Extremophiles (2007) 11:537–541
DOI 10.1007/s00792-007-0061-6

NOTE

Characterization of a thermostable cyclodextrin glucanotransferase from *Pyrococcus furiosus* DSM3638

Myoung-Hee Lee · Sung-Jae Yang · Jung-Woo Kim · Hee-Seob Lee · Jung-Wan Kim · Kwan-Hwa Park

Received: 8 October 2006 / Accepted: 10 December 2006 / Published online: 17 February 2007

Abstract A gene that encodes the enzyme *Pyrococcus furiosus* cyclodextrin glucanotransferase (PFCGT) was cloned in *Escherichia coli*. PFCGT was highly expressed in recombinant *E. coli* after compensation for codon usage bias using the pRARE plasmid. Purified PFCGT was extremely thermostable with an optimal temperature and pH of 95°C and 5.0, respectively, retaining 97% of its activity at 100°C. Incubation at 60°C for 20 min during the purification process led to a 1.5-fold increase in enzymatic activity. A time course assay of the PFCGT reaction with starch indicated that cyclic α-1,4-glucans with DPs greater than 20 were produced at the beginning of the incubation followed by an increase in β-CD. The major final product of PFCGT cyclization was β-CD, and thus the enzyme is a β-CGTase.

Keywords Hyperthermophile · *Pyrococcus furiosus* · *Pyrococcus furiosus* cyclodextrin glucanotransferase (PFCGT)

Communicated by K. Horikoshi.

M.-H. Lee · S.-J. Yang · J.-W. Kim · H.-S. Lee · K.-H. Park (✉)
Center for Agricultural Biomaterials,
and Department of Food Science and Biotechnology,
School of Agricultural Biotechnology,
Seoul National University,
Seoul 151-921, South Korea
e-mail: parkkh@plaza.snu.ac.kr

J.-W. Kim
Department of Biology, University of Incheon,
Incheon 402-749, South Korea

Hyperthermophiles tolerate the highest temperatures of any known organism, achieving optimal growth at above 75°C. There has been increasing interest in isolating and analyzing hyperthermophiles such as *Thermotoga maritima*, *Pyrococcus furiosus*, and *Sulfolobus solfataricus* as potential sources of thermostable enzymes that function at high temperatures (Bibel et al. 1998; Nelson et al. 1999; Schiraldi and Rosa 2002, Rose et al. 2005). In *P. furiosus*, several enzymes that hydrolyze cyclodextrin have been isolated, including an α-amylase (Dong et al. 1997a), an amylopullulanase (Dong et al. 1997b), and a novel amylolytic enzyme (Yang et al. 2004). Furthermore, a full genome analysis of *P. furiosus* has revealed genes that encode additional amylolytic enzymes, including one that may encode cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19), which belongs to glycoside hydrolase family 13.

CGTase is a unique glycoside hydrolase that forms circular α-(1,4)-linked oligosaccharides (CDs) from starch, which in turn may form inclusion complexes with various hydrophobic molecules (Saenger 1980). The enzyme uses multiple modes of action such as hydrolysis, cyclization, coupling, and disproportionation, and it is used industrially to produce CDs composed of six, seven, and eight glucose residues, forming α-, β-, and γ-CD, respectively. Highly thermostable CGTases, therefore, may be useful in the industrial liquefaction of starch, thereby eliminating the need to pretreat with other amylolytic enzymes. The goals of this study were to clone, express, and characterize a gene that encodes thermostable *P. furiosus* CGTase (PFCGT) in *Escherichia coli.*

Multiple sequence alignment of several amylolytic enzymes of the *P. furiosus* DSM3638 genome revealed

an open reading frame, PF0478, homologous to other CGTases. The putative CGTase gene was amplified from the *P. furiosus* chromosome using two primers (PF-Fco, 5′-TTTCCCAGGTCCATGGGCTATTAT GTTCCA-3′ and PF-Rxo, 5′-AGTAGCGGATGG CTCGAGCTAGAGGCACCA-3′) containing an *Nco*I and an *Xho*I restriction site (underlined), respectively. The PCR product (2.1 kb) was ligated into the expression vector, pTKNd119 (Kim et al. 2004), at the corresponding sites and transformed into *E. coli* MC1061 [F-, *araD139*, *recA13*, (*araABC-leu*)7696, *galU*, *galK*, *lacX74*, *rpsL*, *thi*, *hsdR2*, *mcrB*]. The insert of the resulting plasmid, pTKPFCGT, was sequenced using a BigDye™ terminator cycle sequencing kit for ABI377 PRISM (Perkin–Elmer, Norwalk, CT).

Nucleotide sequences of several CGTases were multiply aligned using CLUSTAL X 1.83 (Thompson et al. 1997), and then a radial unrooted phylogenetic tree was constructed based on the CLUSTAL X bootstrap values (1,000 samples) by the neighbor-joining method (Saitou and Nei 1987) using TreeView v.1.66 (Page 1996). The predicted amino acid sequence of the gene product PFCGT represented a protein with a molecular mass of 80,775 Da and shared 61% identity with CGTase from *Thermococcus kodakaraensis* (Rashid et al. 2002) and only 36% with CGTase from *Thermococcus* sp. B1001 (Tachibana et al. 1999). It shared even lower identity (20–30%) with CGTases from other bacteria. A phylogenetic tree based on the amino acid sequence of various CGTases showed that PFCGT is phylogenetically distant from bacterial CGTases (Fig. 1). Interestingly, PFCGT had a shorter N-terminus and a longer C-terminus than other CGTases. The E domain, which in CGTases is known to bind to starch, was longer than and its sequence was different from those found in other CGTases, even at the residues involved in starch binding; it was most closely related to the E domain of *T. kodakaraensis*. Multiple amino acid sequence alignment revealed that PFCGT also contained the four well-known conserved regions of glycoside hydrolase family 13, but with some variation.

The *pfcgt* gene was cloned in *E. coli* without the signal sequence, but was not well expressed. This was likely due to codon usage bias (Yang et al. 2004). Codons that are rarely used in *E. coli* such as AGA and AGG for arginine (35/35), AUA for isoleucine (27/56), and GGA for glycine (31/51), have been used frequently for *pfcgt*. To compensate for codon usage bias and to enhance the expression of *pfcgt* in *E. coli*, the pRARE plasmid (Novagen, Madison, WI), which carries extra tRNA genes for the aforementioned codons,

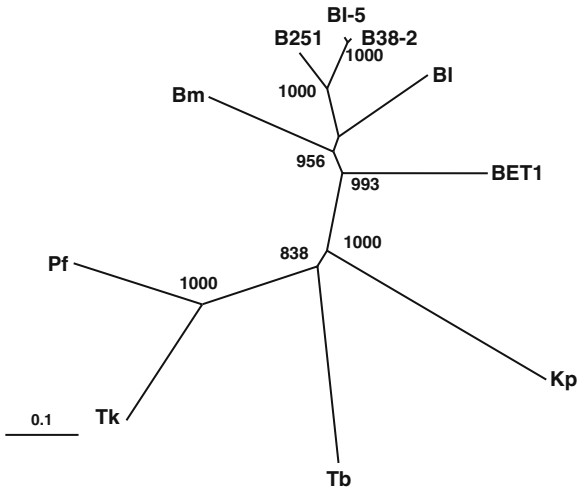

Fig. 1 Phylogenetic relationship among CGTases. Phylip format tree outputs from the CLUSTAL X analysis were visualized with TreeViewPPC based on the distance matrix using the neighbor-joining method. The *bar* in the lower left corner indicates the substitution rate (substitution/site). Bootstrap values (based on 1,000 bootstrap trials) are shown at each node. The unrooted phylogenetic tree was built from entire sequences of the following CGTases: BET1 represents CGTase from *Bacillus stearothermophilus* ET1 (GenBank gi:4099126), *BI-5* from alkalophilic *Bacillus* sp. I-5 (gi:39933005), *Kp* from *Klebsiella pneumoniae* AS-22 (gi:149178), *B251* from *B. circulans* 251 (gi:510491), *B38-2* from *Bacillus* sp. 38-2 (gi:216247), *Bm* from *B. macerans* (gi:39624), *Bl* from *B. licheniformis* (gi:39565), *Tb* from *Thermococcus* sp. strain B1001 (gi:6552351), *Tk* from *T. kodakaraensis* KOD1 (gi:17298172), and *Pf* from *Pyrococcus furiosus* (gi:1468319)

was introduced into *E. coli* harboring pTKPFCGT. The enhanced expression of *pfcgt* led to a significant amount of PFCGT accumulation.

PFCGT was successfully purified from pTKPFCGT-carrying *E. coli* using a four-step procedure that included heat treatment. *E. coli* harboring pTKPFCGT was grown in LB broth supplemented with kanamycin (100 μg/ml) and chloramphenicol (30 μg/ml) at 37°C for 19 h with agitation (200 rpm). The cells were harvested via centrifugation and then sonicated (VC-600; Sonics & Materials Inc., Newtown, CT). The extract was incubated at 60°C for 20 min then applied to a Q-Sepharose column (10 × 1.0 cm), a DEAE-Toyopearl column, and a gel permeation chromatography (GPC) column (30 × 1.0 cm) using an AKTA FPLC purifier system (Amersham–Pharmacia, Uppsala, Sweden). The anion exchange columns were equilibrated with 50 mM Tris–HCl (pH 7.5 or 9.0), and the proteins were eluted with a linear gradient of NaCl (0–1.0 M). When heat treatment was omitted, the yield of PFCGT was very poor. High temperatures facilitate the folding of thermostable proteins (Rahman et al. 1997, 1998; Siddiqui et al. 1998) and may also be required for the

Table 1 Effects of heat treatment on CGTase activity

Time (min)	Relative activity[a] (%)					
	40°C	50°C	60°C	70°C	80°C	90°C
0			100			
5			119			
10	80.6	107	147	88.1	46.8	43.9
20			159			
30			131			
40			83.9			

[a] Refolded CGTases were heated for 10–40 min at the indicated temperatures (40–90°C). Cyclization activity was measured at 50°C

proper folding of secreted enzymes because accessory proteins such as chaperonins are absent in the extracellular environment.

The effect of heat treatment on PFCGT was examined by incubating the cell lysate for 10 min at various temperatures. After treatment, the samples were centrifuged, and the enzyme activity in the supernatant was measured using the phenolphthalein method (Kaneko et al. 1987) with 4% soluble starch as the substrate in 50 mM sodium acetate buffer (pH 5.0) at 50°C to avoid additional heat effects (Table 1). Most enzymes from mesophiles are generally thermolabile and lose activity upon heat treatment. In contrast, PFCGT activity increased during incubation at 60°C for up to 30 min (Table 1). Heat treatment for 20 min at 60°C increased its activity by 1.5-fold.

Recent studies have indicated that high temperatures have significant effects on forming a suitable protein structure. For example, glutamate dehydrogenase (GDH) from *P. kodakaraensis* (Rahman et al. 1997, 1998) or *P. furiosus* (Rice et al. 1996), and D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *T. maritima* (Schultes and Jaenicke 1991) required heat treatment in vitro for proper protein folding. Recombinant thermostable proteins purified in the absence of heat treatment may be in an intermediate stage of protein folding. In the present study, the intermediate form of PFCGT also seemed to be functional because the unheated enzyme possessed cyclization activity.

The estimated molecular mass of PFCGT on a 10% SDS-PAGE gel (65 kDa; Fig. 2) was smaller than that predicted from the amino acid sequence of the gene (81 kDa). Similar abnormal mobility on SDS-PAGE gels has previously been observed among hyperthermophilic starch-degrading enzymes (Tachibana et al. 1996, 1999; Rashid et al. 2002) and is likely the result of incomplete denaturation of the proteins by the loading buffer. The mobility of the active band

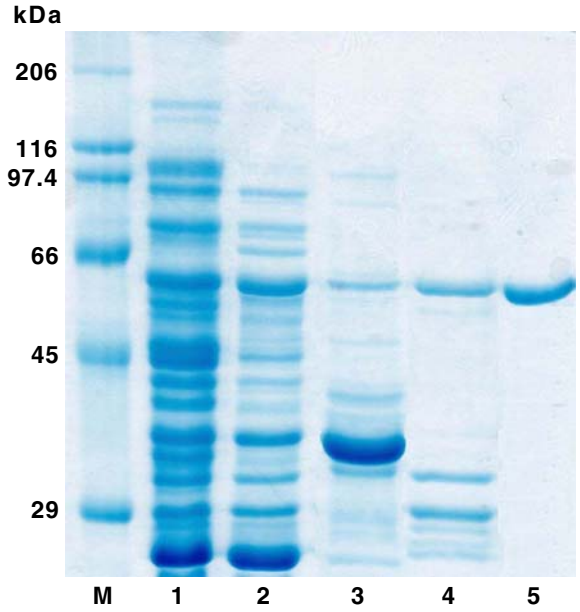

Fig. 2 SDS-PAGE of PFCGT at each purification step from the *E. coli* lysate. Lane M represents standard proteins; lane 1, crude cell extract; lane 2, cell extract after heat treatment; lane 3, partially purified PFCGT after Q-Sepharose column chromatography; lane 4, partially purified PFCGT after DEAE-Toyopearl column chromatography; lane 5, purified PFCGT after gel permeation chromatography (GPC)

confirmed by staining for starch-degrading activity was correlated with that determined by staining with Coomassie brilliant blue, indicating that purified PFCGT was definitely a CGTase (data not shown). Furthermore, the first six amino acids of purified PFCGT (MGYYVP) determined by protein sequencing (Model 491; Perkin–Elmer) were identical to those deduced from the *pfcgt* gene (data not shown).

The optimal reaction temperature and pH for PFCGT were 95°C and 5.0, respectively. The enzyme retained 97% of its activity at 100°C and 60% at 105°C, but the residual activity was greatly reduced to below 45% at 110°C. The half-lives of the enzyme at 85, 90, and 95°C were 142, 85, and 46 min, respectively (data not shown). CGTase from *T. kodakaraensis* (Rashid et al. 2002), which has the highest similarity to PFCGT, had an optimal temperature of 80°C. None of the bacterial CGTases were as thermostable as PFCGT (Schmid 1989). To our knowledge, PFCGT is the second most thermostable CGTase known, following the CGTase from the *Thermococcus* B1001 strain, which has an optimal temperature of 110°C.

A time course assay of PFCGT reactivity at 90°C was performed to clarify the enzyme's modes of action. Soluble starch (4%) was incubated with 0.1 U of the purified enzyme for up to 360 min, and the cyclic

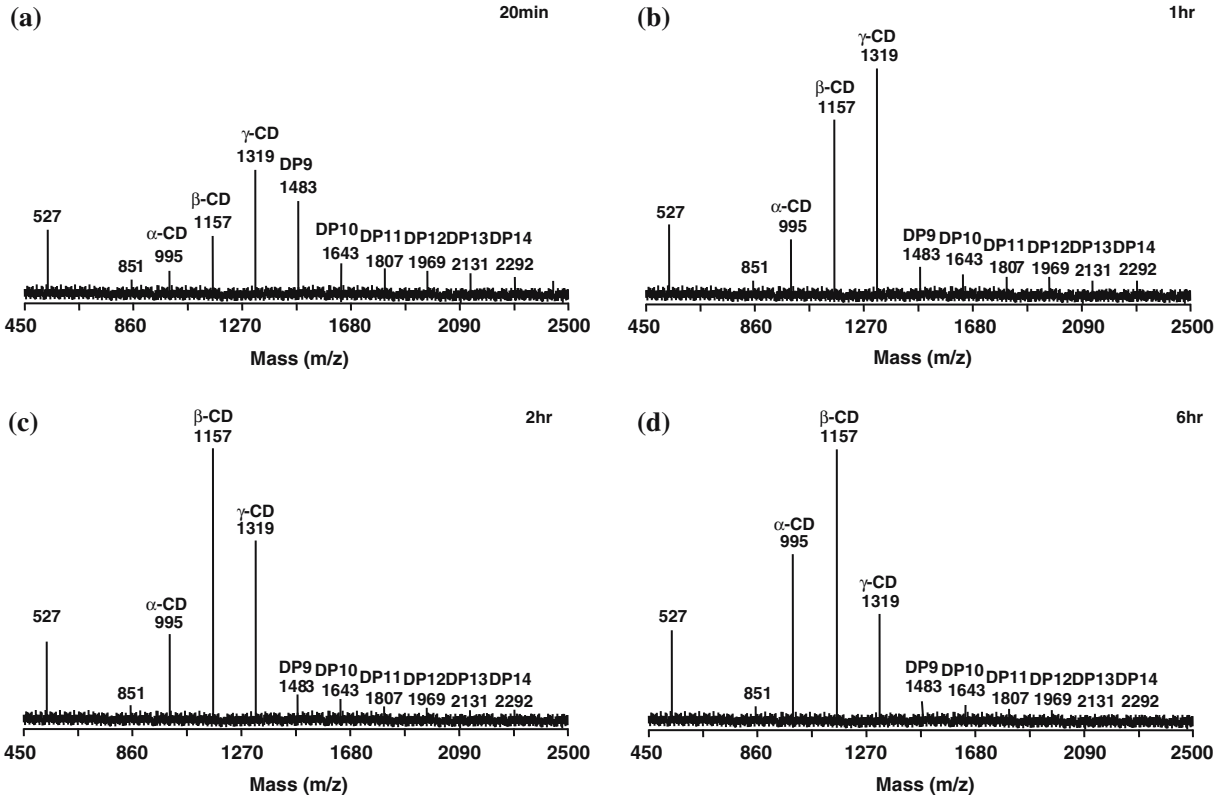

Fig. 3 Time course analysis of *β*-amylase-resistant molecules using MALDI TOF-MS. Soluble starch (4%) was incubated with purified PFCGT (0.1 U/ml) at 90°C for different time periods (**a**–**d**). After *β*-amylase treatment, *β*-amylase-resistant molecules were purified and subjected to TOF-MS analysis. *Numbers* above each peak indicate the molecular mass (in Daltons) of the molecule plus the mass of Na^+ (23 Da)

α-1,4-glucans were recovered from the solution and purified. Beta-Amylase was added to the reaction solution following PFCGT inactivation and incubated at 40°C for 6 h to remove any noncyclic glucans. This process degrades noncyclic glucans into maltose. Pure cyclic α-1,4-glucans were precipitated by adding 10 volumes of ethanol and then freeze-dried. Many peaks of cyclic α-1,4-glucans with DPs greater than 20 were detected when the mixtures taken at each time point were analyzed using high performance anion exchange chromatography and MALDI-TOF mass spectrometer (Voyager™ DE; Perceptive Biosystem, Framingham, MA). Cyclic α-1,4-glucans increased after 20 min of incubation (data not shown) but remained constant as the reaction proceeded, while *β*-CD continued to increase (Fig. 3a–c). The major final product of the PFCGT reaction was *β*-CD (Fig. 3d).

Given these results, it is likely that PFCGT targeted any α-1,4-linkage and then transferred the newly formed reducing end of the substrate either to the nonreducing end of a separate linear acceptor molecule, to glucose (intermolecular transglycosylation or disproportionation reaction), or to its own nonreducing end (intramolecular transglycosylation or cyclization reaction). This random cyclization reaction produces a wide variety of cyclic α-1,4-glucans. The reversibility of these reactions allows large cyclic molecules to be re-linearized by transglycosylation, which leads to the production of smaller cyclic molecules. The equilibrium of the whole reaction tends to be toward the formation of α- or *β*-CD as the final major products.

Acknowledgments We thank A. M. Grunden of North Carolina State University, USA, for donating the *P. furiosus* DSM3638 genomic DNA used in the study. This study was supported by the Marine and Extreme Genome Research Center Program, Ministry of Marine Affairs and Fisheries, Republic of Korea.

References

Bibel M, Brettl C, Gosslar U, Kriegshäuser G, Liebl W (1998) Isolation and analysis of genes for amylolytic enzymes of the hyperthermophilic bacterium *Thermotoga maritima*. FEMS Microbiol Lett 158:9–15

Dong G, Vieille C, Savchenko A, Zeikus JG (1997a) Cloning, sequencing, and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and biochemical

characterization of the recombinant enzyme. Appl Environ Microbiol 63:3569–3576

Dong G, Vieille C, Zeikus JG (1997b) Cloning, sequencing, and expression of the gene encoding amylopullulanase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. Appl Environ Microbiol 63:3577–3584

Kaneko T, Kato T, Nakamura N, Horikoshi K (1987) Spectrophotometric determination of cyclization activity of β-cyclodextrin-forming cyclomaltodextrin glucanotransferase. J Jpn Soc Starch Sci 34:45–48

Kim YW, Lee SS, Warren RAJ, Withers SG (2004) Directed evolution of a glycosynthase from *Agrobacterium* sp. increases its catalytic activity dramatically and expands its substrate repertoire. J Biol Chem 279:42787–42793

Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Nelson WC, Ketchum KA, McDonald L, Utterback TR, Malek JA, Linher KD, Garrett MM, Stewart AM, Cotton MD, Pratt MS, Phillips CA, Richardson D, Heidelberg J, Sutton GG, Fleischmann RD, Eisen JA, White O, Salzberg SL, Smith HO, Venter JC, Fraser CM (1999) Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. Nature 399:323–329

Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Rahman RNZA, Fujiwara S, Takagi M, Kanaya S, Imanaka T (1997) Effect of heat treatment on proper oligomeric structure formation of thermostable glutamate dehydrogenase from a hyperthermophilic archaeon. Biochem Biophys Res Commun 241:646–652

Rahman RNZA, Fujiwara S, Nakamura H, Takagi M, Imanaka T (1998) Ion pairs involved for maintaining a thermostable structure of glutamate dehydrogenase (GDH) from a hyperthermophilic archaeon. Biochem Biophys Res Commun 248:920–926

Rashid N, Cornista J, Ezaki S, Fukui T, Atomi H, Imanaka T (2002) Characterization of an archaeal cyclodextrin glucanotransferase with a novel C-terminal domain. J Bacteriol 184:777–784

Rice DW, Yip KSP, Stillman TJ, Britton KL, Fuentes A, Connerton I, Pasquo A, Acandurra R, Engel PC (1996) Insights into the molecular basis of thermal stability from the structure determination of *Pyrococcus furiosus* glutamate dehydrogenase. FEMS Microbiol Rev 18:105–117

Rose NN, Li D, Park JT, Cha HJ, Kim MS, Kim JW, Park KH (2005) Characterization and application of a novel thermostable glucoamylase cloned from a hyperthermophilic archaeon *Sulfolobus tokodaii*. Food Sci Biotechnol 14:860–865

Saenger W (1980) Cyclodextrin inclusion compounds in research and industry. Angew Chem Int Ed 19:344–362

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Schiraldi C, Rosa MD (2002) The production of biocatalysts and biomolecules from extremophiles. Trends Biotechnol 20: 515–521

Schmid G (1989) Cyclodextrin glycosyltransferase production: yield enhancement by overexpression of cloned genes. Tibtech 7:244–248

Schultes V, Jaenicke R (1991) Folding intermediates of hyperthermophilic D-glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima* are trapped at low temperature. FEBS Lett 290:235–238

Siddiqui MA, Fujiwara S, Takagi M, Imanaka T (1998) In vitro heat effect on heterooligomeric subunit assembly of thermostable indolepyruvate ferredoxin oxidoreductase. FEBS Lett 434:372–376

Tachibana Y, Leclere MM, Fujiwara S, Takagi M, Imanaka T (1996) Cloning and expression of the α-amylase gene from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1, and characterization of the enzyme. J Ferment Bioeng 82:224–232

Tachibana Y, Kuramura A, Shirasaka N, Suzuki Y, Yamamoto T, Fujiwara S, Takagi M, Imanaka T (1999) Purification and characterization of an extremely thermostable cyclomaltodextrin glucanotransferase from a newly isolated hyperthermophilic archaeon, a *Thermococcus* sp. Appl Environ Microbiol 65:1991–1997

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Yang SJ, Lee HS, Park CS, Kim YR, Moon TW, Park KH (2004) Enzymatic analysis of an amylolytic enzyme from the hyperthermophilic archaeon *Pyrococcus furiosus* reveals its novel catalytic properties as both an α-amylase and a cyclodextrin-hydrolyzing enzyme. Appl Environ Microbiol 70:5988–5995