0273-2289/1559–0291 (Online)/07/136–140/41–56/$30.00

Purification and Characterization of a Cyclomaltodextrin Glucanotransferase From *Paenibacillus campinasensis* Strain H69-3

Heloiza Ferreira Alves-Prado,[1,2] Eleni Gomes,[1] and Roberto da Silva*,[1]

[1]UNESP—State University of São Paulo, Biochemistry and Applied Microbiology Laboratory, Rua Cristóvão Colombo n 2265, 15054-000, São José do Rio Preto, SP, Brazil, E-mail: dasilva@ibilce.unesp.br; and [2]UNESP—State University of São Paulo, Biology Institute, Rio Claro, SP

Abstract

A cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19) from a newly isolated alkalophilic and moderately thermophilic *Paenibacillus campinasensis* strain H69-3 was purified as a homogeneous protein from culture supernatant. Cyclomaltodextrin glucanotransferase was produced during submerged fermentation at 45°C and purified by gel filtration on Sephadex G50 ion exchange using a Q-Sepharose column and ion exchange using a Mono-Q column. The molecular weight of the purified enzyme was 70 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the p*I* was 5.3. The optimum pH for enzyme activity was 6.5, and it was stable in the pH range 6.0–11.5. The optimum temperature was 65°C at pH 6.5, and it was thermally stable up to 60°C without substrate during 1 h in the presence of 10 m*M* $CaCl_2$. The enzyme activity increased in the presence of Co^{2+}, Ba^{2+}, and Mn^{2+}. Using maltodextrin as substrate, the K_m and K_{cat} were 1.65 mg/mL and 347.9 μmol/mg·min, respectively.

Index Entries: CGTase characterization; CGTase purification; cyclomaltodextrin glucanotransferase; thermostable CGTase.

Introduction

Cyclomaltodextrin glucanotransferase (CGTase; EC 2.4.1.19) is a member of the α-amylase family (family 13) of glycosyl hydrolase *(1)*. CGTase can also hydrolyze glucan chains in a manner similar to α-amylases, but differs in its ability to form cyclodextrins (CD) as reaction products. CDs are formed from starch molecules through intramolecular transglycosylation (cyclization) and can be made up of 6–8 glucan residues, α-, β-, and γ-CD, respectively. This enzyme is in fact multifunctional; besides cyclization reaction it

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

displays intermolecular transglycosylation (coupling and disproportionation) and hydrolytic activity on starch and CDs *(2–4)*. CDs are doughnut-shaped molecules with a hydrophilic outer surface and a relatively hydrophobic cavity. Owing to their ability to form inclusion complexes with many organic molecules, CDs have become increasingly useful in pharmacy, food, cosmetics, agriculture, analytical chemistry, and biotechnology. They can be used to capture flavors and odors, stabilize volatile compounds, improve the solubility of hydrophobic substances, and protect substances against undesirable modifications *(2–7)*.

CGTases are produced extracellularly by a variety of bacteria mainly by the alkalophilic, mesophilic, and thermophilic *Bacillus* genus *(4,8)*. However, other producers have also been reported such as *Klebsiella* sp. *(9,10)*, *Brevibacterium* sp. *(11)*, *Paenibacillus* sp. *(12–15)*, *Thermoanaerobacter* sp. *(16,17)*, *Thermoanaerobacterium* sp. *(18)*, *Thermococcus* sp. *(19)*, and *Thermoactinomyces* sp. *(20)*. Most of these CGTase producers are able to produce a mixture of CGTase types, mainly α-CD and/or β-CD.

As the majority of the CGTases studied are produced by mesophilic microorganisms, they possess low-thermostability. In the CD production process, therefore, it is necessary to add a thermostable α-amylase during the liquefaction step carried out at high-temperature (95–105°C). On completion of liquefaction, the stream is cooled to 50–55°C for the CD production by CGTase *(16)*. Thermostable CGTases would make it less necessary to reduce the temperature for CGTase action, and this would decrease the cost of final CD production. On the other hand, the high production cost of CGTase and CDs is considered the limiting factor in CD applications on an industrial scale. Research aimed at decreasing the cost of CGTase production is, therefore, necessary if commercial use of CDs is to become economically feasible. The search for a thermophilic CGTase producing microorganism with high-thermostability is thus of commercial interest.

We have isolated a new CGTase producer from soil cassava crop samples, which grows at 45°C and is classified as *P. campinasensis* strain H69-3 (henceforth referred to as H69-3). Although many studies have been carried out on detection of CGTase in different microorganisms, few reports have described CGTase from the alkalophilic thermophilic *P. campinasensis*. Therefore, in the present study we report its classification and the purification and characterization of its CGTase for the first time.

Materials and Methods

Materials

CDs (α-, β-, and γ-CD), maltodextrin, phenolphthalein, orange methyl, and bovine serum albumin were purchased from Sigma (St. Louis, MO). Yeast extract, peptone, and agar were obtained from Difco (Detroit, MI). Soluble starch and all other chemicals of analytical grade were obtained

from Merck (Darmstadt, Germany). Resins for enzyme purification were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Microorganism Isolation and Identification

The bacterial strain H69-3 was isolated from soil cropped with cassava in São José do Rio Preto, SP—Brazil, as described in our previous work *(15)*. The phylogenetic properties of H69-3 were determined with the help of the *Centro Pluridisciplinar de Pesquisas Químicas, Biológicase Agrícolas* (Unicamp, Campinas, SP, Brazil) using molecular techniques of the sequencing and phylogenetic identification analysis of the 16S rRNA gene fragments. The 16S rDNA sequence was amplified by polymerase chain reaction (PCR), using as template the genomic DNA that was isolated off the H69-3 strain. The primers used in PCR were a p27 forward primer and a p1525 reverse primer that corresponded to a homologous conserved region of the 16S rRNA gene in bacteria.

The amplified PCR product was purified and sequenced directly on a MegaBACE 1000 (Amersham Biosciences) automatic sequenator. The forward primers p10 and 765 and reverse primers 782 and p1100 were used during sequencing. The 16S rDNA sequence was compared with known sequences on databases from Ribosomal Database Project (RDP, WI; http://www.cme.msu.edu/RDP/html) and GenBank (http://www.ncbi.nlm.nih.gov/). The 16S rDNA sequences related to H69-3 sequence were selected for phylogenetic analysis. The evaluative distance matrices were calculated with the Kimura model *(21)* and the phylogenetic tree was built using the *Neighbor-Joining* method *(22)* in accordance with analysis software from RDP.

Assay of CGTase

CGTase activity was measured as β-CD forming activity based on phenolphthalein method *(23)* with slight modifications as described in Alves-Prado et al. *(20)*. One unit of CGTase activity was defined as the amount of enzyme that produced 1 μmol of β-CD per min.

Protein Determination

Protein concentration was estimated according to the Hartree-Lowry method, using bovine serum albumin as standard *(24)*.

Purification of CGTase

The culture medium used for CGTase production was based on the proposal by Nakamura and Horikoshi *(25)* with some modifications: soluble starch 10.0 g/L, peptone 5.0 g/L, yeast extract 5.0 g/L, K_2HPO_4 1.0 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, Na_2CO_3 5 g/L (separately sterilized), pH 9.6.

The CGTase was produced by cultures of H69-3 in 500-mL Erlenmeyer flasks, containing 80 mL culture medium with soluble starch as substrate. The cultures were incubated on a rotary shaker at 45°C, for 48 h at 200 cycles per min. The cells were removed from the medium by centrifugation at 10000*g* for 15 min at 5°C. Supernatant containing crude CGTase was concentrated by ultrafiltration using the Pellicon® system (Millipore, Beldford, MA). The concentrated CGTase was subjected to gel filtration chromatography on a Sephadex superfine G-50 column (2.6×100 cm^2) that had been preequilibrated with 20 m*M* Tris-HCl buffer (pH 7.5), containing 20 m*M* NaCl. Elution was carried out by the same buffer at a flow rate of 0.3 mL/min at room temperature, and 5 mL fractions were collected using a fraction collector (Pharmacia Biotech Frac-100, Sweden). The CGTase-containing fractions were spin-concentrated using Centricon® YM10 tubes (amicon bioseparations, Millipore, Beldford, MA) with 10 kDa cutting membrane and purified to homogeneity on an ÄKTA purifier system (Pharmacia Biotech, Sweden). The following chromatographic steps were performed using a Q-Sepharose® Fast Flow column (5.0×10.0 cm^2) followed by a Mono-Q HR 5/5 column with bed volume of 1.0 mL (Pharmacia Biotech, Sweden) using 20 m*M* Tris-HCl buffer (pH 7.5). The purity of the CGTase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of Protein Molecular Weight

The molecular weight of the pure protein was estimated by SDS-PAGE according to Laemmli *(26)*. The SDS-PAGE was performed on a 10% homogeneous gel using the Mini-Cell electrophoresis apparatus (BioRad Laboratories, Richmond, CA). The gel was stained by means of the Blum silver-staining method *(27)*. BenchMarker™ Protein Ladder (Invitrogen) was used as standard.

Activity Gel

The native gel was performed on a 10% homogeneous gel using the Mini-Cell electrophoresis apparatus (BioRad Laboratories) in accordance to Laemmli *(26)*, but without SDS. Part of the gel was stained by means of the Blum silver-staining method *(27)* and other part of this gel was incubated on soluble starch solution 1% overnight, rotating at 5°C and after this, the gel was stained with I-IK solution 1%.

Isoelectric Focusing

Isoelectric focusing (p*I*) was performed in an Ettan IPGphor II two-dimensional electrophoresis apparatus (Amersham Biociences), using a strip holder with a p*I* scale from 3.0 to 10.0 for focusing and a 12% (w/v) acrylamide gel for the second dimension. During isoelectric focusing, the strip

holder was submitted to rehydration loading together with the enzyme sample for 12 h. It was subjected to different voltage steps, with the first step carrier in 500 Vh, the second step carrier in 1000 Vh, and the third step carrier in 12,500 Vh. After focusing, the strip holder was transferred to a polyacrylamide gel (SDS-PAGE) for the two-dimensional. SDS-PAGE was carried out at a constant voltage of 90 V for the first half hour and 220 V for the next 4 h at 25 mA. The gel was stained by the Blum silver-staining method *(27)*.

Kinetic Parameters

The kinetic parameters were determined by incubating the pure enzyme in maltodextrin with DE 13.0–17.0 (Aldrich, Milwaukee, WI) (from 0 to 10 mg/mL) in 50 m*M* acetate buffer, pH 6.5, at 60°C, for 10 min under phenolphthalein assay conditions. The values of K_m and V_{max} were estimated by fitting the data to a Michaelis-Menten model using the GraFit program, version 5.0 (Erithacus Software).

Effect of pH and Temperature on Activity and Stability of the Enzyme

The optimum pH of the pure CGTase was determined by measuring activity at 60°C using MacIlvaine buffer (pH 2.5–8.0) and glycine–NaOH buffer, 0.1 *M* (pH 8.0–11.5). The reaction was carried out using the CGTase assay as previously mentioned. Optimal pH was the pH where the enzyme displayed its maximal activity, which was considered 100% activity. The optimum temperature of the pure enzyme was determined by incubating the reaction mixture of the CGTase assay in different temperatures, ranging from 5 to 90°C for 10 min. Optimal temperature was the temperature where the enzyme displayed its maximal activity, which was considered 100% activity.

The pH stability was determined by incubating the CGTase preparation without substrate in the same buffer systems used previously, at 25°C for 24 h. The remaining activity was assayed under standard conditions at 60°C. The temperature stability was checked by subjecting the enzyme, without substrate, at various temperatures at 5°C up to 90°C for 60 min and then cooling in ice before measuring the residual activity under standard conditions at 60°C and pH 6.5.

Results and Discussion

Microorganism Isolation

The bacterial strain H69-3 showed high CGTase activity and growth at 45°C. The strain H69-3 was identified according to 16S rDNA sequence comparisons and correlation with the physiological characteristics of this isolate. H69-3 has rod-shaped cells and Gram coloration was variable. During early growth phase Gram-positive cells could be observed, but

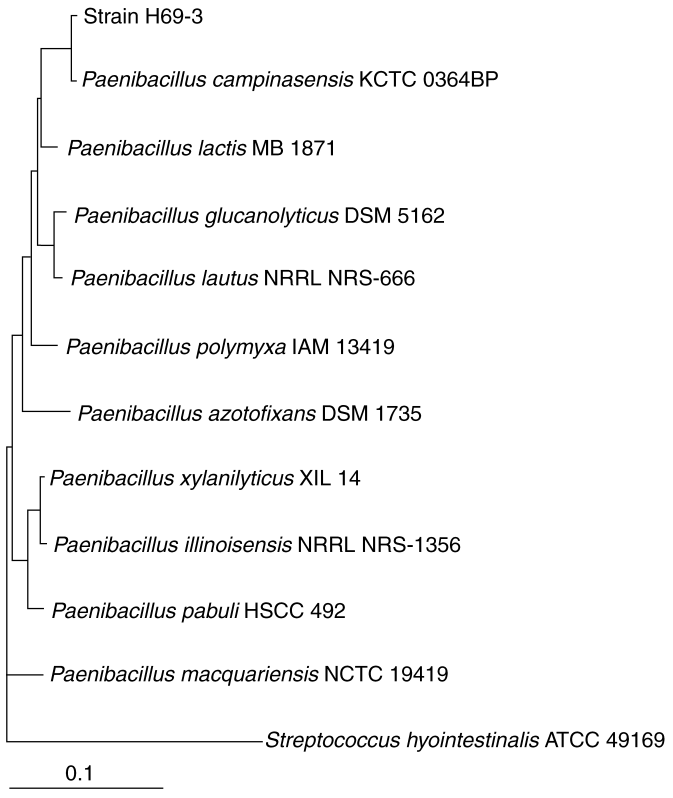

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequence, of the H69-3 strain and other related microorganisms. The *Neighbor-Joining* method was used and *Streptococcus hyointestinalis* was used as the out-group. The GeneBank accession numbers of the additional 16S rRNA sequences used are as follows: *P. campinasensis* KCTC 0364BP (AF021924); *P. lactis* MB 1871 (AY257868); *P. glucanolyticus* DSM 5162 (D78470); *P. lautus* NRRL NRS-666 (D78473); *P. polymyxa* IAM 13419 (D16276); *P. azotofixans* DSM 1735 (X77846); *P. xylanilyticus* XIL14 (AY427832); *P. illinoisensis* NRRL NRS-1356 (D85397); *P. pabuli* HSCC 492 (AB045094); *P. macquariensis* NCTC 10419 (X60625).

after 50 h of growth, only Gram-negative rods were visible. This microorganism was facultative anaerobic, positive for catalase reaction and nitrite to nitrate reduction as well as negative to oxidase reaction. The cellular growth at 30°C, 37°C, 40°C, 50°C, and 55°C was observed for nutrient medium in pH6.8. These physiological properties indicated the gender of the strain H69-3, but only with 16S rRNA was possible to identify correctly this strain. The partial 16S rRNA sequence of H69-3, with 892 pb revealed 99% similarity with sequences of the *P. campinasensis* strain 324 *(28)* and other *Paenibacillus* sp. strains included in RDP and GenBank. Similarity of 94 and 96% was found with other *Paenibacillus* sp., such as *P. lactis* and *P. pabuli.* Phylogenetic analysis and physiological characteristics confirmed the similarity found in databases and clearly grouped the strain H69-3 with *P. campinasensis* (Fig. 1). Based on these results, the studied

microorganism (strain H69-3) was classified as *P. campinasensis* strain H69-3. The partial 16S rDNA sequence has been deposited in the GeneBank and has been assigned the accession number DQ153080.

CGTase Purification

After 50 h of fermentation, the supernatant from the *P. campinasensis* strain H69-3 culture was used for purification of CGTase in four steps. The supernatant containing crude enzyme was first concentrated by ultrafiltration and subsequently purified by gel filtration and anion exchange (Table 1). These steps resulted in a 115-fold purification with a yield of 13.3%, and specific activity of 8.1 U/mg of protein (Table 1). The purification steps resulted in one homogeneous band on a silver-stained SDS-PAGE (Fig. 2A). A native polyacrylamide gel for activity staining also gave the purified CGTase one single band in the presence of soluble starch (Fig. 2B).

Previously, CGTase purification from *Bacillus* sp. AL-6 was performed using starch adsorption chromatography and two DEAE–Sephadex A-50 chromatographies, with a yield of 14.4% and a 230-fold purification, similar to the yield *(29)*. In another study, CGTase from the *P. campinasensis* (*B. firmus*) strain 324 was purified by ion exchange- and affinity-chromatography resulting in a 26.6% yield and a 90-fold purification *(30)*. Finally, process chromatography was used to purify the CGTase from *P. illinoisensis* ST-12K using a DEAE-cellulose column and a Butyl-Toyopearl column, resulting in a 4.5-fold purification and a 27% yield *(13)*.

Molecular Weight

The molecular weight of the purified CGTase was estimated by electrophoretic mobility under denaturing conditions to be 70 kDa (Fig. 2A). This molecular weight is in agreement with the majority of purified CGTase reported in literature, which is between 70–88kDa *(4,8–13,25,29–31)*. However, in some cases, CGTase has been reported to have a lower molecular weight, such as those from *B. lentus* (33 kDa) *(32)*, *B. coagulans* (36 kDa) *(33)*, and *Bacillus* sp. 1919 (42 kDa) *(34)*. On the other hand, CGTases with a higher molecular weight have also been reported, such as those from *B. agaradhaerens* (110 kDa) *(35)*, *B. licheniformis* (144 kDa) *(36)*, and *Thermoanaerobacter* sp. (103 kDa) *(16, 17)* (Table 2). The variation of the p*I* values among studied CGTases can be owing to adaptations from environmental properties of the screening place. Different environments can lead to genetic sequence changes of this enzyme during the evolutionary process *(40)*.

Isoelectric Focusing

The p*I* of the purified CGTase was 5.3. The p*I* exhibited by this CGTase is slightly acidic, similar to CGTase from *B. stearothermophilus (37)*, which has a p*I* value of 5.0. Some CGTases have shown high acidic p*I* values, such as CGTase from *Brevibacterium* sp. *(11)* with a p*I* value of 2.8 and from

Table 1
Summary of Purification Results

Step	Total volume (mL)	Total protein (mg)	Total CGTase activity (U)	Specific activity (U/mg)	Fold purification	Percent yield
Crude enzyme (supernatant)	350.0	1393.0	91.0	0.07	–	100
Concentration by ultrafiltration (Pelicon)	40.0	316.0	87.2	0.28	4.0	95.8
Gel filtration Sephadex G-50	123	32.0	49.2	1.54	22.0	54.1
Ion exchange (Q-Sepharose)	70	2.8	39.9	14.25	203.6	43.8
Ion exchange (Mono-Q column)	7.5	1.5	12.1	8.07	115.3	13.3

Enzymatic activity determined by phenolphthalein method.

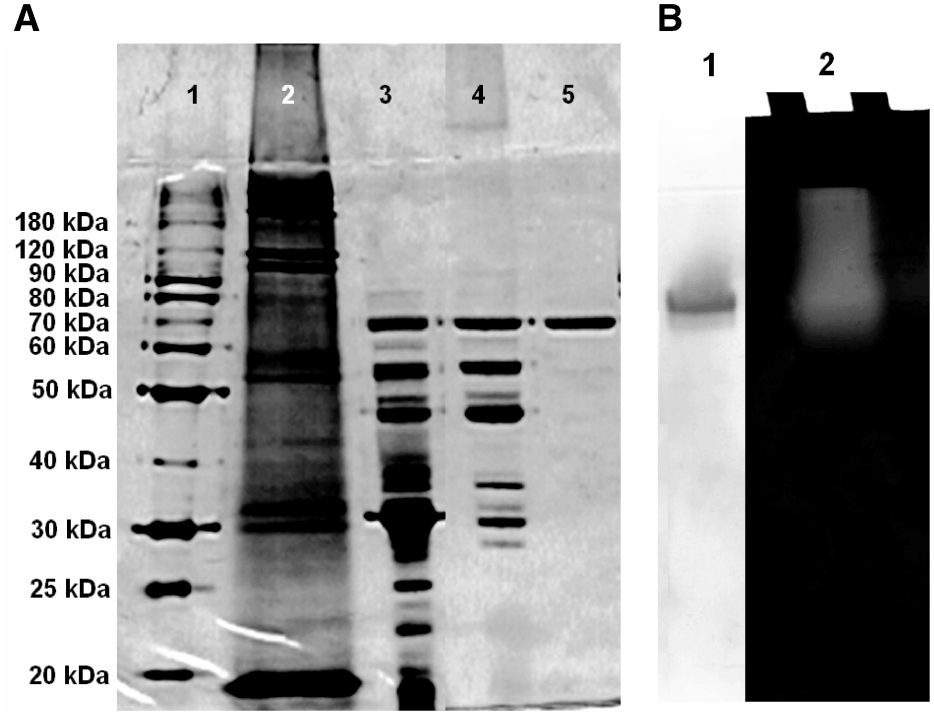

Fig. 2. CGTase from *P. campinasensis* strain H69-3 **(A)** determination of molecular weight on SDS-PAGE. Lane 1, molecular weight marker; lane 2, crude enzyme; lane 3, elution from gel filtration; lane 4, Q-Sepharose fast flow elution; and lane 5, purified CGTase (Mono-Q elution). **(B)** Native gel, 1: silver stained and 2: amylolytic activity I-KI stained.

Table 2
Comparison Between the Properties of CGTase From the *P. campinasensis* Strain H69-3 and From Some Other CGTase Producers

Strain	Molecular weight (kDa)	Optimum temperature (°C)	Stability temperature (°C)		Optimum pH	Stability pH	K_m (mg/mL)	*p*I	References
			+Ca^{+2}	–Ca^{+2}					
Alkalophilic *Bacillus* sp. ATCC 21783	85	50	60	70	7.0 and 8.0–9.0	6.0–9.0	–	–	*25*
Bacillus sp. AL-6	74	55–60	40	–	8.0	5.0–8.0	–	–	*29*
Bacillus sp. 1919	42	55–60	50	–	4.0	5.0–8.0	–	–	*34*
Bacillus sp. G1	75	60	60	70	6.0	7.0–9.0	0.15[a]	8.8	*31*
B. autolyticus 11149	68	60	40	–	5.0–6.0	5.0–9.0		3.0–4.0	*38*
B. agaradhaerens LS-3C	110	55	30	40	9.0	5.0–11.4	18.0[b] and 21.2[c]	6.9	*35*
B. circulans E192	78	60	–	45	5.5–5.8	6.0–9.0	0.70[b] and 0.57[d]	6.9 and 6.7	*39*
B. coagulans	36	65	65	–	6.5	5.0–10.0	–	–	*33*
B. lentus	33	45–50	–	55	6.5–7.5	6.5–8.5	–	–	*32*
B. firmus (NCIM 5119)	78	65	30	–	5.5–8.5	7.0–11.0	1.21[c]	–	*41*
B. licheniformis	144	–	60	–	5.0–6.0	6.0–8.0	–	–	*36*
B. stearothermophilus ET1	66.8	80	60	70	6.0	6.0–8.0	–	5.0	*37*
Brevibacterium sp. n 9605	75	45	30	50	10.0	6.0–8.0	–	2.8	*11*
K. pneumoniae AS-22	72	45	35	–	7.0–7.5	5.5–9.0	1.35[c]	7 and 3	*10*
Paenibacillus sp. F8	72	50	40	50	7.5	6.0–8.0	–	–	*12*
Thermoactinomyces sp. INMIA—A-561	64	–	70	–	6.0–7.0	5.5–8.5	–	–	*20*
Thermoanaerobacter sp.-ATCC53627	103	95	80	–	5.0	5.0–6.7	–	–	*16,17*
P. campinasensis H69-3	70	65	55	60	6.5	6.0–11.0	1.65[b]	5.3	This work

[a]β-cyclodextrin.
[b]maltodextrin.
[c]Soluble starch.
[d]Wheat starch.

B. autolyticus (38) with a p*I* s value of 3.0 and 4.0. However, other CGTases have a high basic p*I*, such as that from *Bacillus* sp. G1 *(31)* with a p*I* of 8.8. There are also neutral p*I* CGTases, such as *B. agaradhaerens* LS-3C *(35)* with a p*I* value of 6.9, and *B. circulans* E192 *(39)* with p*I* values of 6.9 and 6.7 (Table 2).

Kinetic Parameters

The K_m and V_{max} values obtained were 1.69 ± 0.39 mg/mL and 4.97 ± 0.30 μmol/min·mg, respectively. K_m values ranging from 0.15 to 21.2 mg/mL and V_{max} values ranging from 7.4 to 249 U/mg have previously been reported for few CGTases *(10, 30, 31, 35, 39, 41)*. However, only the *B. agaradhaerens* LS-3C *(35) and B. circulans* E192 *(39)* used maltodextrin as substrate. The K_m of CGTase from *P. campinasensis* strain H69-3 was larger than *B. circulans* E192 (0.7 mg/mL) and smaller than CGTase form *B. agaradhaerens* LS-3C (18.0 mg/mL). As K_m might be correlated with the affinity for substrate, by comparison it is possible to infer a good affinity of the CGTase of *P. campinasensis* strain H69-3 for maltodextrin. The K_{cat} and K_{cat}/K_m were calculated and it was 347.9 μ*M*/mg·min and 205.9 min/μmol, respectively. The K_{cat} value exhibited by this CGTase was larger than those obtained by CGTases from *Klebsiella pneumoniae* AS-22 (249 μ*M*/mg·min) *(10)* and *B. firmus* (145.17 μ*M*/mg·min) *(41)*. However, here any conclusion might be obtained, as the used substrates were not the same.

Effect of pH on Activity and Stability of the Enzyme

The activity CGTase was determined at varying pH values ranging from 2.5 to 11.5 at 60°C. The purified CGTase was highly active between pH values 5.5 and 7.0 with maximum activity at pH 6.5 in MacIlvaine buffer (Fig. 3). The optimum pH value suggests that, in order to effect the cyclization reaction, CGTase from the *P. campinasensis* strain H69-3 needs a pH near to neutral. This optimum pH was also noted for CGTase from *B. lentus (32)* and *B. coagulans (33)*. The pH stability was determined by incubating the crude CGTase on different pH values, ranging from 2.5 to 12.0 for 24 h at 25°C. Then, the residual activity was measured at standard activity conditions. The purified CGTase was found to be stable over a wide range of pH (6.0–11.0) after 24 h of incubation at 25°C (Fig. 3).

Effect of Temperature On Activity and Stability of The Enzyme

The activity of pure CGTase was measured at temperatures between 30°C and 80°C at pH 6.5. The enzyme exhibited maximum activity at temperatures between 60°C and 65°C (Fig. 4). The effect of temperature on stability of pure CGTase was also investigated. The enzyme was incubated for 1 h at various temperatures (30–70°C) followed by measurement of residual activity under standard assay conditions. Hundred percent CGTase activity was maintained up to 55°C, indicating good thermal stability (Fig. 4). The

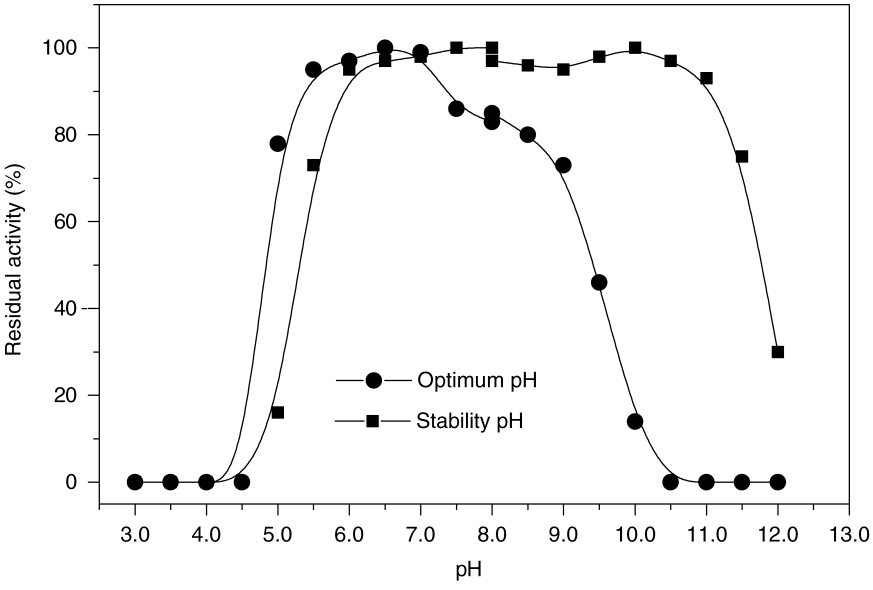

Fig. 3. Effect of pH on CGTase activity (—•—) and CGTase stability (—▪—) from the *P. campinasensis* strain H69-3. The buffers used were: MacIlvaine (pH 3.0–8.0) and glycine–NaOH (pH 8.0–11.5).

enzyme was completely inactive at 80°C. The thermal stability increased when the CGTase was incubated in the presence of the Ca^{2+}. About 90% of CGTase activity was maintained up to 60°C when it was incubated with 10 m*M* $CaCl_2$ (Fig. 5). Ion Ca^{2+} has been used as enzyme stabilizer and these results were similar to that obtained by Chung *(37)* studying CGTase from *B. stearothermophilus* ET1.

Effect of Metal Ions and Chemicals on CGTase Activity

The enzyme was incubated with a number of salts and reagents, at 5 m*M* and 1 m*M*, in 100 m*M* acetate buffer, pH 6.5 at 25°C for 60 min. A sample of the mixture was used to determine residual activity under standard assay conditions, while maintaining salt and reagent concentrations. The results are summarized in Table 3. Considering the presence of metallic ions and other reagents, the response of CGTase activity from the *P. campinasensis* strain H69-3 was similar to that reported for other CGTases *(29,35)*. This CGTase was strongly inhibited by Ag^{1+}, Al^{3+}, Cr^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{3+}, $NH_4{}^{2+}$, Sn^{2+}, and β-CD. It was slightly inhibited by Cd^{1+}, Pb^{2+}, Zn^{2+}, Zn^{3+}, Sr^{2+}, SDS, and γ-CD with 20–70% of the activity. CGTase activity was not inhibited in the presence of Mg^{2+}, K^{1+}, Ca^{2+}, Na^{1+}, Ni^{2+} ethylenediamine tetra acetic acid, phenylmethylsulfonyl fluoride, sodium *m*-arsenite, sodium azide, 2-mercaptoethanol, dithiothreitol, and α-CD and it was enhanced in the presence of Co^{2+}, Ba^{2+}, and Mn^{2+}.

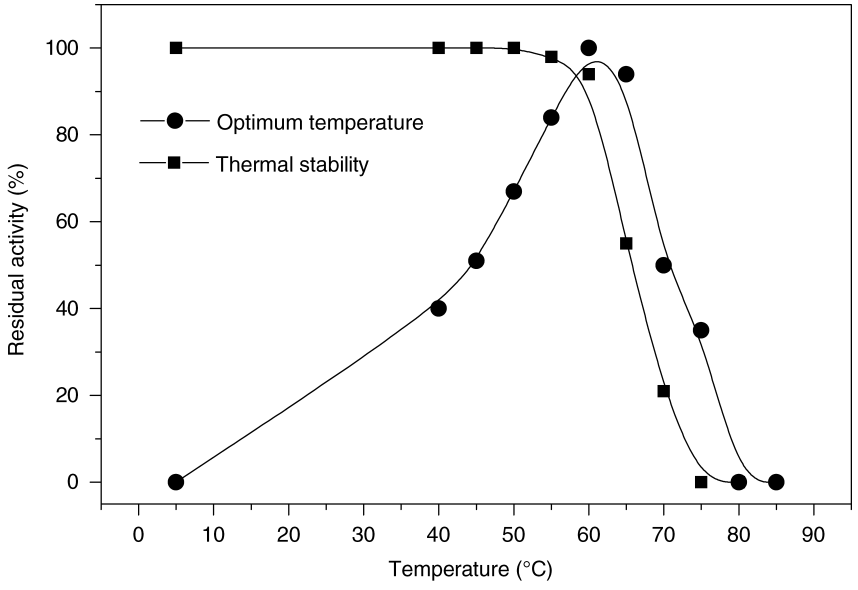

Fig. 4. Effect of temperature on CGTase activity (—•—) and CGTase stability (—▪—) from the *P. campinasensis* strain H69-3.

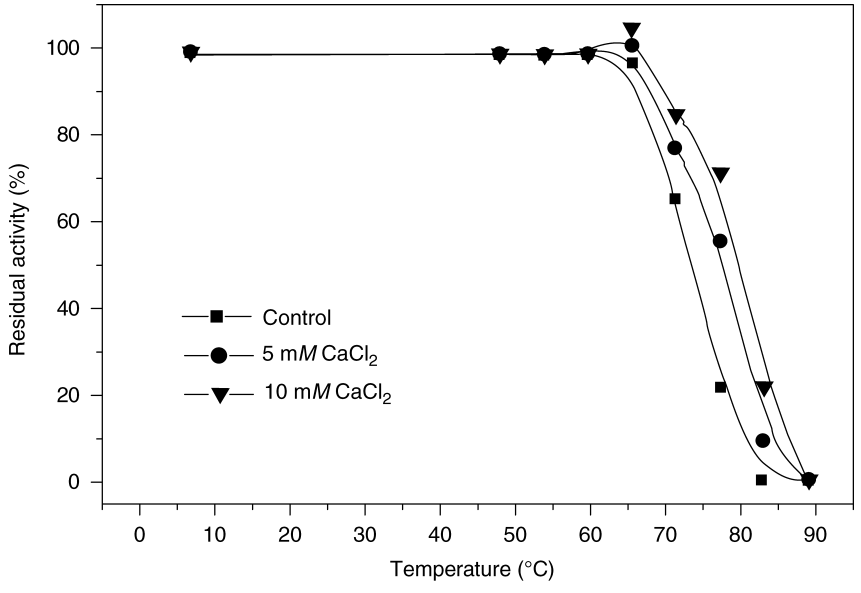

Fig. 5. Effect of $CaCl_2$ on temperature stability of CGTase from *P. campinasensis* strain H69-3. (—▪—) control, without $CaCl_2$; (—•—) 5 m*M* $CaCl_2$ and (—▲—) 10 m*M* $CaCl_2$.

Unlike our results, the activity of CGTases from *Bacillus* AL-6 *(29)* was not inhibited by Fe^{2+}; the same was reported from *P. campinasensis* strain 324 (*B. firmus*) *(30)*, *B. firmus (42)*, and *B. autolyticus (38)*. The activity was increased using the CGTase from *Brevibacterium* sp. *(22)* and *Bacillus* sp. G1

Table 3
Effects of various reagents on CGTase activity
from *P. campinasensis* strain H69-3

Reagent (1 m*M*)	Residual activity (%)
None	100
SDS[a]	70.0
EDTA[b]	84.0
PMSF[c]	95.0
Sodium azide	96.5
Sodium *m*-arsenite	97.0
2-mercaptoethanol	98.0
Dithiothreitol	106.5
α-CD	90.0
β-CD	0
γ-CD	69.0

[a] Sodium dodecyl sulfate.
[b]Ethylenediaminetetra acetic acid.
[c]Phenylmethylsulfonyl fluoride.

(31). It has been reported that Cu^{2+} has a significant inhibitory effect on CGTases from *Bacillus* AL-6 *(29)*, *B. firmus (42)*, *Brevibacterium* sp. *(11)*, and B. *agaradhaerens (35)*, whereas CGTases from *B. autolyticus (38)* and *P. campinasensis* strain 324 (*B. firmus*) *(30)* were not inhibited in presence of the Cu^{2+} at 2 m*M*. Also, the CGTase activity from B. *agaradhaerens (35)*, *B. autolyticus (38)*, and *Brevibacterium* sp. *(11)* was maintained in presence of the Zn^{2+}, Pb^{2+}, and Sr^{2+}, unlike the CGTase from *P. campinasensis* strain H69-3. Metal ions such as Hg^{2+}, Ag^{1+}, Zn^{2+}, and Cu^{2+} have been reported as inhibitors of thermostable α-amylases. As one of the mechanism of protein thermostabilization is by formation of disulfite bridges, the presence of cysteine residues are higher in themostable molecules. These metal ions (Hg^{2+}, Ag^{1+}, and Cu^{2+}) have affinity to the sulfhydryl groups. So, these ions inhibit the enzymatic activity of some proteins by oxidation of the functional cysteine residue groups *(43)*. The almost complete inhibition of CGTase activity from the *P. campinasensis* strain H69-3 by Hg^{2+}, Ag^{1+}, and Cu^{2+} reinforces the hypothesis that the thiol group is essential for the enzymatic activity of the CGTase. About the CD type effect on the CGTase activity, the presence of β-CD and γ-CD has exhibited an inhibitory effect for the CGTase from *B. autolyticus (39)*. In the case of CGTase activity from B. *agaradhaerens (35)*, a slight increase in the presence of β-CD is reported. In common with the studied CGTase, the majority of the CGTase reported present stimulated activity in the presence of Ca^{2+} that also has been reported to be a stabilizer of thermal denaturation *(11,12,21,30,35,38)*.

Conclusions

The CGTase from new producer *P. campinasensis* strain H69-3 isolated from soil cassava crop was purified and characterized with molecular weight of 70 kDa, optimum pH at 6.5, and optimum temperature at 65°C. So, this bacteria *P. campinasensis* strain H69-3 proved to be a viable alternative microorganism for CGTase production. The moderate optimum temperature and thermostability of the purified CGTase would be advantageous in certain industrial applications.

Acknowledgments

The authors are grateful to the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP) for its financial support. Acknowledgement is due to Dr. Peter James Harris for English-language revision of the text of this article.

References

1. Henrissat, B. (1991), *Biochem. J.* **280,** 309–316.
2. Bender, H. (1986), *Adv. Biotechnol. Process* **6,** 31–71.
3. Szejtli, J. (1997), *J. Mater. Chem.* **7,** 575–587.
4. Tonkova, A. (1998), *Enzyme Microbial. Technol.* **22,** 678–686.
5. Szejtli, J. (1998), *Chem. Rev.* **98,** 1743–1754.
6. Martin del Valle, E. M. (2004), *Process Biochem.* **39,** 1033–1046.
7. Allegre, M. and Deratani, A. (1994), *J. Agroo Food Ind. Hi Technol.* January/February, 9–17.
8. Alves-Prado, H. F., Gomes, E., and DaSilva, R. (2002), *Bol. SBCTA*. **36,** 43–54.
9. Bender, H. (1985), *Carbohydr. Res*. **135,** 291–302.
10. Gawande, B. N. and Patkar, A. Y. (2001), *Enzyme Microbial. Technol.* **28,** 9–10.
11. Mori, S., Hirose, S., Oya, T., and Kitahata, S. (1994), *Biosci. Biotechnol. Biochem.* **58,** 1968–1972.
12. Larsen, K. L., Duedhal-Olisen, L., Christensen, H. J. S., Mathiesen, F., Pedersen, L. H., and Zimmermann, W. (1998), *Carbohydr. Res.* **310,** 211–219.
13. Doukyu, N., Kuwahara, H., and Aono, R. (2003), *Biosci. Biotechnol. Biochem.* **67,** 334–340.
14. Kaulpiboon, J. and Pongsawasdi, P. (2003), *J. Biochem. Mol. Biol.* **36,** 409–416.
15. Alves-Prado, H. F., Gomes, E., and DaSilva, R. (2006), *Appl. Biochem. Biotechnol.* **132–136**.
16. Starnes, R. L. (1990), *Cereal Foods World* **35,** 1094–1099.
17. Zamost, B. L., Nilsen, H. K., and Starnes, R. L. (1991), *J. Ind. Microbiol.* **8,** 71–82.
18. Wind, R. D., Libl, W., Buitelaar, R. M., et al. (1995), *Appl. Environ. Microbiol.* **61,** 1257–1265.
19. Tachibana, Y., Kuramura, A., Shirasaka, N., et al. (1997), *Appl. Environ. Microbiol.* **65,** 1991–1997.
20. Abelyan, V. A., Afyan, K. B., Avakyan, Z. G., Melkumyan, A. G., and Afrikyan, E. G. (1995), *Biochemistry* **60,** 1223–1229.
21. Kimura, M. (1980), *J. Mol. Evol.* **16,** 111–120.
22. Saitou, N. and Nei, M. (1987), *Mol. Biol. Evol*. **4,** 406–425.
23. Mäkelä, M. J., Korpela, T. K., Puisto, J., and Laakso, S. V. (1988), *Agric. Food Chem.* **36,** 83–88.
24. Hartree, E. F. (1972), *Anal. Biochem*. **48,** 422–427.
25. Nakamura, N. and Horikoshi, K. (1976), *Agric. Biol. Chem.* **40,** 1785–1791.
26. Laemmli, U. K. (1970), *Nature* **27,** 680–685.

27. Blum, H., Bier, H., and Gross, H. J. (1987), *Eletrophoresis* **8,** 93–99.
28. Yoon, J. -H., Yim, D. K., Lee, J. -S., et al. (1998), *Int. J. System. Bacteriol.* **48,** 833–837.
29. Fujita, Y., Tsubouchi, H., Inagi, Y., Tomita, K., Ozaki, A., and Nakanishi, K. (1990), *J. Ferment. Bioeng*. **70,** 150–154.
30. Yim, D. G., Sato, H. H., Park, Y. H. E., and Park, Y. K. (1997), *J. Ind. Microbiol. Biotechnol.* **18,** 402–405.
31. Sian, H. K., Said, M., Hassan, O., et al. (2005), *Process Biochem.* **40,** 1101–1111.
32. Sabioni, J. G. and Park, Y. K. (1992), *Starch/Stärke* **44,** 225–229.
33. Akimura, K., Yagi, T., and Yamamoto, S. (1991), *J. Fermen. Bioeng*. **71,** 322–328.
34. Abelyan, V. A., Avakyan, A. G., Melkumyan, A. G., Balayan, A. M., Uzunyan, L. V., and Gasparyan, A. V. (1992), *Biochem.* **57,** 285–291.
35. Martins, R. F. and Hatti-Kaul, R. (2002), *Enzyme Microbial Technol.* **30,** 116–124.
36. Aoki, H., Yu, E. K. C., and Misawa, M. (1988), Novel Cyclodextrin Glycosyltransferase and its use in Manufacturing Cyclodextrin. US Patent 87-35952, 27p.
37. Chung, H. J., Yoon, S. H., Lee, M. J., et al. (1998), *J. Agric. Food Chem.* **46,** 952–959.
38. Tomita, K., Kaneda, M., Kawamura, K., and Nakanishi, K. (1993), *J. Fermen. Bioeng.* **75,** 89–92.
39. Bovetto, L. J., Backer, D. P., Villette, J. R., Sicard, P. J., and Bouquelet, S. J. -L. (1992), *Biotechnol. Appl. Biochem.* **15,** 48–58.
40. Janecek, S. (2002) *Biologia. Bratislava.* **57,** 29–41.
41. Gawande, B. N., Goel, A., Patkar, A. Y., and Nene, S. N. (1999), *Appl. Microbiol. Biotechnol.* **51,** 504–509.
42. Higuti, I. H., Grande, S. W., Sacco, R., and Nascimento, A. J. (2003), *Brazilian Arch. Biol. Technol.* **46,** 183–186.
43. Vieille, C. and Zeikus, G. J. (2001), *Microbiol. Mol. Biol. Rev.* **65,** 1–43.