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ORIGINAL PAPER

Cyclodextrin glycosyltransferase: a key enzyme in the assimilation of starch by the halophilic archaeon *Haloferax mediterranei*

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Abstract A cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) was successfully isolated and characterized from the halophilic archaeon *Haloferax mediterranei*. The enzyme is a monomer with a molecular mass of 77 kDa and optimum activity at 55°C, pH 7.5 and 1.5 M NaCl. The enzyme displayed many activities related to the degradation and transformation of starch. Cyclization was found to be the predominant activity, yielding a mixture of cyclodextrins, mainly α-CD, followed by hydrolysis and to a lesser extent coupling and disproportionation activities. Gene encoding *H. mediterranei* CGTase was cloned and heterologously overexpressed. Sequence analysis revealed an open reading frame of 2142 bp that encodes a protein of 713 amino acids. The amino acid sequence displayed high homology with those belonging to the α-amylase family. The CGTase is secreted to the extracellular medium by the Tat pathway. Upstream of the CGTase gene, four maltose ABC transporter genes have been sequenced (*malE*, *malF*, *malG*, *malK*). The expression of the CGTase gene yielded a fully active CGTase with similar kinetic behavior to the wild-type enzyme. The *H. mediterranei* CGTase is the first halophilic archaeal CGTase characterized, sequenced and expressed.

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Introduction

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a member of the glycosyl hydrolases family 13 (Henrissat 1991; Bourne and Henrissat 2001; Cantarel et al. 2009). It includes a variety of enzymes which share the same mechanism of catalysis. This family 13 has been subdivided into 35 subfamilies on the basis of their sequences. The CGTases are classified within the subfamily GH13_2 (Stam et al. 2006). At the last review (July 2011) the database CAZy (Carbohydrate Active Enzymes, http://www.cazy.org) (Cantarel et al. 2009) included a total of 38 CGTases, of which only 4 have been described in the Domain *Archaea* and none of them belonging to a halophilic archaeon.

CGTase is the only one capable of catalyzing reversible inter- and intramolecular α-1,4 transglycosylation reactions from starch. The CGTase carries out four different activities: hydrolysis, cyclization, coupling and disproportionation (van der Veen et al. 2000). Of these, the cyclization reaction is the predominant, where an intramolecular transglycosylation occurs between a reducing end of a sugar molecule to a non-reducing end of the same chain to form a cyclic compound called cyclodextrin (CD). These CDs are cyclic non-reducing oligosaccharides consisting of 6 (α-CD), 7 (*β*-CD), 8 (*γ*-CD) or more glucose units linked by α-1,4 glycosidic bonds. All known CGTases produce a mixture of CDs in different concentration ratios depending on the microbial enzyme source, and are designated α-, *β*- or *γ*-CGTase depending on the relative levels produced (Qi and Zimmermann 2005).

The CDs molecules have a hydrophilic outer surface and a relatively hydrophobic cavity. Due to this particular structure, CDs are able to form inclusion complexes with a wide variety of compounds. Molecules encapsulated in CDs have beneficial changes in their chemical and physical properties. This property has been used to solubilize hydrophobic molecules in water or to stabilize volatile or labile substances (Singh et al. 2002). According to these features, the CDs are used in the food, cosmetic, pharmaceutical, agricultural and chemical industries (Lezcano et al. 2002; Szente and Szejtli 2004; Numanoglu et al. 2007; van de Manakker et al. 2009).

Many biotechnological processes including in the food, chemical and pharmaceutical industries, are carried out under extreme conditions (high temperatures, high salinity, high pressure or extremes of pH). So that in recent years, the production of enzymes that are stable and active under these conditions have attracted great interest. Due to their nature, the halophilic archaeon *H. mediterranei* could be used as a source of enzymes for industrial applications, as it is able to grow in a wide range of salt concentrations ($[Na^+]$ = 1.0–5.2 M) and temperatures (20–55°C) (Bowers and Wiegel 2011). Among these enzymes we find amylases, nucleases and proteases that are currently being used in different processes coming from other haloarchaeal microorganisms (Eichler 2001; Oren 2002). In this paper, we report the purification, characterization, sequencing and heterologous expression, of the CGTase from *H. mediterranei*.

Materials and methods

Enzyme production and purification of *H. mediterranei* CGTase

H. mediterranei strain R4 (ATCC 33500) was grown at 42°C to stationary phase in minimal medium with 25% (w/v) salts (Rodríguez-Valera et al. 1980), 1% (w/v) ammonium acetate, supplemented with 0.2% (w/v) soluble potato starch, and pH 7.2. Cells were harvested by centrifugation at 9000 rpm for 1 h at 4°C and supernatant was used as crude enzyme preparation.

All the purification steps were carried out at room temperature:

- Step 1: Tangential ultrafiltration. Supernatant was concentrated using VivaFlow 200 filtration system with a 30 kDa cut-off membrane (Vivascience).
- Step 2: β-CD-Sepharose 6B chromatography. This column was prepared by coupling β-CD (Sigma) to epoxy-activated Sepharose 6B (GE Healthcare). The column was equilibrated with buffer A (20 mM Tris–HCl, pH 7.5, 3 M NaCl, and 5 mM $CaCl_2$) and the concentrate from step 1 was subjected to chromatography. The column was washed with buffer A and protein elution was carried out with an increasing linear gradient of 0–3 mg/ml β-CD in buffer A at a flow rate of 30 ml/h. Fractions of 2 ml were collected and those containing amylolytic activity were combined. β-CD was then removed by filtration through a 30 kDa cut-off membrane (Millipore) to avoid interferences in the activity measurement methods.
- Step 3: Sephacryl S-200 chromatography. The fractions from the preceding step were loaded on a Sephacryl S-200 column (HiPrep 10/60 Ge Healthcare) equilibrated with buffer B (20 mM Tris–HCl, pH 7.5, 2 M NaCl, and 5 mM $CaCl_2$). The sample was eluted with the same buffer B at a flow rate of 0.2 ml/min and fractions of 2 ml were collected. Amylolytic activity was measured and the fractions were combined. To determine the molecular mass of the enzyme by gel filtration, the CGTase and protein standards were eluted in the presence of salt. The purified protein was stored at 4°C. The quantification of protein concentration was performed by Bradford method using bovine serum albumin as standard (Bradford 1976).

CGTase activity assay

The activity assays were performed by triplicate at 55°C for 20 min.

The cyclization activity was determined by different dyes. α-, β- and γ-CD form inclusion complexes with methyl orange, phenolphthalein and bromocresol green, respectively. The production of CDs was analyzed spectrophotometrically by the absorbance decreasing at 490 nm in the case of α-CD (Hirai et al. 1981) and 552 nm for β-CD (Penninga et al. 1995), and by the increase in absorbance at 630 nm for γ-CD (Kato and Horikoshi 1984). The reaction mixture contained potato starch solution 1% (w/v) in 0.1 M Bis-Tris propane, pH 7.0, 1.5 M NaCl buffer (buffer C). Standard curves were obtained using α-, β- and γ-CD (Sigma) at different concentrations. One unit of cyclization activity (U) is defined as the amount of enzyme that produces 1 µmol of α-, β- or γ-CD (depending on the assay carried out).

To determine the coupling activity, a protocol based on the use of α- or β-CD as donor and methyl-α-D-glucopyranoside as acceptor was used (Nakamura et al. 1994a). The γ-coupling activity cannot be quantified by this method because γ-CD interferes in the analysis (Alcalde et al. 2003). Reaction mixture contained 10 mM α-/β-cyclodextrin (according to the assay performed) and 400 mM methyl-α-D-glucopyranoside. The quantity of glucose produced was detected by the GOD-PAP reagent (Randox) and

determined by a glucose standard curve. One unit of α- or β-coupling activity (U) is defined as the amount of enzyme that catalyzes the disappearance of 1 μmol of α- or β-CD (as the test performed) per minute at the reaction conditions.

To establish the disproportionation activity we used maltose as an acceptor sugar and *p*-nitrophenyl-α-D-maltoheptaoside-4-6-O-ethylidene (EPS) (Randox) as donor sugar. This substrate is locked in its non-reducing end eliminating the possibility of acting as an acceptor. It also contains a *p*-nitrophenyl group at the reducing end to quantify the reaction. The release of *p*-nitrophenyl group occurred by the addition of 1 U of α-glucosidase (Randox) and it is determined by measuring the absorbance at 401 nm (Nakamura et al. 1994b). Reaction mixture contained 50 mM maltose and 22 mM EPS. One unit of disproportionation activity (U) is the amount of enzyme required to produce 1 μmol of *p*-nitrophenol per minute at the reaction conditions.

Hydrolysis activity was tested quantifying the amount of reducing sugars released by the action of CGTase on starch according to the DNS method of Miller (1959). The reaction mixture contained potato starch solution 1% (w/v) in buffer C. A standard curve was obtained using maltose at different concentrations. One unit of hydrolysis activity (U) was defined as the amount of enzyme that produces 1 μmol of reducing sugar per minute under the assay conditions.

Effect of salt concentration, pH and temperature on CGTase hydrolysis activity

The effect of salt concentration on enzyme activity was tested by measuring the activity at 50°C for 20 min in 20 mM Tris–HCl, pH 7.5, buffers containing different NaCl concentrations (0–4 M). For the test performed in the absence of salt, protein samples were previously dialyzed against 20 mM Tris–HCl, pH 7.5 buffer.

For pH studies the enzyme activity was measured at 50°C for 20 min using different buffers with 1.5 M NaCl: 0.1 M sodium citrate for pH from 4.5 to 6.5, 0.1 M Bis-Tris for pH from 6.0 to 7.0, and 0.1 M Bis-Tris propane for pH from 6.5 to 9.0.

The assays to study the effect of temperature on the CGTase activity were carried out in buffer C for 20 min at different temperatures (20–80°C).

Effect of salt concentration and temperature on CGTase stability

The stability of the CGTase at different temperatures and salt concentrations was determined by incubating the enzyme in buffers containing the NaCl concentrations studied (1.0, 2.0 and 3.0 M) at diverse temperatures (50, 60, 70 and 80°C). At different times aliquots were collected and cooled on ice, and their hydrolytic activities were measured and referred to the initial activity of the sample.

Effect of EDTA and metal ions on CGTase activity

Pure enzyme was incubated for 1 h at room temperature in the presence of increasing concentrations of the chelating agent EDTA (0.5–50 mM). The hydrolytic activity of the different samples was measured and compared with the initial activity. When the enzyme inactivation was achieved the sample was dialyzed against 20 mM Tris–HCl, pH 7.5, 2 M NaCl, buffer previously treated with Chelex (Sigma) to remove ions traces. The dialyzed sample was incubated for different times (15 min, 22 and 72 h) at room temperature in the presence of increasing concentrations of $CaCl_2$: from 0.5 to 10, 50, 100 and 200 mM. The hydrolytic activity was measured and compared with the initial activity. The same assay was performed to test the effect of other ions in the recovery of enzyme activity. The CGTase was also incubated with 10 mM $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$.

Substrate specificity

Different substrates were used to analyze the CGTase specificity: potato starch, pullulan, glycogen, α-CD, β-CD and γ-CD (Sigma). The reaction was carried out at 55°C for 20 h and the hydrolytic activity was measured.

Determination of kinetic parameters

Kinetic parameters V_{max} and K_m of the hydrolysis and β-cyclization reactions were calculated by incubating the enzyme at different potato starch concentrations (10–150 mM) in buffer C for 20 min at 55°C. The determination of kinetic parameters was carried out by fitting the data to a Lineweaver–Burk equation.

Analysis by nanoelectrospray LC/MS of the purified CGTase

Purified CGTase was dialyzed against 100 mM ammonium bicarbonate pH 8.5, at 4°C for at least 12 h and concentrated to 30 μg/ml. Lyophilized trypsin (Sigma) was reconstituted by dissolving in 1 mM HCl to a concentration of 1 mg/ml. The dialyzed protein was then digested with trypsin 1:20 (trypsin:enzyme) at 37°C for 18 h. The digested sample was lyophilized and sent to Agilent Technology (Germany) to be analyzed using a HPLC/nanoLC/Trap with a mobile phase of 0.1% (v/v) formic acid in water. The sample was processed using the SpectrumMill software with the NCBI database.

Preparation of *H. mediterranei* DNA and PCR amplification

DNA from *H. mediterranei* was obtained from an exponential phase culture as described by Ausubel et al. (1995). Degenerate primers were designed based upon amino acid sequences of peptides obtained after digestion of the purified CGTase with trypsin. The PCR fragment was sequenced and compared with database. This PCR fragment was labeled with digoxigenin to be used as a probe in the library screening of the gene.

Library screening and DNA sequencing

To find the CGTase gene a *H. mediterranei* genomic DNA library in λ phage was used. For library screening, plaque hybridization was performed using the PCR fragment labeled with digoxigenin as a probe. Positive λ phages were isolated after third screening and DNA was purified by "Lambda DNA Maxi Kit" (Qiagen). Nucleotide sequencing was performed by the method of *primer walking*. DNA and amino acid sequences were analyzed using the available bioinformatic software on the ExPASy server (Expertise Protein Analysis System) and the EMBOSS software package of EMBnet/CNB (European Molecular Biology Network).

Expression of the CGTase gene in *Escherichia coli*

The CGTase gene was amplified from the λ phage DNA using oligonucleotides designed including sequences corresponding to the amino and carboxyl terminus of the *H. mediterranei* CGTase and the restriction sites of *Nde*I and *Bam*HI, to direct cloning into the expression vector pET-3a (Novagen). The PCR fragment was inserted in the pSTBlue-1 vector (Novagen) and the resulting clone was transformed into *E. coli* Novablue (Novagen) cells. The recombinant plasmid (pSTBlue-1-CGTase) was digested with *Nde*I and *Bam*HI (Fermentas) and ligated into expression vector pET-3a. This recombinant plasmid (pET-3a-CGTase) was propagated in *E. coli* NovaBlue cells before to transform *E. coli* BL21 (DE3) (Novagen) cells used as expression host.

Cells of *E. coli* BL21(DE3) containing the recombinant plasmid pET-3a-CGTase were grown to an OD_{600} of 0.5–1 at 22 or 37°C in Luria–Bertani medium with 100 μg/ml ampicillin. The expression of the gene was induced by addition of 0.4 mM isopropyl-β-D-thiogalactoside (IPTG). The cellular fractions were isolated as described by Pire et al. (2001).

Purification and characterization of recombinant CGTase

Purification of recombinant CGTase was performed by a single chromatographic step at room temperature from the soluble fraction isolated before. This fraction was loaded on a Sepharose 6B-β-CD column which was developed as described for wild-type CGTase. The purified enzyme solution was stored at 4°C.

The recombinant enzyme was characterized as previously described for the wild-type CGTase.

Nucleotide sequences

The nucleotide sequences of the genes studied in this work were submitted to the GenBank database with the following accession numbers: AJ876899 (*CGTase*), AM411531 (*malE*), AM411532 (*malF*), AM411533 (*malG*) and AM411534 (*malK*).

Results

Purification of *H. mediterranei* CGTase

The enzyme was successfully purified to homogeneity through three chromatographic steps at room temperature. Data for this purification procedure are summarized in Table 1. The CGTase was purified about fivefold with a β-cyclization specific activity of 48 U/mg.

On the SDS-PAGE a single band of approximately 82 kDa was observed after the last chromatographic step (Fig. 1). This size is similar to those observed for other CGTases characterized in the *Archaea* Domain, such as *Pyrococcus furiosus* of 81 kDa (Lee et al. 2007) and

Table 1 Purification steps of CGTase from *H. mediterranei*

Purification step	Volume (ml)	Total units	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude supernatant	1000	310	30.5	10.2	100	1
Tangential ultrafiltration	50	102	5.0	20.5	31	2
β-CD-Sepharose 6B	30	37	1.3	28.0	12	3
Sephacryl S-200	15	7	0.2	48.0	3	5

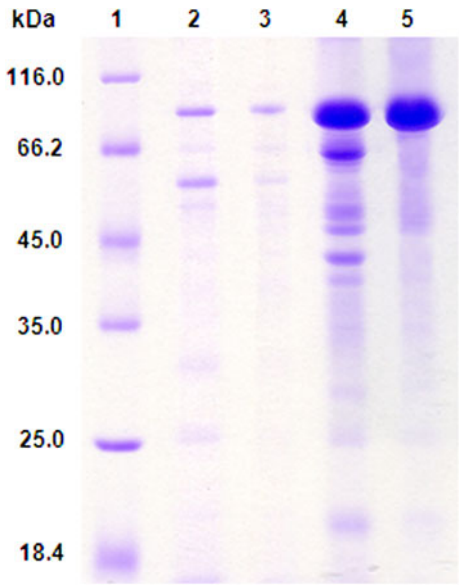

Fig. 1 SDS-PAGE of the purification of *H. mediterranei* CGTase. *Lane 1* molecular weight marker. *Lane 2* crude supernatant. *Lane 3* tangential ultrafiltration. *Lane 4* β-CD-Sepharose 6B. *Lane 5* Sephacryl S-200

Table 2 CGTase activities from *H. mediterranei*

Activity	(U/mg)
Hydrolysis	49.3
α-Cyclization	88
β-Cyclization	48
γ-Cyclization	34
α-Coupling	0.022
β-Coupling	0.49
Disproportionation	4.4

Pyrococcus kodakaraensis of 79 kDa (Rashid et al. 2002). However, it should be noted that SDS-PAGE overestimates the molecular weight of halophilic proteins as they migrate less due to the high content of negative amino acids on its surface (Lanyi 1974; Bonete et al. 1996; Madern et al. 2000).

Gel filtration chromatography in Sephacryl S-200 was also performed to determine the molecular mass of this protein. In the case of *H. mediterranei*, the protein eluted at a volume larger than that expected for its size, indicating that the protein presents some interaction with the column. This behavior has also been observed in the α-amylase characterized in *Haloarcula hispanica* (Hutcheon et al. 2005). The molecular mass estimated by this method was 17 ± 2 kDa.

CGTase activity assays

In relation to the four activities that characterize this type of enzyme, the cyclization is the predominant activity followed by hydrolysis, while the coupling and disproportionation activities are very low in *H. mediterranei* CGTase (Table 2). CGTases from other sources are reported to produce a mixture of α-, β- and γ-CD (Qi and Zimmermann 2005). The halophilic CGTase produces mainly α-CD, followed by β-CD and γ-CD, with a ratio determined by spectrophotometric assays of 1:0.6:0.3, respectively.

Effect of salt concentration, pH and temperature on CGTase hydrolysis activity

Salt concentration, pH and temperature, have a great influence on the enzyme activity, since they are factors that alter the tertiary and quaternary structure of proteins. The stability of a protein depends on its interaction with other proteins and molecules of water or salts included in the solution in which it is located. If a protein is halophilic, it will be more stable and active in a solution with high salt concentration (Madern et al. 2000). *H. mediterranei* CGTase shows its maximum hydrolysis activity in a buffer with 1.5 M NaCl and it keeps its activity even at low salt concentrations (Fig. 2a). The protein retains up to 65% of its activity at 0.5 M NaCl. A similar behavior can be observed in other halophilic proteins, as glucose dehydrogenase from *H. mediterranei* (Bonete et al. 1996).

The optimum pH value is in the range 6.5–7.0 (Fig. 2b). Over 60% of this activity is lost at pH below 5.0 and above 8.0. There is a great diversity in terms of pH optimum of CGTase because of the wide variety of microorganisms in which this enzyme has been isolated. Moreover, enzymes characterized from *H.mediterranei* also show optimal pH of 7–8, such as nitrite reductase (Martínez-Espinosa et al. 2001) or glucose dehydrogenase (Pire 1998).

At pH 7.0 the optimal temperature is 55°C (Fig. 2c). At temperatures of 35 and 60°C the enzyme retains 50% of its activity. This high optimal temperature is a common feature in enzymes from halophilic microorganisms and is often accompanied by a high thermostability.

Effect of salt concentration and temperature on CGTase stability

Thermostability studies were carried out by incubating the enzyme at diverse temperatures in different salt concentration. Generally, at low salt concentration halophilic proteins are less stable, and high temperatures can contribute to their destabilization under these conditions. At high salt concentration halophilic proteins are stable, however, their stability can be perturbed through several factors. *H. mediterranei* CGTase was quite stable, requiring temperatures of 70 and 80°C to denature independently of salt concentration (Fig. 3). The protein presents higher

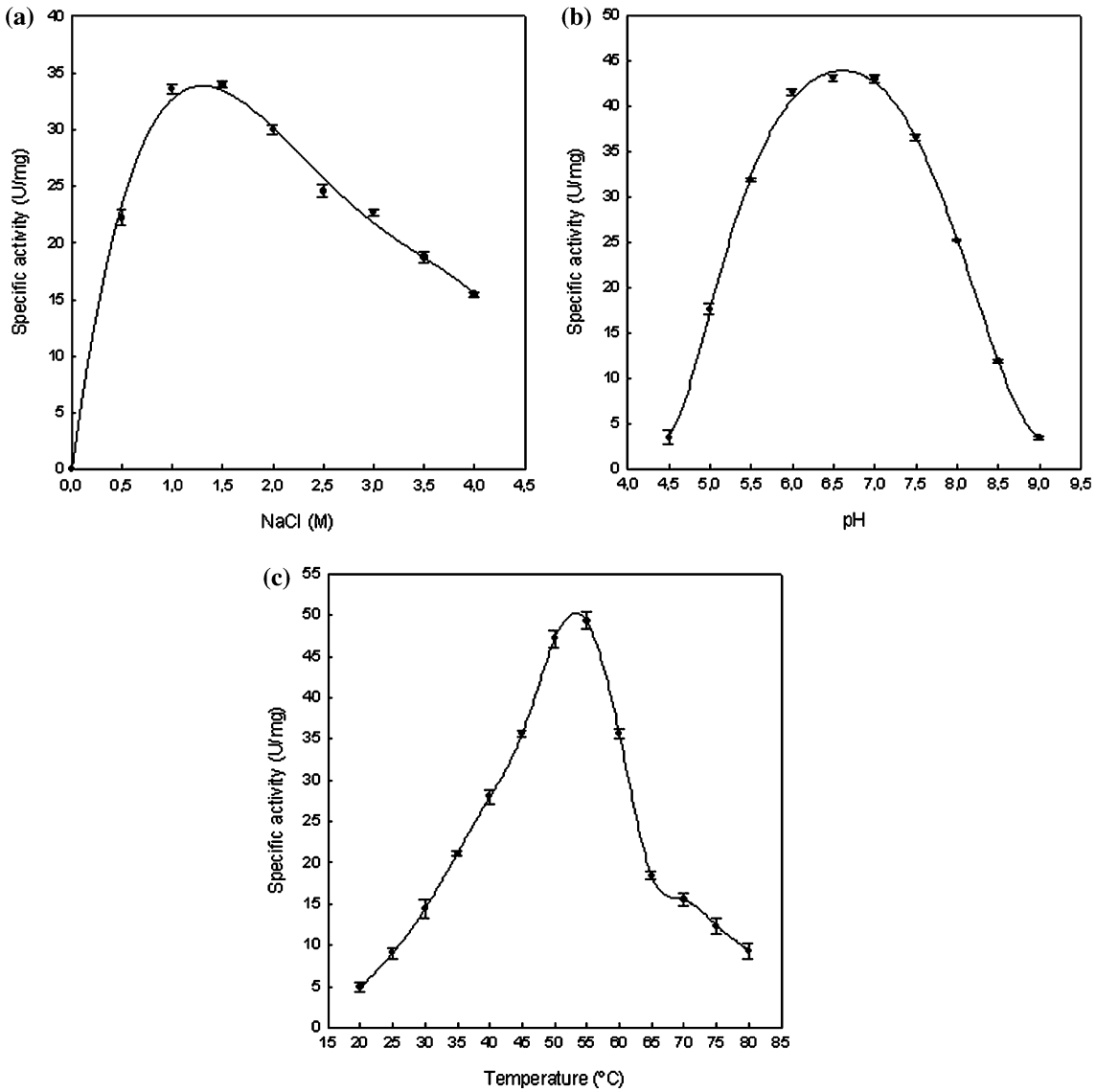

Fig. 2 Influence of salt concentration (**a**), pH (**b**) and temperature (**c**) on *H. mediterranei* CGTase activity

thermostability with increasing concentration of NaCl in the buffer. At a temperature of 60°C the half-life time of the enzyme is increased 15-fold from 1 to 3 M NaCl. At 50°C the differences between the half-life times of the enzyme are not very significant because proteins are stable for weeks in these conditions. This pattern is also observed in other halophilic enzymes studied by our research group (Bonete et al. 2007).

Effect of EDTA and metal ions on CGTase activity

To check whether the enzyme requires metal ions that can participate in its stability or activity as occurs in enzymes belonging to the α-amylase family (van der Maarel et al. 2002), the protein was incubated for 1 h at room temperature in presence of increasing concentrations of EDTA. The results show that the enzyme is completely inactive at concentrations of 10 mM EDTA, as in the CGTase of *P. kodakaraensis* in which the activity decreases by 70% in the presence of this concentration of EDTA (Rashid et al. 2002). When the loss of activity was attained, the recovery of its activity at different $CaCl_2$ concentrations for different times was evaluated. The best results were obtained at a concentration of 10 mM $CaCl_2$ after 72 h, although in that conditions the CGTase could only recover 19% of its activity. The recovered activity decreased at higher concentrations of $CaCl_2$, obtaining only 10% at 50 mM and hardly recovered activity at 200 mM. This same behavior has been observed in the CGTase of *P. kodakaraensis* (Rashid et al. 2002) and *H. mediterranei* α-amylase (Pérez-Pomares et al. 2003). In the same way, recovery of CGTase activity was determined by the addition of $MgCl_2$, $MnCl_2$,

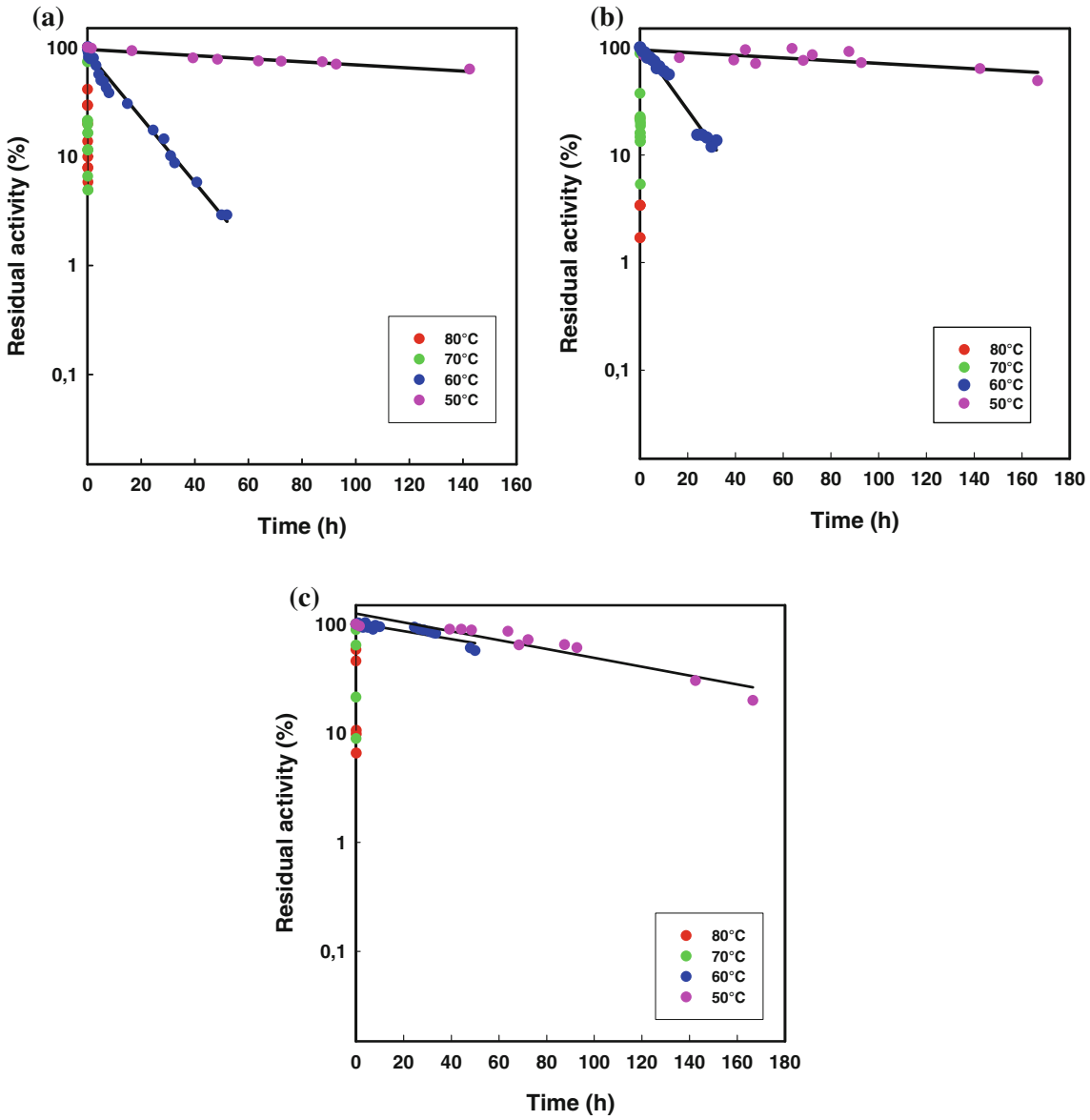

Fig. 3 Thermostability of *H. mediterranei* CGTase at a concentration of 1 M NaCl (**a**), 2 M NaCl (**b**) and 3 M NaCl (**c**)

$NiCl_2$ and $ZnCl_2$ to a concentration of 10 mM for 72 h. With $MnCl_2$ and $MgCl_2$ the recovered activity was 4 and 3%, respectively, and no activity was recovered in the presence of $NiCl_2$ and $ZnCl_2$.

The data show that this CGTase specifically requires calcium ion for stability and/or activity as in enzymes belonging to the α-amylase family (MacGregor et al. 2001; van der Maarel et al. 2002).

Substrate specificity

The results are shown in the Table 3, the highest hydrolysis activity was obtained using potato starch as substrate. Among the cyclodextrins, the CGTase shows greater activity with γ-CD, because of the large number of units of glucose present in its structure. The low activity obtained using pullulan as substrate confirms that the enzyme is not

Table 3 Hydrolysis activities of *H. mediterranei* CGTase on various substrates

Substrate	Activity (U/mg)	Relative activity (%)
Soluble starch	73.0	100
Pullulan	4.6	6
Glycogen	17.5	24
α-CD	1.3	2
β-CD	7.5	10
γ-CD	20.6	28

a pullulanase because it is not capable of hydrolyzing α-1,6 glycosidic linkages present in this substrate.

Determination of kinetic parameters

Kinetic parameters were obtained using potato starch as substrate and determining the activity of hydrolysis and cyclization to β-CD. The V_{max} and K_m values obtained were 106 ± 4 U/mg and 14.3 ± 0.8 g/l, respectively, with hydrolysis activity, and 161 ± 5 U/mg and 17.2 ± 0.6 g/l, respectively, with β-cyclization activity. The kinetic constants determined for both activities are of the same order as those obtained for other CGTase characterized (Martins and Hatti-Kaul 2002). The comparison between the constants determined for each of the activities shows that the results obtained for the cyclization activity of β-CD are slightly higher than those obtained for the activity of hydrolysis.

Analysis by nanoelectrospray LC/MS of the purified CGTase

Nanoelectrospray analysis by LC/MS of purified *H. mediterranei* CGTase has confirmed that the enzyme was a cyclomaltodextrin glucanotransferase. Peptides obtained were compared with databases and those which had higher homology with enzymes belonging to the α-amylase family, were used to search for the gene sequence in a phage λ genomic library of *H. mediterranei*.

Library screening and DNA sequencing

PCR product of approximately 200 bp was successfully amplified from *H. mediterranei* genomic DNA as described in "Materials and methods". The amino acid sequence deduced from the nucleotide sequence of the PCR product exhibited high similarity with enzymes belonging to α-amylase family. Therefore, this PCR fragment was labeled with digoxigenin to be used as a probe in the library screening of the gene.

DNA was isolated from one positive phage and nucleotide sequencing was performed by the method of *primer walking*. With this technique a region of 7898 bp was sequenced. This region corresponds to five open reading frames that encoded five different proteins related to starch metabolism and maltose transport (Fig. 4). In the second frame, in 3′-5′ direction *malK* gene was identified as maltose ABC transporter ATP-binding protein. In the first frame in 5′-3′ direction *malE* and *malF* genes were identified as maltose ABC transporter maltose-binding protein and maltose ABC transporter permease, respectively. In the second frame in 5′-3′ direction *malG* gene was identified as the second maltose ABC transporter permease. And finally, the *cgt* gene again in the first frame in 5′-3′ direction was identified as a CGTase.

Three putative TATA box signals and BRE sequences were identified in the sequenced region based on the consensus sequences for halophilic archaeal microorganisms (Palmer and Daniels 1995; Soppa 1999; Brenneis et al. 2007). The CGTase and the ATPase (*malK*) genes could be transcribed each one separately, while the maltose transporter genes (*malE*, *malF* and *malG*) could be transcribed as a single transcriptional unit. No obvious Shine–Dalgarno sequence was found in the regions immediately upstream from the genes.

Analysis of the CGTase sequence

A signal peptide was identified in the CGTase gene by the TATFIND program (Rose et al. 2002). This region preceding the N-terminus of the mature enzyme showed the characteristics of a typical protein that is translocated by the Tat pathway (twin-arginine translocation) with the consensus sequence S/T-R-R-X-F-L-K (X is any amino acid).

The molecular mass estimated from the deduced amino acid sequence of the mature CGTase was 77 kDa. This value was similar to the molecular mass estimated from the SDS-PAGE. This CGTase is rich in acidic amino acids with a 16% Asp and Glu content, which is also the case in other halophilic enzymes. This has been described as a mechanism of adaptation to hypersaline environments because the negative charges contributed to the solubility and stability of the proteins (Mevarech et al. 2000). The increased number of acidic amino acids results in a decrease in the content of Lys and aliphatic residues, and an increase in hydrophobic residues Gly, Ala and Val (Madern et al. 2000; Esclapez et al. 2005).

The alignment of the CGTase sequence with other homolog enzymes suggests that the enzyme from *H. mediterranei*

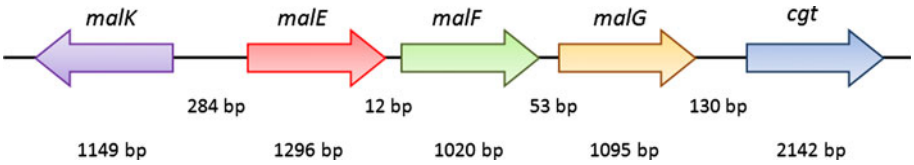

Fig. 4 Genomic organization of CGTase and maltose ABC transporter genes of *H. mediterranei*

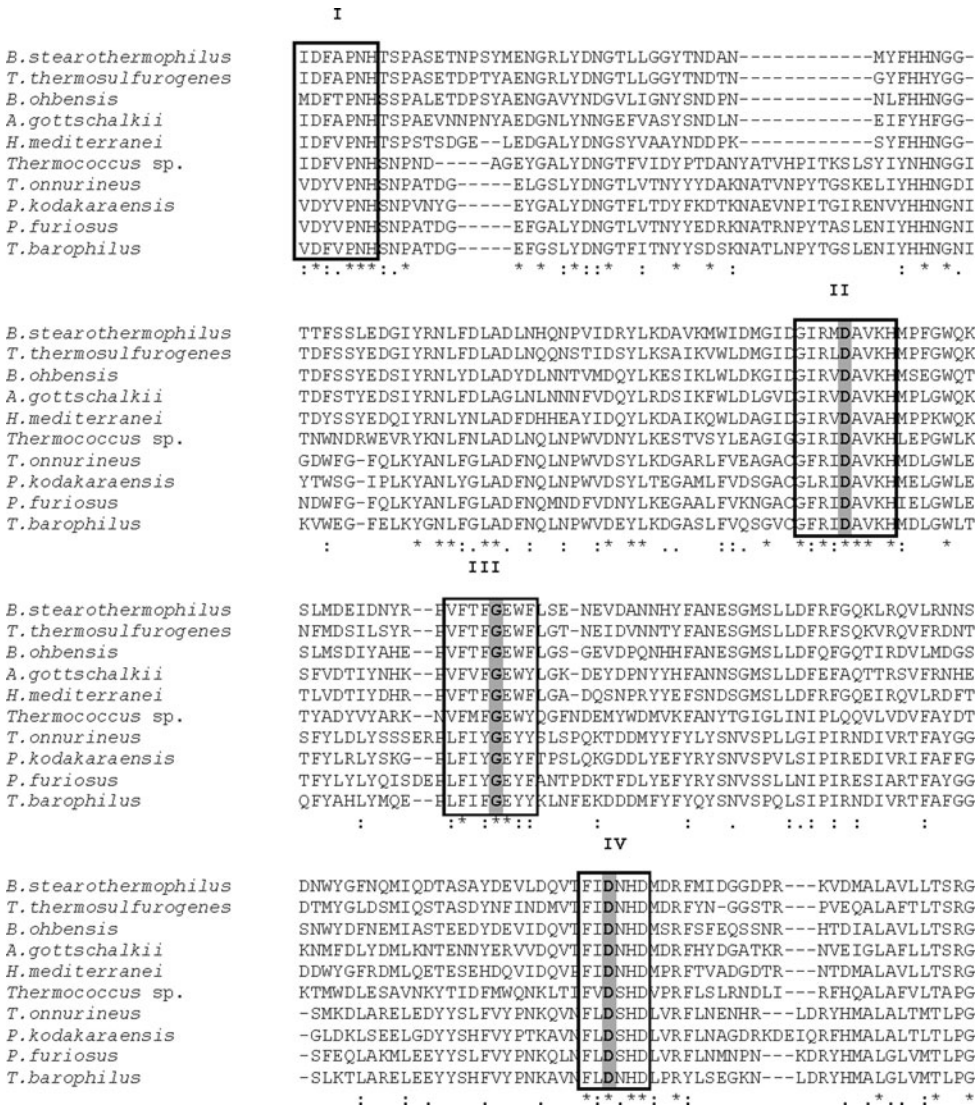

Fig. 5 Alignment of the amino acid sequence corresponding to the CGTase of *H. mediterranei* with other homologue enzymes from *Thermococcus* sp. (Q9UWN2), *Bacillus stearothermophilus* (P31797), *Thermoanaerobacter thermosulfurogenes* (P26827), *B. ohbensis* (P27036), *Anaerobranca gottschalkii* (Q5ZEQ7), *P. kodakaraensis* (Q5JHI5), *P. furiosus* (Q3HUR2), *Thermococcus onnurineus* (B6YXX8) and *T. barophilus* (B5IS54). Identical (*asterisks*), strongly similar (*colon*) and weakly similar amino acids (*dots*) are marked in the sequence. The four highly conserved regions are *boxed* and the catalytic residues are *shaded*

contains the four conserved regions that have been identified in the α-amylase family (MacGregor et al. 2001): XDXXXNH ($_{142}$IDFVPNH$_{148}$), GXRXDXXZZ ($_{231}$GIRVDAVAH$_{239}$), XXX(G/A)EZZZ ($_{259}$FTFGEWFL$_{266}$), and XXBBHD ($_{329}$FIDNHD$_{334}$); where X is usually a hydrophobic residue, B a hydrophilic residue and Z a residue important for the specificity of the enzyme (Fig. 5). Three of the four regions contain active site residues, Asp235 and Asp331 in regions II and IV, respectively, and Glu263 in region III. In addition, the regions I and IV also contain two conserved His.

The starch binding domain (SBD) in the *H. mediterranei* CGTase consists of 106 residues (from amino acid 583 to 688) of which 11 are strictly conserved (Svensson et al. 1989; Machovič and Janeček 2006):

Thr600 Gly603 Gly610 Leu615 Gly616 Trp618 Pro636 Trp638 Lys653 Trp665 Asn670

Four of these 11 residues, Trp618, Lys653, Trp665 and Asn670, are part of the first binding site of maltose, while Thr600, Gly603 and Trp638 are part of the second maltose binding site. The other residues, Gly610, Leu615, Gly616 and Pro636, are not directly involved in starch binding, but are probably necessary for structural support of this domain.

We have obtained a tertiary structure model of *H. mediterranei* CGTase by homology with the sequences from crystallized CGTases (PDB-1CYG, PDB-1UKQ, PDB-1PAM, PDB-1I75). In this model the five domains typical of this group of enzymes have been identified.

Expression of the CGTase gene in *E. coli*

The gene encoding the mature CGTase was ligated to pET-3a and the recombinant plasmid was used to transform *E. coli* BL21(DE3) cells as described in "Materials and methods". After 3 h of induction with IPTG at 37°C, all

fractions were isolated to localize the CGTase in *E. coli*. No obvious improvement in the expression level was seen at 22°C compared to 37°C. SDS-PAGE of the proteins of the induced culture revealed that the overexpressed protein was obtained mainly in the soluble cytoplasmatic fraction.

Purification and characterization of recombinant CGTase

The isolated soluble fraction was incubated for 5–6 h at room temperature to obtain fully active CGTase. During this incubation time, the activity was gradually recovered since the enzyme is in a medium with high salt concentration that promotes its correct folding and stability.

The purification was carried out by a single chromatographic step on β-CD-Sepharose 6B column at room temperature (Fig. 6). Data for this purification procedure are shown in Table 4. The recombinant CGTase was purified 40-fold with a β-cyclization specific activity of 50 U/mg. This specific activity was in the same order as that obtained in the purification of the wild-type CGTase. The yield greatly improved in the purification of the recombinant enzyme, with up to 13 times higher heterologous expression obtained.

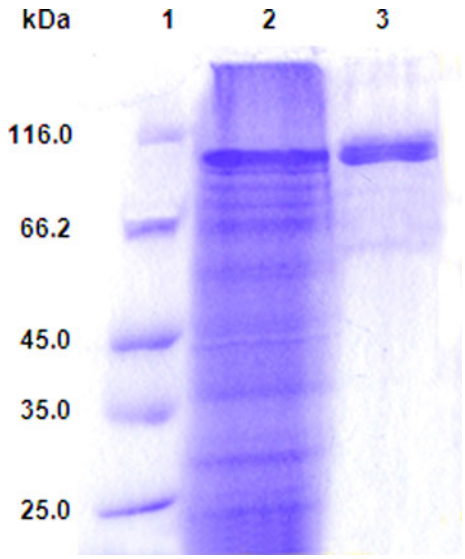

Fig. 6 SDS-PAGE of the purification of *H. mediterranei* recombinant CGTase. *Lane 1* molecular weight marker. *Lane 2* soluble fraction. *Lane 3* β-CD-Sepharose 6B

In a Sephacryl S-200 column the recombinant CGTase showed the same behavior as the wild-type one. Recombinant CGTase presented the same elution volume, confirming that this type of enzyme interacts with the matrix.

All the results achieved in the characterization of the recombinant CGTase agree with those obtained for the wild-type one. Both enzymes show similar kinetic behavior (Table 5) and characteristics.

Discussion

The *H. mediterranei* CGTase is the first halophilic archaea CGTase characterized with biochemical and molecular techniques. This enzyme is a monomeric extracellular protein of approximately 77 kDa with optimum activity at 55°C, pH 7.5 and 1.5 M NaCl. It shows a high tolerance to saline conditions, showing activity even at low salt concentrations as 0.5 M NaCl where it retains 65% of its activity. Like other halophilic enzymes, *H. mediterranei* CGTase showed a higher thermostability with increasing salt concentration of buffer in which it is located. Dym et al.(1995) found from the crystal structure of *Haloarcula marismortui* malate dehydrogenase that several structural motifs which confer halophilic character are the same than those that contribute to the stability of thermophilic enzymes. The high optimal temperature of halophilic enzymes in general, can be considered as an adaptive response to high temperatures that these microorganisms have to bear in their natural habitats where they are exposed to intense solar radiation.

CGTase was completely inactivated by treating it with the chelating agent EDTA, suggesting the existence of a metal essential for the maintenance of its activity. The enzyme recovered only 19% of this activity when it was incubated with 10 mM $CaCl_2$ for 72 h. This fact indicated that the enzyme requires calcium ion for stability and/or activity as in enzymes belonging to the α-amylase family.

Cyclization is the predominant activity followed by hydrolysis in the CGTase of *H. mediterranei*, while the coupling and disproportionation activities are very low. Like the other characterized CGTase this enzyme produces

Table 4 Purification steps of recombinant CGTase from *H. mediterranei*

Purification step	Volume (ml)	Total units	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Soluble fraction	50	27.5	22.0	1.25	100	1.0
β-CD-Sepharose 6B	30	13.0	0.26	50.00	47	40.0

Table 5 Kinetic parameters of *H. mediterranei* CGTase

Activity	CGTase	K_m (g/l)	V_{max} (U/mg)	$K_{cat} \times 10^{-3}$ (min^{-1})	$K_{cat}/K_m \times 10^{-3}$ ($l\ g^{-1}\ min^{-1}$)
Hydrolysis	Wild type	14.3 ± 0.8	106 ± 4	8.07 ± 0.02	0.57 ± 0.03
	Recombinant	22 ± 1	125 ± 6	9.55 ± 0.01	0.43 ± 0.02
β-Cyclization	Wild type	17.2 ± 0.6	161 ± 5	12.26 ± 0.02	0.71 ± 0.03
	Recombinant	21 ± 1	188 ± 7	14.36 ± 0.01	0.68 ± 0.03

a mixture of cyclodextrins, mainly α-CD (α-CD > β-CD > γ-CD).

The sequence of the CGTase gene consists of 2142 bp encoding 713 amino acids. In this sequence, we have identified the four regions conserved in the family of α-amylase and the starch binding site. The CGTase shows the typical characteristics of halophilic enzymes, showing a low isoelectric point, an increase of negatively charged amino acids and a decrease in Lys content. The sequence analysis also revealed the presence of a signal peptide and the signal sequence of Tat protein family, which indicates that the CGTase of *H. mediterranei* could be transported by this system to the extracellular medium. The extensive use of this pathway by halophilic archaea suggests an evolutionary adaptation to extremely high-salt conditions (Rose et al. 2002). To maintain osmotic balance, halophilic archaea accumulate high concentrations of ions K^+ in the cytoplasm (Madern et al. 2000). To prevent aggregation and to be stable under these conditions, proteins fold rapidly acquiring a structural conformation in which acidic residues are exposed on the surface of the protein. The Tat pathway allows the proteins to be folded in the cytoplasm before their secretion.

Upstream of the CGTase gene, four adjacent genes have been sequenced. These genes belong to a maltose ABC transporter and show the typical characteristics of these transporters. Using the consensus sequences for TATA boxes of halophiles we have proposed that the CGTase and ATPase (*malK*) genes are transcribed each one separately, while the other transporter genes (*malE*, *MalF* and *MalG*) are transcribed as one transcriptional unit.

The CGTases and α-amylases share 30% amino acid sequence identities. Crystal structures of both type of enzymes show a clear similarity in the N-terminal of approximately 400 residues, which are a catalytic domain in the form of a $(\alpha/\beta)_8$ barrel (MacGregor et al. 2001). This structure is organized by eight parallel β-strands surrounded by eight α-helices and includes domains A, B, and C. The catalytic triad, formed by three acidic residues, in *H. mediterranei* CGTase is located in the A domain (from amino acid 41 to 413, whereas the role of the other two domains is not yet clear. It is possible that the B domain (from amino acid 153 to 191) participates in substrate or Ca^{+2} binding and the C domain (from amino acid 414 to 503) stabilizes the A domain. In contrast to the α-amylases (45–55 kDa), CGTase (70–75 kDa) have two additional C-terminal domains involved in binding of starch (D and E domains). These domains have an antiparallel β-sheet structure and the role of the D domain (from amino acid 504 to 585) is still unclear. In the E domain (from amino acid 586 to 688), two maltose binding sites have been described that are part of an upper structure, the SBD which is located in a remote area of the active site in the C-terminal region. In the SBD the enzyme interacts with the substrate, the substrate is oriented toward the active site in the catalytic domain and the surface of the starch granule is altered (Janeček et al. 2003). In the classification of carbohydrate-binding sites (CBM), it is considered that the SBD belongs to the family 20 (BCM 20) (Machovič and Janeček 2006).

Halophilic protein expression in mesophilic guests such as *E. coli* has the disadvantage that usually the protein is obtained in the form of inclusion bodies, requiring a further step of protein refolding. This is because these proteins require the presence of high salt concentrations to be active, soluble and stable. However, the *H. mediterranei* CGTase is expressed in active form in the soluble cytoplasmatic fraction and can be purified in a single chromatographic step. The recombinant enzyme has a similar kinetic behavior and characteristics to the wild-type one. At the moment, only four thermophilic archaea CGTases have been expressed in a heterologous system. Therefore, the *H. mediterranei* CGTase is the first expressed in this system that belongs to a halophilic archaea.

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