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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1. Introduction

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

Xylose reductase is a member of the aldo-keto reductase (AKR) superfamily which is made up of 14 different families and approximately 120 members with a majority of dual cofactor type enzymes [8]. *Candida tenuis* XR (CtXR) is one of these enzymes. Its crystal structure has been determined at different levels of resolution and its 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 (GAA→GCA), E223D (GAA→GAC), and S271A (TCC→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* DH5α. 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* DH5α 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 $(\alpha/\beta)_8$ 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 Glu^{227} and Asn^{276} in CtXR, which equal to Glu^{223} and Asn^{272} in PsXR, primarily mediate the interactions with the adenosine ribose 2'- and 3'-hydroxy groups. As shown in Fig. 1,

Glu^{223} represents the essential part NADH binding where contacts by bidentate hydrogen bond with both of the hydroxy groups. Similar interactions have been seen in many other NADH-binding protein structures [2, 3]. However, The structurally equivalent residues Asp^{216} and Val^{264} in aldose reductase in human (AR) are unable to fulfill the equivalent roles, Asp^{216} is required for high affinity binding of NADPH by forming two salt linkages with Lys^{21} and Lys^{262} and fastening the loop over the co-substrate [24].

Glu^{227} and Lys^{274} in CtXR makes water-mediated interactions each other and with the 3'-hydroxy group in the case of $NADP^+$-bound structures. In the absence of a negatively charged phosphate, Glu^{227} side chain is able to rotate into a favourable conformation to accept a 2.64 Å hydrogen bond contact with the 2'-hydroxy group and a 2.65 Å hydrogen bond with the 3'-hydroxy group when NAD^+ is bound. The root mean square deviations of the Cα values between NAD^+- and $NADP^+$-bound models was calculated in CtXR. The largest conformational change is seen in residues 274–280, which corresponding to 270-276 in PsXR, and then residues 225–229, which corresponding to 221-225 in PsXR, a short helical region that appears at the end of β7. The largest main-chain shift is seen in Ser^{275}, which corresponding to Ser^{271} in PsXR, moves 2.0 Å in response to the miss contact of the phosphate group of NADPH [10]. Furthermore, Glu^{223} of PsXR was subjected to a mutation trial and the result revealed that alteration of this site might further inhibit NADH binding [13]. In addition, from the 3D structure model of PsXR, it was reported that Glu^{223} and Phe^{236} can form 3 and 2 hydrogen bonds with NAD^+, respectively [19].

Considering the property as described above, the mutations were designed based on sequence alignment of some strictly NADPH dependent analogous enzymes in the 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 Glu^{223} mutants

We applied Glu^{223} 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 Glu^{223} 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.

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146 3.3 Improvement of enzyme activities with double mutants

147 We previously reported that the mutation of Lys^{270} and Arg^{276} 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 Glu^{223} 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 Glu^{223} mutants. A combination of site-directed mutations of the residues Glu^{223} 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 Glu^{223} 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 $\mu M^{-1}/min^{-1}$, 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 Glu^{223} and Ser^{271}
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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References

[1] O. Bengtsson, B. Hahn-Hagerdal, M.F. Gorwa-Grauslund, Xylose reductase from *Pichia stipitis* with altered coenzyme preference improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*, Biotechnol. Biofuels 2 (2009) 9.

[2] O. Carugo, P. Argos, NADP-dependent enzymes. 1. Conserved stereochemistry of cofactor binding, Proteins Struct. Funct. and Genet. 28 (1997) 10-28.

[3] O. Carugo, P. Argos, NADP-dependent enzymes. 2. Evolution of the mono- and dinucleotide binding domains, Proteins Struct. Funct. and Genet. 28 (1997) 29-40.

[4] A. Eliasson, C. Christensson, C.F. Wahlbom, B. Hahn-Hagerdal, Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures, Appl. Environ. Microbiol. 66 (2000) 3381-3386.

[5] A. Eliasson, J.H.S. Hofmeyr, S. Pedler, B. Hahn-Hagerdal, The xylose reductase/xylitol dehydrogenase/xylulokinase ratio affects product formation in recombinant xylose-utilising *Saccharomyces cerevisiae*, Enzyme and Microbial. Technology 29 (2001) 288-297.

[6] T.W. Jeffries, Y.S. Jin, Metabolic engineering for improved fermentation of pentoses by yeasts, Appl. Microbiol. Biotechnol. 63 (2004) 495-509.

[7] H. Jeppsson, S. Yu, B. Hahn-Hagerdal, Xylulose and glucose fermentation by *Saccharomyces cerevisiae* in chemostat culture, Appl. Environ. Microbiol. 62 (1996) 1705-1709.

[8] J.M. Jez, T.M. Penning, The aldo-keto reductase (AKR) superfamily: an update, Chem. Biol. Interact. 130-132 (2001) 499-525.

[9] K.L. Kavanagh, M. Klimacek, B. Nidetzky, D.K. Wilson, The structure of apo and

holo forms of xylose reductase, a dimeric aldo-keto reductase from *Candida tenuis*, Biochemistry 41 (2002) 8785-8795.

[10] K.L. Kavanagh, M. Klimacek, B. Nidetzky, D.K. Wilson, Structure of xylose reductase bound to NAD^+ and the basis for single and dual co-substrate specificity in family 2 aldo-keto reductases, Biochem. J. 373 (2003) 319-326.

[11] R.A. Laskowski, M.W. Macarthur, D.S. Moss, J.M. Thornton, Procheck - a Program to Check the Stereochemical Quality of Protein Structures, Journal of Applied Crystallography 26 (1993) 283-291.

[12] S. Leitgeb, B. Petschacher, D.K. Wilson, B. Nidetzky, Fine tuning of coenzyme specificity in family 2 aldo-keto reductases revealed by crystal structures of the Lys-274 → Arg mutant of *Candida tenuis* xylose reductase (AKR2B5) bound to NAD^+ and $NADP^+$, Febs Letters 579 (2005) 763-767.

[13] L. Liang, J. Zhang, Z. Lin, Altering coenzyme specificity of *Pichia stipitis* xylose reductase by the semi-rational approach CASTing, Microb. Cell Fact. 6 (2007) 36.

[14] A. Matsushika, H. Inoue, S. Watanabe, T. Kodaki, K. Makino, S. Sawayama, Efficient bioethanol production by a recombinant flocculent *Saccharomyces cerevisiae* strain with a genome-integrated $NADP^+$-dependent xylitol dehydrogenase gene, Appl. Environ. Microbiol. 75 (2009) 3818-3822.

[15] A. Matsushika, S. Watanabe, T. Kodaki, K. Makino, S. Sawayama, Bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing xylose reductase, $NADP^+$-dependent xylitol dehydrogenase, and xylulokinase, J. Biosci. Bioeng. 105 (2008) 296-299.

[16] B. Petschacher, B. Nidetzky, Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*, Microb. Cell Fact. 7 (2008) 9.

[17] M. Rizzi, K. Harwart, P. Erlemann, N.A. Buithanh, H. Dellweg, Purification and Properties of the NAD^+-Xylitol-Dehydrogenase from the Yeast *Pichia stipitis*, Journal of Fermentation and Bioengineering 67 (1989) 20-24.

[18] C. Verduyn, R. Van Kleef, J. Frank, H. Schreuder, J.P. Van Dijken, W.A. Scheffers, Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*, Biochem. J. 226 (1985) 669-677.

[19] J.F. Wang, D.Q. Wei, Y. Lin, Y.H. Wang, H.L. Du, Y.X. Li, K.C. Chou, Insights from modeling the 3D structure of NAD(P)H-dependent D-xylose reductase of *Pichia stipitis* and its binding interactions with NAD and NADP, Biochem. Biophys. Res. Commun. 359 (2007) 323-329.

[20] S. Watanabe, A. Abu Saleh, S.P. Pack, N. Annaluru, T. Kodaki, K. Makino, Ethanol

production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*, Microbiology 153 (2007) 3044-3054.

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

[22] S. Watanabe, S.P. Pack, A.A. Saleh, N. Annaluru, T. Kodaki, K. Makino, The positive effect of the decreased NADPH-preferring activity of xylose reductase from *Pichia stipitis* on ethanol production using xylose-fermenting recombinant *Saccharomyces cerevisiae*, Biosci. Biotechnol. Biochem. 71 (2007) 1365-1369.

[23] S. Watanabe, A.A. Saleh, S.P. Pack, N. Annaluru, T. Kodaki, K. Makino, Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein engineered $NADP^+$-dependent xylitol dehydrogenase, J. Biotechnol. 130 (2007) 316-319.

[24] D.K. Wilson, K.M. Bohren, K.H. Gabbay, F.A. Quiocho, An unlikely sugar substrate site in the 1.65 A structure of the human aldose reductase holoenzyme implicated in diabetic complications, Science 257 (1992) 81-84.

[25] D.K. Wilson, K.L. Kavanagh, M. Klimacek, B. Nidetzky, The xylose reductase (AKR2B5) structure: homology and divergence from other aldo-keto reductases

288 and opportunities for protein engineering, Chem. Biol. Interact. 143-144 (2003)
289 515-521.

Table1
The mutation designs of the PsXR enzyme

Enzyme	Accession No. or mutant	Organism	Coenzyme preference	Amino Acid Sequence 222 223 224 270 271 272
PsXR	CAA42072	*Pichia stipitis*	NADPH	V E L K **S** N
AKR1 B7[§]	P21300	*Mus musculus*	NADPH[♠]	P D R * * V
2, 5 DKGRA[*]	AAA83534	*Corynebacterium sp*.	NADPH[♠]	Y D * * * V
AR	P15121	*Homo sapiens*	NADPH[♠]	P D R * * V
XR	O94735	*Pichia guilliermondi*	NADPH	* * * * * N
XR	Q6Y0Z3	*Candida parapsilosis*	NADH	L * M * * S
XR	O74237	*Candida tenuis*	NADPH	* * M * * L
XR	P87039	*Candida tropicalis*	NADPH	L * * * * N
PsXR	E223A	*P. stipitis*	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

	Kinetic parameters							
	NADPH				NADH			
Enzymes	K_m xylose [a] [mM]	K_m [b] [μM]	k_{cat} [b] [Min^{-1}]	k_{cat}/K_m [$\mu M^{-1}/min^{-1}$]	K_m xylose [a] [mM]	K_m [b] [μM]	k_{cat} [b] [min^{-1}]	k_{cat}/K_m [$\mu M^{-1}/min^{-1}$]
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

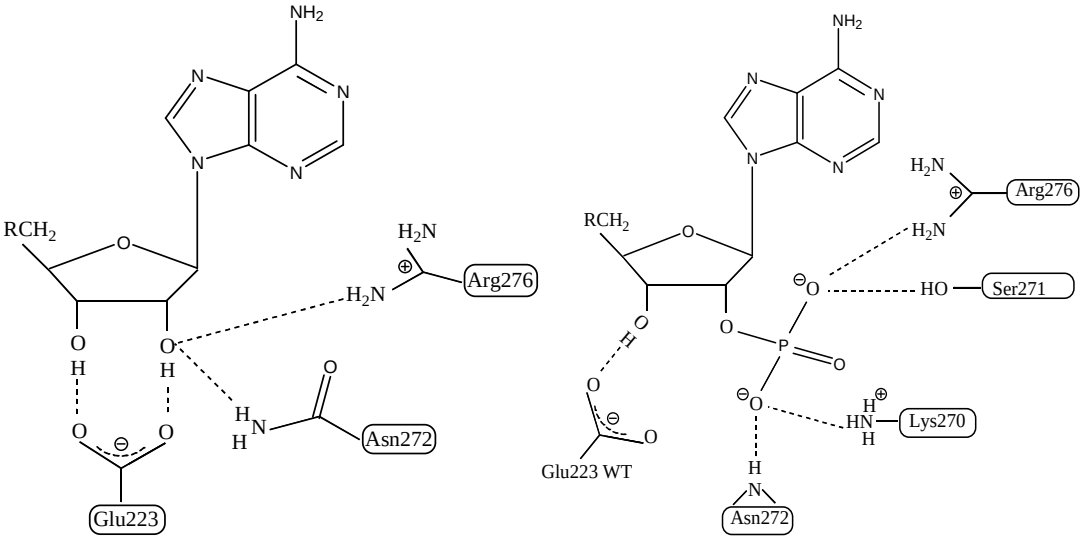

Fig. 1. Schematic diagrams showing the predicted interactions of wild-type PsXR; Left-hand panel: adenosine 2'- and 3'- hydroxy groups in the complex with NAD^+ and Right-hand panel: adenosine 2'- and 3'- hydroxy groups in the complex with $NADP^+$ based on the coenzyme binding sites in CtXR [10].

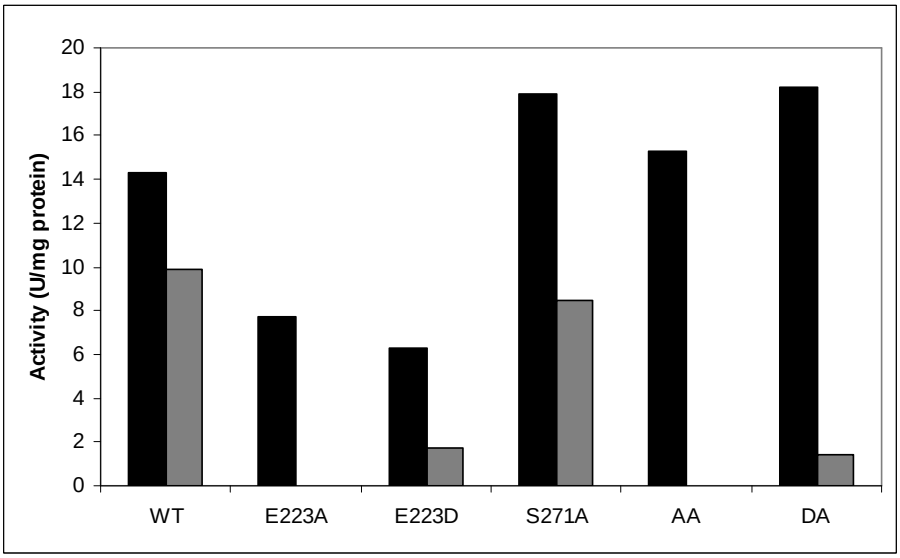

Fig. 2. Enzyme activities of PsXR wild-type and mutated enzymes. Black and grey bars indicated activities with NADPH and NADH respectively; Values are average ± SD, n=3.