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Mutational analysis of the interaction between a potential inhibitor luteolin and enoyl-ACP reductase (FabI) from *Salmonella enterica*

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ABSTRACT

Salmonella enterica is the main cause of food-borne disease worldwide and the emergence of antibiotic-resistant *Salmonella* strains has become a major public health concern. To combat the resistant pathogens, screening of new antibacterials with novel targets or mechanisms of action is very urgent. Luteolin, a traditional Chinese medicine monomer, was proven to be an uncompetitive inhibitor of FabI, the sole enoyl-ACP reductase (ENR) from *S. enterica* (SeFabI), with the inhibition constant (K_i) of 15.1 ± 0.3 μM. Three missense mutations SeFabI[G93V], SeFabI[G93S], and SeFabI[Y156F] were designed to investigate the structure–activity relationship between the inhibitor and the SeFabI target. The specific activities and substrate affinities of SeFabI[G93V] and SeFabI[G93S] were similar to the wild-type SeFabI, while SeFabI[Y156F] lost the substrate catalytic activity, which was consistent with the mechanism for catalytic activity of FabI from *Escherichia coli* (EcFabI) described previously. SeFabI[G93V] mutation showed high level luteolin resistance, which was consistent with the studies in *E. coli* by triclosan. Interestingly, the SeFabI[G93S] showed both luteolin sensitivity and triclosan resistance, and the difference could be explained by the structure discrepancy between luteolin and triclosan. These data imply that the Gly-93 and Tyr-156 are key amino acid residues for luteolin in the active site of the target. As a phytochemical, it has been certified to be safe, thus luteolin would be able to develop as a lead compound for combating resistant bacteria.

1. Introduction

Salmonellosis, caused by the bacteria *Salmonella*, is one of the most common and widely distributed foodborne diseases. Since the beginning of the 1990s, *Salmonella* isolates which are resistant to a range of antimicrobials, including first-choice agents for the treatment of humans, have emerged and are threatening to become a serious public health problem. The resistances result from the use of antimicrobials both in human and animal husbandry. Multi-drug resistances to critically important antimicrobials are compounding the problems. To combat the antibiotic-resistant pathogens, the effective strategy is to identify new antibacterials that function through novel targets or new mechanisms of action [1,2].

Fatty acid synthesis (FAS) pathway is an essential process that supplies precursors for the assembly of important cellular components, including lipopolysaccharides, lipoproteins, phospholipids and the cell envelope. FAS pathways are divided into two distinct forms: FAS-I and FAS-II [3]. Eukaryotes synthesize fatty acid using a multifunctional enzyme complex (FAS-I) [4], while the FAS-II found in prokaryotes consists of individual enzymes that catalyze each step of fatty chain elongation. Subsequently, the obvious differences in the overall architectures between the FAS-I and FAS-II support the proposal that enzymes of FAS-II pathway are selectively targets for the development of novel antibacterials [5].

In particular, the enoyl-ACP reductase (ENR), which catalyzes the last reaction in each round of elongation circle, has become an important target. There are four isoforms, FabI [6], FabK [7], FabL [8] and FabV [9], of ENR among various prokaryotes. For most bacteria, FabI is the unique ENR and shares high overall structural homology, and its variability exists mainly in a mobile loop of amino acids close to the active site (the substrate binding loop) [10]. There are many exciting findings of artificially synthesized inhibitors [6,11–15], including a broad-spectrum antibacterial tri-

Abbreviations: CoA, coenzyme A; DMSO, dimethyl sulfoxide; EcFabI, FabI from *Escherichia coli*; ENR, enoyl-acyl carrier protein reductase; FAS, bacterial fatty acid synthesis; IC_{50}, concentration giving 50% inhibition of activity; IPTG, isopropyl-β-D-thiogalactoside; K_i, inhibition constant; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide reduced form; OE-PCR, overlap extension PCR; PCR, polymerase chain reaction; RMSD, root mean square deviation; SeFabI, FabI from *Salmonella enterica*.

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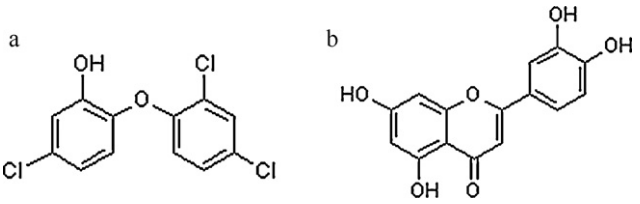

Fig. 1. The structure of (a) triclosan and (b) luteolin.

closan (Fig. 1a) [11,13], which is widely employed in many personal care products, i.e. deodorants, soaps, hand washes and toothpastes. It is a slow, tight-binding inhibitor of FabI, interacting specifically with the enzyme/NAD^+ product complex. However, triclosan is never been used for systemic therapeutic purposes due to its toxicity. Nowadays, numerous investigations have focused on the natural origin FabI inhibitors [16,17], which represent a source of relatively nontoxic. Meanwhile, several detailed kinetic studies coupled with high-resolution crystal structures have provided a solid foundation for the further development of new antibacterials.

In our previous research, the flavonoid luteolin (its structure is shown in Fig. 1b) was proven to be an uncompetitive inhibitor of FabI from *Escherichia coli* (EcFabI) by structure-based virtual screening and experiments [18]. In the present study, mutational analysis of the luteolin-binding region of FabI from *Salmonella enterica* (SeFabI) coupled with cofactor NAD^+ was addressed. The structure–activity studies will lay a solid foundation for further improvement the inhibition activity by structure modification of the phytochemical.

2. Materials and methods

2.1. Materials

Triclosan, crotonyl-CoA, NADH, isopropyl-β-D-thiogalactoside (IPTG) and kanamycin were bought from Sigma–Aldrich. Luteolin with purity 98% up by HPLC method was purchased from Shanghai Tauto Biotech Co., Ltd. (http://www.tautobiotech.com/en/Products_04.htm). His-bind Ni^{2+}-NTA resin was obtained from Invitrogen, while other molecular biology reagents were provided by Takara Biotechnology Co. The purity of all other chemicals was analytical grade.

2.2. Bacterial strains, plasmids and primers

Bacterial strains, plasmids and primers used in this study are listed in Table S1.

2.3. Cloning, expression and purification of SeFabI

The *fabI* gene (GeneID: 6950964) from the *S. enterica* MGSC B090004 was amplified by PCR using the primer pair FabI(F) and FabI(R) (Table S1). The PCR product was digested with *Nde* I and *Hin*d III and cloned into pET-28b(+) at the same restriction sites, so that a His tag was encoded at N-terminus of the coding sequence. The construction sequence was confirmed by DNA sequencing, and the recombinant plasmid pET-*fabI* was then transformed into *E. coli* strain BL21(DE3) competent cells to obtain the SeFabI expression strain BESeI. A single colony of BESeI was used to inoculate into 5 mL of Luria-Bertani (LB) medium containing 30 μg/mL kanamycin, and the culture was grown at 37 °C overnight with shaking. The overnight culture was then inoculated into 500 mL of LB medium containing kanamycin (30 μg/mL) and continuously incubated at 37 °C with vigorous shaking. When the optical density at 600 nm (OD_{600}) reached approximately 1.0, IPTG was added at the final concentration of 1 mM to induce the expression of SeFabI. Subsequently, the culture was shaken at 25 °C for another 16 h. The cells were harvested and resuspended in 30 mL of His binding buffer [20 mM Tris–HCl, 500 mM NaCl, and 10 mM imidazole (pH 7.9)] and lysed by sonication. The cell lysate was removed by centrifugation at 12,000 × *g* for 60 min, and the supernatant was loaded onto a His binding column containing 4 mL of Ni^{2+}-NTA resin. The column was washed with 40 mL of binding buffer, followed by 30 mL of wash buffer [20 mM Tris–HCl, 500 mM NaCl, and 20 mM imidazole (pH 7.9)], and SeFabI was finally eluted by 30 mL of elute buffer [20 mM Tris–HCl, 500 mM NaCl, and 250 mM imidazole (pH 7.9)]. Fractions containing SeFabI were collected, and imidazole was removed by dialysis against 20 mM Tris–HCl (pH 7.5), containing 10% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.002% Triton X-100. The purity of the enzyme was checked by 12% SDS-PAGE, which gave an apparent molecular mass of ~28 kDa. Protein concentration was assayed by the dye-binding method (Bradford assay) with bovine serum albumin as the standard [19,20].

2.4. PCR-based site-directed mutagenesis

The SeFabI[G93V], SeFabI[G93S], and SeFabI[Y156F] site-directed mutageneses were designed through OE-PCR with the pET-*fabI* as the DNA template [21]. The procedure of the OE-PCR was as follows: (1) in separate PCR reactions, two fragments of *fabI* were amplified by using, for each reaction, one universal [FabI(F) or FabI(R)] and one mutagenic primer [G93V(F) or G93V(R)]. The amplified products were then purified by a gel extraction kit. (2) The resulting DNA fragments were mixed to obtain the full-length reassembled DNA with the mutation site by OE-PCR without primers. (3) The entire DNA was synthesized by PCR with outermost primers and template DNA from step (2). After that, the *fabI* mutations were inserted into pET-28b(+). The sequence of each mutant plasmid was confirmed by DNA sequencing, and the expression and purification of each SeFabI mutant followed the same protocol that was described above for the wild-type SeFabI protein.

2.5. Enzyme assays and kinetic analysis of wild-type and mutant SeFabIs

Various FabI activities were determined by monitoring the oxidation of NADH to NAD^+, which was monitored at 340 nm (ε^{M}_{340} = 6220 M^{-1} cm^{-1}) for 10 min at 30 °C [22]. The standard reaction mixture contained 200 μM crotonyl-CoA, 100 μM NADH, 5 nM enzyme, and luteolin or triclosan in 20 mM Tris–HCl, 150 mM NaCl buffer (pH 7.5) in a total volume of 100 μL. The reaction was initiated by adding the substrate crotonyl-CoA. All the inhibitor compounds were dissolved in DMSO. The concentration of DMSO in all assays was maintained at 5%, which did not significantly affect SeFabI activity according to the control reaction mixture.

The kinetic parameters, K_m (Michaelis constant) and k_{cat} (catalytic constant) of the mutant enzymes for crotonyl-CoA and NADH, were measured for comparison of the specificity constants, k_{cat}/K_m, with that of wild-type SeFabI. The reactions were carried out by changing the concentrations of crotonyl-CoA (50–400 μM) at several fixed concentrations of NADH (50, 100 and 250 μM) or by varying the NADH concentrations (25–250 μM) at several fixed concentrations of crotonyl-CoA (50, 100 and 200 μM). Kinetic parameters were calculated by fitting the data to the Michaelis-Menten equation (Eq. (1)):

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

k_{cat} values were obtained by using the relationship between k_{cat} and V_{max} ($V_{max} = k_{cat}[E]$) [23].