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Identification of an Extracellular Thermostable Glycosyl Hydrolase Family 13 α-Amylase from *Thermotoga neapolitana*

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We cloned the gene for an extracellular α-amylase, AmyE, from the hyperthermophilic bacterium *Thermotoga neapolitana* and expressed it in *Escherichia coli*. The molecular mass of the enzyme was 92 kDa as a monomer. Maximum activity was observed at pH 6.5 and temperature 75°C and the enzyme was highly thermostable. AmyE hydrolyzed the typical substrates for α-amylase, including soluble starch, amylopectin, and maltooligosaccharides. The hydrolytic pattern of AmyE was similar to that of a typical α-amylase; however, unlike most of the calcium (Ca^{2+})-dependent α-amylases, the activity of AmyE was unaffected by Ca^{2+}. The specific activities of AmyE towards various substrates indicated that the enzyme preferred maltooligosaccharides which have more than four glucose residues. AmyE could not hydrolyze maltose and maltotriose. When maltoheptaose was incubated with AmyE at the various time courses, the products consisting of maltose through maltopentaose was evenly formed indicating that the enzyme acts in an endo-fashion. The specific activity of AmyE (7.4 U/mg at 75°C, pH 6.5, with starch as the substrate) was extremely lower than that of other extracellular α-amylases, which indicates that AmyE may cooperate with other highly active extracellular α-amylases for the breakdown of the starch or α-glucans into maltose and maltotriose before transport into the cell in the members of *Thermotoga* sp.

Keywords: GH 13 α-amylase, hyperthermophiles, thermostability, *Thermotoga neapolitana*

A number of thermostable and thermoactive enzymes produced by hyperthermophiles has generated a great interest for studies in the field of biotechnology. These enzymes are excellent catalysts for the hydrolysis (under industrially relevant conditions) of both simple and complex plant polymers that have potential as renewable energy sources. Among the free-living prokaryotes known to date, members of the genus *Thermotoga* have been reported to have the largest number of genes encoding carbohydrate-active enzymes (Nelson *et al*., 1999). The members of the genus *Thermotoga* are strictly anaerobic fermentative heterotrophs that grow on a variety of simple and complex sugars, including ribose, xylose, glucose, galactose, sucrose, maltose, lactose, starch, and glycogen. *Thermotoga neapolitana* and *T. maritima* have been reported to catalyze the hydrolysis of a wide variety of α-linked glucans via fermentative metabolism (Duffaud *et al*., 1997; Ruile *et al*., 1997; Bibel *et al*., 1998; Miller *et al*., 2001; Nguyen *et al*., 2001; Chhabra *et al*., 2002, 2003; Veith *et al*., 2003; Conners *et al*., 2005). Our research interest includes investigating the enzymatic properties of the specific enzymes that are involved in the breakdown and utilization of starch. The vast majority of starch-hydrolyzing α-amylases are classified into glycoside hydrolase family 13 (GH 13) (Henrissat, 1991). Some α-amylases are also classified into GH 57. The latter enzyme family has fewer members than those in GH 13, and enzymes belonging to GH 57 have not been investigated in detail.

Among the starch-degrading enzymes produced by the members belonging to *Thermotogales*, three α-amylases have been described in the literature thus far. These amylases include one extracellular lipoprotein AmyA (KEGG Database entry TM1840) and two intracellular enzymes AmyB (TM1650 and TM1650 homolog from *T. neapolitana*) and AmyC (TM1438) (Liebl *et al*., 1997; Lim *et al*., 2003; Ballschmiter *et al*., 2006; Park *et al*., 2010). AmyA is a calcium ion (Ca^{2+})-dependent membrane-bound α-amylase that hydrolyzes starch extracellularly in an endo-fashion, but the low activity of this enzyme prevents the complete breakdown of the starch. AmyA also exhibits less activity for the hydrolysis of more highly branched polysaccharides such as glycogen, amylopectin, and pullulan (Schumann *et al*., 1991; Liebl *et al*., 1997). The activity of intracellular α-amylases AmyB and AmyC appears to complement that of the α-amylase which hydrolyzes amylose and starch, and the catalytic properties of these enzymes have been studied (Lim *et al*., 2003; Ballschmiter *et al*., 2006; Park *et al*., 2010). The two intracellular α-amylases are thought to be involved in the breakdown of maltodextrin into maltose and glucose, which are further utilized as energy sources or used for production of storage polysaccharides like glycogen, but the physiological roles of these enzymes remain to be clarified.

In this study, we have studied an α-amylase from *T. neapolitana* in addition to the three α-amylases that have been previously reported. The gene encoding the putative α-amylase (AmyE) from *T. neapolitana* was cloned and expressed in *E. coli* for examining the biochemical and catalytic properties of the enzyme. The enzymatic characteristics indicate that the putative α-amylase, AmyE, is an endo-acting extracellular

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α-amylase that produces various sizes of maltooligosaccharides from hydrolysis of starch or α-linked glucans.

Materials and Methods

Bacterial strains and culture conditions

T. neapolitana KCCM 41025 was obtained from the Korean Culture Center of Microorganisms (KCCM) and was cultivated under anaerobic conditions as described previously (Park *et al*., 2005). *E. coli* MC1061 was used as the heterologous host for protein overexpression. *E. coli* transformants were grown at 37°C in Luria-Bertani (LB) broth [1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl] containing ampicillin (50 μg/ml). The plasmid p6xHis119 was used as the cloning and expression vector (Kim *et al*., 1999).

Sequencing and identification of the gene encoding an α-amylase from *T. neapolitana*

In a previous study, a β-glucan glucohydrolase gene cluster containing the partial open reading frame (ORF) of a putative α-amylase was identified from the chromosome database of *T. neapolitana* KCCM 41025 (Yernool *et al*., 2000). To determine the full-length sequence of the α-amylase gene in the cluster, we amplified the downstream DNA region by primer walking using only a specific primer 5′-TGCT ACTTCTTGGCATGACGTT-3′ derived from the known sequence of α-amylase. The amplified 400-bp fragment was isolated for nucleotide sequencing to confirm the sequences. On the basis of the data from the sequenced DNA regions and BLAST search, we designed primers for determining the complete ORF of a putative α-amylase. The 2 sense primers designed on the basis of data from sequencing of DNA regions by primer walking were 5′-AACAGAGAGAAATGAATGTT TACCACTGGAC-3′ and 5′-GACAATTTTATCCAATAAAGAAGG AGCAGGTC-3′, and the antisense primer designed on the basis of the sequence of Tpet_0954 gene encoding an extracellular solute-binding protein from the chromosome database of *T. petrophila* RKU-1 was 5′-TATCTGGGTACTCTGGTGGAACATCTC-3′. The complete ORF of a putative α-amylase that was identified was designated as *amyE*. For cloning and expression of the putative α-amylase, *amyE* was amplified from the genomic DNA using 5′-CAAACATATGTCT TGGCATGACGTTGTA-3′ (sense) and 5′-CTTTGCATGCCTATTT TTCGATTTTAAT-3′ (antisense) (the underlined regions indicate the *Nde*I and *Sph*I sites, respectively). The polymerase chain reaction (PCR) product was digested using the restriction enzymes *Nde*I and *Sph*I and was cloned into the plasmid p6xHis119 digested using the same restriction enzymes to construct the expression plasmid p6xHis119-AmyE. For efficient expression of the enzyme in *E. coli*, we deleted 54 nucleotides in the amino terminal region that corresponded to the signal peptide from ATG start codon.

Expression and purification of the recombinant AmyE enzyme

The recombinant *E. coli* MC1061 strain carrying p6xHis119-AmyE constitutively expressed recombinant AmyE without IPTG induction. After the *E. coli* transformants were grown in an ampicillin-supplemented LB broth overnight, they were harvested by centrifugation (10,000×g, 20 min, 4°C) and resuspended in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 5 mM imidazole. Cells were disrupted by 3 passages through a French pressure cell (American Instruments, USA). The crude cell extract was centrifuged at 12,000×g (20 min, 4°C) to remove cell debris. Subsequently, the supernatant was incubated at 80°C for 30 min to denature the thermolabile host proteins and centrifuged again at 12,000×g (20 min, 4°C) to remove the denatured protein from the extract. After removing the precipitant, AmyE with the 6-His tag was purified by performing affinity chromatography by using a nickel-nitrilotriacetic acid (Ni-NTA) column (GE Healthcare/Amersham, Germany). The molecular mass of the native enzyme was estimated by gel-filtration chromatography by using a Sephacryl S-200 HR 16/60 column equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. Invertase (270 kDa), α-amylase (200 kDa), alcohol dehydrogenase (150 kDa), amyloglucosidase (97 kDa), and bovine serum albumin (66 kDa) were used as the standard proteins, and blue dextran was used to calculate the void volume. The purity of the recombinant AmyE protein was determined by SDS-PAGE. The protein concentration was determined according to the Bradford method (Bradford, 1976) by using bovine serum albumin as a standard.

EC	Enzyme	I	II (*)	III (*)	IV (*)
2.4.1.19	Cyclodextrin glucanotransferase	135- DFAPNH	225- .GIRVDAVKHM	253- FIFGEWF.	323- FIDNHDMDRFK
3.2.1.1	Taka_α-amylase A	117- DVVANH	202- DGLRIDTVKHV	226- YCIGEVLD	297- FVENHDNPRFA
3.2.1.133	Maltogenic α-amylase	127- DFVPNH	221- .GLRIDAVKHF	249- FLVGEWY.	321- FTDNHDMSRFL
3.2.1.133	Maltogenic amylase	242- DAVFNH	324- DGWRLDVANEI	353- YILGEIWR	419- LLGSHDTPRLL
3.2.1.135	Neopullulanase	239- DAVFNH	321- DGWRLDVANEV	356- LIVGEIWH	416- LLDSHDTERFL
3.2.1.10	Oligo-1,6-glucosidase	98- DLVVNH	195- .GFRMDVINFI	251- MTVGEMPG	324- YWNNHDQPRVV
3.2.1.60	Maltotetraohydrolase	112- DVVPNH	189- GGFRFDFVRGY	215- FCVGELWK	288- FVDNHDTGYSP
3.2.1.68	Isoamylase	292- DVVYNH	371- DGFRFDLASVL	431- VVRVEWSV	525- IYVTQDANDFS
	Amy13A	141- DLVINH	223- .DFRIDAAKHT	272- ILVGEVFS	323- FLENHDLHRFF
	Amy13B	98- DMVLNH	164- DGFRCDVAGLV	193- IWLSETHD	252- FLENHDQPRIA
	AmyE	138- DIVVNH	239- DGFRVDTAMYV	272- IAFGEAWL	344- FIDNHDMERFI

Fig. 1. Comparison of the conserved region of the amino acid sequence of AmyE and those of other amylolytic enzymes belonging to GH 13. The invariant sequences are highlighted in black-and-white, and inversion and the catalytic triad are indicated by asterisks. The highly conserved sequences are shown within boxes. Amy13A, *T. maritima* extracellular α-amylase (CAA72194); Amy13B, *T. maritima* intracellular α-amylase (AAD36717); AmyE, *T. neapolitana* extracellular α-amylase (ADN95584); *A. oryzae* Taka-α-amylase A (BAA00336); *B. circulans* strain 8, cyclodextrin glucanotransferase (CAA48401); *B. cereus*, oligo-1,6-glucosidase (CAA37583); *Pseudomonas stutzeri* maltotetraohydrolase (AAA25707); *P. amyloderamosa* isoamylase (AAA25854); *T. vulgaris* R-47 neopullulanase (BAA02473); *Thermus* sp. IM6501 maltogenic amylase (AAC15072); *B. stearothermophilus* maltogenic α-amylase (AAA22233). Multiple sequence alignment was performed using CLUSTAL W2 and was visualized using ESPript (http://espript.ibcp.fr/ESPript/ESPript/).

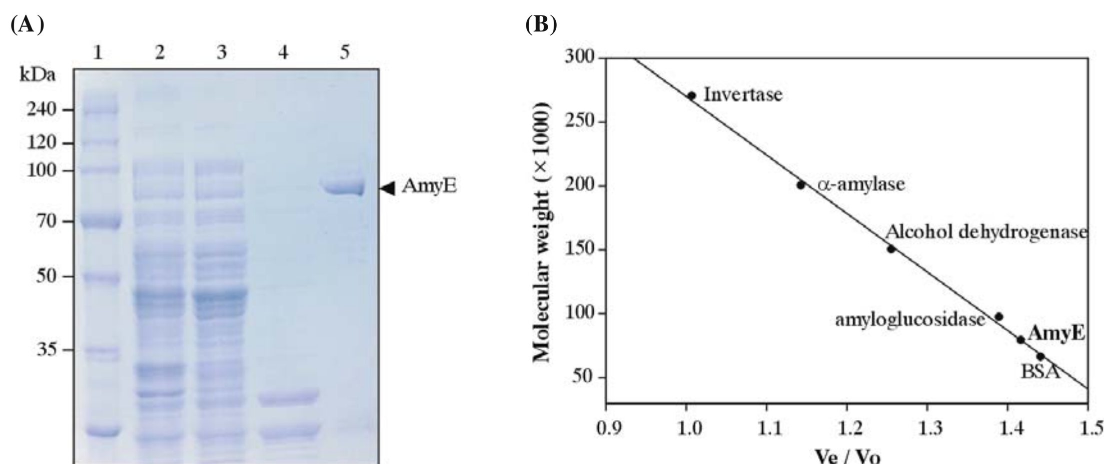

Fig. 2. SDS-PAGE analysis of the enzyme obtained during each purification step. SDS-PAGE was performed at each purification step. Lanes: 1, molecular mass markers; 2, total proteins after cell disruption; 3, soluble proteins (crude extract); 4, proteins after heat treatment; 5, proteins after Ni-NTA column chromatography. (B) Determination of molecular mass of AmyE by gel-filtration chromatography. The molecular mass of the native AmyE was determined by analyzing the molecular mass of standard proteins eluted during chromatography performed using a Sephacryl S-200 HR 16/60 column.

Enzyme assay

Enzyme activity was assayed at 75°C in 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 6.5) by measuring the amount of reducing sugar released from the hydrolysis of soluble starch (Waffenschmidt and Jaenicke, 1987). The reaction mixture (0.5 ml) consisted of 0.1 ml of 1% (w/v) soluble starch as a substrate and 0.03 ml of enzyme solution (20 U/ml). The reaction mixture was incubated for 90 min at 75°C to facilitate the enzymatic reaction, and the reaction was terminated by quenching on ice. An aliquot (100 μl) of the reaction mixture was added to 0.5 ml of the copper-bicinchoninate working reagent and 0.4 ml of distilled water. The reaction mixture was incubated at 80°C for 35 min and then cooled on ice for 15 min. The color that developed was measured at 570 nm, and the specific activity was calculated using maltose as a standard. One unit of AmyE was defined as the amount of enzyme that released 1 nmol of reducing sugar equivalents per minute under the described test conditions.

Effects of pH and temperature on the activity of AmyE

The influence of pH on the activity of AmyE was measured at 75°C in sodium acetate (pH 4.5 to 5.5), MES (pH 5.5 to 6.5), HEPES (pH 6.5 to 8.0), and Tris-HCl (pH 8.0 to 9.0). The pH of each buffer system was adjusted at 75°C according to the standard enzyme assay conditions described above. The influence of temperature on the activity of AmyE was determined in 50 mM MES buffer (pH 6.5) at a temperature range from 50-90°C.

The thermostability of the enzyme was determined by incubating the enzyme solution (4 mg/ml in 50 mM MES buffer, pH 6.5) at 75, 80, and 90°C. Aliquots were collected every hour and immediately placed in an ice-water bath to halt enzymatic activity. The residual soluble starch-hydrolyzing activities of the aliquots were measured at the optimal temperature conditions. The effect of Ca^{2+} on enzyme activity and stability was also examined. The enzyme activity was determined according to the above mentioned methods after the addition of 10 mM $CaCl_2$, and the thermostability of the enzyme was examined at 90°C in the presence of 10 mM $CaCl_2$.

Hydrolytic patterns of AmyE

To determine the hydrolytic patterns of AmyE, we incubated purified AmyE (5 U) with 0.5 ml of 0.5% (w/v) maltooligosaccharides (maltotriose to maltoheptaose), soluble starch, amylopectin, pullulan, glycogen, α-cyclodextrin, and β-cyclodextrin in 50 mM MES buffer (pH 6.5) at 75°C for 16 h. The resulting hydrolysis products were analyzed by thin-layer chromatography (TLC) on Whatman K5F silica gel plates (Whatman, UK) by using 1-butanol/ethanol/H_2O (5:5:3, v/v/v) as the solvent system. After irrigating twice, the TLC plate was dried and visualized by dipping it into a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol and then heating it for 5 min at 120°C (Robyt and Mukerjea 1994).

Results and Discussion

Cloning and sequence analysis of the *amyE* gene

The partial sequence of the *amyE* gene was determined using a degenerate primer derived from the nucleotide sequence of the CTN_0781 gene submitted to GenBank (Accession no. CP000916). BLAST search showed that the partial DNA sequence of *amyE* had a high degree of homology with that

Table 1. Purification of AmyE expressed in *E. coli*

Purification	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	9,240	25,480	2.8	1.0	100
Heat treatment	2,150	9,890	4.6	1.6	39
Ni-NTA	667	4,936	7.4	2.7	19

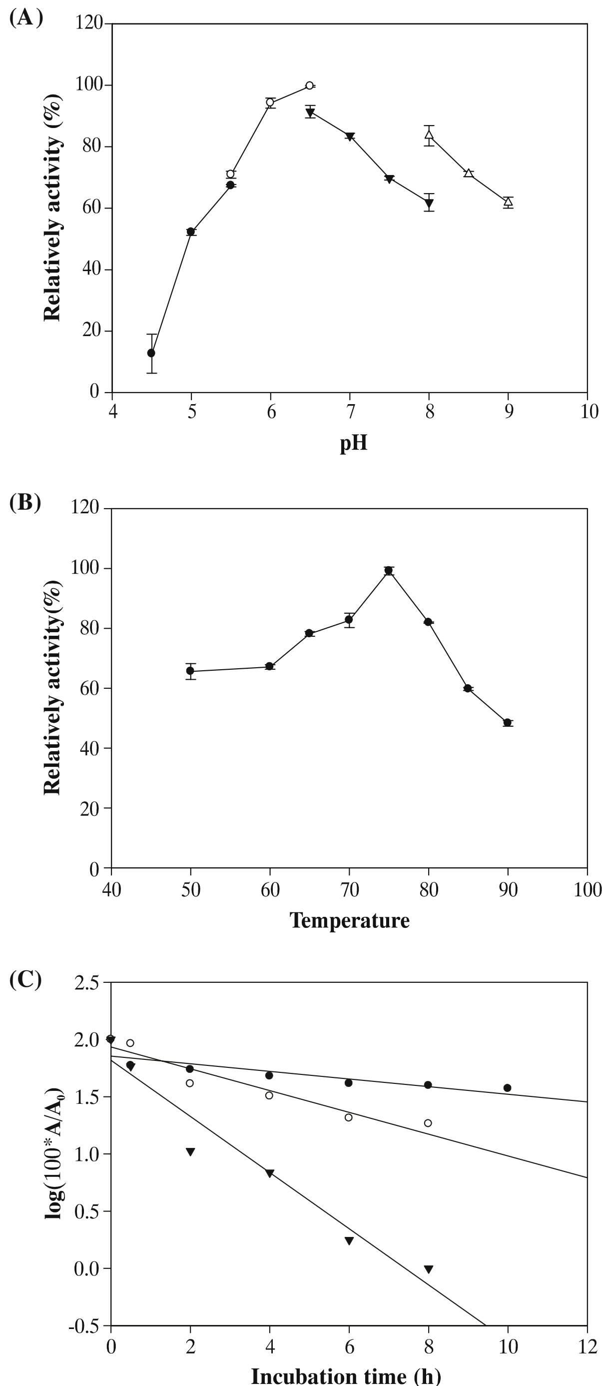

Fig. 3. Effect of pH and temperature on the activity and stability of AmyE. (A) For the determination of optimal pH for enzyme activity, we used the following buffers: 50 mM sodium acetate (●), pH 4.5 to 5.5; 50 mM MES (○), pH 5.5 to 6.5; 50 mM HEPES (▼), pH 6.5 to 8.0; 50 mM Tris-HCl (△), pH 8.0 to 9.0. The enzyme activity was assayed at 75°C for 90 min in 50 mM of the different buffers. (B) To determine optimal temperature, we measured the enzyme activity at the temperatures indicated in the standard assay procedures. (C) To determine the thermostability of AmyE, we incubated the purified enzyme at 75°C (●), 80°C (○), and 90°C (▼) in 50 mM MES buffer (pH 6.5). After various time intervals, samples were withdrawn and the residual activities were measured at 75°C as indicated in the standard assay procedure.

of the Tpet_0953 gene encoding α-amylase from *T. petrophila* RKU-1. The remaining sequence was determined by using primers derived from the initially sequenced DNA regions and the known sequence of the Tpet_0954 gene, which is adjacent to the Tpet_0953 gene. The remaining sequence we solved by primer walking method was cloned into pGEM-T and sequenced. After the new sequence was solved, the new primer was constructed and continued to read the remaining sequence. The complete ORF was finally cloned into pGEM-T and then confirmed. The *amyE* gene consisted of 2,448 base pairs and encoded a protein consisting of 815 amino acids with a predicted molecular mass of 91,799 Da (GenBank accession no. HQ207193). The signal peptide of AmyE protein comprised 18 residues, which indicates that this α-amylase was an extracellular enzyme. The complete ORF reveals that in *amyE*, the initiation codon ATG was located 334 nucleotides upstream of that of CTN_0781, which has been annotated in the whole genome sequencing of *T. neapolitana* DSM4359.

Comparison of the amino acid sequence of AmyE with various amylolytic enzymes

The deduced amino acid sequence of *amyE* shows 76% identity with the sequences of putative α-amylases from *T. petrophila* RKU-1 and *T. naphthophila* RKU-10, 76% identity with an α-amylase AmyA from *Marinitoga piezophila* KA3, and 57% identity with a putative α-amylase from *Fervidobacterium nodosum* Rt17-B1. Although 4 well-known conserved regions (I, II, III, and IV), including invariant catalytic residues of GH 13 were evident, the amino acid sequence of *amyE* exhibited unexpectedly low sequence similarity (5-10% identity) with other amylolytic enzymes of GH 13, including α-glucosidase (EC 3.2.1.20) (Peist *et al*., 1996), α-amylase (EC 3.2.1.1) (Stam *et al*., 2006), oligo-1,6-glucosidase (EC 3.2.1.10) (Oslancová and Janecek, 2002), maltogenic amylase (EC 3.2.1.133) (Park *et al*., 2000), and CD-/pullulan hydrolyzing enzymes (Park *et al*., 2000; Lee *et al*., 2002) (Fig. 1). The complete amino acid sequence of AmyE was completely different from that of the two α-amylases (AmyA and AmyB) previously characterized from *Thermotoga* sp. (Liebl *et al*., 1997; Park *et al*., 2010). The simulated three-dimensional structure of AmyE showed that N-terminal domain of AmyE has 33% identity to the structure of α-amylase 2 from *Thermoactinomyces vulgaris* R-47 (Ohtaki *et al*., 2004). However, the C-terminal domain was not matched with any other known structures. Although the expected three-dimensional structure of AmyE is locally similar to the known α-amylase, the remarkable differences of C-terminal part and the variations in the amino acid sequences among GH 13 enzymes implies that AmyE might be a new type of an amylolytic enzyme.

Expression of AmyE in *E. coli*

The AmyE protein was highly expressed in *E. coli* MC1061 harboring p6XHis119-AmyE. Heat-treatment (80°C for 30 min) efficiently excluded substantial amounts of thermolabile host proteins from cell-free extracts, and the protein was further purified by chromatography by using Ni-NTA column. The purified enzyme had a specific activity of 7.4 U/mg and showed a single band corresponding to 92 kDa in SDS-PAGE (Fig. 2A and Table 1). The molecular mass of the enzyme

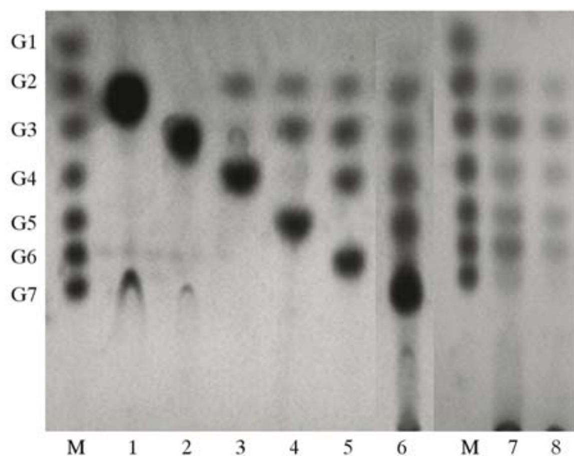

Fig. 4. Hydrolytic patterns of different substrates of AmyE. Lanes: M, maltooligosaccharide standards (glucose to maltoheptaose); 1, maltose; 2, maltotriose; 3, maltotetraose; 4, maltopentaose; 5, maltohexaose; 6, maltoheptaose; 7, soluble starch; 8, amylopectin. AmyE was incubated with the various substrates at a concentration of 0.5% (w/v) at 75°C for 16 h.

determined by gel-filtration chromatography was same as that determined by SDS-PAGE (Fig. 2B), which indicated that the enzyme is monomeric. These findings are consistent with the findings from prediction of the sequence analysis of AmyE, which lacks an extra N-terminal domain that is known to be involved in the oligomerization of the enzyme (Kim *et al.*, 2001).

Enzymatic properties of AmyE

The pH range in which the recombinant AmyE was active was determined using soluble starch as the substrate. As shown in Fig. 3A, maximum enzymatic activity was observed at pH 6.5. AmyE is active in a broad temperature range from 50°C to 90°C (Fig. 3B). The optimal temperature for AmyE was approximately 75°C. AmyE exhibited a high degree of thermostability; almost the entire activity of the enzyme was retained after 10 h of incubation at 75°C. The half-lives of AmyE were determined to be 2.6 and 0.4 h at 80°C and 90°C, respectively (Fig. 3C). We examined the effects of Ca^{2+} on the enzyme activity and thermostability. The enzyme was not activated by the presence of up to 10 mM Ca^{2+}, and its thermostability was not also affected (data not shown).

The substrate specificity of AmyE was examined using various substrates typically used for α-amylases, such as maltooligosaccharides, soluble starch, amylopectin, glycogen, pullulan, α-cyclodextrin, and β-cyclodextrin. AmyE hydrolyzed soluble starch, amylopectin, and maltooligosaccharides of various sizes (Fig. 4). In contrast, pullulan, α-cyclodextrin, and β-cyclodextrin were barely degraded by AmyE (data not shown). The hydrolytic pattern of AmyE was also confirmed by measuring the specific activity of AmyE (Table 2). The specific activities of AmyE indicated that the enzyme preferred maltooligosaccharides which have more than four glucose residues. AmyE could not hydrolyze maltose and maltotriose. AmyE-mediated hydrolysis of maltotetraose resulted in maltose, and the hydrolysis of maltopentaose, maltohexaose, and maltoheptaose yielded the mixtures of maltose, maltotriose, maltotetraose, and maltopentaose.

To understand the detailed mechanism of action of AmyE, the hydrolytic pattern was further investigated as a function of time by using maltoheptaose as a substrate. AmyE was incubated with maltoheptaose at 75°C for various reaction times ranging from 0 min to 36 h to investigate the changes in the reaction products. Maltotetraose and maltotriose were produced first after 4 h, and then, maltopentaose, maltotetraose, maltotriose, and maltose were almost equally produced after 8 h (Fig. 5). Glucose and maltohexaose were not observed up to 36 h, indicating that the enzyme could not attack the non-reducing or reducing end of the substrate. Because the enzymatic activity of AmyE was extremely lower than that

Table 2. Substrate specificity of AmyE towards various α-linked glucans

Substrate	Specific activity (U/mg)
Starch	2.8
Amylose	1.2
Amylopectin	1.4
Glycogen	ND
Pullulan	ND
α-Cyclodextrin	0.12
β-Cyclodextrin	0.15
Maltose	ND[a]
Maltotriose	ND
Maltotetraose	19
Maltopentaose	22
Maltohexaose	28
Maltoheptaose	39

[a] ND, not detected

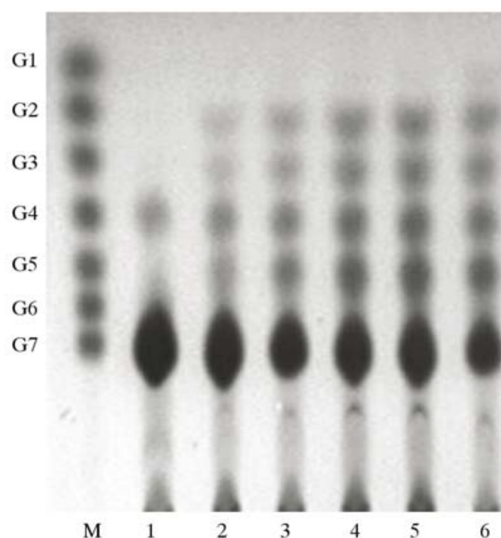

Fig. 5. AmyE hydrolysis products of maltoheptaose as a function of reaction time. Lanes: M, maltooligosaccharide standards (glucose to maltoheptaose); 1-6, hydrolysis products of maltoheptaose at different reaction times (4, 8, 12, 16, 24, 36 h, respectively). AmyE was incubated with 0.5% (w/v) maltoheptaose at 75°C.

of other known α-amylases, complete hydrolysis of the substrates was not observed after 36 h. The hydrolytic pattern of various AmyE substrates indicates that AmyE hydrolyzes maltooligosaccharides in an endo-fashion.

We cloned and expressed a putative α-amylase *amyE* gene from *T. neapolitana*. Although the specific activity of the enzyme was low, the substrate specificity of the enzyme indicated that the enzyme was an unidentified extracellular α-amylase with high thermostability. The pathway for utilization of α-linked glucan in *Thermotoga* sp. has been extensively studied (Bibel *et al*., 1998; Chhabra *et al*., 2003; Conners *et al*., 2005). An extracellular α-amylase, AmyA, has been characterized from *T. maritima*, and its homologous proteins have also been identified in other *Thermotoga* sp. by sequence alignment. AmyA showed much higher activities than AmyE for the hydrolysis of α-linked glucans. Both the enzymes hydrolyzed the substrates in an endo-fashion, a characteristic of most extracellular enzymes. Our results indicate that AmyE may be involved in the utilization of starch or α-glucans in *Thermotoga* sp. by assisting the highly active extracellular α-amylase AmyA. Both enzymes perform the reactions necessary to break down the complex polysaccharides into maltose and maltotriose for transport into the cell. Binding of maltose and maltotriose involves maltose or maltodextrin binding proteins. Following transport into the cell, hydrolysis likely involves the α-glucosidase, which is active on maltose and maltotriose but not starch, amylopectin or amylose (Bibel *et al*., 1998). Further studies should be performed to understand the physiological role of this extracellular enzyme in *Thermotoga* sp.

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