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ORIGINAL RESEARCH PAPER

Characterization of a tryptophan 6-halogenase from *Streptomyces toxytricini*

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Abstract Tryptophan (Trp) halogenases are found in various bacteria and play an important role in natural product biosynthesis. Analysis of the genome of *Streptomyces toxytricini* NRRL 15443 revealed an ORF, *stth,* encoding a putative Trp halogenase within a non-ribosomal peptide synthetase gene cluster. This gene was cloned into pET28a and functionally over-expressed in *Escherichia coli*. The enzyme halogenated both L- and D-Trp to yield the corresponding 6-chlorinated derivatives. The optimum activity was at 40°C and pH 6 giving k_{cat}/K_M value of STTH of 72,000 min^{-1} M^{-1}. The enzyme also used bromide to yield 6-bromo-Trp.

Keywords Brominase · Chlorinase · *Streptomyces toxytricini* · Tryptophan · Tryptophan 6-halogenase

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Introduction

More than 4,000 halogenated natural products have been reported, many of which possess significant biological activities, such as chloramphenicol (antibacterial), vancomycin (antibacterial) and rebeccamycin (anticancer). Flavin-dependent halogenases are a group of enzymes frequently involved in natural product biosynthesis. The most widely reported are tryptophan (Trp) halogenases that specifically halogenate Trp at particular positions to yield corresponding halogenated products, which are then used to synthesize halogen-containing natural products. Recently, the Trp 5- and 7-halogenases from soil bacteria have been successfully engineered into the medicinal plant *Catharanthus roseus* to generate novel halogenated plant alkaloids (Runguphan et al. 2010). This suggested that Trp halogenases are a useful tool in engineered biosynthesis of halogenated molecules.

Streptomyces toxytricini NRRL 15443 is an industrially important actinobacterium that is well known for its production of lipstatin, a potent pancreatic lipase inhibitor and direct precursor of the anti-obesity drug orlistat. Our group has recently sequenced the genome of this strain. Bioinformatic analysis of the genome sequencing results revealed a putative flavin-dependent halogenase gene (*stth*) and an adjacent flavin reductase gene (*stfre*). In this paper, we report functional characterization and enzymatic studies of this halogenase.

Materials and methods

Strains, media and culture conditions

Streptomyces toxytricini NRRL 15443 was grown in YM medium (yeast extract/malt extract) at 28°C. *Escherichia coli* XL1-Blue and BL21 CodonPlus (DE3)-RIL (hereafter referred to RIL) were used for routine cloning and protein expression, respectively. The *E. coli* strains were grown in LB medium at 37°C except that 28°C is used for protein expression.

Cloning of *stth* from the genomic DNA of *S. toxytricini*

The strain was grown in YM medium at 250 rpm and 28°C for 4 days and the genomic DNA was extracted from the mycelia. Phusion high-fidelity DNA polymerase (New England BioLabs) and a set of primers including *stth*-5′-*Nde*I (AA*CATATG*AACACGAGA AATCCGGA) and *stth*-3′-*Bam*HI (AA*GGATCC*TC AGACACGCTGCCCCATCG) were used to clone the target gene from the genomic DNA. The introduced restriction sites are shown in italics. The PCR program used for amplification of the *stth* gene consisted of an initial denaturation at 98°C for 5 min, 20 cycles of touchdown program (98°C for 30 s, annealing at 80°C for 40 s, decreasing 0.5°C per cycle, and extension at 72°C for 90 s), 20 cycles of regular program (98°C for 30 s, annealing at 70°C for 40 s, and extension at 72°C for 90 s), followed by a final extension at 72°C for 10 min. The PCR product was ligated into the pJET1.2 vector to yield pZJ90. The 1,572 bp gene (GenBank accession number HQ844046) was confirmed by sequencing, which was subsequently ligated into pET28a between the *Nde*I and *Bam*HI sites to yield the expression plasmid pZJ95.

Amino acid sequence alignment and phylogenetic analysis of STTH and nine known flavin-dependent halogenases

The deduced product of *stth* contains 523 amino acids. Nine known flavin-dependent halogenases including Rdc2 (GenBank HQ149729) from *Pochonia chlamydosporia* ATCC 16683 (Zeng and Zhan 2010), ChlA (DictyBase DDB0306268) from *Dictyostelium discoideum* (Neumann et al. 2010), PyrH (GenBank AAU95674) from *S. rugosporus* NRRL 21084 (Zehner et al. 2005), MibH (GenBank ADK32563) from *Microbispora corallina* NRRL 30420 (Foulston and Bibb 2010), Thal (GenBank ABK79936) from *S. albogriseolus* MJ286-76/F7 (Seibold et al. 2006), PrnA (GenBank U74493) from *Pseudomonas fluorescens* (Dong et al. 2005), RebH (GenBank CAC93722) from *Lechevalieria aerocolonigenes* ATCC 39243 (Yeh et al. 2005), as well as KtzR (GenBank ABV56598) and KtzQ (GenBank ABV56597) from *Kutzneria* sp. 744 (Fujimori et al. 2007). Multiple sequences alignment was performed using ClustalW2. Phylogenetic analysis of the halogenases was conducted using phylogeny.fr (Dereeper et al. 2008).

Production and purification of His_6-tagged STTH

For production of His_6-tagged STTH, *E. coli* RIL/pZJ95 was grown in LB medium containing 50 μg kanamycin/ml and 25 μg chloramphenicol/ml. After the OD_{600} reached 0.4–0.6, the culture was induced by 100 μM IPTG and maintained at 28°C for an additional 18 h. Cells were harvested by centrifugation, re-suspended in the lysis buffer consisting of 20 mM Tris/HCl (pH 7.9) and 0.5 M NaCl, and disrupted by sonication. Cell debris was removed from the crude cell lysate by centrifugation at 48,320×*g* and 4°C for 30 min. STTH was purified by nickel-nitrilotriacetic acid (Ni–NTA) agarose column chromatography. The purified His_6-tagged protein was concentrated and desalted against buffer A (50 mM Tris/HCl, pH 7.9, 2 mM EDTA and 1 mM DTT) with centrifugal filter devices. The purity of STTH (59 kDa) was checked by SDS-PAGE.

Halogenation assays

Fre is a flavin reductase from *E. coli* BL21(DE3) and it was expressed and purified as previously reported (Zeng and Zhan 2010). A typical 100 μl reaction contained 9.6 mM NADH, 100 μM FAD, 10 mM NaCl, 1 mM L-Trp, 10 μM Fre, and 5.5 μM STTH in 100 mM phosphate buffer (pH 6.0) and was kept at 28°C for 1 h. Reactions were quenched by 100 μl of methanol. The mixtures were centrifuged at ~20,000×*g* for 5 min to remove the precipitated proteins. The samples were analyzed on an Agilent 6130 single quadrupole LC–MS using an Agilent Eclipse Plus C18 reversed-phase analytical column (5 μm, 4.6 × 250 mm^2) at 280 nm. A gradient of acetonitrile/water (1:9–5.8:4.2, v/v) containing 0.1%

trifluoroacetic acid was at 1 ml min^{-1} and programmed over 15 min.

To test other halogen donors, NaCl in the reaction mixtures was replaced by NaF, NaBr or NaI. The reaction mixtures were analyzed using the same method as described above. ESIMS (+) for 6-bromo-L-Trp: $[M + H]^+$ *m/z* 283 and 285 (1:1).

In vitro enzymatic preparation of 6-chloro-L-Trp

A 10 ml reaction was set up to isolate the halogenated product of L-Trp. The reaction mixture was incubated at 28°C for 12 h. The product was purified by HPLC using the same conditions as described above. A total of 1.2 mg of 6-chloro-L-Trp was isolated in pure form. ^{1}H NMR (D_2O, 300 MHz): δ 7.65 (1H, d, $J = 8.4$ Hz, H-4), 7.55 (1H, d, $J = 1.7$ Hz, H-7), 7.31 (1H, s, H-2), 7.17 (1H, dd, $J = 8.4$, 1.7 Hz, H-5), 4.13 (1H, m, H-α), 3.47 (1H, dd, $J = 15.5$, 5.1 Hz, Ha-β), 3.34 (1H, dd, $J = 15.5$, 10.6 Hz, Hb-β); ESIMS (+): $[M + H]^+$ *m/z* 239 and 241 (3:1).

Determination of the optimum temperature and pH

STTH was assayed from 20 to 55°C for up to 1 h and at pH 4.5–8.5 at the optimum temperature. Reactions were quenched and analyzed by HPLC. All reactions were performed in triplicate.

Kinetic analysis of 6-chlorination of L-Trp by STTH

To measure the k_{cat} and K_m values for the 6-chlorination, a series of 100 µl reactions containing 9.6 mM NADH, 100 µM FAD, 10 mM NaCl, 10 µM Fre, and 5.5 µM STTH in 100 mM phosphate buffer (pH 6.0) with varying amount of L-Trp (2.5–15 µM) were run. The reaction components were mixed thoroughly and maintained at 40°C for 1 min. STTH was then added to initiate the reaction. After 15 min, the reactions were quenched and quantified by HPLC. Reactions were run in triplicate and the steady-state parameters k_{cat} and K_m were determined by nonlinear fitting of Michaelis–Menten equation.

Results and discussion

BLAST analysis of STTH

BLAST analysis of the deduced amino acid sequence of STTH revealed numerous Trp halogenases, among which KtzR showed 76% identity and 88% similarity to STTH (Fig. 1). The two crucial motifs that are highly conserved in most flavin-dependent halogenases (Zehner et al. 2005) were identified in STTH (Fig. 1). The GxGxxG motif near the *N*-terminus is the flavin binding site, while WxWxIP in the middle of the sequence is proposed to prevent the enzyme from catalyzing a monooxygenase reaction. KtzR is a Trp 6-halogenase involved in kutzneride biosynthesis. Although, KtzR can use L-Trp as a substrate to synthesize 6-chloro-L-Trp, KtzR has a ~120 fold preference for 7-chloro-L-Trp over L-Trp to generate 6,7-dichloro-L-Trp, suggesting that the former is the natural substrate of this enzyme (Heemstra and Walsh 2008). A putative flavin reductase gene, *stfre*, was found next to *stth* and its amino acid sequence is highly similar to KtzS (70% identity and 77% similarity), the partner enzyme of KtzR. STFRE is proposed to synthesize $FADH_2$ from FAD and NADH for the halogenation.

Phylogenetic analysis of STTH and homologues

Phylogenetic analysis of STTH and nine reported flavin-dependent halogenases was conducted. Multiple sequences alignment of these ten proteins is shown in supplementary Fig. 1. All these halogenases contain the GxGxxG flavin binding site, whereas, only the eight bacterial halogenases have the WxWxIP motif. The enzymes were divided into two major groups in the phylogenetic tree (Fig. 2). The first group contains the two recently identified eukaryotic halogenases, Rdc2 and ChlA, while all bacterial Trp halogenases belong to the other group. Rdc2 and ChlA have been reported to be involved in radicicol biosynthesis (Zeng and Zhan 2010) and formation of the polyketide-derived morphogen, differentiation-inducing factor 1 (DIF-1) (Neumann et al. 2010), respectively. In the group of bacterial halogenases, STTH fell into the same clade as KtzR, but not the other Trp 6-halogenase Thal. PyrH (Zehner et al. 2005) and MibH (Foulston and Bibb 2010) are Trp 5-halogenase involved in the biosynthesis of pyrroindomycin and lantibiotic, respectively. PrnA (Dong et al. 2005), RebH (Yeh

Fig. 1 Amino acid sequence alignment of STTH with KtzR. The two proteins are highly similar and both contain the two conserved motifs, GxGxxG and WxWxIP (*boxed*)

et al. 2005) and KtzQ (Fujimori et al. 2007) have been previously characterized as Trp 7-halogenase. Apparently, grouping of the bacterial Trp halogenases in the phylogenetic tree is not completely consistent with the enzymatic properties, suggesting that it is difficult to predict the regiospecificity of unknown halogenases only based on the amino acid sequence.

Functional characterization of STTH

The His_6-tagged STTH was expressed in *E. coli* RIL, purified to homogeneity by Ni–NTA chromatography, and checked by SDS-PAGE (Fig. 3). Most of the overexpressed protein was in the soluble fraction (lane 1) and showed the correct size of 59 kDa. The yield of STTH was 7.7 mg/l.

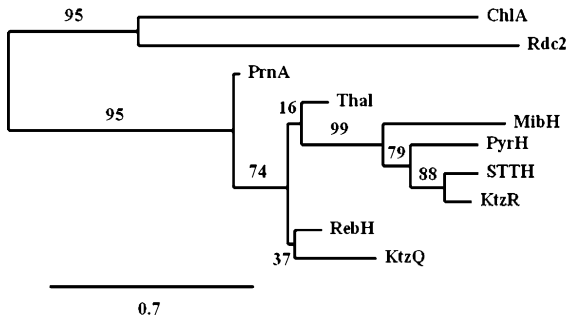

Fig. 2 Phylogenetic analysis of STTH and nine other flavin-dependent halogenases involved in natural product biosynthesis. ChlA and Rdc2 are two eukaryotic flavin-dependent halogenases, while others are from bacteria. STTH showed the closest relationship to KtzR, while Thal, the other bacterial Trp 6-halogenase was not in the same clade. Grouping of the halogenases is not always consistent with the function and regiospecificity of the enzymes

We next examined the activity of STTH. It is known that a flavin reductase is required to generate $FADH_2$ for the function of flavin-dependent halogenases. Although the *stfre* gene was found in the gene cluster, previous researches have shown that flavin reductases are not specific and can be replaced by other flavin reductases. Thus, we use Fre from *E. coli* as the partner enzyme, which we have previously used for the study of Rdc2 (Zeng and Zhan 2010). As shown in Fig. 4(a), L-Trp was converted into a less polar compound ($R_t = 8.5$ min). This product showed apparent bathochromic shift of the UV absorbance (Supplementary Fig. 2), which is typical for halogenated derivatives. ESIMS showed two quasimolecular ion peaks $[M + H]^+$ at *m/z* 239 and 241 at a ratio of 3:1 (Supplementary Fig. 3), indicating that this is a mono-chlorinated derivative of L-Trp. We also tested D-Trp as a substrate, and the same result was observed (data not shown), confirming that this enzyme is a Trp halogenase that accepts both L- and D-Trp as the substrates.

Various Trp halogenases have been reported and different enzymes may introduce the chlorine atom into Trp at different positions. To identify the structure of the halogenated product of L-Trp by STTH, we scaled up the reaction and isolated 1.2 mg of the product. NMR analysis revealed four low-field proton signals from the indole ring, including two

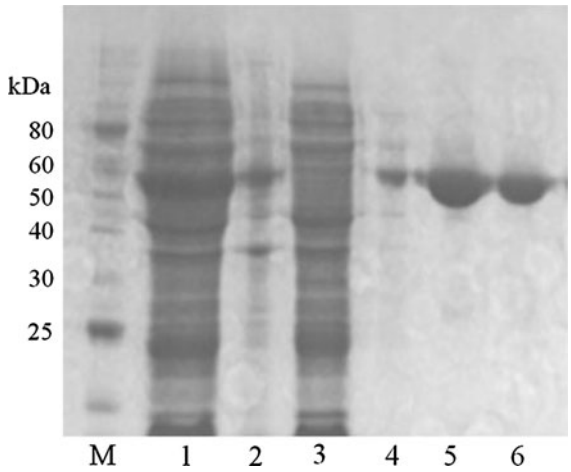

Fig. 3 SDS-PAGE analysis of expression and purification of STTH. *M*: protein ladder; *1*: soluble fraction; *2*: insoluble fraction; *3*: flow through; *4*: elution fraction by buffer A; *5*: elution fraction by buffer A containing 10 mM imidazole; *6*: elution fraction by buffer A containing 250 mM imidazole. The induced broth of *E. coli* RIL/pZJ95 was centrifuged and the pellets were re-suspended in the lysis buffer and lysed by sonication. The lysate was separated by Ni–NTA chromatography. The pure protein came off the column when 10 and 250 mM imidazole were applied. Gels were stained with coomassie brilliant blue R-250

doublets, respectively at δ 7.65 ($J = 8.4$ Hz) and 7.55 ($J = 1.7$ Hz), a double doublet at δ 7.17 ($J = 8.4$ and 1.7 Hz) and a singlet at δ 7.31, which indicated that 5-CH or 6-CH of L-Trp has been substituted by a chlorine atom. A further comparison of the NMR data with the literature (Li et al. 2004) confirmed that this product is 6-chloro-L-Trp. Thus, STTH was characterized as Trp 6-halogenase (Fig. 4b).

To test whether STTH can take other halides as halogen donor, we tested NaF, NaBr and NaI as the substrate and found that STTH can incorporate bromine into L-Trp to yield 6-bromo-L-Trp (Fig. 4a), as indicated by the $[M + H]^+$ peaks at *m/z* 283 and 285 at a ratio of 1:1 (Supplementary Fig. 3), suggesting that STTH is also a brominase.

Optimum reaction conditions for 6-halogenation of L-Trp catalyzed by STTH

With L-Trp as the substrate, enzymatic activity of STTH was highest at 40°C (see Fig. 5a) and at pH 6, (see Fig. 5b).

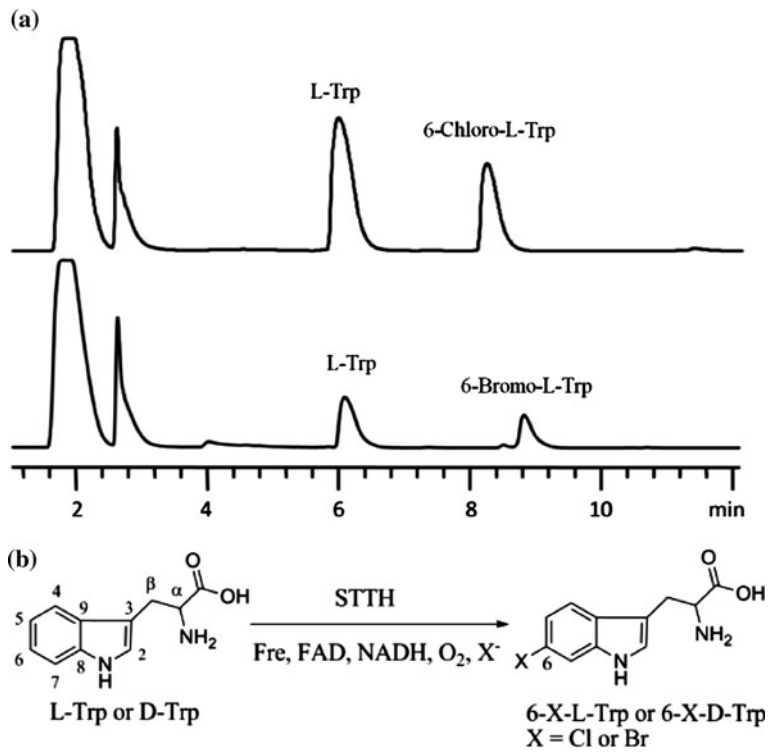

Fig. 4 **a** HPLC analysis of chlorination (*top*) and bromination (*bottom*) of L-Trp catalyzed by STTH. The reaction mixtures were analyzed by HPLC using an Agilent Eclipse Plus C18 reversed-phase analytical column (5 μm, 4.6 × 250 mm^2) at 280 nm. The substrate was converted into a new chlorinated product ($R_t = 8.5$ min) and a brominated product ($R_t = 9.3$ min). **b** Halogenation of Trp by STTH. Both L-Trp and D-Trp can be halogenated at C-6 to yield the corresponding chlorinated and brominated derivatives

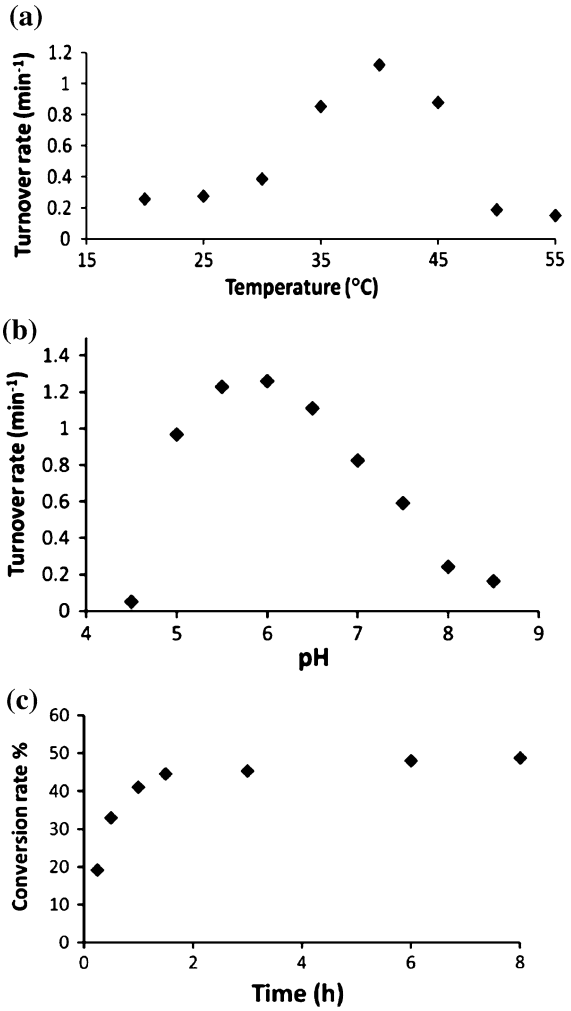

Fig. 5 **a** Temperature dependence of the 6-halogenation of L-Trp catalyzed by STTH. The reactions were conducted at pH 6 and eight different temperatures including 20, 25, 30, 35, 40, 45, 50 and 55°C for 1 h. The turnover rate is calculated based on the product formation during the reaction period. **b** pH Dependence of the 6-halogenation of L-Trp catalyzed by STTH. The reactions were conducted at 40°C in phosphate buffers with various pH values including 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 for 1 h. The turnover rate is calculated based on the product formation during the reaction period. **c** Time-course analysis of the 6-halogenation of L-Trp catalyzed by STTH at 40°C and pH 6. The reactions were quenched at different time points including 0.25, 0.5, 1, 1.5, 3, 6, and 8 h. The conversion rates were analyzed by HPLC. The data shown are representative of three independent experiments

Kinetic analysis of 6-halogenation of L-Trp catalyzed by STTH

The highest initial reaction velocity was obtained at 0.5 h (see Fig. 5c). No further product was formed after 1.5 h. To measure the steady-state kinetic parameters, we chose 0.5 h as the reaction time. The k_{cat} was 1.53 ± 0.12 min^{-1}, and the K_m was 21 ± 3 μM. Thus, the average k_{cat}/K_m value was determined to be 71,942 min^{-1} M^{-1}, which is much more efficient than KtzR that showed a k_{cat}/K_m value of 12,281 min^{-1} M^{-1} when its preferred substrate 7-chloro-L-Trp was used (Heemstra and Walsh 2008).

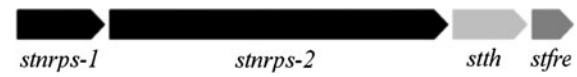

Fig. 6 A NRPS gene cluster from *S. toxytricini* NRRL 15443 containing four genes encoding STTH, STFRE, and two unknown NRPSs. STTH and STFRE may play an important role in supplying 6-chloro-L-Trp as the biosynthetic precursor for the NRPSs

To our knowledge, this is the third characterized Trp 6-halogenase. The physiological role of this halogenase in the host is unclear. A further check into the genes next to *stth* and *stfre* revealed a nonribosomal peptide synthetase (NRPS) gene cluster that contains two NRPSs, STNRPS-1 and STNRPS-2 (Fig. 6). It was reported that higher percentages of halogenase gene-positive microorganisms than halogenase gene-negative ones contained polyketide synthase genes and/or NRPS genes or displayed antimicrobial activities, indicating the genetic and physiological potentials of halogenases for producing secondary metabolites (Gao and Huang 2009). We propose that STTH and STFRE work concertedly to generate 6-chloro-L-Trp which might be used as the biosynthetic precursor by the NRPSs to synthesize chlorine-containing natural products. The functional study on the putative NRPSs is currently under way.

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