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ORIGINAL RESEARCH PAPER

A mutant α-amylase with only part of the catalytic domain and its structural implication

T. Ke · X. D. Ma · P. H. Mao · X. Jin · S. J. Chen · Y. Li · L. X. Ma · G. Y. He

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Abstract A truncated mutant α-amylase, Xa-S2, was obtained from *Xanthomonas campestris* wild type α-amylases (Xa-WT) through random mutagenesis that contained 167 amino acid residues (approx 65% shorter than that of Xa-WT). Secondary structure prediction implied that Xa-S2, would be unable to form the whole $(\beta/\alpha)_8$-barrel catalytic domain and did not have the three conserved catalytic residues of wild type α-amylase, but it still displays the starch-hydrolyzing activity. Xa-S2 was prepared, characterized and compared to the recombinant wild-type enzymes. The K_m for starch was 32 mg/ml; activity was optimal at pH 6.2 and 30°C. In contrast, the K_m for starch of Xa-WT was 8 mg/ml and optimal enzyme activity was at pH 6.0–6.2 and 45–50°C. Our results suggested that Xa-S2 is a new amylase with a minimal catalytic domain for hydrolyzing substrates with of α-1,4-glucosidic bonds.

Keywords α-amylase · $(\beta/\alpha)_8$-barrel catalytic domain · Mutagenesis · Truncated mutant

Introduction

α-Amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) is present in microorganisms, animals and plants and has important industrial applications. It catalyzes the hydrolysis of α-1,4-glucosidic bonds of glycogen, starch and related polysaccharides or oligosaccharides (Davies and Henrissat 1995). Several α-amylases from different species have been crystallized and analyzed by X-ray diffraction. α-Amylase contains three domains: (1) the central catalytic domain, A, containing an amino-terminal $(\beta/\alpha)_8$-barrel structure; (2) a smaller domain, B, present as a loop between the third β-strand and the third α-helix of the $(\beta/\alpha)_8$-barrel; and (3) a C domain at the *C*-terminus, consisting of antiparallel β-strands (Janecek et al. 1997). The central catalytic domain contains three critical catalytic residues located at the bottom of the active site cleft, that are invariant throughout the α-amylase family members and the substitution of any one of

T. Ke and X. D. Ma contributed equally to this work

T. Ke · Y. Li · G. Y. He (✉)
China-UK HUST-RRes Genetic Engineering and Genomics Joint Laboratory, Huazhong University of Science and Technology (HUST), Wuhan, Hubei 430074, China
e-mail: guangyuan.he@bbsrc.ac.uk

X. D. Ma · S. J. Chen · L. X. Ma
College of Life Science, Hubei University, Wuhan, Hubei 430062, China

P. H. Mao · X. Jin
Ion Beam Biotechnology Center, Xinjiang University, Urumqi, Xinjiang 830008, China

three catalytic residues inactivates the enzyme (Svensson and Sùrgaard 1993).

To investigate the structure-function relationship of α-amylase, we constructed a random mutagenesis library via the nucleotide analogue substitution method and have obtained some mutant amylases. Among these, a truncated mutant with low amylase activity attracted our interest. It has only a small part of the whole $(\beta/\alpha)_8$ barrel catalytic domain, and lacks the three conserved regions where the three catalytic amino acid residues are located. Nevertheless, it still retains catalytic activity towards soluble starch. Earlier results showed that no functional α-amylase without the $(\beta/\alpha)_8$ barrel structure has been obtained in the laboratory or from nature (Marco et al. 1996). Here, we suggest that the functional mutant amylase, containing only part of the (β/α) subunits should be a good tool for better understanding of the catalytic properties of $(\beta/\alpha)_8$-barrel proteins. In this investigation, the truncated amylase (Xa-S2) and the wild type amylase (Xa-WT) have been expressed in *E. coli* and purified to compare their kinetic parameters and enzyme properties. Based on the analysis of primary structure alignment between Xa-S2 and Xa-WT, we predict important features of the secondary structure of this new amylase, and discuss the implications of this structure.

Materials and methods

Bacterial strains, materials, plasmids, and growth conditions

Escherichia coli DH5α and BL21 (DE3) were used as the host for gene manipulation and for expression of truncated amylase and wild-type Xa, respectively. pET30a was used as an expression vector, and pBluescript II KS(+) was used as a mutagenesis and sequencing vector. The *E. coli* transformants were cultured at 37°C in LB broth that contained either 100 μg ampicillin/ml or 50 μg kanamycin/ml. Mutants were screened on LB agar plates that contained 1% (w/v) soluble starch and 0.01% (w/v) Trypan Blue. Chemicals were purchased from Sigma Chemical Co. unless otherwise stated. Restriction enzymes were obtained from New England Biolabs, Polymerase EX-Taq and ligase were purchased from Takara Co. The oligonucleotides (Table 1) were synthesized by Shanghai Sangon Biological Engineering Technology Co. (Shanghai, China).

Molecular techniques and sequencing

Enzyme digestion, ligation, transformation and recovery of PCR products were all carried out according to the methods described by Sambrook et al. (1989). Sequencing of the α-amylase genes was carried out by TaKaRa Co. (Dalin, China).

In vitro random mutagenesis and mutant library construction

The in vitro random mutagenesis method and the construction of mutant libraries method were carried out as previously described (Ma et al. 2004). The wild type amylase gene and mutant amylase gene selected from the mutant library were sequenced before constructed them to the expression vector of *E. coli*.

Construction of expression plasmids

A 1.4-kb DNA fragment containing the open reading frame (ORF) of the Xa-WT gene was PCR amplified using primer 1 and primer 2 which contain a *Kpn*I and a *Sac*I site, respectively. Primers 1 and 3 were used to amplify the Xa-S2 gene. Primers 1 and 4 were used to amplify the Xa-S2-1 (deleted 96 bp encoding the *C*-terminal 32 amino acids of Xa-S2) gene. Following double digestion with *Kpn*I and a *Sac*I, the PCR products were then ligated into pET30a at the corresponding sites, which yielded plasmid pXAWT (Xa-WT), pXAS2 (Xa-S2), pXAS2-1 (Xa-S2-1), and then, they were transformed into *E. coli* BL21 (DE3).

Table 1 Primers used in this work

Number	Sequence
1	5′-TTGGTACCATGCACGCCACCTCT-3′
2	5′-GCGAGCTCAACGTCGCCACATAC-3′
3	5′GCGAGCTCACTGGCGCTCAAAAAG-3′
4	5′-GCGAGCTCAGTAATTGAGGTCCGA-3′

Preparation of crude enzyme

For hyper-expression of the α-amylase gene, the transformant harboring a recombinant plasmid with the α-amylase gene was first propagated at 37°C overnight in 5 ml LBkan. 0.5 ml of the culture was then transfered to 50 ml LBkan and incubated at 37°C until OD_{600} = 0.4–0.6. After this, 1 mM IPTG was added and the culture was incubated at 26°C for 2 h. Cells were harvested by centrifugation (3,000 × *g*, 10 min, and 4°C), the pellets were resuspended in 6 ml of 10 mM phosphate buffer (pH 7.8) and the cell suspensions were then subjected to ultrasonication (200 W, 10 min). After centrifugation again, the crude enzyme was obtained in the supernatant.

Enzyme purification, SDS–PAGE, activity assays and protein determination

Each protein was fused to an *N*-terminal His_6-tag, and purified to homogeneity (as judged by SDS-PAGE analysis) using Ni-chelate affinity chromatography. α-Amylase activity was assayed by a modified starch-iodine method (Yoo et al. 1987). The hydrolytic activities of the wild-type and mutant proteins were assayed at their optimal temperatures and pH value using 0.5% (w/v) soluble starch in 50 mM sodium acetate. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 mg soluble starch per minute under relevant conditions. The total protein concentration was quantified using the Bradford protein assay (Bio-Rad).

Nucleotide sequence accession numbers

The nucleotide sequence for the wild type α-amylase and the truncated amylase Xa-S2's genes were deposited in the GenBank databases under accession number of **AF482991** and **AY167891** respectively.

Results

Purification of a-amylase from *E. coli*

To compare the kinetic parameters and enzyme properties of Xa-S2 and Xa-WT, the two amylases gene PCR product were digested with *Kpn*I and a *Sac*I and the fragments was ligated into the same sites of the the *E. coli* expression vector pET30a. Protein expression was initiated by the addition of IPTG (1 mM). After the crude enzymes had been obtained, the proteins were expressed in *E. coli* and purified to homogeneily. Their molecular masses were estimated by SDS-PAGE. The results shown in Fig. 1 indicate 22.3 kDa for Xa-S2 and 56.2 kDa for Xa-WT.

Comparison between the enzyme characteristics of Xa-S2 and Xa-WT

As shown in Fig. 2, both amylases were optimally active at pH 6–6.2 and at 30°C for the Xa-S2, at 45–50°C for Xa-WT. The activities of Xa-S2 and Xa-WT were increased to 1.4-fold and 1.1-fold respectively at the presence of 2 mM $CaCl_2$.

Kinetic studies of the enzymes

The kinetic parameters of both Xa-WT and Xa-S2 were studied by using soluble starch as substrate. The V_{max} value of Xa-S2 was 2 U/mg, which was 57-fold lower than that of Xa-WT (113 U/mg). The K_m value of Xa-S2 was 32 mg/ml, which was 4-fold higher than that of Xa-WT (8 mg/ml). Thus the affinity to starch of Xa-S2 is lower than that of the wild type amylase. The

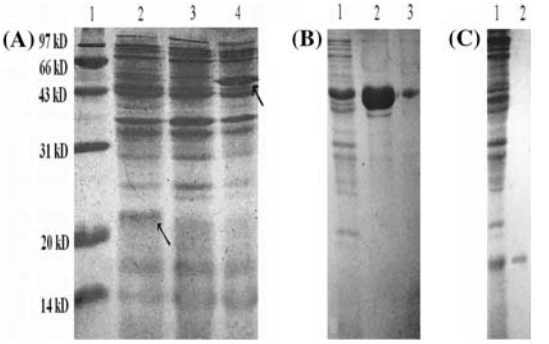

Fig. 1 SDS-PAGE analysis of Xa-WT and Xa-S2. (**A**) Lane 1, protein size marker; Lane 2, *E. coli* BL21 (pXAS2) with IPTG induction; Lane 3, *E. coli* BL21 (pET30a); Lane 4, *E. coli* BL21 (pXAWT) with IPTG induction; (**B**) Lane 1, *E. coli* BL21 (pXAWT) with IPTG induction; Lanes 2 and 3, purified Xa-WT; (**C**) Lane 1, *E. coli* BL21 (pXAS2) with IPTG induction; Lane 2, purified Xa-S2

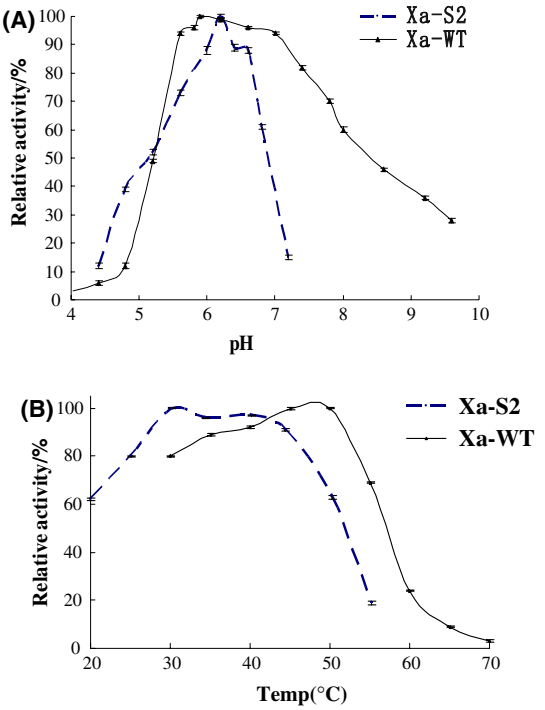

Fig. 2 Effects of pH (**A**) and temperature (**B**) on enzyme activity. All experiments were done in triplicate. Data points represent the average of duplicate experiments. (**A**) Specific activitis of Xa-WT was determined at 30°C; (**B**) Specific activitis of Xa-S2 was determined at pH 6.2

V_{max}/K_m value of Xa-WT is 240-fold higher than that of Xa-S2.

Alignment of amino acid sequences and secondary structure prediction of Xa-S2 and Xa-WT

The Xa-WT gene had a single open reading frame composed of 1,425 nucleotides encoding 475 amino acids, including a signal peptide region composed of 35 residues at the *N*-terminus. Four highly conserved regions in the α-amylase family and three invariant catalytic residues were identified through alignment with other well-known α-amylases (Hu et al. 1992) based on the deduced amino acid sequences (see Fig. 3). The Xa-S2 gene originated from the wild type amylase gene via a G base insertion, which resulted in a reading frame shift. The single open reading frame for Xa-S2 was composed of 501 nucleotides encoding 167 amino acid residues. The comparative alignment of amino acid sequences in Xa-S2 and Xa-WT showed that the *N*-terminal 135 amino acids of Xa-S2 were identical with those of Xa-WT. The molecular weights of Xa-WT and Xa-S2 calculated from their amino acid sequences by computering deduction were 52.3 and 18.4 kDa, respectively. Analysis of the secondary structure and three-dimensional structure prediction result (the HMMSTR program in http://www.bio-info.rpi.edu/~bystrc/hmmstr) showed that the two enzymes shared similar secondary structure elements in the *N*-terminal sequence, including $\beta1$, $\alpha1$, $\beta2$, $\alpha2$ and $\beta3$ of the $(\beta/\alpha)_8$-barrel (domain A). The $\beta2$ and $\beta3$ of Xa-S2 are longer than those of Xa-WT. In addition, Xa-S2 also had a short β-sheet and a helix composed of the original domain B and 32 carboxyterminal residues at the *C*-terminal end. From this analysis we conclude that Xa-S2 has a new configuration, and obviously unable to form a barrel domain. Compared with the structures of wild type amylase, the truncated amylase appeared to only have one domain, including $(\beta/\alpha)_1$ and $(\beta/\alpha)_2$ subunits in the *N*-terminus, followed by a β-sheet and a new α-helix at the *C*-terminal end.

Analysis of Xa mutants

To investigate the role of the *C*-terminal region of Xa-S2, we constructed a new truncated mutant Xa-S2-1, containing only 135 amino acid residues of the *N*-terminus of Xa-S2 via PCR amplification using primer1 and primer 4 (see Table 1). Specifically, 32 amino acid residues that are not present in the indigenous wild type protein at the *C*-terminal end of Xa-S2 were deleted. We found that Xa-S2-1 did not display any hydrolytic activity toward the soluble starch. Different activities between the truncated Xa-S2 and Xa-S2-1 indicate that the new 32 amino acid residues at the *C*-terminus may play a very important role in keeping the enzyme activity or the stability of configuration.

Discussion

The $(\beta/\alpha)_8$-barrel scaffold is one of the most versatile protein folds adopted by a large number of enzymes and has raised much interest in the

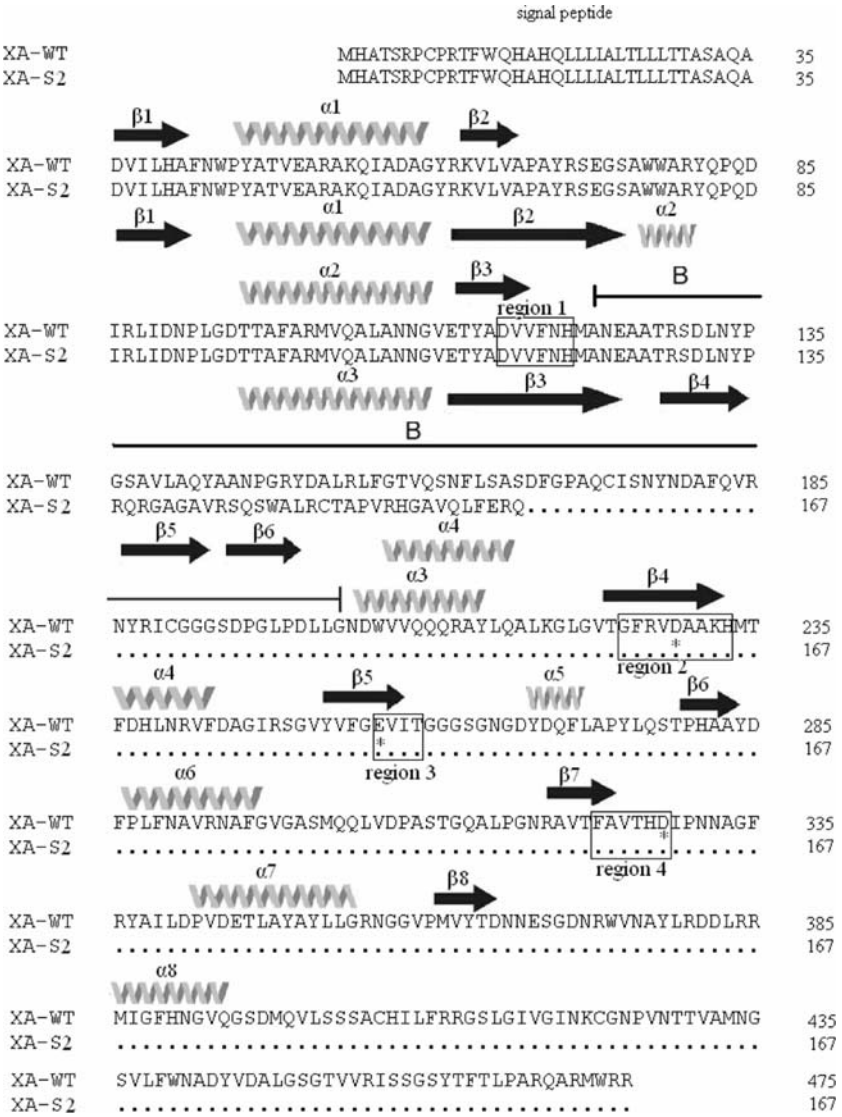

Fig. 3 Structure-based sequence alignment between Xa-WT and Xa-S2 and their secondary structure prediction. The secondary structures are predicted using the HMMSTR program in http://www.bioinfo.rpi.edu/~bystrc/hmmstr. The catalytically important residues are marked by a star. Four regions (1, 2, 3, and 4) that are highly conserved in the α-amylase family are in boxes. The β-strands and helixes of the amino-terminal $(\beta/\alpha)_8$-barrel (domain A) are indicated by black arrows and helices. Domain B between β3 and α3 is indicated by a black line

structural, enzymological and evolutionary properties of this fold (Wierenga 2001). Recently, the $(\beta/\alpha)_4$-half-barrel was identified as a possible structural subdomain and the smallest evolutionary unit of the proteins which contained $(\beta/\alpha)_8$ barrel domain (Lang et al. 2000). A interesting question arises as to whether the $(\beta/\alpha)_4$-half-barrel is the minimal subdomain to maintain the enzyme functions of this type of protein. All structural and functional data obtained to date support the conclusion that the whole of the $(\beta/\alpha)_8$-barrel catalytic domain is essential for amylase activity. The functional amylase Xa-S2 described in this report, containing only the $\beta\alpha\beta\alpha\beta$ unit, is the smallest subdomain of active α-amylases identified to date. The symmetry analyses of the $(\beta/\alpha)_8$-barrel suggest that the smallest possible unit is the $\beta\alpha\beta\alpha$ unit (Wierenga 2001). Our results suggest that the smallest symmetrical unit may serve as a functional unit for the enzymes. We also anticipate that it is possible to constructed artificial functional amylase containing from 2 to 8 (β/α) subunits.

Throughout the α-amylase family, three catalytic residues appear to be required for functionality of α-amylase. Substitution of any of these residues causes completely loss of activity. In the case of Xa-S2 which lost all the three catalytic

residues (D194, E221 and D293) of the wild type amylase, the remaining amylase catalytic activity may be most contributed to the highly conserved residue Asp (D81 on the β3-strand) located in a strictly conserved structural motif (region1) in the α-amylase family, which has been recognized as an important residue for substrate binding site (Svensson 1994). We suggest that this residue may also play a very important role in the wild type amylase.

With regard to the enzyme characteristics, our results suggest that both amylases are Ca^{2+}-stimulated. Xa-S2 is less thermostable than the Xa-WT. The affinity and the Vmax of the Xa-S2 were much lower than those of the Xa-WT, presumably due to the loss of the three catalytic residues and the change of the structural configurations.

Based on the experimental results described above, we suggest that the $(\beta/\alpha)_8$-barrel domain provides a stabilized structural scaffold and substrate binding site for α-amylase, and that it can be separated into several functional subunits. The mutant Xa-S2 provides a new avenue to getting the insight into structure-function relationships of barrel domains. The folding of the structure, the position of the subsites for substrate (the position where the substrate is cleaved, Davies et al. 1997) and the catalytic reaction mechanism of this new enzyme call for the further investigation.

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