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Cloning, Expression, and Characterization of Thermostable Region of Amylopullulanase Gene from *Thermoanaerobacter ethanolicus* 39E

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Abstract

The bifunctional activities of α-amylase and pullulanase are found in the cloned recombinant amylopullulanase. It was encoded in a 2.9-kb DNA fragment that was amplified using polymerase chain reaction from the chromosomal DNA of *Thermoanaerobacter ethanolicus* 39E. An estimated 109-kDa recombinant protein was obtained from the cloned gene under the prokaryotic expression system. The optimum pH of the recombinant amylopullulanase was 6.0. The most stable pH for the α-amylase and pullulanase activity was 5.5 and 5.0, respectively. The optimum temperature for the α-amylase activity was 90°C, while its most stable temperature was 80°C. Regarding pullulanase activity, the optimum temperature and its most stable temperature were found to be 80 and 75°C, respectively. Pullulan was found to be the best substrate for the enzyme. The enzyme was activated and stabilized by the presence of Ca^{2+}, whereas EDTA, *N*-bromosuccinimide, and α-cyclodextrin inhibited its bifunctional activities. A malto-2–4-oligosaccharide was the major product obtained from the enzymatic reaction on soluble starch, amylose, amylopectin, and glycogen. A single maltotriose product was found in the pullulan hydrolysis reaction using this recombinant amylopullulanase. Kinetic analysis of the enzyme indicated that the K_m values of α-amylase and pullulanase were 1.38 and 3.79 mg/mL, respectively, while the V_{max} values were 39 and 98 μmol/(min · mg of protein), respectively.

Index Entries: Amylopullulanase; *Thermoanaerobacter ethanolicus*; α-amylase; *Escherichia coli*; *apu* gene.

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Introduction

Starch is composed of glucose units linked through α-1,4 linkages and α-1,6 linkages to form linear and branched chains of varying lengths. The enzymatic saccharification of starch is catalyzed by several enzymes. α-Amylase (EC 3.2.1.1) degrades starch exclusively at α-1,4 glucosidic linkages in an endocleavage manner to yield maltose, maltooligosaccharides, and α-limit dextrins. Pullulanase (EC 3.2.1.41) hydrolyzes the α-1,6-glucosidic linkages of pullulan as well as the α-limited dextrins and therefore has debranching activities. When used in combination with α-amylases and pullulanases, starch can be hydrolyzed into a mixture composed of maltose saccharides without α-limited dextrins.

Many industries, such as the food, chemical, detergent, and textile industries, employ microbial amylolytic enzymes to convert starch into different sugar products. Attention is focused on the extreme thermostability and thermoactivity of amylases and pullulanases for use in the bioprocessing of starch into maltose, glucose, fructose, and various sugar syrups *(1–3)*, because starch saccharification processes are usually carried out at >60°C to obtain a higher substrate solubility and to prevent the interference of microorganism growth. Thermoanaerobic bacteria are of considerable interest as producers of thermostable amylolytic enzymes *(4,5)*. Amylopullulanase with a dual specificity toward α-1,4- and α-1,6-glucosidic linkages of starch and related polysaccharides has been found in *Thermoanaerobacter ethanolicus*. This enzyme has been purified to homogeneity. The biochemical characteristics of the purified amylopullulanase indicated that it is thermostable, with a half-life of 40 min at 90°C (pH 6.0) in the presence of Ca^{2+} *(6)*. The gene encoding amylopullulanase (*apu*) has been cloned, sequenced, and expressed in *Escherichia coli (7)*. The expressed recombinant protein in *E. coli* had an M_r of 160,000. Nested deletion mutagenesis has confirmed that the conserved regions of the enzyme were localized within a 2.9-kb gene fragment, which encodes an M_r 100,000 protein having the dual activities and thermostability of the native enzyme *(7)*. The exact regions and their nucleotide sequence have not been reported.

In this study, a pair of gene-specific primers was designed and polymerase chain reaction (PCR) was performed to obtain the 2.9-kb conserved region of the *apu* gene. Recombinant DNA cloning and protein expression using the pET20b(+) expression vector in *E. coli* BL21(DE3) pLysS were carried out. The biochemical properties of this site-directed deletion mutant of recombinant amylopullulanase are reported.

Materials and Methods

Bacterial Strains and Plasmid

T. ethanolicus 39E (ATCC no. 53033) was purchased from American Type Culture Collection and was used as a source of chromosomal DNA for *apu* gene cloning. *E. coli* Novablue (*endA1 hsdR17* [$r_{k12}^{-}m_{k12}^{+}$] *supE44 thi-1*

recA1 gyrA96 relA1 lac {F' *proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15*::*Tn10* [Tet^R]}) and *E. coli* BL21 (DE3) pLysS (F'*opmT hsdS*$_B$ [r_B^-m$_B^-$] *gal dcm* [λcI*ts*857 *ind1 Sam7 nin5 lac*UV5-T7 *gene*1] pLysS [Cm^R]) were used for the cloning and expression of the recombinant *T. ethanolicus* amylopullanase, respectively. The plasmid pET20b (+) was used as both the cloning and expression vector (Novagen, Madison, WI).

Media and Growth Conditions

T. ethanolicus 39E was grown anaerobically for 16 h in ATCC1118-broth as described in the ATCC catalog at 60°C. *E. coli* was grown in LB medium (1% peptone, 0.5% yeast extract, 1% NaCl) at 37°C. The antibiotic ampicillin was used at 100 μg/mL, if needed.

DNA Manipulations

Chromosomal DNA from *T. ethanolicus* 39E was prepared by the method described by Marmur *(8)*. Plasmid DNA preparation, restriction analysis, and PCR were performed by the standard techniques *(9,10)*. DNA was recovered from agarose gel with a Qiaquick gel extraction kit (Qiagen, Germany). Recombinant DNA techniques were carried out by the methods described by Ausubel et al. *(9)* and Sambrook et al. *(10)*.

Cloning of Thermostable Region of apu *Gene Using PCR*

A pair of primers encoding the putative thermostable region of the *apu* gene was designed and synthesized with an Applied Biosystems 380A DNA synthesizer. The genomic DNA was mixed with the Apu N_1C_1 primer pairs (Apu N_1C_1 sense primer: 5'CGGGATCCGTTAAGCTTGCATCTT GATTCAGATTCTG 3'; Apu N_1C_1 antisense primer: 5' CCGCTCGAGCAT ATTTTCCCCTTGGCCAGG 3') and subjected to a PCR amplification with 35 cycles at 55°C as the annealing temperature. Amplified PCR products were analyzed by 0.8% agarose gel electrophoresis, and DNA fragments were recovered from the gel using the Qiaquick gel extraction kit (Qiagen). The isolated and purified DNA fragments were sequenced with a forward (Apu N_1C_1 sense primer) and a reverse (Apu N_1C_1 antisense primer) sequencing primer using the Sanger's dideoxy chain-termination method *(11)* employing the Sequenase Version 2.0 kit (USB, Cleveland, OH). The sequence data were analyzed by comparison with the reported *T. ethanolicus* 39E *apu* gene sequence *(7)*.

Expression of T. ethanolicus *39E* Apu *Thermostable Region (TetApuN$_1$C$_1$) Gene in* E. coli

Plasmid pTetApuN_1C_1 was constructed by inserting a PCR product encoding a thermostable region of *apu* gene from *T. ethanolicus* 39E genomic DNA using the primer pairs (Apu N_1C_1 sense and antisense primers) into the pET20b (+) vector at the *Bam*HI and *Xho*I sites in which the N- and C-terminals of the protein Tet$ApuN_1C_1$ were located, respectively. *E. coli*

BL21 (DE3) pLysS cells harboring plasmid pTetApu N_1C_1 were induced with 0.4 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) at the midexponential phase (OD_{600}: 0.6–0.8) and incubated further for 7 h at 25°C *(12)*. The cells (from a 50-mL culture) were centrifuged, washed, and resuspended in 6 mL of binding buffer (20 m*M* Tris-HCl, pH 7.9; 0.5 *M* NaCl; 5 m*M* imidazole). Cell lysates were obtained by sonicating cells in a Sonicator XL-2020 (Heat System-Ultra Sonics) at 30% power for 6 min at 4°C. Cell debris was removed by centrifuging at 10,000*g* for 15 min, and the protein concentration was measured using the dye-binding method of Bradford *(13)*, with bovine serum albumin as the standard. The supernatant fraction was applied to a His-Bind Resin column (Novagen), and the recombinant TetApuN_1C_1 was purified by metal ion affinity chromatography *(14)*. The homogeneity of purified TetApuN_1C_1 protein was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) *(15)*.

Enzyme Assay

α-Amylase and pullulanase activities were assayed in terms of the release of reducing sugars from starch (soluble starch from potato; Sigma, St. Louis, MO) and pullulan (Sigma). Assay mixtures (0.5 mL) containing 0.2% (w/v) substrate in 50 m*M* sodium acetate buffer (pH 6.0) were incubated at 90 (for amylase activity) or 80°C (for pullulanase activity) for 30 min. The reactions were stopped by heating at 100°C for 10 min. Reducing sugars were assayed with 3',5'-dinitrosalicylic acid reagent. One unit of activity was defined as the amount of enzyme liberating 1 µmol of reducing sugars/min *(7)*.

Analysis of Substrate Hydrolysis Products

Thin-layer chromatography (TLC) using the silica gel 60F254 plate (Merck, Germany) was employed for qualitative analysis of the reaction products in the substrate hydrolysates. A butanol:ethanol:water (5:3:2 [v/v/v]) mixture was used as the eluent *(16)*.

Thermostability Studies

The purified recombinant enzyme was incubated at various temperatures in 50 m*M* sodium acetate buffer (pH 6.0) containing 5 m*M* $CaCl_2$ for 30 min, and the residual enzyme activity was immediately measured using the method already described *(7)*.

Effects of Metal Ions and Other Reagents

Purified enzymes were first treated with various metal ions or chemicals in 50 m*M* sodium acetate buffer (pH 6.0) at 30°C for 30 min. The remaining enzyme activity was monitored using the activity assay as already described *(7)*.

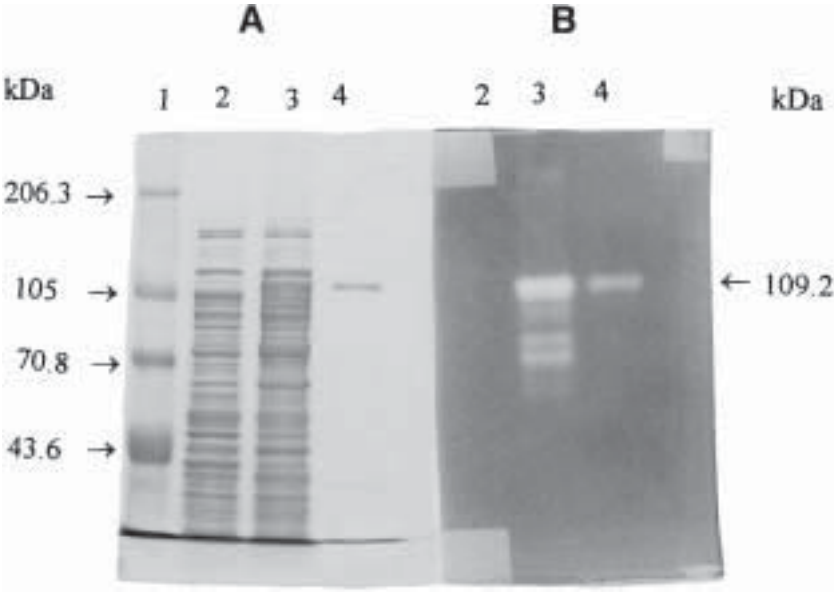

Fig. 1. SDS-PAGE and zymogram of recombinant *T. ethanolicus* 39E Amylopullulanase (TetApuN$_1$C$_1$). Cells were harvested after 0.4 m*M* IPTG induction for 7 h at 25°C. Proteins were separated by SDS-PAGE with 7.5% gel. **(A)** Coomassie brilliant blue staining; **(B)** activity staining. Lane 1, protein molecular weight marker; lane 2, *E. coli* BL21(DE3)pLysS containing pET-20b(+); lane 3, unpurified TetApuN$_1$C$_1$ amylopullulanase; lane 4, TetApuN$_1$C$_1$ amylopullulanase purified with His-Bind resin.

Results

Cloning and Sequencing of TetApuN$_1$C$_1$ Gene

A DNA fragment of approx 2.9 kb was produced at the annealing temperature of 55°C by 35 PCR cycles using the ApuN$_1$C$_1$ sense and antisense primer pairs. The nucleotide sequence of this 2.9-kb DNA was obtained by cycle sequencing and confirmed with the reported one *(7)*. The nucleotide sequence of the thermostable region of the *T. ethanolicus* 39E *apu* gene encodes a polypeptide of 955 amino acids with a calculated M_r of 109 kDa.

Expression and Purification of Recombinant TetApuN$_1$C$_1$ from E. coli

The recombinant TetApuN$_1$C$_1$ enzyme expressed from *E. coli* was analyzed by SDS-PAGE. The apparent mol wt of 109 kDa of the recombinant TetApuN$_1$C$_1$ is consistent with the mol wt calculated from the deduced open reading frame (ORF). The recombinant TetApuN$_1$C$_1$ protein was produced after induction by 0.4 m*M* IPTG for 7 h at 25°C (Fig. 1A, lane 3). An efficient one-step affinity purification using the His-Bind Resin for the recombinant TetApuN$_1$C$_1$ (Fig. 1A, lane 4) and α-amylase activity staining on SDS-PAGE were performed (Fig. 1B, lane 4).

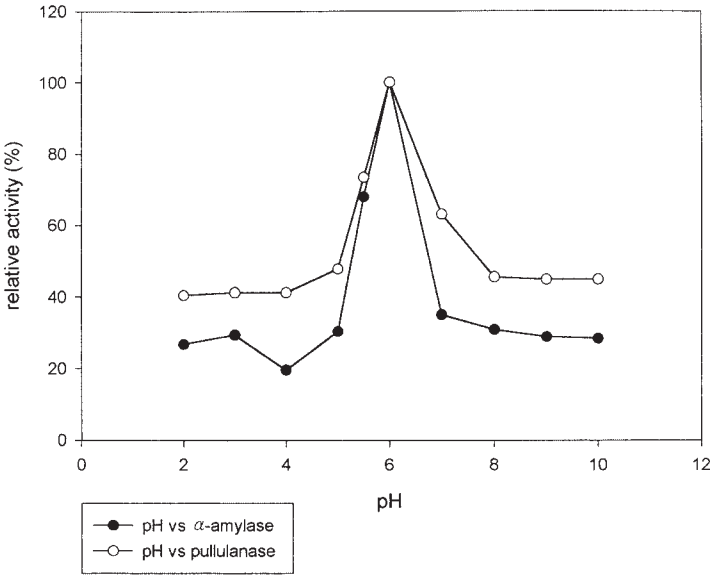

Fig. 2. Effect of pH on α-amylase (●) and pullulanase (○) activities of recombinant TetApu N_1C_1 amylopullulanase expressed from *E. coli* BL21(DE3)pLysS. The enzyme activities were assayed by observing the formation of reducing sugar from the substrates, and the activity was expressed as the relative activity to the maximum activity. Glycine-HCl buffer, pH 2.0; acetate buffer, pH 3.0–6.0; phosphate buffer, pH 7.0–8.0; borate buffer, pH 9.0–10.0.

Physicochemical Properties of Recombinant TetApuN_1C_1

The pH effects on the α-amylase and pullulanase activities of the recombinant TetApuN_1C_1 are shown in Fig. 2. The thermostable region of *T. ethanolicus* 39E Apu (TetApuN_1C_1) had an optimum pH of 6.0 for both activities. The enzyme was active over a pH range of 3.0–6.0 for either α-amylase or pullulanase activity (Fig. 3). The optimum temperature of the purified recombinant enzyme for α-amylase and pullulanase activities was found at 90 and 80°C, respectively (Fig. 4). The recombinant TetApuN_1C_1 was active between 30 and 90°C. A rapid decrease in both α-amylase and pullulanase activities was observed at 100°C (Fig. 5).

Effects of Metal Ions and Other Reagents

Divalent cations such as Mg^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, and Fe^{2+} inhibited the α-amylase activity of the enzyme, whereas Ca^{2+}, Ba^{2+}, Mn^{2+}, and Co^{2+} increased the α-amylase activity of the enzyme. Similarly, Ca^{2+}, Ba^{2+}, and Mn^{2+} increased the pullulanase activity of the enzyme, whereas Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, and Fe^{2+} decreased pullulanase activity (Table 1). Both activities of the recombinant TetApuN_1C_1 were protected from heat denaturation by the presence of the 5 m*M* Ca^{2+} ion during the assay period time of 60 min (Fig. 6). These activities were consistent with the strong inhibition of both activities of the enzyme by the metal ions' chelator EDTA (Table 2).

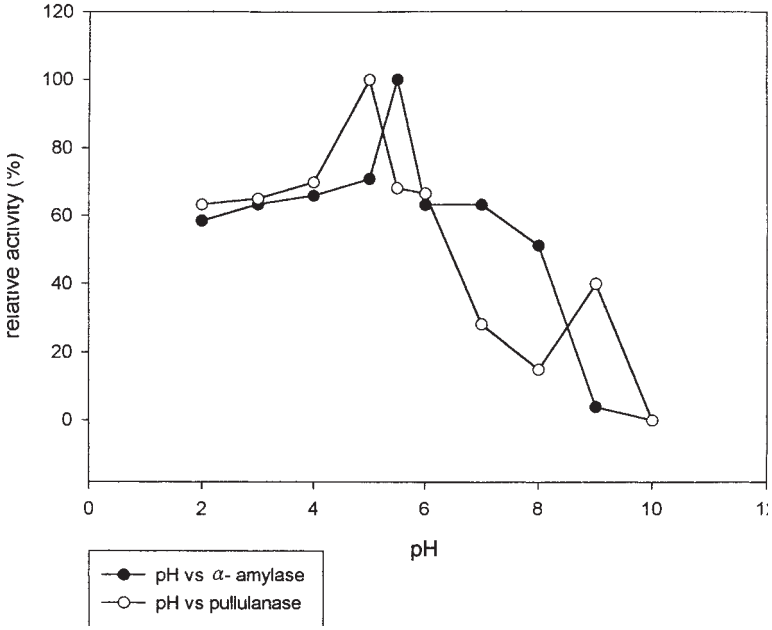

Fig. 3. pH stability of α-amylase (●) and pullulanase (○) activities from recombinant TetApuN$_1$C$_1$ amylopullulanase expressed from *E. coli* BL21(DE3)pLysS. The enzyme was dialyzed with buffer at various pHs for 24 h at 4°C, and then the buffer was changed to optimum pH. The enzyme activities were assayed as described in Fig. 2.

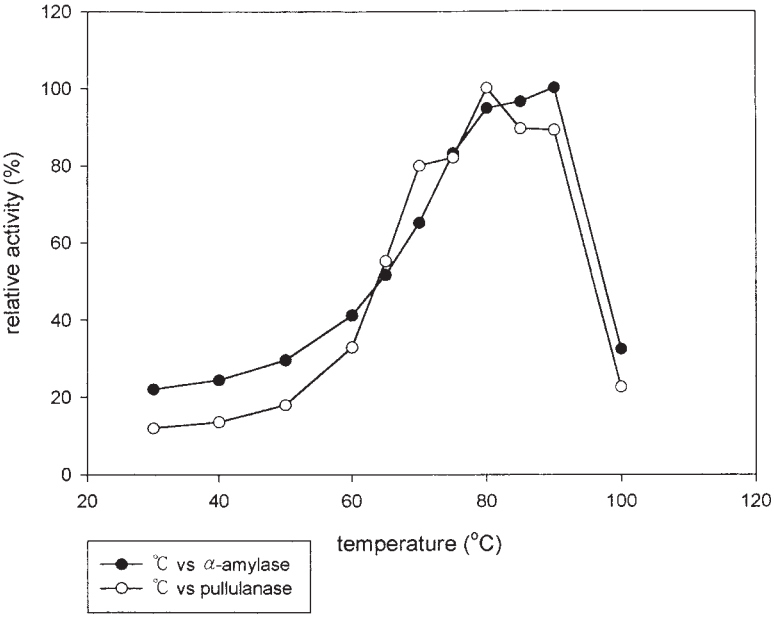

Fig. 4. Effect of temperature on α-amylase (●) and pullulanase (○) activities of recombinant TetApuN$_1$C$_1$ amylopullulanase expressed from *E. coli* BL21(DE3)pLysS.

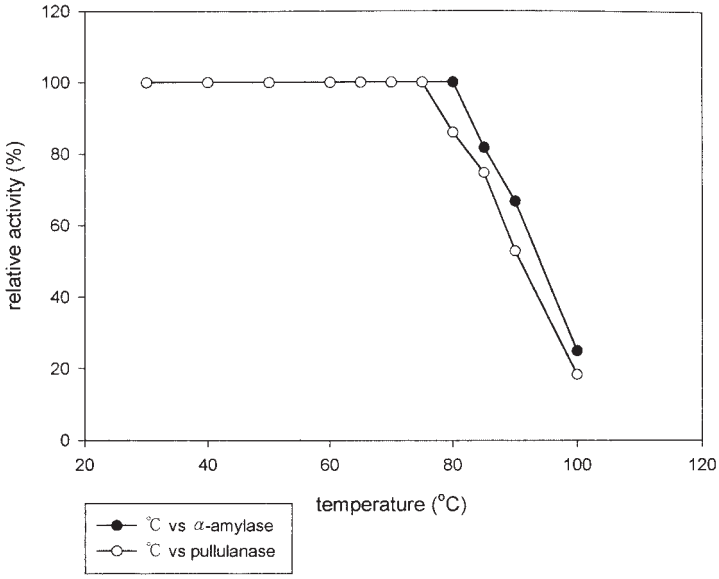

Fig. 5. Thermostability of α-amylase (●) and pullulanase (○) activities from recombinant TetApuN$_1$C$_1$ amylopullulanase expressed from *E. coli* BL21(DE3)pLysS.

Table 1
Effect of Various Metal Ions on Activity
of Recombinant *T. ethanolicus* 39E Amylopullulanase

Metal ions (5 m*M*)[a]	Relative activity (%)	
	α-Amylase	Pullulanase
None	100	100
Ca^{2+}	197	169
Ba^{2+}	144	104
Mg^{2+}	84	95
Mn^{2+}	154	149
Co^{2+}	115	94
Hg^{2+}	48	35
Cu^{2+}	96	58
Ni^{2+}	64	46
Zn^{2+}	57	38
Fe^{2+}	74	44

[a]Metal ions were used as chloride salts except for Ni^{2+} (sulfate).

A known substrate competitor, α-cyclodextrin, inhibited both activities of the purified recombinant enzyme. The enzyme was also sensitive to protein denaturants such as urea and guanidine-HCl. Purified recombinant TetApuN$_1$C$_1$ was strongly inhibited by 0.1 m*M* *N*-bromosuccinimide, suggesting that the tryptophan amino acid residue may be required for both α-amylase and pullulanase activities (Table 2).

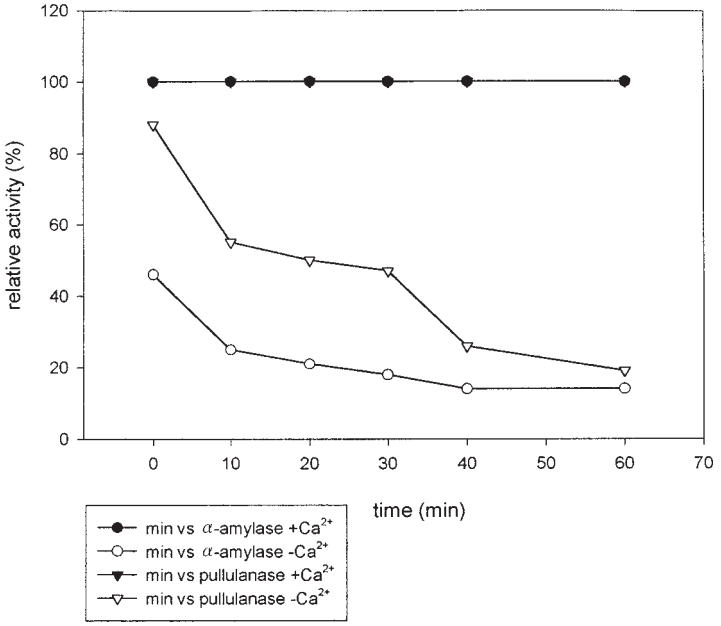

Fig. 6. Effect of presence (●, α-amylase; ▼, pullulanase) and absence (○, α-amylase; ▽, pullulanase) of calcium ion on thermostability of α-amylase and pullulanase activities from recombinant TetApuN$_1$C$_1$ amylopullulanase. Enzyme solution in 50 m*M* acetate buffer, pH 6.0, with or without 5 m*M* calcium ion was preincubated at 80°C for different times. The remaining activity was assayed by observing the formation of reducing sugar after the addition of substrate.

Table 2
Effect of Various Chemicals on Activity
of Recombinant TetApuN$_1$C$_1$ Amylopullulanase

Chemical	Concentration (final)	Relative activity (%)	
		α-Amylase	Pullulanase
None	—	100	100
EDTA	10 m*M*	0	23
α-Cyclodextrin	10 m*M*	43	36
Urea	1 *M*	36	51
Urea	5 *M*	0	5
Guanidine-HCl	1 *M*	13	21
N-bromosuccinimide	0.1 m*M*	9	6

Catalytic Properties, Substrate Specificity, and Hydrolysis Products Analysis

TLC results for the hydrolysis products of various α-glucans showed that both α-1,6- and α-1,4-glucosidic linkages were cleaved by the purified recombinant TetApuN$_1$C$_1$ of *T. ethanolicus* 39E (Fig. 7). The activity toward

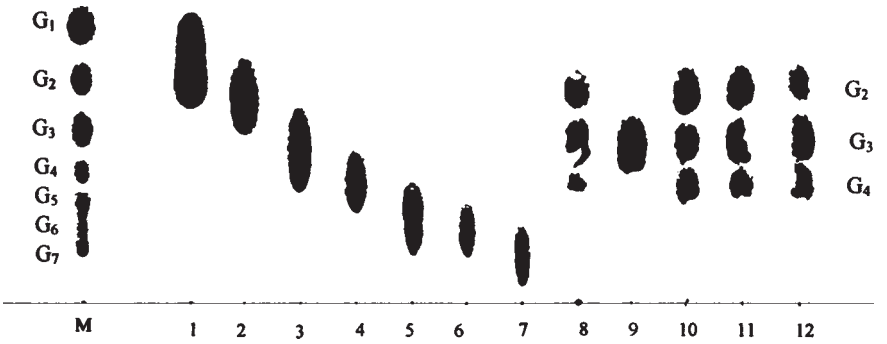

Fig. 7. TLC analysis of hydrolysis products from different substrates by purified recombinant TetApuN$_1$C$_1$ enzyme. Lane M, G$_1$–G$_7$ maltooligosaccharide markers; lanes 1–7, G$_1$–G$_7$; lane 8, soluble starch; lane 9, pullulan; lane 10, potato amylose; lane 11, amylopectin; lane 12, glycogen.

Table 3
Substrate Specificity of Purified
Recombinant TetApuN$_1$C$_1$ Amylopullulanase

Substrate	Relative activity (%)
Pullulan	100
Soluble starch	63.5
Potato amylose	65.4
Amylopectin	52.5
Glycogen (from oyster)	25.5

pullulan was higher than that toward the α-1,4-glucan amylose (Table 3). Interestingly, the recombinant TetApuN$_1$C$_1$ had a relative lower activity on amylopectin and glycogen substrates that have similar α-1,6-glucosidic linkages to pullulan structures. An oligosaccharide with a degree of polymerization (DP) of DP3 was detected as the final product of pullulan hydrolysis by the recombinant TetApuN$_1$C$_1$. The trisaccharide was identified as maltotriose by TLC analysis of the substrate hydrolysates (Fig. 7). The K_m values of the recombinant TetApuN$_1$C$_1$ with soluble starch and pullulan as substrates for α-amylase and pullulanase activities were 1.38 and 3.79 mg/mL, respectively, and the V_{max} values of the α-amylase and pullulanase were 39 and 98 μmol/(min · mg of protein), respectively.

Discussion

In a previous study by Mathupala et al. *(7)*, the amylopullulanase of *T. ethanolicus* 39E was purified to homogeneity as a monomer (M_r = 140,000) with a catalytic optimum temperature of 90°C. Ca^{2+} is required for the stability of the enzyme, which has a half-life of 40 min at 90°C (pH 6.0). The full-length Apu of *T. ethanolicus* 39E was encoded in a single ORF 4443 bp

in length, corresponding to 1481 amino acids, with an estimated mol wt of 162,780. In addition, the smallest gene fragment of *apu* from *T. ethanolicus* 39E capable of having both α-amylase and pullulanase activities and thermostability was within 2.9 kb in length. Nested deletion mutants analysis of Apu activities from this 2.9-kb gene fragment–encoded protein located its 5'- and 3'-terminal regions, but their exact corresponding amino acids were not reported *(7)*.

In the present study, the thermostable and enzymatically active region of *apu* was cloned using PCR and expressed in *E. coli* using the pET-20b(+) expression system. The biochemical and physicochemical properties of the recombinant TetApuN_1C_1 consistently supported the previous findings by Mathupala et al. *(7)*. That is, the conserved region is localized within the 2.9-kb gene fragment, which encodes an M_r 100,000 protein and maintains the dual activities and thermostability of the native enzyme. Further deletion from either the N- or C-terminus of Tet ApuN_1C_1 was conducted, and the corresponding mutants were all enzymatically inactive (unpublished results). In general, the recombinant Tet ApuN_1C_1-encoded *apu* gene from *T. ethanolicus* 39E exhibited similar physiochemical characteristics as the full-length Apu did except the optimal temperatures for amylase and pullulanase activities were found at 90 and 80°C, respectively (Fig. 4). The different optimal temperatures may be caused by the whole molecule of amylopullulanase perturbed by this site-directed deletion mutagenesis. More structural and functional analysis data of Tet ApuN_1C_1 are needed to explain this deviation.

Since the amino acid residues constituting the catalytic triad of amylopullulanase, Asp^{597}, Glu^{626}, and Asp^{703}, are within this 2.9-kb gene fragment and are located within close proximity to each other, they may form a single active site for the dual activities of the enzyme. Furthermore, this region contains sequence motifs similar to each of the four conserved regions identified on α-amylases. This is in contrast to the dual active sites proposed for the α-amylase-pullulanase from other organisms *(17,18)*. Our study provides direct evidence that the *apu* gene of *T. ethanolicus* 39E can tolerate the substantial deletions from its 5' and 3' ends and that it maintains its dual enzymatic α-amylase and pullulanase activities. Our study may also indicate that the amylopullulanase of *T. ethanolicus* 39E is an enzyme with greater flexibility toward various polymeric substrates. This is contrary to the amylases from other organisms, which are highly specific toward either α-1,4 or α-1,6 linkages. The broad substrate specificity and tolerance of deletion (flexibility) of this enzyme suggest that the amylopullulanase isolated from the thermophilic anaerobes may be among the first microorganisms to evolve on Earth, and that α-amylases and pullulanases may have evolved from this type of precursor enzyme. As a result, this enzyme may contribute more in protein engineering studies to alter the substrate or product specificity of amylases or pullulanases, by changing the substrate-binding or catalytic residues.

Acknowledgment

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