論文の要旨
（Thesis Summary）

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Enzyme engineering of phosphite dehydrogenase and its applications for biotechnology

亜リン酸デヒドロゲナーゼの酵素工学的改変とバイオテクノロジーへの応用

Introduction

Phosphorus (P) is an indispensable nutrient for all living organisms. It is assimilated into essential cellular constituents such as nucleic acids, lipids, cofactors, and various metabolites. Basically, almost all biologically available P sources are in the form of phosphate (H_3PO_4, Pi) and its esters, in which the P valence is +5. Nonetheless, a limited number of bacteria can metabolize other reduced P compounds such as phosphite (H_3PO_3, Pt: P valence +3), and less commonly, hypophosphite (H_3PO_2, HPt: P valence +1), via oxidizing to Pi prior to P metabolism. The metabolic pathway of selective uptake and oxidation of Pt and HPt was established using the soil bacterium *Pseudomonas stutzeri* WM88, which possesses *htx* and *ptx* operons for utilizing HPt and Pt, respectively. HPt is taken up via the HtxBCDE transporter, which is then oxidized to Pt by HPt dioxygenase (HtxA). Phosphite dehydrogenase (PtxD) is an NAD-dependent oxidoreductase, which catalyzes the oxidation of phosphite (Pt) to phosphate (Pi) with concomitant reduction of NAD. In addition to its unique enzymatic reaction, considering the scarcity of Pt-metabolizable organism and Pt in the environment, PtxD enzyme offers several interesting biotechnology applications: i) a cost-effective NADH regeneration system due to the low cost of Pt and the strong thermodynamic driving force for Pt-oxidation, ii) contamination management that conferring host cells a competitive growth advantage on Pt-containing growth conditions, and iii) engineering of P metabolic pathway for a biological containment measure. However, the currently available PtxD enzymes are NAD-dependent, making these applications unfeasible or less effective.

With these in mind, this study aimed to expand the biotechnological applications of PtxD isolated from *Ralstonia* sp. (RsPtxD) by: I) deploying the Pt-dependent biocontainment strategy to the model cyanobacterium *Synechococcus elongatus* PCC 7942 (Syn 7942) by expressing RsPtxD and HtxBCDE proteins and evaluated its effect and II) engineering the cofactor specificity of RsPtxD toward NADP for developing a highly effective and universal NAD(P)H regeneration system.

Articles

I. Biological containment application of RsPtxD for Cyanobacteria (Supplement 1)

Cyanobacteria have recently become attractive bioproduction hosts due to their ability to convert CO_2 into useful compounds. Recent advances in genetic and metabolic engineering have further expanded their potential applications such as agriculture, medical uses, and environmental technology. However, concerns regarding biosafety issues and the risk of contamination in outdoor culture conditions remain to be resolved. To overcome such limitations, we deployed the biocontainment strategy based on Pt dependency that was initially established in a heterotrophic bacterium *Escherichia coli*. Exogenous expression of RsPtxD and hypophosphite transporter (HtxBCDE) gene that exclusively transports Pt, not Pi confer the ability of Syn 7942 to metabolize Pt as a sole P source. After that, identifying and disrupting the indigenous Pi transporters, *pstSCAB* (Synpcc7942_2441 to 2445) and *pit* (Synpcc7942_0184) resulted in strain failed to grow on any media containing various types of Pi compounds other than Pt. The Pt-dependent strain did not yield any escape mutants under non-permissive growth conditions for at least 28 days with an assay

detection limit of 3.6×10^{-11} per colony forming unit, and rapidly lost viability in the absence of Pt. Furthermore, the growth of Pt-dependent strain could easily outcompete wild-type cyanobacteria on Pt medium, which revealed that this strain could dominate in cultures containing Pt as the sole P source. Thus, we could successfully apply this strategy to photosynthetic cyanobacteria. However, the contained cyanobacteria strain showed reduced cell growth (30% reduction in cell growth compared to the wild-type strain in Pi medium), possibly due to intracellular NADH/NAD redox imbalances caused by Pt oxidation because wild-type RsPtxD strictly use the less abundant NAD(H) than NADP(H) in cyanobacteria. To maintain optimal balance and sustain robust growth of Pt-dependent strains, engineering the cofactor specificity of RsPtxD toward NADP was considered as one of the solutions.

II. Engineering cofactor specificity of RsPtxD toward NADP (Supplement 2)

Engineering cofactor specificity of RsPtxD is also useful for *in vitro* applications. NADP-dependent enzymes have been extensively used as catalysts for the chemical, pharmaceutical, and energy industries. However, their dependence on the costly cofactor, NAD(P)H remains a challenge that must be addressed. To overcome such limitations, cofactors are regenerated using cheaply available sacrificial substrates.

In this study, we engineered the cofactor specificity of RsPtxD for an effective NADPH regeneration system. Replacement of the five amino acid residues, Cys174–Pro178, located at the C-terminus of the ß7-strand in the Rossman-fold domain of RsPtxD, by site-directed mutagenesis, resulting in four mutants with replaced amino acids, $RsPtxD_{AR}$ (D175A/P176R), $RsPtxD_{HAR}$ (C174H/D175A/P176R), $RsPtxD_{HARKA}$ (C174H/D175A/P176R/I177K/P178A), and $RsPtxD_{HARRA}$ (C174H/D175A/P176R/I177R/P178A). The kinetic parameters of all mutants revealed that NADP preference has been increased significantly with the highest catalytic efficiency for NADP $(K_{cat}/K_M)^{NADP}$ was 44.1 mM^{-1} min^{-1} attributed with the lowered K_M^{NADP} 2.9 ± 0.5 of $RsPtxD_{HARRA}$, which was superior compared with the previously reported phosphite dehydrogenases. Moreover, preincubation of $RsPtxD_{HARRA}$ mutant in the presence of 0.5 mM NADP exhibited protection from thermal inactivation at 45°C for up to 6 h and high tolerance to organic solvents. We also demonstrated the effect of $RsPtxD_{HARRA}$ as an NADPH regeneration system in the coupled reaction of chiral conversion of 3-dehydroshikimate (3-DHS) to shikimic acid (SA) by the thermophilic shikimate dehydrogenase of *Thermus thermophilus* HB8 at 45°C. The wild-type RsPtxD could not support SA production at 45°C, suggesting that it did not regenerate NADPH. Whereas $RsPtxD_{HARRA}$ supported almost the full conversion of 3-DHS to SA for 90 min with a total turnover number (TTN) of 78, suggesting that it regenerated NADPH without losing its activity at 45°C. Considering these properties of the RsPtxD mutants, the higher affinity toward NADP makes them especially promising not only for *in vitro* NADPH regeneration but also for *in vivo*, such as heterologous expression of RsPtxD mutants in cyanobacteria and using NAD(P) cofactors might solve the challenge abovementioned.