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Site-directed mutagenesis of a yeast gene for improvement of enzyme thermostability

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ABSTRACT

Enzyme stability is one of the critical factors to construct an efficient biological conversion system. Xylitol dehydrogenase (XDH) from *Pichia stipitis* is one of the key enzymes for bio-ethanol fermentation system from xylose. Previously, we tried to improve thermostability of XDH by introduction of structural zinc into the enzyme and successfully obtained a mutant, named C4 mutant, with an increased unfolding temperature (J. Biol. Chem., 280:10340-10349, 2005). We focused on further improvement of the thermostability of XDH in this study and employed subsequent site directed mutagenesis in structural zinc binding region for stabilizing the structural zinc binding loop. Two variants (C4/F98R and C4/E101F) showed higher thermostability than C4 mutant judged by thermal inactivation of enzyme activity and thermal transition temperature.

INTRODUCTION

We are attempting to construct an efficient ethanol fermentation system from biomass. *Saccharomyces cerevisiae* is the favoured ethanol-producing microorganism but it can't metabolize xylose which is one of major sugars included in biomass. Xylose consuming yeasts such as *Pichia stipitis* can metabolize through expression of xylose reductase (XR; alditol:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.21) and xylitol dehydrogenase (XDH; xylitol:NAD$^+$-2-oxidoreductase; EC 1.1.1.9). XR catalyzes the reduction of xylose to xylitol whereas XDH oxidizes xylitol to xylulose. However, *Pichia stipitis* is susceptible to ethanol and obliges stumpy and carefully controlled oxygenation. These disadvantages thwart its usage for industrial ethanol production. *S. cerevisiae* strains producing XR and XDH from *Pichia stipitis* in addition to expression of homologous xylulokinase (XK, ATP D-xylulose 5-phosphotransferase; EC 2.7.1.17) are able to produce ethanol from xylose.[1] However, the recombinant yeast system has not yet been applied to the industrial bio-process mainly due to the unfavourable xylitol excretion.

XDH belongs to polyol dehydrogenase (PDH) sub-family which comes under medium-chain dehydrogenase /reductase (MDR) super family. Many MDRs has one zinc atom at the catalytic site for enzyme activity, and some of the enzymes have additional zinc atom which is recognized as structural zinc. The significant role of the structural zinc is yet to discover but it was proposed to stabilize tertiary structure of the enzyme.

Recently, we have developed a new approach to increase thermostability of the XDH by introduction of structural zinc. Because four cysteine residues are necessary for structural zinc binding, we employed site directed mutagenesis for conversion of the residues Ser96, Ser99, Tyr102 with Cys to obtain C4 Mutant.[2] We chose *Bemisia argentifolli* ketose reductase (sorbitol dehydrogenase) as a reference protein which belongs to the same family as *P. stipitis* XDH and has 60% primary sequence similarity with *P. stipitis* XDH.[3] Based on these results, we have anticipated that the structural zinc binding loop is conserved in *P. stipitis* XDH.

In this study, we focused on further improvement of *P. stipitis* XDH thermostability by introduction of subsequent site directed mutation. We designed mutants by comparing the primary sequences at structural zinc binding region with other members of the PDH family.

RESULTS AND DISCUSSION

Previously, we constructed a triple mutant (C4 mutant) of *P. stipitis* XDH and introduced structural zinc into the protein. Thermostability (T_m) of the C4 mutant has been improved compared to the wild type.[2] In this study, we designed another 4 mutations at structural zinc binding region based on amino acid sequences comparison with the other members in the family. *B. argentifolii* SDH was taken as a reference enzyme for this study because of its high similarity with *P. stipitis* XDH. Site-directed mutagenesis was employed subsequently to examine the role of P95, F98, E101 and H112 at structural zinc binding region. We constructed four mutants (C4/P95S, C4/F98R, C4/E101F and C4/H112) and nucleotide exchange of each mutant was confirmed with DNA sequencing by dideoxynucleotide chain-termination method. All recombinant proteins were expressed in *E. coli* and purified by AKTA FPLC system. Purification efficiency and expression levels of mutants were confirmed by SDS-PAGE and Western blot analysis.

Table 1. Thermal Inactivation of recombinant *P. stipitis* XDHs.

	Temperature of half-inactivation (°C)
C4	46.1±0.3
C4/P95S	37.4±0.2
C4/F98R	53.1±0.1
C4/E101F	50.9±0.1
C4/H112D	44.0±0.2

Thermostability of the mutants: Thermal stability against irreversible inactivation of the mutated enzymes was examined by analyzing the residual activity after heat treatment at various temperatures (Table 1). Variants C4/F98R and C4/E101 showed higher thermostability than C4, whereas C4/P95S and C4H112D showed less thermostability than C4. We also analyzed thermal denaturation temperature (T_m) by measuring the ellipticity at 220 nm and confirmed the same tendency as thermostability of enzyme activities (Table 2). The T_m of C4/F98R and C4/E101F increased 4.2 and 3.0 °C, respectively, whereas that of C4/P95S and C4/H112D decreased 4.0 °C and 0.5 °C, respectively. Thus C4/F98R was shown to be the best mutant in four mutants constructed in this study.

Table 2. Thermal transition temperature of recombinant *P. stipitis* XDHs.

	Thermal transition temperature (°C)
C4	47.5±0.2
C4/P95S	43.5±0.8
C4/F98R	51.7±0.3
C4/E101F	50.5±0.5
C4/H112D	47.0±1.0

CONCLUSION

In this study, we have successfully improved thermostability of XDH by subsequent site directed mutagenesis around the structural zinc region. Thermostability of C4/F98R and C4/E101F mutants has been improved compared to the C4 and remaining mutants C4/P95S and C4/H112D showed lower thermostability than C4. Our results revealed that the zinc binding loop region is also playing a prominent role in influencing the thermostability of *P. stipitis* XDH. To find out the factors which cause for further improvement of thermostability of the recombinant XDHs, we are in progress in building a model structure of XDH on the basis of the refined crystal structure of *B. argentifolli* sorbitol dehydrogenase.

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