Fermentative production of ophthalmic acid

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Research aims

Escherichia coli YggS is a highly conserved pyridoxal phosphate (PLP)-binding protein whose biochemical function is unknown. We found that a *yggS*-deficient *E. coli* strain (Δ*yggS*) accumulates an unusual metabolite, ophthalmic acid (γ-L-glutamyl-L-2-aminobutyryl-glycine, Fig. 1)[1)]. This Δ*yggS* mutant is the first microorganism that accumulates high levels of ophthalmic acid. Ophthalmic acid is known to be an oxidative stress biomarker and a potent agonist of a calcium-sensing receptor that elicits the *kokumi* taste. Although ophthalmic acid has potential uses in research and industrial applications, it is currently very expensive, and no fermentative production method has been reported. In the present study, we tried to develop the first known fermentative method for the production of ophthalmic acid.

Methods

Intracellular ophthalmic acid concentrations for Δ*yggS* strains overexpressing threonine dehydratase (IlvA), alanine-valine transaminase (avtA), γ-glutamyltransferase (GshA), or glutathione synthase (GshB) were determined. We constructed a γ-glutamyltransferase (Ggt)-deficient strain, Δ*yggS*/*ggt*, using the Δ*yggS* strain.

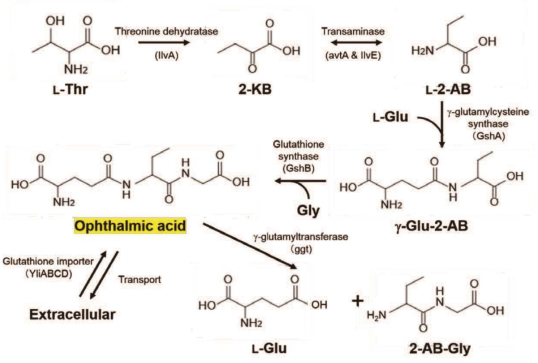

Fig. 1 Putative metabolic pathway of ophthalmic acid

A mutant strain (Δ*yggS*/*ggt*/*yliA-B*) lacking a glutathione importer (Δ*yliA-B*) was then generated, using the Δ*yggS*/*ggt* strain. Plasmids that express *gshA* and *gshB*, and that express mutated *gshA* and *gshB*, were constructed and then introduced into the Δ*yggS*/*ggt* strain. These *E. coli* cells were cultivated in a synthetic M9-glucose medium, in the presence or absence of amino acids (0–1.0 mM of L-Glu, L-2-AB, and Gly). Total amounts of ophthalmic acid were quantified by HPLC.

Results

Ophthalmic acid is probably synthesized via sequential reactions catalyzed by GshA and GshB in *E. coli*. IlvA and AvtA are expected to participate in L-2-aminobutyrate (L-2-AB) biosynthesis (Fig. 1). IlvA catalyzes the dehydration of L-Thr to generate 2-ketobutyrate (2-KB) and ammonia. AvtA converts 2-KB to L-2-AB, and vice versa. Overexpression of these enzymes is potentially capable of elevating intracellular ophthalmic acid levels. As expected, expression of plasmid-borne *gshA* and *gshB* in the Δ*yggS* strain further increased (~20-fold) intracellular ophthalmic acid levels; however, the expression of plasmid-borne *ilvA* and *avtA* did not.

In the Δ*yggS* strain, ophthalmic acid levels increased during cultivation, and reached maximal concentrations when the culture reached the early stationary phase. Ophthalmic acid levels then dropped to below the detection limit during the late stationary phase (Fig. 2), indicating the presence of some ophthalmic acid-metabolizing enzyme(s). We speculated that Ggt is the primary enzyme responsible for the degradation of ophthalmic acid. Therefore, we constructed a double-mutant of *yggS* and *ggt* (Δ*yggS*/*ggt*) and analyzed its cultivation time-dependent production of ophthalmic acid. As shown in Fig. 2, the *ggt* mutation completely suppressed the cultivation time-dependent decreases in ophthalmic acid levels.

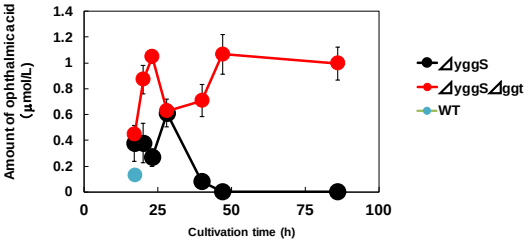

Fig. 2 Effect of *ggt* mutation on the cultivation time-dependent ophthalmic acid accumulation

In *E. coli*, a directed fermentation method for producing glutathione was reported that used an *E. coli* strain overexpressing *gshA* and *gshB*, and lacking *ggt* and glutathione importers (*yliA-B*). Therefore, we constructed a triple-mutant lacking *yggS*, *ggt*, and

yliA-B. However, no significant increases in ophthalmic acid productivity were observed for the strain containing the glutathione importer mutation (data not shown).

We then constructed a *gshA* and *gshB* co-expression vector. In addition, to confer resistance to product inhibition, a point mutation was introduced into *gshA*, and a plasmids that express the mutated *gshA* (*gshA*[S495F]) and *gshB* were constructed. These plasmids were introduced into Δ*yggS*/*ggt*, and this transfected strain was cultivated in M9 medium in the presence or absence of amino acids (0–1.0 mM of L-Glu, L-2-AB, and Gly). In the M9 medium (without amino acids), co-expression of *gshA* and *gshB* slightly increased ophthalmic acid productivity, but supplementation with amino acids significantly increased ophthalmic acid productivity. In the presence of 1 mM of L-Glu, L-2-AB, and Gly, the *E. coli* Δ*yggS*/*ggt* strain overexpressing *gshA* and *gshB* produced 114 μmol/L of ophthalmic acid. In the same conditions, the *E. coli* strain overexpressing *gshA*(S495F) and *gshB* produced 186 μmol/L of ophthalmic acid (Fig. 3).

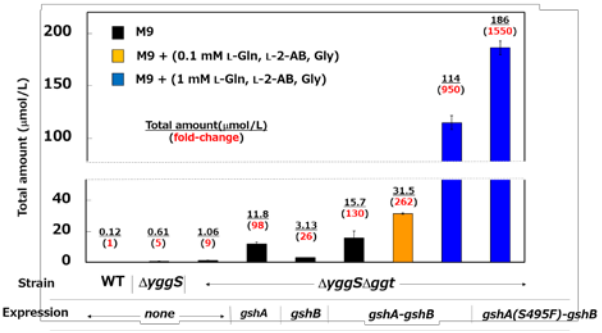

Fig. 3 Effect of *ggt* mutation and *gshA*/*gshB* overexpression on the ophthalmic acid production

Conclusion

In this study, we developed the first known fermentative method for producing ophthalmic acid, using with a *yggS*-deficient *E. coli* strain. We found that overexpression of *gshA* and *gshB* was effective in increasing ophthalmic acid production. The *ggt* mutation completely suppressed the cultivation time-dependent decrease of ophthalmic acid, demonstrating that Ggt is the primary enzyme responsible for the degradation of ophthalmic acid. We also found that the *E. coli* Δ*yggS*/*ggt* strain overexpressing *gshA*(S495F) and *gshB* produced ~50 mg/L of ophthalmic acid in an M9 medium that was supplemented with 1 mM of L-Glu, L-2-AB, and Gly. Our results reveal the method for further fermentative production of ophthalmic acid.

References

1) Ito T., Yamauchi A., Hemmi H., Yoshimura T. (2016) Ophthalmic acid accumulation in an *Escherichia coli* mutant lacking the conserved pyridoxal 5′-phosphate-binding protein YggS. *Journal of Bioscience and Bioengineering*. In press.