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REVIEW

Novel Heterogeneous Carbohydrase Reaction Systems for the Direct Conversion of Insoluble Carbohydrates: Reaction Characteristics and their Applications

LEE, YONG-HYUN* AND DONG-CHAN PARK

Department of Genetic Engineering, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea

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Abstract Most carbohydrates exist in nature in an insoluble state, which reduces their susceptibility towards various carbohydrases. Accordingly, they require intensive pretreatment for structural modification to enhance an enzyme reaction. The direct conversion of insoluble carbohydrates has distinct advantages for special types of reaction, especially exo-type carbohydrase; however, its application is limited due to structural constraints. This paper introduces two novel heterogeneous enzyme reaction systems for direct conversion of insoluble carbohydrates; one is an attrition coupled enzyme reaction system containing attrition-milling media for enhancing the enzyme reaction, and the other is a heterogeneous enzyme reaction system using extruded starch as an insoluble substrate. The direct conversion of typically insoluble carbohydrates, including cellulose, starch, and chitin with their corresponding carbohydrases, including cellulase, amylase, chitinase, and cyclodextrin glucanotransferase, was carried out using two proposed enzyme reaction systems. The conceptual features of the systems, their reaction characteristics and mechanism, and the industrial applications of the various carbohydrates are analyzed in this review.

Key words: Heterogeneous enzyme reaction systems, attrition coupled enzyme reaction system, insoluble extruded starch, direct conversion, hydrolysis, transglycosylation, insoluble carbohydrates, cellulose, starch, chitin, carbohydrases, cellulase, amylase, cyclodextrin glucanotransferase, chitinase, glucose, maltose, maltooligosaccharides, HFCS, cyclodextrin, transglycosylated stevioside, chitooligosaccharides, maltitol, xylitol

Most carbohydrates that are composed of highly polymerized granular structures naturally exist in an insoluble state. Accordingly, their susceptibility towards various carbohydrases is somewhat limited. The major structural features of polymerized carbohydrates influencing against an enzymatic reaction include the degree of crystallinity, accessible surface area, degree of polymerization, and conformational rigidity, as well as the presence of other extraneous compounds [4, 6-8, 20, 36, 43, 53].

To increase the susceptibility of insoluble substrates to a carbohydrase reaction, insoluble carbohydrates must be intensively pretreated. Typical pretreatment methods include physical treatment to reduce particle size and crystallinity, chemical treatments to swell the compact structure, and biological treatments for liquefaction using an enzyme [4, 43, 51]. The direct conversion of native or insoluble carbohydrates is advantageous for certain specific reactions, in particular for exo-type carbohydrases that act directly on the end of the micellar chain of insoluble substrates. Furthermore, it can minimize the accumulation of undesirable compounds produced during the pretreatment and enzyme reaction which require the use of pretreated substrates.

Two novel heterogeneous enzyme reaction systems for the direct conversions of insoluble state carbohydrates have been proposed. These systems have been applied to various insoluble carbohydrates, including cellulose, starch and chitin, using the corresponding carbohydrases, including cellulase, amylases, cyclodextrin glucanotransferase (CGTase) and chitinase, for the production of glucose, maltose, chitooligosaccharides, cyclodextrin (CD), and various transglycosylated saccharides, respectively.

The first system is an attrition coupled enzyme reaction using a bioattritor, or a mechano-enzyme reaction system, where solid attrition-milling media are added to a mixture of insoluble substrate-enzyme to achieve the simultaneous effects of enzymatic catalysis and the physical impact of the milling media. The other system is a reaction system utilizing swollen extruded starch as insoluble substrates, which possess an expanded micelle structure and yet exhibit the characteristics of a nearly water insoluble

**Corresponding author*
Phone: 82-53-950-5384; Fax: 82-53-959-8314;
E-mail: leeyh@bh.kyungpook.ac.kr

suspension, where in effect they can produce a heterogeneous enzyme reaction system.

The above two reaction systems were applied to the enzyme reactions typically carried out by carbohydrase, including complete or partial hydrolysis, cyclization, and intermolecular transglycosylation. Finally, the reaction characteristics, mechanisms, and the practical applications of the various carbohydrase reactions are all reviewed in this article.

Attrition Coupled Enzyme Reaction System for the Direct Conversion of Insoluble Carbohydrates

Conceptual illustration of an attrition coupled enzyme reaction system. Figure 1 depicts a schematic illustration of an attrition coupled enzyme reaction system, where a solid attrition-milling media is added to a mixture of insoluble substrate and enzyme, along with the relative sizes of insoluble substrates, enzymes, and attrition-milling media. The cooperative actions of the carbohydrase reaction and mechanical impact of the attrition-milling media induce the structural transformation of the insoluble substrate, which in turn stimulates the carbohydrase reaction.

Hydrolysis of cellulose, raw starch, and crystalline chitin. The enzymatic hydrolysis of cellulose, raw starch, and chitin can be considered as the most common enzymatic reaction. The enzymatic hydrolysis of pure cellulose by simultaneous wet milling was performed initially by Shafizadeh and coworkers [13, 44], who observed a substantially increased hydrolysis due to the continuous generation of new accessible sites on the cellulose particles as the enzyme reaction proceeded. The hydrolysis of cellulose in a new type reactor, the so-called attrition bioreactor, was also carried out by Lee and coworkers [54, 56], who achieved a high rate and extensive saccharification without any significant deactivation of the cellulase during the attrition and milling.

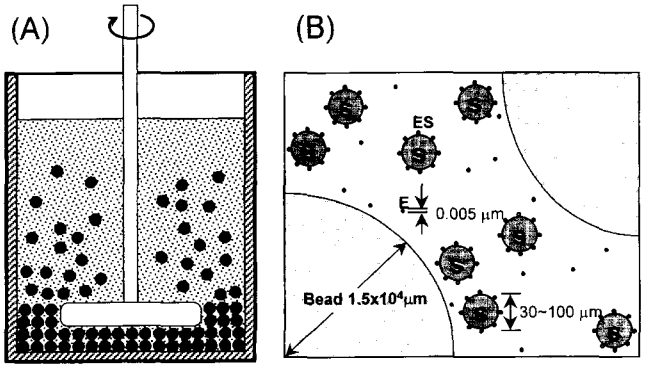

Fig. 1. Schematic illustration of attrition coupled enzyme reaction system (A) and conceptual model for the enzymatic hydrolysis of insoluble carbohydrates (B).
S, substrate; E, enzyme; ES, enzyme substrate complex.

A similar reactor system was also applied by Lee *et al.* [42] for the saccharification of corn stover which delignified using an organosolve method, and was further expanded to the enzymatic hydrolysis of pulp mill sludge [23] and the hydrolysis of α-cellulose [32]. Figure 2A illustrates a progress profile of the hydrolysis of α-cellulose catalyzed by Celluclast, a crude cellulase obtained from *Trichoderma viride*, which indicates an 80% hydrolysis after 24 h, demonstrating the effectiveness of an attrition coupled enzyme reaction system for the hydrolysis of cellulose.

The enzymatic hydrolysis of raw starch was carried out by a two-stage process; first, liquefaction by heating with thermostable α-amylase, and then saccharification by glucoamylase [8, 51]. The direct hydrolysis of raw naked barley [33] and raw corn starch [29] without liquefaction were attempted by Lee and Jo using an attrition coupled enzyme reaction system. As shown in Fig. 2B, the raw corn starch was almost 90% saccharified after 8 h and almost completely saccharified after 24 h by enzyme mixture of liquefying α-amylase from *Bacillus licheniformis* and saccharifying glucoamylase from *Aspergillus niger*. However, the raw corn starch without a milling media indicated a very low rate of saccharification and yielded only about 50% after 24 h. The hydrolysis of raw starch in the bioattritor indicated a substantially high glucose concentration when compared with that of conventional liquefied starch. The activities of the α-amylase and glucoamylase showed a relatively high stability against the physical impact of the attrition-milling media during the reaction, maintaining amylase activity of 75% after 24 h. Ca^{2+} played an essential role as an enzyme stabilizer preventing the enzyme deactivation caused by the physical impact of the attrition-milling media [34].

The naked barley was also effectively saccharified in an attrition coupled reaction system. Accordingly, after partial or complete hydrolysis, it has potential as a fermentation substrate in the production of alcoholic fuel [11]. The resulting hydrolyzate of the naked barley showed a superior fermentability compared to that obtained from a conventional saccharification process using a liquefaction stage, especially in the earlier stage of fermentation.

The production of a highly pure concentrated glucose solution through the fed-batch-wise addition of corn starch, that can be used for isomerization of glucose to fructose without condensation of the hydrolyzate, was attempted by Park and Lee [48]; 400 g/l of raw corn starch was added fed-batch-wise, which is an extremely high concentration compared to viscous liquefied starch. Using this method, 398 g/l of highly pure glucose solution, with a purity of 98%, was obtained. An innovative integrated process for the production of high fructose corn syrup (HFCS) was proposed [48] by combining the

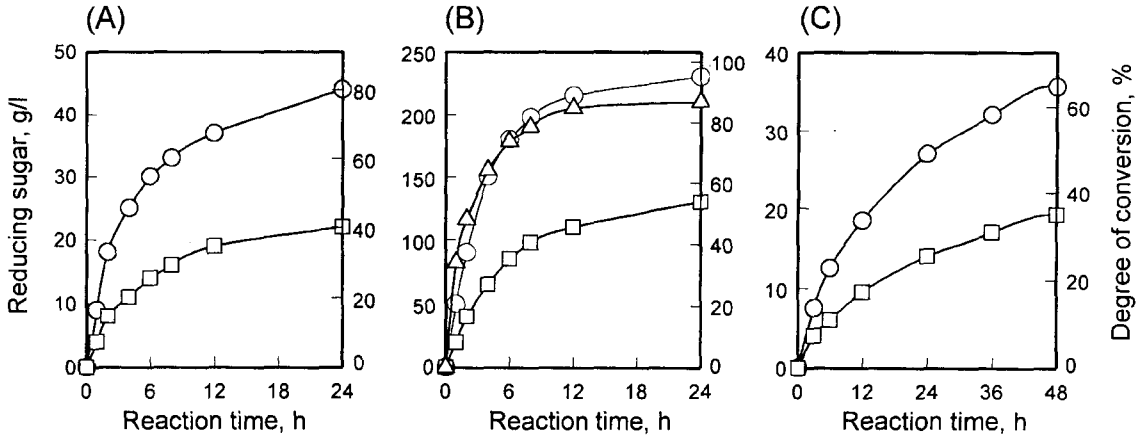

Fig. 2. Comparison of the progress of the hydrolysis reaction of cellulose (A), raw starch (B), and chitin (C) in an attrition coupled enzyme reaction system containing an attrition-milling media (○) and without a milling media (□).

(A) Cellulose (50 g/l) was hydrolyzed with 1,206 IU/l of cellulase (Celluclast, Novo Nordisk Co.) in the presence of 600 g/l of glass beads (diameter 3 mm), mixing at 550 rpm, at pH 4.8, and 55°C. (B) Raw corn starch (220 g/l) was hydrolyzed with a mixture of the enzyme, 1,200 AGU/l of saccharifying (AMG 300L, Novo Nordisk Co.) and 320 KNU/l of liquefying enzyme (Termamyl 120L, Novo Nordisk Co.), in the presence of 300 g/l of glass beads, mixing at 200 rpm, at pH 3.8, and 55°C. Symbol △, liquefied starch, DE value of 10, obtained from liquefaction at 120°C for 20 min by 100 KNU/l of Termamyl. (C) Native crystalline chitin (50 g/l) was hydrolyzed with 1,500 units/l of crude chitinase (from *Aspergillus fumigatus* JC-19), in the presence of 400 g/l of glass beads, mixing at 250 rpm, at pH 7.0, and 50°C.

hydrolysis process in an attrition coupled enzyme reaction system with the isomerization process without the condensation of the hydrolyzate.

The direct production of maltose from raw starch using a maltose-forming α-amylase from *Aspergillus oryzae* also showed a comparable reaction rate to that of a conventional process utilizing liquefied starch; however, the maltose content was substantially increased [31]. Approximately 132 g/l of reducing sugar containing 95 g/l of maltose, equivalent to 72%, was obtained from 150 g/l of raw starch after 24 h reaction.

The crystalline nature of chitin is unsusceptible to hydrolysis by chitinase [43], therefore, Lee *et al.* [41] hydrolyzed it in an attrition coupled enzyme reaction system using crude chitinase from *Aspergillus fumigatus*. It was also successfully hydrolyzed to *N*-acetyl-glucosamine as shown in Fig. 2C; this method of hydrolysis yielded 1.86 more than without the milling media. The chitinase also exhibited a high stability, and sustained an enzyme activity of 80% after 24 h in the presence of Ca^{2+} as the enzyme stabilizer.

Direct conversion of raw starch to cyclodextrin by cyclodextrin glucanotransferase. CD production was conventionally carried out with completely or partially liquefied soluble starch by various types of CGTase [3, 14, 55]. The direct conversion of raw corn starch to CD in an attrition coupled enzyme reaction system using CGTase from *Bacillus* sp. BE101 was studied by Lee and Kim [21] and using CGTase from *Bacillus macerans* by Han and Lee [9]. Consequently, a higher CD yield and production rate were obtained compare to the conventional method utilizing partially liquefied starch. In particular, high purity-CD was produced without the accumulation of undesirable maltooligosaccharides.

The energy consumption in the attrition bioreactor system was estimated by Lee and Kim [21] to be about 25% of that required for the liquefaction of starch. They also analyzed the effect of organic solvents on the production of CD in an attrition bioreactor [22], and proposed an ultrafiltration membrane bioreactor system, which recycles milled corn starch and reduces the product inhibition while reutilizing the CGTase, thereby efficiently increasing the conversion yield [16].

Transglycosylation reaction using raw starch as the glucosyl donor with cyclodextrin glucanotransferase. Transglycosylation catalyzes the transfer reaction of sugar molecules from glucosyl donors to acceptors, and it has been widely utilized for the synthesis of new functional carbohydrate materials [19]. An attrition coupled enzyme reaction system was also applied to an intermolecular transglycosylation reaction of CGTase from *Bacillus macerans* using raw corn starch as the glucosyl donor, and various saccharides and glycosides as the glucosyl acceptor [40]. Glucose, xylose, maltose, sucrose, and stevioside were all identified as good acceptors for the transglycosylation reaction by CGTase when using raw starch as the glucosyl donor. In the case of stevioside, a similar rate of reaction with yet a substantially higher yield of transglycosylation was obtained compared to the conventional liquefied starch process, although there were signs of a limited formation of oligosaccharides.

The transglycosylation mechanism of stevioside in an attrition coupled reaction system was investigated by comparing the effect of an organic solvent on the

formation of intermediate compounds including CD. The transglycosylation reaction occurs via two steps; initially, the transformation from raw starch to CD, and then the transglycosylation of glucosyl residues in CD molecules to stevioside, according to the random sequential bireactant mechanism [1]. Kinetic models by which the progress of the transglycosylation reaction can be predicted have been developed for the optimization and scale-up of the reactor and process [45].

Structural features of insoluble carbohydrates in an attrition coupled enzyme reaction system. To explain the enhanced reaction mechanism, the structural features of cellulose, raw corn starch, and chitin were visualized using a scanning electron microscope [9, 12, 24, 32, 41, 47]. Figure 3 compares the granular structures of corn starch in a cyclization reaction with CGTase, with and without the attrition-milling media [9]; the intergenic microcrystalline structure of the starch granule did not change significantly as a result of the simple mechanical impact of the attrition-milling media without any enzyme action (Fig. 3A). Also, the starch granule was not fragmented to any recognizable level by the carbohydrase action alone; instead, a cavity was formed on the surface of the granules (Figs. 3B and 3C). However, the starch granules were extensively fragmented into many small particles as the enzyme reaction proceeded (Figs. 3D~3F) through the simultaneous actions of the attrition milling media and enzyme, a similar phenomena to that found in cellulose [32] and chitin [41].

The changes of crystalline structures of cellulose [32], raw corn starch [12, 47], and crystalline chitin [41] were observed by X-ray diffraction, however, no significant changes were observed with attritional motion. The enhanced reaction may be more closely related to the increment of the accessible surface area than to the destruction of the crystalline structure.

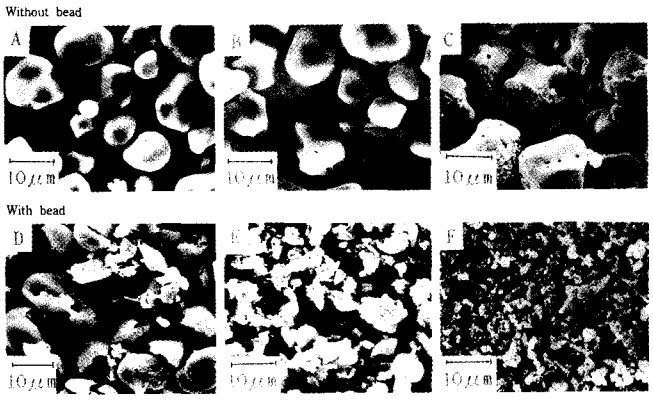

Fig. 3. Granular structures of raw corn starch subjected to a cyclization reaction of cyclodextrin glucanotransferase.

Granular structures of corn starch after the enzyme reaction without beads (A, B, C) and with beads in the attrition coupled enzyme reaction system (D, E, F) after 4, 12, and 24 h, respectively.

Reactor development for an attrition coupled reaction system. Lee and coworkers designed several bioreactors for attrition coupled enzyme reaction systems in order to select the most suitable one for the enzymatic hydrolysis of cellulose and raw starch. Three types of bioreactors, (1) a vertical impeller type reactor using an impeller for agitation, (2) a horizontal paddle type reactor mixing with a parallely located paddle, and (3) a tumbling drum type reactor mixing the attrition media by rotating the buffled drum itself, were designed for the saccharification of α-cellulose [30, 32, 35, 49] and raw starch [29, 50].

The comparative efficiency showed that the horizontal paddle type bioreactor was identified as having the most appropriate structural features for scaled-up industrial use for cellulose hydrolysis when considering effectiveness and energy consumption [49]. Meanwhile, the tumbling drum type reactor was recommended for the scale-up for saccharification of raw starch [29, 50]; however, further research is needed for a scaled-up system to clarify certain controversial conclusions.

Heterogeneous Enzyme Reaction System Using Insoluble Extruded Starch as a Substrate

Conceptual illustration of a heterogeneous enzyme reaction system using insoluble extruded starch as a substrate. The direct conversion of insoluble carbohydrates shows distinct advantages for some specific carbohydrase reactions as discussed previously. Similarly, a heterogeneous enzyme reaction system was generated utilizing an extruded raw starch to increase the susceptibility towards enzymed, while it remained in the insoluble state, and was practically composed of a heterogeneous enzyme reaction system [10, 52].

The granular structure of extruded starch observed using a SEM, and its hypothetical expanded swollen micellar structure, is shown in Fig. 4. The mean diameter of raw starch increased around 3~5 times after extrusion, corresponding to an increment of 9~25 times of the surface area. This structural feature is more favorable for adsorption and surface reaction with various carbohydrases, especially with exo-type amylase, which acts from the nonreducing end of a starch molecule. The direct reaction on the surface of extruded starch makes a precise reaction from the nonreducing end of extruded starch more feasible and avoids the accumulation of undesirable oligosaccharides formed as a byproduct of either the reaction or the liquefaction stage.

Maltose forming reaction by fungal α-amylase. A heterogeneous enzyme reaction system using extruded starch as the substrate was applied for the production of highly pure and concentrated maltose using maltose-forming α-amylase from *Aspergillus oryzae* by Lee *et al.* [28]. A substantially increased maltose forming reaction

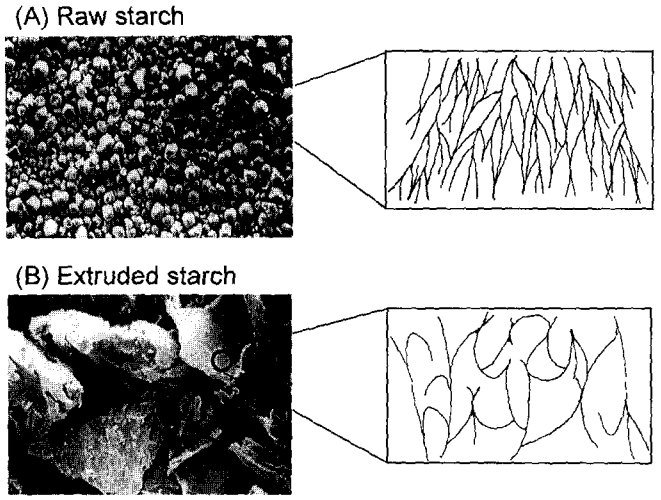

Fig. 4. Scanning electron micrographs of granules of raw (A) and extruded (B) corn starch, and their conceptual micellar structures.

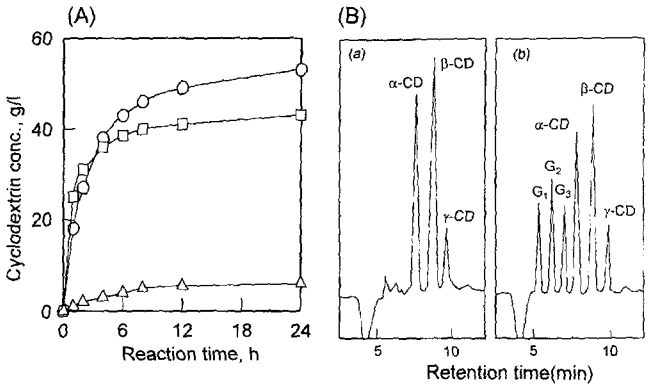

Fig. 5. Comparison of a cyclodextrin forming reaction (A) and its HPLC chromatogram (B) from extruded starch and liquefied starch.

(A) CD production reaction was carried out with 100 g/l of starch, 900 units/l of CGTase (Amano Pharmaceutical Co.), mixing at 200 rpm, at pH 6.0, and 50°C. Symbols : △, raw starch; ○, swollen extruded starch; □, liquefied starch. (B) HPLC chromatogram of the reaction mixture obtained from extruded starch (a) and liquefied starch (b) after 12 h.

was produced compared to the reaction using liquefied starch, and an especially high maltose containing hydrolyzate resulted; these were due to a decrease in the production of maltotriose and maltooligosaccharides caused by the end-wise mode of reaction.

High maltose concentration could be obtained by the fed-batch-wise addition of extremely high concentration of extruded starch of 700 g/l, which is an impossible level to achieve if viscous liquefied starch is used. Consequently, a maltose concentration of 465 g/l with a purity of 70% (w/w) and a conversion yield of 63%, were achieved [15]. A higher reaction rate, yield, and purity of maltose were all obtained from extruded starch compared with the conventional method utilizing liquefied starch, which suggests a promising prospect for the commercial production of maltose.

Cyclodextrin formation reaction using cyclodextrin glucanotransferase. Lee and Park [25, 26] applied this reaction system for CD production instead of partially liquefied starch. The reaction profile of CD formation from raw starch, liquefied starch, and insoluble swollen extruded starch are compared in Fig. 5A. 54 g/l of CD was produced after 24 h under optimal reaction conditions, 100 g/l of extruded starch, 900 units/l of CGTase, and pH 6.0, compared to 45 g/l of liquefied starch.

As can be seen in the HPLC chromatograms for liquefied starch in Fig. 5B, a significant amount of glucose, maltose, and other oligosaccharides were accumulated, perhaps due in part to the liquefaction step and in part to the reaction residuals. Whereas, CD was the main product for extruded starch without any significant amount of oligosaccharides, demonstrating the reaction characteristics of the end-wise acting CGTase.

Kinetic models were derived considering the structural features [20, 37-39, 53] of extruded starch, including the degree of gelatinization, accessible surface area, adsorption of CGTase, and product inhibition of CD, which could reasonably predict CD production from swollen starch, and could be utilized for process development and optimization [2].

Transglycosylation reaction of saccharides and glucosides using CGTase. A transglycosylation reaction of CGTase, using extruded starch as the glucosyl donor and mono-, di-saccharide, and glucosides as the acceptors, was performed by Park *et al.* [27, 46]. The transglycosylation yield from the extruded starch also increased substantially compared to liquefied starch for various acceptors. Monosaccharides, such as, glucose, xylose, and sorbose, which have the same configuration of C2-, C3-, and C4-OH as D-glucopyranoside, perform well as acceptors. Furthermore, various glucosides, which contain glucose molecules in their structure, such as stevioside, hesperidin, and salicin, also perform well as acceptors. With stevioside, a similar transglycosylation rate and an increased yield were observed compared to using liquefied starch as the glucosyl donor, and the accumulation of maltooligosaccharides was also minimized [46].

Sugar alcohols, such as maltitol, xylitol, and inositol, can also be effectively used as acceptors in the heterogeneous enzyme reaction system which utilizes extruded starch as the glucosyl donor [17]. The produced glucosylated sugar alcohols can be useful as functional oligosaccharides that are low cariogenic, calorie free, and stimulate the growth of *Bifidobacterium* [18].

Reaction mechanism of exo-type amylases on swollen starch. Extruded starch suspended in water in a colloidal state has a swollen structure, and its microcrystalline structure is corrupted and also possesses a significantly increased accessible surface area for a carbohydrase

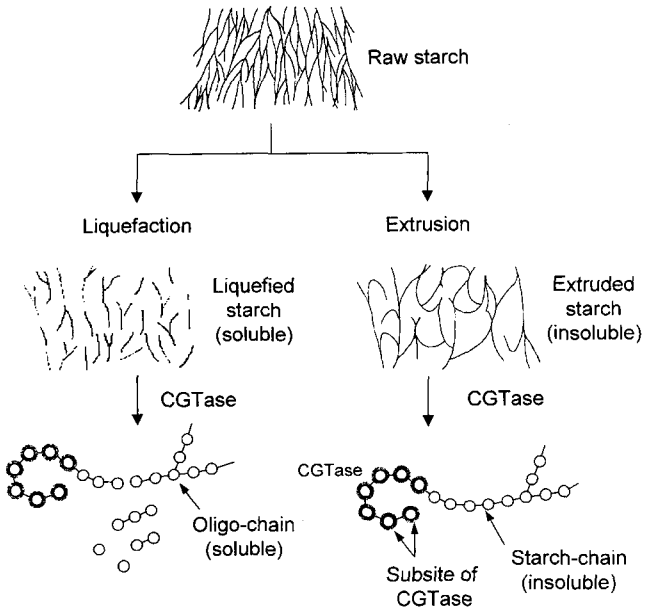

Fig. 6. Mechanistic models for cyclodextrin production using cyclodextrin glucanotransferase from a micelle chain of swollen extruded starch and liquefied starch.

reaction. Furthermore, swollen starch further fragments into many small particles as the enzyme reaction proceeds, generating new surfaces for a carbohydrase reaction, including the maltose forming reaction, CD production reaction, and transglycosylation reaction [15, 27, 39, 46].

Figure 6 illustrates the schematic representations of the micellar organization of raw, extruded, and liquefied starch, indicating the expected schematic mechanism that carried out from the micelle chain of swollen extruded starch and soluble liquefied starch by CGTase, respectively. The exo-wise enzyme reactions on partially digested liquefied starch proceed exo-wisely from the end of different sized dextrins. Consequently, a significant amount of glucose, maltose, and other maltooligosaccharides remain as reaction residuals. Meanwhile, exo-wisely acting carbohydrase, such as CGTase and maltose-forming fungal α-amylase, initiate reactions from the nonreducing end of exposed starch chains on the surface of insoluble swollen starch. Accordingly, the unreacted residual starch will remain in an insoluble state rather than oligosaccharides, and consequently, highly pure CD and maltose can be obtained. This indicates the distinct characteristics of exo-wisely acting enzymes in heterogeneous enzyme reaction systems using both insoluble carbohydrates and swollen extruded starch as substrates.

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