Appl Biochem Biotechnol (2011) 165:1047–1056
DOI 10.1007/s12010-011-9319-7

Biochemical Characterization of Two Truncated Forms of Amylopullulanase from *Thermoanaerobacterium saccharolyticum* NTOU1 to Identify Its Enzymatically Active Region

Fu-Pang Lin · Hsiu-Yen Ma · Hui-Ju Lin · Shiu-Mei Liu · Wen-Shyong Tzou

Received: 9 February 2011 / Accepted: 27 June 2011 /
Published online: 13 July 2011

Abstract The enzymatically active region of amylopullulanase from *Thermoanaerobacterium saccharolyticum* NTOU1 (TsaNTOU1Apu) was identified by truncation mutagenesis. Two truncated TsaNTOU1Apu enzymes, TsaNTOU1ApuM957 and TsaNTOU1ApuK885, were selected and characterized. Both TsaNTOU1ApuM957 and TsaNTOU1ApuK885 showed similar specific activities toward various substrates. The overall catalytic efficiency (k_{cat}/apparent K_m) for the soluble starch or pullulan substrate, however, was 20–25% lower in TsaNTOU1ApuK885 than in TsaNTOU1ApuM957. Both truncated enzymes exhibited similar thermostability and substrate-binding ability against the raw starch. The fluorescence and circular dichroism spectrometry studies indicated that TsaNTOU1ApuK885 retained an active folding conformation similar to that of TsaNTOU1ApuM957. These results indicate that a large part of the TsaNTOU1Apu, such as the C-terminal carbohydrate-binding module family 20, the second fibronectin type III, and a portion of the first FnIII motifs, could be removed without causing a serious aberrant structural change or a dramatic decrease in hydrolysis of soluble starch and pullulan.

Keywords *Thermoanaerobacterium saccharolyticum* NTOU1 · Amylopullulanase · Truncation mutagenesis · Circular dichroism

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

F.-P. Lin · H.-J. Lin · W.-S. Tzou
Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan

H.-Y. Ma · S.-M. Liu
Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan

F.-P. Lin (✉)
Center of Excellence for Marine Bioenvironment and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan
e-mail: fpl5505@mail.ntou.edu.tw

Introduction

Glycoside hydrolases (GHases) are classified on the basis of similarities in amino acid sequences, which are available from analyses of several thousands of protein sequences, and are grouped into 118 families [1]. The carbohydrate-active enzymes (CAZy) web system (http://www.cazy.org, 1998–2008) provides information on how individual enzymes are grouped into families according to their sequence-structural similarities and catalytic machineries [1, 2]. As such, amylopullulanase (Apu; pullulanase type II, E.C.3.2.1.1/41) cleaves both α-1,4- and α-1,6-glycosidic linkages in starch, pullulan, amylopectin, and other related oligosaccharides. Maltotriose and small oligosaccharides are the major products in Apu-catalyzed reactions. Amylopullulanases isolated from a wide variety of microorganisms, especially from thermophiles, have great potential for industrial applications [3]. Five archael amylopullulanases belong to the GHase family 57 (GH-57) [4]. The remaining amylopullulanases, including those from *Thermoanaerobacter pseudoethanolicus* (formerly, *Thermoanaerobacter ethanolicus* 39E, TetApu) [5] and *Thermoanaerobacterium saccharolyticum* NTOU1 (TsaNTOU1Apu, GenBank accession number FJ919954), have motif structures, catalytic sites, and a general acid–base catalytic mechanism that are similar to those of the GHase family 13 (GH-13). The GH-57 and GH-13 families of amylopullulanases are both characterized by their large diversity of gene sequences and lengths. Enzyme sizes can range from <400 to >1,500 amino acid residues in length [1].

Distinctive motifs are present in the GH-13 amylopullulanases family. For example, domain structures for TetApu include the cyclomaltodextrin and pullulan-degrading enzyme N terminus domain, the α-amylase catalytic domain $(\alpha/\beta)_8$ barrel core, and the C-terminal region, which contains 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) [6]. The diversity, structure, and function of several CBM20 families that bind to starch have been extensively analyzed and reviewed [7–10]. The FnIII domain is composed of a seven-stranded beta sandwich, similar to the immunoglobulin fold, and usually occurs in multiple copies in intracellular, extracellular, and membrane-spanning proteins. The FnIII domains can be involved in accessory binding between the enzyme and the polysaccharide substrates in certain GHases [11–13]. Furthermore, the AamyC domain is a C-terminal, all-beta domain of α-amylase and, as proposed, participates in disentanglement of α-glucan chains in starch, along with the other surface-binding sites in the $(\alpha/\beta)_8$ 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 has been demonstrated in the barley α-amylase isozyme I [14]. The α-amylase catalytic domain, $(\alpha/\beta)_8$ TIM barrel, has four conserved regions, contains 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 homodimeric/tetrameric/dodecameric interactions (http://www.cazy.org, 1998–2008).

T. saccharolyticum NTOU1, which was isolated from the hydrothermal vent near the Quiashan Island of Taiwan, is a thermophilic eubacterium with an optimum growth temperature of 70 °C. To localize the functional region of the TsaNTOU1Apu enzyme, we applied truncation mutagenesis. The biochemical properties of the two truncated amylopullulanases, TsaNTOU1ApuM957 (amino acids L105-M1061) and its C-terminal

truncated mutant TsaNTOU1ApuK885 (amino acids L105-K989), were selected and biochemically compared in detail. The enzyme kinetics, substrate-binding and hydrolysis abilities, molecular spectroscopies, and thermal unfoldings were examined.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

T. saccharolyticum NTOU1 (BCRC910455, Bioresource Collection Research Center, Institute of Food Industry and Development, Hsinchu, Taiwan), obtained from Professor Shiu-Mei Liu, was grown anaerobically in liquid broth at 70 °C for 16 h, as described by Wiegel et al. [15], and was used for TsaNTOU1Apu gene cloning. *Escherichia coli* NovaBlue and Rosetta (DE3) pLysS (Novagen, Madison, WI, USA) were used as the cloning host and expression host, respectively. The plasmid pET20b(+) (Novagen) was used as both the cloning vector and the expression vector. *E. coli* was grown in LB broth containing 1% peptone, 0.5% yeast extract, and 1% NaCl, at 37 °C. Ampicillin (100 μg ml^{-1}), chloramphenicol (34 μg ml^{-1}), or both were added to the LB broth when needed.

DNA Manipulations

T. saccharolyticum NTOU1 chromosomal DNA was prepared according to the method described by Marmur [16]. Other recombinant DNA techniques were performed according to standard procedures [17].

Cloning of TsaNTOU1ApuM957 and Its C-Terminal Truncated Genes

The putative and enzymatically active region of 2.9 kb from the TsaNTOU1Apu gene (GenBank accession number FJ919954) was cloned by polymerase chain reaction (PCR) from *T. saccharolyticum* NTOU1 genomic DNA. The sense primer was TsaNTOU1ApuM957N1, 5′-CATGCCATGGCACTAACGCTGAACTTAG CAAATGATTCA-3′, and the antisense primer was TsaNTOU1ApuM957C1, 5′-ATAAGAATGCGGCCGCCATATTTGGACCTTGA CCAGTGC-3′. With the use of the CAZy web system, the TsaNTOU1ApuM957 C-terminal truncated genes were constructed according to the C-terminal amino acid sequences predicted from the various domain structures of TsaNTOU1ApuM957. The 3′-end primers were designed as follows: TsaNTOU1ApuA908 (5′-ATAAGAATGCGGCCGCTGCTACTGCAT CATTAC-3′), TsaNTOU1ApuK885 (5′-ATAAGAATGCGGCCGCTTTTAATCCATTT GTAAC-3′), TsaNTOU1ApuG861 (5′-ATAAGAATGCGGCCGCACCACCTTCTACT GACGA-3′), TsaNTOU1ApuR855 (5′-ATAAGAATGCGGCCGCACGATATATATTGTATCC-3′), and TsaNTOU1ApuA822 (5′-ATAAGAATGCGGCCGCAGCAGTTAAATCTTGCCC-3′). All TsaNTOU1ApuM957 and their C-terminal truncated genes clones were sequenced to ensure that no point mutations occurred during the PCR cloning.

Recombinant Gene Expression and Protein Purification

The recombinant target enzymes were obtained from the expression host, *E. coli* Rosetta (DE3) pLysS, in LB broth that contained ampicillin and chloramphenicol with 0.4 mM (final concentration) isopropyl-1-thio-β-D-galactopyranoside induction at 25 °C for 4 h.

The soluble recombinant amylopullulanases were further purified with BD TALON spin columns, designed for 6× His-Tag protein affinity purification (BD Biosciences Clontech, Palo Alto, CA, USA). The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 12%) and the zymogram, using 1% soluble starch as the substrate, were simultaneously conducted for protein homogeneity and activity estimation [18, 19].

Biochemical Characterizations of the Recombinant Amylopullulanases

Amylopullulanase activity was assayed by using either soluble starch or pullulan (Sigma P4516) as substrates [19]. The concentration of the reducing sugars liberated from the enzymatic reaction mixture at 70 °C was spectrophotometrically quantified with 3,5-dinitrosalicylic acid reagent at $OD_{640\ nm}$ (Amersham Ultrospec 2100 pro, Biochrom, Cambridge, England). Protein concentrations were determined by using bovine serum albumin as the standard [20]. 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, temperature, and metal ions on the enzymatic activities of TsaNTOU1ApuM957 and TsaNTOU1ApuK885 were performed as described [18]. The optimum temperatures for both enzymes were measured at the range of 40 °C to 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 both from 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 TsaNTOU1ApuM957 and TsaNTOU1ApuK885 in 20 μL buffer A at 30–90 °C for 30 min. The residual enzyme activity was measured. The first-order reaction was chosen for thermal inactivation of each enzyme, and the half-lives ($t_{1/2}$) were measured. The corresponding k_{inact} values, the rate constant of thermal inactivation, 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 ($k_{inact} = Ae^{-E_a/RT}$), in which A is 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 (5–50 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.

TLC Analysis of Hydrolysis Products

Thin-layer chromatography 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).

Spectrometry

The fluorescence spectroscopy of TsaNTOU1ApuM957 and TsaNTOU1ApuK885 was conducted at 25 °C with a Hitachi F-2500 spectrofluorometer (Hitachi). The circular dichroism (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.

Results

TsaNTOU1Apu has typical motifs that are present in the GH-13 amylopullulanase family. Truncation mutagenesis of TsaNTOU1Apu revealed that its functional region was within the TsaNTOU1ApuK885 molecule (Fig. 1).TsaNTOU1ApuM957 and TsaNTOU1ApuK885 were selected as the largest and smallest truncated molecules of TsaNTOU1Apu, respectively, that retained their enzymatically active region. Both truncated molecules were expressed from *E. coli* and His-Tag affinity purified. The protein homogeneities of purified TsaNTOU1ApuM957 and TsaNTOU1ApuK885 were investigated by 12% SDS-PAGE and their zymograms are shown (Fig. 2). TsaNTOU1ApuM957 and TsaNTOU1ApuK885 showed similar activities against substrates of soluble starch, amylose, glycogen, pullulan, and amylopectin and produced the same hydrolysis

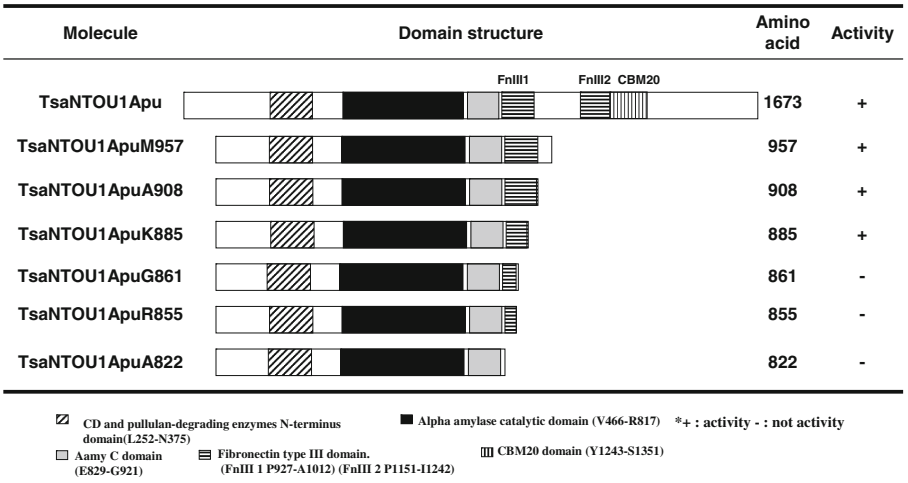

Fig. 1 Schematic representations of molecular structures of amylopullulanases from *T. saccharolyticum* NTOU1

products, maltose and maltotriose, from these substrates (data not shown). The optimum pH of 6.0 or 5.0 for amylase or pullulanase activity, respectively, and the optimum temperature, 70 °C, were determined (data not shown). The effects of C-terminal truncation of TsaNTOU1ApuM957 on enzyme properties, such as metal ion effects and raw starch binding, are similar (data not shown). Kinetic parameters for enzyme catalysis were obtained (Table 1).

There was no apparent difference in the temperature sensitivity of the enzyme activity between the two enzymes when analyzed at 30–90 °C (data not shown). Furthermore, the thermoinactivation analysis indicated that TsaNTOU1ApuK885 has an activation energy (E_a) value of 293.6 J/mol K while TsaNTOU1ApuM957 has an E_a of 313.1 J/mol K within the temperature range of 70–80 °C (Fig. 3). The thermal unfolding of the enzymes was also monitored by CD spectrometry. Apparently, TsaNTOU1ApuK885 exhibited thermal unfolding similar to that of TsaNTOU1ApuM957, and both exhibited a one-step melting curve. The onset of denaturation (T_d) was approximately 72 °C for TsaNTOU1ApuM957 and 71 °C for TsaNTOU1ApuK885 (Fig. 4). The structural integrities of TsaNTOU1ApuM957 and TsaNTOU1ApuK885 were analyzed by fluorescence and CD spectrometry. Figure 5 illustrates the fluorescence emission spectra of the native forms of both recombinant amylopullulanases, with a maximum emission peak at 342.5 nm. The secondary protein structures of TsaNTOU1ApuM957 and TsaNTOU1ApuK885 were also compared by far-UV CD spectroscopy. The global structures of these enzymes were indistinguishable from each other, as judged by both spectrometric methods (data not shown).

Discussion

The amylopullulanase gene (*apu*) of the thermophilic anaerobic bacterium *T. saccharolyticum* B6A-RI was first reported with the GenBank accession number of L07762. This gene consists of 1,288 amino acids and has an N-terminal signal peptide of 35 amino acids [21]. The genome of *T. saccharolyticum* NTOU1 was sequenced and was analyzed by

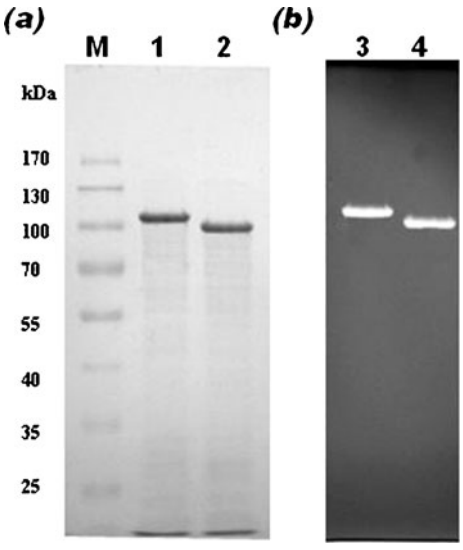

Fig. 2 SDS-PAGE analysis (**a**) of the His-Tag affinity-purified recombinant TsaNTOU1ApuM957 (*lane 1*) and TsaNTOU1ApuK885 (*lane 2*) stained with gel code blue (*Pierce*). Zymogram (**b**) of the amylase activity of TsaNTOU1ApuM957 (*lane 3*) and TsaNTOU1ApuK885 (*lane 4*) using soluble starch as the substrate. *Lane M* is a broad range prestained protein marker (One Star Biotechnology)

Table 1 Kinetic parameters of TsaNTOU1ApuM957 and TsaNTOU1ApuK885 using soluble starch and pullulan as the substrates

Substrate	Enzyme	Apparent K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/apparent K_m (s^{-1} ml/mg)
Starch	TsaNTOU1ApuM957	7.38±0.37	877.87±2.02	118.95
	TsaNTOU1ApuK885	6.02±0.20	574.67±7.87	95.50
Pullulan	TsaNTOU1ApuM957	12.96±0.38	1751.27±37.98	135.12
	TsaNTOU1ApuK885	17.99±0.49	1828.42±43.58	101.61

Kinetic parameters (apparent 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

Professor Wen-Shyong Tzou (F.-P. Lin, unpublished results). The gene fragment containing the putative alpha amylase catalytic region of 1,673 amino acids was identified, and further study was carried out in this laboratory. The overall amino acid homology of Apu between *T. saccharolyticum* NTOU1 and *T. saccharolyticum* B6A-RI was 68.6%. However, no detailed information about the molecular characteristics of *T. saccharolyticum* B6A-RI Apu have been reported since that time. The active enzyme could be derived from the precursor molecule after protein processing, such as proteolytic cleavages at the N- or C-terminal region. In this study, TsaNTOU1ApuM957 and TsaNTOU1ApuK885 were artificially derived from TsaNTOU1Apu in vitro under conditions that maintained similar enzyme properties for each. The thermostable and enzymatically active regions of thermophilic amylopullulanases have been reported to be encoded within the full-length open reading frame gene fragment [6, 18, 19].

Studies of the roles of C-terminal domains of TetApuM955 in enzyme catalysis in this laboratory proved that the larger C-terminal region of TetApu was not essential [6]. The necessity of the two C-terminal FnIIIs and putative CBM20 modules of TetApu for enzyme properties has been further investigated, revealing that the putative CBM20 and both FnIII motifs of TetApu were all disposable [6, 18] (Lin, unpublished results). The amino acid sequence alignments of GH-13 amylopullulanases between TsaNTOU1Apu and TetApu 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

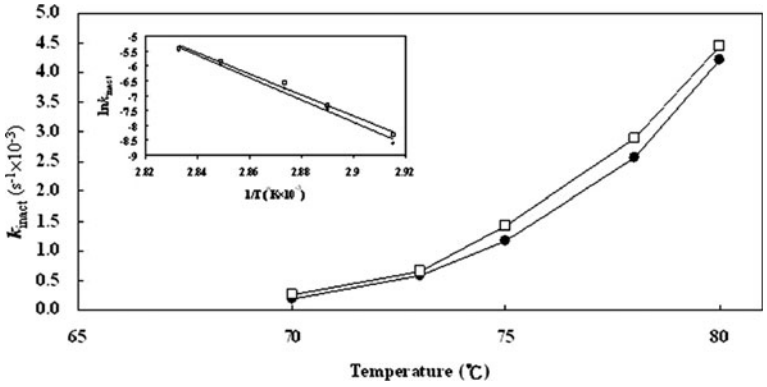

Fig. 3 Temperature dependence of k_{inact} for pullulanase hydrolysis using TsaNTOU1ApuM957 and TsaNTOU1ApuK885. *Inset* Arrhenius plots of ln k_{inact} vs. reciprocal absolute temperature for the two amylopullulanases

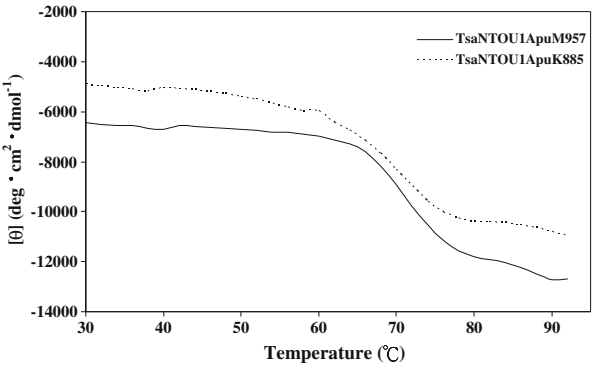

Fig. 4 Thermal unfolding of TsaNTOU1ApuM957 and TsaNTOU1ApuK885 monitored by CD spectroscopy

terminus domain of TsaNTOU1Apu (L252-N375) exhibited 81% similarities to that of TetApu (L253-N376). The α-amylase catalytic domain of TsaNTOU1Apu (V466-R817) contained the four highly conserved regions identified in the amylolytic enzymes and showed a similarity of 83% to that of TetApu (Q390-R820) domains. The α-amylase C-terminal all-beta domain (AamyC domain) of TsaNTOU1Apu (E829-G921) also exhibited a high similarity (69%) to that of TetApu (E832-Q923) regions. Finally, a substantial amino acid similarity (69–74%) was found in both FnIII regions between two Apu molecules (TsaNTOU1Apu first FnIII (FnIII1), P927-A1012; second fibronectin type III (FnIII2), P1156-I1242; TetApu FnIII1, N924-A1013; FnIII2, D1156-T1249).

The present study, however, indicated that TsaNTOU1Apu could afford the loss of only the FnIII2 and CBM20 motifs, but not the whole FnIII1 motif. 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. Further deletion of the FnIII1 motif in TsaNTOU1ApuG861 (55%), TsaNTOU1R855 (61%), and TsaNTOU1ApuA822 (100%) molecules resulted in the complete loss of their enzymatic activities (Fig. 1). The reason for this different phenomenon between TsaNTOU1ApuM957 and TetApuM955 is currently unclear; further studies are required for clarification.

FnIIIs are postulated to be linkers that regulate interactions between the catalytic and substrate-binding modules. The effects of FnIII on enzyme properties have been investigated in other GHases [11, 22, 23]. As such, the FnIII motifs of *Bacillus circulans* chitinase (BcChiA1) were required for the efficient hydrolysis of the insoluble chitin substrate. In contrast, the FnIII motifs of *Bacillus licheniformis* chitinase (BlChi1) were disposable. Kataeva et al. suggest that interactions between FnIII and CBM modify the surface of FnIII, promoting more efficient cellulose hydrolysis [11]. Spectroscopic analysis

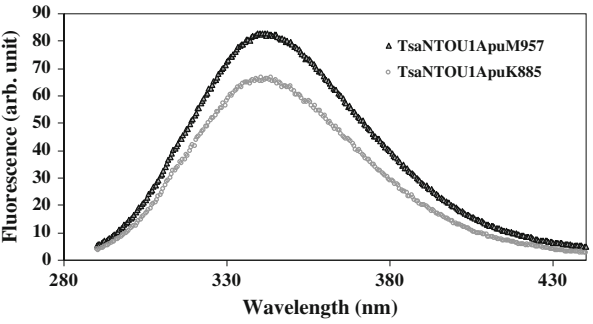

Fig. 5 Fluorometry of TsaNTOU1ApuM957 and TsaNTOU1ApuK885

of TsaNTOU1ApuM957 and TsaNTOU1ApuK885 indicated that the amylopullulanases were indistinguishable from each other, suggesting both molecules were in active conformation even after further partial deletion of FnIII1. Therefore, currently, defining the exact role of FnIII on the GH-13 amylopullulanases properties is controversial. More studies are required for this clarification. Still, the role of the AamyC module, as proposed, is to help enzyme binding, specifically with the insoluble substrate, by maintaining the correct enzyme–substrate interactions through important aromatic residues at nearby locations [14, 24]. The AamyC module is located next to the FnIII1 domain of TsaNTOU1Apu. More importantly, the possibility that the AamyC module contributed to substrate binding with the enzyme or to other interactions (cation binding) is still unverified. A current hypothesis postulates that the FnIII1 motif preserves catalytic activity by multivalent binding between the catalytic domain and the substrate (with possible AamyC motif involvement), in the absence of the FnIII2 and CBM20 motifs in TsaNTOU1ApuK885. This hypothesis could be supported analogously by the results of AamyC deletion mutants of either TetApuR715 or TetApuK791, in which both mutants completely lost their enzymatic activities [6].

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 [24]. The secondary structure-based alignment of CBM modules from CBM48, CBM20, and CBM21 revealed that several residues were consistently conserved. The GH-13 branching enzymes possess the aromatic residues that correspond with the two tryptophans forming the evolutionary conserved starch-binding site 1 in CBM20 [10, 25]. The catalytic domain of TsaNTOU1ApuK885 could possibly interact with the substrate via the aromatic residue contributions located in the N-terminal region of cyclomaltodextrin and pullulan-degrading enzymes (L252-N375) for insoluble raw starch binding and hydrolysis. Coincidentally, there are three Trp, four Tyr, and seven Phe aromatic residues in the L252-N375 region of TsaNTOU1ApuK885. Therefore, efficient substrate hydrolysis would not be totally dependent on the C-terminal FnIII2 and CBM20 motifs. As shown by the present study, the C-terminal K885 truncation of TsaNTOU1Apu did not abolish the enzyme's insoluble raw starch-binding and hydrolyzing abilities. In fact, the variety of motif organizations in different GHases could help the enzymes cope with 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, 1998–2008). In particular, the C-terminal CBM37 module of cellulase, which plays a significant role in *Ruminococcus albus* adhesion to cellulose, has been recently recognized [26]. The necessity of these motifs in the enzymatic hydrolysis in other GHases has also been studied [6, 27–29]. Since TsaNTOU1Apu is an extracellular enzyme that has to be exported via a general extracellular protein transport system, the C-terminal region of TsaNTOU1Apu could provide a peptide signal recognized by the secretion machinery for the efficient transport of the enzyme outside the cell membrane. This possibility could be confirmed in future studies.

In conclusion, the molecular characteristics of TsaNTOU1Apu was illustrated by truncation mutagenesis, and biochemical characterization of two selected truncation mutants, TsaNTOU1ApuM957 and TsaNTOU1ApuK885, elucidated the essential functions performed by its C-terminal modules. The necessity of the portion of the FnIII1 of TsaNTOU1Apu required for the enzymatic properties, as in TsaNTOU1ApuK885, was illustrated. The putative roles played by the cyclomaltodextrin and pullulan-degrading enzyme region (L252-N375), the AamyC motif, and the first FnIII (FnIII1) on the TsaNTOU1Apu enzyme properties were also proposed.

Acknowledgments We thank Dr. Chin-Pan Chen of the Institute of Biomedical Science, Academia Sinica, for his considerable help in the CD spectroscopy analyses. This work was supported by the National Science Council, Taiwan (grant NSC 94-2313-B019-029) and the Center of Excellence for Marine Bioenvironment and Biotechnology (CMBB), National Taiwan Ocean University (grant 99529001A8).

References

1. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., & Henrissat, B. (2009). *Nucleic Acid Research, 37*, D233–D238.
2. Coutinho, P. M., & Henrissat, B. (1999). In H. J. Gilbert, G. Davies, B. Henrissat, & B. Svensson (Eds.), *Recent advances in carbohydrate bioengineering, carbohydrate-active enzymes: An integrated database approach* (pp. 3–12). Cambridge: Royal Society of Chemistry.
3. Vieille, C., & Zeikus, J. G. (2001). *Microbiology and Molecular Biology Reviews, 65*, 1–43.
4. Janecek, S. (2005). *Biologia, 60*(Suppl. 16), 177–184.
5. Mathupala, S. P., Lowe, S. E., Podkovyrov, S. M., & Zeikus, J. G. (1993). *Journal of Biological Chemistry, 268*, 16332–16344.
6. Lin, H. Y., Chuang, H. H., & Lin, F. P. (2008). *Extremophiles, 12*, 641–650.
7. Machovic, M., Svensson, B., MacGregor, E. A., & Janecek, S. (2005). *FEBS Journal, 272*, 5497–5513.
8. Boraston, A. B., & Bolam, D. N. (2004). *Biochemical Journal, 382*, 769–781.
9. Rodriguez-Sanoja, R., Oviedo, N., & Sanchez, S. (2005). *Current Opinion in Microbiology, 8*, 260–267.
10. Christiansen, C., Hachem, M. A., Janeček, S., Viksø-Nielsen, A., Blennow, A., & Svensson, B. B. (2009). *FEBS Journal, 276*, 5006–5029.
11. Kataeva, I. A., Ronald, D. S., Ashit, S., Larry, T. W., Xin-Liang, L., & Lars, G. L. (2002). *Applied and Environmental Microbiology, 68*, 4292–4300.
12. Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B., & Watanabe, T. (1999). *Biochemical Journal, 343*, 587–596.
13. Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S., & Tanaka, H. (1994). *Journal of Bacteriology, 176*, 4465–4472.
14. Robert, X., Haser, R., Gottschalk, T. E., Ratajczak, F., Driguez, H., Svensson, B., et al. (2003). *Structure, 11*, 973–984.
15. Wiegel, J., Ljungdahl, L. G., & Rawson, J. R. (1979). *Journal of Bacteriology, 139*, 800–810.
16. Marmur, J. J. (1961). *Journal of Molecular Biology, 3*, 208–218.
17. Sambrook, J. J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory.
18. Lin, F. P., & Leu, K. L. (2002). *Applied Biochemistry and Biotechnology, 97*, 33–44.
19. Mathupala, S. P., & Zeikus, J. G. (1993). *Applied Microbiology and Biotechnology, 39*, 487–493.
20. Bradford, M. (1976). *Analytical Biochemistry, 72*, 248–254.
21. Ramesh, M. V., Podkovyrov, S. M., Lowe, S., & Zeikus, J. G. (1994). *Microbiology, 60*, 94–101.
22. Chuang, H. H., Lin, H. Y., & Lin, F. P. (2008). *FEBS Journal, 275*, 2240–2254.
23. Watanabe, T., Ariga, Y., Sato, U., Toratani, T., Hashimoto, M., Nikaidou, N., et al. (2003). *Biochemical Journal, 376*, 237–244.
24. Bozonnet, S., Jensen, M. T., Nielsen, M. M., Aghajari, N., Jensen, M. H., Kramhøft, B., et al. (2007). *FEBS Journal, 274*, 5055–5067.
25. Machovič, M., & Janeček, Š. (2008). *Biologia, 63*(6), 1057–1068.
26. Rakotoarivonina, H., Terrie, C., Chambon, C., Forano, E., & Mosoni, P. (2009). *Archives of Microbiology, 191*, 379–388.
27. Erra-Pujada, M., Chang-Pi-Hin, F., Debeire, P., Duchiron, F., & O'Donohue, M. J. (2001). *Biotechnology Letters, 23*, 1273–1277.
28. Lo, H. F., Lin, L. L., Chiang, W. Y., Chie, M. C., Hsu, W. H., & Chang, C. T. (2002). *Archives of Microbiology, 178*, 115–123.
29. Kim, J. H., Michihiro, S., Hisayo, O., Yoshikatsu, M., Eiichiro, F., & Mitsuo, Y. (2009). *Journal of Biosciences and Bioengineering, 107*, 124–129.