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Engineering disulfide bonds in *Selenomonas ruminantium* β-xylosidase by experimental and computational methods

Ehsan Dehnavi[a], Mehrnoosh Fathi-Roudsari[b], Sako Mirzaie[c], Seyed Shahriar Arab[d], Seyed Omid Ranaei Siadat[e,f,**], Khosro Khajeh[a,*]

[a] Department of Biochemistry, Faculty of Biological Science, Tarbiat Modares University, Tehran, Iran
[b] National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[c] Department of Biochemistry, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran
[d] Department of Biophysics, Faculty of Biological Science, Tarbiat Modares University, Tehran, Iran
[e] Protein Engineering Laboratory, Protein Research Center (PRC), Shahid Beheshti University, GC, Tehran, Iran
[f] Nanobiotechnology Engineering Laboratory, Faculty of Engineering and New Technologies, Shahid Beheshti University, GC, Tehran, Iran

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ABSTRACT

Homotetrameric β-xylosidase from *Selenomonas ruminantium* (SXA) is one of the most efficient enzymes known for the hydrolysis of cell wall hemicellulose. SXA shows a rapid rate of activity loss at temperatures above 50 °C. In this study, we have introduced two inter-subunit disulfide bridges with one point mutation. Lys237 was chosen to be replaced with cysteine since it interacts with the same residue in the opposite subunit. While pH optimum, temperature profile and catalytic efficiency of the mutated variant were similar to the native enzyme, the mutated enzyme showed about 40% increase in thermal stability at 55 °C. Our results showed that introduction of a single residue mutation in structure of SXA results in appearance of two disulfide bonds at dimer-dimer interface of the enzyme. Coarse-grained molecular dynamics (CG-MD) simulations also proved lower amounts of root mean square fluctuation (RMSF) for position 237 and potential energy for mutated SXA. Based these results, we suggest that choosing a correct residue for mutation in multi subunit proteins results in multiple site conversions which equals to several simultaneous mutations. Furthermore, CG-MD simulation in agreement with experimental methods showed higher thermostability of mutated SXA which proved applicability of this simulation for thermostability analysis.

1. Introduction

β-D-Xylosidase/α-L-arabinofuranosidase from the ruminal anaerobic bacterium *Selenomonas ruminantium* (SXA) is a bifunctional glycoside hydrolases (belonging glycoside hydrolases family 43) which simultaneously shows β-D-xylosidase and α-L-arabinofuranosidase activities (EC.3.2.1.37) [1]. The enzyme cooperates with other members of glycoside hydrolases family including endo-xylanase (EC.3.2.1.8) and accessory enzymes like α-L-arabinofuranosidase and α-glucoronidase to degrade xylan polymers. Xylan is a major structural component commonly found in the plant cell wall hemicelluloses [2]. Due to superior catalytic efficiency and dual function, SXA has potential utility in industrial processes such as depolymerization of complex carbohydrates and production of ethanol fuel and other bio-products [3]. Nevertheless, the applicability of this enzyme in industrial applications is restricted due to its low stability at temperatures above 50 °C. The three dimensional structure of SXA has been determined by Brunzelle and his colleagues in 2008. Tertiary structure of *Selenomonas ruminantium* β-D-xylosidase exhibited a homotetrameric structure composed of two dimers with the A + B dimer turned at 90° against C + D dimer [4].

Reinforcing the subunit contact interface by introduction of additional interactions is one of the best strategies for increasing thermal stability in oligomeric proteins [5]. Disulfide bonds are among substantial physical forces which can maintain the tertiary structure of proteins even in unfavorable condition. Not only some examples of increasing thermostability of tetrameric enzymes due to introduction of inter-subunit disulfide bridges are reported [6–9] but also, inter-subunit disulfide engineering has been used as a sta-

* Corresponding author.
** Corresponding author at: Protein Engineering Laboratory, Protein Research Center (PRC), Shahid Beheshti University, GC, Tehran, Iran.
E-mail addresses: o_ranaei@sbu.ac.ir (S.O. Ranaei Siadat), khajeh@modares.ac.ir, khajeh_k@yahoo.com (K. Khajeh).

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bilization strategy by several studies in dimeric enzymes [10,11]. Here, we have tried to improve SXA thermal stability through introducing inter-subunit disulfide bonds at dimer-dimer interface of the enzyme. A site directed mutagenesis strategy was used for conversion of a single lysine residue to cysteine. The native and mutant variants were expressed in *Pichia pastoris*. Kinetic and thermostability parameters of mutant enzyme were compared with the wild type enzyme. In addition to experimental data, coarse grained molecular dynamics simulations were operated to simulate and analyze the mutant and wild-type SXA stability and flexibility in silico.

2. Materials and methods

2.1. Materials

PCR reagents and materials were prepared from Sinaclon Bioscience (Iran). All restriction enzymes, DNA marker and T4 DNA ligase enzyme were purchased from Vivantis (Malaysia). Protein markers prepared from Vivantis and Thermo scientific companies. Primers were synthesized by Sinaclon Bioscience (Iran). DTNB was prepared from Thermo scientific (USA). Yeast growth and induction media were purchased from Invitrogen (USA).

2.2. Plasmids, bacterial strains and growth condition

Escherichia coli strain DH5α was used for cloning and propagation of plasmids. *Pichia pastoris* strain GS115 and pPinkα-HC expression vector were obtained from Invitrogen (USA). *E. coli* cells were grown in Luria and Bertani (LB) medium at appropriate conditions (37 °C with 200 rpm shaking). Yeast extract Peptone Dextrose (YPD) and buffered Glycerol-complex (BMGY) were used as *Pichia pastoris* growth media. The growth condition was adjusted to 30 °C and 300 rpm shaking. The induction was carried out in buffered methanol complex medium (BMMY). Unless otherwise stated, standard DNA methodologies, according to the Sambrook laboratory manual were used [12].

2.3. Site of mutagenesis

Crystal structure of SXA (PDB, 3C2U) was used as model in order to select the appropriate sites of disulfide bond formation. Mutation Proposer on Disulfide Bonds (MPDB) program (bioinf.modarec.ac.ir/software/mpdb) was used to find a list of amino acids potentiates to form a disulfide bond. Advanced mode of the software with C_β-C_β lower than 4.8 Å and inter-chain bonds was used. Among several predicted disulfide bonds, Lysine237 was selected for creation of inter-subunit disulfide bridge. Structures of the native and engineered enzymes were analyzed by YASARA program. Stability changes following mutating Lysine237 to cysteine were examined by FoldX force field (http://foldxsuite.crg.eu) in YASARA molecular graphics suite (http://foldxyasara.switchlab.org). In addition, energy minimization of the wild type and mutated enzymes were obtained by Hyperchem 7.5 molecular modeling program (Hypercube, Inc.).

2.4. Site directed mutagenesis and DNA manipulation

The synthetic SXA gene (GenBank Accession No. JF193553.1) in pMK-RQ cloning vector was used as a template for site directed mutagenesis. The single point mutation (K237C) was introduced into the SXA gene according to QuikChange site-directed mutagenesis method. Oligonucleotide primers 5′-CCGCTTGG**TGT**GAGGTTCACAACCCATTG-3′ and 5′-TGTGAACCTC**ACA**CCAAGCGGACAACAAAGG-3′ were used to replace Lys237 with Cys. The sites of mutation in forward and reverse primers are shown in bold. The methylated (parental) DNA molecules were degraded using *Dpn*I restriction enzyme. Digestion was carried out at 37 °C for 90 min followed by transformation of the products into *DH5α* competent cells. Transformants were screened on ampicillin containing medium (100 μg/ml). The authenticity of mutation was confirmed by DNA Sequencing (MWG, Germany).

2.5. Pichia pastoris transformation and screening

Native and mutated genes were subcloned into the *Xho*I/*Kpn*I cloning sites of pPinkα-HC expression vector under the control of the AOX1 promoter. The desired genes were placed downstream of α- mating factor secretion signal originating from the yeast *Saccharomyces cerevisiae*. Recombinant native (pPinkα-HC-SXA) and mutated (pPinkα-HC-SXA-K237C) plasmids were linearized by *Bsp*T1 restriction enzyme to target the integration of the expression cassettes into the *TRP2* locus of *P. pastoris*. The linearized expression vectors were transformed into *P. pastoris* GS115 by electroporation (BTX, ECM630 at 1800 V, 200 Ω and 25 μF) following Invitrogen protocol. Selected colonies were used for colony PCR using specific *sxa* primers (Forward: 5′-TCTCTCGAGTCAAAGGCAACAAACTGT-3′ and Reverse: 5′-GGTACCGCATGCCTACTACTTTCTT-3′) to confirm the authenticity of the integration site. The PCR cycling program was as follows: 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 90 s at 72 °C; and 1 cycle of 5 min at 72 °C.

2.6. Expression of recombinant xylosidases and purification

Expression of wild type and mutant K237C SXA were performed according to our previous report [13]. After four days expression of xylosidase in shake flask, the yeast cells were collected by centrifugation (3000*g* for 5 min at 4° C). Then, crude supernatant was concentrated 10- fold by ammonium sulfate precipitation. Ammonium sulfate equal to 80% saturation was slowly added to the supernatant while stirring at 4 °C. Stirring was continued overnight in 4 °C. To pellet out the proteins, centrifugation was carried out at maximum speed for 10 min at 4 °C. The precipitated proteins were resuspended in citrate buffer pH 4.8 (xylosidase assay buffer) and subjected to dialysis at 4 °C against the same buffer. final purification was carried out by size exclusion chromatography on a Sephadex S-200 column (Amersham Pharmacia Biotech, Sweden) equilibrated with 50 mM potassium phosphate buffer pH 4.8 containing 150 mM sodium chloride. The protein was eluted at a flow rate of 0.8 ml/min^{-1}. Active fractions were combined and used as the purified xylosidase for further analysis. Native and K237C mutant enzymes were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with and without 2-mercaptoethanol (2ME) as a reducing agent. The amount of total protein was determined by the Bradford method using bovine serum albumin (BSA) as standard.

2.7. Titration of SH group

The concentration of free SH groups was determined by Ellman's method [14] using dithionitrobenzoic acid (DTNB) in 0.1 M sodium phosphate buffer (pH 8) containing 1 mM EDTA according to Thermo Scientific manual. In order to increase the accessibility of the thiol groups to DTNB, the proteins were unfolded by addition of guanidine hydrochloride (GuHCl) up to 6 M for 15 min. The number of SH-groups was calculated based on molar absorptivity using maximum absorbance at 412 nm and the extinction coefficient of 14,150 and 13,700 M^{-1} cm^{-1} for the native and denaturing conditions, respectively.