**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 Ikeuchi¹,²,*

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

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

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 (kcat/Km) 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.

**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 encoded by the *s6pdh* gene in apple (Kanayama et al., 1992), and NAD-dependent S6PDH (EC 1.1.1.140) is encoded by *srlD2* 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 *srlD2* 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, phosphonatase, 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.
Detection of active sorbitol-6-phosphate phosphatase in the haloacid dehalogenase-like hydrolase superfamily

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 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 sp. PCC 6803, which was reported to be a sucrose-phosphatase (Lunn, 2002).

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 (μmol·min⁻¹·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).

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.
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 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.

**Fig. 3.** Lineweaver-Burk plots of phosphatase activity of HADs for sorbitol-6-phosphate.

**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 \times 10^4 | 2-Deoxyglucose-6-P | 0.61 | 33 | 5.4 \times 10^4 |
| HAD2 (YfbT) | 3.9 | 63 | 1.6 \times 10^4 | Glucose-6-P | 1.8 | 13 | 7.1 \times 10^3 |
| HAD4 (YihX) | --- | ND | --- | Glucose-1-P | 0.24 | 1.4 | 5.9 \times 10^3 |
| HAD6 (YqeB) | --- | ND | --- | Fructose-1-P | 1.7 | 20 | 2.0 \times 10^4 |
| HAD12 (YbiV) | 2.3 | 12 | 5.3 \times 10^3 | Fructose-1-P | 1.4 | 111 | 8.0 \times 10^4 |
| HAD13 (YidA) | 4.5 | 14 | 3.2 \times 10^3 | Erythrose-4-P | 0.019 | 19 | 1.0 \times 10^6 |
| Slr0953 | --- | ND | --- | Sucrose-6-P | 0.0075 | 21 | 2.8 \times 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.
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 μmol-min⁻¹·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 $p$NPP 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 $sdpd$-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 HAD12 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 (YbtT), HAD12 (YbiV), and HAD13 (YidA). The sorbitol-6-phosphate activity was also detected for yeast HADs, but the best $k_{cat}/K_m$ value was exhibited by YNL010W and was only $2.7 \times 10^3$ (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 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.jbt.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)8521-2.

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.M605494200.

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, 535–557, 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/jea.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.