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Enhancement of Selectivity for Producing γ-Cyclodextrin

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Abstract

The production of cyclodextrins (CDs) by cyclodextrin-glycosyl-transferase (CGTase) from *Bacillus firmus* was studied, with respect to the effect of the source of starch upon CD yield and on the selectivity for producing γ-CD. Cyclodextrin production tests were run for 24 h at 50°C, pH 8.0, and 1 mg/L of CGTase, and substrates were maltodextrin or the starches of rice, potato, cassava, and corn hydrolyzed up to D.E. 10. Cornstarch was the best substrate for producing γ-CD. Later, glycyrrhizin (2.5% [w/v]), which forms a stable complex with γ-CD, was added to the cornstarch reaction medium and increased the yield of γ-CD to about four times that produced with only maltodextrin, but the total yield of CDs remained practically unchanged. Therefore, the results showed that the studied CGTase is capable of giving relatively high yield of γ-CD in the presence of glycyrrhizin as complexant and cornstarch as substrate.

Index Entries: Cyclodextrin-glycosyl-transferase; cyclodextrin; hydrolyzed starches; glycyrrhizin; cornstarch.

Introduction

Cyclodextrins (CDs) are oligosaccharides constituted by a variable number of units of glucose (generally from 6 to 8), linked by α-1,4 bonds. The most common are α-, β-, and γ-CD. CDs have a truncated cone form with an interior cavity whose size and form are determined by the number of glucose units *(1,2)*. This interior is relatively nonpolar compared with the water, and therefore CD easily forms inclusion complexes with organic substances *(3)*. The inclusion of a substance results in physical and chemical modifications that can increase the stability of the guest molecule and/or improve its solubility *(1,4)*. This characteristic associated with the nontoxic

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effect of the CDs makes them an interesting prospect for a varied number of industrial applications, e.g., in foods, medicines, cosmetics, perfumes, and agricultural products *(5)*.

CDs are usually produced from starch by the action of the enzyme cyclodextrin-glycosyl-transferase (CGTase). This enzyme not only transforms starch into CDs but also catalyzes the transfer of CDs to appropriate acceptors, and the disproportionation of linear dextrins *(6)*. A mixture of the α-, β-, and γ-CD is usually produced; however, the ratio of these CDs formed is dependent on the source of the CGTase enzyme and the reaction time. The γ-CD is a CD of special interest because it presents the advantage of having a larger diameter cavity, which allows the formation of inclusion complexes with large molecules of modern pharmaceutical application, and it has a larger solubility in water than α-CD and a much larger solubility than that of β-CD. The higher solubility of γ-CD facilitates the preparation of more concentrated solutions of active molecules *(3)*. However, α- and β-CD are more frequently produced on an industrial scale because of the low production of γ-CD by microbial CGTases *(7)*. Research has been aimed at the objective of obtaining a larger production of γ-CD, and one of the ways to achieve this goal is to prevent the destruction of γ-CD by reversible reactions that may occur during its production. This is possible through the formation of a stable complex of γ-CD with an appropriate substance *(8)*. Based on results obtained by Tsuchiyama et al. *(6,9)*, Sato and Yagi *(10)*, and Okabe et al. *(11)*, we decided to use glycyrrhizin as complexant for obtaining higher γ-CD selectivity. Because of the formation of a complex between the product γ-CD and the complexant glycyrrhizin, this CD becomes inaccessible to the reverse reactions that cause opening of the CD ring. This shifts the balance of the reaction toward the formation of γ-CD. Glycyrrhizin is an extract of the plant Glycyrrhiza glabra that has high sweetening power and is used in some candies, tobacco, syrups, and elixirs *(12,13)*. Also known as licorice, glycyrrhizin is a nontoxic complexant, which has 100% selectivity in favor of forming a complex with γ-CD *(11)*.

In this study, the effect of the starch source on the yield of CDs was studied with the aim of increasing the selectivity for γ-CD. Production of CDs was carried out by the CGTase of *Bacillus firmus*, strain number 37, isolated from Brazilian soil *(14)*. Also, the influence of glycyrrhizin on the selectivity for the production of γ-CD by maltodextrin and cornstarch was investigated.

Materials and Methods

Cyclodextrin Production Test

A batch reactor with a capacity of 100 mL was used for the production of CDs with the purified CGTase from *B. firmus*, strain 37. Conditions were 50°C and about 1 mg/L of pure enzyme (80 mmol of β-CD/[min · mg of protein]), and the substrate solution contained 10% (w/v) maltodextrin (Dextrin 10, Fluka [Buchs, Switzerland] article 31412) or the starches

(Claspar, Maringá, PR, Brazil) of rice, potato, cassava, and corn hydrolyzed up to D.E. 10, Tris-HCl buffer, pH 8.0 (0.01 *M*), and $CaCl_2$ (5 m*M*). The test was run for 24 h, and samples were taken at regular intervals and boiled for 5 min, after which they were assayed for α-, β-, and γ-CD.

Analysis of CDs

CDs were measured by high-performance liquid chromatography (HPLC) with a differential infrared refractometer detector, using a hydrophobic interaction column (Licrosphere RP C18; ISCO, Australia). The mobile phase was water:methanol (92:8%) with a flow rate of 1 mL/min *(15)*.

Hydrolysis of the Starches up to D.E. 10

The starches of rice, potato, cassava, and corn were partially hydrolyzed up to D.E. 10. The preparation of hydrolyzed starch solutions followed the methodology of Lima et al. *(16)* using α-amylase (Termamyl 120L) from Novo Nordisk, Denmark.

Selectivity Test for Producing γ-CD

To verify the possibility of increasing the selectivity for production of γ-CD by purified CGTase from *B. firmus*, glycyrrhizin was used as complexant. Conditions were 50°C and about 1 mg/L of pure enzyme, and the substrate solution contained 10% (w/v) maltodextrin or cornstarch hydrolyzed up to D.E. 10, Tris-HCl buffer, pH 8.0 (0.01 *M*), $CaCl_2$ (5 m*M*), and 2.5% (w/v) glycyrrhizin. The test was run for 24 h, and samples were taken at regular intervals and boiled for 5 min, after which they were assayed for α-, β-, and γ-CD. The pH of the glycyrrhizin solution was adjusted to 8.0 with NaOH (2 *N*) before the solution was added to the reaction medium. The CDs were measured by HPLC, as previously mentioned. The presence of glycyrrhizin in the solution to be assayed by HPLC did not interfere with this method.

Results and Discussion

Effect of the Starch Source on CD Yield

Figure 1 shows the yields of CDs obtained from maltodextrin (standard substrate) and the starches of rice, potato, corn, and cassava by purified CGTase, in the 24-h batch test. Figure 2 shows CD yield after 24 h of reaction. Cornstarch was the best substrate for producing both β- and γ-CD. The purified CGTase produced 1.7 times more γ-CD and 1.4 times more β-CD from cornstarch than from maltodextrin. Therefore, besides cornstarch giving the highest yield of total CDs (increment of 65% in comparison with the yield obtained with maltodextrin), it also gave the highest yield of γ-CD (increment of 87%).

After 24 h, the purified CGTase produced, α-, β-, and γ-CD in a proportion of 1:188:29 when maltodextrin D.E. 10 was used as substrate. There-

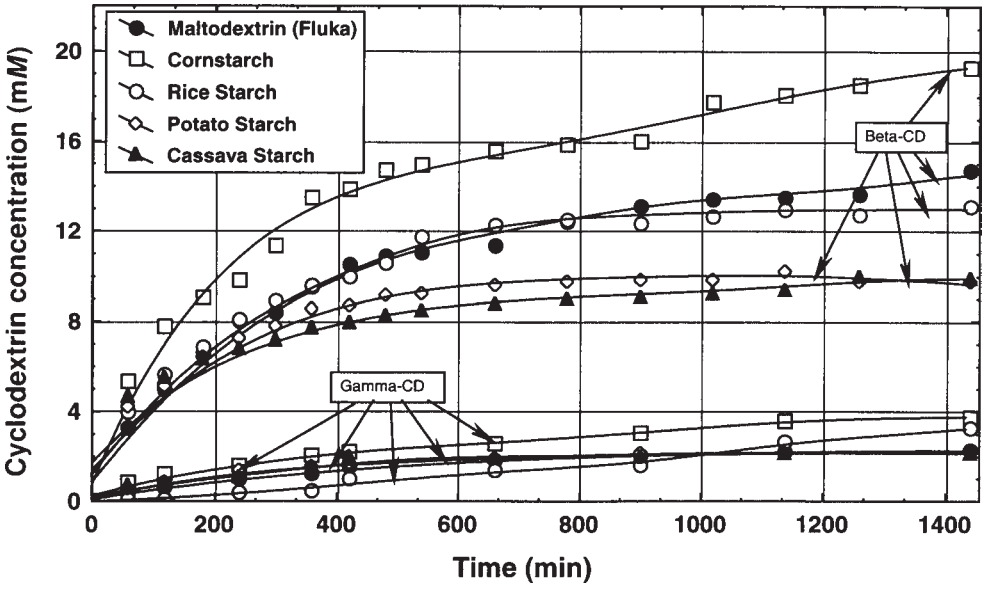

Fig. 1. Effect of different sources of starch (10% [w/v]) on CD production by purified CGTase. Batch test run for 24 h at 50°C, pH 8.0, and about 1 mg/L of pure enzyme.

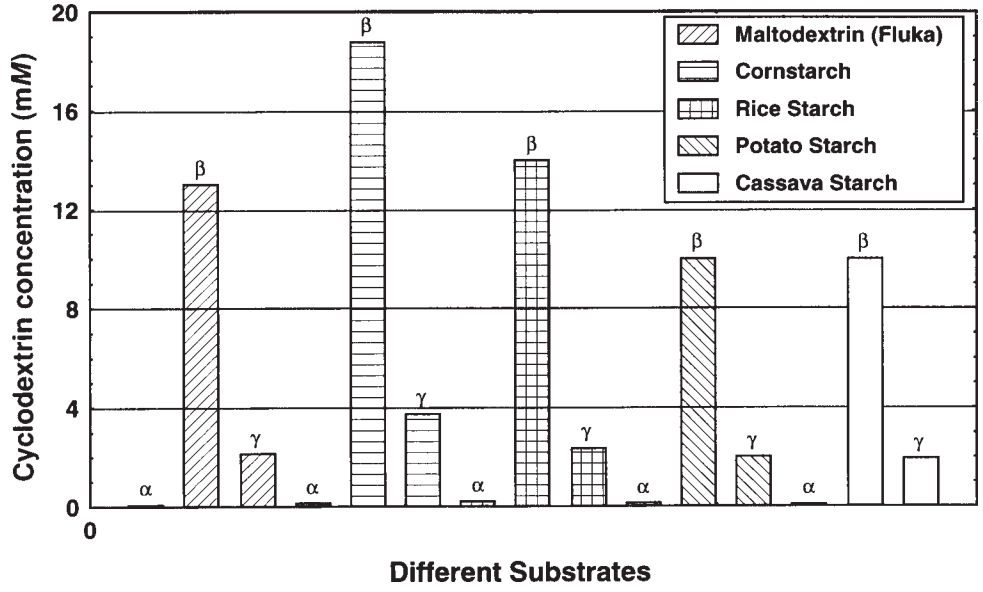

Fig. 2. Effect of different sources of starch (10% [w/v]) on CDs produced after 24 h by purified CGTase. Batch test run at 50°C, pH 8.0, and about 1 mg/L of pure enzyme.

fore, this enzyme is characterized as a β-CGTase owing to the higher yield of β-CD *(14)*. This result suggests that this enzyme has potential for industrial use for the specific production of β-CD.

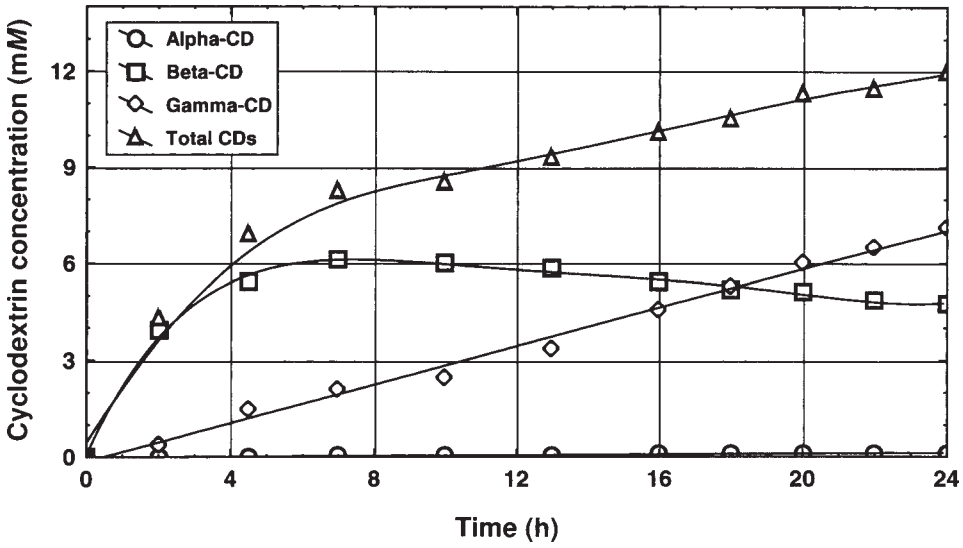

Fig. 3. Production of CDs from a mixture of 10% (w/v) maltodextrin and 2.5% (w/v) glycyrrhizin using purified CGTase. Batch test run for 24 h at 50°C, pH 8.0, and about 1 mg/L of pure enzyme.

Out of several substrates tested by Mori et al. *(17)*, cornstarch was also shown to produce the highest yield of total CD (36%). Another substrate they studied that gave a relative high yield of CDs was the starch of sweet potato (35%).

High Yield of γ-CD in the Presence of the Complexant

Figure 3 shows the production of CDs from a mixture of maltodextrin 10% (w/v) and glycyrrhizin 2.5% (w/v) by purified CGTase, in the 24-h batch test. β-CD concentration increased up to approx 6 h of the test, and after this period, it began to decrease. The concentration of γ-CD has increased during the whole assay. After approx 18 h, the γ-CD concentration exceeded the β-CD concentration. Production of α-CD began only after 7 h of assay, and later it increased during the whole test period, although with a very small yield. Figure 3 also shows that after 24 h, γ-CD production was 45% greater when a mixture of 10% (w/v) maltodextrin and 2.5% (w/v) glycyrrhizin was used. A higher yield of γ-CD was accompanied by a decrease in β-CD in the same proportion. In this medium, the amount of γ-CD formed after 24 h was about 59% of the total CD yield, and β-CD reached 40%. These results compare favorably with 15% of γ-CD and 85% of β-CD when the reaction medium contained only 10% (w/v) maltodextrin. However, in the case of using only 10% (w/v) maltodextrin, the production of total CDs was 15 m*M* after 24 h of assay, whereas 10% (w/v) maltodextrin plus 2.5% (w/v) glycyrrhizin gave a total CD production of 12 m*M*. Therefore, with glycyrrhizin there was a 45% higher yield of γ-CD, albeit accompanied by a 20% reduction in the total production of CDs.

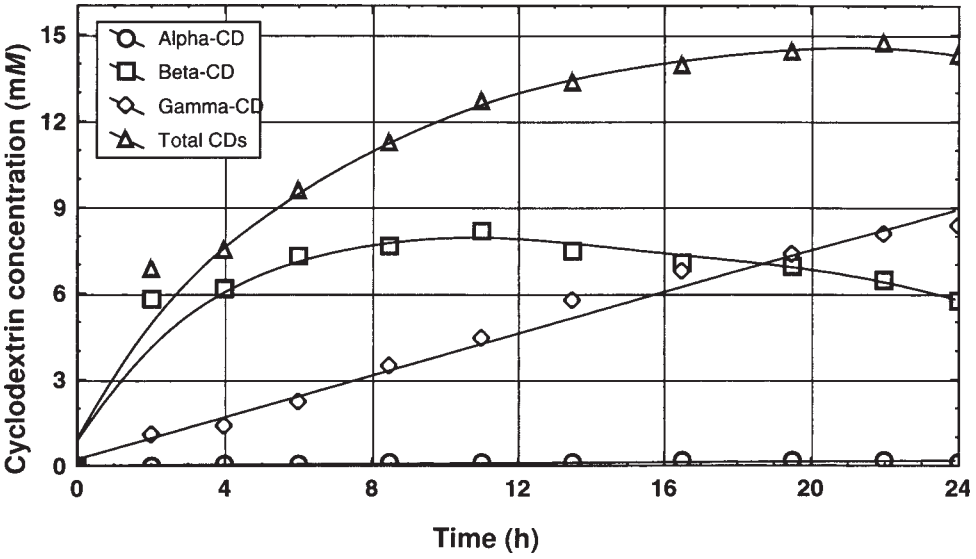

Fig. 4. Production of CDs from a mixture of 10% (w/v) cornstarch and 2.5% (w/v) glycyrrhizin using purified CGTase. Batch test run for 24 h at 50°C, pH 8.0, and about 1 mg/L of pure enzyme.

The use of glycyrrhizin as complexant reached the objective of increasing the selectivity for production of γ-CD, obtaining an excellent result. The data of Sato and Yagi *(10)* show that for production of CDs, glycyrrhizin was effective only when used with CGTase from *Bacillus ohbensis*. For CGTases from *Bacillus macerans* or *Bacillus circulans*, glycyrrhizin did not have any significant effect. Therefore, it seems that the inhibition of the reverse reaction by the formation of the inclusion complex γ-CD/glycyrrhizin is dependent on the source of the enzyme.

High Yield of γ-CD in the Presence of Complexant and Cornstarch

Since a 20% reduction in the total production of CDs was observed with 10% (w/v) maltodextrin plus 2.5% (w/v) glycyrrhizin, an alternative substrate was used to search for improvements in total yield of CD. The same methodology described under Selectivity Test for Producing γ-CD was used. Maltodextrin was substituted with cornstarch hydrolyzed to D.E. 10. Figures 4 and 5 give the results.

With cornstarch plus glycyrrhizin, the total production of CDs reached 14 m*M*. This result corresponds to an increase of about 17% in relation to that obtained with maltodextrin plus glycyrrhizin, which reached 12 m*M*. It was observed that the production of the three CDs increased, and the proportion among them was practically the same as that obtained with maltodextrin plus glycyrrhizin. Therefore, the selectivity was similar, but the yield was higher. γ-CD production with cornstarch plus glycyrrhizin also exceeded β-CD production after 18 h of assay (Fig. 4). With cornstarch

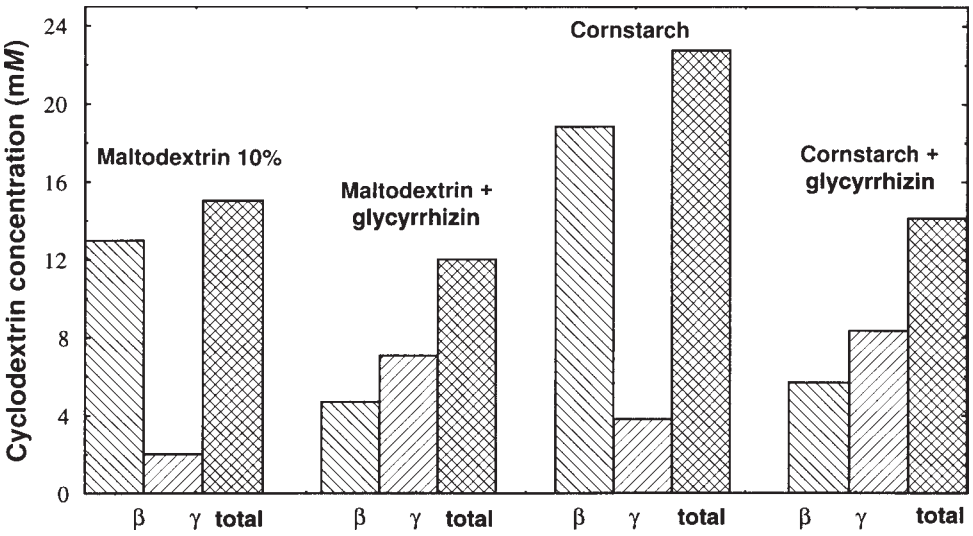

Fig. 5. Comparison between β- and γ-CD produced after 24 h by purified CGTase in different reaction media containing either maltodextrin or cornstarch, with and without 2.5% (w/v) glycyrrhizin. Batch test run at 50°C, pH 8.0, and about 1 mg/L of pure enzyme.

plus glycyrrhizin, the amount of CDs produced after 24 h of test reached 8.4 m*M* for γ-CD and 5.7 m*M* for β-CD. These values compare favorably with 7.1 m*M* for γ-CD and 4.8 m*M* for β-CD obtained when maltodextrin plus glycyrrhizin was used. On the other hand, in relation to the medium containing only maltodextrin, there was a small reduction of about 6% in the total production of CDs by cornstarch plus glycyrrhizin (Fig. 5), and in relation to cornstarch without glycyrrhizin, total CD yield was reduced by 38%, albeit with a 118% increase in γ-CD. Different proportions of starch and glycyrrhizin, as well as longer reaction times, should be investigated to try to achieve still higher yields of γ-CD.

Conclusion

In spite of CGTase from *B. firmus* strain 37 being a β-CGTase, this enzyme is capable of producing more γ-CD than β-CD when in the presence of a complexant such as glycyrrhizin. Cornstarch was the best substrate for giving higher yields of γ-CD with this enzyme. Selectivity for γ-CD in the presence of 2.5% (w/v) glycyrrhizin achieved 60%, and γ-CD yield was nearly 11% in relation to the starch loaded into the reactor. As we have shown, the use of glycyrrhizin might lead to a successful industrial process, because the minimum γ-CD yield for a process to be economically feasible is 10% *(17)*.

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