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Effect of introducing a disulphide bond between the A and C domains on the activity and stability of *Saccharomycopsis fibuligera* R64 α-amylase

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ABSTRACT

Native enzyme and a mutant containing an extra disulphide bridge of recombinant *Saccharomycopsis fibuligera* R64 α-amylase, designated as Sfamy01 and Sfamy02, respectively, have successfully been overexpressed in the yeast *Pichia pastoris* KM71H. The purified α-amylase variants demonstrated starch hydrolysis resulting in a mixture of maltose, maltotriose, and glucose, similar to the wild type enzyme. Introduction of the disulphide bridge shifted the melting temperature (T_M) from 54.5 to 56 °C and nearly tripled the enzyme half-life time at 65 °C. The two variants have similar k_{cat}/K_M values. Similarly, inhibition by acarbose was only slightly affected, with the IC50 of Sfamy02 for acarbose being 40 ± 3.4 μM, while that of Sfamy01 was 31 ± 3.9 μM. On the other hand, the IC50 of Sfamy02 for EDTA was 0.45 mM, nearly two times lower than that of Sfamy01 at 0.77 mM. These results show that the introduction of a disulphide bridge had little effect on the enzyme activity, but made the enzyme more susceptible to calcium ion extraction. Altogether, the new disulphide bridge improved the enzyme stability without affecting its activity, although minor changes in the active site environment cannot be excluded.

1. Introduction

α-Amylase (α-1,4-glucan-4-glucanohydrolase, EC. 3.2.1.1) hydrolyzes starch into linear and branched oligosaccharides (Vihinen and Mantsala, 1989). The enzyme has been employed in industries that utilize starch processing, such as in the production of food, pharmaceuticals, detergents, textile, paper, and bioethanol (Pandey et al., 2000). More recently, the enzyme has also been tested for medical applications (McCue and Shetty, 2004).

Saccharomycopsis fibuligera, a yeast commonly used in the fermentation of rice and cassava, is one of the best α-amylase producers (Chi et al., 2009). α-Amylases from this organism (Sfamys) demonstrate raw-starch degradation (Hasan et al., 2008; Hostinová et al., 2010) and are active at pH 5.0–6.0, which is about the pH of starch slurry (Yamashita et al., 1985; Gogoi et al., 1987; Sukara et al., 1998; Hostinova, 2002; Hasan et al., 2008). Sfamys are active at a temperature range of 50–65 °C, and, in the case of Sfamy R64, can

Abbreviations: Aotamy, Aspergillus oryzae Taka-amylase; Anamy, Aspergillus niger α-amylase; DNS, 3,5-Dinitrosalicylic acid; EDTA, ethylene diamine tetra-acetate; MES, 2-(N-morpholino) ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PEGylation, modification with polyethylene glycol; SDS, sodium dodecyl sulphate; Sfamy, *Saccharomycopsis fibuligera* α-amylase.

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be stored up to months at room temperature without losing activity (Ismaya et al., 2013). α-Amylases that are active under conditions of industrial processing are highly desired, and therefore Sfamy has been modified to improve its stability against e.g. temperature, chelating agents, and proteolytic inactivation. Previously, a Sfamy from *S. fibuligera* strain R64 (Sfamy R64) was chemically modified by PEG. The residual activity of the PEGylated enzyme was doubled at higher temperatures and the enzyme was more resistant toward chelating agents and proteolytic digestion (Ismaya et al., 2013).

Sfamy R64 (GenBank accession no. **HQ172905**) contains 494 amino acid residues (including the 26-amino-acid signal peptide) that share high sequence identity to *Aspergillus* α-amylases of which three-dimensional structures have been elucidated (Brzozowski and Davies, 1997; Vujicic-Zagar and Dijkstra, 2006). The amino acid sequences of yeast α-amylases are highly homologous. For instance, the amino acid sequence of mature Sfamy R64 (468 amino acids) shares 87 and 85% similarity (54 and 52% identity) to those of the mature α-amylases from *A. niger* and *A. oryzae*, respectively. The crystal structure of *A. oryzae* α-amylase has indeed been used as a model for the structural study of yeast α-amylases (Matsui et al., 1994). The *A. oryzae* α-amylase structure consists of three domains, which are assembled into two separate entities, the integrated A-B domains and the C domain (Janecek et al., 1997). Domain A contains the classical $(\beta/\alpha)_8$-TIM barrel motif (Janecek and Sevcik, 1999), while domains B and C are more diverse. The A-B entity contains the catalytic domain while the C domain has been associated with the binding of starch substrates. These two entities are connected by a long surface loop, allowing the C domain to be flexible during the catalysis. A calcium ion, an essential cofactor of α-amylase, is bound in the A-B domain interface.

Previous results showed that modification by chemical agents to introduce stronger hydrophobic interactions in the interface between the A and C domains yielded modified enzymes with lower activities (Ismaya et al., 2013), which could be due to an increase in the rigidity of the enzyme molecule (Vieille and Zeikus, 1996). On the other hand, the lower enzyme activity could also be due to perturbations at the active site of the enzyme. Therefore, we made a modification directed solely to the A-C domain interface by introducing a disulphide bond between these two domains. Such an approach has successfully been carried out for e.g. *Rhizopus chinensis* lipase, where the $t_{1/2}$ value at 60 °C was increased by up to 11-fold and the T_M by 7 °C (Yu et al., 2012), and *Drosophila melanogaster* acetylcholinesterase (Siadat et al., 2006).

The structures of *A. niger* and *A. oryzae* α-amylases suggest the presence of structurally conserved disulphide bonds between Cys57-Cys65, Cys177-191, Cys267-Cys310, Cys462-Cys493 (the Sfamy R64 amino acid sequence numbering will be used henceforth) in the catalytic domain. Disruption of these bonds causes disintegration of the catalytic domain, making Sfamy R64 more susceptible to proteolytic digestion (Ismaya et al., 2013), demonstrating the power of disulphide bonds to maintain domain integrity. On account of the initial finding of improved enzyme stability due to better molecular packing in the region between the A and C domains (Ismaya et al., 2013), introducing a disulphide bond in the domain interface region appeared an appealing approach to stabilize the enzyme. Following the success of overexpressing Sfamy R64 in the yeast *Pichia pastoris* KM71H, designated as Sfamy01, the amino acid residues at positions 336 and 437 were mutated into cysteines to promote a new disulphide bond. The resulting mutant, designated as Sfamy02, showed similar activity and pH optimum, but appeared to have improved optimum working temperature, melting temperature, as well as reduced susceptibility toward inactivation by acarbose.

2. Materials and methods

2.1. Bacterial and yeast strains

Escherichia coli TOP10 for cloning and site directed mutagenesis, and *Pichia pastoris* KM71H (*arg4 aox1::ARG4*) for expression of the recombinant protein were purchased from Invitrogen (Carlsbad, Germany).

2.2. Homology modeling of Sfamy R64 and search for candidate residues for mutation

A homology model of Sfamy01 was generated with SWISS-MODEL (Bordoli et al., 2009), using the amino acid sequence of Sfamy R64 and the structure of *A. oryzae* α-amylase (PDB accession no. **7TAA**). The structural geometry of the model was checked with PROCHECK (Laskowski et al., 1993). The Sfamy model was manually evaluated using Coot (Emsley et al., 2010). Candidate residues for mutation were searched using SSBOND (Hazes and Dijkstra, 1988) using Taka-amylase as the template, or selected manually based on the Sfamy R64 homology model. The graphic representation of the model was prepared with PyMOL (DeLano, 2008).

2.3. Cloning of Sfamy into pPICZαA

The Sfamy gene encoding residues 21-494 was amplified by PCR using chromosomal DNA of *S. fibuligera R64* as the template and primers A1F (GAA TTC GTG ACT CTA TTC AAA AGA GAA A) and A2R (TCT AGA CAT GAA CAA TGT CAG AAG C). A fragment of ~1.4 kb was recovered and ligated into the pGEMT cloning vector prior to subcloning into pPICZαA, in which the *Sfamy* open reading frame was fused with α-mating factor. The cloning of *Sfamy* R64 into pPICZαA-Sfamy was confirmed by nucleotide sequence analysis.

2.4. Site-directed mutagenesis

Mutations were introduced sequentially into *Sfamy*, using the pPICZαA-Sfamy plasmid as the template, employing the primers A3F (CAG CGA CCA ATG CTT GAT TTC TAA TG) and A4R (CAT TAG AAA TCA AGC ATT GGT CGC TG) to generate the first mutation, designated as pPICZαA-SfamyS336C. The resulting plasmid was first transformed into *E. coli* TOP10 for propagation. The second mutation (S437C) was carried out using pPICZαA-SfamyS336C as the template, and A5F (CAA CCT TGG TTG CAG CGG TTC TT) and A6R (AAG AAC CGC TGC AAC CAA GGT TG) as the primers. The mutations were confirmed by a sequencing analysis.

2.5. Transformation of P. pastoris

The transformation was carried out using the Pichia EasyComp™ Kit according to the manufacturer's instruction (Invitrogen, Carlsbad, Germany). The yeast cells carrying *Sfamy* R64 were grown on a YPD agar plate containing 100 μg/mL zeocin, 1% of starch and 0.5% of methanol for 3 days at 30 °C. α-Amylase was expressed in YPD medium containing 1% of starch for 3 days at 30 °C, supplemented with 0.5% methanol supplemented daily. Iodine solution was added to this starch containing culture to check for α-amylase activity.

2.6. Production and purification of recombinant α-amylase

Sfamy01 was produced in a fed-batch culture in a 1.5 L vessel (Eyela, Tokyo, Japan) at 30 °C for three days, aeration rate of 1.0 vvm (volume of gas per volume of media per minute) (47–64% of air saturation), mixing rate of 200 rpm, with daily methanol supplementation at 1.5% (v/v). Sfamy02 was produced in a shake flask