Short Communication

Detection of active sorbitol-6-phosphate phosphatase in the haloacid dehalogenase-like hydrolase superfamily

(Received November 27, 2017; Accepted December 25, 2017; J-STAGE Advance publication date: May 8, 2018)

Taejun Chin¹ and Masahiko Ikeuchi^{1,2,*}

¹ Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan

² Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

Sorbitol-6-phosphatase (EC 3.1.3.50) catalyzes sorbitol production from sorbitol-6-phosphate in certain organisms, but has not been identified unequivocally. We screened the activity of the haloacid dehalogenase-like hydrolases (HAD) superfamily and identified four HAD proteins from *Escherichia coli* as sorbitol-6-phosphatase. Of these proteins, HAD2 (YfbT) exhibited catalytic activity (k_{cat}/K_m) that was better than that of the previously reported “preferred” substrate. HAD1 (YniC) and HAD2 exhibited higher sorbitol-6-phosphatase activity than that of HAD12 (YbiV) and HAD13 (YidA). Therefore, genes of HAD may be useful for metabolic engineering of effective sorbitol production.

Key Words: cyanobacteria; *Escherichia coli*; haloacid dehalogenase-like hydrolase; phosphatase; sorbitol-6-phosphate

Sorbitol is one of the major sugar alcohols and has numerous uses, such as its use as an industrial sweetener and humectant. Biologically, sorbitol is a primary photosynthate and translocated carbohydrate found in *Rosacea* plants (Loescher, 1987) and a waste product of anaerobic metabolism in lactic acid bacteria (Monedero et al., 2010). In these organisms, sorbitol is produced from glucose-6-phosphate or fructose-6-phosphate by a two-step reaction mediated by NAD(P)-dependent sorbitol-6-phosphate dehydrogenase (S6PDH) and sorbitol-6-phosphatase (Fig. 1): NADP-dependent S6PDH (EC 1.1.1.200) is en-

coded by the *s6pdh* gene in apple (Kanayama et al., 1992), and NAD-dependent S6PDH (EC 1.1.1.140) is encoded by *srID2* in lactic acid bacteria (Kleerebezem et al., 2003). However, a gene for the sorbitol-6-phosphatase has not yet been identified in any organism, although its enzyme activity has been detected in plant extracts (Grant and Rees, 1981; Zhou et al., 2003). In metabolic engineering, sorbitol production has been reported following the introduction of only *s6pdh* or *srID2* into yeast, *Escherichia coli*, and cyanobacteria (Chin et al., 2018; Shen et al., 1999). In these cases, some intrinsic enzyme(s) must hydrolyze the phosphate ester of the intermediate, sorbitol-6-phosphate, which is a non-natural metabolite. The sorbitol production could still be improved by the introduction of an additional sorbitol-6-phosphatase gene, although we do not know whether the dephosphorylation step is rate-limited or not. Moreover, we noticed that the expression of *s6pdh* in a cyanobacterium *Synechocystis* sp. PCC 6803 induced toxicity that affected the growth. Co-expression of sorbitol-6-phosphatase may prevent such a defect.

Because *E. coli* cells produced sorbitol only when the apple *s6pdh* is overexpressed, potential phosphatase genes may be screened in its genome. We chose the haloacid dehalogenase-like hydrolase (HAD) superfamily, which includes numerous related proteins with phosphatase, phosphonate, dehalogenase, phosphoglucomutase, and ATPase activities and a fairly broad specificity (Burroughs et al., 2006; Koonin and Tatusov, 1994). These proteins are widely distributed in almost all organisms, although their physiological roles have remained unclear. There are 28 and 45 genes for the HAD enzymes in the genomes of *E. coli* and the yeast *Saccharomyces cerevisiae*, respec-

*Corresponding author: Masahiko Ikeuchi, Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan; Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan.
E-mail: mikeuchi@bio.c.u-tokyo.ac.jp

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Fig. 1. Sorbitol biosynthesis by the two-step enzymatic reactions.

Sorbitol is produced by a yet unidentified phosphatase from sorbitol-6-phosphate, which is supplied from glucose-6-phosphate or fructose-6-phosphate by NAD(P)-dependent S6PDH.

Fig. 2. SDS-PAGE analysis of *E. coli* HAD1 expression and purification.

The arrow indicates the His-tagged HAD1 (YniC) protein at 24.3 kDa. M, marker; S, supernatant of cell extracts; P, pellet of cell extracts; F, flow-through fraction of the Ni²⁺ chromatography; H, purified HAD1 fraction.

tively. In particular, the specificity and kinetic parameters of the phosphatase activity of the soluble HADs of these organisms have been extensively characterized (Kuznetsova et al., 2006, 2015). As a result, these HADs have been grouped into several categories, depending on their substrate specificity. However, the activity of *E. coli* HADs for sorbitol-6-phosphate has not been reported. In this study, we focused on six *E. coli* HADs, HAD1 (YniC), HAD2 (YfbT), HAD4 (YihX), HAD6 (YqaB), HAD12 (YbiV), and HAD13 (YidA) for screening of the sorbitol-6-phosphatase activity, because these enzymes exhibited dephosphorylation (esterase) activity preferentially against some sugar phosphates similar to sorbitol-6-phosphate (Kuznetsova et al., 2006). We also prepared a single HAD-like protein (Slr0953) of a cyanobacterium *Synechocystis* sp. PCC 6803, which was reported to be a sucrose-phosphatase (Lunn, 2002).

Genes of the target HADs were amplified using a

Table 1. Screening of phosphatase activities of several HADs from *E. coli* and *Synechocystis* for sorbitol-6-phosphate and pNPP.

HADs	Sorbitol-6-P	pNPP	
		This study	Kuznetsova et al. (2006)
HAD1 (YniC)	6.1	0.48	0.60
HAD2 (YfbT)	9.3	0.86	1.1
HAD4 (YihX)	ND	0.50	0.22
HAD6 (YqaB)	ND	0.65	0.84
HAD12 (YbiV)	2.9	3.8	2.4
HAD13 (YidA)	2.0	0.38	0.70
Slr0953 (SPP)	ND	0.12	—

Phosphatase activities ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein) are shown. Assays were performed in the presence of substrates (0.25 mM sorbitol-6-phosphate or 10 mM pNPP). Reference activities of pNPP (Kuznetsova et al. (2006)) are also shown. Slr0953 is sucrose-6-phosphate phosphatase (SPP), which is a sole member of the HAD superfamily in the cyanobacterium *Synechocystis* sp. PCC 6803. ND indicates that activity was not detected even at higher concentrations of sorbitol-6-phosphate (0.25–10 mM).

polymerase chain reaction with the PrimeSTAR Max DNA polymerase (TaKaRa Bio, Japan) and the genome DNA of *E. coli* JM109 or a glucose-tolerant substrain of *Synechocystis* sp. PCC 6803, and then they were cloned into the expression vector pET28a (Merck, Germany) using the In-Fusion HD cloning kit reagents (TaKaRa Bio, Japan). N-terminal His-tag of HAD proteins was derived from the pET28a vector. *E. coli* C41 (DE3) harboring pET28a-HAD were cultured in 1 L of LB medium with 0.1 mM isopropyl β -D-1-thiogalactopyranoside at 37°C for 3 h for expression. After disrupting the cells using a French press, each His-tagged protein was purified using nickel affinity chromatography as described previously (Maeda et al., 2014). The protein purity was confirmed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue R-250 staining. All six HADs of *E. coli* and the Slr0953 of *Synechocystis* were successfully purified into homogeneous soluble proteins (Fig. S1). Figure 2 shows the typical profile of the purified HAD1. Proteins were quantitated using the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

The phosphatase activity was assayed as follows: 200 μL of the reaction mixture containing 50 mM HEPES-KOH (pH 7.0), 2 mM MgCl₂, 1 μg of each purified pro-

Fig. 3. Lineweaver-Burk plots of phosphatase activity of HADs for sorbitol-6-phosphate. Assays were performed using 1 μg each of purified HAD1 (A), HAD2 (B), HAD12 (C), or HAD13 (D) in the reaction mixture containing 50 mM HEPES-KOH (pH 7.0) and 2 mM MgCl₂.

Table 2. Kinetic parameters of selected *E. coli* HADs for sorbitol-6-phosphate and the reported preferred substrates.

HADs	Sorbitol-6-P (present study)			Preferred substrate (previous studies)			
	K _m (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (s ⁻¹ M ⁻¹)	Compound	K _m (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (s ⁻¹ M ⁻¹)
HAD1 (YniC)	1.8	21	1.2 × 10 ⁴	2-Deoxyglucose-6-P	0.61	33	5.4 × 10 ⁴
HAD2 (YfbT)	3.9	63	1.6 × 10 ⁴	Glucose-6-P	1.8	13	7.1 × 10 ³
HAD4 (YihX)	—	ND	—	Glucose-1-P	0.24	1.4	5.9 × 10 ³
HAD6 (YqaB)	—	ND	—	Fructose-1-P	1.7	20	2.0 × 10 ⁴
HAD12 (YbiV)	2.3	12	5.3 × 10 ³	Fructose-1-P	1.4	111	8.0 × 10 ⁴
HAD13 (YidA)	4.5	14	3.2 × 10 ³	Erythrose-4-P	0.019	19	1.0 × 10 ⁶
Slr0953	—	ND	—	Sucrose-6-P	0.0075	21	2.8 × 10 ⁶

Kinetic parameters were estimated from the data in Fig. 2. Parameters for the preferred substrates are from Kuznetsova et al. (2006). Slr0953 is sucrose-6-phosphate phosphatase, which is the sole member of the HAD superfamily in the cyanobacterium *Synechocystis* sp. PCC 6803. The kinetic parameters for sucrose-6-phosphate are from Lunn (2002). P, phosphate. ND, not detected.

tein, and a predetermined concentration of D-sorbitol-6-phosphate barium salt (Sigma-Aldrich, Germany) or 10 mM *p*-nitrophenylphosphoric acid disodium salt (*p*NPP) (Nacalai Tesque, Japan) was incubated at 30°C for 10 min. The liberated inorganic phosphate was determined using a Malachite Green phosphate assay kit (BioAssay Systems, USA), which is based on color development at 620 nm. Interestingly, HAD1, HAD2, HAD12, and HAD13 but

not HAD4 and HAD6 showed phosphatase activity for the fixed concentration (0.25 mM) of sorbitol-6-phosphate (Table 1). The phosphatase activities against *p*NPP, which is a general phosphatase substrate, were also confirmed in all purified *E. coli* HADs at levels comparable to those reported in a previous study (Kuznetsova et al., 2006). To further evaluate the sorbitol-6-phosphatase activity, the enzyme kinetic parameters of HAD1, HAD2, HAD12, and

HAD13 were determined (Fig. 3). The K_m values and the maximal activities of the four HADs were in the range of 1.8–4.5 mM and 24–158 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein, respectively. It is also noteworthy that no phosphatase activity was detected for HAD4 and HAD6 even at higher concentrations of sorbitol-6-phosphate, which attributed the near maximal activity to the other *E. coli* HADs (Fig. 3). The HAD member of *Synechocystis* (Slr0953) exhibited low activity for pNPP but showed no activity for sorbitol-6-phosphate (Table 1). This finding eliminates the possibility that the sorbitol production was assisted by Slr0953 in the *s6pdh*-expressing cyanobacteria.

Table 2 summarizes their kinetic parameters for sorbitol-6-phosphate as well as the “preferred” substrates that showed the highest catalytic efficiencies (k_{cat}/K_m) in the previous report (Kuznetsova et al., 2006; Lunn, 2002). The K_m value of HAD1 was the lowest, but somewhat comparable to those of HAD12, HAD2, and HAD13. The k_{cat} value of HAD2 was highest, followed by that of HAD1, which was low, and those of HAD12 and HAD13 were even lower. The results indicated that the k_{cat}/K_m values of HAD2 and HAD1 were high while those of HAD12 and HAD13 were low. When these values were compared with those in the literature, the k_{cat}/K_m value of HAD2 for sorbitol-6-phosphate was higher than that for glucose-6-phosphate, while the values of HAD1, HAD12, and HAD13 were lower than those for the preferred substrates. The k_{cat}/K_m values of HAD13 and Slr0953 for the preferred substrates were much higher than those for sorbitol-6-phosphate, showing their high specificity for the preferred substrates.

Thus, we identified the following four HADs of *E. coli* as sorbitol-6-phosphatases: HAD1 (YniC), HAD2 (YfbT), HAD12 (YbiV), and HAD13 (YidA). The sorbitol-6-phosphatase activity was also detected for yeast HADs, but the best k_{cat}/K_m value was exhibited by YNL010W and was only 2.7×10^3 (Kuznetsova et al., 2015), which is lower than those of the four HADs of *E. coli* (Table 2). Therefore, HAD1 and HAD2 are currently the most plausible candidates for overexpression to enhance the dephosphorylation of sorbitol-6-phosphate in the engineered organisms for sorbitol production. It might also be worth expressing HAD12 and HAD13 to improve the sorbitol production, although they may also exhibit other promiscuous activities.

The physiological roles of numerous HADs that have phosphatase activity with broad specificity have not yet been fully elucidated. These include HAD1 of *E. coli* and DOG1 and DOG2 of yeast, which are both known to be essential for acquiring resistance against the toxic substance 2-deoxyglucose (Kuznetsova et al., 2006; Randez-Gil et al., 1995; Sanz et al., 1994). The discovery that these HADs exhibit phosphatase activity with 2-deoxyglucose-6-phosphate as the preferred substrate strongly suggest that the generation, or accumulation, of non-natural 2-deoxyglucose-6-phosphate is similarly toxic to *E. coli* and yeast. The broad substrate specificity of such HADs could be profitable if they are prepared for certain non-natural compounds. Indeed, we observed that the co-expression of *E. coli* HAD1 reduced the growth toxicity induced by sorbitol production in cyanobacteria (unpublished results).

HAD1 may be a multifunctional scavenger that reduces the accumulation of phosphate esters of non-natural compounds in *E. coli*. In this context, it is strange that *E. coli* HAD2 acts preferentially on the important metabolite, glucose-6-phosphate. However, our data demonstrated that HAD2 could serve as a sorbitol-6-phosphatase rather than a glucose-6-phosphatase, which is consistent with the presumed dogma of scavenging toxic non-natural compounds. Moreover, we observed that the growth of *E. coli* was not markedly retarded by the overexpression of HAD2. These results suggest that HAD2 may also serve as a scavenger, implying that its overexpression might also facilitate the detoxification of the side effects of sorbitol production in recombinant cyanobacteria. Co-expression of HAD2 in cyanobacteria is now in progress.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research and the GCOE program “From the Earth to Earths” from MEXT and CREST from JST (to M.I.).

Supplementary Materials

Supplementary figure is available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

References

- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254, doi:10.1016/0003-2697(76)90527-3.
- Burroughs, A. M., Allen, K. N., Dunaway-Mariano, D., and Aravind, L. (2006) Evolutionary genomics of the HAD superfamily: understanding the structural adaptations and catalytic diversity in a superfamily of phosphoesterases and allied enzymes. *J. Mol. Biol.*, **361**, 1003–1034, doi:10.1016/j.jmb.2006.06.049.
- Chin, T., Okuda, Y., and Ikeuchi, M. (2018) Sorbitol production and optimization of photosynthetic supply in the cyanobacterium *Synechocystis* PCC 6803. *J. Biotechnol.*, doi:10.1016/j.jbiotec.2018.04.004 (in press).
- Grant, C. R. and Rees, T. ap (1981) Sorbitol metabolism by apple seedlings. *Phytochemistry*, **20**, 1505–1511, doi:10.1016/S0031-9422(00)98521-2.
- Kanayama, Y., Mori, H., Imaseki, H., and Yamaki, S. (1992) Nucleotide sequence of a cDNA encoding NADP-sorbitol-6-phosphate dehydrogenase from apple. *Plant Physiol.*, **100**, 1607–1608, doi:10.1104/pp.100.3.1607.
- Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O. P. et al. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA*, **100**, 1990–1995, doi:10.1073/pnas.0337704100.
- Koonin, E. V. and Tatusov, R. L. (1994) Computer analysis of bacterial haloacid dehalogenases defines a large superfamily of hydrolases with diverse specificity. *J. Mol. Biol.*, **244**, 125–132, doi:10.1006/jmbi.1994.1711.
- Kuznetsova, E., Proudfoot, M., Gonzalez, C. F., Brown, G., Omelchenko, M. V. et al. (2006) Genome-wide analysis of substrate specificities of the *Escherichia coli* haloacid dehalogenase-like phosphatase family. *J. Biol. Chem.*, **281**, 36149–36161, doi:10.1074/jbc.M605449200.
- Kuznetsova, E., Nocek, B., Brown, G., Makarova, K. S., Flick, R. et al. (2015) Functional diversity of haloacid dehalogenase superfamily phosphatases from *Saccharomyces cerevisiae*: biochemical, structural, and evolutionary insights. *J. Biol. Chem.*, **290**, 18678–18698, doi:10.1074/jbc.M115.657916.
- Loescher, W. H. (1987) Physiology and metabolism of sugar alcohols in higher plants. *Physiol. Plant.*, **70**, 553–557, doi:10.1111/j.1399-3054.1987.tb02857.x.

- Lunn, J. E. (2002) Evolution of sucrose synthesis. *Plant Physiol.*, **128**, 1490–1500, doi:10.1104/pp.010898.
- Maeda, K., Narikawa, R., and Ikeuchi, M. (2014) CugP is a novel ubiquitous non-GalU-type bacterial UDP-glucose pyrophosphorylase found in cyanobacteria. *J. Bacteriol.*, **196**, 2348–2354, doi:10.1128/JB.01591-14.
- Monedero, V., Pérez-Martínez, G., and Yebra, M. J. (2010) Perspectives of engineering lactic acid bacteria for biotechnological polyol production. *Appl. Microbiol. Biotechnol.*, **86**, 1003–1015, doi:10.1007/s00253-010-2494-6.
- Randez-Gil, F., Blasco, A., Prieto, J. A., and Sanz, P. (1995) DOG^{R1} and DOG^{R2}: Two genes from *Saccharomyces cerevisiae* that confer 2-deoxyglucose resistance when overexpressed. *Yeast*, **11**, 1233–1240, doi:10.1002/yea.320111303.
- Sanz, P., Randez-Gil, F., and Prieto, J. A. (1994) Molecular characterization of a gene that confers 2-deoxyglucose resistance in yeast. *Yeast*, **10**, 1195–1202, doi:10.1002/yea.320100907.
- Shen, B., Hohmann, S., Jensen, R. G., and Bohnert, H. J. (1999) Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. *Plant Physiol.*, **121**, 45–52, doi:10.1104/pp.121.1.45.
- Zhou, R., Cheng, L., and Wayne, R. (2003) Purification and characterization of sorbitol-6-phosphate phosphatase from apple leaves. *Plant Sci.*, **165**, 227–232, doi:10.1016/S0168-9452(03)00166-3.