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ORIGINAL PAPER

Biochemical characterization of engineered amylopullulanase from *Thermoanaerobacter ethanolicus* 39E-implicating the non-necessity of its 100 C-terminal amino acid residues

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Abstract The functional and structural significance of the C-terminal region of *Thermoanaerobacter ethanolicus* 39E amylopullulanase (TetApu) was explored using C-terminal truncation mutagenesis. Comparative studies between the engineered full-length (TetApuM955) and its truncated mutant (TetApuR855) included initial rate kinetics, fluorescence and CD spectrometric properties, substrate-binding and hydrolysis abilities, thermostability, and thermodenaturation kinetics. Kinetic analyses revealed that the overall catalytic efficiency, k_{cat}/K_m, was slightly decreased for the truncated enzymes toward the soluble starch or pullulan substrate. Changes to the substrate affinity, K_m, and turnover rate, k_{cat}, varied in different directions for both types of substrates between TetApuM955 and TetApuR855. TetApuR855 exhibited a higher thermostability than TetApuM955, and retained similar substrate-binding ability and hydrolyzing efficiency against the raw starch substrate as TetApuM955 did. Fluorescence spectroscopy indicated that TetApuR855 retained an active folding conformation similar to TetApuM955. A CD-melting unfolding study was able to distinguish between TetApuM955 and TetApuR855 by the higher apparent transition temperature in TetApuR855. These results indicate that up to 100 amino acid residues, including most of the C-terminal fibronectin typeIII (FnIII) motif of TetApuM955, could be further removed without causing a seriously aberrant change in structure and a dramatic decrease in soluble starch and pullulan hydrolysis.

Keywords *Thermoanaerobacter ethanolicus* 39E · Amylopullulanase · C-terminal truncation · Thermostability · Circular dichroism

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Introduction

Glycosyl hydrolases (GHases) constitute one large group of diversified saccharides metabolizing enzymes existed on Earth. A classification of GHases based on amino-acid sequence similarities was originally utilized from the analysis of several hundred sequences and was grouped into 35 families (Henrissat 1991). Nowadays, the CAZy web-system was available for individual enzymes to form their own families according to their sequence-structural similarities and catalytic machineries. Almost 100 of different glycosyl hydrolase families were currently classified (Coutinho and Henrissat 1999). Amylopullulanase (Apu, pullulanase type II, E.C.3.2.1.41) cleaves both α-1,4- and α-1,6-glycosidic linkages in starch, pullulan, amylopectin, and related oligosaccharides. Maltotriose and small oligosaccharides are the major products from pullulan or starch, respectively, in the Apu catalyzed reactions. A large number of amylopullulanases have been isolated from a wide variety of microorganisms, particularly thermophiles, because scientific interest in this thermophilic enzyme has been largely motivated by its industrial applications (Vieille and Zeikus 2001). Amylopullulanases from five archaeal members, *Pyrococcus furiosus* (Dong et al. 1997), *Thermococcus litoralis* (Brown and Kelly 1993), *P. woesei* (Rudiger et al. 1995), *T. celer* (Canganella et al. 1994), and

T. hydrothermalis (Erra-Pujada et al. 1999) belong to the GHase family 57 (GH-57) (Janecek 2005). On the other hand, amylopullulanases from the rest of other thermophiles including *Thermoanaerobacter ethanolicus* 39E (TetApu; Mathupala and Zeikus 1993), shared similar motif structures, catalytic sites, and the same general acid-base catalytic mechanism as the GHase family 13 (GH-13). Amylopullulanases belonging to either the GH-57 or GH-13 family feature in their large diversity in gene sequences and lengths. Enzymes with varied sizes can be ranged from <400 to >1,500 amino acid residues in length. The molecular structure information for GH-57 is poor except for the 3D structure of 4-α-glucanotransferase from *Thermococcus litoralis*, which reveals a $(\alpha/\beta)_7$-barrel fold (an incomplete TIM barrel; Imamura et al. 2003), and the identifying the conserved sequence regions (Zona et al. 2004). By contrast, GH-13 family of enzymes has a well-characterized core, $(\beta/\alpha)_8$ TIM barrel, the catalytic residues, Glu and Asp, and utilizes the retaining mechanisms for α-glycosidic bond cleavage (Kuriki and Imanaka 1999; MacGregor et al. 2001; Vieille and Zeikus 2001; van der Maarel et al. 2002).

The GH-13 family amylopullulanases showed distinctive motifs within the enzyme molecule. For example, TetApu has distinct domain structures including cyclomaltodextrin (CD) and pullulan-degrading enzymes N terminus domain, α-amylase catalytic domain $(\beta/\alpha)_8$ barrel core, one AamyC, two fibronectin type III (FnIII), and one putative carbohydrate-binding module 20 (CBM 20) domains located at the C-terminal region. The CBM is an ancillary module of 40–200 amino acids with a discrete fold that possesses carbohydrate-binding activity. The CBM does not have any catalytic activity against the carbohydrate substrate but allows the interaction between the insoluble substrate and the enzyme by bringing the substrate to the active site in the catalytic domain and disrupting the surface of the starch granule. Consequently, it improves the substrate hydrolysis. CBMs are currently divided into families based on amino acid sequence similarity and are defined as 39 families. CBMs display large variation in ligand specificity for recognizing crystalline cellulose, non-crystalline cellulose, chitin, beta-1,3-glucans and beta-1,3-1,4-mixed linkage glucans, xylan, mannan, galactan and starch. The structure and biochemistry of several family 20 CBMs, which bind to starch, have been analyzed extensively and reviewed (Machovic et al. 2005; Boraston et al. 2004; Rodriguez-Sanoja et al. 2005). FnIII consists of a seven-stranded β sandwich similar to the immunoglobulin fold. It usually occurs in multiple copies in intracellular, extracellular, and membrane-spanning proteins (Little et al. 1994). Bacterial FnIII are present only in the extracellular GHases (Gilkes et al. 1991; Hansen 1992) and are remarkably sporadic in many GHase families. The possible functions of FnIII domains involving accessory binding between the enzyme and the polysaccharide substrates have been investigated and proposed in certain GHases (Kataeva et al. 2002; Suzuki et al. 1999; Watanabe et al. 1994). The AamyC is a C-terminal all β-domain of α-amylase and is proposed to participate in disentanglement of α-glucan chains in starch with the other surface binding site in $(\beta/\alpha)_8$ TIM barrel domain by selecting and orienting the substrate chains for hydrolysis at the active sites. The role of AamyC in securing the proper position of the enzyme on the compact substrate was demonstrated in a barley α-amylase isozyme 1 (Robert et al. 2003).

In earlier studies, the thermostable and enzymatically active region of *T. ethanolicus* 39E amylopullanase encoded within a 2.9-kb gene fragment (amino acids L106-M1060 of TetApu) was cloned and expressed in *Escherichia coli* in this laboratory. Biochemical characteristics of this truncated TetApu have been studied and reported (Lin and Leu 2002). It is significant to note that large parts of the N- and C-terminal regions of the native full-length TetApu (4,443 bp, amino acids M1-L1481 of TetApu) are not essential for the activity and thermostability of the enzyme (Mathupala et al. 1993; Lin and Leu 2002). To further understand the roles of C-terminal domains in the enzyme catalysis, the larger C-terminal truncation mutants were constructed through the proteolytic cleavage approach. These larger truncated TetApu genes were expressed in *E. coli*. The properties of the engineered amylopullulanase, TetApuM955 (amino acids L106-M1060 of TetApu), and its C-terminal truncated mutant, TetApuR855 (amino acids L106-R960 of TetApu), were compared in detail. We examined the enzyme kinetics, substrate-binding and hydrolysis abilities, molecular structures, and thermodynamics of heat denaturation. The objective of this study is to clarify the necessity of the involvement of C-terminal domain boundary region of TetApuM955 in the enzyme substrate binding and hydrolysis.

Materials and methods

Bacterial strains, plasmids, and culture conditions

T. ethanolicus 39E [from American Type Culture Collection (ATCC), Manassas, VA, USA] was used as a source of chromosomal DNA for *TetApu* gene cloning. *E. coli* NovaBlue and Rosetta (DE3) pLysS (Novagen, Madison, WI, USA) were used as the cloning host and expression host, respectively. The pET20b(+) (Novagen) was used as both the cloning and expression vector. *T. ethanolicus* 39E was grown anaerobically in ATCC1118 broth as described in the

ATCC catalog at 60°C for 16 h. *E. coli* was grown in an LB broth (1% peptone, 0.5% yeast extract, 1% NaCl) at 37°C. Afterwards, ampicillin (100 μg ml^{-1}) or chloramphenicol (34 μg ml^{-1}) or both was added to the LB broth as needed.

DNA manipulations

Chromosomal DNA from *T. ethanolicus* 39E was prepared by the same method described by Marmur (1961). Other recombinant DNA techniques were performed through standard methods (Ausubel et al. 1993; Sambrook et al. 1989).

Cloning of *TetApuM955* and *TetApuR855* amylopullulanase genes

The thermostable and enzymatically active region of *TetApu* gene (2.9 kb) was subcloned by PCR from *T. ethanolicus* 39E genomic DNA by referring to the reported sequence (Mathupala et al. 1993; Lin and Leu 2002). *TetApuM955* with a hexahistidine tag at the C terminus using the sense primer apuN3, 5′-GGAATTCCATATGTTAA GCTTGCATCTTGATTC-3′, with a *Nde*I restriction site and the antisense primer N1C1, 5′-CCGCTCGAGCATA TTTTCCCCTTGGCCAGG-3′, with an *Xho*I restriction site, was cloned into the pET-20b(+) plasmid by PCR. The *TetApuR855* gene was constructed based on the N terminus amino acid sequence of L–S–L–H–L and the C terminus of TetApuR855, which was predicted based on the ESI Q-TOF protein identification analyses of the autoproteolytic forms of TetApuM955 and the SDS-gel separated target protein with an apparent molecular weight of 85 kDa, which was estimated from the reference protein markers. Several putative C-termini of TetApuM955 autoproteolytic derivatives were approached by mimicking this autoproteolysis event with the trypsin-like cleavage reaction. One of them, TetApuR855, was chosen and its gene 3′-end primer was designed as apuR855, 5′-CCGCTCGAGGCGGTAAATGT TATAACTTACTGCT-3′. Both the cloned *TetApuM955* and *TetApuR855* genes were sequenced to ensure that no point mutations occurred during the PCR cloning.

Protein expression and purification

For expression, the recombinant plasmids were transformed into the expression host *E. coli* Rosetta(DE3)pLysS and clones were cultured in an LB broth containing ampicillin and chloramphenicol at 37°C until A_{600} of 0.6 was reached. Protein expression was then induced by the addition of 0.4 mM (final concentration) isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 25°C for 4 h. The cells were harvested by centrifugation and lysed in B-PER bacterial protein extraction reagent (Pierce, Rockford, IL, USA). The crude protein lysates containing the soluble recombinant amylopullulanases were obtained after 10,000×*g* centrifugation for 15 min and was further purified using BD TALON spin columns designed for 6× His-tag protein affinity purification (BD Biosciences Clontech, Palo Alto, CA, USA). SDS/PAGE (12%) (Laemmli 1970) and zymogram using 1% soluble starch as the substrate were simultaneously conducted for protein homogeneity and activity estimation (Lin and Leu 2002).

Biochemical characterization of the recombinant amylopullulanase

The amylopullulanase activity was determined using either soluble starch or pullulan (Sigma P4516) as the substrates (Mathupala and Zeikus 1993). In brief, an appropriate amount of purified enzyme (approximately 1 μg) added in the reaction mixture (100 μL) contained 1% soluble starch or pullulan in a 50 mM sodium acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ (Buffer A) was incubated for 30 min at the desired temperature. Amylase and pullulanase activities were then measured by the concentration of reduced sugars liberated into the reaction mixture at 80°C (for the amylase activity) or 75°C (for the pullulanase activity). The reaction was terminated by adding 400 μL of 3,5-dinitrosalicylic acid reagent and was heated at 99°C for 10 min. The colored products were measured by spectrophotometry at $OD_{640\ nm}$ (Amersham Ultrospec 1100 pro, Biochrom, Cambridge, England). Protein concentrations were determined (Bradford 1976) using bovine serum albumin as the standard. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reduced sugar as glucose per minute. The effects of pH, temperature, and metal ions on the enzyme activities of TetApuM955 and TetApuR855 were performed as described (Lin and Leu 2002). The optimum temperatures of both enzymes were measured at temperatures ranging from 30 to 99°C with 1.3 μg enzyme in 20 μL Buffer A for 1% soluble starch or pullulan. The effects of different metal ions (1 mM) on the activities of TetApuM955 and TetApuR855 were assessed after incubation with different metal ions at 30°C for 30 min. Kinetic parameters were measured for the soluble starch or pullulan substrates. In brief, the reaction mixture (100 μL) with 1 μg TetApuM955 or TetApuR855, containing various concentrations (5–50 mg/ml) of soluble starch or pullulan in Buffer A, was incubated at 80°C (for amylase activity) or 75°C (for pullulanase activity) for the desired time (5–30 min), respectively, and was terminated as described above. The colored products were quantitated by spectrophotometry. Enzyme assays were run in triplicates. K_m and k_{cat} values were analyzed from both extrapolated intercepts of Lineweaver–Burk plots and direct non-linear least squares curve fitting to the

Michaelis–Menten equation. The linearity of the plot obtained from both methods was confirmed by the correlation coefficient value ($r^2 \geq 0.998$). Thermostability was performed by adding one microgram each of TetApuM955 and TetApuR855 in the 20 μL Buffer A and was heat-treated at 30–99°C for 30 min. The residual enzyme activities were measured. For thermoinactivation kinetics analyses, reaction mixtures were heat-treated for temperature-dependent (90–99°C) denaturation for various periods of time (1–60 min). A first-order reaction was chosen for the thermoinactivation of each enzyme, and the half-lives ($t_{1/2}$) were measured. The corresponding k_{inact} values (rate constant of thermoinactivation) were calculated with the equation $t_{1/2} = 0.693/k_{inact}$. The activation energy of thermoinactivation was calculated from the slope ($-E_a/R$) of Arrhenius plots ($k_{inact} = Ae^{-Ea/RT}$), where A represents the Arrhenius constant, E_a is the activation energy, R is the gas constant, and T is the absolute temperature.

Raw starch-binding assay

Different amounts (5–80 μg) of each enzyme and 2-mg raw starch (amylomaize VII starch, American-Maize Products, Stamford, CT) were mixed in a final volume of 0.2 mL ddH_2O (pH 6.5) for 1 h at 4°C under constant shaking. The supernatant containing unadsorbed protein was collected through centrifugation, and the protein concentration was determined. Bovine serum albumin was used as a background control for nonspecific adsorption. The difference between the amount of protein added and that in the supernatant was estimated as the raw starch-bound protein.

TLC analysis of hydrolysis products

TLC of hydrolysis products from different substrates by TetApuM955 and TetApuR855 was performed. Each reaction containing 1% substrate and 3 μg enzyme was incubated at 80°C for soluble starch and glycogen (Sigma, G8751) or 75°C for pullulan in 100 μL Buffer A for 24 h. The hydrolysis products were analyzed in silica gel plates (Kiesel gel 60 F254; Merck, Rahway, NJ) with *n*butanol–ethanol–water (5:3:2 by volume) as the mobile phase. The reference materials and their final concentrations used were maltose (20 mM), maltotriose (20 mM), maltotetraose (10 mM), maltopentaose (10 mM), maltohexaose (10 mM), and maltoheptaose (10 mM). The plates were sprayed with ethanol containing H_2SO_4 (10% by volume) and were heated at 95°C for 10 min to visualize the carbohydrate spots.

Spectrometry

The fluorescence emission spectra of TetApuM955 and TetApuR855 were obtained with a Hitachi F-2500 spectrofluorometer (Hitachi) at 25°C with 1 × 1 cm cuvettes. Excitation spectra were taken at 282 nm, and emission spectra were recorded at 300–430 nm, with a 4-nm slit for both spectra. Samples with a protein concentration of 15 μg/mL in a final volume of 0.5 mL in 50 mM sodium acetate buffer (pH 6.0) were measured. CD spectrometry was performed using an Aviv CD 202 spectrophotometer (Aviv, Lakewood, NJ, USA). The wavelength scan data were recorded using 0.1-cm path length quartz cuvettes. The far-UV CD spectra were measured at 190–260 nm and 25°C. The averages of the triplicate scans were obtained, and all CD spectra were corrected against their respective buffer blanks. The protein concentrations were 0.14 mg mL^{-1} in 10 mM sodium phosphate buffer (pH 7.0). After background subtraction and smoothing, all the CD signals were converted into mean residue ellipticity (degree cm^2 $dmol^{-1}$). Thermal unfolding transitions were determined by monitoring the changes in the dichroic intensity at 201 nm as a function of temperature. Thermal denaturation was studied in the range of 30–100°C at 2°C increments. The denaturation process was characterized by determining the midpoint of denaturation temperature (T_d) when half of the protein molecules are in a denatured state.

Results

Domain structures of TetApu

Multiple amino acid sequence alignments of GH-13 amylopullulanases among *T. ethanolicus* 39E (TetApu; Mathupala et al. 1993), *T. thermohydrosulfuricum* (Tth Apu; Melasniemi et al. 1990), *Geobacillus kaustophilus* HTA426 (Gka Apu; Takami et al. 2004), and *Bacillus* sp. XAL601 (Bac Apu; Lee et al. 1994), showed distinctive domains (Fig. 1) and their overall amino acid sequence similarities were 77.8, 50.3 and 54.8%, respectively. The amino acid sequence of the CD and pullulan-degrading enzymes N terminus domain of TetApu (L253-N376) exhibited 96.7, 41.1 and 41.9% similarities to those of TthApu (L253-N376), GkaApu (L248-T401), and BacApu (L346-T469), respectively. The α-amylase catalytic domain of TetApu (Q390-R820) showed similarities of 50.9, 29.4 and 29.2%, respectively, to TthApu (Q390-R821), GkaApu (K453-R872), and BacApu (Q480-R940) domains. The Aamy C domain of TetApu (E832-Q923) also exhibited high similarities (90.2, 42.4 and 40.2%) to TthApu (E833-I919), GkaApu (K885-T966), and BacApu (E953-T1034) regions, respectively. Finally, the less amino acid similarity (12.2–33.3%) was found in both FnIII regions among these Apu molecules [TetApu FnIII(1), N924-A1013; FnIII(2), D1156-T1249; TthApu FnIII(1),

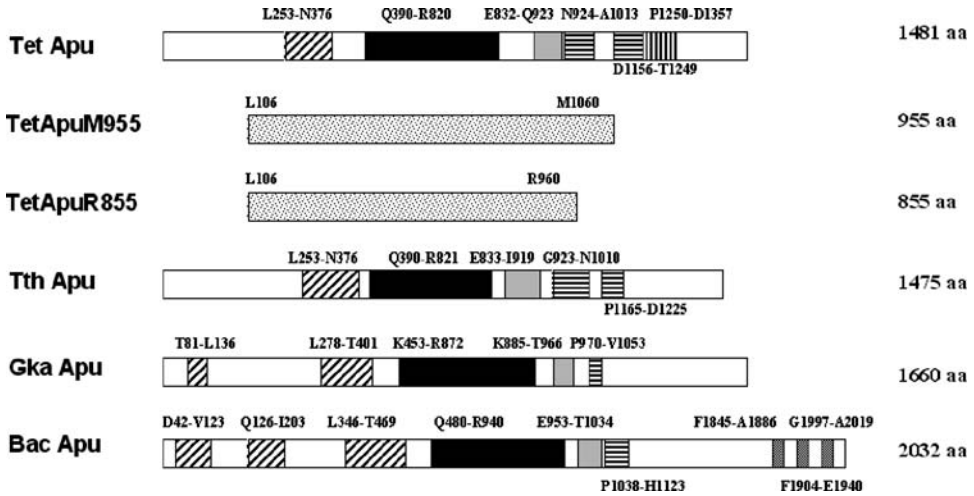

Fig. 1 Schematic representations of conserved domain structures in various GH-13 family amylopullulanases. Tet Apu, *T. ethanolicus 39E* APU precursor gi: 728871; TetApuM955 and TetApuR855, *T. ethanolicus 39E* truncated APU; TthApu, *T. thermohydrosulfuricum* APU precursor gi:114076; GkaApu, *Geobacillus kaustophilus* HTA426 APU gi:56421715; BacApu, *Bacillus* sp. XAL601 alpha-amylase-pullulanase gi:460687 were indicated. Symbols are: *diagonal lines* CD and pullulan-degrading enzymes N terminus domain; *black bar* Alpha amylase catalytic domain; *grey bar*, amy C domain; *horizontal lines*, Fibronectin type III domain; *vertical lines*, CBM20 domain; *dotted lines*, S-layer homology domain

G923-N1010; FnIII(2), P1165-D1225; GkaApu FnIII, P970-V1053; and BacApu FnIII, P1038-H1123]. The Apu four conserved regions and active-site Asp597 (Region II), Glu626 (Region III), and Asp703 (Region IV) residues as well as important aromatic residues Trp594 and Trp628, were all present in TetApuM955 and TetApuR855 (data not shown).

Construction, expression and purification of TetApuM955 and TetApuR855

The 2883-bp DNA fragment encoding TetApuM955 with a signal peptide of 13 amino acids and a $(His)_6$-tag at the C terminus was cloned and the calculated molecular mass of 109,216 Da was deduced from the open reading frame. The N-terminal amino acid sequencing of TetApuM955 revealed that the N-terminal 13-amino acid signal peptide had been cleaved off during protein synthesis in the pET expression system. The predicted molecular mass of 107,818 Da of the matured recombinant TetApuM955 was in good agreement with the apparent molecular weight estimated from the SDS/PAGE. The smaller as well as enzymatically active molecules were found from the autoproteolytic cleavage reaction of TetApuM955 after overnight storage. Their N-terminal amino acid sequences as F–Y–Y–N–Y were determined by Edman degradation. ESI Q-TOF protein identity analyses confirmed that the autoproteolytic derivatives were exactly originated from TetApuM955. Because the mechanism of this autoproteolysis of TetApuM955 was currently not known, the trypsin-like cleavage reaction was preliminarily proposed and mimicked for TetApuM955 autoproteolysis. Several trypsin recognition sites such as Lys or Arg within the open reading frame of TetApuM955 were accordingly selected based on the SDS/PAGE apparent molecular weight of these smaller autoproteolytic derivatives for the possible locations of their C-termini. TetApuR855, having a theoretically calculated molecular mass of approximate 85 KDa, was chosen and its 5′- and 3′-end primers were designed. *TetApuR855* was then cloned through PCR techniques. The protein homogeneity of His-tag affinity purified TetApuM955 and TetApuR855 was investigated by 12% SDS/PAGE, and their zymograms are confirmed (data not shown).

Biochemical characterizations of TetApuM955 and TetApuR855

Biochemical characterizations of TetApuM955 and TetApuR855 including enzyme specific activity against substrates of soluble starch, pullulan, and raw starch (Table 1) and hydrolysis products from starch, pullulan and glycogen (Fig. 2) were presented and they were almost not changed. The optimum pH 6.0, and temperature, 75–80°C, for TetApuM955 and TetApuR855 were the same. The effects of C-terminal truncation of TetApuM955 on the enzyme properties such as metal ions effects (data not shown), raw starch binding (Fig. 3), and kinetic parameters (Table 2) were obtained. Kinetic parameters were showing no significant differences toward either starch or pullulan

Table 1 Specific activities of TetApuM955 and TetApuR855 for different substrates

Enzyme	Specific activities (Units/mole enzyme)		
	Substrates		
	Soluble starch	Pullulan	Raw starch
TetApuM955	1.194×10^{10}	2.342×10^{10}	1.186×10^{9}
TetApuR855	1.174×10^{10}	2.430×10^{10}	1.175×10^{9}

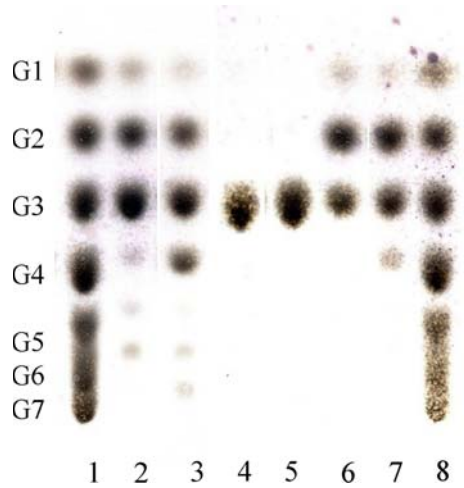

Fig. 2 TLC of hydrolysis products from different substrates by TetApuM955 and TetApuR855. Lane 1 and 8, G1-G7 maltooligosaccharide markers; lane 2, glycogen/TetApuM955; lane 3, glycogen/TetApuR855; lane 4, pullulan/TetApuM955; lane 5, pullulan/TetApuR855; lane 6, starch/TetApuM955; lane 7, starch/TetApuR855 were shown, respectively

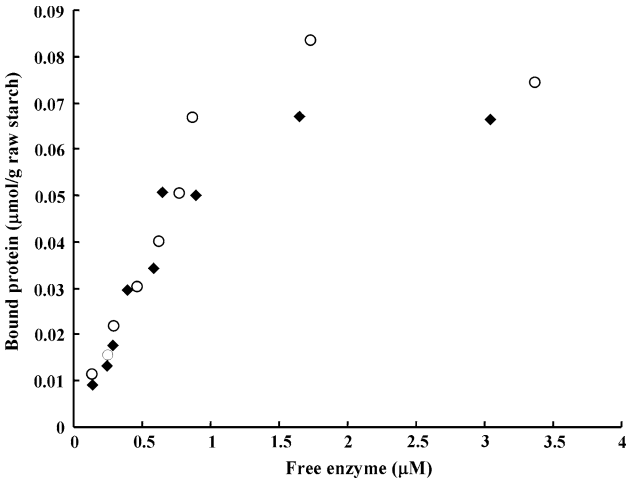

Fig. 3 Equilibrium isotherms for the binding of TetApuM955(*filled diamond*) and TetApuR855(*open circle*) to the raw starch. Data were the average of triplicates

Table 2 Kinetic parameters of TetApuM955 and TetApuR855

Substrate	Enzyme	K_m (mg/mL)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mL/mg)
Starch	TetApuM955	5.2 ± 0.1	298.3 ± 3.5	57.6
	TetApuR855	6.0 ± 0.1	313.3 ± 5.1	52.7
Pullulan	TetApuM955	11.6 ± 1.0	828.3 ± 35.8	71.2
	TetApuR855	16.0 ± 2.7	1009.7 ± 84.3	63.3

substrate between both enzymes. TetApuR855 had higher k_{cat} values, but a lower affinity for both the soluble starch and pullulan substrates. A slight decrease but statistically insignificant in the catalytic efficency (k_{cat}/K_m) of TetApuR855 was observed (Table 2).

Thermal stability of TetApuR855

There was no apparent difference in temperature sensitivity between the two enzymes when the temperature was kept at ≤80°C for the enzymatic reaction, but a significant difference in thermostability occurred when assayed at temperatures above 90°C. TetApuR855 was more thermostable than TetApuM955 (data not shown). In addition, thermoinactivation kinetics analysis indicated that TetApuR855 had the higher activation energy (E_a) than TetApuM955, 299.0 versus 292.9 $kJmol^{-1}$, for the thermoinactivation process within the temperature range of 90–99°C (Fig. 4). Furthermore, the thermal unfolding of both enzymes was monitored by CD spectrometry. The thermal unfolding curve of TetApuR855 was similar to TetApuM955. Apparently, TetApuR855 exhibited a higher thermal unfolding and both enzymes exhibited a one-step melting curve. The onset of denaturation (T_d) was approximately 88°C for TetApuM955; and $T_d = 90$°C for TetApuR855 (Fig. 5).

Spectrometric properties

The structural integrities of TetApuM955 and TetApuR855 were analyzed by fluorescence and CD spectrometry. Figure 6 illustrates the fluorescence emission spectra of the native forms of both recombinant amylopullulanases with a maximum emission peak at 340.5 nm. They are alike very well. After unfolding in 8 M urea, the fluorescence spectra of both denatured enzymes showed an almost similar overlapping spectroscopic pattern. All of the urea-denatured enzymes were then renatured through centrifugation in a desalting column for urea removal. The spectra of the renatured TetApuM955 and TetApuR855 were again highly similar with each other (data not shown). The activities of the native and renatured enzymes were determined simultaneously, and similar enzymatic

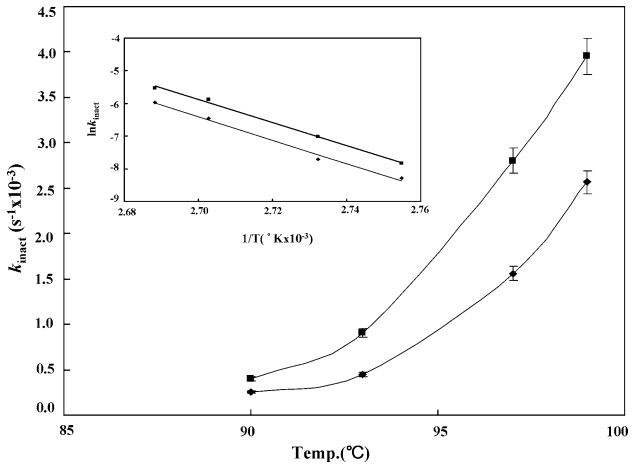

Fig. 4 Temperature dependence of k_{inact} for soluble starch hydrolysis using TetApuM955 (*filled square*) and TetApuR855 (*filled diamond*) (Inset) Arrhenius plots of $\ln k_{inact}$ vs reciprocal absolute temperature for the two amylopullulanases

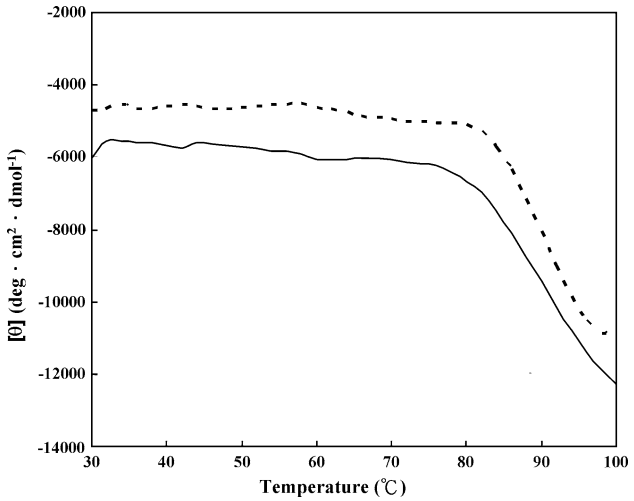

Fig. 5 Thermal unfolding of TetApuM955 (*thin line*) and TetApuR855 (*dotted line*) monitored by CD spectroscopy

activities compared to the native enzyme were recovered for the renatured enzymes. The protein secondary structures of TetApuM955 and TetApuR855 were also compared using far-UV CD spectroscopy. The CD spectra did not decrease in intensity after C-terminal deletion of up to 100 amino acid residues of TetApuM955 (data not shown). The overall structures of both enzymes were indistinguishable as judged from both spectrometry methods.

Discussion

The active amylopullulanases could be derived from the precursor molecule after protein processing at the N- or

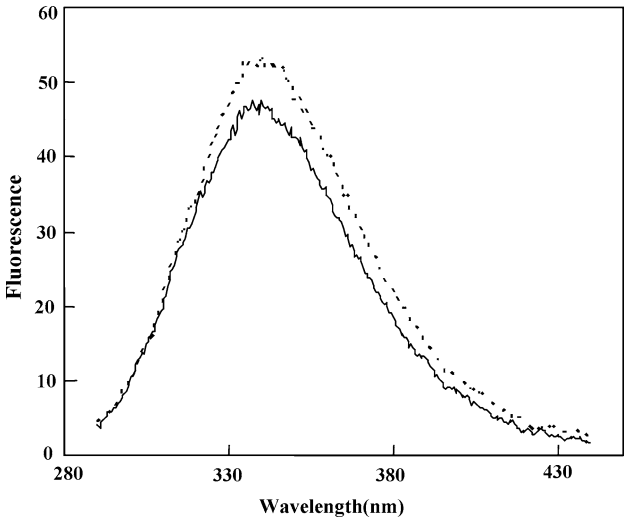

Fig. 6 Fluorescence emission spectra of TetApuM955 (*thin line*) and TetApuR855 (*dotted line*)

C-terminal region. In this study, TetApuR855 was proteolytically produced from TetApuM955 in vitro, while maintaining almost the same enzyme properties. From its N terminus to the start of the C-terminal deletion region, TetApuR855 maintained four conserved regions and catalytic domain of the amylopullulanase. The catalytic active-site amino acids, Asp597, Glu626, and Asp703 in addition to the high level of amino acid sequence similarity among the catalytic domains, Aamy C domains, and cyclodextrin and pullulan-degrading enzymes N terminus domains are all present in the engineered TetApuR855 and TetApuM955 amylopullulanases (Fig. 1). The necessity of C terminal FnIII and putative CBM20 domains of TetApu for enzymic properties has been initially investigated by both N- and C-terminal truncations in this laboratory. Results have revealed that the putative CBM20 and one FnIII motifs of TetApu were disposable without significantly affecting its enzymatic properties (Lin and Leu 2002). The present study consistently indicated that a larger C-terminal truncation of TetApuM955 including almost the entire second FnIII domain was also permitted.

Bacterial FnIIIs were present only in the extracellular glycosyl hydrolyases such as polygalacturonidases, cellulases, chitinases, pullulanases, and amylases (Little et al. 1994). FnIIIs were postulated to serve as linkers regulating interactions between catalytic and substrate-binding modules. The roles of FnIII on the enzymatic properties have been investigated in other GHases such as chitinases. Results, however, are inconclusive. The *B. circulans* chitinase (BcChiA1) requires its FnIII motifs to achieve efficient hydrolysis of the insoluble chitin substrate (Watanabe et al. 1994). Yet, the FnIII motifs of *B. licheniformis* chitinase (BlChi1) was disposable (Chuang et al.

2008). Kataeva et al. (2002) also suggested that the FnIII domain aided the hydrolysis of the cellulose by modifying its surface. This effect is enhanced by the presence of CBDIII (a family III carbohydrate-binding domain), which increases the concentration of the FnIII on the cellulose surface. To our knowledge, there have been no reports regarding the roles of FnIII in other thermophilic amylopullulanases. Thus, the present study was worthy and supported the previous finding that the C-terminal region consisting one FnIII and putative CBM20 domains was not essential for TetApu enzyme activity (Lin and Leu 2002). Spectroscopy analysis of TetApuM955 and TetApuR855 indicated that they were almost indistinguishable. Therefore, the observed differences, though not significant, of the kinetic parameters between TetApuR855 and TetApuM955 may not, in part, be caused by the disruption of the structural integrity of the enzyme. On the other hand, TetApuR855 demonstrated higher thermal unfolding temperature than TetApuM955. The reason for this is not currently clear without more molecular structure studies.

The function of AamyC module has not yet been assigned but it has been proposed to help the enzyme in binding specifically with the insoluble substrate. Therefore, it enhance the hydrolysis efficiency of the substrate molecule by maintaining the correct enzyme-substrate interactions through important aromatic residues located nearby (Robert et al. 2003; Bozonnet et al. 2007). The AamyC module is located next to the second FnIII domain of TetApuM955. The deletion of almost the entire second FnIII of TetApuM955 did not significantly decrease the insoluble raw starch-binding ability (Fig. 3). It was possible that the AamyC domain contributed importantly to the adsorption to starch granules in the absence of FnIII and CBM20 motifs in TetApuR855 for preserving the catalytic activity by multivalent binding between the catalytic domain and the substrate in the enzymatic degradation of polysaccharides. The above hypothesis may be supported by our preliminary results of AamyC partial deletion mutants, TetApuR715 and TetApuK791. Both of them completely lost their enzymatic activities.

The noncatalytic regions of bacterial GHases can be involved with the substrate binding by other interaction forces contributed from aromatic residues such as Trp, Tyr, and Phe (Watanabe et al. 2003). The catalytic domain of TetApuR855 can somehow interact with the substrate via contributions from these residues located in the N-terminal region for insoluble raw starch binding and hydrolysis, and does not absolutely depend on the FnIII and CBM20 motifs. The present study showing that C-terminal R855 truncation of TetApuM955 did not abolish the enzyme against insoluble raw starch-binding and hydrolyzing ability of TetApuR855 provided another example. The variety of motif organization in GHases from different biospheres could make the enzyme more suitable for meeting various requirements in their own environments. C-terminal motifs such as CBM, FnIII, and SLH (surface layer homology) motif-bearing domains have been found in other GHases. SLH motif was speculated to be responsible for the anchoring of proteins to the cell surface, possibly by interacting with peptidoglycan or with other wall-associated polymers in certain bacteria (Lemaire et al. 1995; Olabarria et al. 1996; Sara et al. 1998). The necessity of these motifs for the enzymatic hydrolysis has been studied. For example, up to 98 amino acids from the C-terminal end of *Bacillus* sp strain TS-23 α-amylase could be deleted without significantly affecting the raw-starch hydrolytic activity or thermal stability (Lo et al. 2002). Similar findings were also observed in GH-57 amylopullulanase from *Thermococcus hydrothermalis* (Erra-Pujada et al. 2001). In order to clarify the necessity of the boundary region of the second intact FnIII for the enzyme activity, the complete FnIII deletion mutant of TetApuR855, TetApuQ818, was currently constructed and enzymatic properties of this mutant will be investigated in the near future.

Because TetApu is an extracellular enzyme and has to be exported via a general extracellular protein transport system, the C-terminal region could encode a peptide recognized by the secretory machinery for efficiently transporting the enzyme outside the cell membrane. Evidences for this are yet to be confirmed in future studies.

In conclusion, the molecular characteristics of engineered TetApuM955 and TetApuR855 were studied for the possible function played by its C-terminal FnIII module on the properties of the enzyme. The necessity of the whole second FnIII of TetApuM955 was not absolutely required for the enzymatic properties. Studies of effects from the series of C-terminal truncations of TetApuR855 will illustrate the boundary region of the enzyme that accounts for the interaction between the C-terminal region and the catalytic domain of TetApuR855.

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