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Cycloamylose (Cyclodextrin) Glucanotransferase Degrades Intact Granules of Potato Raw Starch

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Cycloamylose (cyclodextrin) glucanotransferase (EC 2.4.1.19, CGTase) originated from *Bacillus macerans* degraded intact granules of potato raw starch and converted them into cyclodextrins (CDs). The degradation required sufficient stirring of starch–CGTase suspension. The morphology of the degraded starch granules was unique; that is, the inner part of the granules was observed by scanning electron microscope to be more susceptible to CGTase than the outer part. Effects of pH, temperature, starch concentration, and enzyme amount on CD production were studied.

Keywords: Cycloamylose (cyclodextrin); glucanotransferase; raw starch; enzymatic; degradation

INTRODUCTION

Cyclodextrins (CDs) are nonreducing, α -(1 \rightarrow 4) linked cyclic oligosaccharides, which are industrially produced from liquefied starch by utilizing the action of cycloamylose (cyclodextrin) glucanotransferase (CGTase, EC 2.4.1.19). α -, β -, and γ -CDs are composed of 6, 7, and 8 units of α -glucopyranoside, respectively. CD is a doughnut-shaped molecule, having a hydrophobic cavity. CD forms inclusion complexes with organic compounds and improves their physical properties such as solubility in water and their chemical properties such as stability against oxidation, discoloration, and vaporization (Bender and Komiyama, 1978).

CDs have been used in food, cosmetics, and pharmaceuticals. However, at present, the high cost for cyclodextrin production imposes limitations on the extensive use of CDs, especially in food industries.

In industrial cyclodextrin production, potato raw starch suspension (~10%) is, first, liquefied, that is, heated at ~65 °C over the gelatinization temperature of starch in the presence of starch-liquefying enzyme such as CGTase. The liquefied starch is then reacted again with CGTase at ~50 °C, and CDs are produced. If raw starch could be directly degraded by CGTase, the liquefaction process could be omitted and the production cost could be reduced.

On the other hand, it has been reported that there exist raw starch binding sites, namely, specific homologous sequences of amino acid, in raw starch degrading amylases, such as α -amylases, β -amylases, and glucoamylases, and that these amylases actually degraded raw starch (Svensson, 1988; Svensson et al., 1989). CGTase was also reported to have a raw starch binding site (Svensson, 1988; Svensson et al., 1989). However, raw starch degradability of CGTases has not yet been clarified, although some studies regarding nonintact, namely damaged, starch degradation by CGTase can be

found. For example, milled maize starch was used to produce CDs by using CGTase from *Bacillus* sp. BE 101 in an attrition bioreactor (Lee et al., 1991) and in an ultrafiltration membrane bioreactor (Kim et al., 1993).

We assumed that CGTase could degrade intact granules of raw starch as in the case of nonintact starch under an appropriate reaction condition. In this study, intact granules of potato raw starch and CGTase from *Bacillus macerans* were employed to investigate the possibility of intact starch degradation by CGTase.

MATERIALS AND METHODS

Materials. Potato raw starch was supplied from Hokuren (Hokkaido, Japan). CGTase from *B. macerans* was kindly donated by Amano Pharmaceutical Co., Ltd. (Aichi, Japan). CGTase was purified by forced-affinity chromatography (Kobayashi et al., 1997), and the purity was electrophoretically assured. Soluble starch (for biochemical use) was purchased from Wako Pure Chemical Industries, Ltd. (Japan). Standard α -, β -, and γ -CDs were donated by Ensui Sugar Refining Co., Ltd. (presently, Bio Research Corp. of Yokohama, Yokohama, Japan). All other chemicals were of reagent grade. Heat-treated starch (HT starch) was prepared by heating raw starch suspension (10% w/w) in boiling water for 10 min with manual shaking; the suspension was then cooled to 50 °C and applied for reaction.

Dextrinizing Activity of CGTase. Dextrinizing activity of CGTase for soluble starch was determined according to the method of Kim et al. (1993). Soluble starch (0.2% w/v) was dissolved in 20 mM phosphate buffer ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.0). The solution was incubated with 50 μL of enzyme solution at 45 °C for 10 min. Adding 4 mL of 0.2 N HCl stopped the reaction. After 0.5 mL of iodine reagent (0.02% w/v I_2 , 0.2% w/v KI) was added to the mixture, the absorbance at 700 nm (blue) was spectrophotometrically measured. One unit of dextrinizing activity was defined as the amount of enzyme that reduced the intensity of the original blue color by 10% per minute.

CGTase Reaction. Reactions were conducted on two scales: 10 g and 100 g. The 10 g scale reaction was adopted to determine the yield of CDs after 24 h. Potato raw starch (1.0 g) was suspended in 8.90 g of 20 mM phosphate buffer ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 5.5) containing 0.02% w/v NaN_3 using a 20 mL screw vial. For adjusting to the other pH values, two H_3PO_4 , NaH_2PO_4 , Na_2HPO_4 , and NaOH solutions (20 mM) containing 0.02% w/v NaN_3 were mixed. CGTase solution [0.10

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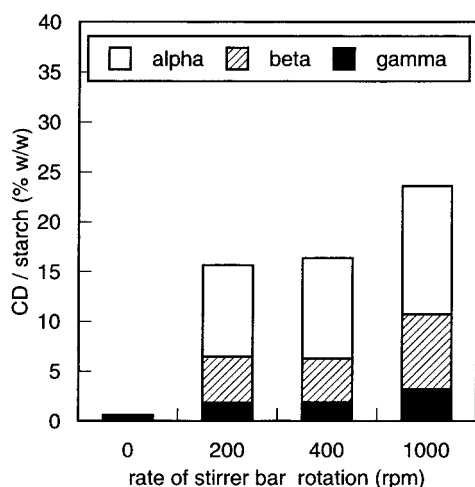


Figure 1. Effect of the rate of the stirrer bar rotation on yields of CDs from intact granules of potato raw starch.

g (200 units)] was added to the suspension, and the reaction was started. Reaction mixtures were stirred with a magnetic bar at a selected rate of stirrer bar rotation. Reactions were mainly carried out under this standard condition (10% w/w starch in the buffer solution of pH 5.5, 200 units of CGTase, stirred at 1000 rpm, 50.0 °C for 24 h) or otherwise as mentioned. One hundred microliters of the reaction mixture was taken at a specified time and centrifuged at 10000 rpm for 30 s. The supernatant (0.015 g) was immediately mixed with 1.00 g of 0.1 N acetic acid to stop the reaction. The resulting solution was filtered with a 0.45 μ m Millipore filter and applied to HPLC analysis. The 100 g scale reaction was conducted only to measure a time course of the reaction. The composition of the 100 g scale mixture was the same as that of the 10 g scale mixture with a larger amount by 10 times. One hundred microliters of reaction mixture was taken at each selected time. The successive procedure was the same as that of the 10 g scale. Every reaction was repeated twice, and each average value was used.

HPLC Analysis. Concentrations of CDs were determined by HPLC using an ODS column (YMC-Pack ODS-AQ-312-3, 6.0 mm ϕ \times 150 mm, YMC, Tokyo, Japan). Methanol/H₂O (5% v/v) was eluted at a flow rate of 1.0 mL/min. CDs were detected by reflective index, and peak area was calibrated with standard CDs.

Scanning Electron Microscopy. Starch granules after CGTase reaction were observed using a scanning electron microscope JSM-880 (JOEL, Japan) at 5 kV. The starch suspension after CGTase reaction (24 h) was centrifuged (500 rpm for 3 min), and the supernatant was discarded. The precipitate was shaken with an excess amount of distilled water and again centrifuged. After the second rinse, the starch was dried in vacuo at 45 °C overnight. The resulting starch granules were put on a sample plate with carbon paste, covered with spattered gold (25 nm thick), and applied to the microscopic observation.

RESULTS AND DISCUSSION

We examined whether CGTase could degrade intact granules of potato raw starch or not. The reactions were conducted for 24 h on the 10 g scale at selected stirring rates. As shown in Figure 1, ~25% w/w (grams of CD per gram of starch) of the intact granule was found to be degraded and converted into CDs with stirring at 1000 rpm, which was the maximal limit of the stirrer, but little (2% w/w) was degraded without stirring. This indicated that shear stress caused by stirring might have played an important role in allowing CGTase to act on intact granules. Therefore, all reactions hereafter were conducted with stirring at 1000 rpm.

Figure 2 shows a comparison of time courses of CD production using (A) raw starch and (B) HT starch. In the degradation of raw starch, α -CD was mainly produced at an early stage before 24 h, and β -CD was dominant afterwards. In the case of HT starch, α -CD was dominant throughout. In the case of raw starch, yield of total CDs of the 100 g scale reaction at 24 h was <20% w/w, although that of the 10 g scale reaction at 24 h in Figure 1 reached 25% w/w. This implies that the scale-up of the reaction system may have changed the stirring condition between the 10 g scale and the 100 g scale. CDs were produced more slowly from intact starch than from HT starch at the early stage. As CGTase is adsorbed to heat-moisture-treated starch granule (Kobayashi et al., 1978), probably by its raw starch binding site (Svensson, 1988; Svensson et al., 1989), adsorption of CGTase on intact starch granule might retard its successive attacks on neighboring granules.

CGTase from *B. macerans* degraded intact granules of potato raw starch and converted them into CDs. The morphology of degraded potato starch granules was investigated by using a scanning electron microscope. Figure 3 shows SEM photographs of representative degraded granules at the same reaction stage. Several degraded granules with unique patterns were observed among a few hundred intact-looking precipitated granules. Most degraded granules could have been removed as the supernatant as the precipitation was isolated by centrifugation. Two patterns of degradation were observed: a broken-eggshell-like pattern and a spongy one. As seen in Figure 3A,B, CGTase seems to degrade partly on the surface and for the most part inside the granules and leave a broken-eggshell-like surface. The inner part is likely to be more susceptible to the enzyme than the outer part of the granules. In Figure 3C,D, starch granules were degraded on a part of the surface with spongy erosion. These two morphological patterns of degradation were completely different from those exhibited by the other raw starch degrading amylases acting on starch granules of potato and other plants. For example, potato starch was eroded by α -amylase gradually from the outer part (Taniguchi et al., 1982; Taniguchi and Maruyama, 1985). Some raw starches such as maize starch are eroded with holes (Fuwa, 1977; Fuwa et al., 1979; Kainuma et al., 1985). CGTase catalyzes multiple reactions (hydrolysis, cyclization, coupling, and disproportionation), whereas other amylases such as α -amylase, β -amylase, and glucoamylase only catalyze *endo*- or *exo*-hydrolyses. Therefore, it is suggested that the unique morphology of the degraded potato starch granules might be associated with the unique action mechanism of CGTase.

In the absence of the enzyme, we did not find any starch granules with enhanced artifacts. Furthermore, as starch granules that had been stirred for 24 h were reacted with CGTase under no stirring, CDs were little observed as well as in the case in Figure 1. Therefore, the stirring procedure in this study could neither mechanically damage native starch granules further nor promote damage-facilitated enzyme digestion.

For establishing an optimal reaction condition, effects of pH, temperature, starch concentration, and enzyme amount on CD yield should be key factors.

The effect of pH on yield of CDs is shown in Figure 4. Optimum CD yields were observed between pH 5.5 and 8.0. CDs were not produced at pH > 11. Figure 5 shows

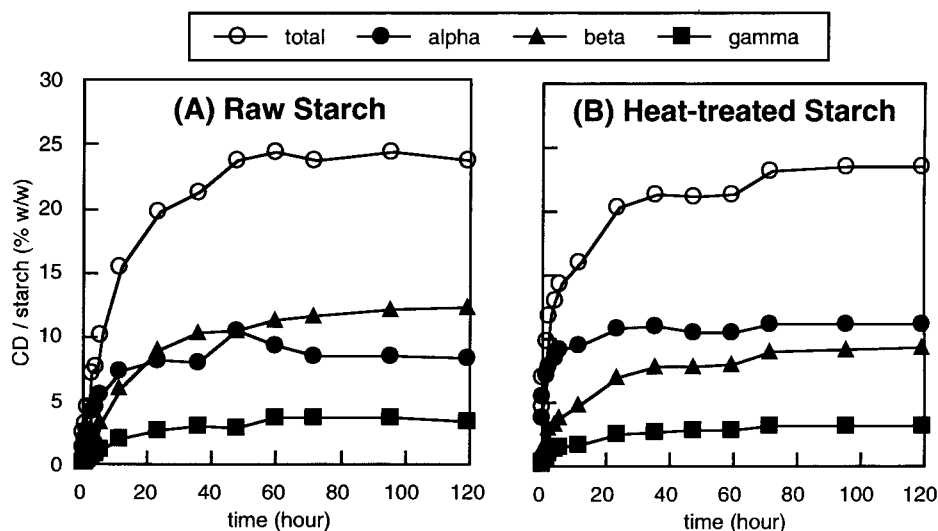


Figure 2. Time dependence of yields of CDs from (A) raw or (B) heat-treated potato starches. A portion of the sample was periodically taken from each reaction batch of the 100 g scale.

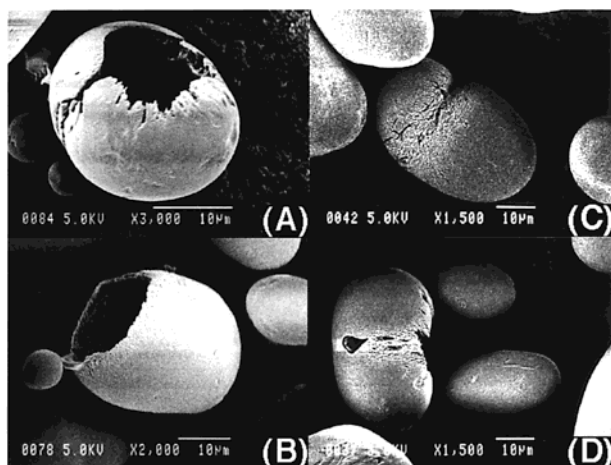


Figure 3. Scanning electron micrographs of potato starch granules degraded by CGTase. The granules were taken from one batch. Two patterns of degradation were observed: (A and B) eggshell-like erosion; (C and D) spongy erosion.

the effect of reaction temperature on yield of CDs. Optimum temperatures for CD production existed between 40 and 55 °C, and the yield of CDs decreased as temperature increased over 60 °C. Even at 70 °C, CGTase was a little active. Potato raw starch is reported to gelatinize at 61 °C (Hizukuri, 1969) or to begin gelatinization at temperatures between 56 and 66 °C (Leach, 1965); therefore, potato starch could gelatinize over 55.0 °C in this reaction.

Kobayashi et al. (1978) has purified CGTase (*B. macerans*) through adsorption of CGTase onto heat-moisture-treated starch granules and investigated the characteristics of the enzyme by employing soluble starch as the substrate. According to that paper, the enzyme stability (1 h at 25 °C) was completely lost at pH >10.0. Furthermore, the residual activity (thermal treatment for 15 min) of the enzyme decreased a little at 50 °C and disappeared at >65 °C. Their results should not be directly compared with those in this study because the experimental conditions are not identical. However, their results can be referred to as the minimum requirements for inactivating CGTase. As shown in Figure 2, CDs were produced even after 24 h, indicating that CGTase was active at least for 24 h at 50 °C. In addition, as presented in Figures 4 and 5, the

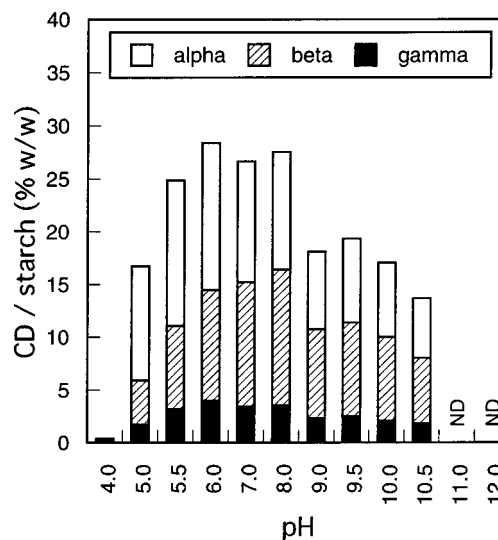


Figure 4. Effect of pH on yields of CDs. The standard reaction condition (see Materials and Methods) was modified in terms of pH value. ND represents "not detected".

enzyme did not completely inactivate even at pH 10.0 and 10.5 and at 65 and 70 °C. These indicate that CGTase might be stabilized in our study by its adsorption to raw starch granules and to HT starch, respectively. The HT starch was prepared by heating starch suspension in boiling water for 10 min while the suspension was manually shaken. This procedure might be insufficient for the complete gelatinization and retain some crystalline part. Heat-moisture-treated starch adsorbs CGTase (Kobayashi et al., 1978), and heat-moisture-treated starch shows higher crystallinity than raw starch (Osman, 1967). The crystalline part of raw starch might have adsorbed CGTase as well as the partly gelatinized HT starch.

Yield of CDs was dependent on starch concentration (Figure 6). CDs yielded per gram of starch decreased monotonically, as starch concentration increased from 5 to 40% w/w. However, the amount of total CD in one batch (grams of CD per gram of suspension) leveled off at 3.3% w/w at starch concentrations >30% w/w. In terms of CD productivity, optimal starch concentration will be >30% w/w. This may depend on the other operation factors such as pH and temperature. With

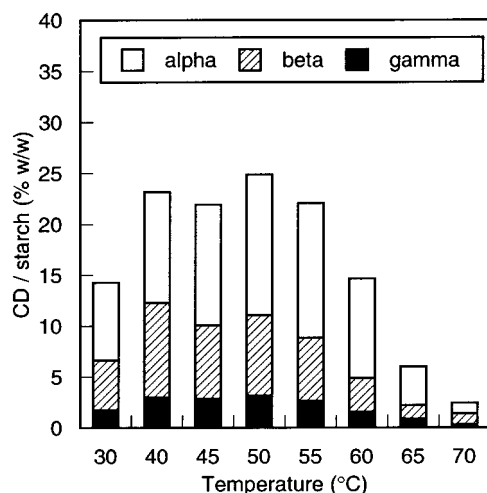


Figure 5. Effect of temperature on yields of CDs. The standard reaction condition (see Materials and Methods) was modified in terms of temperature.

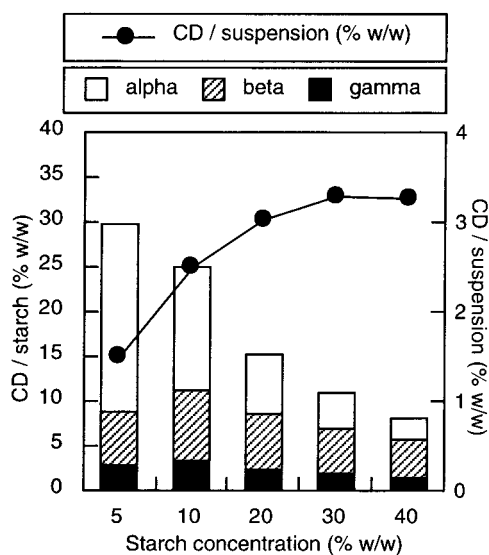


Figure 6. Effect of starch concentration on yields of CDs. The standard reaction condition (see Materials and Methods) was modified regarding starch concentration. Total yield of CDs in one batch (grams of CD/gram of suspension, % w/w) was calculated by reducing the total amount of CDs by the weight of reacting suspension.

increasing starch concentration, however, stirring of the suspension will be more difficult and energy-consuming in a larger scale. Therefore, optimal starch concentration should be determined by CD productivity and working efficiency.

The effect of enzyme amount on CD yield is presented in Figure 7. CGTase was added up to 2000 units/g of starch, and this was 10 times as much as the standard amount (200 units/g of starch) in this study. As the enzyme amount increased, CD yield also increased up to 35.1% w/w. The optimal amount of investigated CGTase should be decided by taking into consideration the price of the enzyme.

In conclusion, for an efficient CD production in our system, reaction conditions should be set between pH 5.5 and 8.0, between 40 and 55 °C, around 30% w/w starch concentration, and at higher CGTase concentration. More vigorous stirring of the reaction mixture might further raise the optimum yields of CDs. To employ our method, a nonthermal sterilization process

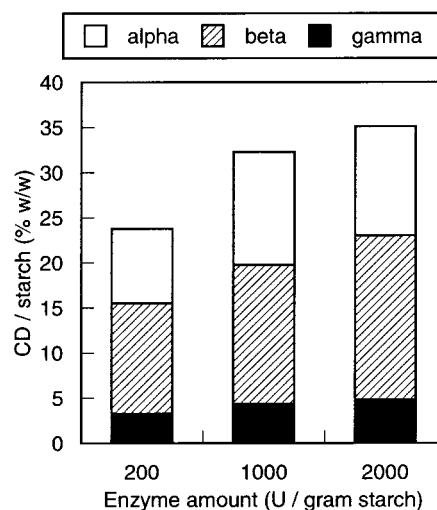


Figure 7. Effect of enzyme amount on yields of CDs. The standard reaction condition (see Materials and Methods) was modified in terms of enzyme amount and reaction time (120 h).

(e.g., γ -ray irradiation) may be necessary to avoid contamination from nonsterilized starch suspension. The feasibility of the parent process would depend on the cost of the nonthermal sterilization process. Further study on scale-up will also be necessary to apply these results to industrial CD production.

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