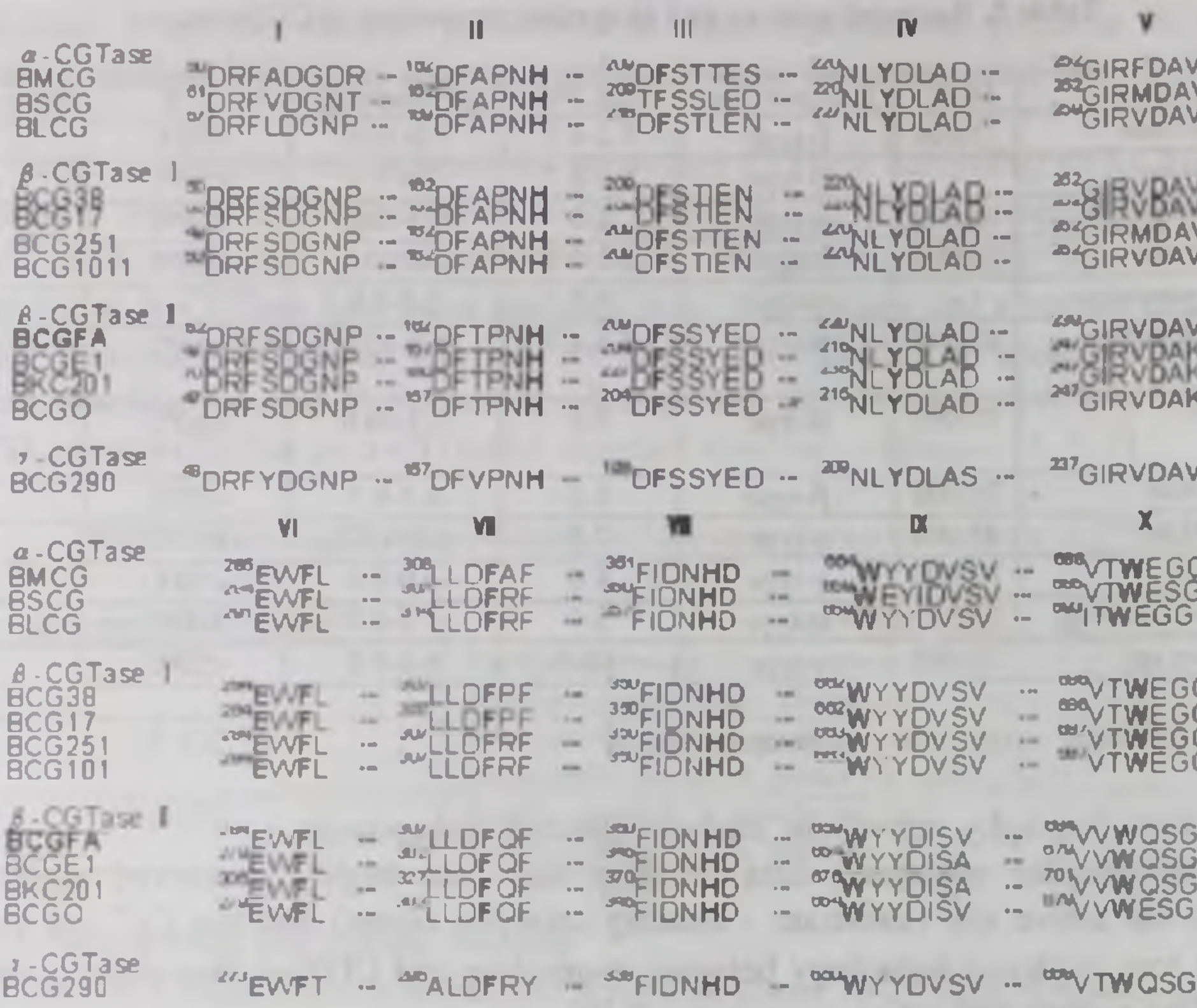

Figure 2. Comparison of the deduced amino acid sequences of the CGTase gene from *Bacillus firmus* var. *alkalophilus* with other CGTases.

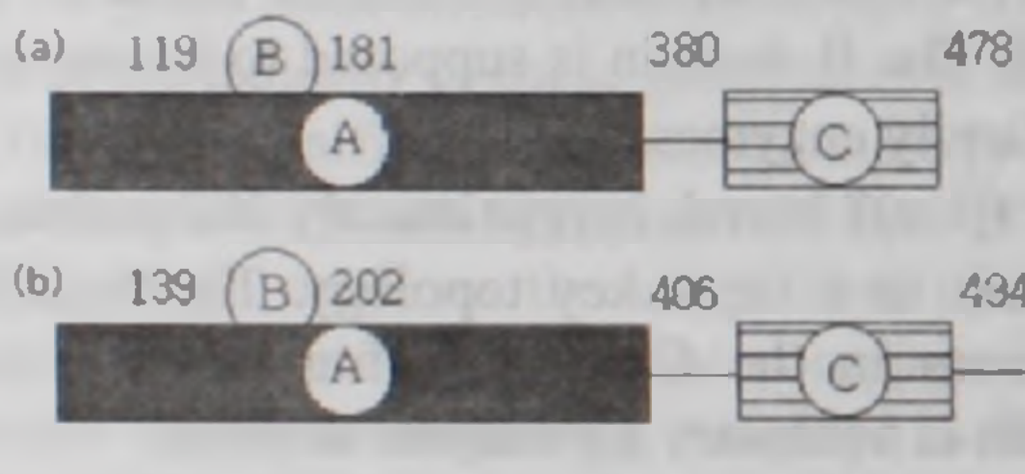

Figure 3. Schematic protein domain arrangement of (a) taka-amylase and (b) CGTase

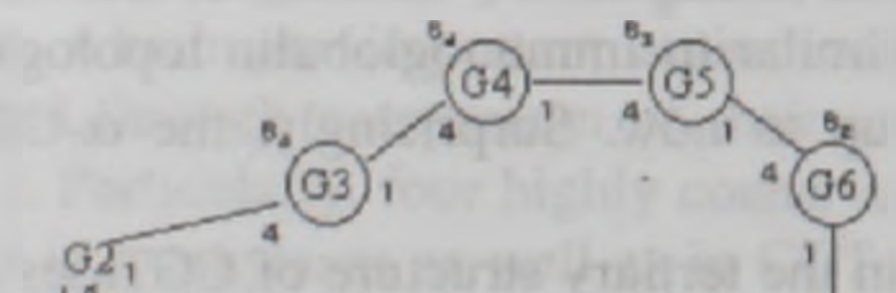

Figure 4. Model for the active site of CGTase: S refers to the subsites for binding of a glucopyranosyl residue (G) which are α-1,4-linked. The "cleavage" and "coupling" site is between subsites S_1 and S_1'.

In our laboratory, in order to identify the catalytic functions of E-domain, especially MBS1, site-directed mutation on MBS1 of E-domain from β-CGTases gene of *B. firmus* var. *alkalophilius* was cared out, thereby several mutant β-CGTases was obtained. Our recent work using site-directed mutagenesis showed that MBS1 of E-domain plays a more critical role in the cyclization reaction forming CDs from starch than in the coupling or starch-hydrolysis reactions as shown in Figure 11 [12].

Three-dimensional structures of α-amylase and CGTases on X-ray crystallographic data reveal a common principle in the set up of their catalytic domains. The N-terminal domain A, a(β/α)8 barrel. This common structure motif and the four highly conserved primary sequences, which are part of the active site, implies that both enzymes are a common catalytic mechanism. However, distinct variations in β-strand-loop structures are believed to be responsible for different product specificity in different enzymes.

Klein *et al.*[13] have proposed that the active center of CGTase contains seven subsites designated from the nonreducing end to the reducing end, as S5 through S2` as shown in Figure 4. The cleavage point is located between sub-site S1 and S1`. The acidic amino acids Asp-229, Glu-328 are arranged next to the cleavage point and play an essential role in catalysis.

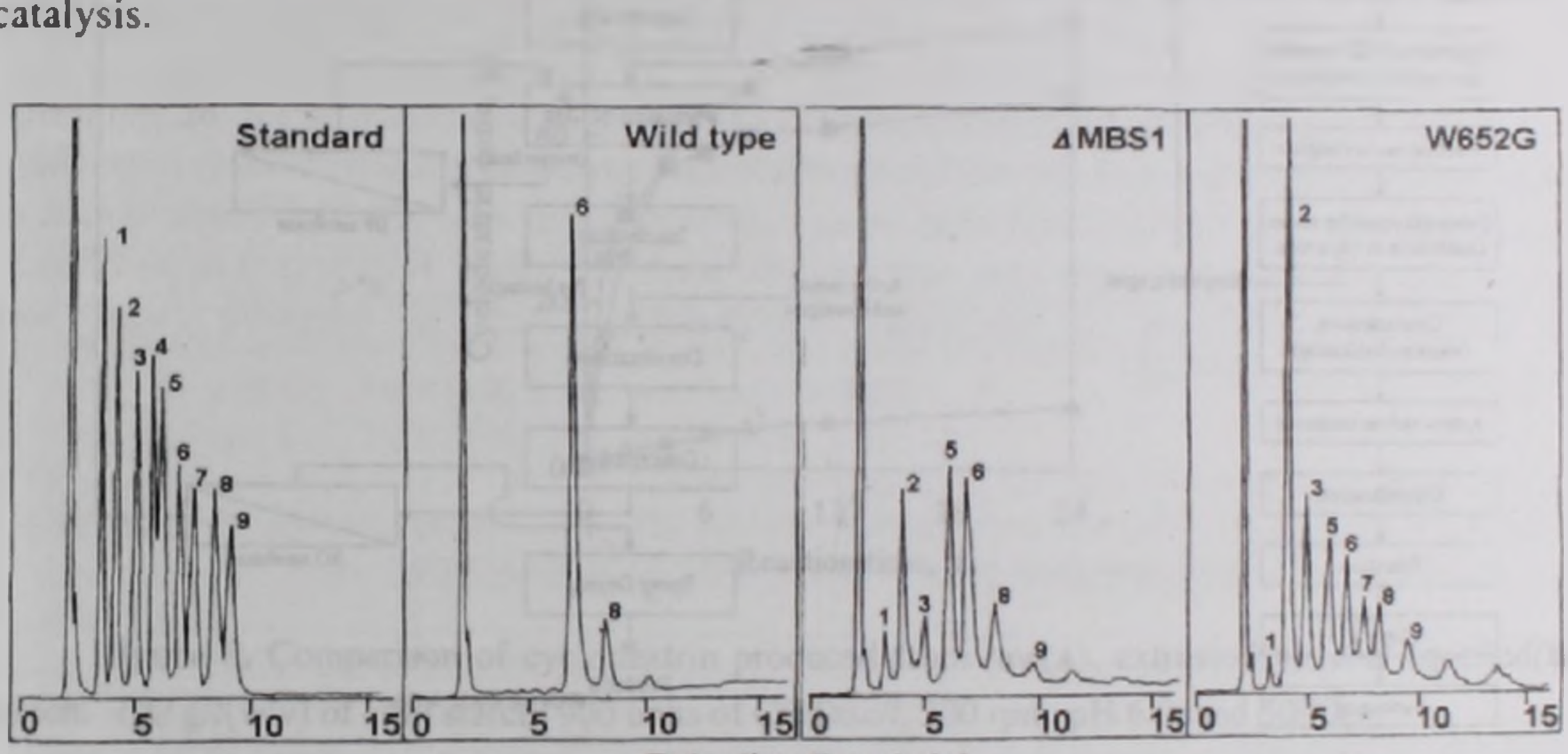

Figure 5. HPLC chromatogram of CDs and maltooligosaccharides produced from soluble starch by wild-type, MBS1, and W652G β-CGTases.

Wild-type, MBS1, and W652G β-CGTases (0.1 unit/ml cyclization activity) were reacted with 5.0 %(w/v) of soluble starch dissolved in 50 mM Tris-Maleic acid-NaOH buffer (pH 6.0) at 50°C for 12 hours. Peak 1 : Glucose (G1), 2 : maltose (G2), 3 : Maltotriose (G3), 4 : α-CD, 5 : maltotetraose (G4), 6 : β-CD, 7 : maltopentaose (G5), 8 : γ-CD, 9 : maltohexaose (G6)

Industrial Processes for Production of Cyclodextrins

Production of CDs derived from starch has been carried out by two stages; liquefaction or partial cyclization of starch by liquefying amylase or CGTase and then cyclization of the liquefied starch by CGTase [14]. And also, surfactant, solvents, and PEG etc. as complexants are added into reaction in order to enhance the CD conversion yield and production rate in solvent process developed in the late 1960s from work by Corn Products Corporation, a US-based food company. Figure 6. shows the scheme of a typical solvent process for production of CDs.

However, the addition of solvents may be cause the safety in foods and pharmaceutical applications and need the complicated process to recycle the solvent. Therefore, CDs produced by solvent process are restricted the application of certain field because remained the solvent in DE cavity. In the beginning of the 1970s, to overcome the problems of solvent process, a process for manufacturing CDs using chromatography or ultrafiltration system instead of the use of any organic solvent in Japan [15,16]. Fig .7. shows manufacturing procedure for cyclodextrin using ultrafiltration and reverse osmosis membrane of Dnsuiko Co. in Japan.

However, above traditional enzyme reaction system utilizing liquefied starch as the substrate for follow-up enzyme reaction by various carbohydrase has the following shortcomings, such as, high energy consumption, low product yield, and complexities of separation and purification of products because the residual oligosaccharides formed at liquefaction step and remained after main reaction by various carbohydrases.

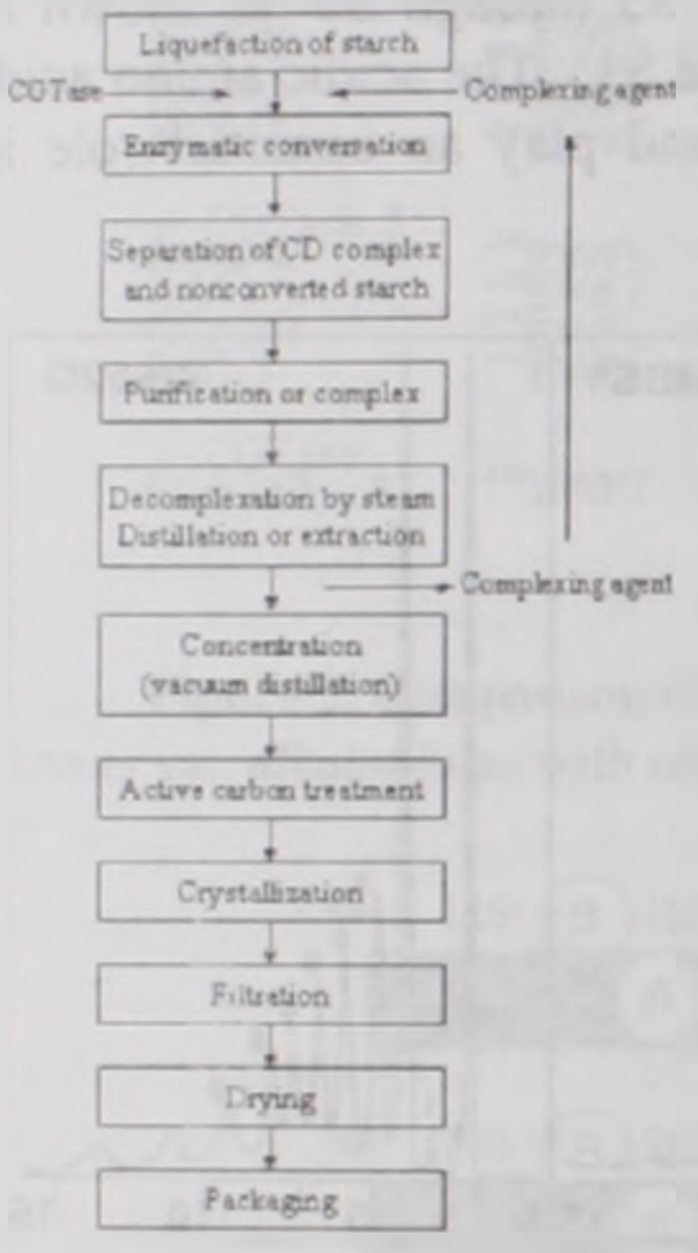

Figure 6. Solvent process for production of cyclodextrin

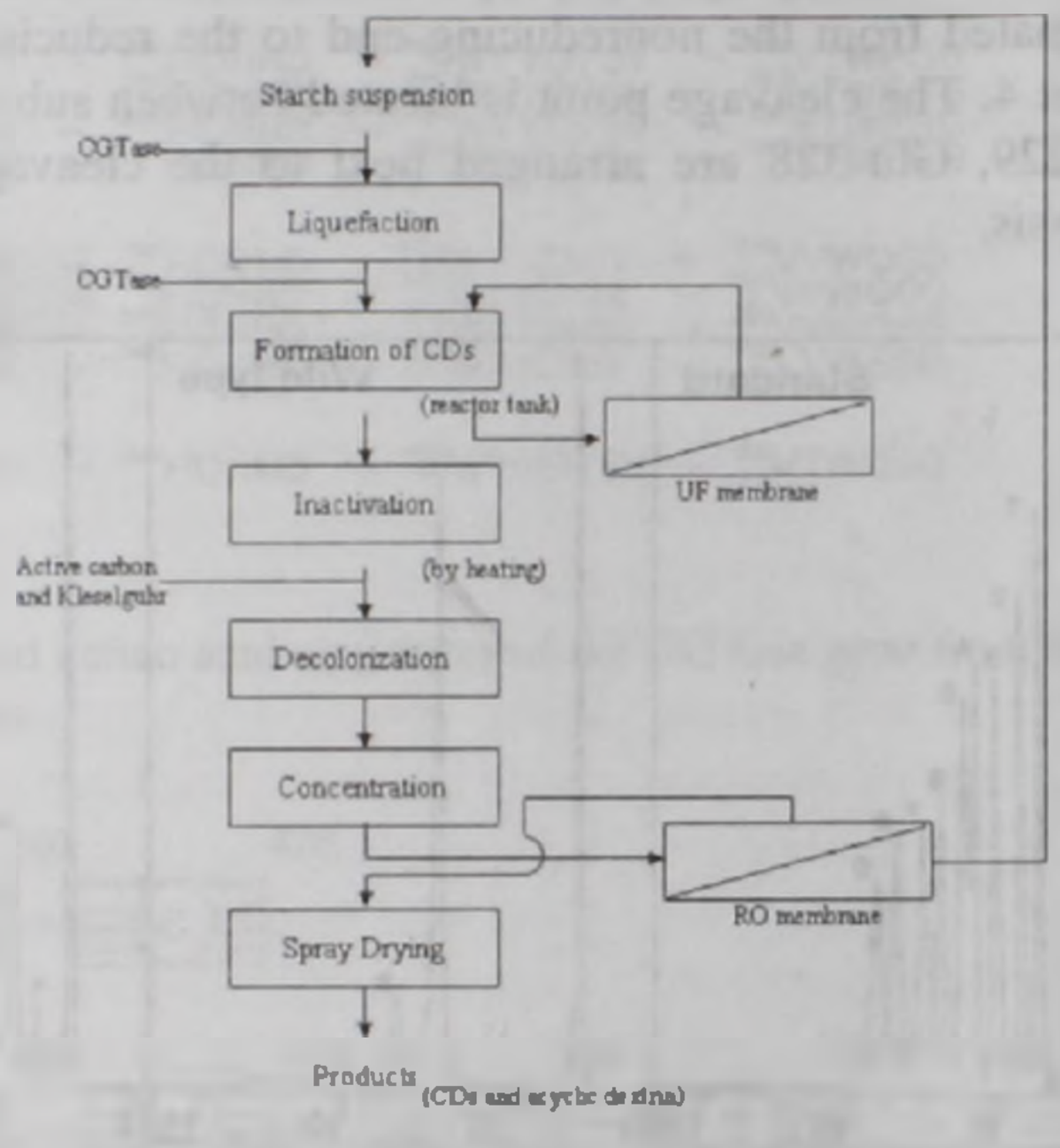

Figure 7. Manufacturing procedure for cyclodextrin using ultrafiltration and reverse osmosis membrane

If the above products can be produced from insoluble raw starch directly without liquefaction of starch, it can be expected that the high purity of products can be obtained without accumulation of undesirable oligosaccharides, for enzyme reaction may be carried out directly from the glycosyl residues in the surface of raw starch. Also, the easy separation of residual insoluble starch by simple separation process will facilitate the purification of various products. However, because the raw starch exists as the compact crystalline structure, the enzyme reaction rate and yield of products from raw starch would be too low for industrial implication. Therefore the structure modification of raw starch is required to increase the susceptibility to enzymes.

In order to increase the susceptibility to various carbohydrases, the raw starch was extruded instead of liquefaction by cooking and liquefying enzyme. The extrusion starch

exists as nearly water insoluble suspension state, the intermediate states between water soluble liquefied and insoluble raw starch, therefore the practical heterogeneous enzyme reaction system composed of soluble enzyme and nearly insoluble substrate could be maintained. In this way the advantages of direct production of CD, maltose, and transglycosylated stevioside from surface of swollen extrusion starch may be achieved.

In our laboratory, characteristics of carbohydrase reaction in heterogeneous enzyme reaction system utilizing swollen extrusion starch were investigated [17-23]. The progresses of enzyme reaction of CD production using raw starch, liquefied starch, and swollen extrusion starch, were compared. The highest concentration of 54 g/l CDs (total of α-, β-, and γ-CD) was obtained after 24hours for extrusion starch, compared to 45 g/l of that of liquefied starch. Meanwhile only 6 g/l of CD was produced after 24 hours from raw starch indicating that CD production reaction from raw starch is very limited. Figure 8 also showed that the rate of CD synthesis from swollen extrusion starch was slightly lower at the initial stage of reaction compared with that of liquefied starch, however increased steadily, and them exceeded from after 4 hours of reaction.

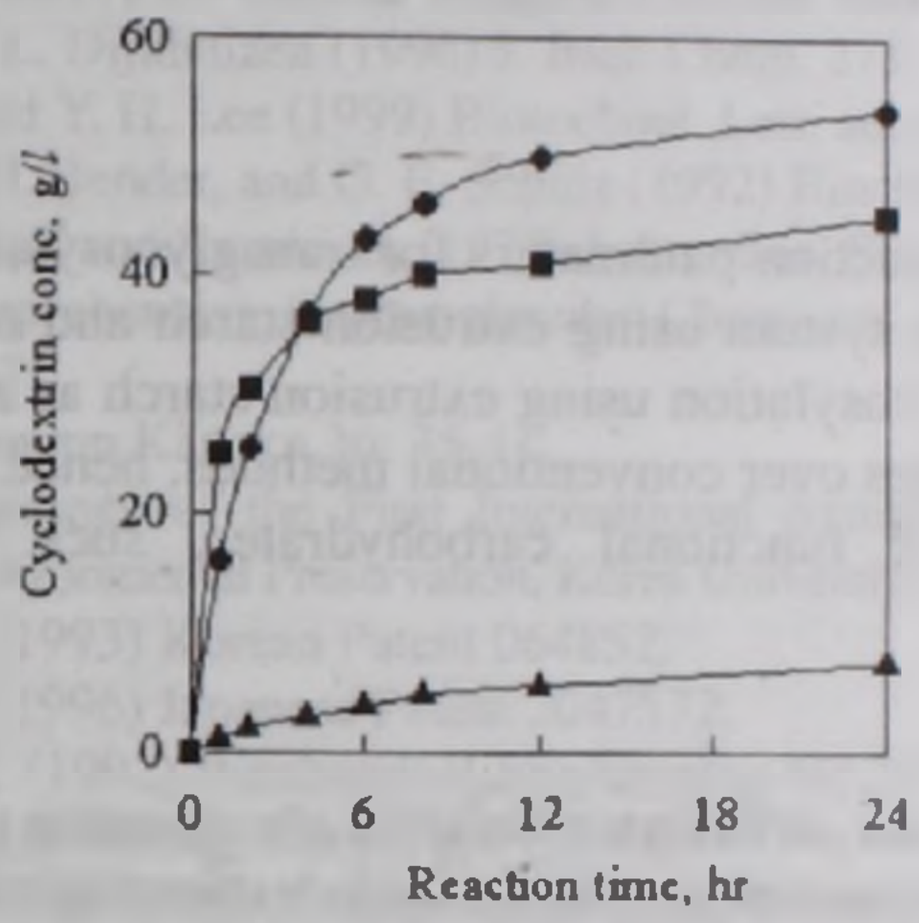

Figure 8. Comparison of cyclodextrin produced from raw(▲), extrusion(●), and liquefied(■) starch. 100 g/l(w/v) of corn starch, 900 units of CGTase/l, 200 rpm, pH 6.0, and 50°C.

As shown in Figure 9 in the case of swollen extrusion starch, CD was mainly produced without accumulation of any significant amount of maltooligosacchries as can be seen in HPLC chromatogram. Above characteristics may be explained by the action of CGTase that synthesize CD from the non-reducing ends of extrusion starch, and the extrusion starch existed in swollen granular structure without significant fragmentation, so the formation of other maltooligosaccharides was depressed.

Also the un-reacted residual starch could be easily separated by simple unit operation, such as certifugation which will facilitate recovery and purification of CD produced after enzyme reaction. It was also suggested that the spent CGTase remained in the reaction mixture could be recovered for reutilization by adsorbing on fresh swollen extrusion starch. Above facts suggested that the CD production in heterogeneous enzyme reaction system utilizing the swollen extrusion starch seems to have potential advantages for CD production in industrial scale, and further study needed to be conducted.

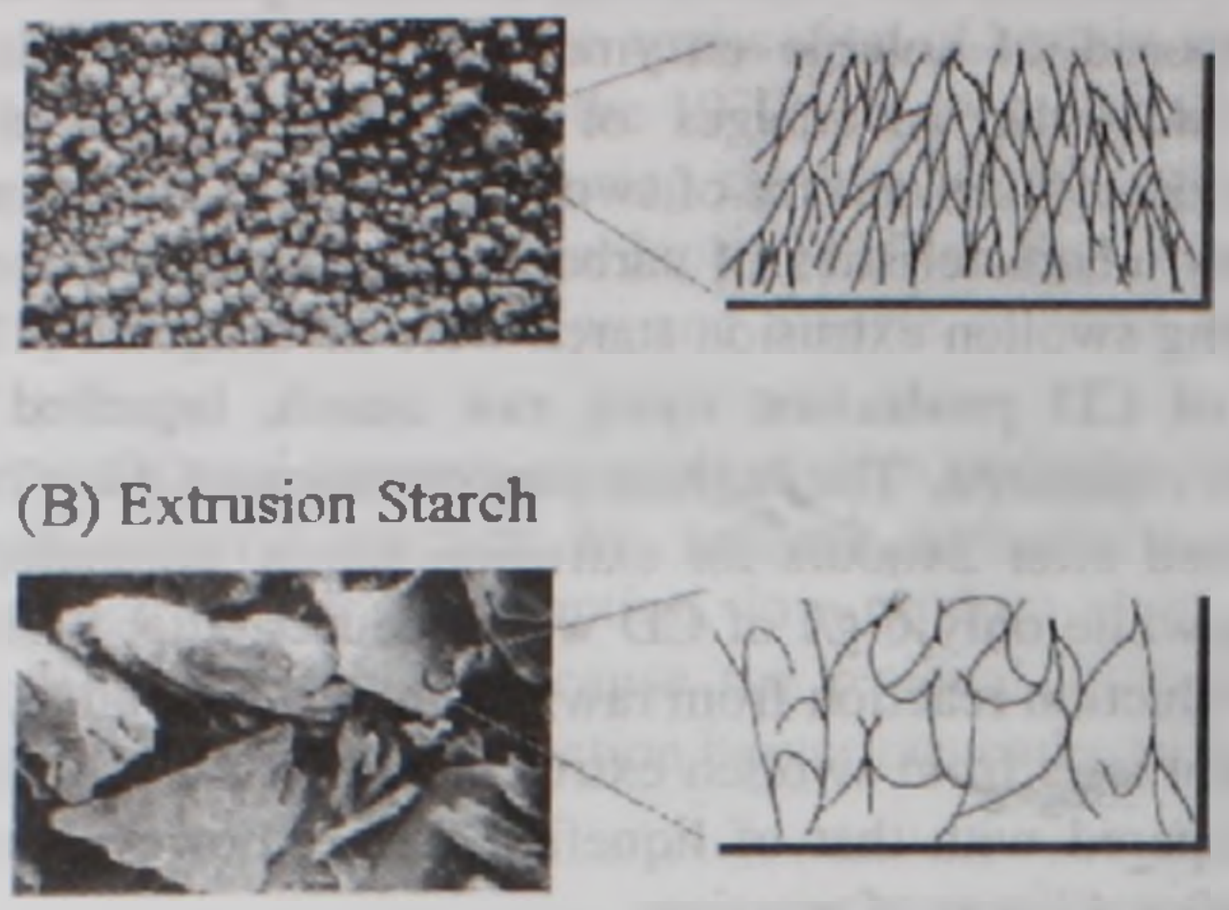

Figure 9. Scanning electron microscopic photograms and hypothetical micellar structures of raw and extrusion starch.

Table 3 compares the reaction parameters for transglycosylation reaction of stevioside in the heterogeneous reaction system using extrusion starch and conventional system using liquefied starch. The transglycosylation using extrusion starch as a glycosyl donor seems to have many potential advantages over conventional methods, hence, is expected to be utilized for industrial production of functional carbohydrates, such as coupling sugar and transglycosylated stevioside.

Table 3. Comparison of the main performance variables of convention process and heterogeneous enzyme reaction systems for cyclodextrin production

	Conventional process	Heterogeneous enzyme reaction system	
		Bioattritor	Extrusion starch
Starch concentration (g/l)	100	100	100
Cyclodextrin concentration (g/l)	45	48	54
Yield of cyclodextrin	0 45	0.48	0.54
Half reaction time (hr)*	2 0	6.2	3.2
Separable residual starch (g/l_	-	51	43
Purity of total cyclodextrin (%)	65	>95	>95
Glucose concentration (g/l)	16	<1	<1
Maltooligosaccharide concentration (g/l)	8	<1	<1
α-CD: β-CD: γ-CD ratio	1 9:2.6:1.0	1.6:3.9:1.0	1.9:4 3:1.0
Separation of residual maltooligosaccharides	Required	Not required	Not required
Separation of residual starch	Difficult	Easy	Easy
Separation and purification of cyclodextrin	Difficult	Easy	Easy

REFERENCES

1. Szejtli, J. (1997) Comprehensive Supramolecular Chemistry vol.3 Cyclodextrins (J.Szejtli and T. Osa eds). pp.1-3.
2. The Korean Genetic Engineering Research Association (1986) Cyclodextrin (Technical Information of Genetic Engineering 60). Seoul, pp.2-40.
3. Kitahata, S. and Okada, S. (1982) J. Jpn. Soc. Starch Sci. 29: 13-18.
4. Schimid, G. (1989) Tibtech. 7: 244-248.
5. Do, E.J., Shin, H.K., Kim, C., and Lee.Y.H. J. Microbiol. Biotechnol. 3: 78-85.
6. Bender, H. (1982) Carbohydr. Res. 101: 279-285.
7. Kobayashi, S. (1996) Enzymes for Carbohydrate Engineering (K.H. Park, J.F. Robyt, and Y.K.Choi, eds). Elsivier Science B.V., Amsterdam. pp. 23-41.
8. Kobayashi, S., Watanabe, N., Nakasjima, K., Sjiota, M., and Yatake, T. (1995) Oyo Toshitsu Kagaaku 42: 203-210.
9. Lawson, C. L., R. van Montfor, B. Strokopytov, H. J. Rozeboom, K.H. Kalk, G.E. de Bries, K. Penninga, L. Kijkhuizen, and B.W. Dijkstra. (1994) J. Mol. Biol. 236: 590-600.
10. Tonkoba. A. (1998) Enzyme Microbial Technol. 22: 678-686.
11. Penninga, D., B.A. van der Veen, R.M.A. Knegtel, S.A.F.T. van Hijum, H. J. Rozeboom, K.H. Kalk, B.W. Kijkstra, and L. Dijkhuizen (1996) J. Biol. Chem. 271: 32777-32784.
12. Shin, H. D., T. H. Park and Y. H. Lee (1999) Biotechnol. Lett. submitted.
13. Klein, C., J. Hoolender, H. Bender, and G.E. Schulz (1992) Biochemistry 31: 8740-8746.
14. Kobayashi, S., Kainuma, K. and Suzuki, S. (1975) J. Jpn. Soc. Starch Sci. 28: 132-141.
15. Schimid, G. (1997) Comprehensive Supramolecular Chemistry vol.3 Cyclodextrins (J. Szejtli and T. Osa eds). pp. 41-57.
16. Hashimoto, H. (1989) Denpun Kagaku 36: 35-41.
17. Lee, Y.H. (1992) Proceedings of the First International Symposium on the Development of Natural Resources and Environmental Preservation, Korea University, Seoul, p.104 (1992).
18. Lee, Y.H. and D.C. Park (1993) Korean Patent 064852.
19. Lee, Y.H. and D.C. Park (1996) Japanese Patent 2047572.
20. Lee, Y.H. and Park, D.C. (1992) Biochemical Engineering for 2001 (Furusakim S., Endo, I. and Matsuno, R., eds.), Springer-Verlag, Tokyo, pp. 127-129.
21. Lee, Y.H. (1994) Proceedings of the Second Korea-China Biotechnology Symposium, Seoul, 42-56.
22. Lee, Y.H. and Park, K.C. (1996) Enzymes for Carbohydrate Engineering (Park, K.H., Robyt, J.F. and Choi, Y.D., eds.) Elsevier Science B.V., Amsterdam, pp. 171-188.
23. Lee, Y.H. and Park, D.C.(1999) J. Microbial. Biotechnol, 9: 1-8.