Available online at www.sciencedirect.com
ScienceDirect
Journal of Biotechnology 129 (2007) 717–722
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Short communication

Thermostabilization of *Pichia stipitis* xylitol dehydrogenase by mutation of structural zinc-binding loop

Narayana Annaluru [a], Seiya Watanabe [a,b,c], Seung Pil Pack [a,b], Ahmed Abu Saleh [a], Tsutomu Kodaki [a,b], Keisuke Makino [a,b,d,*]

[a] *Institute of Advanced Energy, Kyoto University, Gokasho, Uji 611-0011, Japan*
[b] *CREST, JST (Japan Science and Technology Agency), Kyoto University, Gokasho, Uji 611-0011, Japan*
[c] *Faculty of Engineering, Kyoto University, Kyotodaigaku-katsura, Saikyo-ku, Kyoto 615-8530, Japan*
[d] *International Innovation Center, Kyoto University, Yoshidahonmachi, Sakyo-ku, Kyoto 606-8501, Japan*

Received 23 December 2006; received in revised form 1 February 2007; accepted 5 February 2007

Abstract

Xylitol dehydrogenase from *Pichia stipitis* (PsXDH) is one of the key enzymes for the bio-ethanol fermentation system from xylose. Previously, we constructed the C4 mutant (S96C/S99C/Y102C) with enhanced thermostability by introduction of structural zinc. In this study, for further improvement of PsXDH thermostability, we constructed the appropriate structural zinc-binding loop by comparison with other polyol dehydrogenase family members. A high thermostability of PsXDH was obtained by subsequent site-directed mutagenesis of the structural zinc-binding loop. The best mutant in this study (C4/F98R/E101F) showed a 10.8 °C higher thermal transition temperature (T_{CD}) and 20.8 °C higher half denaturation temperature ($T_{1/2}$) compared with wild-type.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Thermostability; Xylitol dehydrogenase; Structural zinc; Site-directed mutagenesis

1. Introduction

Improvement of protein thermostability is one of the major concerns of protein engineers since the protein structure has evolved and has been optimized for its function but not essentially for its stability. Several engineering approaches such as random mutagenesis, rational design, and the consensus approach using sequence databases and statistics have been employed to significantly modify the thermostability of a protein (Lee and Vasmatzis, 1997; Van den Burg and Eijsink, 2002; Bornscheuer and Pohl, 2001; Lehmann and Wyss, 2001). However, there is no standard or general procedure for protein thermostabilization, because the factors governing protein thermostability are not usually similar in all proteins (Pace et al., 1996; Vieille and Zeikus, 2001).

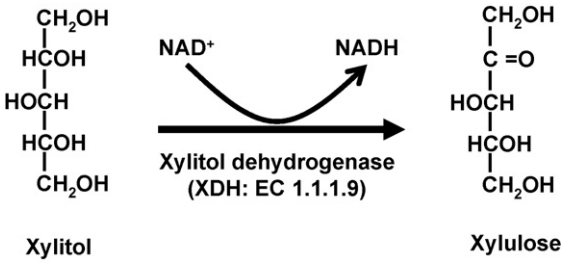
Xylitol dehydrogenase from *Pichia stipitis* (PsXDH) plays an important role in the xylose metabolic pathway (Scheme 1) (Ingram et al., 1998; Jeffries and Jin, 2004). Raising the thermostability of XDH has a significant impact on fermentation efficiency in the biomass to bio-ethanol conversion system. However, the present protein engineering approaches mentioned above have difficulties when using PsXDH. That is, the random mutagenesis method demands an appropriate selection system (Giver et al., 1998), lack of three-dimensional (3D) structure of PsXDH precludes rational design (Mooers et al., 2003) and without any reports on XDHs from thermophilic microorganisms, we cannot employ the consensus method.

In a recent report, we constructed PsXDH with structural zinc (C4 mutant) by site-directed substitution of Ser96, Ser99, and Tyr102 with cysteine based on the fact that some members of the polyol dehydrogenase (PDH) family contain structural zinc, which is bound tetrahedrally to four sulfur atoms of

Abbreviations: XDH, xylitol dehydrogenase; PsXDH, XDH from *Pichia stipitis*; MDR, medium-chain dehydrogenase/reductase; PDH, polyol dehydrogenase; SDH, sorbitol dehydrogenase; CD, circular dichroism; $T_{1/2}$, half denaturation temperature; T_{CD}, thermal transition temperature; *M. morganii*, *Morganella morganii*; *S. pombe*, *Schizosaccharomyces pombe*; *E. japonica*, *Eriobotrya japonica*; *H. jecorina*, *Hypocrea jecorina*

* Corresponding author at: Institute of Advanced Energy, Kyoto University, Gokasho, Uji 611-0011, Japan. Tel.: +81 774 38 3517; fax: +81 774 38 3524.
E-mail address: kmak@iae.kyoto-u.ac.jp (K. Makino).

0168-1656/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2007.02.006



Scheme 1. Conversion of xylitol to xylulose by xylitol dehydrogenase.

cysteine in the loop (Watanabe et al., 2005), as shown in Fig. 1. The C4 mutant showed a 4.5 °C higher transition temperature (T_{CD}) than wild-type PsXDH, indicating that the mutation of the structural zinc-binding loop could be effective in increasing the thermostability of PsXDH. In the present study, with a vision that refinement of the zinc-binding loop may be involved in PsXDH thermostabilization, we carried out subsequent site-directed mutagenesis around the structural zinc loop. The resultant best mutant (C4/F98R/E101F) increased 6.3 °C in T_{CD} and 9.9 °C in $T_{1/2}$ compared with the PsXDH C4 mutant.

2. Materials and methods

2.1. Construction of plasmids and site-directed mutagenesis

Mutants C4/P95S, C4/F98R, C4/E101F, C4/H112D, and C4/F98R/E101F were constructed by PCR-based site-directed mutagenesis using a Quick Change Site-Directed Mutagenesis Kit (Stratagene). Details of the mutant primers are described in Table 1. The amplified PCR product was digested with Dpn1 and transformed into *Escherichia coli* DH5α. DNA sequencing was carried out using the Dual CyDye^{TM} Terminator Sequencing Kit (Veritas) and appropriate primers with Long-Read Tower, UBC DNA sequencer (Amersham Biosciences).

2.2. Expression and purification of recombinant PsXDHs

$(His)_6$-tagged recombinant PsXDHs were expressed from pQE-81L in *E. coli* DH5α as a host. After cell lysis and centrifugation, all variants were found in the supernatant and were purified with a Ni-NTA Superflow column (Qiagen). All chromatography was carried out using the ÄKTA purifier system (GE Healthcare) at 4 °C as described previously (Watanabe et al., 2005). SDS-polyacrylamide gel electrophoresis (PAGE) was carried out in 12% slab gels by the method of Laemmli (1970).

2.3. Protein sequences analysis and alignment

The Protein-BLAST program of the National Center for Biotechnology Information, USA (http://www.ncbi.nlm.nih.gov/) was used for homology searches and analysis of the protein sequence of PsXDH. Among the matched candidates, members with highly noteworthy six matches, were selected and compared as listed in Table 2. For multiple-sequence alignment, the Clustal W program was employed, sponsored by the Bioinformatics Center of Kyoto University, Japan (http://www.genome.jp/). In addition, GENETYX 7.0 (Software Development, Tokyo, Japan) was used for sequence analysis.

2.4. Enzyme assay

PsXDH activity was analyzed spectrophotometrically by measuring the reduction of NAD^+ at 340 nm and 35 °C (Rizzi

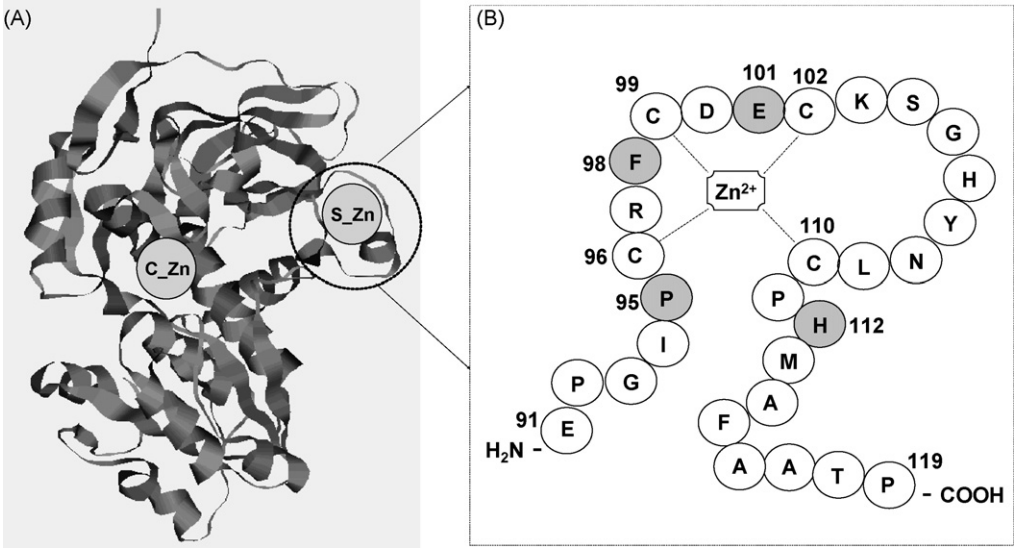


Fig. 1. (A) Catalytic zinc (C_Zn) and structural zinc (S_Zn) on the proposed 3D-model of PsXDH C4 mutant and the estimated region (from E91 to P1 19) of structural zinc-binding loop of PsXDH C4 mutant. Since the proposed 3D-model of PsXDH C4 mutant was obtained by homology modeling (on-line Swiss-Model provided by ExPASy proteomics server) using BaSDH (pdb code; 1e3j) as template, it is not accurate structure, but it is drawn here for understanding our research strategy. Structural zinc loop region and their positions were estimated by homology search results based on six members of two-zinc-containing PDH (polyol dehydrogenase) enzymes, which were found similar to PsXDH as aligned in Group-2Z of Table 2. (B) Four cysteine residues (C96, C99, C102, and C110) are involved in structural zinc binding. P95, F98, E101, and H112 in gray circle were selected as mutagenesis targets for designing more thermostable PsXDH and their mutants were analyzed in this study.

Table 1
Designation of *Pichia stipitis* XDH variants

Variant	Mutations	Template	Primer[a]
C4[b]	S96C/S99C/Y102C	Wild-type	
C4/P95S	P95S/S96C/S99C/Y102C	C4	ACCAGGTATT**TCC**TGTAGATTC
C4/F98R	S96C/F98R/S99C/Y102C	C4	GTATTCCATGTAGA**CGT**TGTGACGAATGTAAG
C4/E101F	S96C/S99C/E101F/Y102C	C4	GTAGATTCTGTGAC**TTC**TGTAAGAGCGGTC
C4/H112D	S96C/S99C/Y102C/H112D	C4	CTTGTGTCCT**GAC**ATGGCCTTC
C4/F98R/E101F	S96C/F98R/S99C/E101F/Y102C	C4/E101F	GTATTCCATGTAGA**CGT**TGTGACTTCTGTAAG

[a] Only sense primers are shown. The nucleobases in bold and underlined in these primers are code for amino acid substitutions.
[b] C4 mutant was constructed in previous study (Watanabe et al., 2005).

et al., 1989). The standard assay mixture is composed of 50 mM $MgCl_2$ and 300 mM xylitol in 50 mM Tris–HCl buffer (pH 9.0). All reactions were started with the addition of 100 μl of 20 mM NAD^+ solution to a final volume of 1.0 ml (standard assay condition). One unit of enzyme activity refers to 1 μmol of NADH produced per 1 min. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

2.5. Measurement of thermostability against thermal denaturation

To determine the thermostability of PsXDH, purified mutant enzymes were dialyzed and diluted with 50 mM sodium phosphate buffer (pH 8.0) containing 2 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) (referred to as Buffer A), and then were incubated for 10 min at various temperatures, ranging from 20 °C to 60 °C. After incubation, the samples were chilled for 10 min in ice and then the remaining activity was determined under the standard assay conditions. The half denaturation temperature ($T_{1/2}$) was obtained by estimating the temperature at which the remaining activity was half the initial activity in the experimental data points versus temperature.

2.6. Circular dichroism (CD) measurements

CD measurements were performed with a spectropolarimeter model J-720 (JASCO, Tokyo, Japan). The loss of α-helical structure in PsXDH mutants was observed by recording the CD signal at 220 nm as a function of temperature. Enzyme samples were dialyzed and diluted at concentrations of 1 mg ml^{-1} with Buffer A. During the experiment, the temperature of the sample changed at a rate of 1 °C min^{-1} between 10 °C and 70 °C with a PTC 348WI programmable temperature-controller (JASCO). The temperature of the sample was controlled during the measurements in a constant N_2 circulating chamber. The temperature scale in the final plots represents the temperature actually measured by the sensor in the immediate vicinity of the sample. A quartz cuvette with a 2 mm path length was used for CD measurements. The thermal transition temperature (T_{CD}) was obtained by estimating the midpoint of the transition in the experimental data points of the CD signal versus temperature.

Table 2
Partial sequence alignment of the polyol dehydrogenase subfamily

Group	Abbreviation[a]	Accession No	Source organism		Structural zinc binding region[b]	Enzyme[c]
Group-1Z[d]	PsXDH	P22144	*P. stipitis*	91 -	EPGIPSRFSDEYKSGHYNLCPHMAFAATP	XDH
	GmXDH	AAC24597	*G. mastotermitis*	93 -	EPGVPSRHSDEYKSGRYNLCPHMAFAATP	XDH
	ScXDH	NP_013171	*S. cerevisiae*	94 -	EPGIPDRFSPEMKEGRYNLDPNLKFAATP	XDH
	MmXDH	Q59545	*M. morganii*	90 -	EPGIPDLQSPQSRAGIYNLDPAVRFWATP	XDH
	ScSDH	NP_012693	*S. cerevisiae*	93 -	EPGVPSRYSDETKEGRYNLCPHMAFAATP	SDH
	RaSDH	S16132	Rat	113 -	EPGVPREVDEYCKIGRYNLTPTIFFCATP	SDH
	CsSDH	AAB69288	*Callithrix* sp.	95 -	EPGAPRETDEFCKTGRYNLSPTIFFCATP	SDH
	ShSDH	S10065	Sheep	92 -	QPGAPRQTDEFCKIGRYNLSPTIFFCATP	SDH
	ApSDH	AAL37294	Apple	113 -	EPGVPREVDEYCKIGRYNLTPTIFFCATP	SDH
	HuSDH	NP_003095	Human	94 -	EPGAPRENDEFCKMGRYNLSPSIFFCATP	SDH
	BsSDH	Q06004	*B. subtilis*	95 -	EPGVTCGRCEACKEGRYNLCPDVQFLATP	SDH
Group-2Z[d]	BaSDH	AAD02817	*B. argentifolii*	91 -	EPGVPCRRCQFCKEGKYNLCPDLTFCATP	SDH
	SpSDH	S35981	*S. pombe*	92 -	EPGCVCRLCDYCRSGRYNLCPHMEFAATP	SDH
	SwSDH	Q02912	Silkworm	90 -	EPTQPCRSCELCKRGKYNLCVEPRYCSSM	SDH
	PeSDH	BAA94084	Peach	107 -	EPGISCWRCEQCKGGRYNLCPDMKFFATP	SDH
	EjSDH	BAA95897	*E. japonica*	114 -	EPGISCKRCNLCKQGRYNLCRKMKFFGSP	SDH
	HjLADH	AAL08944	*H. jecorina*	116 -	EPNIICNACEPCLTGRYNGCEKVEFLSTP	LADH
	C4 mutant	--	--	91 -	EPGIPCRFCDECKSGHYNLCPHMAFAATP[e]	XDH

[a] Abbreviations were used for only this study. [b] Cysteine in black background is the residue involved in structural zinc-binding. [c] Enzyme abbreviation: XDH, xylitol dehydrogenase; SDH, sorbitol dehydrogenase; LADH, L-arabinitol 4-dehydrogenase. [d] The members of Group-1Z contain only catalytic zinc and the ones of Group-2Z contain structural zinc as well as catalytic zinc. [e] P95, F98, E101, and H112 underlined in gray background are the target amino acids selected in this study for PsXDH thermostabilization.

3. Results and discussion

3.1. Selection of amino acid residues for mutation of PsXDH

P. stipitis XDH (PsXDH) belongs to the polyol dehydrogenase (PDH) subfamily which comes under the medium-chain dehydrogenase/reductase (MDR) superfamily (Nordling et al., 2002; Riveros-Rosas et al., 2003). Many PDHs have one zinc atom at the catalytic site for enzyme activity, and some of the enzymes possess an additional zinc atom, known as structural zinc. As listed in Table 2, 16 enzyme members of the PDH subfamily, which show a high homology match with PsXDH, were aligned. Among them, six enzymes (BaSDH, SpSDH, SwSDH, PeSDH, EjSDH, and HjLADH) were estimated to possess additional structural zinc (although BsSDH possesses four cysteine residues, it was known to possess only catalytic zinc) (Ng et al., 1992) and their zinc-binding loop regions were identified. Other members that do not possess structural zinc including PsXDH were also observed to have a similar loop region possibly for structural zinc introduction (Watanabe et al., 2005). It was found that two residues existed between the first three cysteine residues in the loop region of six enzymes and there were seven residues between the third and fourth cysteine residue in all the members without any sequence gaps in this region. In a previous report, based on the results of bioinformatic homology searches, we succeeded in introducing the structural zinc into PsXDH and obtained its C4 mutant by site-directed mutagenesis substitution of Ser96, Ser99, and Tyr102 by cysteine (Watanabe et al., 2005) (Fig. 1). Considering the result that such structural zinc introduction into the loop region is effective for PsXDH thermostabilization, we attempted the further refinement of the loop by site-directed mutagenesis of C4 mutant and selected four mutation sites Pro95, Phe98, Glu10l and His112 as follows.

First, Pro95 was selected to examine whether proline near the first cysteine influenced the structural zinc-binding region. As shown in Table 2, in the group containing one zinc (Group-1Z), proline was kept as the consensus residue; however, in the group containing two zinc atoms (Group-2Z), some diversity exists. Pro95 was substituted with serine because two enzymes such as SwSDH and PeSDH possess serine in the first position. Next, Phe98 was selected because the members belonging to Group-1Z and Group-2Z showed various tendencies in that position. Considering the sequence information of three members of Group-2Z (BaSDH, SwSDH, and PeSDH), the designed mutation was Phe98 with arginine. Then for Glu10l, there is no negatively charged residues at this position in Group-2Z, although in Group-1Z, several residues were observed regardless of non-charged and charged residue (the charged one is glutamate). Therefore the Glu10l was changed with a nonpolar amino acid, in particular, phenylalanine, by referencing the amino acid sequence of BaSDH. Finally, His112 was considered for mutagenesis site because several different amino acids were observed at this position irrespective of Group-1Z and Group-2Z. Although three members of Group-2Z (SpSDH, EjSDH, and HjLADH) possess positively charged amino acids such as lysine and histidine, the other three members possess negatively charged residues of glutamate and aspartate at that position. To investigate whether the charge differences at the position had a significant effect on stabilization of the zinc-binding loop of PsXDH, we substituted the His112 with aspartate.

3.2. Effect of the designed mutations on PsXDH thermostability

All recombinant PsXDHs with the $(His)_6$-tag attached to their N-termini were expressed in *E. coli* and purified using Ni^{2+}-chelating affinity column chromatography. SDS-PAGE analysis revealed that the purities of the recombinant enzymes were up to 90–95% (data not shown).

Thermo stabilities of the four mutants were analyzed by measuring the remaining activities at 35 °C after heat treatment at various temperatures (Fig. 2). The thermal inactivation processes of C4 and the other mutants were irreversible under these conditions. $T_{1/2}$, the half denaturation temperature of the C4 mutant was 46.1 °C, which is 10.9 °C higher than $T_{1/2}$ of the wild-type (Watanabe et al., 2005). Among the four mutants, C4/F98R and C4/E101F were showed the significant thermostabilization, compared with the C4 mutant; 7.0 °C and 4.8 °C in $T_{1/2}$ higher than C4 mutant. On the other hand, C4/P95S and C4/H112D mutants were showed lower $T_{1/2}$ than C4 mutant by 8.7 °C and 2.1 °C, respectively (Table 3). Estimation of thermostability by CD measurement (T_{CD}) was also analogous to the heat-inactivation experiment; increase of 4.2 °C and 3.0 °C in C4/F98R and C4/E101F and decrease of 4.0 °C and 0.5 °C in C4/P95S and C4/H112D mutants, compared with the C4 mutant (Table 3). These results suggested clearly that F98R and E101F mutations in the structural zinc loop region are effective in increasing the thermostability of PsXDH.

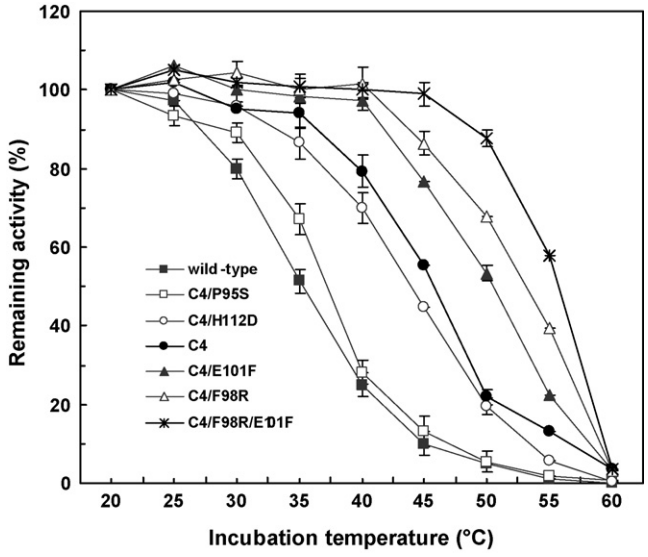


Fig. 2. Temperature-dependent profile of the remaining activity of PsXDH variants and wild-type. Recombinant purified enzymes were dialyzed against Buffer A and incubated 10 min at various temperatures, ranging from 20 °C to 60 °C. After incubation, the enzyme samples were chilled for 10 min in ice, and then their residual activity was determined at 35 °C. Enzyme activities of wild-type and mutant PsXDHs were compared as the relative values (means ± S.D., $n = 3$) in a percentage of the initial activity.

Table 3
Specific activity and thermostability of *Pichia stipitis* XDH mutants

Protein	Specific activity (min^{-1})	Half denaturation temperature, $T_{1/2}$ [a] (°C)	Thermal transition temperature, T_{CD} [b] (°C)
Wild-type	1110 ± 90	35.2 ± 0.30 (0.0)[c]	43.0 ± 0.10 (0.0)[c]
C4	1440 ± 30	46.1 ± 0.31 (+10.9) (0.0)[d]	47.5 ± 0.09 (+4.5) (0.0)[d]
C4/P95S	1220 ± 80	37.4 ± 0.24 (+2.2) (−8.7)	43.5 ± 0.08 (+0.5) (−4.0)
C4/F98R	1510 ± 40	53.1 ± 0.15 (+17.9) (+7.0)	51.7 ± 0.09 (+8.7) (+4.2)
C4/E101F	1550 ± 80	50.9 ± 0.14 (+15.7) (+4.8)	50.5 ± 0.06 (+7.5) (+3.0)
C4/H112D	1360 ± 50	44.0 ± 0.26 (+8.8) (−2.1)	47.0 ± 0.08 (+4.0) (−0.5)
C4/F98R/E101F	1620 ± 60	56.0 ± 0.31 (+20.8) (+9.9)	53.8 ± 0.05 (+10.8) (+6.3)

[a] The apparent half denaturation temperature obtained from Fig. 2.
[b] The temperature from the transition profile monitored by CD at 220 nm.
[c] The difference from the wild-type PsXDH enzyme.
[d] The difference from the PsXDH C4 mutant.

Based on the results we also constructed the C4/F98R/E101F mutant to examine the synergic effect of combination of two successive mutations on PsXDH thermostabilization. The obtained PsXDH mutant (C4/F98R/E101F) is the best in thermostability, in which T_{CD} was 10.8 °C and 6.3 °C higher than the wild-type and C4 mutant, respectively (Table 3). In the case of $T_{1/2}$, the C4/F98R/E101F mutant was also found to possess higher thermostability than wild-type and C4 mutant by 20.8 °C and 9.9 °C, respectively (Fig. 2 and Table 3).

There is one inconsistency why PsXDH mutants possess different types of relationships between $T_{1/2}$ and T_{CD} values: $T_{1/2} < T_{CD}$ or $T_{CD} < T_{1/2}$ (Table 3). It is noteworthy that C4/F98R, C4/E101F single and C4/F98R/E101F double mutants ($T_{CD} < T_{1/2}$) show significantly higher thermostability and specific activity than the C4 mutant (and wild-type). Since heat inactivation experiment is related to the amount of an "active form" protein, the $T_{1/2}$ values of these mutants may increase unexpectedly but positively, compared with other mutants.

3.3. Mutation of structural zinc loop for PsXDH thermostabilization; implications and hypothesis

Beginning with the information that PsXDH C4 mutant containing structural zinc possessed thermostability (Watanabe et al., 2005), we designed mutagenesis strategies for refining the structural zinc-binding loop and obtained dramatically increased thermostability of PsXDH. As shown in Table 3, all the specific activities of PsXDH variants were not significantly influenced by the designed mutagenesis, and those with increased thermostability showed higher specific activities than wild-type. Even though successful PsXDH mutants with high thermostability were obtained, the lack of PsXDH crystal structure prevented to indicate the definite factors involved in refinement effect of the zinc-binding loop. However, our mutation approach for the structural zinc loop region is feasible for designing thermostable enzymes with unknown 3D structure and insufficient consensus information, in particular, members belonging to the PDH subfamily. In addition, from these results, some useful information on PsXDH structure may be deduced as follows.

First, we identified the important residue positions around the zinc-binding loop in terms of their influence on zinc-binding loop stabilization. As shown in Fig. 2 and Table 3, C4/P95S showed similar $T_{1/2}$ and T_{CD} values to those of wild-type, while H112D showed similar $T_{1/2}$ and T_{CD} values to those of C4 mutant. These results indicated that Pro95 might be an essential residue to maintain the thermostability of PsXDH. In contrast, the position of His112 is not so strongly correlated with PsXDH thermostability even though the charge was converted from a positive to a negative one. Next, it should be noted that the substituted arginine (from Phe98) and phenylalanine (from Glu10l) contribute to the increased thermostability of PsXDH together with the C4 mutation. From the structural information for BaSDH, a member of the PDH family and which has a known 3D structure containing structural zinc, the region of the structural zinc-binding loop was known to be involved in stabilizing the dimer–dimer interaction (Banfield et al., 2001). In the zinc-binding loop of BaSDH, the same Arg98 and Phe10l exist and participate in the interface interactions of BaSDH subunits. From all of the mutagenesis results obtained here and consideration of the BaSDH structure, it was hypothesized that PsXDH may possess a similar loop structure or loop interaction to BaSDH because the important amino acids and substituted ones for thermostabilizing PsXDH follow the same pattern as the structural zinc loop sequence of BaSDH. We are now carrying out more mutagenesis experiments to elucidate the factors involved in PsXDH thermostabilization, in particular, the similarity of loop structure and the role of loop interaction in subunit complexation, based on the above hypothesis.

On the other hand, recombinant *Saccharomyces* strains producing xylose reductase (XR) and XDH from *P. stipitis* have been constructed for ethanol production from xylose (Ostergaard et al., 2000; Zaldivar et al., 2001). Walfridsson et al. (1997) showed that the overexpression of XDH in *Saccharomyces cerevisiae* may reduce the amount of xylitol excreted, which influences ethanol production yield. Producing thermostable XDH is capable of increasing the intercellular expression level. The PsXDH mutant, C4/F98R/E101F, with high thermostability, could be a good candidate for the development of a highly efficient ethanol fermentation system.

4. Conclusion

Since the 3D structure of PsXDH is not known and sufficient consensus information on thermophilic XDHs are not available,

the improvement of PsXDH thermostability has remained a difficult topic. In the present study, we succeeded in obtaining dramatically increased thermostable PsXDH variants by refining the structural zinc-binding loop (10.8 °C higher thermal transition temperature (T_{CD}) and 20.8 °C higher half denaturation temperature ($T_{1/2}$) than wild-type) without affecting their activities. Although the relationship between mutation of the loop and PsXDH thermostabilization has not been fully elucidated, our loop-mutation approach can be an useful guideline for designing thermostable enzymes with unknown 3D structure and insufficient consensus information, in particular, members belonging to the PDH subfamily. In addition, the thermostable PsXDH variants obtained here can contribute to improving fermentation efficiency in the xylose to bio-ethanol conversion system.

Acknowledgements

This work was supported by the Center of Excellence (COE) program for the "Establishment of COE on Sustainable Energy System," a Grant-in-Aid for Scientific Research, and Grants for Regional Science and Technology Promotion "Kyoto Nanotechnology Cluster" project from the Ministry of Education, Science, Sports and Culture, Japan. This work was also supported by CREST and "Research for Promoting Technological Seeds" of the Japan Science and Technology Corporation and by New Energy and Industrial Technology Development Organization.

References

Banfield, M.J., Salvucci, M.E., Baker, E.N., Smith, C.A., 2001. Crystal structure of the NADP(H)-dependent ketose reductase from *Bemisia argentifolii* at 2.3 Å resolution. J. Mol. Biol. 306, 239–250.

Bornscheuer, U.T., Pohl, M., 2001. Improved biocatalysts by directed evolution and rational protein design. Curr. Opin. Chem. Biol. 5, 137–143.

Giver, L., Gershenson, A., Freskgard, P., Arnold, F.H., 1998. Directed evolution of a thermostable esterase. Proc. Natl. Acad. Sci. U.S.A. 95, 12809–12813.

Ingram, L.O., Gomez, P.F., Lai, X., Moniruzzaman, M., Wood, B.E., Yomano, L.P., York, S.W., 1998. Metabolic engineering of bacteria for ethanol production. Biotechnol. Bioeng. 58, 204–214.

Jeffries, T.W., Jin, Y.S., 2004. Metabolic engineering for improved fermentation of pentoses by yeasts. Appl. Microbiol. Biotechnol. 63, 495–509.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227, 680–685.

Lee, B., Vasmatzis, G., 1997. Stabilization of protein structures. Curr. Opin. Biotechnol. 8, 423–428.

Lehmann, M., Wyss, M., 2001. Engineering proteins for thermostability: the use of sequence alignments *versus* rational design and directed evolution. Curr. Opin. Biotechnol. 12, 371–375.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mooers, B.H., Datta, D., Baase, W.A., Zollars, E.S., Mayo, S.L., Matthews, B.W., 2003. Repacking the core of T4 lysozyme by automated design. J. Mol. Biol. 332, 741–756.

Ng, K., Ye, R., Wu, X.C., Wong, S.L., 1992. Sorbitol dehydrogenase from *Bacillus subtilis*. purification, characterization, and gene cloning. J. Biol. Chem. 267, 24989–24994.

Nordling, E., Jornvall, H., Persson, B., 2002. Medium-chain dehydrogenases/reductases (MDR) family characterizations including genome comparisons and active site modelling. Eur. J. Biochem. 269, 4267–4276.

Ostergaard, S., Olsson, L., Nielsen, J., 2000. Metabolic engineering of *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 64, 34–50.

Pace, C.N., Shirley, B.A., McNutt, M., Gajiwala, K., 1996. Forces contributing to the conformational stability of proteins. FASEB J. 10, 75–83.

Riveros-Rosas, H., Julian-Sanchez, A., Villalobos-Molina, R., Pardo, J.P., Pina, E., 2003. Diversity, taxonomy and evolution of medium-chain dehydrogenase/reductase superfamily. Eur. J. Biochem. 270, 3309–3334.

Rizzi, M., Harwart, K., Erlemann, P., Bui-Thanh, N.A., Dellweg, H., 1989. Purification and properties of the NAD^+-xylitol-dehydrogenase from the yeast *Pichia stipitis*. J. Ferment. Bioeng. 67, 20–24.

Van den Burg, B., Eijsink, V.G., 2002. Selection of mutations for increased protein stability. Curr. Opin. Biotechnol. 13, 333–337.

Vieille, C., Zeikus, G.J., 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol. Mol. Biol. Rev. 65, 1–43.

Walfridsson, M., Anderlund, M., Bao, X., Hahn-Hägerdal, B., 1997. Expression of different levels of enzymes from the *Pichia stipitis XYL1* and *XYL2* genes in *Saccharomyces cerevisiae* and its effects on product formation during xylose utilisation. Appl. Microbiol. Biotechnol. 48, 218–224.

Watanabe, S., Kodaki, T., Makino, K., 2005. Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc. J. Biol. Chem. 280, 10340–10349.

Zaldivar, J., Nielsen, J., Olsson, L., 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl. Microbiol. Biotechnol. 56, 17–34.