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Purification and Properties of a New Thermostable Cyclodextrin Glucanotransferase from *Bacillus pseudalcaliphilus* 8SB

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Abstract A new cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) from an alkaliphilic halotolerant *Bacillus pseudalcaliphilus* 8SB was studied in respect to its γ-cyclizing activity. An efficient conversion of a raw corn starch into only two types of cyclodextrins (β- and γ-CD) was achieved by the purified enzyme. Crude enzyme obtained by ultrafiltration was purified up to fivefold by starch adsorption with a recovery of 62% activity. The enzyme was a monomer with a molecular mass 71 kDa estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE. The CGTase exhibited two pH optima, at pH 6.0 and 8.0, and was at most active at 60 °C and pH 8.0. The enzyme retained more than 80% of its initial activity in a wide pH range, from 5.0 to 11.0. The CGTase was strongly inhibited by 15 mM Cu^{2+}, Fe^{2+}, Ag^{+}, and Zn^{2+}, while some metal ions, such as Ca^{2+}, Na^{+}, K^{+}, and Mo^{7+}, exerted a stimulating effect in concentration of 5 mM. The important feature of the studied CGTase was its high thermal stability: the enzyme retained almost 100% of its initial activity after 2 h of heating at 40–60 °C; its half-life was 2 h at 70 °C in the presence of 5 mM Ca^{2+}. The achieved 50.7% conversion of raw corn starch into 81.6% β- and 18.4% γ-CDs after 24 h enzyme reaction at 60 °C and pH 8.0 makes *B. pseudalcaliphilus* 8SB CGTase industrially important enzyme for cyclodextrin production.

Keywords Cyclodextrin glucanotransferase · Starch conversion · Cyclodextrins · *Bacillus pseudalcaliphilus* · Halotolerant alkaliphilic bacilli

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Introduction

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is a unique enzyme with the potential for environmental protection, food, chemical, and pharmaceutical industries because of its ability to form cyclodextrins (CDs) from starch and related α-1,4-glucans by the cyclization reaction. The enzymatic product is usually a mixture of cyclodextrins, including mainly α-, β-, and γ-cyclodextrin consisting of six, seven, and eight glucose units, respectively, and trace amount of large-ring cyclodextrins [1].

Cyclodextrins are cyclic oligosaccharides composed of 6–60 glucose units [2], possessing a hydrophilic outside and hydrophobic central cavity. CDs can encapsulate various organic and inorganic compounds in their hydrophobic internal cavities and can greatly modify the physical and chemical properties of the guest molecule. This, their property, has resulted in the growing importance of the applications of CDs: in food, pharmaceutical, agriculture, and chromatographic techniques [3]; in environmental protection for removal of highly toxic substances from soil, water, and atmosphere [4]; in food industry for removal of cholesterol from eggs and dairy products and of phenolic compounds from fruits and vegetable juices [5], and for aflatoxin detection in food samples [6]; in chemical industry for the synthesis of organic nano-polymers [7]; and in pharmaceutical industry for increase of the aqueous solubility, bioavailability, and stability of the different drugs and also for reduction or elimination of unpleasant smells or tastes or to convert oils and liquid drugs into microcrystalline powders [8].

Compared to α- and β-cyclodextrins, γ-cyclodextrin possesses a larger internal cavity and can accommodate a wider variety of large organic compounds such as macrocycles and steroids [3]; it has higher water solubility and more bioavailability which makes it preferable for some applications in the food and pharmaceutical industries.

To date, most CGTases studied are characterized as α- and β-CGTases and only few as γ-CGTase [1, 9–11]. The market share of γ-cyclodextrin is considerably small because of its low yield and high price. Therefore, finding a producer that generates a large amount of γ-cyclodextrin is of great industrial interest.

Recently, we have reported the isolation of two new CGTase producers from Bulgarian habitats. One of them is a halotolerant obligate alkaliphile, identified as *Bacillus pseudalcaliphilus* 8SB growing at pH 9.0–11.0, 40 °C, in the presence of 2–10% NaCl [12]. *B. pseudalcaliphilus* 8SB CGTase converted starch into β- and γ-CDs and produced γ-CD efficiently. The established high values of γ-cyclizing activity compared to those of the known γ-CGTases without any manipulations of the strain or the enzyme reaction were the reason to perform all analyses in respect to γ-cyclizing activity. Having in mind the great interest to CGTases generating γ-cyclodextrin, the present work reports the obtaining of a thermostable CGTase in relation to its γ-cyclizing activity from the halotolerant alkaliphilic *B. pseudalcaliphilus* 8SB and its application for cyclodextrin production from raw corn starch.

Materials and Methods

Materials

Peptone and yeast extract were products of Oxoid (Basingstoke, UK). Soluble starch (according to Zullkowsky) for CGTase assay was purchased from Fluka (Germany). Gamma-

cyclodextrin for a standard curve and Bromocresol green (BCG) were purchased from AppliChem GmbH, Germany. Protein marker for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE was a product from BioLabs (New England, P7702S). All other chemicals used were reagent grade. A commercial insoluble corn starch (Bulgaria) was used for a cyclodextrin production by the purified enzyme.

Microorganism and Cultivation

Strain *Bacillus* sp. 8SB was isolated from a sample containing mineral water and soil from Sapareva Bania region, Bulgaria and identified as *B. pseudalcaliphilus* according to the 16S rRNA gene sequence (deposited to the NCBI GenBank database under accession number EF 589780) as well as the phenotypic properties [12]. The strain was deposited to the National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria under number 8703. The seed and production media were with the same composition: soluble starch, 2 $g l^{-1}$; peptone, 5 $g l^{-1}$; yeast extract, 5 $g l^{-1}$; $MgSO_4$, 0.2 $g l^{-1}$; and K_2HPO_4, 1 $g l^{-1}$. Sterile sodium carbonate was added to adjust the medium to pH 9.8–10.0 after autoclaving. The strain was cultivated overnight in 100 ml alkaline medium on a shaker (New Brunswick, USA, 240 rpm) at 40 °C. This culture was then inoculated into 500-ml flask containing 100 ml of the same medium (2%, *v*/*v*; OD_{650nm} 1.5–1.7) and cultivated at 40 °C and 240 rpm for 24 h. The bacterial cells were removed by centrifugation at 5,000×*g*, 4 °C for 30 min, and the clear supernatant was used for obtaining the purified enzyme.

Enzyme Purification

The CGTase was concentrated and partially purified from a cell-free culture supernatant by ultrafiltration using Millipore Ultrafiltration System (Bedford, MA, USA) and polyethersulfone membrane PBTK, 30 kDa (Millipore, MA, USA). The concentrate obtained was used as a crude enzyme. The further purification step was adsorption to the insoluble corn starch (commercial product, Bulgaria). Insoluble corn starch (10%, *w*/*v*) and 30% (*w*/*v*) ammonium sulfate in respect to 0.65 mg ml^{-1} protein content of the crude enzyme were added. The procedure was extended 1 h at 8 °C under a continuous agitation to allow enzyme adsorption. The mixture was centrifuged at 4,500×*g* for 10 min, and the residue was washed twice with cold water. In order to separate the enzyme from starch, the residue was incubated with phosphate buffer (67 mM, pH 6.0) containing 1 mM β-CD for 30 min at 37 °C with shaking followed by centrifugation. The obtained eluate 1 was separated, and the second elution with the same buffer was repeated once (eluate 2). Both eluates were mixed and concentrated by using of Millipore stirred ultrafiltration cell (model 8050) and polyethersulfone membrane PBTK, 30 kDa (Millipore, MA, USA).The protein concentration was determined based on the Bradford method [13] using bovine serum albumin as a standard, and the enzyme purity was assessed by SDS-PAGE and native PAGE.

Enzyme Assay

Gamma-CGTase cyclizing activity was determined by the method of Kato and Horikoshi [14] with some modifications, as follows. A reaction mixture comprising 450 μl of 4% (*w*/*v*) soluble starch (Fluka) in 67 mM phosphate buffer (pH 8.0) and 50 μl of the enzyme was incubated at 60 °C for 20 min. The reaction was terminated by the addition of 500 μl

0.1 N HCl and then 100 μl of 5 mM BCG in 20% (*v*/*v*) ethanol was added. The reaction mixture was incubated at room temperature for 20 min to facilitate the formation of inclusion complex between γ-CD and BCG. After incubation, 2 ml citrate buffer (pH 4.2; 0.1 M citric acid and 0.2 M di-sodium hydrogen phosphate) was added. The amount of γ-CD in the mixture was determined spectrophotometrically by measuring the absorbance at 630 nm against the blank in which the sample was substituted by water. One unit of γ-CGTase activity was defined as the amount of enzyme that formed 1 μmol of γ-CD per minute under these conditions.

Enzyme Characterization

The SDS-PAGE was performed according to Laemmli [15] on a vertical slab polyacrylamide gel 10% (*w*/*v*) using electrophoresis apparatus SCIE-PLAS TV 100, UK. The sample was onefold diluted by a sample buffer containing 40 mM Tris–HCl (pH 6.8), glycerol 4% (*w*/*v*), SDS 2% (*w*/*v*), 2 mM β-mercaptoethanol 0.01% (*v*/*v*), and bromophenol blue 0.01% (*w*/*v*) and boiled for 5 min. Protein marker, broad range (2–212 kDa) from BioLabs, containing myosin (212 kDa), MBP-β-galactosidase (158 kDa), β-galactosidase (116 kDa), phosphorylase b (97.2 kDa), serum albumin bovine (66.4 kDa), glutamic dehydrogenase (55.6 kDa), MBP2 (42.7 kDa), thioredoxin reductase (34.6 kDa), triosephosphate isomerase (27 kDa), trypsin inhibitor (20 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa), was used as standard. The migration was realized under 100 V tension and 20 mA current intensity by a generator (LKB, BROMMA, Sweden) during 2.5 h. Proteins were stained 15 min in a 0.1% Coomassie brilliant blue R250 (ethanol/acetic acid/distilled water). PAGE of the native (non-denaturated) enzyme was performed using the same procedure as for SDS-PAGE but without heat of the sample and use of distilled water instead of SDS solution.

The optimum pH and stability of the purified enzyme was studied over a pH range of 4.0–11.0 at 60 °C. The investigation of the pH stability was performed by pre-incubation of the enzyme for 1 and 2 h at 25 °C in different buffers, and the residual activity was determined under standard assay conditions (phosphate buffer pH 8.0; 60 °C; 20 min reaction time). The following buffers were used: 0.1 M Na-acetate–CH_3COOH (pH 4.0–5.0), 1/15 M potassium–sodium phosphate (pH 6.0–8.0), and 0.1 M glycine–NaOH–NaCl (pH 9.0–11.0). The temperature profile and thermal stability of the enzyme were studied over the range 50–80 and 40–70 °C, respectively. The thermal stability was measured after pre-incubating the enzyme under different temperatures for 2 h, and then, the residual activity was determined under standard assay conditions (pH 8.0, 60 °C). The non-heated enzyme was considered as 100%. The effect of 5 and 15 mM various metal ions and reagents on enzyme activity was determined after pre-incubation at 25 °C for 1 and 2 h.

Cyclodextrin Quantification

The enzyme was incubated with 4% (*w*/*v*) insoluble raw corn starch (Bulgarian product) in phosphate buffer, pH 8.0 at 60 °C. The cyclodextrin formation was estimated after 24 h enzyme reaction without any additives. At defined time, the enzyme effect was stopped by placing the samples in boiling water. The quantity of cyclodextrins formed was analyzed by HPLC system Waters Alliance (RI Detector 2414) with YMC-Pack-ODS-AQ column (150×4.6 mm) at 30 °C. Samples were applied to the column after a membrane filtration (0.20 μm, Minisart). The mobile phase was methanol/water (7:93, *v*/*v*) with flow rate of 1.0 ml min^{-1}. Injection volume was 50 μl.

Results and Discussion

Purification of the γ-CGTase

The concentrated crude enzyme after ultrafiltration was purified 5.2-fold with a yield of 62.4% by starch adsorption (Table 1). The adsorption of crude enzyme was optimized by varying starch and ammonium sulfate concentrations (in respect to 0.65 mg ml^{-1} protein content) and by using of phosphate buffers with pH 6.0 or 8.0, containing β- or γ-CD for enzyme elution. The established optimal conditions for starch adsorption were 10% (*w*/*v*) insoluble corn starch, 30% (*w*/*v*) ammonium sulfate, and double elution with 67 mM phosphate buffer (pH 6.0) containing 1 mM β-CD.

The used strain *B. pseudalcaliphilus* 8SB is a halotolerant obligate alkaliphile, and by its cultivation in an alkaline medium containing 8% NaCl, almost pure enzyme in the culture liquid was produced. On that account, the ultrafiltration became needless and a direct adsorption of the centrifuged cell-free supernatant on starch was performed (Table 1). In this case, the purified enzyme was with a very high specific activity (92.3 Umg^{-1}) and the yield was approximately the same (65.2%) compared to the results obtained after cultivation of the strain in an alkaline medium without NaCl. It makes an impression that in both cases (use of a production medium with or without NaCl), the purified enzymes exhibited a high activity compared to other bacterial CGTases reported as γ-GTases (Table 2). The denoted CGTase activities in the Table 2 were determined under almost identical reaction conditions. Moreover, some γ-CGTases studied have been purified by several steps including ultrafiltration; starch adsorption; and DEAE-Sephadex, DEAE-Sepharose, DEAE-Cellulofine, and Octil-Cellulofine chromatographies, unlike the proposed two-step purification procedure for *B. pseudalcaliphilus* 8SB CGTase providing 62% enzyme yield, compared to other combinations of starch adsorption and ion exchange chromatography resulting in 3.8% or 26% recovery of the enzyme [11, 16].

Molecular Weight of the Enzyme

Both native and denaturated PAGE indicated a molecular weight of 71 kDa, and this observation suggests that the enzyme is a monomer (Fig. 1). Most of the reported γ-CGTases are with molecular weight between 64 and 78 kDa [10, 11, 17, 18].

Table 1 Purification of CGTase from *B. pseudalcaliphilus* 8SB

Purification step	Volume (ml)	Activity (U ml^{-1})	Protein (mg ml^{-1})	Specific activity (U mg^{-1} protein)	Fold purification	Yield (%)
Bacterial cultivation without NaCl						
Crude enzyme after ultrafiltration	70	5.54±0.08	0.60±0.02	9.2	1.0	100.0
Starch adsorption (eluate 1+eluate 2)	42	5.76±0.10	0.12±0.01	48.0	5.2	62.4
Bacterial cultivation in the presence of 8% NaCl						
Centrifuged culture liquid	40	2.55±0.14	0.09±0.005	28.3	1.0	100.0
Starch adsorption (eluate 1+eluate 2)	24	2.77±0.11	0.03±0.001	92.3	3.3	65.2

Table 2 Comparison of the activities of purified γ-CGTases

Producer	Specific γ-CGTase activity (U mg^{-1})	References
Bacillus clarkii 7364	5.41	[10]
Thermoanaerobacter sp. 501	131	[26]
Recombinant *Bacillus subtilis* KN2; CGTase825-6	5.57	[11]
Bacillus pseudalcaliphilus 8SB cultivated without NaCL	48.0	Present work
Bacillus pseudalcaliphilus 8SB cultivated with 8% NaCL	92.3	Present work

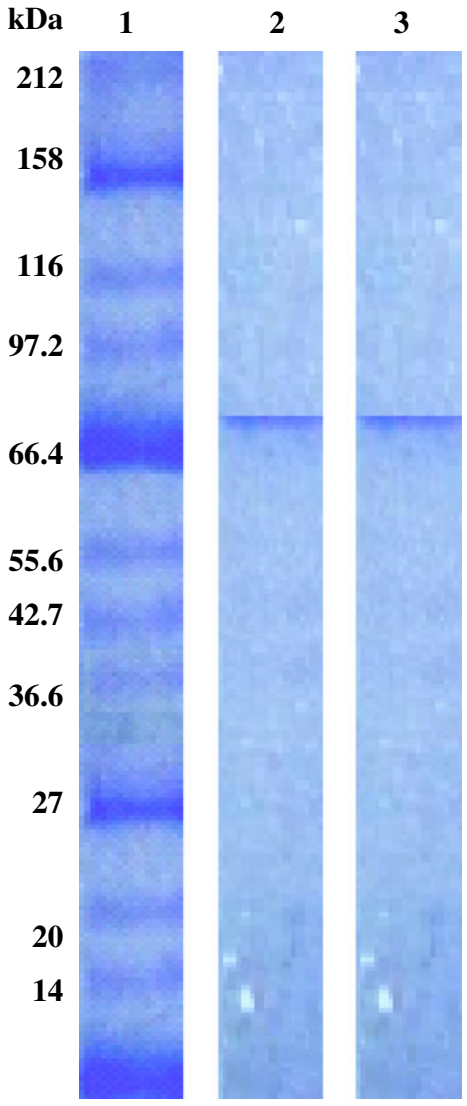

Fig. 1 SDS-PAGE and PAGE analyses of the purified CGTase from *B. pseudalcaliphilus* 8SB on a 10% acrylamide gel. *Lane 1*, protein marker; *lane 2*, native PAGE of the purified CGTase; *lane 3*, SDS-PAGE of the purified CGTase

Temperature and pH Profiles of the Enzyme

The temperature profile of the enzyme was studied under standard assay conditions at various temperatures and pH 6.0 (Fig. 2). The enzyme was optimally active at 60 °C. The optimum temperature was similar to that of the γ-CGTase from *Bacillus clarkii* 7364 [10] and higher than that from *Bacillus macorous* WSHO2-06 and *Bacillus* sp. G-825-6 [11, 18].

The effect of pH on γ-CGTase activity was established by determination of the enzyme activity at varying pH values ranging from 4.0 to 11.0 at 60 °C (Fig. 3). The purified enzyme exhibited two pH peaks at pH 6.0 and 8.0, similar to CGTase from alkaliphilic *Bacillus* sp. G-825-6 with pH optimum 8.0–10.0 [11] and *Bacillus* sp. AL-6 with pH optimum for γ-CD formation 7–10 [9] and different from those of *B. clarkii* 7364 with pH optima 10.5–11.0 [10].

Effect of Various Reagents on γ-CGTase Activity

The studied CGTase showed a significant stability in the presence of 5 mM various metal ions and reagents after 1 h pre-treatment (Table 3). The enzyme was strongly inhibited by 15 mM Cu^{2+}, Fe^{2+}, Ag^{+}, and Zn^{2+} after a 2-h pre-treatment. Some metal ions, such as Ca^{2+}, Na^{+}, K^{+}, and Mo^{7+}, exerted a stimulating effect in concentration of 5 mM. A similar inhibitory effect of Cu^{2+}, Fe^{2+}, and Zn^{2+} and a positive influence of Ca^{2+}, Na^{+}, and K^{+} have been reported for γ-CD formation of CGTase from *Bacillus* sp. AL-6 [9].

Temperature and pH Stability of the Enzyme

The enzyme retained more than 80% of its initial activity in a wide pH range, 5.0–11.0, after a 1-h pre-incubation in appropriate buffers (Fig. 4). The studied CGTase could be

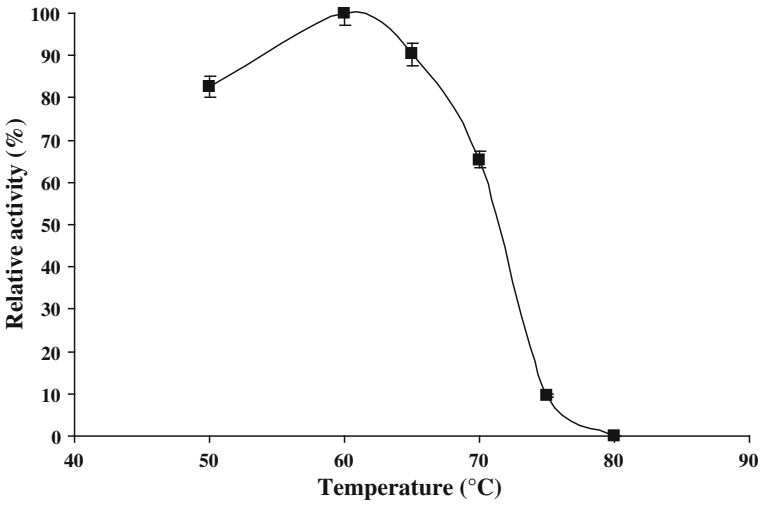

Fig. 2 Temperature profile of the purified CGTase from *B. pseudalcaliphilus* 8SB. The enzyme activity corresponding to 100% was 5.7 Uml^{-1}. Standard deviations are shown as *bars*

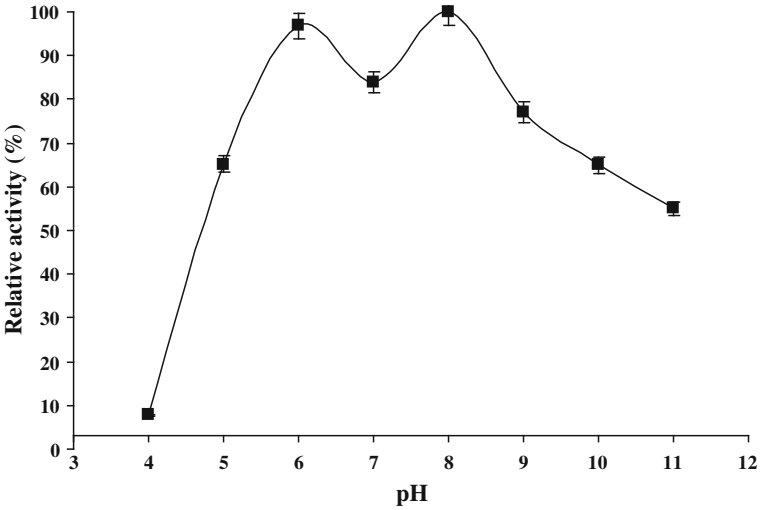

Fig. 3 pH profile of the purified CGTase from *B. pseudalcaliphilus* 8SB. The enzyme activity corresponding to 100% was 5.6 Uml^{-1}. Standard deviations are shown as *bars*

successfully applied in the denoted pH range unlike from other *Bacillus* γ-CGTases possessing more limited pH range for starch conversion, such as CGTase 825-6, stable in pH 7.0–12.0 after 30-min treatment [11], and γ-CGTase from *B. macorous* WSHO2-06,

Table 3 Effect of metal ions and chemical reagents on the γ-cyclizing activity of CGTase from *B. pseudalcaliphilus* 8SB

Metal ions and reagents	Residual activity (%)			
	5 mM ions or reagents		15 mM ions or reagents	
	1-h treatment	2-h treatment	1-h treatment	2-h treatment
None	100.0[a]	100.0	100.0	100.0
$CoCl_2$	90.3±0.26	80.4±0.30	50.0±0.45	50.0±0.40
$CaCl_2$	120.4±0.32	107.8±0.39	92.0±0.45	90.0±0.47
$ZnSO_4$	84.2±0.30	82.3±0.40	45.1±0.43	29.4±0.30
$FeSO_4$	93.9±0.47	93.9±0.71	27.4±0.40	0
$CuSO_4$	82.3±0.25	78.4±0.21	0	0
$MgSO_4$	98.0±0.20	98.0±0.47	66.5±0.45	66.5±0.26
NaCl	100.0±0.26	90.0±0.49	86.2±0.40	84.2±0.43
KCl	111.0±0.25	95.6±0.15	84.3±0.30	84.2±0.38
$AgNO_3$	70.8±0.25	49.0±0.20	17.7±0.36	15.7±0.25
$(NH_4)_6Mo_7O_{24}$	115.6±0.46	111.7±0.32	84.2±0.42	76.5±0.40
EDTA	93.9±0.10	93.9±0.10	84.2±0.26	82.3±0.25
NaN_3	111.7±0.36	103.9 ±0.43	92.0±0.26	92.0±0.26

[a] The enzyme activity corresponding to 100% was 5.65 Uml^{-1}

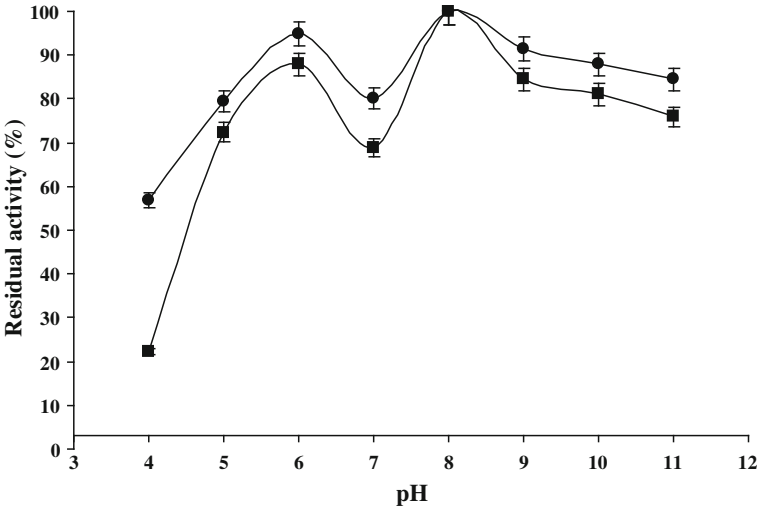

Fig. 4 pH stability of the purified CGTase from *B. pseudalcaliphilus* 8SB. The enzyme activity corresponding to 100% was 5.8 $U ml^{-1}$. Standard deviations are shown as *bars*; *solid circle*, after 1 h of pre-treatment; *solid square*, after 2 h of pre-treatment

stable in pH 5.0–8.0 [1]. The pH stability of the proposal CGTase was similar to that from *B. clarkii* 7364, completely stable over the pH range 6.0–11.0 [10].

The important feature of the new CGTase was its high thermal stability: the enzyme retained 92–95% of its initial activity after 2 h heating at 40–60 °C; its half-life was 2 h at 70 °C in the presence of 5 mM Ca^{2+} (Fig. 5). The reported known γ-CGTases retain its original activity upon heating to the temperatures 30–50 °C [1, 9–11, 17].

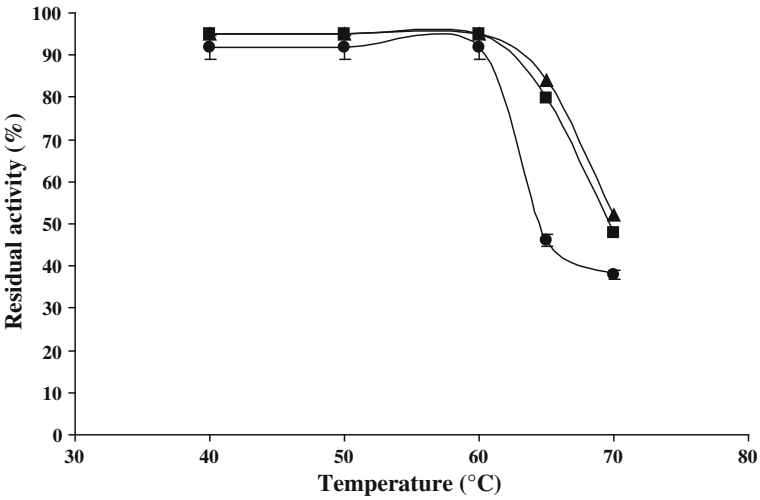

Fig. 5 Temperature stability of the purified CGTase from *B. pseudalcaliphilus* 8SB. The non-heated enzyme was considered as 100% (5.54 $U ml^{-1}$). Standard deviations are shown as bars; *solid circle*, enzyme; *solid square*, enzyme+5 mM Ca^{2+}; *solid triangle*, enzyme+5 mM Ca^{2}+1% starch

Cyclodextrin Production

The purified CGTase from *B. pseudalcaliphilus* 8SB converted a raw insoluble corn starch (4%, *w*/*v*) into only two types of CDs, β- and γ-CD (Table 4). The increase of the enzyme concentration led to enhanced yield of γ-CD and starch conversion. The new CGTase produced a relatively large amount of γ-cyclodextrin (18.4%) at 60 °C without any additives and converted 50.7% of a raw corn starch into cyclodextrins (16.573 mg ml^{-1} β-CD and 3.725 mg ml^{-1} γ-CD) after a 24-h starch hydrolysis in the presence of 6.0 U of enzyme per gram substrate.

The studied CGTase could be compared to γ-CGTase from *B. clarkii* 7364 converting 13.7% of pre-gelatinized potato starch [10] and γ-CGTase 825-6 producing primarily γ-CD at pH 10.0, namely 7.2 mg ml^{-1} γ-CD and 3.5 mg ml^{-1} β-CD from 10% soluble starch with conversion of 10.7% [11]. The advantage of the new CGTase was its effective action on raw insoluble starch (50.7% conversion) at high temperatures of 60–65 °C and a relatively high content of the formed γ-CD (3.7 mg ml^{-1}), unlike the most other studies where the high production of γ-CD has been achieved by using of special reaction conditions, such as a heat or pullulanase pre-treatment of the starch; addition of some additives such as glucose [19], glycyrrhzic acid [20], toluene, cyclododecanone [19], and ethanol [21, 22]; or by genetic manipulations [23, 24].

Conclusions

A new CGTase from an alkaliphilic halotolerant *B. pseudalcaliphilus* 8SB was studied in respect to its γ-cyclizing activity. The enzyme exhibits some distinct features valuable for an industrial starch conversion into cyclodextrins, such as a wide range of pH stability (5.0–11.0), a significant resistance to 5 mM various metal ions and reagents, and a high thermal stability (92–95% residual activity after 2 h heating at 40–60 °C; half-life 2 h at 70 °C in the presence of 5 mM Ca^{2+}). The last important feature of the studied enzyme arranges it among a few known CGTases stable at temperatures over the 60 °C. A majority of the reported γ-CGTases retain its original activity upon heating to the temperatures 30–50 °C [1, 9–11, 17]. The properties of the studied enzyme, important for CD production, as pH and thermal stability, were related to those of a thermoalkali-stable CGTase from the anaerobic thermoalkaliphilic bacterium *Anaerobranca gottschalkii* [25].

Table 4 Cyclodextrin production by the purified CGTase from *B. pseudalcaliphilus* 8SB after a 24-h enzyme reaction using 4% (*w*/*v*) insoluble raw corn starch

Enzyme concentration	β-CD		γ-CD		Starch conversion into CDs (%)
	mg ml^{-1}	%[a]	mg ml^{-1}	%[b]	
0.5 Ug^{-1} starch	0.287	90.0	0.032	10.0	0.8
1.0 Ug^{-1} starch	0.842	88.2	0.113	11.8	2.4
2.0 Ug^{-1} starch	5.229	85.5	0.887	14.5	15.3
3.5 Ug^{-1} starch	14.517	83.6	2.847	16.4	43.4
6.0 Ug^{-1} starch	16.573	81.6	3.725	18.4	50.7

[a] Quantity of β-cyclodextrin towards the total cyclodextrin content

[b] Quantity of γ-cyclodextrin towards the total cyclodextrin content

The ability of the new enzyme to convert insoluble raw corn starch into cyclodextrins and to generate a relatively large amount of γ-cyclodextrin could be of a great industrial interest. The achieved quantity of γ-CD without additives after a 24-h enzyme reaction could be significantly improved by cloning and extracellular expression of the *cgt* gene in a suitable host. These studies are in progress for obtaining of γ-CGTase with industrial important properties.

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