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Identification and characterization of a novel alkaline α-amylase Amy703 belonging to a new clade from *Bacillus pseudofirmus*

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Abstract Alkaline α-amylases are of great interest in desizing processes and detergent industries. Here, an alkaline α-amylase gene *amy*703 from an alkaliphilic *Bacillus pseudofirmus* strain was cloned and sequenced. Its encoding product, Amy703, might represent a new clade of α-amylase family, because it shared only 35 % highest identity with all amylases characterized up to date and was not clustered into any subfamilies with amylase activity in glycoside hydrolase family 13. Heterologous expression and characterization of Amy703 showed that it is a metalloenzyme with maximal activity at 40 °C and pH 9.0. Its activity was significantly enhanced by 2- and 2.48-fold at the presence of 10 mM Ca^{2+} and Mg^{2+}, respectively, while Hg^{2+} was a strong inhibitor of Amy703. Amy703 has a higher affinity (K_m = 3.92 mg/ml) for soluble starch compared to many other alkaline amylases. The computer modeling of its structure indicated that Amy703 contains typical amylase domains and a loop region appearing to bind the substrates. Site-directed mutagenesis suggested that a conserved residue Glu550 was essential for the activity of Amy703, and proposed it working together with other two residues to constitute a catalytic triad (Asp521, Glu550, and Asp615).

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Introduction

The α-amylases (EC3.2.1.1) belong to glycoside hydrolase family 13 (GH13) capable of hydrolyzing the internal α-1, 4-*o*-glycosidic bonds of starch and related polymers [37]. α-Amylases are widely distributed in plants, animals, and microorganisms. However, amylases from microbial sources have dominated applications in industries, due to their capability of extracellular secretion for economical bulk production, ease processing optimization, and simple genetic manipulation to improve desired enzymatic properties [16].

α-Amylase family enzymes have four well-known highly conserved sequence regions to form substrate-binding and catalytic sites, and three additional relatively conserved regions, based on exhaustive analyses of α-amylases and related enzymes, were proposed by Janeček [21]. More and more structural elucidation of α-amylases supported that α-amylases consist of three domains including N-terminal $(\beta/\alpha)_8$ or TIM (triosephosphate isomerase) barrel structure (domain A), a long loop (domain B) and C-terminal extension (domain C) [32]. All members in GH13 possess a TIM barrel catalytic domain, which three highly conserved residues (Asp-Glu-Asp) were proved to constitute a catalytic triad [23, 38].

α-Amylases hydrolyze starch and related polymers to produce α-anomeric monosaccharides and oligosaccharides or form α-glucosidic linkages by transglycosylations. Therefore, they have been applied widely in pharmaceutical, food, and fermentation industries for many decades [9, 16, 37].

Table 1 Primers used in this study

Name	Primers (5′-3′)
Amy-F	ATGGCAGGAGACCAAGTATATGATAAAGT
Amy-R	TTATTTCTGACCTCGCTTGTCACTC
Amy703-F	CGCGGATCCGCAGGAGACCAAGTATATGATAAAGTCG
Amy703-R	CCGCTCGAGTTATTTCTGACCTCGCTTGTCACTC
st-Amy703-F	TTGGCGcGGTATGGGACAGC
st-Amy703-R	CCATACCgCGCCAAATACATACAT

Primers Amy703-F and Amy703-R contained *Bam*HI and *Xho*I restriction sites are *underlined*. Primers st-Amy703-F and st-Amy703-R with 15 bp overlapping, and the mutated bases are shown in *lowercase letters*

Textile desizing also need largely α-amylases to remove starch, a strengthening agent, from the fabric, so that the high-quality fibers and low pollution can be achieved, as well the addition of α-amylases in detergents is advantageous to protect the fabric under milder conditions and lower temperatures [1]. However, several properties of commonly used α-amylases, such as high alkali-instability and low catalytic efficiency, have become obstacles to their applications, because textile desizing and detergents auxiliaries were performed at high pH values. Thus, great interest has been attracted to search for alkaline amylases with high alkali-stability and low cost.

In the present study, an alkaline α-amylase gene *amy703* from a newly isolated amylase-producing strain *Bacillus pseudofirmus* 703 was cloned and expressed in *Escherichia coli* system [27]. The biochemical properties of the recombinant alkaline α-amylase Amy703 were then characterized. Moreover, its phylogenic status was analyzed by comparing with 13 subfamilies within GH13 family with well-known or supposed α-amylases activity. Finally, a combination of these data, computer modeling of its structures, and site-directed mutagenesis supported Amy703 to be a novel alkaline α-amylase.

Materials and methods

Plasmids, strains, chemicals, and medium

The strain of *B. pseudofirmus* 703 was isolated from a paddy field at Hubei, China [27]. *Escherichia coli* XL10-Gold and BL21 (DE3) were obtained from Stratagene as the cloning host and expression host, respectively. The plasmid pET28a from Stratagene was used as the vector for the construction of the expression plasmid. While pMD18-T was purchased from TaKaRa (Dalian, China) for DNA sequencing. The plasmids and strains were selected and maintained in LB medium containing ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

The synthesis of DNA primers and DNA sequencing were performed by GenScript Co. Ltd (Nanjing, China). Restriction enzymes, ExTaq DNA polymerase, and T_4 DNA ligase were obtained from TakaRa (Dalian, China). All chemicals and reagents were analytical grade and commercially available.

Cloning of α-amylase gene *amy703* from *Bacillus pseudofirmus* 703

The genomic DNAs from the overnight culture of *B. pseudofirmus* 703 at 30 °C in Horikoshi-I medium [20] were extracted as described previously [33] and used for *amy703* amplification. A pair of primers (Amy-F and Amy-R, in Table 1) for *amy*703 amplification were designed based on the sequence of the predicted α-amylase of *B. pseudofirmus* OF4, a closest strain to *B. pseudofirmus* 703 [27]. Subsequently, *amy703* was amplified in a PCR amplifier (Bio-Rad, Hercules, CA, USA) using a 25-circling reaction composed of 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 3 min. The resulting 2,676-bp PCR product was ligated to pMD18-T and sequenced (its accession number in GenBank database is KF451925).

Construction of the expression plasmid

The α-amylase gene Amy703 without signal peptide was amplified by PCR using gene-specific primers Amy703-F and Amy703-R (Table 1). The PCR product was digested with *Bam*HI and *Xho*I, and then cloned into the expression vector pET28a. The recombinant plasmid (namely as pET28a-*amy703*) was confirmed by PCR and restriction enzyme digestion.

Expression and purification of recombinant α-amylase Amy703

The recombinant plasmid pET28a-*amy703* was transformed into *E. coli* BL21 (DE3) firstly. After cultivation in LB-kanamycin medium at 37 °C and 200 rpm until the $OD_{600} = 0.6$, the expression of Amy703 was induced with isopropylthiogalactoside (IPTG) at a final concentration of 0.5 mM for continuing 12-h cultivation at 18 °C. Cells were harvested by centrifugation, followed by ultrasonication. Then, His•Bind Kits (Novagen) were used to purify the His-tagged Amy703 following the manufacturer's instructions.

The purified protein was filtrated through a membrane with 50-kDa pores to concentrate and remove the other protein under 50 kDa. Then, the dialysis was conducted

against 50 mM Tris–HCl buffer (pH 9.0). All enzyme purification steps were carried out at 4 °C with 10 mM calcium ions added in the buffer in order to stabilize the α-amylase. The molecular weight and homogeneity of the protein were evaluated by SDS-PAGE.

Enzyme assays of α-amylase Amy703

The α-amylase activity was evaluated by measuring the amount of reducing sugar released in the enzymatic hydrolysis reactions using 1 % (w/v) soluble starch (Sigma) as substrate, following a modified dinitrosalicylic acid method [14]. One unit of α-amylase was defined as the amount of enzyme of releasing 1 μmol of reducing sugar per minute under the assay conditions, and glucose was used as a standard. The protein concentration was determined by Bradford assay using bovine serum albumin (BSA) as the standard [7].

Effects of temperature and pH on the enzyme activity and stability

The optimal temperature for activity of Amy703 was assessed at different temperatures ranging from 30 to 55 °C for 15 min in Tris–HCl buffer (50 mM, pH 9.0). The thermostability was determined by pre-incubating the enzyme for 20 min under specific conditions (30–50 °C), followed by residual activity determination at pH 9.0 and 40 °C.

The effects of pH on the enzyme activity were determined at different pH values by varying the buffer used to dissolved the substrate in the standard assay: 50 mM Tris–HCl buffer (pH 7.5–9.0) and 50 mM glycine–NaOH buffer (pH 9.0–11.0). The pH stability of α-amylase was studied by incubating the Amy703 at different pH values ranging from 8.0 to 10.5 at 4 °C for 12 h. The residual activity was then measured at pH 9.0 and 40 °C.

Effects of metal ions on enzyme activity

The effects of 10 mM various metal ions on the activity of Amy703 were determined under optimal conditions (pH 9.0, 40 °C). The activity of enzyme without any addition of metal ions was set as 100 %.

Substrate specificity

The substrate specificity of purified Amy703 was tested at 40 °C with soluble starch, amylose from potato, amylopectin from potato, amylopectin from maize, α-cyclodextrin, pullulan, glycogen from rat liver (purchased from Sigma) at a concentration of 1 % (w/v) in Tris–HCl (50 mM, pH 9.0) as substrates. The amount of reducing sugar produced was measured as mentioned above.

Determination of kinetic parameters

Enzymatic assay for determining kinetic parameters was performed in Tris–HCl buffer (50 mM, pH 9.0) at 40 °C using soluble starch at different concentrations from 1 to 10 mg/ml. The Eadie–Hofstee plots were used to calculate kinetic parameters K_m and V_{max} according to the enzyme reactions.

Sequence analysis and homology modeling

Protein sequence similarity were analyzed using Blast analysis [2]. Multiple sequence alignments were performed by ClustalX with default parameters, and the alignment result was exported by ESPript [15, 40]. The phylogenetic tree was constructed using Mega5.2 based on the neighbor-joining method [39]. The signal peptide was identified using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP).

Homology modeling of Amy703 was conducted by SWISS-MODEL program using default parameters [4], debranching enzyme NPDE from cyanobacterium *Nostoc punctiforme* (PDB: 2wkgA) shared 29 % identity of amino acid sequence was chosen as the best structure template by SWISS-MODEL program automatically. The simulated structure was validated by Verify 3D [13]. All 3D structures of Amy703 were visualized by Pymol Molecular Graphics System [11].

Site-directed mutagenesis

Glu(550) of Amy703 was mutated to Ala by site-directed mutagenesis method. Briefly, inverse polymerase chain reaction amplification of entire circular pET28a-*amy703* was performed using mutagenic primers st-Amy703-F and st-Amy703-R (Table 1). The PCR products were purified by agarose gel purification kit, and then digested with *Dpn*I. The *Dpn*I-treated products were transformed into competent *E. coli* XL10-Gold. Two transformants were sent to be sequenced by T7 promoter primer. At last, the mutated recombinant plasmid was transformed into *E. coli* BL21 (DE3), and transformant was test for the expression of amylase. The subsequent enzymatic assay followed the procedures as above.

Results

Cloning and bioinformatics analysis of α-amylase Amy703

A 2,607-bp α-amylase-encoding gene *amy703* was cloned from *B. pseudofirmus* 703, an alkaliphilic strain that showed alkaline α-amylase activity by plate assay [27].

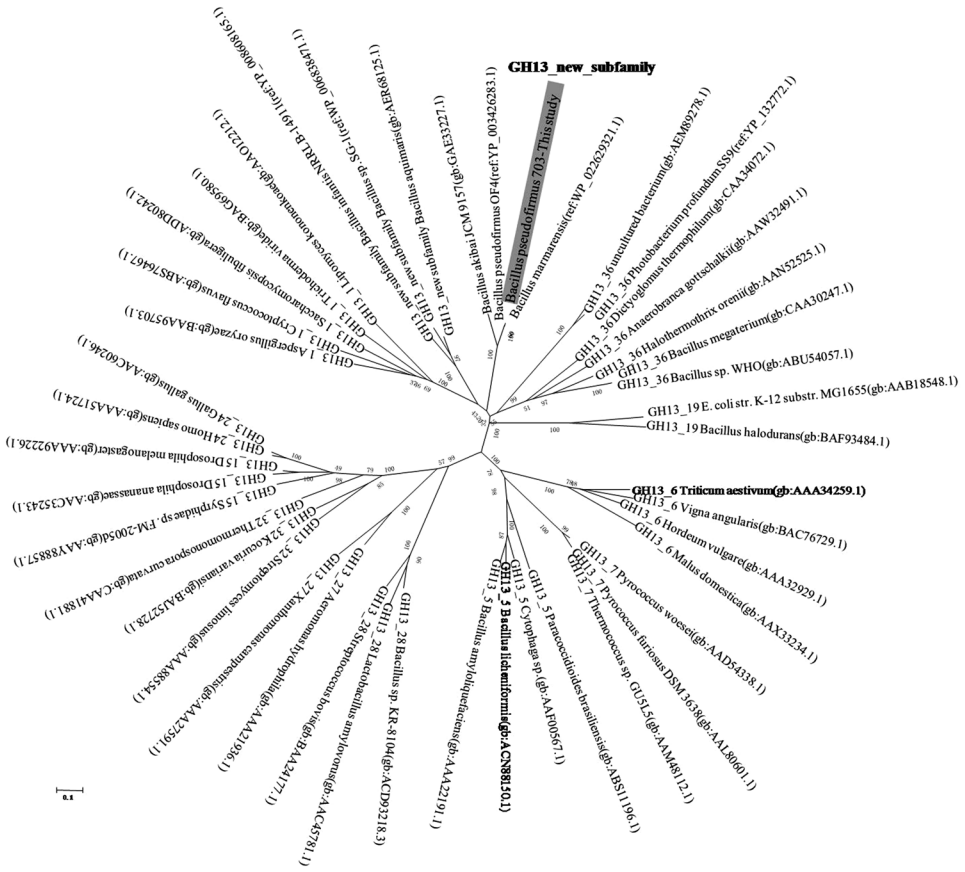

Fig. 1 Establishment of the phylogenetic tree of Amy703 α-Amylase from *Bacillus pseudofirmus* 703 and some other α-amylases from bacilli are not clustered into any other previously known GH13 subfamilies with amylase activity. The individual α-amylases are represented by the GH13 subfamily number, their producers, and the GenBank accession numbers. Sequence alignment was performed by ClustalX and the tree was created by program MEGA5.2 using un-rooted neighbor-joining method

A 23-residue signal peptide in the N-terminal of *amy*703 detected by web-based SignalP 4.1. Blast analysis showed that Amy703 shared the highest identity (35 %) to AmyB (GenBank accession number: CAA31586.1) from *Dictyoglomus thermophilum* [19] among all amylases characterized functionally and enzymatically. We also observed that Amy703 has a higher identity to alpha amylase catalytic region in *B. pseudofirmus* OF4 (97 %, GenBank accession number: ADC49391.1) and to α-amylase in *Halobacillus halophilus* DSM 2266 (51 %, GenBank accession number: CCG46443.1) respectively, but both of which have not yet been verified experimentally.

To elucidate the evolutionary status of Amy703, a phylogenetic tree was established based on alignment of Amy703 with α-amylases in different subfamilies of family GH13 using MEGA5.2. To date, a total of 40 subfamilies of GH13 have been found in the Carbohydrate-Active enzyme (CAZy) database (http://www.cazy.org/) [8], 12 subfamilies within GH13 family have been reported with α-amylase activity (or, in a few cases, putative α-amylase), includes GH13_1, GH13_5, GH13_6, GH13_7, GH13_15, GH13_19, GH13_24, GH13_27, GH13_28, GH13_32, GH13_36, and GH13_39 [31]. The number of subfamilies with α-amylase activity is still increasing, a raw starch-degrading α-amylase from *Bacillus aquimaris* MKSC 6.2 recently be classified as a new cluster, might represent a novel GH13 subfamily [31], although it has not been collected by the CAZy database since the data update period. Based on these sequences, plus three unclassified but the highest homologous sequences of Amy703, the phylogenetic tree (Fig. 1) was constructed to show that Amy703 and putative homologous are not clustered into any existing subfamilies of GH13, implying that they constitute a distinct clade of α-amylases.

To predict the conserved regions and the catalytic triad of Amy703, multiple sequence alignments were performed based on six best-matched amylases characterized enzymatically (GenBank accession number ACA48225.1, AAX85453.1, P14898.2, ADN80732.1, AFM43699.1, BAN63122.1) as well as the debranching enzyme NPDE, whose 3D structure is available, from cyanobacterium *Nostoc punctiforme* (PDB: 2WKG), which was chosen as the structure template of Amy703. Figure 2 shows seven conserved regions, which is similar to the reports of Refs. [23, 45]. Among them, region I contains three highly conserved amino acid residues (Asp-Asn-His) that were verified previously to be critical for the stability and activity of amylases. Region II contains a catalytic nucleophile residue Asp521 and invariant residue Arg519, both of which were highly conserved in all α-amylases and believed to be also

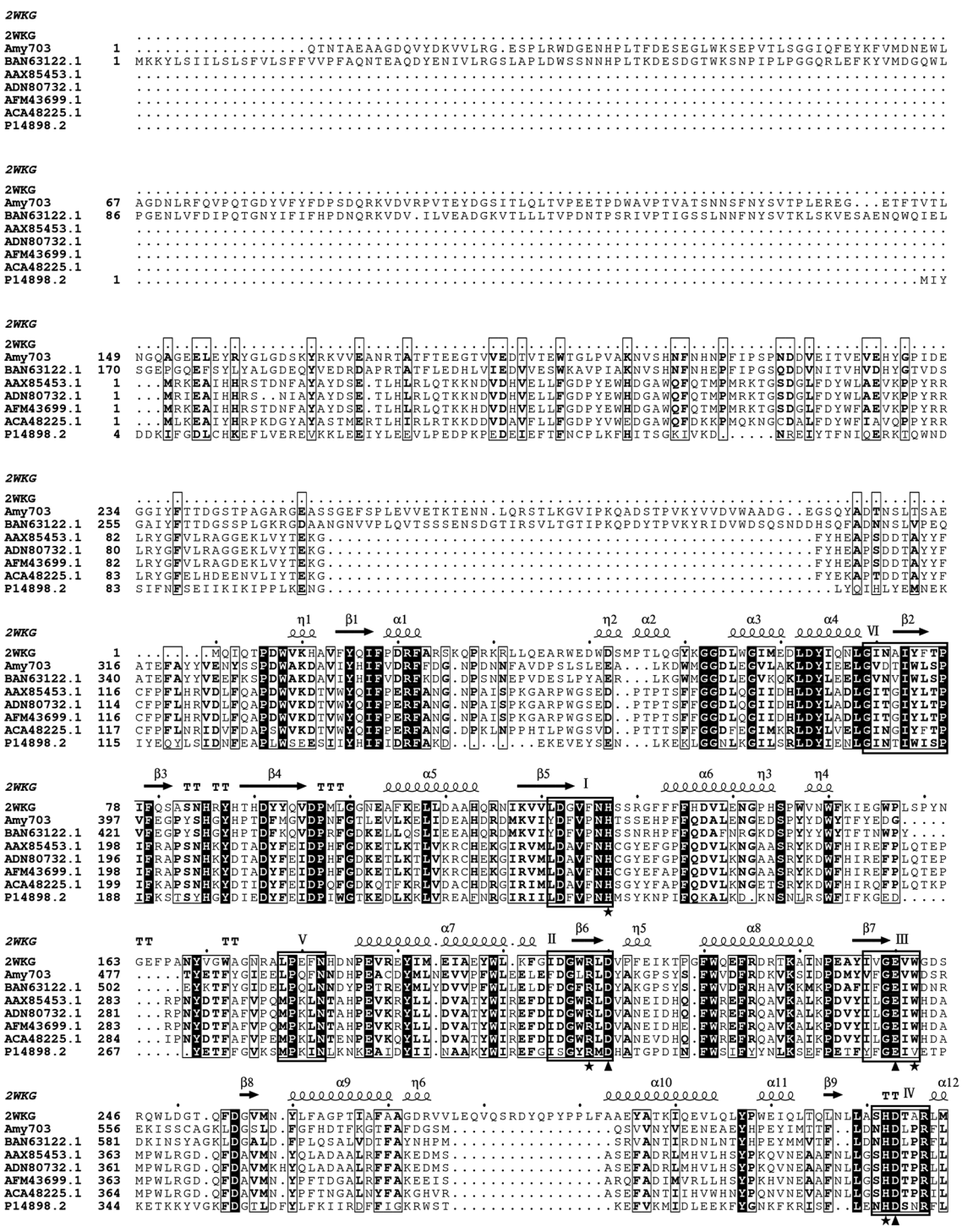

Fig. 2 Multiple sequence alignment of Amy703 orthologies. The accession numbers represent alpha-amylase from *Bacillus* sp. AAH-31 (BAN63122.1), amylase from *Bacillus* sp. WPD616 (ACA48225.1), cyclodextrinase from uncultured bacterium (AAX85453.1), amyB from *Dictyglomus thermophilum* (P14898.2), maltogenic dextrinase from *Bacillus* sp. (ADN80732.1), and maltogenic-amylase from *Geobacillus thermoleovorans* (AFM43699.1). 2WKG_A represents the debranching enzyme NPDE from cyanobacterium *Nostoc punctiforme*, which was chosen as the best structure template of Amy703. Strictly conserved residues are indicated in *white letters on a grey background*, and conservatively substituted residues are *boxed*. The conserved regions are shown in *black boxes*. The residues equivalent to the putative catalytic triad are indicated by a *solid triangle* and the other four highly conserved residues are indicated by a *black asterisk*. These residues are believed to play a vitally important role in the interaction of Amy703 with substrate

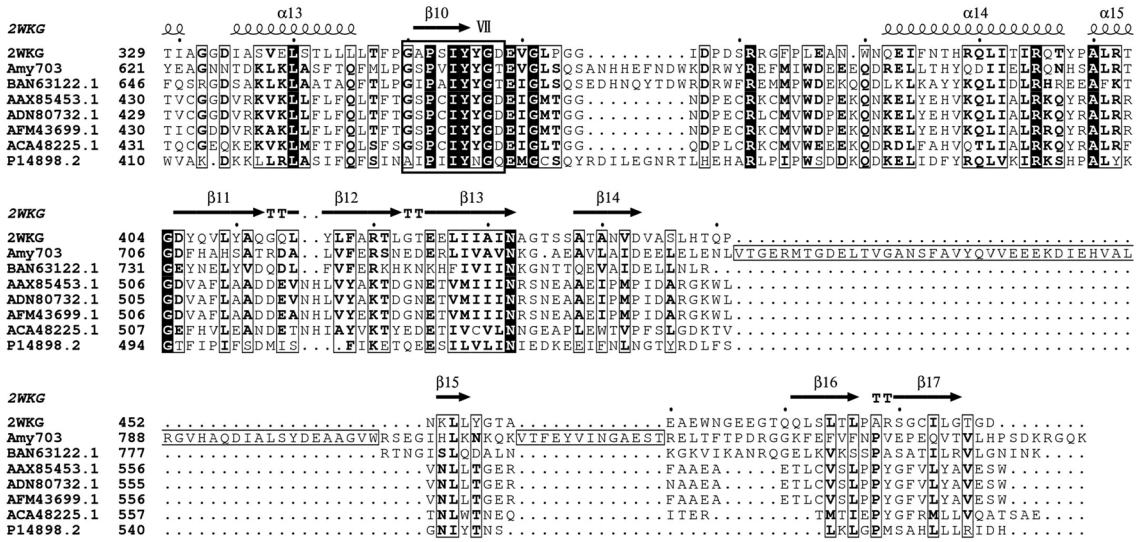

Fig. 2 continued

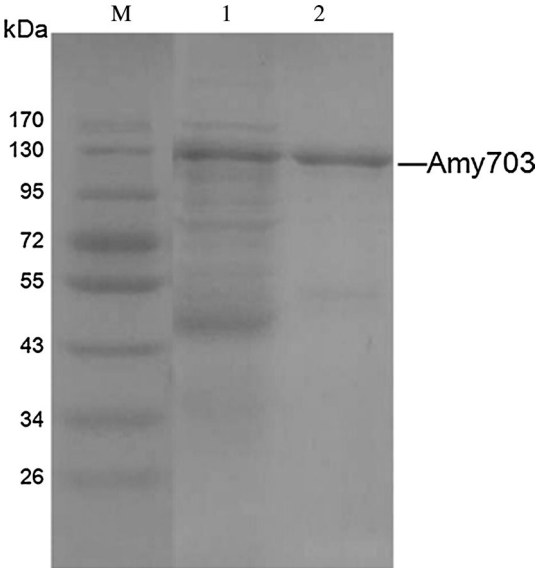

Fig. 3 SDS-PAGE analysis of the recombinant Amy703. Samples were resolved on a 12 % polyacrylamide gel and then stained with Coomassie Blue R-250. *M* Protein marker; *1* Lysate of induced *E. coli* BL21 (DE3) pET28a-*amy703* cells; *2* Purification on Ni–NTA affinity column

Table 2 Purification steps of recombinant amylase Amy703

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude enzyme	35.4	51.11	1.44	1	100
Ni-column	6.05	29.8	4.93	3.42	58.3

indispensable for catalytic activity of amylase. Region III includes the catalytic proton donor Glu550 and an another conserved residue Trp552 that were significant for the geometry of the substrate binding groove, and the tryptophan residue positioned two residues succeeding the catalytic proton donor (Glu550 in Amy703) plays a vitally important role in substrate specificity, and be reported to make hydrophobic stacking interactions with glucose moieties [12, 28]. Region IV contains three fully conserved residues, of which Asp615, the catalytic residue, and His614 are postulated to make hydrogen bonds with substrate [29]. Region V contains two fully conserved residues Pro489 and Asn492, and it was proposed before that this region seems to be very characteristic for enzyme specificity, there is an evolution that occurs from QPDLN in oligo-1,6-glucosidase group to MPKLN in neopullulanase group via the MPDLN for the members of the α-amylase family [21], and this region has also been reported to be involved in binding of the structural calcium ion [6]. In Amy703, the Ca^{2+}-binding aspartate (corresponding to Asp206 in Taka-amylase A) was substituted by glutamine, which means Amy703 may have a different Ca^{2+}-binding position. Region VI is characterized by a glycine residue followed by seven amino-acid residues and then a conserved proline. This region plays a role in distinguishing CGTases from α-amylases and is evolutionarily important for the $(\beta/\alpha)_8$ –barrel fold [22]. Region VII contains four highly conserved residues, Pro644, Ile646, Tyr647, and Gly649, and other residues were relatively conserved, while it has been reported that invariant residues conserved throughout all the α-amylase family members were nonexistent in this region [21].

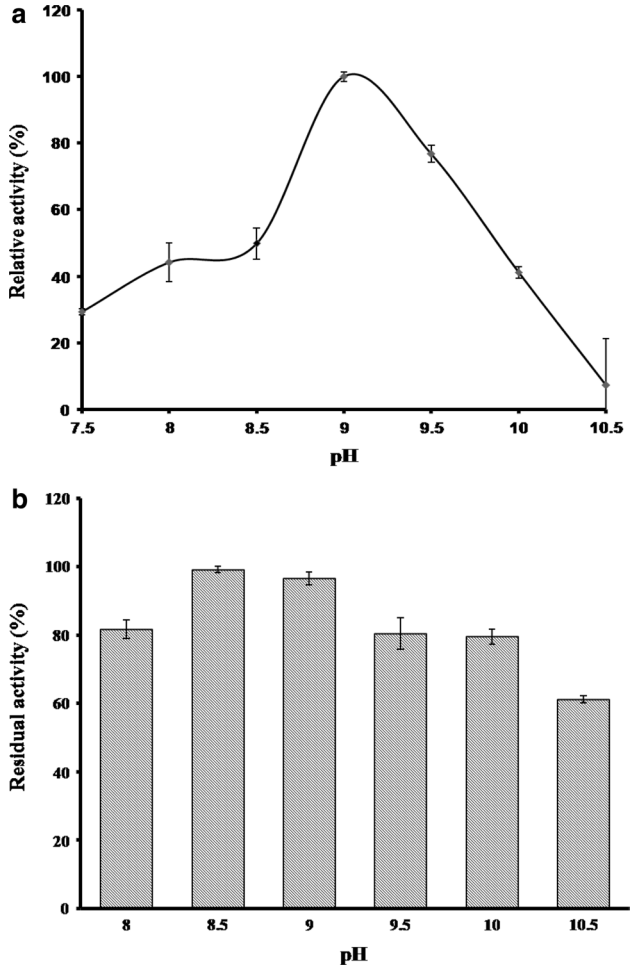

Fig. 4 Effects of pH values on the activity and stability of Amy703. **a** Effect of pH on the activity of Amy703. The reaction was conducted at 40 °C with soluble starch as substrate in different buffers: 0.5 mM Tris–HCl (pH 7.5–9.0) and 0.5 mM glycine–NaOH (pH 9.0–10.5). **b** Effect of pH on the stability of Amy703. The stability of Amy703 was determined at different pH values ranging from 8.0 to 10.5 (0.5 mM Tris–HCl or 0.5 mM glycine–NaOH) at 4 °C for 12 h. After incubation, the residual activity was measured at pH 9.0 and 40 °C. Each value of the assay was the arithmetic mean of triplicate measurements

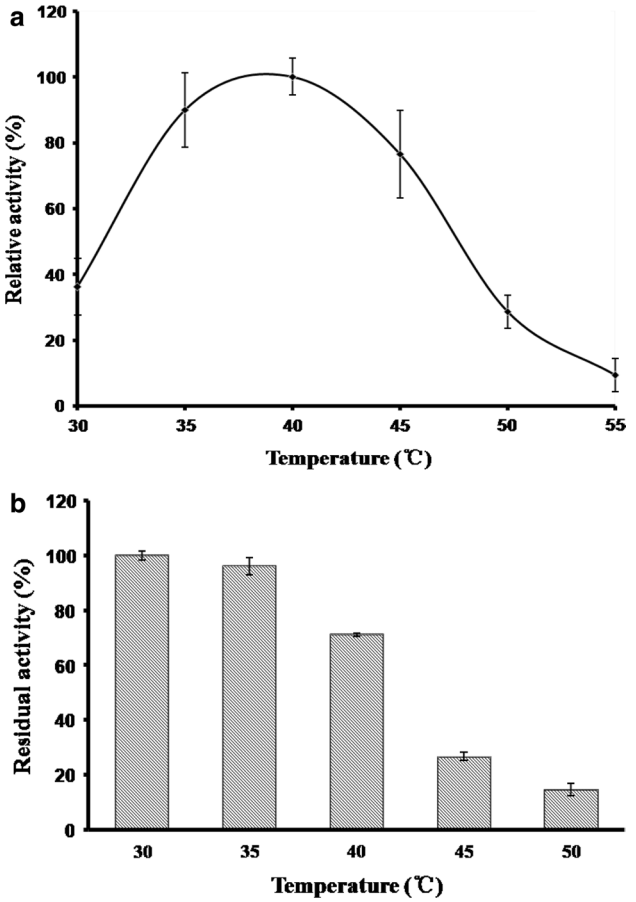

Fig. 5 Effect of temperature on the activity and stability of Amy703. **a** Effect of temperature on the activity of Amy703. To determine the optimal temperature of Amy703, the reaction was conducted from 30 to 55 °C in 0.5 mM Tris–HCl buffer (pH 9.0). **b** Effect of temperature on the stability of Amy703. The thermal stability of Amy703 was determined at the indicated temperature in 0.5 mM Tris–HCl buffer (pH 9.0) for 20 min. After incubation, the residual activity of Amy703 was measured at pH 9.0 and 40 °C. Each value of the assay was the arithmetic mean of triplicate measurements

Expression and purification of the recombinant α-amylase Amy703

We established a prokaryotic expression system (*E. coli* BL21 (DE3)/pET28a-amy703) for inducible expression of the mature α-amylase Amy703 lacking signal peptide. Based on the optimization of induction conditions, recombinant protein could be expressed efficiently in this system and accumulated about 17 % of the total protein in the cell extract. Cell extract was loaded on the Ni–NTA resin, and eluted by imidazole concentration gradient to obtain the purified Amy703. SDS-PAGE analysis revealed that the purified Amy703 migrated as one band with a molecular

Table 3 Effect of metal ions on the activity of Amy703

Metal ions (10 mM)	Relative activity (%)
No addition	100 ± 0.6
Na^+ (NaCl)	70.59 ± 4
Ca^{2+} ($CaCl_2$)	300 ± 2
Mg^{2+} ($MgCl_2$)	348.04 ± 1.1
Ni^{2+} ($NiCl_2$)	14.22 ± 2.5
Li^+ (LiCl)	81.86 ± 2
K^+ (KCl)	83.82 ± 3
Co^{2+} ($CoCl_2$)	135.29 ± 2
Hg^{2+} ($HgCl_2$)	26.29 ± 1.5
EDTA	0 ± 0.6

The assay was taken at optimal conditions (40 °C and pH 9.0) with soluble starch as substrate. No addition means the activity of Amy703 was determined in buffers without the addition of any ions

Table 4 Substrate specificity of Amy703

Substrate	Specific activity (U/mg)
Soluble starch	4.93
Amylopectin from potato	2.432
Amylopectin from maize	2.394
Amylose from potato	0.704
Glycogen from rat liver	0.294
Pullulan	N.D.
α-cyclodextrin	N.D.

For the determination of substrate specificity, purified Amy703 was incubated in Tris–HCl (50 mM, pH 9.0) at 40 °C with different substrates (1 % w/v)

mass of about 110 kDa (Fig. 3), the same as the theoretical value. We also evaluated its amylase activity for each step and found that it appeared quite stable during purification (Table 2).

Characterization of the recombinant α-amylase Amy703

In the subsequent experiments, we determined enzymatic properties of recombinant alkaline α-amylase Amy703. The pH and temperature are critical parameters for amylase application, so the effects of pH and temperature on Amy703 were investigated firstly. Our data showed that its activity increased gradually as the increase of pH from 7.5 to 9.0, while it declined obviously when the pH values were higher than 9.5 (Fig. 4a). As it exhibited maximal activity between 9 and 9.5, and retained about 80 % of its maximal activity between 8.0 and 10.5 after incubation at 4 °C for 12 h, as shown in Fig. 4b, we concluded that it was an alkaline α-amylase, based on the criteria for alkaline amylase [34].

The optimal activity of the purified Amy703 was observed at 40 °C and it still could function with high remaining activity (76 %) at 45 °C and displayed nearly 90 % activity at 35 °C as shown in Fig. 5a. Amy703 retained above 95 % of activity after 20 min incubation at 30 and 35 °C, while it lost activity rapidly above 40 °C (Fig. 5b).

Most of the previously reported amylases are metalloenzyme [16]. In this study, the effects of various metal ions on the activity of Amy703 are shown in Table 3. Its activity was improved significantly by 300 and 348 % at the presence of 10 mM Ca^{2+} and Mg^{2+}, respectively. On the other hand, 10 mM EDTA inhibited completely its enzyme activity, while the activity was regained after addition of Ca^{2+} or Mg^{2+} (>10 mM). These results supported that Amy703 was a Ca^{2+}/Mg^{2+}-dependent metalloenzyme. It was also strongly inhibited by Hg^{2+} and Ni^{2+} with relative lower remaining activity (26 and 14 %, respectively). In addition, K^{+}, Na^{+}, Li^{+}, and Mn^{2+} showed inhibition on Amy703 to a lower extent (Table 3).

The kinetic properties of Amy703 were analyzed using soluble starch as a substrate under the optimal conditions. Based on our data, it is calculated that Amy703 hydrolyzed soluble starch with K_m of 3.92 mg/ml and V_{max} of 17.664 nmol/min/mg. This implies that Amy703 has relatively higher affinity for soluble starch (see "Discussion" section).

Substrate specificity

Amy703 was hydrolytically active on a number of substrates as shown in Table 4. Apart from soluble starch, Amy703 also degraded amylopectin from potato, amylopectin from maize, amylose from potato, and glycogen from rat liver, but there were no detectable activities on α-cyclodextrin and pullulan. The specific activity (U/mg) for soluble starch, amylopectin from maize, amylopectin from potato, amylose from potato and glycogen from rat liver were 4.93, 2.394, 2.432, 0.704, and 0.294, respectively. These results suggest that Amy703 merely catalyze the hydrolysis of α-1, 4-glucosidic bonds in starch and related α-glucans.

Computer modeling and site-directed mutagenesis of Amy703

In order to understand further the mechanistic characteristics of Amy703 for subsequent molecular engineering, we performed the 3D structure simulation by computer modeling of Amy703 (Fig. 6a) using the crystal structure of a debranching enzyme NPDE from cyanobacterium *Nostoc punctiforme* as template [12].

The mimic structure of Amy703 suggested three domains were included in this protein, which were domain A (a typical TIM barrel structure), domain B and C, as well as a loop region protruded away from the catalytic activity center, which was reported previously to bind the substrate in many amylases [12]. The putative catalytic triad residues (Asp521, Glu550, and Asp615) were located at the bottom of the active site cleft, and residue interaction between catalytic triad by hydrogen bonding are shown in Fig. 6b.

We introduced a mutation (Glu550Ala) in Amy703 by site-directed mutagenesis. The subsequent enzymatic assay showed that the point mutation at this residue led to complete loss of activity (data not shown). This implies that Glu550 is essential for Amy703 activity and might be critical for constituting a catalytic triad together with Asp521 and Asp615.

Discussion

Though starch-degrading enzymes are distributed widely throughout the whole biodiversity, it is an increasing

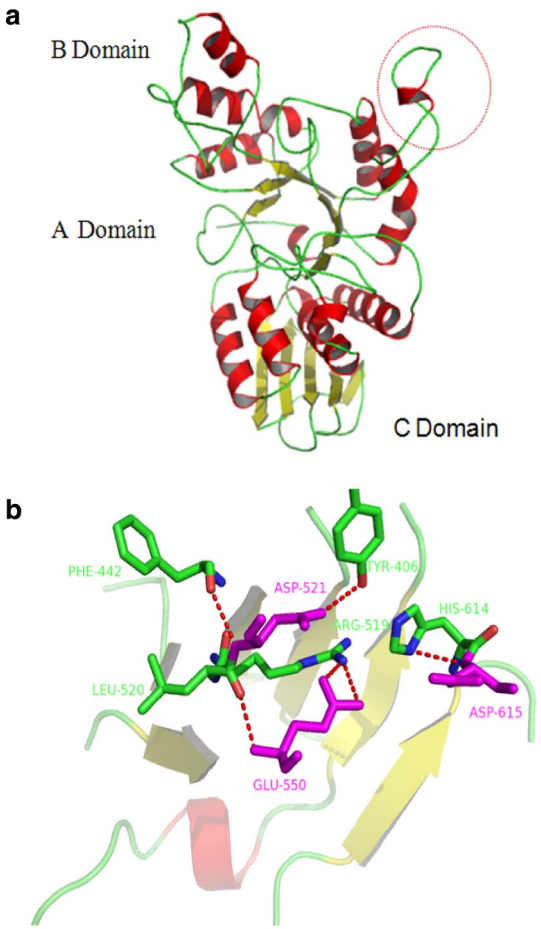

Fig. 6 Homology model of Amy703. The SWISS-MODEL program was performed to construct the 3D structure of Amy703 based on NPDE from cyanobacterium *Nostoc punctiforme* (PDB: 2WGK_A). The visualization of the tertiary structure was done by PyMOL. **a** The homology modeled structure of Amy703. β-strands and α-helices are shown in *yellow* and *red*, respectively. The region involved in substrate binding is highlighted in *red* and circled in *dotted magenta lines*. For clarity, the loops have been smoothed using PyMOL. **b** Active site architecture of Amy703 model. The active site of Amy703 model shown as cartoon, the catalytic triad (Asp521, Glu550, Asp615) shown in *magenta sticks*, residues (Tyr406, Phe442, Arg519, Leu520, His615) interacted by hydrogen bonding with catalytic triad shown as *sticks*, and the hydrogen bond shown in *red dots*. Several other residues in close contact with the active site are shown without labels (color figure online)

interest to screen alkaline amylases, since the huge potential application exists in textile desizing and detergent industries [37]. Therefore, in the present work, we cloned and characterized an alkaline amylase Amy703 from *B. pseudofirmus*. This is the first report to isolate alkaline amylase from *B. pseudofirmus*.

Since Amy703 shared only 35 % sequence identity to its highest homologous amylase reported functionally and occupied a distinct position in the phylogenetic tree, we characterized its biochemical properties and enzymatic parameters. Our data proved that:

Firstly, Amy703 ($K_m = 3.92$ mg/ml) has a relatively higher affinity for soluble starch compared to many other alkaline amylases like amylase from *B. alcalophilus* JN21 (9.64 mg/ml) [44], AmyA (9.0 mg/ml), and AmyB (8.7 mg/ml) from *Alkalimonas amylolytica* [36].

Secondly, Amy703 is an alkaline amylase and shows maximal activity at pH 9.0, which was consistent with some other alkaline amylases [17, 44]. Though alkaline α-amylase from *Bacillus* sp. A3-15 showed an extremely higher optimal pH of 11.0 [3], Amy703 displayed higher stability (80 %) in a wider range of pH (8.0–10.5) for 12 h than alkaline amylase from *Bacillus* sp. A3-15, Amy34 from *Bacillus halodurans* LBK 34 [18], and BLA from *Bacillus* sp. TS-23 [26].

Thirdly, though the optimal temperature of Amy703 was 40 °C, similar to reported alkaline amylases from *Bacillus* sp. IMD370 [41], *Bacillus flexus* XJU3 [35] and *Bacillus* sp. AB04 [5], and even lower than alkaline amylase from *Bacillus alcalophilus* [44], amylases ASKA and ADTA from *Anoxybacillus* species [9], and amylase from *Bacillus* sp. A3-15 [3]. Amy703 was more stable below 40 °C, implying that Amy703 showed superiority for application in the detergent industries of developing countries.

In addition, Amy703 was a metalloenzyme, also supported by the facts that most of α-amylases were metalloenzymes [42], despite several metal ion–independent α-amylases [30]. More interestingly, Amy703 could be enhanced significantly by Ca^{2+}or Mg^{2+} as activators (300 % by Ca^{2+} and 348 % by Mg^{2+}). To our knowledge, it should represent an example being activated at the highest extent by Ca^{2+} or Mg^{2+}, compared to all known α-amylases, including amylase ASKA from *Anoxybacillus* sp. (259 %) [9] and amylase from *Bacillus amyloliquefaciens* P-001 (140 %) [10].

It was also observed that Amy703 was completely inhibited by 10 mM EDTA, similar to some reports [24, 25], which suggested that metal ions were needed for stabilization of the tertiary structure of Amy703. The activity of Amy703 pre-treated by EDTA could completely regained by addition of Ca^{2+}/Mg^{2+}. These results indicated further that Amy703 was Ca^{2+}/Mg^{2+}-dependent metalloenzyme.

Amy703 will probably have a possibility to be applied in textile desizing or detergent industries, because in these processes, Ca^{2+}/Mg^{2+} naturally existing in the circulating water or hard water could efficiently enhance its starch-degradation activity.

Finally, we observed that Hg^{2+} was a strong inhibitor of Amy703. Zhang et al. [43, 46] reported a xylanase XynZG that has a relative activity of 1.7 % when 5 mM Hg^{2+} coexisted, and the inhibition was attributed to oxidization of indole ring by Hg^{2+}. In this study, we observed Amy703

still remain 27 % activity with 10 mM Hg^{2+}. The sequence analysis of amino acid showed that there are 17 tryptophan residues in the total 868 amino acids of Amy703, only one was (Trp552) located in the conserved region, while in the XynZG, there are seven tryptophan residues in the total of 260 amino acids, and six are located at the highly conserved region. This explains why Amy703 can still remain 27 % residual activity with 10 mM Hg^{2+}.

Additionally, bioinformatic analysis proposed the putative catalytic triad (Asp521, Glu550, and Asp615) exists in Amy703. Generally, α-amylases hydrolyze the internal α-(1,4) glucosidic linkages in starch through α-retaining double displacement [16]. According to this mechanism, we deduced the conserved residue Glu550 of Amy703 as an acid/base catalyst, as well Asp521/Asp615 as nucleophiles to bind with OH-2 and OH-3 groups of the substrate through hydrogen bonds. The 3D-modeled structure of Amy703 showed that the putative catalytic residues (Asp521, Glu550, and Asp615) were located at the bottom of the active site cleft, like other amylases. Though Amy703 and the template NPDE shared low sequence identity (29 %) and the residue-based quality of this modeled structure of Amy703 by Verify 3D showed a relative low assessment value that only 87.80 % of the residues had a score >0.2 [12], seven conserved sequence regions clearly existed and residues in these regions were highly conserved, as shown in Fig. 2. Thus, it is possible to have a better understanding of this amylase from its modeled structure. In order to identify the predicted catalytic residues, site-directed mutagenesis of Glu550 was performed and confirmed its essential role in the activity of Amy703 and the catalytic triad.

Alkaline amylases were proposed to have significant economic advantages in several industries, such as desizing and detergent. The Amy703 studied in the present study displayed high alkali-stability and has a relatively low optimum temperature, endowing Amy703 with potential applications in enzyme-based desizing processes and detergent industries.

In conclusion, we identified a novel alkaline amylase Amy703 and characterized it to be a metalloenzyme. Its biochemical properties are suggestive for its potential applications in enzyme-based desizing processes and detergent industries.

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