Engineering the shikimate pathway for biosynthesis of molecules with pharmaceutical activities in E. coli

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Abstract

Engineering the shikimate pathway is a primary approach for biosynthesis of various aromatic compounds, many of which are involved in formation of important compounds with pharmaceutical values. The development of metabolic engineering allows for high-efficiency production of desired molecules derived from the shikimate pathway using engineered microbes as biosynthetic factories. This review summarizes successful and generally applicable strategies for engineering this important pathway in the context of the model bacterium *E. coli* for biosynthesis of molecules with pharmaceutical activities. Similar approaches can also be employed for shikimate pathway engineering in other microorganisms.

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Introduction

The shikimate pathway is a ubiquitously existing pathway in plants, algae, fungi, and bacteria. It is composed of seven enzymatic reactions that combine metabolites phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) to make chorismate (CHR). An essential role of the shikimate pathway for cellular metabolism is to provide precursors for the biosynthesis of aromatic amino acids, including phenylalanine, tyrosine and tryptophan. In plants, the shikimate pathway is also the material source for producing secondary metabolites. The intermediates of the shikimate pathway are also involved in other metabolic pathways such

as petrobactin and quinate biosynthesis [1,2]. Through decades of efforts by biologists, the enzymatic reactions involved in the shikimate pathway have been well elucidated and characterized [3]. Recently, this pathway has attracted increasing research interest from metabolic engineers, as the intermediates and derivatives of the pathway can be used for biosynthesis of a wide range of compounds with various biological activities or chemical properties. Importantly, the dedicated research efforts are powered by the advancement in metabolic engineering that offers sophisticated tools for pathway engineering for making desired molecules with high titer, yield and productivity. This review highlights recent progress of production of bioactive molecules with various pharmaceutical activities by engineering the shikimate pathway in *E. coli*.

How the shikimate pathway works

In *E. coli*, the shikimate pathway starts from the condensation of C3 molecule phosphoenolpyruvate (PEP) and C4 molecule D-erythrose 4-phosphate (E4P) and ends with the formation of chorismate through a series of enzymatic conversions. The schematic of the shikimate pathway in *E. coli* is illustrated in Figure 1. Notably, the conversion from DHS to SHK requires consumption of NADPH or NADH, whereas S3P formation from SHK needs ATP.

Like many other organisms, *E. coli* has a sophisticated regulation system for controlling the flux through the shikimate pathway [4]. For example, TyrR, a DNA-binding transcriptional regulatory protein, negatively regulates the expression of genes *aroF, aroG, aroH* and *aroL* primarily through the use of aromatic amino acids as cofactors [5]. AroG expression has also been found to be regulated by CpxA/CpxR transcriptional regulator [6]. In addition, global regulators, such as leucine-responsive regulatory protein and CRP-cAMP DNA-binding transcriptional dual regulator, are involved in controlling the metabolic flux through the shikimate pathway [7,8]. It has also been found that there is dynamic equilibrium between intermediates of the shikimate pathway, which comprises a challenge for full conversion of intermediates to desired products [9].

Improve metabolic flux through the shikimate pathway

One primary challenge for engineering the shikimate pathway is to improve the availability of the two pathway precursors PEP and E4P. PEP is an intermediate in glycolysis and is also involved in glucose cross-membrane transportation as a phosphoryl group donor. As such, when glucose is used as the carbon source, the metabolic flux through the shikimate pathway is often limited due to the PEP consumption for glucose uptake through the phosphotransferase system (PTS). To this end, utilization of other glucose transport systems or modulation of the PTS system expression have been explored and found to increase the biosynthetic efficiency of the shikimate pathway [10,11]. The other precursor E4P is derived from the pentose phosphate pathway whose metabolic flux varies depending on the growth condition and genetic modifications [12]. Overexpression of the *ppsA* and *tktA* genes is a common strategy to improve the intracellular pools of PEP and E4P, respectively. In addition, balancing the availability of PEP and E4P is also the key to attracting metabolic flux into the shikimate pathway (it should be noted that in addition to the condensation reaction, PEP is also needed in a latter step of the shikimate pathway). There have been excellent studies that investigated the flux distribution between the central metabolism and the shikimate pathway in *E. coli* and highlighted the theoretical limit for using the shikimate pathway for biochemical production [13-15]. Notably, in order to bypass the issue of limited PEP availability, efforts have also been made to utilize pyruvate, rather than PEP, to make the shikimate pathway intermediate DAHP [16]. In addition, glycerol has been found to be an alternative substrate to glucose for supporting the biosynthesis of value-added compounds through the shikimate pathway [17,18].

The complex regulation system of the shikimate pathway is another challenge that needs to be overcome. To this end, recent development of protein engineering and metabolic engineering offers new tools for manipulating the shikimate pathway to attracting more metabolic flux. For example, the biosynthesis of DAHP, the first committed step of the pathway, is one of the primary limiting reactions for the whole pathway. The feedback resistance (fbr) enzymes of AroF, AroG and AroH for this reaction have all been identified, which allowed for significantly higher pathway efficiency by removal of the regulation on this step [19-22]. Pathway regulator TyrR can also be removed to increase the expression of related pathway enzymes [23]. Furthermore, Santos *et al.* identified and utilized global RNA polymerase mutants to engineer the shikimate pathway for tyrosine overproduction [24]. A carbon storage regulator

system (Csr) was also employed to increase the availability of PEP in *E. coli* [25]. The achievements of these studies offer viable options for improving the metabolic flux through the shikimate pathway to produce a variety of different biochemicals, as detailed in the following discussion.

Engineering the shikimate pathway to facilitate the production of molecules with pharmaceutical activities in *E. coli*

There have been extensive research harnessing the power of the shikimate pathway to produce various biochemicals in the context of various organisms. For example, tremendous progress has been made for high-efficiency aromatic amino acid production in *E. coli* [26-28•]. This review focuses on the achievements for heterologous biosynthesis in *E. coli* of a select group of biomolecules with pharmaceutical activities and highlights the strategies for improving the metabolic flux of the shikimate pathway to support the biosynthesis of the target molecules. It should be noted that over-engineering of the shikimate pathway can be detrimental to the biosynthesis performance, as it would consume valuable intracellular resources and impose unnecessary metabolic stress on the host cell (see examples below). Therefore, balancing the metabolic support for the shikimate pathway and the downstream heterologous biosynthetic pathways is of great importance.

Salicylic acid

Salicylic acid (SA) is the active component of aspirin that has been widely used to treat aches, pain, fever and inflammation and to reduce blood clots with long-term use [29]. It has also been used as a required precursor for biosynthesis of natural products such as yersiniabactin in *E. coli* [30]. Heterologous SA production in *E. coli* can be achieved through engineering the shikimate pathway to supply the needed precursor chorismate and isochorismate. Starting from a phenylalanine *E. coli* overproducer, Lin *et al.* utilized a medium copy number plasmid to overexpress genes *ppsA, tktA, aroL* and $aroG^{fbr}$, to improve the metabolic flux towards SA biosynthesis and achieved a SA production of 1.2 g/L [31•]. Interestingly, expression of these genes using a high copy number plasmid reduced the production performance, and deletion of the genes related to the shikimate pathway resulted in impaired growth and lowered production performance. These findings highlight the importance of avoiding over-engineering of the

shikimate pathway. A more recent finding further reported an 11.5 g/L SA production by engineering the shikimate pathway [32▪▪]. Specifically, the removal of PEP-consuming PTS and deletion of genes responsible for PEP to pyruvate conversion were found to have a significant impact on SA precursor supply and thus final production. Based on the same engineering strategies, several other important biochemicals' production was also achieved at gram per liter scale in the same research.

Alkaloid

Alkaloids, mainly sourced from plants, are a large family of natural products with valuable pharmacological activities such as anticancer, antimalarial, antiasthma, analgesic, and many other activities [33,34]. Due to the low efficiency of plant extraction and challenges associated with complex total chemical synthesis, microbial production of alkaloids has been found to be of great research interest. Recently, the heterologous production of benzylisoquinoline alkaloids in *E. coli* has been reported based on engineered tyrosine supply in *E. coli* [35▪▪]. In this report, the metabolite flux to the shikimate pathway was increased through the disruption of *tyrR* and overexpression of *ppsA, tktA, aroG*^{fbr}, and *tyrA*^{fbr}, which offered sufficient tyrosine precursor supply for the successful biosynthesis of (S)-reticuline from glucose. Interestingly, (S)-reticuline production was improved by switching to glycerol as the carbon source, showing a good agreement with the previous findings that glycerol is a good carbon source for the shikimate pathway [17,18]. Tetrahydropapaveroline (THP), another benzylisoquinoline alkaloid, was also produced by a stepwise biosynthesis approach. Specifically, an *E. coli* strain with the engineered shikimate pathway (over-expression of *ppsA*, *tktA*, *tyrA*^{fbr}, *aroG*^{fbr}) was solely dedicated to making the THP precursor dopamine from tyrosine; whereas, a separate strain was responsible for the conversion of dopamine to THP. This modular design was found to improve the productivity of the whole biosynthetic process [36].

Flavonoid

Flavonoids are another sizable family of secondary metabolites with numerous important bioactivities such as anticancer, anti-inflammatory and neuroprotection activity [37,38]. Using a strategy combining the *ppsA*, *tktA* and *aroG*^{fbr} overexpression and *tyrR* deletion, Kim *et al.*

produced two flavonoids (sakuranetin and ponciretin) in *E. coli* by increasing the intracellular tyrosine availability for the biosynthetic pathways [39]. The combination of shikimate pathway engineering and flavonoid biosynthetic enzyme functional expression resulted in a final production of 40 mg/L. Wu *et al.* constructed an eight-step pathway to produce (2S)-pinocembrin directly from glucose via phenylalanine [40••]. The biosynthesis was achieved by splitting the pathway into four modules including one module for *aroF* over-expression to increase the flux through the shikimate pathway. Importantly, it was found that the expression levels of the shikimate pathway module needed to be adjusted and balanced together with the other modules to optimize production.

Naringenin, another flavonoid, has also been successfully produced in *E. coli* [41]. In particular, the shikimate pathway was engineered to produce tyrosine through rational pathway modification and global transcriptional machinery engineering [24]; the resulting tyrosine overproducers were then employed to incorporate the downstream naringenin biosynthesis pathway to achieve de novo production from glucose.

Coumarins

Coumarins and their derivatives, like many other phenylproanoids, are derived from the shikimate pathway and possess various pharmaceutical properties [42,43]. Lin *et al.* reported the heterologous production of two simple coumarins, umbelliferone and scopoletin, using tyrosine as a precursor [44]. In order to maximize production, additional copies of *ppsA, tktA, aroG*^{fbr}, and *tyrA*^{fbr} genes were introduced to the recombinant strain, which improved the shikimate pathway flux and thus the production of scopoletin. In comparison, implementation of the same strategy actually decreased the production of umbelliferone. These findings clearly demonstrate that the optimal strategy for engineering the shikimate pathway in support of bioactive molecules' biosynthesis is case-dependent, and that invariably increasing the expression of the shikimate pathway is not an appropriate engineering approach.

The biosynthesis of 4-hydroxycoumarin has also been achieved by assembly of an artificial pathway involving salicylate as an intermediate [45]. Similar to the strategies highlighted above, *aroL, ppsA, tktA,* and *aroG*^{fbr} were overexpressed in *E. coli* to eliminate the biosynthesis bottle neck of salicylate supply. It was shown that fine-tuning expression level of

these shikimate pathway genes was important for production optimization, as utilization of a high copy number plasmid for expression decreased the production.

Violacein and deoxyviolacein

Rodrigues *et al.* recently reported biosynthesis of antibiotic and anti-tumor agents violacein and deoxyviolacein in engineered *E. coli* [46••]. The biosynthetic route used the shikimate pathway for tryptophan formation and a series of heterologous reactions to convert tryptophan to the final products. As chorismate supply was found limiting the production, the shikimate pathway was engineered by increasing the expression of *aroF*, *aroB*, *aroL*, and *tktA* genes. Such strategy, together with engineering serine biosynthesis, resulted in a better supply of tryptophan and improved production to 710 mg/L of violacein in a fed-batch process. In addition, the same engineering approaches have also been applied to achieve deoxyviolacein production from glycerol [47].

Salvianic acid A

Salvianic acid A is a plant polyphenolic acid with confirmed pharmacological activities [48,49]. As salvianic acid A is derived from tyrosine, its heterologous biosynthesis in *E. coli* involves the shikimate pathway engineering. Yao *et al.* employed a modular engineering approach to specifically increase flux toward tyrosine through the shikimate pathway [50]. It was found that over-expression of $aroG^{fbr}$, $tyrE^{fbr}$, *aroE, ppsA, tktA,* and *glk* (encoding glucose kinase) improved the tyrosine production in *E. coli*. However, additional overexpression of other genes in the shikimate pathway as well as the tyrosine biosynthetic pathway decreased the performance of tyrosine biosynthesis. The best performer strain was then successfully used for provision of the needed intermediate for salvianic acid A and resulted in a production of 7.1 g/L. These findings demonstrated that effective engineering of the shikimate pathway for bioactive molecule production is dependent upon the choice of genes to overexpress. Engineering of unnecessary shikimate pathway genes would actually impact the overall biosynthesis performance negatively.

Conclusions

Despite the fact that functional reconstitution of the heterologous biosynthetic enzymes is essential, engineering the shikimate pathway is equally important for ensuring high-level

production of the target molecules in *E. coli*. In fact, the goal of engineering the shikimate pathway is more than merely providing required aromatic precursors. A well-engineered shikimate pathway requires consideration of several important factors. First, appropriate level of pathway enzyme expression should be identified to prevent unnecessary metabolic burden imposed by over-engineering of the pathway as well as insufficient supply of needed precursors for downstream biosynthesis. In particular, such expression strength optimization should be conducted in the context of complex bioactive molecule formation to balance the biosynthetic needs between endogenous and heterologous metabolism. Second, excessive deletion of competing pathways should be avoided. Even though it would increase the relative metabolic flux through the shikimate pathway, it could impair the growth of the *E. coli* host and thus decrease the production performance. Third, the selection of a suitable carbon source could also help improve the efficiency of the shikimate pathway. As such, the choice of appropriate engineering strategies is case-dependent and yet is the key to successful biosynthesis of a wide variety of biomolecules with pharmaceutical activities.

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Figure 1. Engineering the shikimate pathway for biosynthesis of a variety of molecules with pharmaceutical activities. PYR: pyruvate; G3P: D-glyceraldehyde 3-phosphate; F6P: β-D-fructofuranose 6-phosphate; X5P: D-xylulose 5-phosphate; RI5P: D-ribose 5-phosphate; S7P: D-sedoheptulose 7-phosphate; PEP: phosphoenolpyruvate; E4P: D-erythrose 4-phosphate; DAHP: 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ: 3-dehydroquinate; DHS: 3-dehydroshikimate; SHK: shikimate; S3P: shikimate 3-phosphate; EPSP: 5-enolpyruvylshikimate 3-phosphate; CHR: chorismate. Solid lines indicate a single enzymatic step; dotted lines indicate multiple enzymatic steps. Example pharmaceuticals and their functions for each category of the compounds are given in parentheses.