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A cycloamylose-forming hyperthermostable 4-α-glucanotransferase of *Aquifex aeolicus* expressed in *Escherichia coli*

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Abstract

The gene (*malM*) encoding for 4-α-glucanotransferase (4αGTase) from the hyperthermophilic bacterium *Aquifex aeolicus* was cloned and expressed in *Escherichia coli*. The recombinant enzyme was purified to homogeneity and its behavior towards various substrates was examined. The enzyme was hyperthermostable, exhibiting maximal activity at 90 °C and it retained 70% of its original activity after incubation at 90 °C for 30 min. A low affinity was observed for the enzyme towards maltose (K_m = 71 mM) and the k_{cat}/K_m value for maltotriose was approximately 166 times greater than that observed for maltose but the values did not change significantly with larger maltooligosaccharides. The *A. aeolicus* 4αGTase produced cycloamylose with a minimum degree of polymerization (DP) of 16, whereas the cycloamylose produced by the amylomaltase from the thermophilic bacterium *Thermus aquaticus*, which is also a Type II 4αGTase, produced a cycloamylose with a minimum DP of 22. These findings indicate that the *A. aeolicus* 4αGTase differs from the *T. aquaticus* amylomaltase and the glucanotransferases produced by other hyperthermophilic organisms.

Keywords: *Aquifex aeolicus*; 4-α-Glucanotransferase; Hyperthermostable; Cycloamylose

1. Introduction

4-α-Glucanotransferase (4αGTase) (EC 2.4.1.25) are enzymes that catalyze glucan transfer from one α-1,4-glucan molecule to another α-1,4-glucan or to glucose. This enzyme is also known as the disproportionating enzyme (D-enzyme) in plants and it was first found in potato tubers by Peat et al. [1]. It has since been found in many hyperthermophilic organisms, with varying degrees of substrate specificities [2–4]. 4-α-Glucanotransferase is also named as amylomaltase in bacterial species [5,6], and the crystal structure of the *Thermus aquaticus* amylomaltase has recently been reported [7]. A comparison of the D-enzyme and amylomaltase indicates that these enzymes display significant homology in their amino acid sequences and they display strong similarities in their catalytic properties [8]. These enzymes catalyze an intermolecular transglycosylation reaction called a "disproportionating reaction" and they also catalyze intramolecular transglycosylation, which creates a cyclic glucan (cycloamylose) from a single linear glucan molecule. It has been shown that maltooligosaccharides are effective donors and maltooligosaccharides and glucose serve as acceptors in vitro. Glucosyl or maltosyl groups have been

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established as the major units transferred from the donor molecule [2–10]. Therefore, these enzymes are believed to be part of the starch degradation pathway as the disproportionating of small maltooligosaccharides into larger glucans facilitates their degradation through phosphorylases or hydrolases [11].

Aquifex aeolicus is a hyperthermophilic, hydrogen-oxidizing, microaerophilic, obligate chemolithoautotroph [12], representing the lowest branch in the phylogeny of bacteria [13]. This extremophile has recently generated interest among researchers as a useful source of thermostable enzymes [14–16]. The complete genome sequence of *A. aeolicus* has been determined [17] and it was found to contain 1512 open reading frames (ORFs), one of which (*malM*) encodes for a putative 4-α-glucanotransferase 485 amino acids in length (GenBank accession number: AE000704). Although 4-α-glucanotransferases have been isolated and the genes encoding for this enzyme have been cloned from a number of sources such as potato [18], and thermophilic species such as *Thermotoga maritima* [2], *Thermotoga neapolitana* [3] and *Thermococcus litoralis* [4] the only detailed preparations and size determinations of the cycloamyloses produced are those of the potato D-enzyme [19] and the *T. aquaticus* amylomaltase [6]. To date there have been no reports appearing in the literature regarding the characterization of 4-α-glucanotransferase from the hyperthermophile *A. aeolicus*, a bacterium which has a growth-temperature maximum close to 95 °C. We cloned and expressed the *malM* gene from *A. aeolicus* in *Escherichia coli* and characterized the properties of the recombinant enzyme to examine the use of this enzyme for the efficient production of cycloamylose.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The genomic DNA of *A. aeolicus* was kindly provided by Prof. K.O. Stetter and Prof. R. Hubber from the University of Rosenburg, Germany. pDrive cloning vector was obtained from Qiagen (Hilden, Germany). The expression vector pET-28b(+) was from Novagen (Madison, WI, USA). *E. coli* EZ competent cells (Qiagen) and *E. coli* BL21-CodonPlus-RIL competent cells (Stratagene, La Jolla, CA, USA) were used as hosts for the cloning and expression steps, respectively. Luria–Bertani (LB) medium supplemented with kanamycin (50 μg/ml) was used for the cultivation of the *E. coli* transformants. Maltooligosaccharides were purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan) and potato amylose (Type III) was purchased from Sigma. Glucoamylase, α-amylase and pullulanase were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The recombinant DNA techniques employed for manipulations of DNA are those described by Sambrook et al. [20].

2.2. Cloning and sequencing of the malM gene

Restriction endonucleases were purchased from New England Bio-Labs, USA. The nucleotide sequence of the open reading frame encoding the putative 4-α-glucanotransferase (aq723) was retrieved from GenBank with the accession number: AE000704. The ORF was amplified by the polymerase chain reaction (PCR) using a 5′-forward primer (5′-CATATGAGATTGGCAGGTATTTTACTTC-3′) and a 3′-reverse primer (5′-GAGCTCTTAAACTTCTCT-TCCGTAAATTCCG-3′) which contained *Nde*1 and *Sac*1 restriction sites, respectively (underlined). The expected 1485 bp fragment was obtained by using DNA polymerase (KOD-plus, Toyobo) and a PCR program with 25 cycles comprising the following steps: 98 °C for 1 min, 55 °C for 1 min, and 68 °C for 1 min. The restriction sites were chosen so as to insert an N-terminal hexahistidine (6× His tag) into the construct. The amplified product was cloned into a pDrive vector and the plasmid was transformed into *E. coli* EZ cells. The resulting recombinant plasmid, AF–*malM*–pDrive, was isolated from a positive clone using a QIA Miniprep kit (Qiagen, Germany), and DNA sequencing was performed in both strands to ensure that the nucleotide sequences were identical with those of the *malM* gene. DNA sequencing was performed with an Applied Biosystems 310 Genetic Analyzer using a Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer, Applied Biosystems, Norwalk, CT, USA). The sequence data were analyzed using the GENETYX computer program (Software Development Co., Tokyo, Japan).

2.3. Construction of the expression vector

The *malM* gene was excised from the recombinant plasmid AF–*malM*–pDrive using the restriction enzymes *Nde*1 and *Sac*1 and then ligated to the pET-28b(+) vector, which had been pre-digested with the same pair of restriction enzymes. The ligation process was performed overnight at 16 °C using a ligation kit (High T4 DNA Ligase; TOYOBO Co., Osaka, Japan). *E. coli* BL21-CodonPlus-RIL competent cells were transformed by electroporation with the ligated plasmid. The expression construct (AF–*malM*–pET) obtained from a positive colony was sequenced to confirm that the correct insertion of the ORF into the cloning site had been made.

2.4. Expression and purification of the enzyme

For the expression of *malM*, recombinant *E. coli* BL21-CodonPlus-RIL cells harboring AF–*malM*–pET were grown in LB medium containing kanamycin (50 μg/ml) with shaking at 37 °C. In the exponential growth phase when an $OD_{600\,nm}$ of 0.6 was reached, expression was induced by the addition of isopropyl thiogalactoside (IPTG) to give a final concentration of 0.3 mM. After induction for 4 h, the *E. coli* cells were harvested by centrifugation, suspended in 50 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer (pH 6.6), and then disrupted by sonication (five cycles consisting of 15 s pulses at 35% maximum output with 15 s rests between pulses). After centrifugation (12,000 rpm for 10 min), the supernatant was removed and used as a crude extract.

Unless noted otherwise, the purification steps described below were performed at room temperature. The enzyme was purified first by Ni-NTA agarose metal chelate chromatography followed by Q-Sepharose FF anion exchange chromatography. The crude extract (20 ml) was mixed with Ni-NTA agarose resin (2 ml) on ice with gentle shaking for 20 min. The resin bound to the target protein was then packed into a 2 ml column connected to a FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden) and the unbound proteins were washed off the column with 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM imidazole at a flow rate of 1 ml/min. The elution steps were performed using a linear concentration gradient from 10 to 200 mM imidazole in 50 mM Tris–HCl (pH 8.0) buffer. The fractions possessing enzyme activity were pooled together and dialyzed for 1 h against 2 l of 20 mM MES buffer (pH 6.6) at room temperature. The dialyzed enzyme was then injected onto a Q-Sepharose FF column. The bound proteins were eluted with a linear gradient from 0 to 1 M NaCl in 50 mM MES buffer (pH 6.6). The purity of the protein was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Analysis of the purified protein

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli [21] using 10% acrylamide gel slabs. Samples were boiled for 4 min in the presence of 0.01 M mercaptoethanol and 1% (w/v) SDS. Proteins were stained with 1% (w/v) Coomassie Brilliant Blue R-250 in a methanol/acetic acid/water mixture (50/10/40, v/v/v) and they were destained in a methanol/acetic acid/water mixture (30/10/60, v/v/v). A 10 kDa protein ladder (Life Technologies, GIBCO BRL, Rockville, USA) was used as a molecular weight marker. The protein concentrations were routinely determined spectrophotometrically at 280 nm.

2.6. Enzyme assay

4-α-Glucanotransferase activity was assayed in a reaction mixture with a total volume of 200 μl containing 10 mM maltotriose, 50 mM MES buffer (pH 6.6), and an appropriate amount of enzyme and incubated at 70 °C for 15 min. The amount of glucose released was measured by the glucose oxidase method [22]. One unit of enzyme activity is defined as the amount of enzyme producing 1 μmol of glucose per minute under the assay conditions employed.

2.7. Effects of temperature and pH

The temperature optimum was determined under the standard assay conditions by incubating the reaction mixture at temperatures ranging from 20 to 100 °C. For determinations of thermal stability, the enzyme was pre-incubated for 30 min in 50 mM MES buffer (pH 6.6) at temperatures ranging from 0 to 100 °C. After chilling the sample on ice, the residual activity was determined by the standard assay procedure. In all

cases, the incubations were carried out in closed Eppendorf tubes to prevent evaporation of the solutions.

To determine the optimal pH for enzyme activity, the standard assay mixture was used in 50 mM solutions of the following buffers: sodium acetate (pH 3.8–5.7), 2-(*N*-morpholino)ethane sulfonic acid (pH 5.1–7.2), 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 6.4–8.8), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) (pH 6.5–8.6), Tris–HCl (pH 7.1–9.1) or 3-[cyclohexylamino]-1-propane sulfonic acid (CAPS) (pH 9.5–11.5). The pH stability was determined by pre-incubating the enzyme in 50 mM of the above-named buffers for 30 min at 70 °C. Remaining enzyme activity was then determined under the standard assay conditions.

2.8. Investigation of the kinetic parameters

The reactions were followed by incubating the purified enzyme with appropriate concentrations of substrate (maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, or maltoheptaose) in 50 mM MES buffer (pH 6.6) at 70 °C. Glucose release was measured by the glucose oxidase method at 505 nm on a Beckman DU-640 spectrophotometer. Initial reaction rates were determined at five different substrate concentrations ranging from 0.5 to 2.0 times the K_m value of each substrate. Values for K_m and k_{cat} were obtained by using the nonlinear regression analysis computer program GRAFIT Software (version 4.0) [23].

2.9. Thin-layer chromatography

The reaction products derived from maltooligosaccharides by 4-α-glucanotransferase were identified by thin-layer chromatography (TLC) on silica gel coated plates (silica gel 60; Merck, Darmstadt, Germany). Two microliters of each reaction mixture was spotted onto a plate and developed three times with 75% acetonitrile in water. After drying, the plate was dipped into a solution of methanol/sulfuric acid (95/5, v/v), and the products were visualized by heating in an oven for few minutes.

2.10. Cycloamylose production

Amylose solution was prepared as described by Takaha et al. [19]. For the production of cycloamylose, a reaction mixture (2 ml) containing 2 mg of amylose (Type III), 50 mM MES buffer (pH 6.6), and 0.82 of a unit of the *A. aeolicus* 4αGTase from recombinant *E. coli* was incubated at 70 °C. At selected time intervals, 200 μl of the reaction mixture was removed and transferred into an Eppendorf tube and kept at −20 °C to stop the reaction. Then 20 μl of the reaction mixture was incubated with glucoamylase (6.4 units) with or without α-amylase (0.2 unit) at 40 °C for 3 h. After the reaction was terminated by boiling the solution for 5 min, the released glucose was measured by the glucose oxidase method [22]. The amount of glucoamylase-resistant glucan (cycloamylose) was calculated by subtracting the amount of glucose released by glucoamylase from that released by the combined actions of glucoamylase and α-amylase. The reducing power of the reaction mixture was measured by the method of Waffenschmidt and Jaenicke [24]. To measure the ability of amylose to form a complex with iodine, 50 μl of the reaction solution was mixed with 950 μl of an iodine solution (0.1% I_2 and 1% KI in 3.8 mM HCl), and the absorbance was measured at 660 nm. The glucoamylase-resistant product was deionized using Amberlite MB3 resin, separated by gel filtration using Toyopearl HW40 chromatography, and then concentrated by freeze-drying on a vacuum evaporator. The molecular mass of the cycloamylose was analyzed by “time of flight” (TOF)-mass spectrometry.

3. Results

3.1. Cloning, expression and purification of the enzyme

As described in Section 2, we used PCR to place *Nde*1 and *Sac*1 restriction enzyme sites at the 5′ and 3′ ends of the *malM* open reading frame, respectively, so that the entire open reading frame was cloned and expressed in *E. coli* BL21-CodonPlus-RIL under the control of the inducible phage T7 promoter of the vector pET-28b(+). SDS-PAGE analysis of the cell lysate indicated that a protein of molecular mass 60 kDa was the major protein produced and this protein was not present prior to induction and did not appear in extracts obtained from the parent strain BL21-CodonPus-RIL (data not shown). The apparent

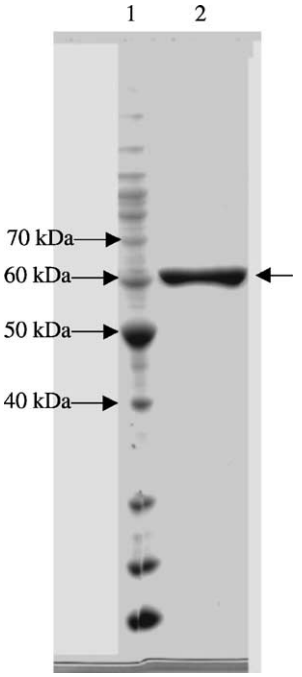

Fig. 1. SDS-PAGE analysis of the *A. aeolicus* 4-α-glucanotransferase. The elute from the Q-Sepharose FF column was run on a 10% polyacrylamide gel stained with Coomassie Brilliant Blue R-250. Molecular weight markers are in line 1. The arrow indicates the purified 4αGTase.

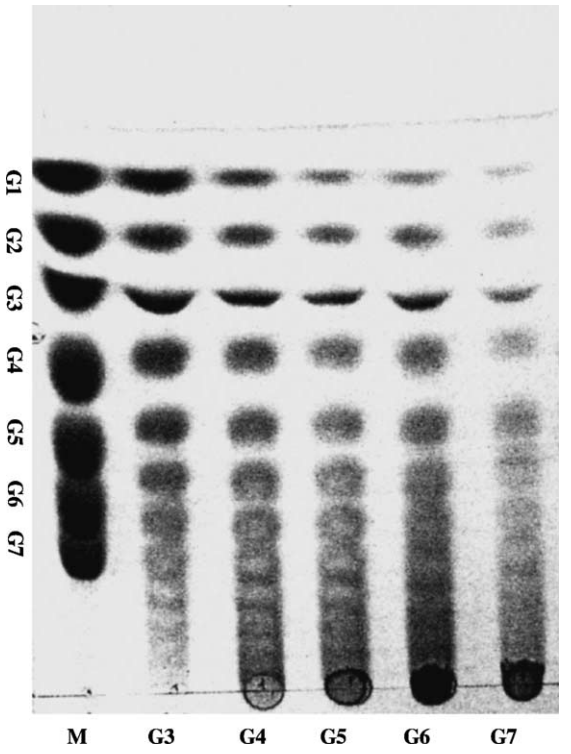

Fig. 2. TLC of the reaction products obtained from the activity of the *A. aeolicus* 4αGTase with different maltooligosaccharides.

molecular mass of the expressed protein as seen by SDS-PAGE was consistent with that of the molecular mass 58,543 Da, a calculation based on the deduced amino acid sequence. The recombinant protein was purified by Ni-NTA agarose column chromatography followed by Q-Sepharose FF column chromatography (Fig. 1). Table 1 summarizes the purification process and indicates that the specific activity of the purified enzyme increased by approximately 58-fold while the recovery was around 27%.

3.2. Transglycosylation activity of the malM enzyme

To confirm the identity of the 4αGTase expressed in *E. coli*, the activity of the purified enzyme toward a range of maltooligosaccharides was investigated. The enzyme was incubated with each maltooligosaccharide at a concentration of 1%, and the reaction products were analyzed by TLC. Fig. 2 shows that after 18 h incubation, all of the reactions containing maltooligosaccharides (G3–G7) showed

Table 1
Summary of the purification of the 4-α-glucanotransferase from *A. aeolicus*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	357	273	0.76	1.0	100
Ni-NTA column	10.1	103	10.2	13.4	38
Q-Sepharose FF	1.68	74.3	44.2	58.1	27

Enzyme activities were measured at 70 °C in a 200 μl reaction mixture containing 10 mM maltotriose.

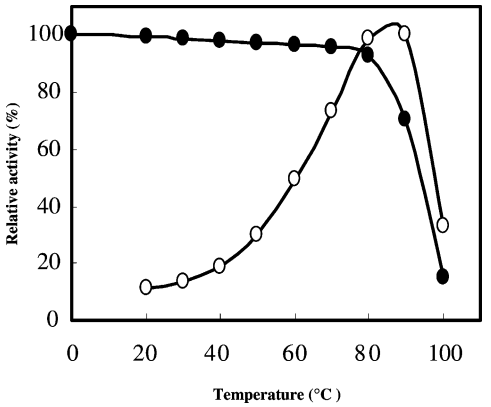

Fig. 3. Effect of temperature on the activity (○) and stability (●) of the 4-α-glucanotransferase. The activity was measured at different temperatures in 50 mM MES buffer, pH 6.6. For stability determinations, enzyme samples were incubated at selected temperatures for 30 min and the remaining activity was determined at 70 °C. The maximal activity level is defined as being 100%.

transglycosylation reactions, producing higher maltooligosaccharides and glucose. When G4–G7 were used as substrates, high molecular mass products were found at the origin on the TLC plate. The enzyme showed neither hydrolyzing nor transferase activity towards α-, β- and γ-cyclodextrins when incubated in the presence or in the absence of glucose (data not shown).

3.3. Enzymatic properties of malM

As shown in Fig. 3, the enzyme showed a temperature optimum of 90 °C. After incubating the purified enzyme for 30 min at selected temperatures between 20 and 100 °C, the residual activity of the enzyme was determined at 70 °C to examine the effects of temperature. The enzyme retained 70% of its original activity after incubation at 90 °C. Under the assay conditions, the pH optimum of the enzyme was observed at a pH value of 6.5. The enzyme was stable between pH values 3 and 11, indicating it is the most stable enzyme of its kind reported so far (Table 2).

Table 3
Kinetic parameters of the *A. aeolicus* 4-α-glucanotransferase with different maltooligosaccharides

Substrate	K_m (mM)	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($(s\,mM)^{-1}$)
Maltose (G2)	71 ± 6	1.3 ± 0.1	0.018
Maltotriose (G3)	6.1 ± 0.4	18 ± 2	3.0
Maltotetraose (G4)	4.5 ± 0.4	17 ± 2	3.8
Maltopentaose (G5)	4.2 ± 0.1	22 ± 2	5.2
Maltohexaose (G6)	4.5 ± 0.4	22 ± 1	4.9
Maltoheptaose (G7)	5.7 ± 0.4	16 ± 1	2.8

The enzyme reaction was carried out at 70 °C in 50 mM MES buffer (pH 6.6).
[a] Based on a molecular mass of 58,543 Da.

3.4. Substrate specificity and kinetic parameters

The substrate specificity and kinetic parameters of the purified 4αGTase were examined with a variety of α-1,4-linked maltooligosaccharides as substrates (maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose) at 70 °C. The results obtained, as shown in Table 3, indicate that the enzyme has a low affinity toward maltose (K_m = 71 mM). In addition, it was found that as the chain length increased

Table 2
Comparison of the characteristics of the *A. aeolicus* 4αGTase and *T. aquaticus* amylomaltase

Characteristic	*A. aeolicus* 4αGTase	*T. aquaticus* amylomaltase[a]
pH optimum	6.6	5.5–6.0
pH stability	3–11	4–10
Temperature optimum	90 °C	75 °C
Temperature stability	Retained 70% activity (90 °C, 30 min)	Retained 60% activity (90 °C, 10 min)
Smallest donor	Maltose	Maltose
Smallest acceptor	Glucose	Glucose
Smallest transferred unit	Glucose	Glucose
Disproportionated products	G, G2, G3, G4, G*n*	G, G2, G3, G4, G*n*
Cyclization reaction	+	+
Smallest CA (DP)	16	22

[a] [6].

the affinity also increased and the lowest K_m value (4.2 mM) was observed with maltopentaose. No significant differences were found in the K_m and k_{cat} values of substrates with chain lengths higher than maltotriose (G3).

3.5. Production and analysis of cycloamylose

To determine whether the *A. aeolicus* 4αGTase was able to catalyze the production of cycloamylose, a Type III amylose (0.1%) from potato was incubated with the enzyme at 70 °C. As shown in Fig. 4, the ability of amylose to form an iodine complex, as determined by measuring the absorbance at 660 nm, decreased rapidly with increasing reaction time. The reducing power of the reaction mixture did not increase significantly with time and it constituted less than 5% of the total reducing sugar present, even after 48 h reaction. These results strongly suggest that the enzyme catalyzed the cyclization of amylose to produce cyloamylose. To confirm that cycloamylose was formed, the reaction mixture was treated with glucoamylase since cycloamylose is resistant to this enzyme. The amount of glucoamylase-resistant glucans increased with time, and approximately 71% of the total sugar was converted into glucoamylase-resistant glucans after 48 h of incubation (Fig. 4). The cyclic nature of the glucoamylase-resistant glucans formed was confirmed and the molecular mass was determined by TOF-mass spectrometry (Fig. 5). The molecular masses of the glucans agreed with the theoretical values for the mono-sodium ions of cyclic glucans with degrees of polymerization (DPs) ranging from 16 to 50. The distribution did not differ with reaction temperatures of 30, 50 and 70 °C.

4. Discussion

In this paper, we describe the characterization and identification of the *malM* gene that encodes for a thermophilic 4-α-glucanotransferase from the hyperthermophilic bacterium *A. aeolicus*. The primary sequence of the putative 4-α-glucanotransferase (AE000704) of *A. aeolicus* was subjected to a psi-blast search at the National Center for Biotechnology Information [25]. This enzyme belongs to the α-amylase super-family 13 and it shows high levels of homology with the *T. aquaticus* amylomaltase (46% identity, [6]), potato D-enzyme (45% identity, [18]) and the amylomaltases of *Synechocystis* sp. (45% identity, [26]), *S. pneumoniae* (42% identity, [27]), *C. butyricum* (43% identity, [28]), and low levels of homology with the *E. coli* amylomaltase (21% identity, [5]). All members of

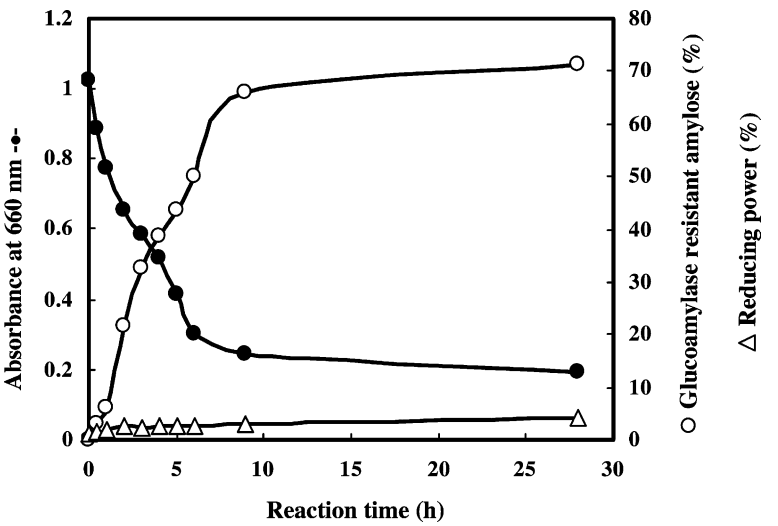

Fig. 4. Reaction of amylose catalyzed by the *A. aeolicus* 4-α-glucanotransferase. The reaction conditions are described in the text. The reducing power (△) due to the presence of glucose derived from the complete digestion of amylose by glucoamylase and α-amylase was defined as 100%.

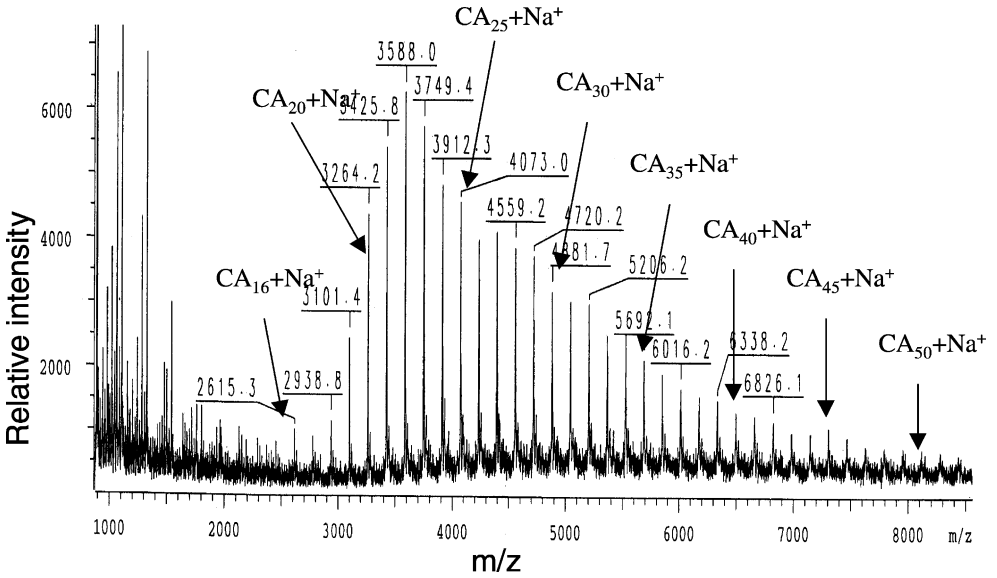

Fig. 5. TOF-mass spectrometry analysis of cycloamyloses obtained from the catalytic activity of the *A. aeolicus* 4αGTase with amylose. CA_n means cycloamylose with a DP = n. The theoretical E/z of $CA_n + Na^+$ is $(162.05n + 22.99)$.

this family contain a characteristic $(\beta/\alpha)_8$-barrel domain and four highly conserved regions [29]. Amino acid alignments indicated that the motifs DHFRGF and EDLG are conserved within the Type II 4αGTase enzyme group (data not shown).

The apparent molecular mass of the thermostable *A. aeolicus* 4αGTase, as determined by SDS-PAGE analysis, was observed to be 60 kDa. The 4αGTases from thermophilic sources such as *T. maritima*, *T. neapolitana*, *T. litoralis*, and a similar enzyme *T. aquaticus* amylomaltase, display optimal temperatures of 70, 85, 90 and 75 °C, respectively [2–4,6]. With an optimum temperature of 90 °C the *A. aeolicus* 4αGTase described herein is one of the most thermostable glucanotransferases reported to date. Based on structural analyses, the major factors that are known to stabilize proteins from hyperthermophiles are the number of ionic bonds, and the presence of salt bridges and hydrogen bonds between charged amino acid residues [30–32]. Consistent with these observations, is the finding that arginine, lysine, aspartic and glutamic acids are the predominant amino acids present in the sequence of the *A. aeolicus* 4αGTase.

An analysis of the kinetic data revealed that the enzyme possessed a low affinity for maltose (K_m = 71 mM) and the k_{cat}/K_m value was 166 times lower than that obtained with maltotriose. When maltooligosaccharides were employed as the substrates, transglycosylation products were generated. In the case of larger maltooligosaccharides (namely G4–G7), high molecular mass products were found at the origin on the TLC plate. The potato D-enzyme exhibits similar activity toward maltooligosaccharides; however, maltose was not detected at the end of the reactions [18]. On the basis of these findings, we concluded that the *malM* enzyme is a 4-α-glucanotransferase. Our studies also established that the smallest donor molecule is maltose, the smallest acceptor molecule is glucose, and that the smallest unit transferred is glucose (Table 2). In this respect the properties of the *A. aeolicus* 4αGTase are similar to those of the amylomaltases of *T. aquaticus* [6] and *E. coli* [5] but it differs from that of the potato D-enzyme which was unable to transfer glucose units [18]. However, the 4αGTase of *T. litoralis* exhibits no similarity to the other glucanotransferases but it can transfer glucose units [4]. The smallest cycloamylose produced by the *A. aeolicus* 4αGTase had a DP of 16—similar to that of the smallest cycloamylose produced by the potato D-enzyme with a DP of 17 [19]. In contrast, the amylomaltase from *T. aquaticus* produces cycloamylose with DP of 22 [6]. Cyclodextrin glucanotransferase (CGTase) also

catalyzes the same reactions, however a major difference is observed in the smallest cyclic α-1,4-glucan produced. The smallest cyclic products produced by the action of CGTases are α-, β- and γ-cyclodextrins with DPs of 6–8 [33]. In contrast, the *A. aeolicus* 4αGTase, like amylomaltase and D-enzyme, did not produce α-, β- and γ-cyclodextrins. The crystal structure of the amylomaltase from *T. aquaticus* has become available quite recently [7], and it indicates that a conserved loop of eight amino acid residues sterically hinders the formation of small cyclic products. These results should therefore contribute to our understanding of the actions of these enzymes.

In conclusion, the recombinant 4αGTase of *A. aeolicus* is a highly thermostable enzyme and it efficiently converts amylose into cycloamylose. The cycloamylose produced is highly soluble in water and it can function as an efficient artificial chaperone in protein refolding [34] and has the potential to be used in the food, pharmaceutical and chemical industries. Therefore, the hyperthermostable 4αGTase of *A. aeolicus* may be of industrial importance for the production of cycloamylose on a large scale.

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