Enzyme and Microbial Technology 53 (2013) 46–54

Contents lists available at SciVerse ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Crystal structure of a compact α-amylase from *Geobacillus thermoleovorans*

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ARTICLE INFO

Article history:
Received 6 September 2012
Received in revised form 1 March 2013
Accepted 4 March 2013

Keywords:
α-Amylase
Crystal structure
Ca^{2+} binding
Acarbose
Substrate binding
Thermostability
Compact fold

ABSTRACT

A truncated form of an α-amylase, GTA, from thermophilic *Geobacillus thermoleovorans* CCB_US3_UF5 was biochemically and structurally characterized. The recombinant GTA, which lacked both the N- and C-terminal transmembrane regions, functioned optimally at 70 °C and pH 6.0. While enzyme activity was not enhanced by the addition of $CaCl_2$, GTA's thermostability was significantly improved in the presence of $CaCl_2$. The structure, in complex with an acarbose-derived pseudo-hexasaccharide, consists of the typical three domains and binds one Ca^{2+} ion. This Ca^{2+} ion was strongly bound and not chelated by EDTA. A predicted second Ca^{2+}-binding site, however, was disordered. With limited subsites, two novel substrate-binding residues, Y147 and Y182, may help increase substrate affinity. No distinct starch-binding domain is present, although two regions rich in aromatic residues have been observed. GTA, with a smaller domain B and several shorter loops compared to other α-amylases, has one of the most compact α-amylase folds that may contribute greatly to its tight Ca^{2+} binding and thermostability.

1. Introduction

α-Amylase (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1) from the glycoside hydrolase family 13 (GH13) is an endoglycosidase, cleaving the α-1,4-glycosidic bonds of α-linked polysaccharides [1,2]. It is a Ca^{2+}-dependent metalloenzyme, and contains one or two Ca^{2+} ions. The GH13 enzymes are characterized by a structurally conserved core with three domains, domains A–C, and a catalytic Asp-Glu-Asp triad. This large family has been divided into subfamilies [3], with 37 presently listed in the Carbohydrate Active Enzymes database (CAZy; http://www.cazy.org) [4]. α-Amylase has significant industrial value as it is extensively used in the production of glucose, fructose and cyclodextrins, as well as playing important roles in a variety of industries such as food, textiles, paper, detergents and biofuel [5,6]. Bacterial α-amylases are also commonly used as anti-staling agents in the baking industry to reduce bread firming [7].

Geobacillus thermoleovorans CCB_US3_UF5, a bacterium isolated from a hot spring in Ulu Slim, Malaysia, was observed to produce an extracellular α-amylase. The bacterium grew optimally at 60 °C, and could survive temperatures up to 70 °C. Its genome [8] is found to encode an α-amylase 513 residues in length. Designated GTA, it is 100% identical to the extracellular α-amylase from *G. thermoleovorans* subsp. *stromboliensis* subsp. nov. (strain Pizzo^T), which hydrolyzes soluble starch into maltose and maltotriose [9]. The thermostable Pizzo^T α-amylase can also adsorb onto and hydrolyze raw starch [9]. Raw starch-digesting enzymes, particularly of microbial sources, are increasingly important because they can help save cost during starch processing [10], as the cooking step to hydrate raw starch granules to open up their crystalline structure for enzymatic digestion can be bypassed.

GTA contains two putative Ca^{2+}-binding sites as deduced from its sequence. However, the Pizzo^T α-amylase showed an activity independent of Ca^{2+}, although addition of $CaCl_2$ was found to

Abbreviations: GTA, *Geobacillus thermoleovorans* α-amylase; gDNA, genomic DNA; ThMA, *Thermus* maltogenic amylase; CGTase, cyclodextrin glycosyltransferase; TVAII, *Thermoactinomyces vulgaris* R-47 α-amylase 2; SBD, starch-binding domain; AMY1, barley α-amylase isozyme 1; Sfamy, *Saccharomycopsis fibuligera* α-amylase; BaqA, *Bacillus aquimaris* MKSC 6.2 α-amylase; FspCMD, *Flavobacterium* sp. no. 92 cyclomaltodextrinase; ASKA, *Anoxybacillus* strain SK3-4 α-amylase; ADTA, *A.* strain DT3-1 α-amylase; BLA, *B. licheniformis* α-amylase; BAA, *B. amyloliquefaciens* α-amylase; LAMY, *Bacillus* KSM-1378 α-amylase; AmyUS100, *G. stearothermophilus* US100 α-amylase; BACΔNC, truncated *Bacillus* sp. strain TS-23 α-amylase.

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http://dx.doi.org/10.1016/j.enzmictec.2013.03.009

enhance its thermostability [9]. Analysis of GTA with SMART [11] predicted the presence of two transmembrane segments flanking both the N (residues 7–26) and C termini (residues 482–504). The N-terminal transmembrane segment represents a putative signal peptide as predicted by SignalP [12], with the cleavage site between A25 and A26. In order to investigate the role of Ca^{2+} and the mechanism of this enzyme, the gene fragment encoding residues 27–480 (excluding the two transmembrane regions) has been cloned, and the recombinant protein has been purified, characterized, and its crystal structure solved in complex with a pseudo-hexasaccharide derived from the inhibitor acarbose.

2. Materials and methods

2.1. Cloning, expression and purification

Genomic DNA (gDNA) was isolated from an overnight culture of *G. thermoleovorans* CCB_US3_UF5, grown in starch-yeast extract-tryptone (SYT) broth at 60 °C and 200 rpm. Using the gDNA as a template, the GTA gene (GenBank: AEV18110), excluding the sequences for the N- and C-terminal transmembrane segments, was amplified by PCR using the forward primer 5′-CCCCATATGGAAAAAGAAGAACGGACGTGG-3′ containing the restriction site *Nde*I (underlined), and the reverse primer 5′-CCCCTCGAGTTATGTTTTCTCCCGTAGCTT-3′ containing *Xho*I. The amplified fragment was cloned into pET-17xb (Novagen), and the recombinant vector was then transformed into *E. coli* BL21(DE3)pLysS (Promega).

The transformed cells were grown in LB broth supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C, and with shaking at 200 rpm. Protein expression was induced with 10 μM isopropyl-β-D-thiogalactopyranoside at OD_{600} around 0.6, and the culture was further incubated overnight at 26 °C and 120 rpm. The cells were then harvested and the pellet sonicated in 50 mM sodium phosphate, 200 mM NaCl, 10 mM PMSF, pH 8.0, and centrifuged at 10,000 × *g* for one hour at 4 °C. Ammonium sulfate powder was added slowly into the supernatant to 55, 60 and 65% saturation and centrifuged. The pellets were resuspended and dialyzed overnight at 4 °C. The resulting sample was then filtered and loaded into the anion exchange column Mono Q 5/50 GL (GE Healthcare), and the recombinant GTA was eluted with a linear gradient of up to 0.2 M NaCl. Final purification of the enzyme was achieved using a HiLoad 16/60 Superdex 200 pg gel filtration column (GE Healthcare).

2.2. Enzymatic assays

Prior to enzymatic assays, the sodium phosphate buffer of the enzyme was exchanged to 50 mM sodium acetate, 200 mM NaCl, pH 5.6. Enzyme activity was measured with the modified DNS method [13]. First, 10 μl of 1 μg/μl purified recombinant GTA was added to 90 μl of 1% soluble starch in 50 mM sodium acetate, 200 mM NaCl, pH 6.0, and incubated at 70 °C for 15 min. Next, 150 μl of DNS solution (10 mM 3,5-dinitrosalicylic acid, 300 mM potassium tartrate, 16 mM NaOH) was added to the mixture and heated at 100 °C for 5 min, cooled and measured at 540 nm. One unit of enzymatic activity is defined as the amount of enzyme required for the liberation of 1 μmol of maltose per ml per minute at 70 °C.

The optimum pH of the enzyme was determined at 70 °C in acetate buffer for pH 3.0–6.0, and in phosphate buffer for pH 7.0–8.0, whereas the optimum temperature was determined at 30–100 °C in acetate buffer at pH 6.0 for 15 min. The effect of Ca^{2+} on the enzymatic activity was determined by adding 1.0–10.0 mM $CaCl_2$ into the reaction mixtures. Thermostability was determined by pre-incubating the enzyme at 60 and 70 °C, with and without 2.0 mM $CaCl_2$, for various time intervals before conducting the enzymatic assays. The effect of EDTA was evaluated by pre-incubating the purified enzyme with 20.0 mM EDTA at 4 °C overnight before assays in 2.0 mM EDTA. It was assayed again after the removal of EDTA through buffer exchange. The kinetic constants, K_m and V_{max}, were measured by estimating the hydrolysis of 1–30 mg ml^{-1} soluble starch using the modified DNS method. The values of K_m and V_{max} were calculated from the Lineweaver–Burk plot. The concentration of GTA was determined using the Bradford protein assay.

2.3. Crystallization, data collection and structure determination

Before crystallization, the purified enzyme was dialyzed against 50 mM Tris–HCl, 200 mM NaCl, pH 8.0, and concentrated to 20 mg ml^{-1}. Using the sitting drop vapour diffusion technique, GTA crystals were grown at 4 °C in 0.1 M sodium citrate pH 5.0, 15% PEG 6000 and 20 mM acarbose. A crystal was flash-cooled to 100 K before diffraction data were collected at wavelength 1.5418 Å on a Rigaku MicroMax-007 HF X-ray generator, equipped with an R-AXIS IV++ area detector, and processed with XDS [14].

Using the crystal structure of TAKA-amylase from *Aspergillus oryzae* (PDB: 7TAA) [15] as an initial model, the structure was solved by molecular replacement with Molrep [16]. Automatic model building with Phenix [17] yielded a structure of about 80% completeness. A complete structure was then manually built and refined using Phenix and Coot [18]. Merohedral twinning with a twin fraction of 0.07 was detected, and the twin operator h,−h−k, −l was introduced during refinement. Data collection and refinement statistics are summarized in Table 1, and the structure has been deposited in the Protein Data Bank as entry 4E2O. Structural comparison was carried out on the Dali server [19], and the sequences of similar structures were manually edited and presented with ESPript [20]. All structural figures were generated with PyMOL (http://www.pymol.org).

Table 1
Data collection and structure refinement statistics.

	GTA
Data collection	
Space group	$P6_1$
Cell dimensions (Å)	$a = b = 122.96$, $c = 55.66$
Resolution (Å)	2.10 (2.16 – 2.10)[a]
R_{merge}	0.083 (0.354)
$I/\sigma(I)$	11.91 (3.14)
Completeness (%)	99.2 (97.0)
Redundancy	3.37 (3.27)
Refinement	
No. of reflections	27917
R_{work}	0.162
R_{free}	0.198
RMSD	
Bond length (Å)	0.007
Bond angle (°)	1.051
B factor (Å^2)	
Protein	22.85
Waters	27.61
Ligands/ions	25.53

[a] Values in parentheses are for the highest resolution shell.

3. Results

3.1. Characterization of GTA

The recombinant GTA had a molecular weight of 53 kDa, close to the 58 kDa of the identical full-length α-amylase from strain Pizzo^T [9]. GTA hydrolyzed soluble starch optimally at 70 °C and pH 6.0 (Fig. 1), also in agreement with the 70 °C and pH 5.6 reported for the Pizzo^T α-amylase [9]. However, GTA retained only 9 and 16%

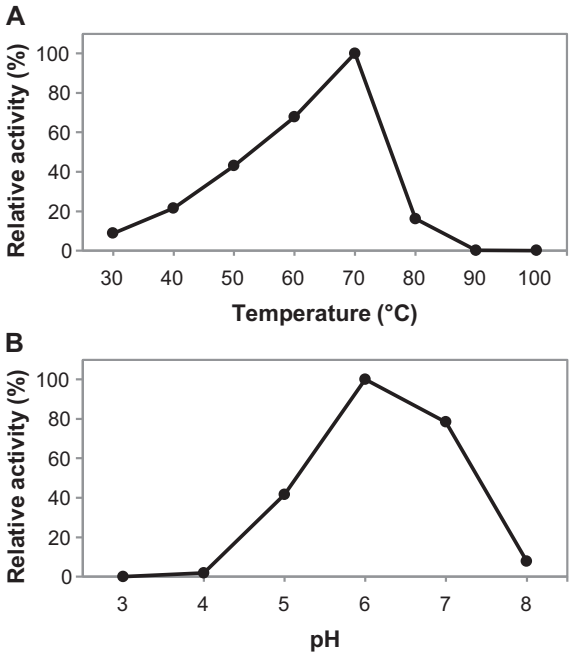

Fig. 1. Effects of (A) temperature and (B) pH on GTA activity. The optimal temperature and pH for the recombinant GTA are 70 °C and 6.0 respectively.