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A Novel Strictly NADPH-Dependent *Pichia stipitis* Xylose Reductase Constructed by Site-directed Mutagenesis

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Abstract

Xylose reductase (XR) and xylitol dehydrogenase (XDH) are the key enzymes for xylose fermentation and have been widely used for construction of a recombinant xylose fermenting yeast. The effective recycling of cofactors between XR and XDH has been thought to be important to achieve effective xylose fermentation. Efforts to alter the coenzyme specificity of XR and HDX by site-directed mutagenesis have been widely made for improvement of efficiency of xylose fermentation. We previously succeeded by protein engineering to improve ethanol production by reversing XDH dependency from NAD⁺ to NADP⁺. In this study, we applied protein engineering to construct a novel strictly NADPH dependent XR from *Pichia stipitis* by site-directed mutagenesis, in order to recycle NADPH between XR and XDH effectively. One double mutant, E223A/S271A showing strict NADPH dependency with 106 % activity of wild-type was generated. A second double mutant, E223D/S271A, showed a 1.27-fold increased activity compared to the wild-type XR with NADPH and almost negligible activity with NADH.

Keywords: Coenzyme specificity, Xylose reductase, Site-directed mutagenesis

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29 1. Introduction

30 Xylose is the second most abundant pentose sugar constituting the lignocellulosic
31 renewable biomass after glucose, and its complete fermentation is economically valuable
32 for producing biofuel from lignocellulosic biomass [6]. Recombinant *S. cerevisiae* can
33 ferment xylose through a fungal pathway involving two heterologous oxidoreductase
34 genes. In this pathway, *Pichia stipitis* xylose reductase (PsXR) (XR; EC 1.1.1.21) [18],
35 which prefers NADPH, reduces xylose to xylitol followed by *P. stipitis* xylitol
36 dehydrogenase (PsXDH), which exclusively requires NAD⁺ (XDH; EC 1.1.1.9) [17],
37 oxidizes xylitol into xylulose. *S. cerevisiae* xylulokinase (XK) (EC 2.7.1.17) naturally
38 phosphorylates xylulose to xylulose-5-phosphate, which is then metabolized by the
39 glycolytic pathway via the pentose phosphate pathway [7]. XK overexpression improves
40 the efficiency of xylose fermentation [4, 5, 15]. Although this fungal pathway is highly
41 expressed in *S. cerevisiae*, the efficiency of ethanol production is somewhat obstructed by
42 the unfavorable accumulation of xylitol due to the imbalance of coenzyme specificities
43 between XR and XDH [6].

44 Xylose reductase is a member of the aldo-keto reductase (AKR) superfamily
45 which is made up of 14 different families and approximately 120 members with a
46 majority of dual cofactor type enzymes [8]. *Candida tenuis* XR (CtXR) is one of these
47 enzymes. Its crystal structure has been determined at different levels of resolution and its
48 binding sites with NAD(P)H were also determined [10,12, 25]. Although only little

structural information of PsXR is available, it has about 76% homology with the CtXR. This high percentage of similarity should provide some clues for manipulation of PsXR [13].

Protein engineering has been widely used to alter the coenzyme specificity of XR and XDH. Since PsXDH accepts only NAD^+ , many researchers reversed the preference of XR to NADH in order to achieve NAD^+/NADH cofactor recycling [1, 13, 16, 20]. On the other hand, we have been working on converting cofactor usage of XDH to NADP^+ from NAD^+ [21]. We previously succeeded to improve the fermentation process and ethanol production by using these XDH mutants [23]. In this study, site-directed mutagenesis of PsXR was performed to construct a strictly NADPH-dependent XR, expecting decreasing or preventing xylitol accumulation and subsequently improving ethanol production.

2. Materials and Methods

2.1 Cloning of the *P. stipitis* Xylose reductase gene and Site-directed mutagenesis

A plasmid, named pHis (WT) harboring the His-tagged wild-type (WT) PsXR gene was constructed as described previously [20]. All XR mutations were introduced by site-directed mutagenesis, using the single round PCR method with *PfuTurbo* DNA polymerase (Stratagene) and the PCR Thermal Cycler PERSONAL (TaKaRa, Otsu, Japan). The codons used for mutations introduced in this study were as follows: E223A ($\text{GAA} \rightarrow \text{GCA}$), E223D ($\text{GAA} \rightarrow \text{GAC}$), and S271A ($\text{TCC} \rightarrow \text{GCC}$). The PCR products were subjected to DpnI restriction enzyme treatment in order to digest the parent DNA strands to prevent transformation of the template plasmid. Only nicked circular mutagenic strands were transformed into *Escherichia coli* DH5 α . Electroporation method

was used to transform plasmids and the mutations were confirmed by DNA sequencing using Applied Biosystems 3031 genetic analyzer and ABI Prism[®] Big Dye[®] Terminator v3.1 Cycle Sequencing kit.

2.2 Overexpression and purification of (His)₆-tagged enzymes

P. stipitis xylose reductase wild-type and mutated genes were expressed in *E. coli* DH5 α and purified as described previously [20]. Purified enzymes were confirmed on 10 % acrylamide SDS-PAGE. Protein concentrations were determined using the Bio RAD Quick Start Bradford 1x Dye Reagent (Bio-Rad Laboratories, CA, USA) by measuring the absorbance at 595 nm with γ -globin as a standard.

2.3 Enzyme assays and Kinetic parameters

Enzyme activities were measured spectrophotometrically as described previously [20] with modification in xylose concentration to 400 mM. The kinetic parameters were calculated by Line Weaver–Burk plots.

3. Result and Discussion

3.1 Speculation and prediction of NAD(P)H binding sites

Crystallographic analyses of many AKRs have revealed that they share a common (α/β)₈ barrel fold, with a highly conserved coenzyme binding pocket at the C-terminus. 90.9 % of the residues are located in the core area and 9.1 % are in the allowed regions [11]. The nicotinamide ring of NAD(P)H is resides in the core of the barrel. Residues Glu²²⁷ and Asn²⁷⁶ in CtXR, which equal to Glu²²³ and Asn²⁷² in PsXR, primarily mediate the interactions with the adenosine ribose 2'- and 3'-hydroxy groups. As shown in Fig. 1,

97 Glu²²³ represents the essential part NADH binding where contacts by bidentate hydrogen
98 bond with both of the hydroxy groups. Similar interactions have been seen in many other
99 NADH-binding protein structures [2, 3]. However, The structurally equivalent residues
100 Asp²¹⁶ and Val²⁶⁴ in aldose reductase in human (AR) are unable to fulfill the equivalent
101 roles, Asp²¹⁶ is required for high affinity binding of NADPH by forming two salt linkages
102 with Lys²¹ and Lys²⁶² and fastening the loop over the co-substrate [24].

103 Glu²²⁷ and Lys²⁷⁴ in CtXR makes water-mediated interactions each other and with
104 the 3'-hydroxy group in the case of NADP⁺-bound structures. In the absence of a
105 negatively charged phosphate, Glu²²⁷ side chain is able to rotate into a favourable
106 conformation to accept a 2.64 Å hydrogen bond contact with the 2'-hydroxy group and a
107 2.65 Å hydrogen bond with the 3'-hydroxy group when NAD⁺ is bound. The root mean
108 square deviations of the C α values between NAD⁺- and NADP⁺-bound models was
109 calculated in CtXR. The largest conformational change is seen in residues 274–280,
110 which corresponding to 270–276 in PsXR, and then residues 225–229, which
111 corresponding to 221–225 in PsXR, a short helical region that appears at the end of β 7.
112 The largest main-chain shift is seen in Ser²⁷⁵, which corresponding to Ser²⁷¹ in PsXR,
113 moves 2.0 Å in response to the miss contact of the phosphate group of NADPH [10].
114 Furthermore, Glu²²³ of PsXR was subjected to a mutation trial and the result revealed that
115 alteration of this site might further inhibit NADH binding [13]. In addition, from the 3D
116 structure model of PsXR, it was reported that Glu²²³ and Phe²³⁶ can form 3 and 2
117 hydrogen bonds with NAD⁺, respectively [19].

118 Considering the property as described above, the mutations were designed based
119 on sequence alignment of some strictly NADPH dependent analogous enzymes in the
120 AKR family, such as AR, as shown in table1, where glutamic acid 223 was substituted by

aspartic acid. Both glutamic and aspartic acid are acidic side chain and fully ionized at neutral pH and able to engage in hydrogen bonds, which is a necessary component for a high affinity xylose binding site [9]. Alanine is a nonpolar side chain that does not bind or give off protons, or participate in hydrogen or ionic bonds. Alanine can be worked as oily or lipid-like that promotes hydrophobic interactions. Accordingly, we apply aspartic acid and alanine to mutation trials instead of PsXR glutamic acid 223.

3.2 Strictly NADPH dependency on Glu²²³ mutants

We applied Glu²²³ residue for mutation trails in order to delete NADH dependency. Although this residue is also shared in NADPH binding, some reports reveal that it contributes more to the affinity of NADH, where it plays a role in the binding site by binding two hydrogen bonds with 2' and 3' hydroxy groups of the adenosine ribose. In addition to changes in hydrogen-bonding of the adenosine, the ribose unmistakably adopts the 3'-*endo* conformation rather than the 2'-*endo* conformation seen in the NADP⁺-bound form [10]. The enzyme activities with NADH were calculated after introduction of Glu²²³ residue mutations (Fig. 2). No activity was detected for E223A with NADH while E223D showed only 17 % of the activity of WT. In addition, catalytic efficiency was decreased to 3.7 % of WT. Their activities with NADPH showed 52 % and 44 % of WT, respectively. The catalytic efficiencies of E223A and E223D were 26 % and 15 % of WT respectively. Although, these ratios were low compared with WT, E223D showed 2.54 and 3.9 fold improvement in NADPH/NADH ratio and k_{cat}/K_m respectively. E223A is a completely NADPH dependent mutant, probably due to the change of 3'-*endo* ribose conformation and miss contact of bidentate hydrogen bonds which was conserved in NAD⁺ binding sites in most members of dual cofactor in AKR family.

145

146 3.3 Improvement of enzyme activities with double mutants

147 We previously reported that the mutation of Lys²⁷⁰ and Arg²⁷⁶ in PsXR improve
148 NADH preference [20], while S271A increased the preference for NADPH [22]. The
149 second rounds of mutations were done based on this data. Accordingly, combination of
150 S271A with Glu²²³ mutants was expected to increase the activity of XR with NADPH. As
151 shown in Fig. 1, S271A mutant showed improved NADPH preference, where the
152 activities with NADPH and NADH were 125% and 85% compared to WT respectively.
153 These data encouraged us to perform further investigations by combining S271A and
154 Glu²²³ mutants. A combination of site-directed mutations of the residues Glu²²³ and
155 S271A produced unique and unprecedented results. The double mutants E223A/S271A
156 (AA) and E223D/S271A (DA) showed improvement in the activities with NADPH
157 compared to single Glu²²³ mutants. As shown in Fig. 2, the activity of the double mutant
158 AA with NADPH was 106% compared to WT. As shown in Table 2, the k_{cat} of WT and
159 AA were 622 and 657 min⁻¹, respectively; their K_{m} for xylose were 97.1 and 226 mM,
160 respectively; and their catalytic efficiencies were 38.6 and 32.4 $\mu\text{M}^{-1}/\text{min}^{-1}$, respectively.
161 On the other hand, the activity of DA showed 15 % WT with NADH (Fig. 1) in addition
162 to K_{m} was increased 12.8-fold and k_{cat} decreased 3-fold (Table 2). As shown in Fig. 2, the
163 activity of DA with NADPH was increased 1.27-fold compared to WT; k_{cat} also increased
164 1.18-fold compared to WT, while catalytic efficiency was decreased to 93 % of WT and
165 K_{m} increased 1.26-fold compared to WT (see table 2). Thus we succeeded to construct a
166 novel strictly NADPH-dependent PsXR by combining the mutation at Glu²²³ and Ser²⁷¹
167 residues.

We previously succeeded in improving xylose fermentation and ethanol production by combining PsXR WT with the mutated PsXDH which accepts only NADP⁺ (i.e., quadruple ARSdR mutant) [23], and overexpression of XK [14, 15]. It may provide further clues for understanding of importance of coenzyme specificities of XR and XDH using the strictly NADPH-dependent PsXR of this study together with the strictly NADP⁺-dependent PsXDH [21]. It could possibly give more efficient xylose fermentation by an effective recycling of coenzymes of NADPH between XR and XDH.

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Table1
The mutation designs of the PsXR enzyme

Enzyme	Accession No. or mutant	Organism	Coenzyme preference	Amino Acid Sequence					
				2	2	2	2	2	2
				2	2	2	7	7	7
				2	3	4	0	1	2
PsXR	CAA42072	<i>Pichia stipitis</i>	NADPH	V	E	L	K	S	N
AKR1 B7 [§]	P21300	<i>Mus musculus</i>	NADPH [†]	P	D	R	*	*	V
2, 5 DKGRA [*]	AAA83534	<i>Corynebacterium sp.</i>	NADPH [†]	Y	D	*	*	*	V
AR	P15121	<i>Homo sapiens</i>	NADPH [†]	P	D	R	*	*	V
XR	O94735	<i>Pichia guilliermondi</i>	NADPH	*	*	*	*	*	N
XR	Q6Y0Z3	<i>Candida parapsilosis</i>	NADH	L	*	M	*	*	S
XR	O74237	<i>Candida tenuis</i>	NADPH	*	*	M	*	*	L
XR	P87039	<i>Candida tropicalis</i>	NADPH	L	*	*	*	*	N
PsXR	E223A	<i>P. stipitis</i>	This work	*	A	*	*	*	*
PsXR	E223D		This work	*	D	*	*	*	*
Ps XR	S271A		This work	*	*	*	*	A	*
Ps XR	AA [†]		This work	*	A	*	*	A	*
Ps XR	DA [†]		This work	*	D	*	*	A	*

Bold letters represent target mutation sites *The same amino acids as PsXR WT
[†] Double mutant E223A/S271A (AA) and E223D/S271A (DA)
[§] Strict NADPH dependent enzyme § Aldo-keto reductase family 1, memberB7
^{*} 2,5-Diketo-D-gluconic acid reductase

Table 2

Kinetic parameters of wild-type and xylose reductase mutants for NADPH- and NADH-dependent reactions

Enzymes	Kinetic parameters							
	NADPH				NADH			
	K_m xylose ^a [mM]	K_m ^b [μM]	k_{cat} ^b [Min ⁻¹]	k_{cat}/K_m [μM ⁻¹ /min ⁻¹]	K_m xylose ^a [mM]	K_m ^b [μM]	k_{cat} ^b [min ⁻¹]	k_{cat}/K_m [μM ⁻¹ /min ⁻¹]
XRWT	97.1 ± 4.8	16.2 ± 1.4	622 ± 22	38.6 ± 2.9	170 ± 23	30.6 ± 1.0	449 ± 22	14.7 ± 1.4
S271A	70.6 ± 8.7	30.1 ± 3.7	874 ± 50	29.0 ± 0.3	180 ± 12	53.3 ± 4.4	480 ± 42	9.00 ± 0.40
E223A	29.8 ± 4.7	35.2 ± 3.7	349 ± 29	9.94 ± 0.44	ND ^d	ND	ND	ND
E223D	114 ± 13	55.4 ± 7.1	314 ± 42	5.65 ± 0.32	376 ± 32	305 ± 11	169 ± 52	0.55 ± 0.15
AA ^c	226 ± 22	17.5 ± 0.7	567 ± 73	32.4 ± 2.7	ND	ND	ND	ND
DA ^c	108 ± 8	20.4 ± 0.4	733 ± 14	36.0 ± 0.3	353 ± 1	391 ± 46	156 ± 29	0.44 ± 0.10

^a Six different concentrations of xylose between 67 and 200 mM were used and NAD(P)H concentration was 150 μM.^b Six different concentrations of NAD(P)H between 50 and 300 μM were used and xylose concentration was 400 mM.^c Double mutants E223A/S271A (AA) and E223D/S271A (DA)^d ND: Not detected

Fig. 1. Schematic diagrams showing the predicted interactions of wild-type PsXR; Left-hand panel: adenosine 2'- and 3'- hydroxy groups in the complex with NAD⁺ and Right-hand panel: adenosine 2'- and 3'- hydroxy groups in the complex with NADP⁺ based on the coenzyme binding sites in CtXR [10].

Fig. 2. Enzyme activities of PsXR wild-type and mutated enzymes. Black and grey bars indicated activities with NADPH and NADH respectively; Values are average $\pm$ SD, n=3.