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ORIGINAL PAPER

Effect of C-terminal truncation on enzyme properties of recombinant amylopullulanase from *Thermoanaerobacter pseudoethanolicus*

Fu-Pang Lin · Yi-Hsuan Ho · Hsu-Yang Lin · Hui-Ju Lin

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Abstract The smallest and enzymatically active molecule, TetApuQ818, was localized within the C-terminal Q818 amino acid residue after serial C-terminal truncation analysis of the recombinant amylopullulanase molecule (TetApuM955) from *Thermoanaerobacter pseudoethanolicus*. Kinetic analyses indicated that the overall catalytic efficiency, k_{cat}/K_m, of TetApuQ818 was 8–32% decreased for the pullulan and the soluble starch substrate, respectively. Changes to the substrate affinity, K_m, and the turnover rate, k_{cat}, were decreased significantly in both enzymatic activities of TetApuQ818. TetApuQ818 exhibited less thermostability than TetApuM955 when the temperature was raised above 85°C, but it had similar substrate-binding ability and hydrolysis products toward various substrates as TetApuM955 did. Both enzymes showed similar spectroscopies of fluorescence and circular dichroism, suggesting the active folding conformation was maintained after this C-terminal Q818 deletion. This study suggested that the binding ability of insoluble starch by TetApuM955 did not rely on the putative C-terminal carbohydrate binding module family 20 (CBM20) and two FnIII regions of TetApu, though the integrity of the AamyC module of TetApuQ818 was required for the enzyme activity.

Keywords *Thermoanaerobacter pseudoethanolicus* · Amylopullulanase · C-terminal truncation mutagenesis · Circular dichroism

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F.-P. Lin (✉) · Y.-H. Ho · H.-J. Lin
Institute of Bioscience and Biotechnology,
National Taiwan Ocean University, Keelung, Taiwan
e-mail: fpl5505@mail.ntou.edu.tw

F.-P. Lin
Department of Life Science, National Taiwan Ocean University,
Keelung, Taiwan

F.-P. Lin
Center of Excellence for Marine Bioenvironment
and Biotechnology, National Taiwan Ocean University,
Keelung, Taiwan

H.-Y. Lin
Department of Health, Food and Drug Administration,
Executive Yuan, Taipei, Taiwan

Introduction

The carbohydrate-active enzymes (CAZy) web system (http://www.cazy.org, 1998–2011) provides information on how glycoside hydrolases (GHases) are grouped into families according to their sequence-structural similarities and catalytic machineries. Currently, GHases are grouped into 128 families (Cantarel et al. 2009). Amylopullulanase (Apu, pullulanase type II, E.C. 3.2.1.1/41) can cleave both α-1,4- and α-1,6-glycosidic linkages in starch, pullulan, amylopectin, and other related oligosaccharides by its single active site with dual activities or by its separate active sites carrying out these hydrolysis (Mathupala et al.1993; Hatada et al. 1996). Maltotriose and small oligosaccharides are the major products from pullulan or starch, respectively, in Apu-catalyzed reactions. Apus isolated from a wide variety of microorganisms, especially from thermophiles, have great potential for industrial applications (Vieille and Zeikus 2001). Currently, six archael Apus such as *Pyrococcus furiosus* (Dong et al. 1997), *Thermococcus litoralis* (Brown and Kelly 1993), *P. woesei*

(Rudiger et al. 1995), *T. celer* (Canganella et al. 1994), *T. hydrothermalis* (Erra-Pujada et al. 1999; Zona et al. 2004), and *T. siculi* (Jiao et al. 2011) were belonged to the glycoside hydrolase family 57 (GH57 Apus) (Janecek 2005). The remaining Apus, either mesophilic or thermophilic, including those from *Thermoanaerobacter pseudoethanolicus* (formerly, *Thermoanaerobacter ethanolicus* 39E, TetApu) (Lin et al. 2008), *Thermoanaerobacter thermohydrosulfuricum* (Melasniemi et al.1990), *Thermoanaerobacterium saccharolyticum* B6ARI (Ramesh Matur et al. 1994), *Thermoanaerobacterium saccharolyticum* NTOU1 (Lin et al. 2011), and *Thermoanaerobacterium thermosulfurigenes* EM1 (Spreinat and Antranikian 1990) have motif structures, catalytic sites, and a general acid–base catalytic mechanism that are similar to those of the GHase family 13 (GH13 Apus). The GH57 archaeal Apus are all thermophilic, but the GH13 Apus are not. Both GH13 and GH57 Apus are characterized by their large diversity of gene sequences and lengths. Sizes of Apus can be ranged from <400 to >1500 amino acid residues (CAZy: http://www.cazy.org; Cantarel et al. 2009).

GH13 Apus have shown distinctive motifs, for example, TetApu which had the cyclomaltodextrin and pullulan-degrading enzyme N-terminus domain, the α-amylase catalytic $(\alpha/\beta)_8$ barrel core, and the C-terminal region containing one α-amylase C-terminal all-beta domain (AamyC), two fibronectin type III (FnIII) domains, and one putative carbohydrate-binding module family 20 domain (CBM20) (Lin et al. 2008). The diversity, structure, and function of several CBM20 families have been extensively analyzed and reviewed (Machovic et al. 2005; Boraston and Bolam 2004; Rodriguez-Sanoja et al. 2005; Christiansen et al. 2009; Janecek et al. 2011). The FnIII domain is similar to the immunoglobulin fold, and usually occurs in multiple copies in intracellular, extracellular, and membrane-spanning proteins. FnIII domains can be involved in accessory binding between the enzyme and the polysaccharide substrates in certain GHases (Kataeva et al. 2002; Suzuki et al. 1999; Watanabe et al. 1994). The AamyC domain, as proposed, may participate in disentanglement of α-glucan chains in starch, along with the other surface-binding sites in the $(\alpha/\beta)_8$ barrel domain, by selecting and orienting the substrate chains for hydrolysis at the active sites. The proposed role of AamyC module in the barley α-amylase isozyme I in securing the proper position of the enzyme on the compact substrate has been investigated (Robert et al. 2003). The α-amylase catalytic domain, $(\alpha/\beta)_8$ barrel, has four conserved regions, which contain catalytic residues Glu and Asp, and utilizes the retaining mechanisms for α-glycosidic bond cleavage. The cyclomaltodextrin and pullulan-degrading enzyme N-terminus domain may be related to the immunoglobulin and/or FnIII superfamilies. These domains are associated with different types of catalytic domains at either the N-terminal or C-terminal end and may be involved in multimeric interactions (http://www.cazy.org, 1998–2011).

PCR mutagenesis has been a useful and successful method to characterize multi-domain proteins and to identify important protein regions. For example, the C-terminal motifs of chitin-binding domain (ChBD) and fibronectin type III (FnIII) of chitinases from *Aeromonas caviae*, *Vibrio parahaemolyticus*, *Bacillus circulans*, *Bacillus licheniformis* and *Serratia marcescens* have been truncated to understand the roles of these modules played in chitinase catalyzed reactions (Watanabe et al. 1994; Chuang and Lin 2007; Chuang et al. 2008; Lin et al. 2001, 2009; FPL unpublished).

In earlier studies, this laboratory has demonstrated that large parts of the N- and C-terminal regions of the native full-length TetApu (4443 bp, amino acids M1-L1481) are not essential for the enzyme activity and thermostability (Mathupala et al. 1993; Lin and Leu 2002). Further truncated mutants, TetApuM955 (amino acid L106-M1060) and TetApuR855 (amino acids L106-R960) were comparatively characterized to show that C-terminal 100 amino acids could be removed without affecting the enzyme active folding and substrate hydrolysis seriously (Lin et al. 2008). Here, further mutagenesis including a total removal of C-terminal FnIII from TetApuM955 was studied for the functional roles of C-terminal regions on enzyme properties of TetApuM955. The biochemical characterizations such as the enzyme kinetics, substrate-binding and hydrolysis abilities, molecular spectroscopies, and thermal unfoldings of TetApuM955 and TetApuQ818, were presented.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Thermoanaerobacter pseudoethanolicus (formerly, *Thermoanaerobacter ethanolicus* 39E, from American Type Culture Collection [ATCC], Manassas, VA, USA) was used as a source of chromosomal DNA for target gene cloning. *E. coli* NovaBlue, Rosetta (DE3) pLysS (Novagen, Madison, WI, USA) and the plasmid pET20b(+) (Novagen) were used as described previously (Lin et al. 2008). *Thermoanaerobacter pseudoethanolicus* was grown anaerobically as described previously (Lin and Leu 2002). *E. coli* was grown in LB broth (1% peptone, 0.5% yeast extract, 1% NaCl) at 37°C. Ampicillin (100 μg ml^{-1}) or chloramphenicol (34 μg ml^{-1}) or both was added to LB broth when needed.

DNA manipulations

Chromosomal DNA preparation from *Thermoanaerobacter pseudoethanolicus* and other recombinant DNA procedures were operated as described previously (Lin et al. 2008).

Cloning of *Thermoanaerobacter pseudoethanolicus* TetApuM955 and its truncated mutants

The thermostable and enzymatically active region of the *apu* gene (2.9 kb, TetApuM955) was previously subcloned by PCR from *Thermoanaerobacter pseudoethanolicus* genomic DNA (Lin and Leu 2002; Lin et al. 2008). The C-terminal truncated mutants of TetApuM955 were constructed based on the C-terminal amino acid sequence of FnIII1 (N819-A908) and AamyC motif (E727-Q818). The 3′-end primers were designed as follows: TetApuK885 (5′-CCGCTCGAGCTTTAGACCATTGGTAACATCTGT A-3′), TetApuR855 (5′-CCGCTCGAGGCGGTAAATGTT ATAACTTACTGCT-3′), TetApuQ818 (5′-CCGCTCGAG CTGTCCTGGATCTGAAATGAGTATAG-3′), TetApuG809 (5′-CCGCTCGAGCCCATCCAATGCCACAAC-3′), and TetApuK791 (5′-CCGCTCGAGCTTACCACTTAAGGC ATCTGTAAAA-3′). The cloned genes were sequenced to ensure that no point mutations occurred during PCR cloning.

Recombinant protein expression and purification

The recombinant target enzymes were produced from *E. coli* Rosetta (DE3) pLysS, in LB broth containing ampicillin and chloramphenicol under 0.4 mM (final concentration) isopropyl-1-thio-β-D-galactopyranoside (IPTG) induction at 25°C for 4 h. The soluble recombinant Apus were affinity purified with BD TALON spin columns (BD Biosciences Clontech, Palo Alto, CA, USA). The SDS-PAGE (12%) 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 Apus

Apu activity was assayed using either soluble starch or pullulan (Sigma P4516) as substrates (Mathupala and Zeikus 1993). The reducing sugars liberated from the enzymatic reaction mixture at 70°C were spectrophotometrically quantified with 3,5-dinitrosalicylic acid reagent at $OD_{640\ nm}$ (Amersham Ultrospec 2100 pro, Biochrom, Cambridge, England). Protein concentrations were determined by Bradford method using bovine serum albumin as the standard (Bradford 1976). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar as glucose per minute. The effects of pH and temperature on the enzymatic activities of TetApuM955 and TetApuQ818 were performed as described (Lin and Leu 2002). The optimum temperatures for both enzymes were measured at the range of 40–90°C with 0.8 μg enzyme in 20 μL of 50 mM sodium acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ (Buffer A) for 1% soluble starch or pullulan. The kinetic parameters were measured for the soluble starch or pullulan substrates. Specifically, the apparent K_m and k_{cat} values were analyzed from both the extrapolated intercepts of Lineweaver–Burk plots and the 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 ($r^2 \geq 0.99$). The thermostability was performed by heating 0.8 μg each of TetApuM955 and TetApuQ818 in 20 μL Buffer A at 30–95°C for 30 min and the residual enzyme activity was measured (Lin et al. 2008).

Raw starch-binding assay

Different amounts (5–50 μg) of each enzyme and 2 mg raw starch substrate (amylomaize VII starch, American-Maize Products, Stamford, CT, USA) were mixed into a final volume of 0.25 mL ddH_2O (pH 6.5) for 1 h at 4°C under constant shaking. After binding, the difference between the amount of protein added and that in the supernatant was estimated as the raw starch-bound protein (Lin et al. 2008).

Hydrolysis product analysis by TLC

Thin layer chromatography (TLC) of the enzymatic hydrolysis products from different substrates was performed with *n*-butanol–ethanol–water (5:3:2 by volume) as the mobile phase in silica gel plates (Kiesel gel 60 F254; Merck, Rahway, NJ, USA). The saccharide markers used were maltose (20 mM), maltotriose (20 mM), maltotetraose (10 mM), maltopentaose (10 mM), maltohexaose (10 mM), and maltoheptaose (10 mM) (Lin et al. 2008).

Fluorescence spectroscopy

The fluorescence spectroscopy of TetApuM955 and TetApuQ818 were conducted at 25°C with a Hitachi F-2500 spectrofluorometer (Hitachi). The CD spectrometry was performed with an Aviv CD 202 spectrophotometer (Aviv, Lakewood, NJ, USA). The far-UV CD spectra were measured within 190–260 nm at 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 0.17 mg mL^{-1} in 10 mM sodium phosphate buffer at pH 7.0. The thermal unfolding transitions were determined by monitoring the changes in the dichroic intensity at 200 nm as a function of temperature. The thermal denaturation was studied within the range of 30–96°C at every 2°C increment. The denaturation process was characterized by determining the midpoint of denaturation temperature (T_d), during which half of the protein molecules were in a denatured state (Lin et al. 2008).

Results

Domain structures of TetApuQ818 and its biochemical characteristics

Multiple amino acid sequence alignments between TetApu and other GH13 Apus have indicated the presence of distinctive motifs (Lin et al. 2008). Previously, truncation mutagenesis analyses of TetApu have revealed that the enzyme active region was within the TetApu R855 molecule (Mathupala et al. 1993; Lin and Leu 2002; Lin et al. 2008). In this study, further C-terminal truncated molecules such as TetApuQ818, TetApuG809 and TetApuK791, were constructed by PCR mutagenesis techniques and were expressed by the heterologous *E.coli*-pET expression system. The crude recombinant enzymes obtained after IPTG induction were analyzed for Apu activity. TetApuQ818 remained active, but both TetApuG809 and TetApuK791 did not show their Apu activities. Therefore, a large scale of recombinant enzyme preparations of TetApuM955 and its truncated derivatives was performed and the target enzymes were His-tag affinity purified. Zymograms were shown after 12% SDS-PAGE (Fig. 1). Among them, both TetApuG809 and TetApuK791 were expressed in a very minute amount and their activities were still not detected (data not shown). Domain structures of TetApuQ818 and other TetApu molecules were summarized in Fig. 2. TetApuM955 and TetApuQ818 were then selected for comparison as the largest and smallest truncated molecules that retained the enzymatically active region for their biochemical characterizations. TetApuM955 and TetApuQ818 showed activities against substrates of soluble starch, amylose, glycogen, pullulan, and amylopectin. Both enzymes produced the same hydrolysis product profile of maltose and maltotriose from these substrates, respectively (Fig. 3, Lin et al. 2008). The optimum pH of 6.0 for both

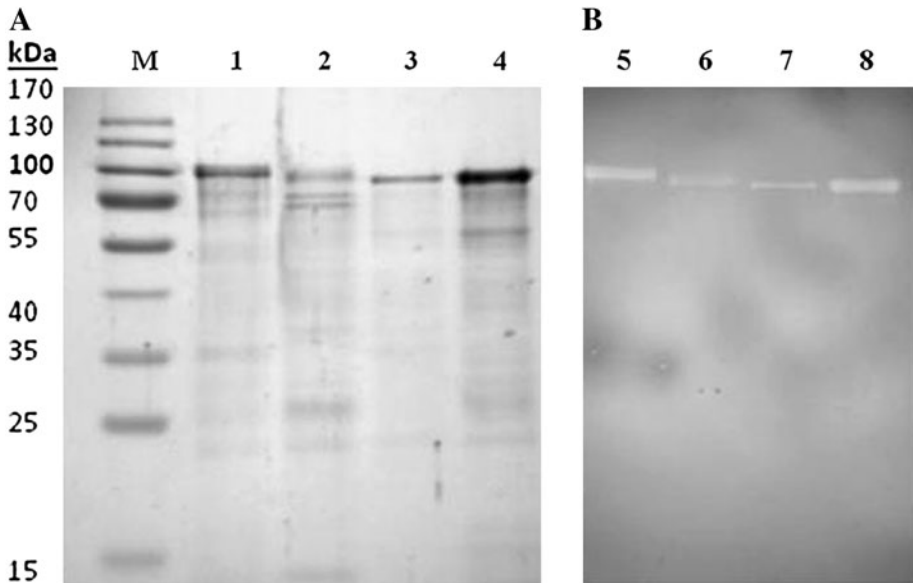

Fig. 1 SDS-PAGE analysis (**a**) of the His-Tag affinity purified recombinant TetApuM955 (*lane 1*), TetApuK885 (*lane 2*), TetApuR855 (*lane 3*) and TetApuQ818 (*lane 4*), stained with Gel Code Blue Stain Reagent (Pierce). Zymogram (**b**) of the amylase activity of TetApuM955 (*lane 5*), TetApuK885 (*lane 6*), TetApuR855 (*lane 7*) and TetApuQ818 (*lane 8*) using the soluble starch as the substrate. *Lane M*, a broad range of prestained protein markers (One Star Biotechnology)

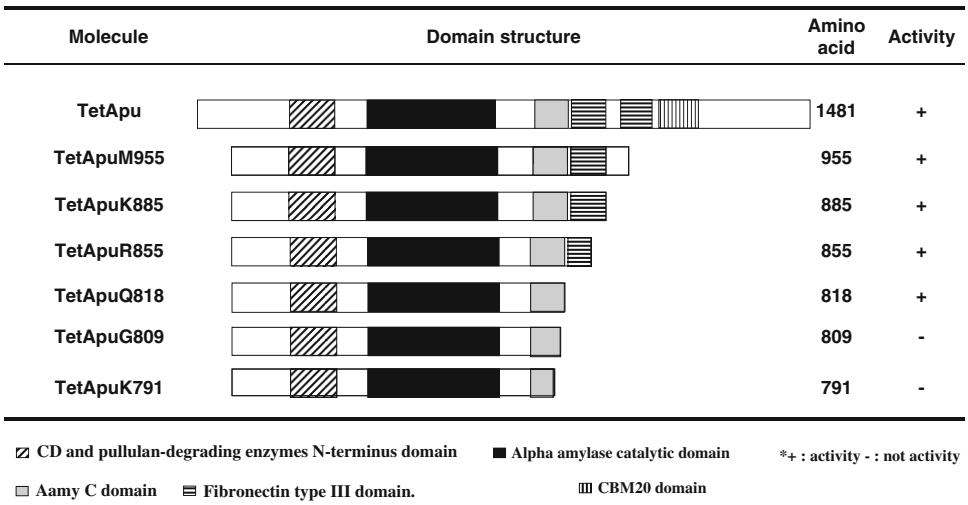

Fig. 2 Putative domain structures of TetApu, TetApuM955 and its C-terminal truncated forms of TetApuK885, TetApuR855, TetApuQ818, TetApuG809 and TetApuK791

amylase and pullulanase activity and the optimum temperature of 75°C were determined for both enzymes (data not shown). There was no apparent difference in the temperature sensitivity of the enzyme activity between the two enzymes when analyzed at 30–80°C, but a significant difference in thermostability occurred at temperatures above 85°C. TetApuQ818 exhibited a lower thermostability than that of TetApuM955 (Fig. 4). The effects of C-terminal Q818 truncation of TetApuM955 on the raw starch binding are not significant (Fig. 5). Kinetic parameters for enzyme catalysis for both enzymes were obtained (Table 1). Both K_m and k_{cat} values of TetApuQ818 enzyme toward soluble starch or pullulan were decreased compared with those of TetApuM955. TetApuQ818 had lower k_{cat} values for both soluble starch and pullulan substrates, whereas a higher affinity for both substrates was observed. There was a 8–32% decrease in k_{cat}/K_m values of TetApuQ818 for both substrates, respectively.

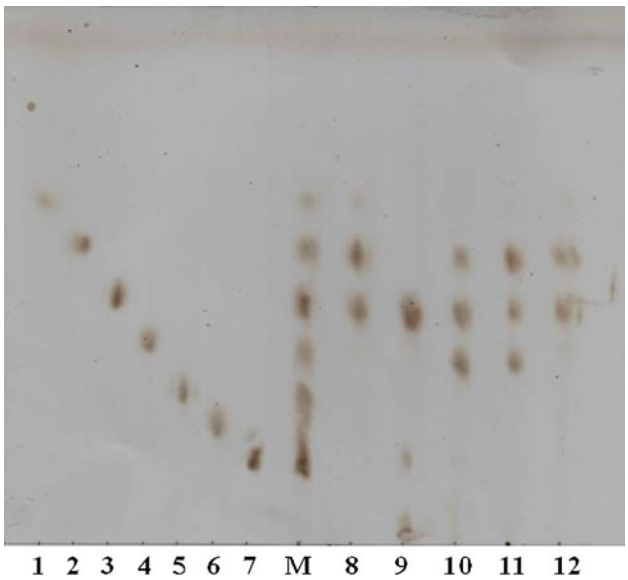

Fig. 3 TLC of hydrolysis products from different substrates by TetApuQ818. *Lanes 1* and *7*, G1–G7 maltooligosaccharide markers; *lane M*, G1–G7 mixture; *lane 8*, soluble starch; *lane 9*, pullulan; *lane 10*, glycogen; *lane 11*, amylose; *lane 12*, amylopectin

Spectrometric properties

Fluorescence and CD spectrometry were used to study the structural integrities of TetApuM955 and TetApuQ818. Both recombinant Apus showed the maximum emission peak at 342.5 nm in the fluorescence emission spectra (Fig. 6). The secondary protein structures of TetApuM955 and TetApuQ818 were also compared by far-UV CD spectroscopy. The global structures of two enzymes were similar from each other, as judged by both spectrometric methods (Fig. 7). The thermal unfolding of the enzymes was also monitored by CD spectrometry. Apparently, TetApuQ818 exhibited thermal unfolding, similar to that of TetApuM955, and both enzymes showed a one-step melting curve. The onset of denaturation (T_d) was approximately the same (Fig. 8).

Discussion

The active Apus could be derived from the precursor molecule after protein processing, such as proteolytic cleavages at the N- or C-terminal region of protein. The enzymatic properties of these derivatives may be changed due to the molecular structure affected possibly by these processing reactions. The necessity of the C-terminal regions of TetApu for enzyme activities has been initially

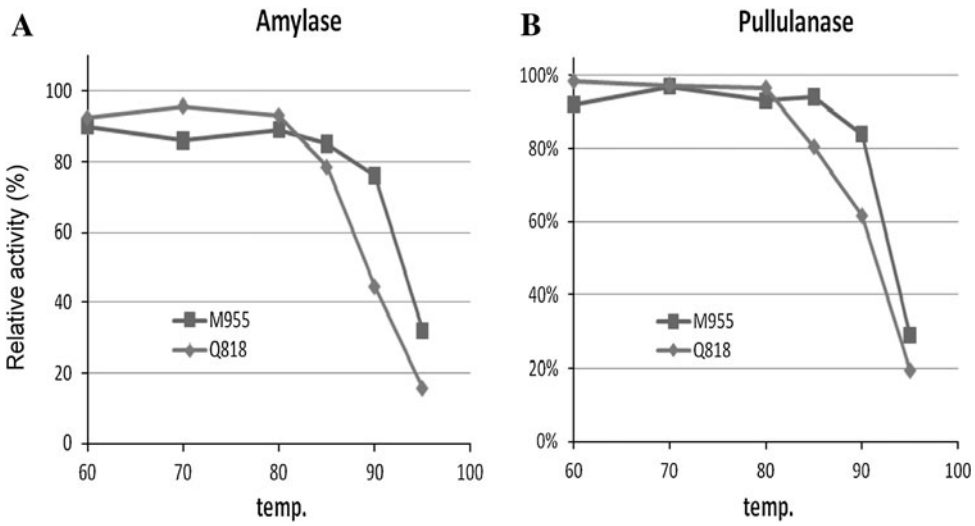

Fig. 4 Thermal stability analyses of TetApuM955 (*filled square*) and TetApuQ818 (*filled diamond*) after 30 min incubation at 60–95°C using soluble starch and pullulan as the substrate

investigated by Mathupala et al. (1993). The exact regions and their nucleotide sequences encoding the thermostable recombinant enzyme with dual activities of α-1,4- and α-1,6-glycosidic cleavages were further identified in this laboratory. Results have revealed that the putative CBM20 and one FnIII motif of TetApu were disposable without significantly affecting its enzymatic properties (Lin and Leu 2002). More truncation mutagenesis studies of TetApu M955 have indicated that a larger C-teminal portion of up to 100 amino acid residues including most of its C-terminal FnIII motifs, i.e. TetApuR855, could be removed without causing a serious change in protein structure and a dramatic decrease in the soluble starch and pullulan hydrolysis (Lin et al. 2008). In this study, not only both the putative CBM20 and two FnIII motifs of TetApu were confirmed to be all disposable, but also the integrity of AamyC domain, which is preceded the second FnIII motif, was tested to be required for TetApuQ818 enzyme activity.

The amino acid sequence alignments between TetApu and TsaNTOU1Apu, both belonging to GH13 Apus, showed similar domain structures with overall amino acid sequence similarities of 79.3%. The amino acid sequence of the cyclomaltodextrin and pullulan-degrading enzyme N-terminal domain of TetApu (L253-N376) exhibited 81% similarities to that of TsaNTOU1Apu (L252-N375). The α-amylase catalytic domain of TetApu (Q390-R820) contained the four highly conserved regions identified in the amylolytic enzymes and showed a similarity of 83% to that of TsaNTOU1Apu (V466-R817). The α-amylase C-terminal all-beta domain (AamyC domain) of TetApu (E832-Q923) also exhibited a high similarity (69%) to that of TsaNTOU1Apu (E829-G921) regions. Finally, a substantial amino acid similarity (69–74%) was found in both FnIII regions between two Apu molecules (TetApu FnIII1, N924-A1013; FnIII2, D1156-T1249 TsaNTOU1Apu FnIII1, P927-A1012; FnIII2, P1156-I1242;) (Lin et al. 2011). TsaNTOU1ApuM957 could allow the FnIII1 motif to be deleted up to 27% in TsaNTOU1ApuK885 without loss of all enzymatic activity or insoluble raw starch-binding ability (Lin et al. 2011). The present study, however, indicated that TetApuM955 could afford the loss of both two FnIIIs and CBM20 motifs without sacrificing its Apu activity, i.e. TetApuQ818 was active. Further deletions of the AamyC motif resulting in TetApuG809 and TetApuK791 molecules caused their enzymatic activity loss. The reason for this different phenomenon between TetApuM955 and TsaNTOU1ApuM957 is currently unclear; further studies are required for clarification.

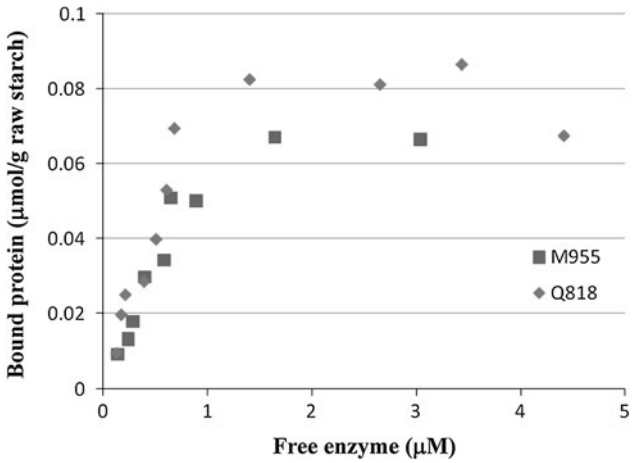

Fig. 5 Equilibrium isotherms for the binding of TetApuM955 (*filled square*) and TetApuQ818 (*filled diamond*) to the raw starch. Assay mixtures contained 2 mg (dry weight) of raw starch, and various amounts of enzymes were incubated in 0.25 mL of ddH_2O (pH 6.5) at 4°C for 1 h under constant shaking. The amount of bound enzyme was calculated from the difference in protein concentration between the enzyme added and the supernatant obtained by centrifugation after binding incubation. Data are the average of two or three replicates

FnIIIs are presumed to be linkers that regulate interactions between the catalytic and substrate-binding modules. The effects of FnIII deletion on enzyme properties have been investigated in other GHases (Kataeva et al. 2002; Chuang et al. 2008; Watanabe et al. 2003). The FnIII motifs of *Bacillus circulans* chitinase (BcChiA1) were required for the efficient hydrolysis of the insoluble chitin substrate. However, the FnIII motifs of *B. licheniformis* chitinase (BlChi1) were disposable. Kataeva et al. (2002) suggest that the efficient cellulose hydrolysis was promoted by modification on the surface of FnIII through interactions between FnIII and CBM. Spectroscopic analysis of TetApuM955 and TetApuQ818 indicated that both were similar from each other, suggesting enzymes were remained in

Table 1 Kinetic parameters of TetApuM955 and TetApuQ818 using soluble starch and pullulan as the substrates

Substrate	Enzyme	K_m (mg/mL)	k_{cat} (s^{-1})	k_{cat}/K_m ($mg^{-1} \times mL \times s^{-1}$)
Starch	TetApuM955	5.2 ± 0.1	298.3 ± 3.5	57.6
	TetApuQ818	3.8 ± 0.1	147.1 ± 1.0	39.0
Pullulan	TetApuM955	11.6 ± 1.0	828.3 ± 35.8	71.2
	TetApuQ818	2.7 ± 0.1	177.3 ± 56.6	65.8

Kinetic parameters (K_m and k_{cat}) were obtained from Lineweaver–Burk plots, which were assessed using a standard linear regression function. The average values of correlation coefficients of $r^2 \geq 0.99$ were obtained from triplicate experiments

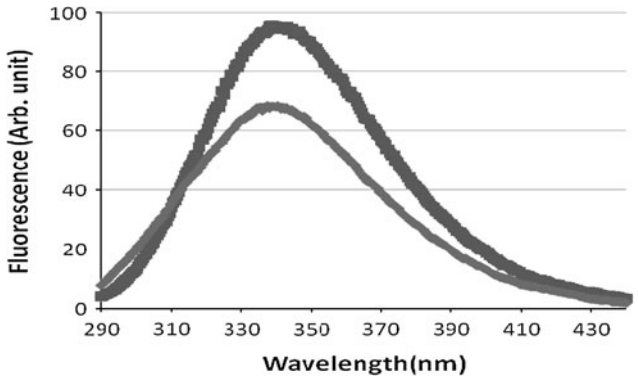

Fig. 6 Fluorescence emission spectra of TetApuM955 (*filled square*) and TetApuQ818 (*filled diamond*). Excitation wavelength was 282 nm and emission spectra were recorded at 300–430 nm. Each enzyme was measured at 15 μg/mL in 20 mM sodium phosphate buffer (pH 7.0)

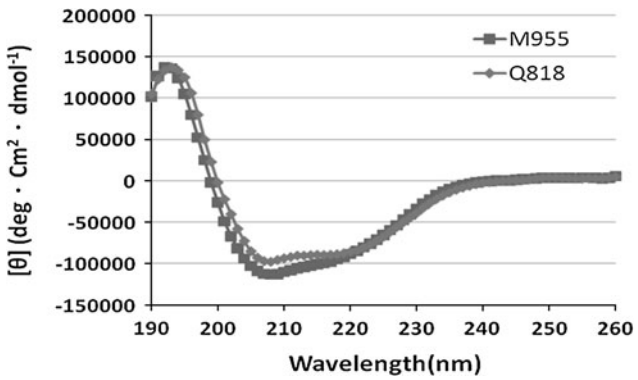

Fig. 7 CD spectra of TetApuM955 (*filled square*), and TetApuQ818 (*filled diamond*) in far-UV region. The CD spectra were obtained at 190–260 nm and 25°C, the protein concentration was 0.17 mg mL^{-1} in 10 mM sodium phosphate buffer (pH 7.0) using 0.1 cm light path CD cell

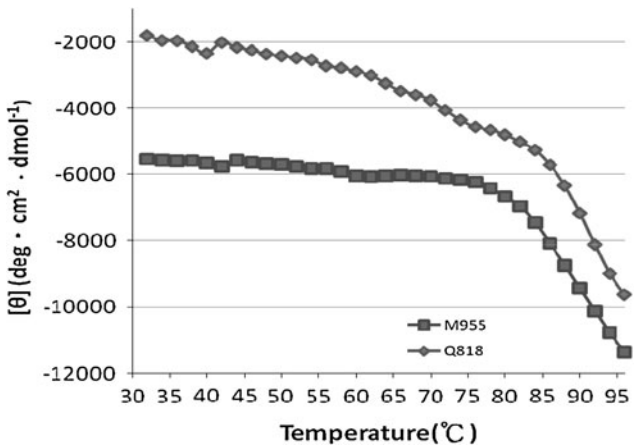

Fig. 8 Thermal unfolding of TetApuM955 (*filled square*) and TetApuQ818 (*filled diamond*) monitored by CD spectroscopy at 201 nm. The CD melting curves were generated by monitoring the changes in the dichroic intensity at 201 nm as a function of temperature. The protein concentration and buffer were the same as in (Fig. 7). Thermal denaturation was measured in the range 30–96°C at 2°C intervals

active foldings even after further deletion of FnIII1. Therefore, the exact role of FnIII on the GH13 Apus properties required more studies for this clarification.

The AamyC module, as proposed previously by Robert et al., might help enzyme binding, specifically with the insoluble substrate, by maintaining the correct enzyme–substrate interactions through important aromatic residues at nearby locations (Robert et al. 2003; Bozonnet et al. 2007). The AamyC module precedes the FnIII1 domain of TetApuM955. The possibility that the AamyC module contributed to substrate binding with the enzyme or to other interactions (cation binding) is still unverified. A hypothesis, proposed in this study, postulated that the integrity of AamyC motif could preserve the enzyme catalytic activity by multivalent binding between the catalytic domain and the substrate when the two FnIIIs and CBM20 motifs are not present in TetApuQ818. This was supported indirectly by the results of AamyC deletion mutants of either TetApuG809 or TetApuK791, in which both mutants lost their enzymatic activities.

The non-catalytic regions of bacterial GHases could be involved indirectly in substrate binding as a result of other interaction forces contributed by the aromatic residues such as Trp, Tyr, and Phe in the substrate-binding domain (Bozonnet et al. 2007). The secondary structure-based alignment of CBM modules from CBM48, CBM20, and CBM21 revealed that several residues were consistently conserved. The GH13 branching enzymes possess the aromatic residues that correspond with the two tryptophans forming the evolutionary conserved starch-binding site1 in CBM20 (Christiansen et al. 2009; Machovič and Janeček 2008; Janecek et al. 2011). The catalytic domain of TetApuQ818 could possibly interact with the substrate via the aromatic residue contributions located in the N-terminal region of cyclomaltodextrin and pullulan-degrading enzymes (L148-N174) for insoluble raw starch binding and hydrolysis. There are 3 Trp, 4 Tyr, and 7 Phe aromatic residues in the L148-N174 region of TetApuQ818, which could have these contribution and may therefore maintain an efficient substrate binding and hydrolysis without the presence of C-terminal FnIII1, FnIII2 and CBM20 motifs.

The variety of motif organizations in different GHases could let the enzymes accommodate their own various requirements in particular environments. C-terminal motifs such as CBM, FnIII, and SLH (surface layer homology) motif-bearing domains were found in other GHases (http://www.cazy.org, 1998–2011). The C-terminal CBM37 module of cellulase, which plays a significant role in *Ruminococcus albus* adhesion to cellulose, has been recently recognized (Rakotoarivonina et al. 2009). The necessity of these motifs in the enzymatic hydrolysis in other GHases has also been studied (Lin et al. 2008; Erra-Pujada et al. 2001; Lo et al. 2002; Kim et al. 2009). Since TetApu is an

extracellular enzyme that has to be exported via a general extracellular protein transport system, the C-terminal region of TetApu could provide a peptide signal recognized by the secretion machinery for the efficient transport of the enzyme outside the cell membrane. Future studies could confirm this possibility.

In conclusion, the molecular characteristics of TetApu was illustrated by truncation mutagenesis, and biochemical characterization of two selected truncation mutants, TetApuM955 and TetApuQ818, indicated the essentiality of the integrate C-terminal AamyC modules. The necessity of the FnIII of TetApuM955 was not absolutely required for the enzyme activity. The putative roles played by the cyclomaltodextrin and pullulan-degrading enzyme region (L148-N174) and the AamyC motif on the TetApuQ818 enzyme properties were discussed.

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References

Boraston AB, Bolam DN (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J 382:769–781

Bozonnet S, Jensen MT, Nielsen MM, Aghajari N, Jensen MH, Kramhøft B, Willemoës M, Tranier S, Haser R, Svensson B (2007) The 'pair of sugar tongs' site on the non-catalytic domain C of barley-amylase participates in substrate binding and activity. FEBS J 274:5055–5067

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brown SH, Kelly RM (1993) Characterization of amylolytic enzymes, having both α-1,4 and α-1,6 hydrolytic activity, from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. Appl Environ Microbiol 59:2614–2621

Canganella F, Andrade CM, Antranikian G (1994) Characterization of amylolytic and pullulytic enzymes from thermophilic archaea and from a new *Fervidobacterium* species. Appl Microbiol Biotechnol 42:239–245

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res 37:D233–D238

Christiansen C, Hachem MA, Janeček S, Viksø-Nielsen A, Blennow A, Svensson BB (2009) The carbohydrate-binding module family 20—diversity, structure, and function. FEBS J 276:5006–5029

Chuang HH, Lin FP (2007) New role of C-terminal 30 amino acids on the insoluble chitin hydrolysis in actively engineered chitinase from *Vibrio parahaemolyticus*. Appl Microbiol Biotechnol 176:123–133

Chuang HH, Lin HY, Lin FP (2008) Biochemical characteristics of C-terminal region of recombinant chitinase from *Bacillus lichenformis*—implication of necessity for enzyme properties. FEBS J 275:2240–2254

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

Erra-Pujada M, Debeire P, Duchiron F, O'Donohue MJ (1999) The type II pullulanase of *Thermococcus hydrothermalis*: molecular characterization of the gene and expression of the catalytic domain. J Bacteriol 181:3284–3287

Erra-Pujada M, Chang-Pi-Hin F, Debeire P, Duchiron F, O'Donohue MJ (2001) Purification and properties of the catalytic domain of the thermostable pullulanase type II from *Thermococcus hydrothermalis*. Biotechnol Lett 23:1273–1277

Hatada Yuji, Igarashi Kazuaki, Ozaki Katsuya, Ara Katsutoshi, Hitomi Jun, Kobayashi Tohru, Kawai Shuji, Watabe Tomoyoshi, Ito Susumu (1996) Amino acid sequence and molecular structure of an alkaline amylopullulanase from *Bacillus* that hydrolyzes α-1,4 and α-1,6 linkages in polysaccharides at different active sites. J Biol Chem 271:24075–24083

Janecek S (2005) Amylolytic families of glycoside hydrolases: focus on the family GH-57. Biologia 60:177–184

Janecek S, Svensson B, MacGregor EAnn (2011) Structural and evolutionary aspects of two families of non-catalytic domains present in starch and glycogen binding proteins from microbes, plants and animals. Enz Microbial Technol 49:429–440

Jiao YL, Wang SJ, Lv MS, Xu JL, Fang YW, Liu S (2011) A GH57 family amylopullulanase from deep-sea *Thermococcus siculi*: expression of the gene and characterization of the recombinant enzyme. Curr Microbiol 62:222–228

Kataeva IA, Ronald DS, Ashit S, Larry TW, Xin-Liang L, Lars GL (2002) The fibronectin type 3-like repeat from the *Clostridium thermocellum* cellobiohydrolases CbhA promotes hydrolysis of cellulose by modifying its surface. Appl Environ Microbiol 68:4292–4300

Kim JH, Michihiro S, Hisayo O, Yoshikatsu M, Eiichiro F, Mitsuo Y (2009) Characterization of the C-terminal truncated form of amylopullulanase from *Lactobacillus plantarum* L137. J Biosci Bioeng 107:124–129

Lin FP, Leu KL (2002) Cloning, expression and characterization of the thermostable region of amylopullulanase gene from *Thermoanaerobacter ethanolicus* 39E. Appl Biochem Biotech 97:33–44

Lin FP, Juang WY, Chang KH, Chen HC (2001) G561 site-directed deletion mutant chitinase from *Aeromonas caviae* is active without its 304C-terninal amino acid residues. Arch Microbiol 175:220–225

Lin HY, Chuang HH, Lin FP (2008) Biochemical characterization of engineered amylopullulanase from *Thermoanaerobacter ethanolicus* 39E-implicating the non-necessity of its 100 C-terminal amino acid residues. Extremophiles 12:641–650

Lin FP, Chuang HH, Liu YH, Hsieh CY, Lin PW, Lin HY (2009) Effects of C-terminal amino acids truncation on enzyme properties of *Aeromonas caviae* D1 chitinase. Arch Microbiol 191:265–273

Lin FP, Ma HY, Lin HJ, Liu SM, Tzou WS (2011) Biochemical characterization of two truncated forms of amylopullulanase from *Thermoanaerobacterium saccharolyticum* NTOU1 to identify its enzymatically active region. Appl Biochem Biotech 165:1047–1056

Lo HF, Lin LL, Chiang WY, Chie MC, Hsu WH, Chang CT (2002) Deletion analysis of the C-terminal region of the α-amylase of *Bacillus* sp. strain TS-23. Arch Microbiol 178:115–123

Machovič M, Janeček Š (2008) Domain evolution in the GH13 pullulanase subfamily with focus on the carbohydrate-binding module family 48. Biologia 63(6):1057–1068

Machovic M, Svensson B, MacGregor EA, Janecek S (2005) A new clan of CBM families based on bioinformatics of starch-binding

domains from families CBM20 and CBM21. FEBS J 272:5497–5513

Mathupala SP, Zeikus JG (1993) Improved purification and biochemical characterization of extracellular amylopullulanase from *Thermoanaerobacter ethanolicus* 39E. Appl Microbiol Biotechnol 39:487–493

Mathupala SP, Lowe SE, Podkovyrov SM, Zeikus JG (1993) Sequencing of the amylopullulanase (apu) gene of *Thermoanaerobacter ethanolicus* 39E, and identification of the active site by site-directed mutagenesis. J Biol Chem 268:16332–16344

Melasniemi H, Paloheimo M, Hemio L (1990) Nucleotide sequence of the α-amylase-pullulanase gene from *Clostridium thermohydrosulfuricum*. J Gen Microbiol 136:447–454

Rakotoarivonina H, Terrie C, Chambon C, Forano E, Mosoni P (2009) Proteomic identification of CBM37-containing cellulases produced by the rumen cellulolytic bacterium *Ruminococcus albus* 20 and their putative involvement in bacterial adhesion to cellulose. Arch Microbiol 191:379–388

Ramesh Matur V, Podkovyrov Sergey M, Lowe Susan E, Gregory ZJ (1994) Cloning and sequencing of the *Thermoanaerobacterium saccharolyticum* B6A-RI *apu* gene and purification and characterization of the amylopullulanase from *Escherichia coli*. Appl Environ Microbiol 60:94–101

Robert X, Haser R, Gottschalk TE, Ratajczak F, Driguez H, Svensson B, Aghajari N (2003) The structure of barley α-amylase isozyme 1 reveals a novel role of domain C in substrate recognition and binding: a pair of sugar tongs. Structure 11:973–984

Rodriguez-Sanoja R, Oviedo N, Sanchez S (2005) Microbial starch-binding domain. Curr Opin Microbiol 8:260–267

Rudiger A, Jorgensen PL, Antranikian C (1995) Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*. Appl Environ Microbiol 61:567–575

Spreinat A, Antranikian G (1990) Purification and properties of a thermostable pullulanase from *Clostridium thermosulfurogenes* EM1 which hydrolyses both α-1,6 and α-1,4 glycosidic bonds. Appl Microbiol Biotechnol 33:511–518

Suzuki K, Taiyoji M, Sugawara N, Nikaidou N, Henrissat B, Watanabe T (1999) The third chitinase gene (ChiC) of *Seratia marcescens* 2170 and the relationship of its product to other bacterial chitinases. Biochem J 343:587–596

Vieille C, Zeikus JG (2001) Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol Mol Biol Rev 65:1–43

Watanabe T, Ito Y, Yamada T, Hashimoto M, Sekine S, Tanaka H (1994) The roles of C-terminal domain and type III domains of chitinases A1 from *Bacillus circulans* WL-12 in chitin degradation. J Bacteriol 176:4465–4472

Watanabe T, Ariga Y, Sato U, Toratani T, Hashimoto M, Nikaidou N, Kezuka Y, Nonaka T, Sugiyama J (2003) Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin. Biochem J 376:237–244

Zona R, Chang-Pi-Hin F, O'Donohue MJ, Janecek S (2004) Bioinformatics of the glycoside hydrolase family 57 and identification of catalytic residues in amylopullulanase from *Thermococcus hydrothermalis*. Eur J Biochem 271:2863–2872