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Characterization of Cyclodextrin Glycosyltransferase from *Bacillus firmus* Strain No. 37

Graciette Matioli,[1] Gisella M. Zanin,[2] and Flávio F. De Moraes*,[2]

[1]Pharmacy and Pharmacology Department and [2]Chemical Engineering Department, State University of Maringá, Av. Colombo, 5790, BL D-90, 87020-900 Maringá, PR, Brazil, E-mail: flavio@maringa.com.br

Abstract

The enzyme cyclodextrin glycosyltransferase (CGTase), EC 2.4.1.19, which produces cyclodextrins (CDs) from starch, was obtained from *Bacillus firmus* strain no. 37 isolated from Brazilian soil and characterized in the soluble form using as substrate 100 g/L of maltodextrin in 0.05 *M* Tris-HCl buffer, 5 m*M* $CaCl_2$, and appropriate buffers. Enzymatic activity and its activation energy were determined as a function of temperature and pH. The activation energy for the production of β- and γ-CD was 7.5 and 9.9 kcal/mol, respectively. The energy of deactivation was 39 kcal/mol. The enzyme showed little thermal deactivation in the temperature range of 35–60°C, and Arrhenius-type equations were obtained for calculating the activity, deactivation, and half-life as a function of temperature. The molecular weight of the enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, giving 77.6 kDa. Results for CGTase activity as a function of temperature gave maximal activity for the production of β-CD at 65°C, pH 6.0, and 71.5 mmol of β-CD/(min · mg of protein), whereas for γ-CD it was 9.1 mmol of γ-CD/(min · mg of protein) at 70°C and pH 8.0. For long contact times, the best use of the enzymatic activity occurs at 60°C or at a lower temperature, and the reaction pH may be selected to increase the yield of a desired CD.

Index Entries: Cyclodextrin glycosyltransferase; cyclodextrins; activation energy; deactivation energy.

Introduction

The cyclodextrins (CDs) are cyclic oligosaccharides formed by residues of glucopyranose linked by α-1,4 bonds. The most common are the

*Author to whom all correspondence and reprint requests should be addressed.

α-, β-, and γ-CDs that present 6, 7, and 8 U of glucopyranose, respectively. CDs are usually produced from starch by the reaction of cyclization of linear chains of glucopyranose by the enzyme cyclodextrin glycosyltransferase (CGTase). A mixture of CDs is usually produced, and the ratio of these CDs formed depends on the origin of the enzyme and on the reaction time *(1,2)*.

CDs have the form of a truncated cone with an interior relatively nonpolar compared to water that allows them to form inclusion complexes with organic substances *(3)*. The formed complex can improve the properties of the complexed molecule such as its solubility and chemical and thermal resistance *(1,4)*. Because of these characteristics, CDs can be used in a range of industrial applications *(5,6)*.

CGTase is a monomeric enzyme, with a molecular weight on the order of 74.5 kDa, that presents a sequence of amino acids that reveals a structural similarity to the enzyme α-amylase *(7)*. In addition to the cyclization reaction, CGTase catalyzes the coupling reaction and the disproportionation of linear maltodextrins *(8)*.

The first microorganism described in the literature as a producer of CGTase was *Bacillus amylobacter*. In 1891, using this microorganism, A. Villiers produced the first Franz Schardinger dextrins *(9)*. In 1903, Schardinger defined the *Bacillus macerans* as a good producer of CGTase *(1)*.

Since the discovery of CGTase in the middle of a culture of *B. macerans* in 1903, the production of that enzyme has been studied in several lineages of bacteria, such as *B. megaterium*, *B. macerans*, *Klebsiella pneumoniae*, and *B. stearothermophillus*. The enzymes obtained from those microorganisms present different properties, such as thermal stability, optimal pH, molecular weight, and capacity for the formation of CDs *(10)*. The great majority of CGTases isolated to date produce preferably α- or β-CD, with traces of γ-CD *(11)*.

In the literature are mentioned more than 15 species of bacteria that produce CGTase, and most of them can be classified into two great groups: (1) α-CGTase, which produces mainly α-CD in the initial instants of the reaction and among the extracellular CGTase isolated, that from *B. macerans* is prominent; and (2) β-CGTase, which produces β-CD initially at a higher ratio *(4)*. CGTases that are γ-CGTase (i.e., produce a higher initial ratio of γ-CD) are rare and avidly sought because of the great interest shown by the pharmaceutical industry *(4,12,13)*. When 10% maltodextrin was used as substrate, the CGTase from *Bacillus firmus*, isolated from Brazilian soil and to be fully characterized in this study, produced γ- to β-CD in the ratio of 0.156 and a small amount of α-CD and therefore, is a β-CGTase *(13)*.

The study of CDs, their production, and application is increasing worldwide, because their applications are numerous. Technologic progress in the production of CDs has resulted in significant reductions in their costs. However, several of their potential applications will become a reality for large-scale use only if their production costs are further reduced *(14)*.

In spite of the common use of CGTases to produce a mixture of CDs, the selection of a strain that would produce a specific CGTase with high selectivity for the β- or γ-CD would greatly facilitate downstream processing and reduce production costs. This is one of the directions for which research on CGTase and selection of strains have been aimed *(10)*.

The present study presents results of the characterization of a CGTase, obtained from an alkalophylic microorganism isolated from soil, regarding its activity as a function of pH and temperature, activation energy, thermal stability, and deactivation energy.

Materials and Methods

Enzyme

CGTase enzyme was obtained from an alkalophylic microorganism isolated from Brazilian soil (*B. firmus*). The microorganism was cultivated in 250 mL of a liquid medium, pH 10.0, with the following composition ([w/v]): 2.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1.0% Na_2CO_3. The cultivation was accomplished at 37°C for 5 d, with agitation at 150 rpm. The cells were removed by centrifugation at 8800*g* for 10 min. The cell-free supernatant received ammonium sulfate (80% of saturation) and the mixture was maintained at 4°C for 48 h. The precipitate obtained was separated by centrifugation at 8800*g* for 20 min under refrigeration. Next, it was dissolved in a 50 m*M* Tris-HCl buffer solution, pH 8.0, purified by biospecific affinity chromatography using β-CD as ligand *(15–17)* and concentrated by ultrafiltration with a 30-kDa cutoff *(17)*. The protein contents were determined by the method of Bradford *(18)*, using bovine serum albumin as standard, giving 0.171 mg of protein/mL of stock solution.

CGTase Activity Assay

One unit of activity corresponds to the amount of CGTase that liberates 1 μmol of β-CD/min in the reaction conditions. The activity assay conditions consisted of a substrate solution containing 10% (w/v) maltodextrin in 50 m*M* Tris-HCl buffer and 5 m*M* $CaCl_2$, pH 8.0, at 50°C. The diluted buffer solutions of enzyme and substrate were separately heated to 50°C. In six test tubes 1 mL of substrate solution was placed and soon after 1 mL of enzyme solution was added. The tubes were agitated and incubated for 30 min at 50°C, and one tube was removed every 5 min. CGTase was inactivated by heating the tubes in boiling water for 10 min *(13,17)*. The reaction time and enzyme dilution were selected in a manner allowing a linear relationship between the formed CD and the time, seeking to reduce the effect of the inhibition of the reaction products, according to criteria established by the method of the initial velocities *(19)*. For assay of the CGTase activity for producing β-CD, a 1:400 dilution of the stock enzyme was used (4.27×10^{-4} mg of protein/mL), whereas for γ-CD a 1:50 dilution

(3.42×10^{-3} mg of protein/mL) was used because of the lower activity of the enzyme for producing γ-CD.

The β-CD produced in the assay was determined by the method of dye extinction, i.e., color reduction that occurs after complexation with β-CD using phenolphthalein at 550 nm *(20)*. The γ-CD concentration was determined by the bromocresol green colorimetric method, and in this case there is an increase in the color of the solution after complexation with γ-CD, which is measured at 620 nm *(20)*.

Enzymatic Activity as a Function of Temperature and pH

The influence of pH and temperature was separately determined for the formation of β-CD and γ-CD, since the amount of γ-CD produced is quite small compared with the β-CD produced. In the β-CD formation test, the substrate solution was prepared in the following pH values: 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 9.5; for γ-CD formation, the pH values were 5.0, 6.0, 7.0, 8.0, 8.5, 9.0, 9.5, and 10.0. Disodium phosphate–citric acid buffer was used for pH 4.0–7.0, and boric acid–potassium chloride was used for pH 8.0–10.0. Final buffer concentration was 50 m*M*. The tests were performed, as in the case of the enzymatic activity assay, at 50°C. The influence of temperature on the activity of the enzyme was determined in the temperature range of 35–70°C with an interval of 5°C. The tests were performed, also as in the case of the enzymatic activity assay, at pH 8.0. The CDs produced were determined as given in CGTase Activity Assay.

Thermal Stability of CGTase

The residual activity of the CGTase, during incubation for 240 min at temperatures from 35 to 80°C with an interval of 5°C, was determined by the method of initial velocities *(19)*. A solution of diluted enzyme (1:40) was prepared in a solution of 10% (w/v) maltodextrin, 50 m*M* Tris-HCl buffer, and 5 m*M* $CaCl_2$, pH 8.0. This diluted enzyme solution was maintained in a selected temperature, and every 40 min a 0.8-mL aliquot was taken and added to 7.2 mL of distilled water. One milliliter of this enzyme solution (now diluted 1:400) was added to the six test tubes followed by the addition of 1 mL of a 20% substrate solution, and the residual activity was determined at 50°C. The substrate solution was prepared at a concentration of 20% (w/v) in 50 m*M* Tris-HCl buffer and 5 m*M* $CaCl_2$, pH 8.0. This procedure was repeated until 240 min of incubation of the enzyme in each selected temperature was completed. The tubes resulting from the residual activity test were maintained at 4°C for later determination of the produced CDs by the colorimetric methods indicated in CGTase Activity Assay *(20)*.

Determination of Molecular Weight

The molecular weight of the CGTase was determined according to Weber and Osborn *(21)*, by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using the molecular weight reference kit

(Pharmacia, Uppsala, Sweden) of six standard proteins with molecular weights ranging from 14.4 to 94 kDa. The relation between log molecular weight and relative mobility was established, and the molecular weight of the CGTase was determined through this relation.

Results and Discussion

Activity of CGTase Enzyme of B. firmus *as Function of pH*

In the determination of the β-CD produced by the CGTase from strain no. 37, as a function of pH, and maintaining the temperature of 50°C, the maximal specific activity occurred at pH 6.0 (Fig. 1A), giving 104.1 mmol of β-CD/(min · mg of protein). This value is in accordance with the range of pH reported in the literature, mainly for CGTases from Bacilli. The same value of optimal pH was not obtained for γ-CD production by the CGTase from strain no. 37. In this case, the maximal specific activity occurred at pH 8.0 (Fig. 1B), giving 5.0 mmol of γ-CD/(min · mg of protein).

The ratio of β-CD and γ-CD formed is presented in Fig. 1C. The relationship of γ-CD/β-CD produced reached a maximum at pH 8.0, within the pH range of 5.0–9.5, and the largest initial relative rate of production of γ-CD at this pH was 0.071.

Kato and Horikoshi *(22)* worked with a strain of Bacillus that produced mainly γ-CD, and whose pH value is close to the value obtained herein. Sato and Yagi *(23)* studied a CGTase of *B. macerans* that displayed a behavior similar to that of the CGTase in the present study, i.e., with different pH values for the maximal production of each type of CD. Existing different pH values for the maximal production of β- and γ-CD imply that the degree of ionization of the different groups of the catalytic site of the enzyme demands different states, and possibly different conformations, for the greatest production of each product. In practice, this verification is quite advantageous, because it propitiates a new method of addressing the production of CDs for increasing the selectivity toward one of the CDs that is accomplished through the choice of appropriate pH.

The optimal pH of other CGTases reported in the literature varies according to the microorganism species that produces the CGTase. For CGTases from Bacilli, the range of optimal pH is quite wide, varying from 4.0, as in the case of the CGTase studied by Techaiyakul et al. *(24)*, to 12, as in the case of the CGTase studied by Horikoshi *(25)*.

Activity of CGTase Enzyme of B. firmus *as Function of Temperature*

Figure 2 presents the activity of the CGTase of strain no. 37, as a function of temperature, for the production of β-CD (Fig. 2A), the production of γ-CD (Fig. 2B), and the ratio of γ-CD/β-CD produced at different temperatures (Fig. 2C). As the temperature increased the ratio of γ-CD/β-CD produced increased (i.e., an increase in the temperature favors the production of γ-CD in relation to β-CD). At 70°C the ratio of γ-CD/β-CD production (0.15) was nearly double that observed at 50°C (0.077).

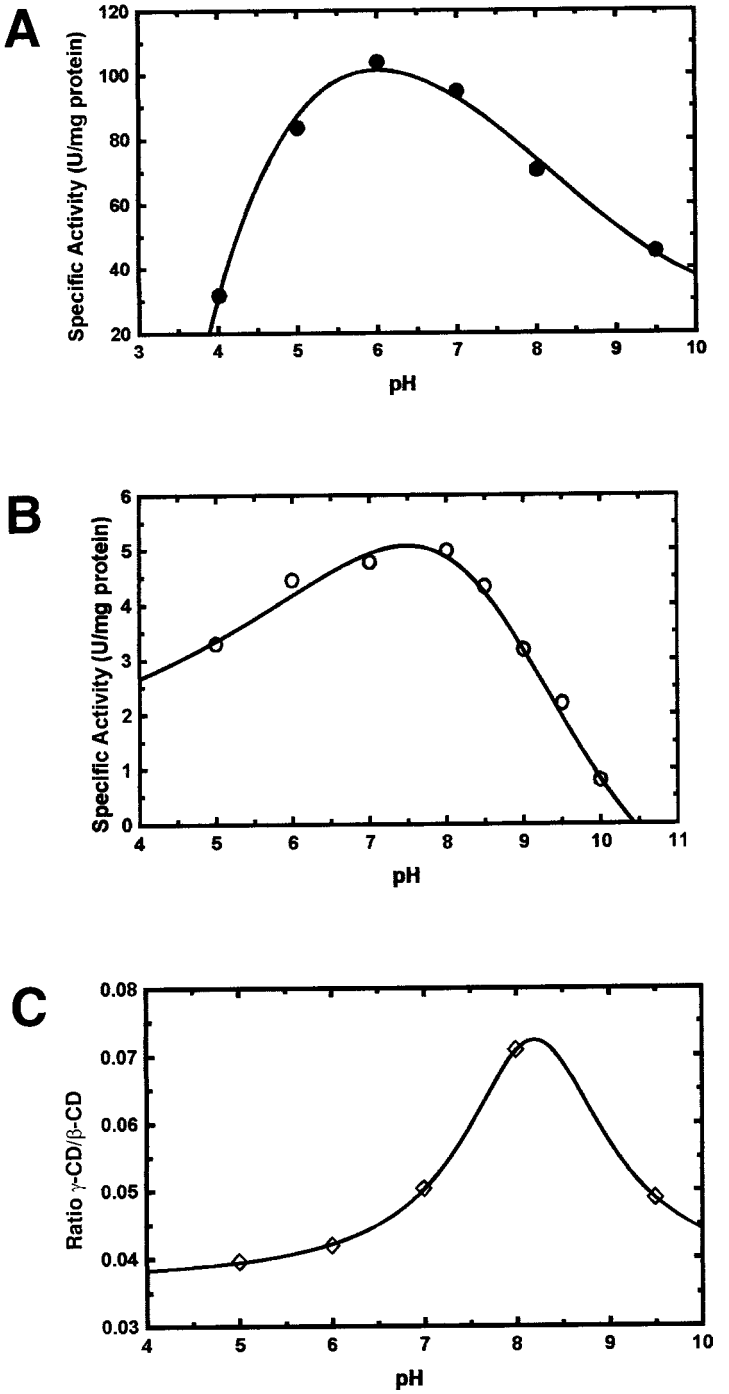

Fig. 1. Specific activity for the production of **(A)** β-CD and **(B)** γ-CD and **(C)** their ratio, γ-CD/β-CD, as a function of pH for the CGTase from *B. firmus* strain no. 37. Conditions: substrate is 10% (w/v) maltodextrin, in 50 m*M* buffer and 5 m*M* $CaCl_2$, 50°C. Buffers: Disodium phosphate–citric acid buffer for pH 4.0–7.0 and boric acid–potassium chloride buffer for pH 8.0–10.0.

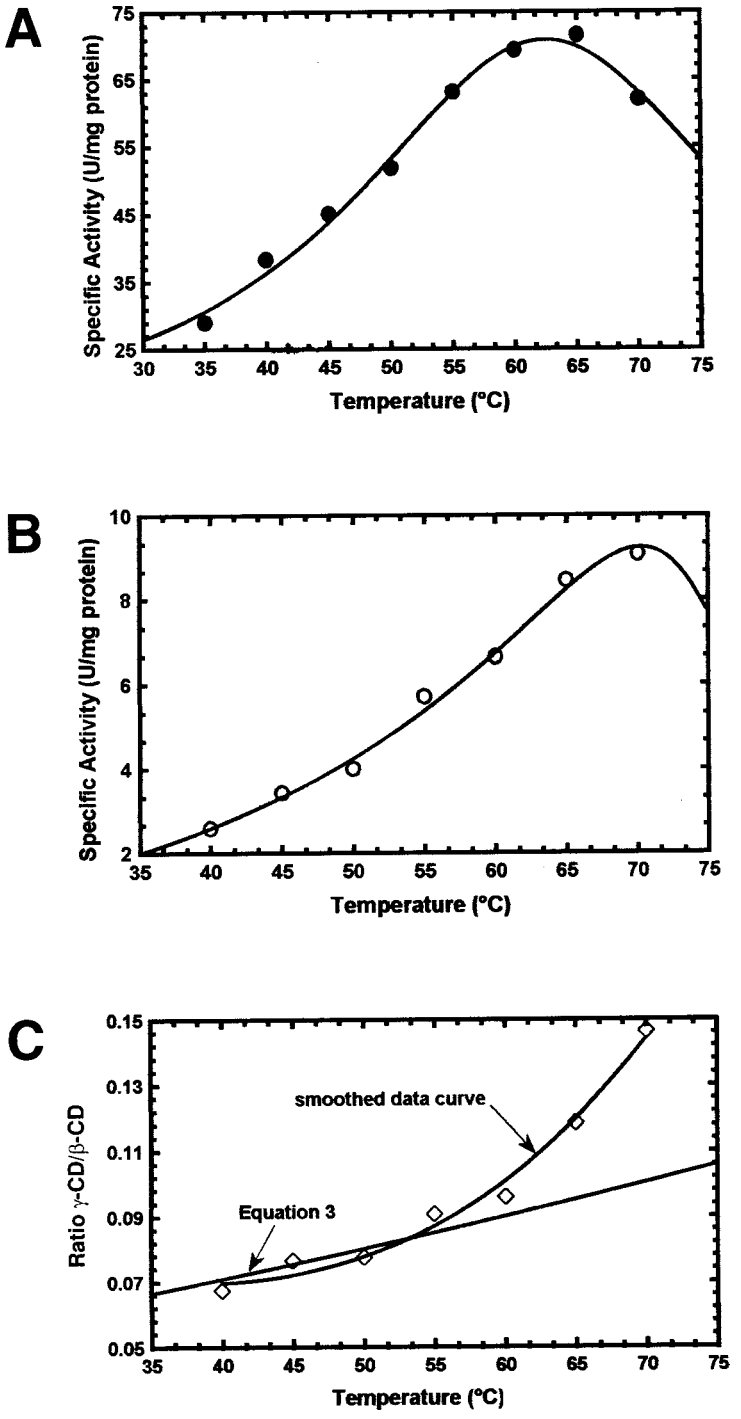

Fig. 2. Specific activity for the production of **(A)** β-CD and **(B)** γ-CD and **(C)** their ratio, γ-CD/β-CD, as a function of temperature for the CGTase from *B. firmus* strain no. 37. Conditions: substrate is 10% (w/v) maltodextrin, in 50 m*M* Tris-HCl buffer and 5 m*M* $CaCl_2$, pH 8.0.

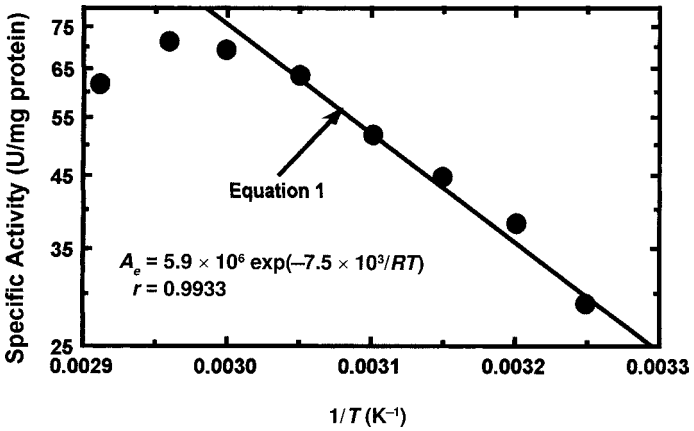

Fig. 3. Arrhenius plot of the specific activity for the production of β-CD as a function of the inverse of the absolute temperature for the CGTase from *B. firmus* strain no. 37. Conditions: substrate is 10% (w/v) maltodextrin, in 50 m*M* Tris-HCl buffer and 5 m*M* $CaCl_2$, pH 8.0.

In the determination of the β-CD produced by the CGTase of strain no. 37, as a function of temperature, the maximal specific activity was found at 65°C (71.5 mmol of β-CD/[min · mg of protein]). For the γ-CD, the maximal specific activity was observed at 70°C (9.1 mmol of γ-CD/[min · mg of protein]). Therefore, both the optimal pH and optimal temperature values, of maximal specific activity of the enzyme, were different for the products β- and γ-CD.

The values of optimal temperature obtained herein are in agreement with those found in the literature, and the optimal temperature for known Bacilli is found in the range of 45–70°C. CGTase of *B. firmus* no. 324, studied by Yim et al. *(26)*, has also shown an optimal temperature of 65°C.

Energy of Activation of CGTase Enzyme of B. firmus

The effect of temperature on an enzymatic reaction can be analyzed through the Arrhenius equation. Therefore, this equation was adjusted to the experimental points of Fig. 2, and it allowed the determination of the activation energy for the reaction of β- (Fig. 3) and γ-CD production (Fig. 4), giving 7.5 and 9.9 kcal/mol, respectively. The larger activation energy found for the reaction of γ-CD production, together with the results presented in Fig. 2C, which show that larger temperatures favor the production of γ-CD, confirms the following general rule: "larger temperatures favor the reactions of larger activation energy" *(27)*.

Within the range of validity, the enzymatic activity for the production of β- and γ-CD and their ratio can be calculated with the adjusted Arrhenius-type Eqs. 1–3 plotted in Figs. 3 and 4:

$$\beta\text{-CD: } A_e = 5.9 \times 10^6 \exp(-7.5 \times 10^3/RT); \quad T \leq 55°C \tag{1}$$

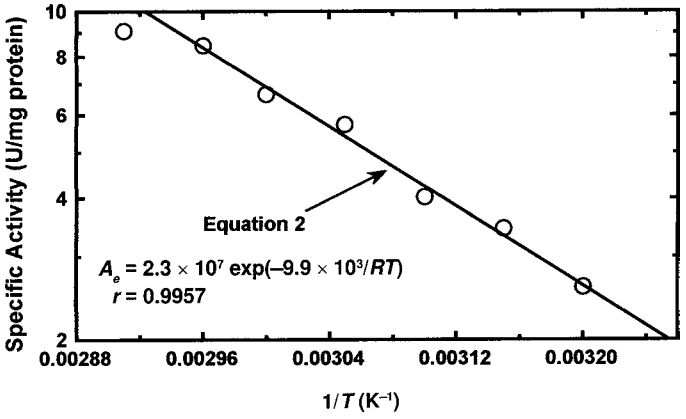

Fig. 4. Arrhenius plot of the specific activity for the production of γ-CD as a function of the inverse of the absolute temperature for the CGTase from *B. firmus* strain no. 37. Conditions: substrate is 10% (w/v) maltodextrin, in 50 m*M* Tris-HCl buffer and 5 m*M* $CaCl_2$, pH 8.0.

$$\gamma\text{-CD: } A_e = 2.3 \times 10^7 \exp(-9.9 \times 10^3/RT); \quad T \leq 65°C \tag{2}$$

$$\gamma\text{-CD}/\beta\text{-CD: production ratio} = 3.9 \exp(-2.5 \times 10^3/RT); \quad T \leq 55°C \tag{3}$$

in which *R* is the ideal gas constant (1.987 cal/[mol·K]) and *T* is the absolute temperature in degrees kelvin.

Thermal Stability of CGTase from B. firmus

Figure 5 presents the residual specific activity for β-CD production as a function of time for the CGTase of *B. firmus* strain no. 37. Although the data presented in Fig. 2 show that the enzyme presents a high specific activity for β-CD production at 65°C, and at 70°C for γ-CD, its thermal stability in these conditions is relatively low, as observed in Fig. 5. This result makes the use of this CGTase unfeasible for periods of time longer than 4 h at 70°C, which was shown as the best temperature for maximizing the ratio of γ- to β-CD production (Fig. 2C). Whereas at 65°C and pH 8.0, the enzyme showed a value of 71.5 U/mg of protein, the specific activity at 60°C and the same pH was equal to 56.4 U/mg of protein, which is 1.2 times less than at 65°C. In spite of the lesser activity at 60°C, it is advisable to use this temperature instead of 65°C because at 60°C, the enzyme presents an activity still relatively high that is associated with a higher thermal stability, which is necessary if the enzyme is to be reacted for long periods such as 24–48 h used in industrial processes. These results are compatible with those obtained by other CGTases *(22)*.

Energy of Thermal Deactivation of the CGTase of B. firmus

Figure 5 shows that the CGTase of *B. firmus* strain no. 37 practically did not show thermal deactivation in the temperature range of 35–60°C for a reaction period of 4 h. At 65°C and above, the enzyme presented increas-

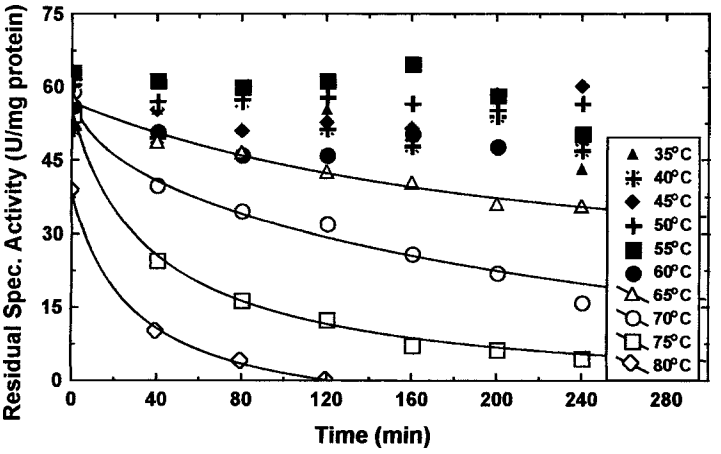

Fig. 5. Residual specific activity for the production of β-CD as a function of time for the CGTase from *B. firmus* strain no. 37. Conditions: substrate is 10% (w/v) maltodextrin, in 50 m*M* Tris-HCl buffer and 5 m*M* $CaCl_2$, pH 8.0; enzyme concentration is 4.3×10^{-4} mg of protein/mL.

ingly higher thermal deactivation, particularly at 80°C, where after 120 min it did not show any residual activity.

The half-life of the CGTase enzyme was calculated by the exponential model *(17,28)*. For the enzyme incubated in a solution of 10% (w/v) maltodextrin, pH 8.0, the half-life was higher than 11 h for temperatures lower than 60°C and dropped to just 40 min at 80°C. For long reaction periods, these results delimit the best use of the enzymatic activity to temperatures lower than 60°C.

It is usually assumed that the kinetics of enzyme thermal deactivation is first order in relation to the concentration of active enzyme, and that the coefficient of thermal inactivation (K_d) is a function of the temperature as given by the Arrhenius law. The slope of the adjusted straight line that correlates to the natural logarithm of the coefficient of thermal inactivation (K_d) with the inverse of the absolute temperature (T) is the energy of thermal deactivation *(17,28)*. This graph is shown in Fig. 6 and the energy of deactivation obtained was 39 kcal/mol. The Arrhenius-type equation adjusted to the data allow the determination of the coefficient of thermal inactivation (K_d) and the half-life ($t_{½}$) for all temperatures *(17,28)*:

$$K_d = 2.1 \times 10^{24} \exp(-39 \times 10^3 / R \cdot T) \quad (4)$$

$$t_{½} = \ln(2) / K_d \quad (5)$$

For example, at 65°C the half-life is 5.2 h whereas at 50°C it is 76.6 h. Additionally, the deactivation of the CGTase enzyme by heating in boiling water (100°C) can now be calculated, and it takes 8.9 min to lower the enzymatic activity to 1% of its initial activity. Therefore, the deactivation of the enzyme by boiling for 10 min was more than satisfactory. This enzyme is less stable

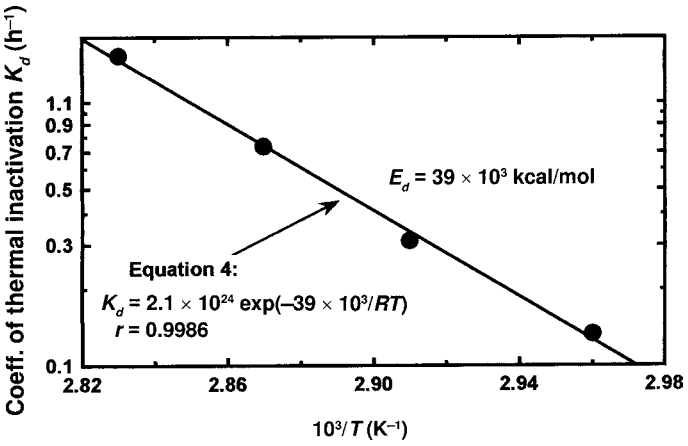

Fig. 6. Arrhenius plot of the residual activity for the production of β-CD as a function of the inverse of the absolute temperature for the CGTase from *B. firmus* strain no. 37. Conditions: substrate is 10% (w/v) maltodextrin, in 50 m*M* Tris-HCl buffer and 5 m*M* $CaCl_2$, pH 8.0; enzyme concentration is 4.3×10^{-4} mg of protein/mL.

than Novo Nordisk (Copenhagen, Denmark) amyloglucosidase, which has an energy of deactivation of 50.6 kcal/mol *(28)*.

Molecular Weight of Enzyme

The molecular weight of the CGTase enzyme was determined by SDS-PAGE and found to be 77.6 kDa. This value is within the usual range of molecular weight obtained for CGTases from different microorganisms (66–80 kDa).

Conclusion

The characterization of the CGTase from *B. firmus* strain no. 37 led to a mol wt of 77.6 kDa. At 50°C the maximum specific activities of this CGTase for β-CD production occurred at pH 6.0, whereas for γ-CD they occurred at pH 8.0. When the pH was fixed at 8.0, the temperatures for maximum specific activities were 65°C for β-CD, giving 71.5 U/mg, and 70°C for γ-CD, giving 9.1 U/mg, respectively. Therefore, both optimum values for pH and temperature for the production of β- and γ-CD were different. This is advantageous because greater production of one of the products can be achieved by appropriate choice of the operating conditions. Although the enzyme was more active at 65°C for the production of β-CD, it was more stable at 60°C. With the CGTase of *B. firmus* strain no. 37, the reactions that produce CDs from the substrate 10% (w/v) maltodextrin at pH 8.0 had an energy of activation of 7.5 and 9.9 kcal/mol for the production of β-CD and γ-CD, respectively. This enzyme when incubated in the same substrate solution had a half-life >11 h for temperatures below 60°C. The energy of thermal deactivation of this enzyme was 39 kcal/mol.

Acknowledgments

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References

1. Szejtli, J. (1988), in *Cyclodextrin Technology*, Szejtli, J., ed., Kluwer Academic, Dordrecht, The Netherlands, pp. 1–78 and 79–185.
2. Bekers, O., Uijtendaal, E. V., Beijnen, J. H., Bult, A., and Underberg, W. J. M. (1991), *Drug Dev. Ind. Pharm.* **17,** 1503–1549.
3. Duchêne, D., Debruères, B., and Brétillon, A. (1984), *Labo-Pharma—Probl. Tech.* **32,** 843–850.
4. Englbrecht, A., Harrer, G., Lebert, M., and Schmid, G. (1990), in *Minutes of the 5th International Symposium on Cyclodextrins*, Duchêne, D., ed., Editions de Santé, Paris, pp. 25–31.
5. Lee, J. H., Choi, K. H., Lee, Y. S., Kwon, I. B., and Yu, J. H. (1992), *Enzyme Microb. Technol.* **14,** 1017–1020.
6. Duchêne, D. (1987), *Cyclodextrins and Their Industrial Uses*, Edition de Santé, Paris.
7. Nakamura, A., Haga, K., and Yamane, K. (1994), *FEBS Lett.* **337,** 6–70.
8. Mattsson, P., Mäkelä, M., and Korpela, T. (1988), in *Proceedings of the Fourth International Symposium on Cyclodextrin*, Huber, O. and Szejtli, J., eds., Kluwer Academic, Dordrecht, The Netherlands, pp. 65–70.
9. French, D. (1957), *Adv. Carbohydr. Chem.* **12,** 189–260.
10. Horikoshi, K. (1988), in *Proceedings of the Fourth International Symposium on Cyclodextrin*, Huber, O. and Szejtli, J., eds., Kluwer Academic, Dordrecht, The Netherlands, pp. 7–17.
11. Jamuna, R., Saswathi, N., Sheelar, R., and Ramakrishina, V. (1993), *Appl. Biochem. Biotecthnol.* **43,** 163–176.
12. Mori, S., Hirose, S., Oya, T., and Kitahata, S. (1994), *Biosci. Biotechnol. Biochem.* **58,** 1968–1972.
13. Matioli, G., Zanin, G. M., Guimarães, M. F., and de Moraes, F. F. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 267–275.
14. Bender, H. (1986), *Adv. Biotechnol. Processes* **6,** 31–71.
15. László, E., Banky, B., Seres, G., and Szejtli, J. (1981), *Starch* **33,** 281–283.
16. Berna, P., de Moraes, F. F., Barbotin, J. N., Thomas, D., and Vijayalaksmi, M. A. (1996), in *Advances in Molecular and Cell Biology*, vol. 15B, Bittar, E. E., series ed., Danielson, B. and Bülow, L., guest eds., JAI Press, pp. 521–535.
17. Matioli, G. (1997), PhD thesis, UFPR, Curitiba, PR, Brazil.
18. Bradford, M. (1976), *Anal. Biochem.* **72,** 248.
19. Dixon, M. and Webb, E. C. (1979), *Enzymes*, 3rd ed., Longman Group Limited, London.
20. Hamon, V. and de Moraes, F. F. (1990), in *Etude Preliminaire a L'immobilisation de L'enzyme CGTase WACKER*, Laboratoire de Tecnologie Enzymatique, Université de Tecnologie de Compiègne.
21. Weber, K. and Osborn, M. (1969), *J. Biol. Chem.* **244,** 4406–4412.
22. Kato, T. and Horikoshi, D. (1986), *Agric. Biol. Chem.* **50,** 2161, 2162.
23. Sato, M. and Yagi, Y. (1991), in *Biotechnology of Amylodextrin Oligosaccharides*, Friedman, R. B., ed., American Chemical Society, Washington, DC, pp. 125–137.
24. Techaiyakul, W., Pongssawasdi, P., and Mongkolkl, P. (1990), in *Minutes of the 5th International Symposium on Cyclodextrins*, Duchêne, D., ed., Editions de Santé, Paris, pp. 50–54.
25. Horikoshi, K. (1971), *Agric. Biol. Chem.* **35,** 1407–1414.
26. Yim, D. G., Sato, H. H., Park, Y. H., and Park, Y. K. (1997), *J. Ind. Microbiol. Biotechnol.* **18,** 402–405.
27. Levenspiel, O. (1972), *Chemical Reaction Engineering*, 2nd ed., John Wiley & Sons, New York, p. 239.
28. Zanin, G. M. and de Moraes, F. F. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 383–394.