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A GH57 Family Amylopullulanase from Deep-Sea *Thermococcus siculi*: Expression of the Gene and Characterization of the Recombinant Enzyme

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Abstract The gene encoding a new extracellular amylopullulanase (type II pullulanase) was cloned from an extremely thermophilic anaerobic archaeon *Thermococcus siculi* strain HJ21 isolated previously from a deep-sea hydrothermal vent. The functional hydrolytic domain of the amylopullulanase (TsiApuN) and its MalE fusion protein (MTsiApuN) were expressed heterologously. The complete amylopullulanase (TsiApu) was also purified from fermentation broth of the strain. The pullulanase and amylase activities of the three enzymes were characterized. TsiApu had optimum temperature of 95°C for the both activities, while MTsiApuN and TsiApuN had a higher optimum temperature of 100°C. The residual total activities of MTsiApuN and TsiApuN were both 89% after incubation at 100°C for 1 h, while that of TsiApu was 70%. For all the three enzymes the optimum pHs for amylase and pullulanase activities were 5.0 and 6.0, respectively. By analyzing enzymatic properties of the three enzymes, this study suggests that the carboxy terminal region of TsiApu might interfere with the thermoactivity. The acidic thermoactive amylopullulanases MTsiApuN and TsiApuN could be further employed for industrial saccharification of starch.

Introduction

To date, industrial processes for starch hydrolysis to glucose mainly rely on enzyme catalysis, usually involving liquefaction and saccharification processes. The conventional condition for liquefaction is about pH 6 and 105°C, while that of the following step saccharification is around pH 4.5 and 60°C [13, 21, 24]. Variation of parameters in the two steps causes handicaps and waste of costs in the industry [15, 23]. Pullulanases (pullulan-6-glucanohydrolase [EC 3.2.1.41]) are interesting in starch processing industry, especially amylopullulanases (type II pullulanases) which are able to degrade both the α-1,6 and α-1,4 glucosidic bonds of starch [12, 17]. Motivated by decreasing the industrial costs and increasing the efficiency, great efforts have been carried to find acidic thermoactive amylolytic enzymes like amylopullulanse and glucoamylase to make liquefaction and saccharification processes combined into one-step-process [2, 13, 23]. Amylopullulanses with high thermoactivity under the enzymatic condition of liquefaction process are desired.

According to CAZy (Carbohydrate-Active enZymes) family classification system based on protein sequence and structure similarity, glycoside hydrolases (GH) have been classified into 115 families [4]. Amylopullulanases involve two groups, thermoactive amylopullulanases belonging to GH57 or GH13 family and mesophilic amylopullulanase usually belonging to GH13 family [14, 26]. Many thermophilic microorganisms, especially *Thermoanaerobacterales* and *Thermococcales* possess GH57 amylopullulanase, such as ThyApu from *Thermococcus hydrothermalis* [7, 8], PfuApu from *Pyrococcus furiosus* [6]. Catalytic mechanism and basis for thermoactivity of GH57 amylopullulanases also have aroused academic interest for research. The issues have long been the hot spots of thermoenzymes research since the sequence of a thermoactive amylase was first reported 15 years ago [9]. To date, the knowledge about thermoactive amylopullulanases especially those belonging

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to GH57 family are still poor and limit the speed of research and industrial application [10].

In previous work, we had isolated an anaerobic archaeon *Thermococcus siculi* strain HJ21 and deposited it in China Center for Type Culture Collection with number M207010 [25]. A Ca^{2+}-independent hyperthermophilic α-amylase THJA from the strain HJ21 had been identified. Enzymatic properties of the enzyme had been characterized and it had been classified into GH13 family. The optimal enzymatic condition of the α-amylase is pH 5 and 95°C. In this study, we identified, characterized, and heterologously expressed a new GH57 amylopullulanase TsiApu from the strain HJ21. The objectives of this study are to explore new GH57 amylopullulanase that could be applied to industrial saccharification in tandem with THJA in lower cost and high efficiency. The function of the C-terminal region of GH57 amylopullulanase is to be investigated by comparing enzyme activities of N-terminal hydrolytic functional region of the new amylopullulanase (TsiApuN), MalE fusion protein of TsiApuN (MTsiApuN) and the complete amylopullulanase (TsiApu).

Materials and Methods

Cloning of TsiApu Gene Fragment TsiApuF

Multiple amino acid sequence alignments of GH57 amylopullulanases among *Thermococcus hydrothermalis* (ThyApu; GenBank gi: 4731920), *Pyrococcus furiosus* (PfuApu; gi: 75993212), *Pyrococcus abyssi* (PabApu; gi: 14520398), *Thermococcus onnurineus* (TonApu; gi: 212224943), *Thermococcus gammatolerans* (TgaApu; gi: 240102660), *Thermococcus kodakarensis* (TkoApu; gi: 57641709), *Thermococcus litoralis* (TliApu; gi: 22759875), were built (data not shown). All the sequences used were retrieved from GenBank (NCBI). To minimize number of the primers designed targeted at the translated nucleotide sequences from conserved motifs, amino acids with low codon degeneracy were preferred. Considering the length of the target fragments appropriate for PCR amplification, we selected two conserved motifs within a distance of 160 residues, QHQPYYY and EFTEQDY as target sites for PCR primers design. According to the preferred usage of G or C base in thermoenzyme genes, we designed 32 primers targeting QHQPYYY and 16 of them were selected as forward primers (FP). Sixteen primers were designed targeting EFTEQDY and eight of them were selected as reverse primers (RP; Table 1). Genomic DNA of *Thermococcus siculi* strain HJ21 was used as template and all PCR reagents (ExTaq, dNTPs, et al.) were from Takara. PCR was run on Tpersonal (Whatman Biometra) under an amplification condition: one cycle of 94°C, 2 min, then 30 cycles of 94°C, 30 s; 65°C, 30 s; 72°C, 30 s, followed by one cycle of 72°C for 5 min. PCR amplicons of predicted molecular size (about 400–500 bp) were cloned into pMD18-T vector (Takara) and verified by sequencing (ABI-3730 sequencer; Applied Biosystems) and analogy against GenBank.

Genome Walking for TsiApu Gene

The strain HJ21 was cultured in conditions reported in our previous work [25]. The cells were harvested at exponential phase of growth. Genomic DNA was isolated with Takara DNA extraction kit. Average molecular size of the genomic DNA was about 25 kbp and concentration was 0.5 μg/μl. Construction of four pools of adaptor-ligated genomic DNA fragments and elongation of the unknown TsiApu gene from both ends of the TsiApuF fragment were performed under instruction of GenomeWalker Universal Kit (Clontech). Primers designed for walking were shown in table 1. A 2-kb amplicon was obtained with primers GSPR22 and AP2 from genomic DNA fragment pool generated by *Pvu* II digestion. A 4 kb amplicon was obtained with primers GSPF22 and AP2 from genomic DNA fragment pool generated by *Dra* I digestion. The two amplicons were sequenced and the sequences were spliced

Table 1 Primers used in the experiments

Products	Primers	Sequences
TsiApuF	FP	CAGCACCAGCCNTAYTAYTAY
	RP	GTAGTCCTGCTCNGTRAAYTC
TsiApuN	NFP	GCGAATTCATGAGGCGGGTGGTTGCCCTAC(*Eco*RI)
	NRP	GCGTCGACTCAGCGCCTCCTTCTGAGGAGG(*Sal*I)
Genome walking for TsiApu gene	GSPR21	GTACTGGCTCAGGTAGTAGGC
	GSPR22	AGTAGTTGTTGGCAGCGTGGAGC
	GSPF21	GACCGCTACACGGAGCTGAAGG
	GSPF22	GCAGAAAGTCGCGGTCACCAACG
	AP1	GTAATACGACTCACTATAGGGC
	AP2	ACTATAGGGCACGCGTGGT

into a 4,056-bp complete TsiApu gene sequence (GenBank accession number EU849120).

Sequence Analysis of TsiApu Protein

Amino acid sequence of TsiApu protein (GenBank gi: 209973469) was compared to other pullulanase sequences in GenBank by using the program BLAST (blastp; NCBI) and submitted to InterPro Scan program (EBML-EBI) for domain search. Amino acid composition and physicochemical parameters of TsiApu protein were analyzed by DNA tool 6.0.122.

Expression and Purification of MTsiApu and TsiApuN

DNA fragment encoding the N-terminal hydrolytic functional region TsiApuN were amplified with PCR primers NFP and NRP (Table 1). A 2338-bp amplicon was double digested with *Eco*RI–*Sal*I, agarose gel purified and ligated into pMAL-c2x (NEB) predigested with *Eco*RI–*Sal*I leading to the pMAN plasmid. It was then transformed into *E. coli* BL21 (DE3; Novagen) strain using conventional calcium chloride method. Positive clones were selected by colony PCR and verified by DNA sequencing of the plasmid pMAN. The recombinant MalE fusion protein MTsiApuN was expressed and purified under the manufacture's instruction of pMAL^{TM} protein fusion and purification system. Homogeneity and molecular size of MTsiApuN were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel. The protein concentrations were determined in triplicate with the Bradford assay (Bio-Rad) with bovine albumin as a standard. The purified MTsiApuN protein was then cleaved by Factor Xa at room temperature overnight. The single TsiApuN protein in the reaction mixture was purified by rebinding MalE to the amylose column. In our further attempt, the complete TsiApu gene was also cloned into pMAL-c2x and pET-22b (+) vectors (NEB) but both failed to be expressed.

Purification of TsiApu

The strain HJ21 was cultured statically with a modified YPS medium at 88°C in sealed bottles flushed with N_2 for 10 h as described previously [25]. The cells, sulfur granules and other impurities were removed by centrifugation for 5 min at 12,000 rpm and filtration with Millipore filter (0.22 μm; Millipore). Ammonium sulfate was added to the supernatant up to 60% saturation (25°C) and kept for overnight precipitation. The precipitate was collected by centrifugation at 12,000 rpm for 1 h and the pellet was suspended in 0.2 M acetate buffer, pH 6.0. Then the suspension was dialyzed against 50 mM acetate buffer (pH 6.0) with a dialysis tubing (100 k MWCO; Spectrum Laboratories). The dialysates were loaded onto a DEAE-52 column (2 × 25 cm; Pharmacia) equilibrated with 20 mM acetate buffer (pH 6.0). The column was washed with the same buffer, and the fractions with amylopullulanase activity were pooled and concentrated by centricon with 100 kDa cut off membrane. The concentrated fraction was applied to Sephadex G-100 gel filtration column (Phamacia) with dimension 2 cm × 70 cm, equilibrated with 20 mM acetate buffer (pH 6.0). The eluted fractions were concentrated and then loaded onto TSK G2000 SW_{XL} HPLC column (30 cm × 7.8 mm; Tosoh) equilibrated with the same buffer. The fraction with enzyme activity was pooled and concentrated by lyophilization and then subjected to SDS–PAGE for verification of homogeneity and molecular weight.

Enzyme Assay

Pullulanase and α-amylase activities of the three enzymes MTsiApuN, TsiApuN, and TsiApu were assayed by measuring the amount of reducing sugar released during hydrolysis of 1% pullulan (Sigma) and soluble starch (from potato; Sigma), respectively. Enzymatic hydrolysis was performed for 15 min in 20 mM acetate buffer at pH 5.0 for amylase activity and pH 6.0 for pullulanase activity in the absence of Ca^{2+} at 95°C for TsiApu, 100°C for MTsiApuN and TsiApuN. Reactions were stopped by immediate cooling in an ice-water bath. Controls without the recombinant enzymes or with MalE protein were used. The amount of reducing sugar produced was measured by the dinitrosalicylic acid method [18]. All assays were performed in triplicates. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol/min of reducing sugar with an appropriate standard (maltotriose for pullulanase activity and glucose for amylase activity) under the assay conditions.

Effect of Temperature, pH, and Metallic Ions on Enzyme Activities

Optimal temperatures and pHs for amylase and pullulanase activities of MTsiApuN, TsiApuN, and TsiApu were determined in 20 mM acetate buffer at different temperatures from 80 to 120°C (by autoclave) and different pHs from 4.0 to 9.0. Thermostability of the three enzymes were examined by incubation at different temperatures (80, 90, 100, and 110°C) for 5 h. Aliquots were withdrawn at regular intervals (1 h) and immediately stored at 4°C for analysis. Remaining activities of the enzymes at different intervals were measured at 95°C in 20 mM acetate buffer at optimum pHs 5.0 and 6.0 for amylase and pullulanase activities, respectively. The activities of the enzymes not

been heat-treated were set to 100% of relative-activity scale.

The effects of pH on amylase and pullulanase activities of the three enzymes were measured at 95°C (for TsiApu) and 100°C (for MTsiApuN and TsiApuN) in 50 mM citric acid buffer (pH 3.0–6.0), 50 mM sodium phosphate buffer (pH 6.0–8.0), and 50 mM Tris–HCl buffer (pH 8.0–9.0) in the absence of Ca^{2+}. pH stabilities of the three enzymes were also examined by measuring the residual activities after incubation for 4 h in gradient Britton-Robinson buffer (pH 4.0–9.0).

Results and Discussion

The multiple amino acid sequence alignment of the GH57 amylopullulanases showed that TsiApu had all the five conserved motifs of GH57 family: 42_HQP, 263_GNVEVT, 315_WAAESA, 409_NYDGSLVYVV, and 566_AEASDWFWW [26]. The alignment exhibited the highest identity of 87% to ThyApu and the lowest identity of 69% to PabApu. All the sequences showed extremely high level of similarity in full length, especially the N-terminal regions which were more conserved than the C-terminal regions. Sequence analysis by SignalP 3.0 Server [1] found that a signal peptide located at the N termini of TsiApu precursor (from 1 to 27 residues). Conserved domains were found by InterPro Scan, a glycoside hydrolase family 57 domain from 36 to 537 residues, two carbohydrate binding domains (CBDs) family 9-like (CBD9-like),one from 791 to 1031 residues and the other from 1037 to 1270 residues), and a Thr-rich region from 1280 to 1342 residues (Fig. 1). CBD9s have been observed in glycoside hydrolases like xylanases, generally consisting of seven beta-strands in two sheets with a Greek key topology, but with an additional beta-strand at the N-terminus [19].

TsiApu had the typical characters in sequence of thermoenzymes, such as more hydrophobic residues (42.8%) than hydrophilic residues (33.3%) and more acidic residues (14.4%) than basic residues (10.7%). For thermostability, the content of leucine residues in TsiApu was highest (9.06%) among all the amino acids and that was a common feature in all the aligned GH57 amylopullulanases.

The insertion of TsiApuN gene into pMAL-c2X vector was verified by DNA sequencing. SDS–PAGE showed that the recombinant protein MTsiApuN existed in non-secreted form. The trace Factor Xa that remained with TsiApuN was less than 0.1% (w/w) by HPLC analysis. Concentrations of purified MTsiApuN and TsiApuN were 100 μg/ml and 80 μg/ml, respectively. Totally 1.5 g lyophilized TsiApu was purified from 35 l of broth. The 1,162-residue MTsiApuN, 773-residue TsiApuN, and 1324-residue mature TsiApu all showed apparent molecular weight in SDS–PAGE consistent with their calculated molecular mass, 131 kDa for MTsiApuN, 89 kDa for TsiApuN, and 148.6 kDa for TsiApu (Fig. 2).

Low-level productivity of TsiApu (1.5 g/35 liter culture) from native strains HJ21 by fermentation cannot meet industrial need. Genetic manipulations could resolve the problem. However, according to previous studies, gene lengths (usually range 3–5 kbp) of GH57 amylopullulanase, frequent usage of hydrophobic amino acids and unknown thermoactivity mechanisms make handicaps for heterologous expression [8, 11].

Studies on the amylopullulanase from *T. siculi* have rarely been reported. In this study, the N-terminal hydrolysis functional region of TsiApu was highly expressed in mesophilic host *E. coli*, whereas the intact gene failed to be expressed. Interestingly, the same failure was reported in expression trails of ThyApu from *Thermococcus hydrothermalis* [8]. The SDS–PAGE analysis of MTsiApuN expression reflected that the signal peptide was not cleaved, which might be caused by the interference of the MalE protein at the N termini of MTsiApuN. Results showed that expression in form of MalE fusion protein could result in high-level expression (0.16 g/l) of MTsiApuN in cytoplasm.

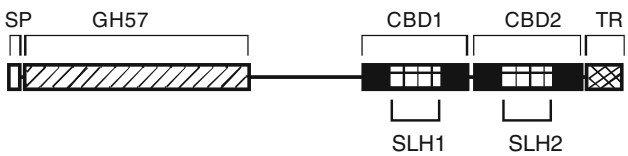

Fig. 1 Predicted multidomain structure of TsiApu. *SP* a signal peptide; *GH57* a glycoside hydrolase family 57 conserved domain; *CBD1* and *CBD2* carbohydrate binding domains (CBDs) family 9-like, two homologous repeats; *TR* a Thr-rich region; *SLH1* and *SLH2* two surface layer homologies

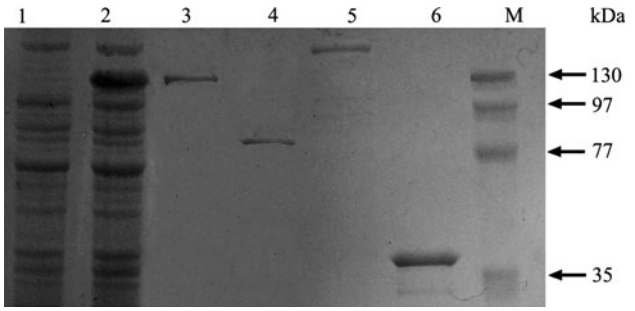

Fig. 2 SDS–PAGE analysis of purified proteins. *Lane 1* BL21-pMAN cell extract, *lane 2* BL21-pMAN cell extract 2 h after IPTG induction, *lane 3* purified MTsiApuN, *lane 4* purified TsiApuN, *lane 5* purified TsiApu, *lane 6* purified MalE protein, *M* molecular mass standard (35 kDa lactase dehydrogenase, 77 kDa siderophilin, 97 kDa phosphorylase B, 130 kDa calmodulin)

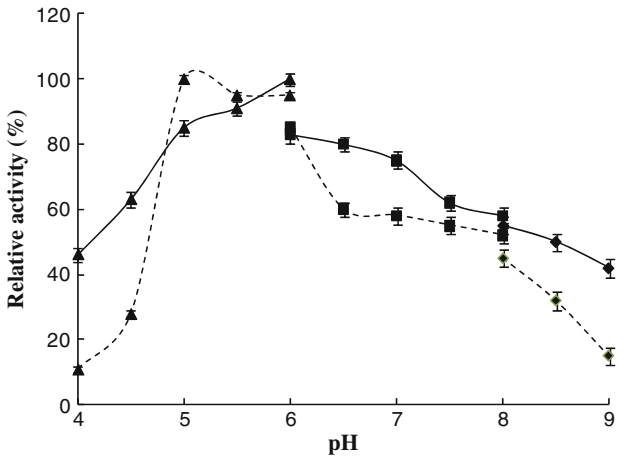

Fig. 3 Effect of pH on amylase (*dashed line*) and pullulanase (*solid line*) activities of TsiApuN. Since the curves of the TsiApu, MTsiApuN, and TsiApuN are close, only the curve of TsiApuN is shown. The following buffers were used: 50 mM citric acid buffer (pH 3.0–6.0; *triangle*), 50 mM sodium phosphate buffer (pH 6.0–8.0; *square*), and 50 mM Tris–HCl buffer (pH 8.0–9.0; *diamond*)

Under the enzyme assay conditions described above and in the absence of Ca^{2+}, the maximum amylase activities of MTsiApuN, TsiApuN, and TsiApu were 6.5, 6.5, and 6.8 U/mg respectively. The maximum pullulanase activities of the above three enzymes were 11.2, 11.0, and 11.3 U/mg, respectively. The enzymes exhibiting both amylase and pullulanase activity confirmed the prediction by amino sequence analysis and was thereby identified as amylopullulanases.

For all the three enzymes, pH optimums of the amylase and pullulanase activities were 5.0 and 6.0, respectively (Fig. 3). The optimal temperatures of TsiApu were 95°C, while MTsiApuN and TsiApuN both showed higher temperature optimum of 100°C (Fig. 4).

MTsiApuN and TsiApuN showed higher thermostability than TsiApu. After incubation at each different temperatures for 1 h, the total residual activities (amylase and pullulanase activities) of MTsiApuN and TsiApuN were both 99, 95, 89, and 56% of the maximum activity at 80, 90, 100, and 110°C, respectively, while that of TsiApu were 96, 91, 70, and 50%, respectively (Fig. 5).

After incubation at optimum temperatures (95°C for TsiApu and 100°C for MTsiApuN and TsiApuN) for 4 h in a pH range from 5 to 6.5, the residual two activities of the three enzymes were above 50% of the maximum activities (Fig. 6). The maximum pH stabilities of all the three enzymes were observed at pH 6.5 for the pullulanase activity and pH 5.5 for the amylase activity, and the two relative activities were both above 90% after 4 h of incubation.

In the absence of Ca^{2+}, the pH (5.0) and temperature optimum (95°C) of amylase activity of MTsiApuN and TsiApuN were in consistent with that of the α-amylase THJA from the strain HJ21 in the same catalytic condition, whereas that of pullulanase activity was different to THJA. However, even under the optimal catalytic condition of THJA, the two recombinant enzymes still have relative activities above 85% (data not shown). The results suggested that the two recombinant enzymes could be used in tandem with the α-amylase THJA in the same catalytic condition, thus provided more feasibility for a one-step process in starch hydrolyzing industry.

For a long time, the thermoactivity property of GH57 has attracted much scientific and industrial interest. To date, catalytic residues of two GH57 amylopullulanases have been experimentally identified, *i.e.* ThyApu from *Thermococcus hydrothermalis* [3, 26] and PfuApu from *Pyrococcus furiosus* [11]. Nevertheless, knowledge about molecular basis of thermoactive amylopullulanases' catalytic activity is still rare, especially the function of the C-terminal domains which are composed of Thr-rich regions and S-layer motifs [22]. The Thr-rich regions have been predicted acting for intensive O glycosylation, while the S-

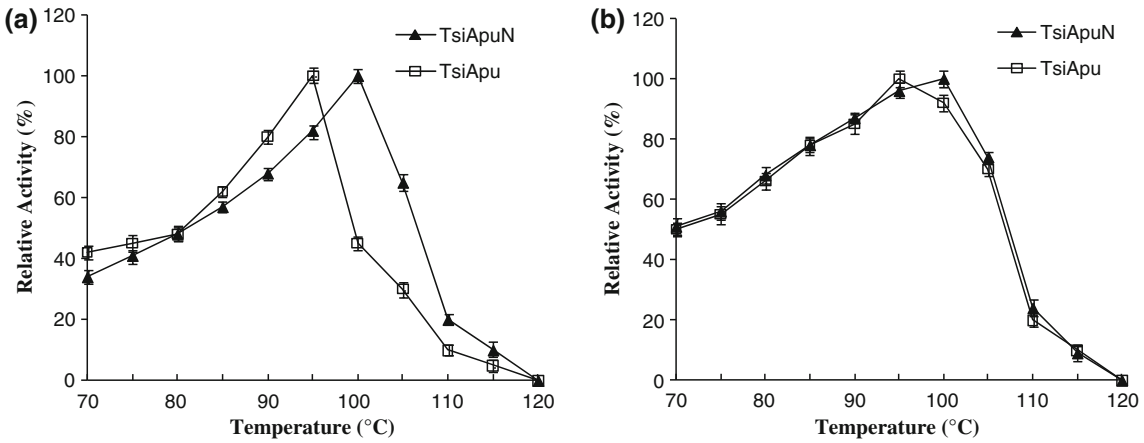

Fig. 4 The optimum temperatures for amylase (**a**) and pullulanase (**b**) activities. TsiApu (*square*), TsiApuN and MTsiApuN (*triangle*)

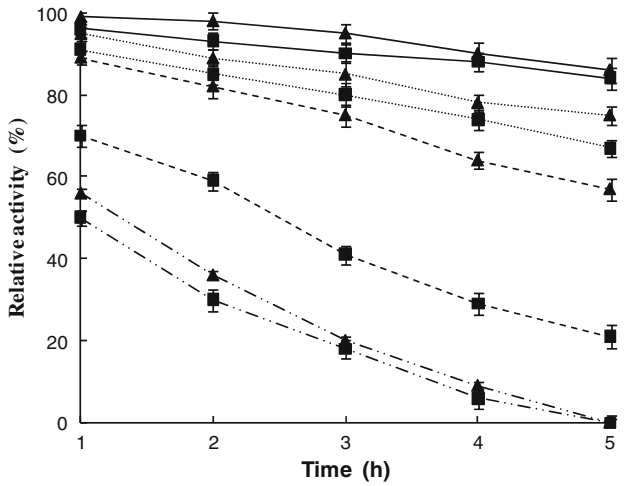

Fig. 5 Effect of temperatures on the total residual activities (amylase and pullulanase activities) of the three enzymes: TsiApu (*square*), MTsiApuN and TsiApuN (*triangle*). Temperatures are 80°C (*solid line*), 90°C (*dotted line*), 100°C (*dashed line*), and 110°C (*dashed dotted line*)

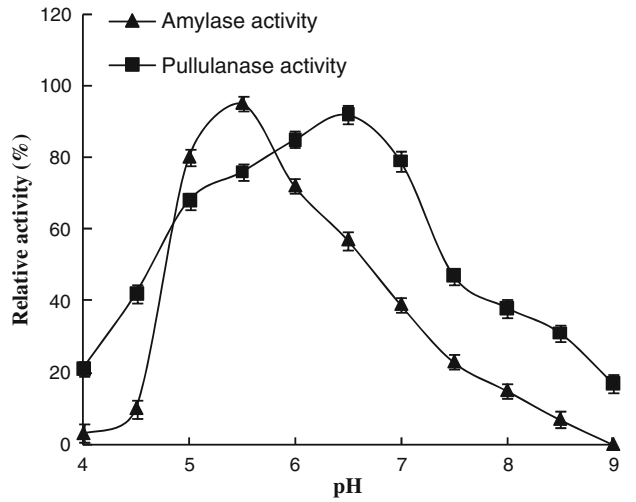

Fig. 6 Effect of pH stability on amylase and pullulanase activities of TsiApuN. Since the curves of the TsiApu, MTsiApuN, and TsiApuN are close, only the curve of TsiApuN is shown

layer motifs have been speculated for the anchoring to the cell envelope or increasing the substrate affinity [5, 20]. In this study, the C-terminal region of TsiApu is composed of Thr-rich regions and S-layer homologies (Fig. 1). MTsiApuN and TsiApuN showed the same catalytic efficiency to the intact enzyme TsiApu, which indicated that the MalE protein fused to the N-termini of TsiApu does not affect function of the catalytic domains. The result is consistent with a conclusion that C-terminal sequence does not necessarily needed for catalytic activity in studies on ThyApu and TetApu (belonging to GH13) from *Thermoanaerobacter ethanolicus* 39E [14, 16]. Besides, MTsiApuN and TsiApuN also showed higher thermoactivity than the intact enzyme TsiApu, which lead us to the conclusion that the C-terminal domains could interfere with the thermoactivity in some way. It indicated that GH57 thermoenzymes might lose the thermoactivity to favor other activities though it is common in thermoenzymes that losing catalytic efficiency to gain thermoactivity.

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