

Directed evolution improves the enzymatic synthesis of L-5-hydroxytryptophan by an engineered tryptophan synthase

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Short Report

Keywords: L-5-hydroxytryptophan, tryptophan synthase, directed evolution

Posted Date: June 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-174454/v1>

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Abstract

L-5-Hydroxytryptophan is an important amino acid that is widely used in food and medicine. In this study, L-5-hydroxytryptophan was synthesized by a modified tryptophan synthase. A direct evolution strategy was applied to engineer tryptophan synthase from *Escherichia coli* to improve the efficiency of L-5-hydroxytryptophan synthesis. Tryptophan synthase was modified by error-prone PCR. A high activity mutant enzyme (V231A/K382G) was obtained by a high-throughput screening method. The activity of mutant enzyme (V231A/K382G) is 3.79 times higher than that of its parent, and k_{cat}/K_m of the mutant enzyme (V231A/K382G) was $4.36 \text{ mM}^{-1} \cdot \text{s}^{-1}$. The mutant enzyme (V231A/K382G) reaction conditions for the production of L-5-hydroxytryptophan were 100 mmol/L L-serine at pH 8.5 and 35°C for 15 h, reaching a yield of L-5-hydroxytryptophan of 86.7%. Directed evolution is an effective strategy to increase the activity of tryptophan synthase.

Introduction

L-5-Hydroxytryptophan is a special amino acid found in both plants and animals. L-5-Hydroxytryptophan increases brain serum concentrations and brain melatonin levels, improves symptoms of spinal cord atrophy and promotes sleep [1]. L-5-Hydroxytryptophan has a good effect on the treatment of depression. Exogenous L-5-hydroxytryptophan crosses the blood-brain barrier, increasing the concentration of serotonin in the brain and reducing the production of cytokines in the body, thus improving depression [2–4]. L-5-Hydroxytryptophan was prepared by plant extraction and biosynthesis. L-5-Hydroxytryptophan from Ghana seeds was adsorbed and separated by ion-exchange resin. The yield of L-5-hydroxytryptophan was 7.95% [5]. L-5-Hydroxytryptophan was extracted from Ghana seeds by hydrothermal reflux extraction, alcohol reflux extraction and ultrasonic water extraction, and L-5-hydroxytryptophan was also extracted from cohosh [6]. L-5-Hydroxytryptophan was prepared by tryptophan hydroxylase, which promoted the synthesis of L-5-hydroxytryptophan by metabolic engineering [7, 8]. The yield of L-5-hydroxytryptophan prepared by the plant extraction method was lower compared with the yield prepared by the biosynthesis method, and the enzymatic reaction was specific with fewer by-products. Chemical synthesis method was easy to bring pollution to the environment. Enzymatic synthesis is an effective strategy to synthesize short-chain flavor esters, phytosteryl lipoate, L- α -galloylglycerol [9–11]. Directed evolution has been widely used in the field of drug synthesis [12, 13]. Directed evolution can promote the enzymatic activity of lipase, xylanase, α -galactosidases, monoamine oxidase and nitrilase [14–19]. L-tryptophan and S-phenyl-L-cysteine were synthesized by tryptophan synthase of *Escherichia coli* k-12, and it was found that tryptophan synthase has the potential to synthesize L-tryptophan and its derivatives [20–22]. In this study, directed evolution of tryptophan synthase was performed by error-prone PCR. L-5-Hydroxytryptophan was synthesized by L-serine and 5-hydroxyindole using tryptophan synthase as the catalyst. This provides a new strategy for the enzymatic synthesis of L-5-hydroxytryptophan.

Materials And Methods

Chemicals

L-Serine, 5-hydroxyindole, peptone, and yeast extract were purchased from Alighting Biochemical Technology Co. Ltd. (Shanghai, China). Isopropyl- β -D-thiogalactoside, pyridoxal phosphate (PLP) were purchased from Sangon Biotech Co., Ltd. (Shanghai). Other chemicals were analytical reagents.

Error-prone PCR mutant library

According to the *trpB* sequence of *E. coli* k-12 tryptophan synthase (ID: 945768) and the MCS site of the pETDuet-1 vector published by the NCBI, two primers were designed using Primer Premier 5.0. *Nco*I and *Eco*RI were introduced at the 5' end of the upstream and downstream primers, respectively. The primers were synthesized by Shanghai Yingjun Biotechnology Co., Ltd.

5'-CCCATATGACAACACTTAAC-3', 5'-CCCGAATTCTTAAGTGGCGTTT-3'

Random mutations were introduced into the *trpBA* gene using the Trans EasyTaq DNA polymerase kit. The PCR system and PCR procedure are described in the instructions.

Screening of tryptophan synthase

A single colony of mutant strain was selected from the petri dish and inoculated into a 96-well shallow orifice plate with 1mL LB liquid medium (35°C). Isopropyl- β -D-thiogalactoside and PLP were added to each well of 96-well shallow orifice plate at 18°C. The fermentation mutant strain was centrifuged after induction (4°C, 4000 r·min⁻¹, 10 min). L-Serine and 5-hydroxyindole were added to the 96-well shallow orifice plate (37°C, 30 min, 200 r·min⁻¹). The absorbance value at 290 nm was measured by ELISA.

Purification of tryptophan synthase

The engineered bacteria of tryptophan synthase were crushed by ultrasonication (600 W, 10 min, 0 °C). The crude enzyme was centrifuged at 5000 r·min⁻¹ for 10 min. The supernatant was collected to obtain the crude tryptophan synthase enzyme liquid. The supernatant of tryptophan synthase were purified by Ni-NTA column using the method reported in the literature [23].

Determination of enzymatic activity

Tryptophan synthase activity was determined by the L-5-hydroxytryptophan concentration. The enzyme reaction conditions for the production of L-5-hydroxytryptophan were 100 mmol/L L-serine at pH 8.5 and 35°C for 15 min. The conversion at this time point was 17%. The concentration of L-5-hydroxytryptophan was determined by an amino acid analyser (L-8900, Japan). Resin column was sulfonic acid type cationic resin column (4.6 mm×60.0 mm). Column temperature was 57.0 °C. Reactor temperature was 130 °C. The flow rate of pump A (elution solution) was 0.40 mL/min. The flow rate of B (ninhydrin solution) was 0.35 mL/min, and the injection volume was 20 μ L. The detection wavelength was 570 nm and 440 nm.

Determination Of Kinetic Parameters

Determination of kinetics parameters was carried out using five L-serine concentrations (0.1 mmol/L, 0.2 mmol/L, 0.3 mmol/L, 0.4 mmol/L and 0.5 mmol/L). Kinetics parameters of tryptophan synthase were obtained through the Lineweaver-Burk methodology.

Preparation of L-5-hydroxytryptophan

The optimum reaction conditions for L-5-hydroxytryptophan synthesis catalysed by tryptophan synthase were studied. The effects of temperature, pH and substrate concentration on tryptophan synthase were investigated. L-5-Hydroxytryptophan was synthesized under the optimal reaction conditions. The optimal conditions and reaction volume for L-5-hydroxytryptophan preparation were L-serine 100 mmol/L, 35°C, pH 8.5 and 1000 ml. The reaction system (1000 ml) containing 10.5 g L-serine, 13.3 g 5-hydroxyindole, 0.01g pyridoxal phosphate and 0.1 g tryptophan synthase. L-5-hydroxytryptophan was analyzed with an FT-IR Spectrometer (Gangdong Sci & Tech. Co., Ltd., Tianjing, China). All treatment of L-5-hydroxytryptophan synthesis catalysed by tryptophan synthase were repeated for three times. SPSS 22.0 was used to analysis of experimental data.

Results And Discussion

Mutant of tryptophan synthase

Random mutant library of tryptophan synthase was constructed by error-prone PCR. The mutation rate of error-prone PCR was 1.2% in this study. The highly enzymatic activity of mutant V231A/K382G was obtained from screening 12,000 mutant strains of tryptophan synthase. The activity of mutant enzyme (V231A/K382G) is 3.79 times higher than that of its parent (Fig. 1), and k_{cat}/K_m of the mutant enzyme (V231A/K382G) was $4.36 \text{ mM}^{-1} \cdot \text{s}^{-1}$ (Table 1). The specific activity of WT was 3876 U/mg. These results indicated that sites 231 and 382 were the key sites affecting the activity of trpBA. The growth of the wild-type strain and V231A/K382G strain was observed under a microscope. There was no difference in morphology between the four strains in Fig. 2 (Wild-type strain; V231A/K382G; D381G; T319S) .

Table 1
Kinetic parameters of tryptophan synthase and its mutants.

Mutations	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ •s ⁻¹)	Fold change over WT
WT	0.33 ± 0.12	0.38 ± 0.13	1.15 ± 0.09	1
V231A/K382G	0.28 ± 0.06	1.22 ± 0.08	4.36 ± 0.11	3.79
D381G	0.31 ± 0.11	0.41 ± 0.09	1.32 ± 0.08	1.14
P257R	0.21 ± 0.03	0.10 ± 0.02	0.47 ± 0.01	0.41
H313F	0.29 ± 0.07	0.32 ± 0.10	1.10 ± 0.05	0.96
L80H	0.25 ± 0.04	0.29 ± 0.07	0.86 ± 0.07	0.75
N64G/S352P	0.23 ± 0.03	0.16 ± 0.02	0.69 ± 0.06	0.60
T319S	0.30 ± 0.07	0.32 ± 0.10	1.06 ± 0.04	0.92
A67M	0.21 ± 0.03	0.12 ± 0.02	0.57 ± 0.01	0.49

Effects of temperature, pH and L-serine on the enzymes

The reaction activity of the mutated enzyme and parental enzyme at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C was investigated. The optimal temperature of the mutated enzyme V231A/K382G and the parental enzyme was approximately 35°C (Fig. 3A). The relative activity of mutant enzyme V231A/K382G was 100% and the relative activity of the parent enzyme was 26%. Under reactions at pH 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, the relative activity of mutant enzyme V231A/K382G was 100% while the relative activity of the parent enzyme was 24% at pH 8.5. The activity of the parent enzyme was still lower than that of the mutant enzyme V231A/K382G (Fig. 3B). tryptophan synthase was reacted with different substrate concentrations of L-serine, and the mutant enzyme of tryptophan synthase V231A/K382G maintained high activity with L-serine 100 mmol/L, while the optimal concentration of L-serine for the parental enzyme was approximately 40 mmol/L (Fig. 3C). The molar concentration ratio of serine to 5-hydroxyindole was 1:1. The optimal concentration of 5-hydroxyindole for the mutant enzyme was 100 mmol/L.

Synthesis of L-5-hydroxytryptophan

L-5-Hydroxytryptophan was synthesized by the mutant enzyme V231A/K382G at L-serine 100 mmol/L, 35°C and pH 8.5. The reactants were collected at 1 h, 2 h, 4 h and 15 h. The content of the product L-5-hydroxytryptophan was determined by an amino acid analyser (L-8900, Japan). The content of L-5-hydroxytryptophan increased with reaction time (Fig. 4). After the reaction, activated carbon was added to the reaction liquid. The filtrate was concentrated and crystallized, and the crystals were separated by centrifugation. The infrared spectrum of L-5-hydroxytryptophan is shown in figure 5, with the introduction

of amino (3400–3500 cm^{-1}) and carboxyl groups (1650–1740 cm^{-1}). L-5-hydroxytryptophan: m.p. 270°C (decomp), $[\alpha]_D^{25} = -32.5^\circ$ ($c = 1, \text{H}_2\text{O}$).

Mutation analysis

Chimera 1.13 was used to analyse the substrate channel space of tryptophan synthase (PDB: 2dh6), and it was found that the amino acid residues of tryptophan synthase at positions 231 and 382 were located in the loop formed by the substrate channel [24]. The amino acid residue at site 231 was mutated from valine to alanine, and the residue at site 382 was mutated from lysine to glycine, which may change the folding direction of the loop, thus changing the size and shape of channel entrance and promoting mutated enzymatic activity (Fig. 6). The function of the β subunit of tryptophan synthase (EC 4.2.1.20) is to use indole and L-serine to synthesize L-tryptophan [25–27]. Directed evolutionary molecular modification for the application of enzyme catalysis has achieved good results [28–29]. Tryptophan synthase from *Pyrococcus furiosus* has been modified by directed evolution and the engineered biocatalyst reacts with a variety of indole analogues and thiophenol [30]. An engineered variant of *Pyrococcus furiosus* tryptophan synthase provides an operationally simple and environmentally benign platform for preparation of β -branched tryptophan building blocks [31]. Directional evolution can reshape the active pocket to improve the substrate specificity of the enzyme and modify its substrate specificity. For a small active pocket, directional evolution can expand the active pocket to accommodate larger substrate molecules. At present, most studies have investigated tryptophan synthase derived from *Pyrococcus furiosus*, but the enzyme activity of this tryptophan synthase is low. The literature has reported that the substrates of tryptophan synthase derived from *Salmonella* include indole derivatives substituted with fluorine, chlorine, bromine and iodine, which can synthesize L-tryptophan derivatives [32]. In this study, L-5-hydroxytryptophan was synthesized by a modified tryptophan synthase. Tryptophan synthase was modified by error-prone PCR. A high-activity mutant enzyme (V231A/K382G) was obtained by a high-throughput screening method.

Conclusion

A highly active mutant of tryptophan synthase was obtained by directed evolution. The activity of mutant enzyme (V231A/K382G) is 3.79 times higher than that of its parent, and the mutant enzyme (V231A/K382G) has good catalytic efficiency (k_{cat}/K_m) of $4.36 \text{ mM}^{-1} \cdot \text{s}^{-1}$. The conditions for L-5-hydroxytryptophan preparation by the mutated enzyme were an L-serine 100 mmol/L, 35°C, pH of 8.5 for 15 h, with the yield of L-5-hydroxytryptophan reaching 86.7%. Directed evolution is an effective strategy to increase the catalytic activity of tryptophan synthase.

Declarations

Ethical Approval Compliance with Ethical Standards.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Author contributions LSX conceived and carried out experimental work and drafted the manuscript. TTL, BLJ and ZYH contributed to data interpretation and reviewed and edited the manuscript. QC and QXX conceived and supervised experimental work, secured funding, and edited the manuscript.

Funding This work was supported by Natural Science Research Project in Anhui Province (KJ2020A0729). Project of Suzhou University (2018XJXS02; 2019XJSN05; 2019XJZY10), Research Project in Suzhou university (2019ykf13, 2019YKF14). Open Fund project of National Engineering Laboratory (NEL-SCRT 002).

Competing Interests The authors declare that they have no conflicts of interest.

Availability of data and materials

The datasets used and/or analysed in the current study are available from the corresponding author upon reasonable request.

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Figures

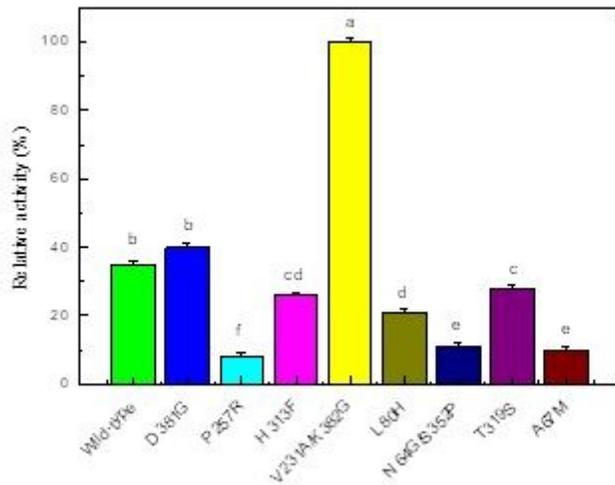


Figure 1

Relative activities of the mutants of tryptophan synthase. The reaction conditions for the mutated enzyme were an L-serine concentration of 100 mmol/L, a reaction temperature of 35°C, and a pH of 8.5 for 15 h. The meaning of different letters in the figure legend indicates significant differences.

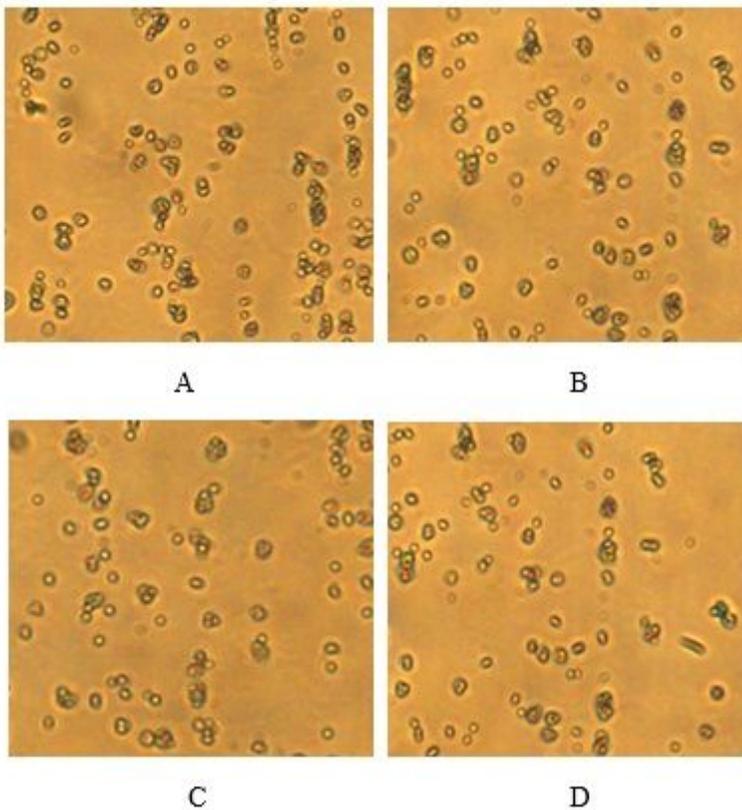


Figure 2

Microbial morphology of the mutants of tryptophan synthase. (A: Wild-type strain; B:V231A/K382G; C:D381G; D:T319S)

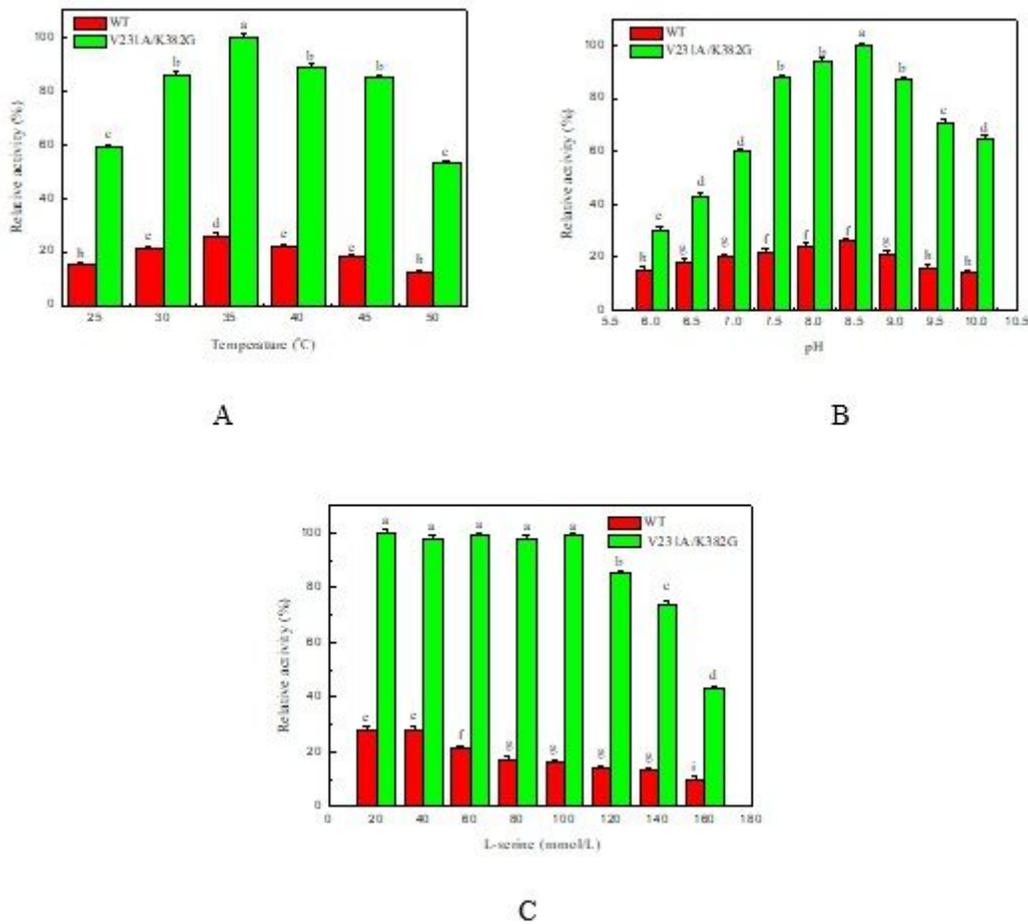


Figure 3

Effects of temperature, pH, and L-serine on the activity of the tryptophan synthase mutant. A: The reaction conditions for the mutated enzyme were an L-serine concentration of 100 mmol/L and a pH of 8.5 for 15 h. B: The reaction conditions for the mutated enzyme were an L-serine concentration of 100 mmol/L at 35°C for 15 h. C: The reaction conditions for the mutated enzyme were 35°C at pH of 8.5 for 15 h. The meaning of different letters in the figure legend indicates significant differences.

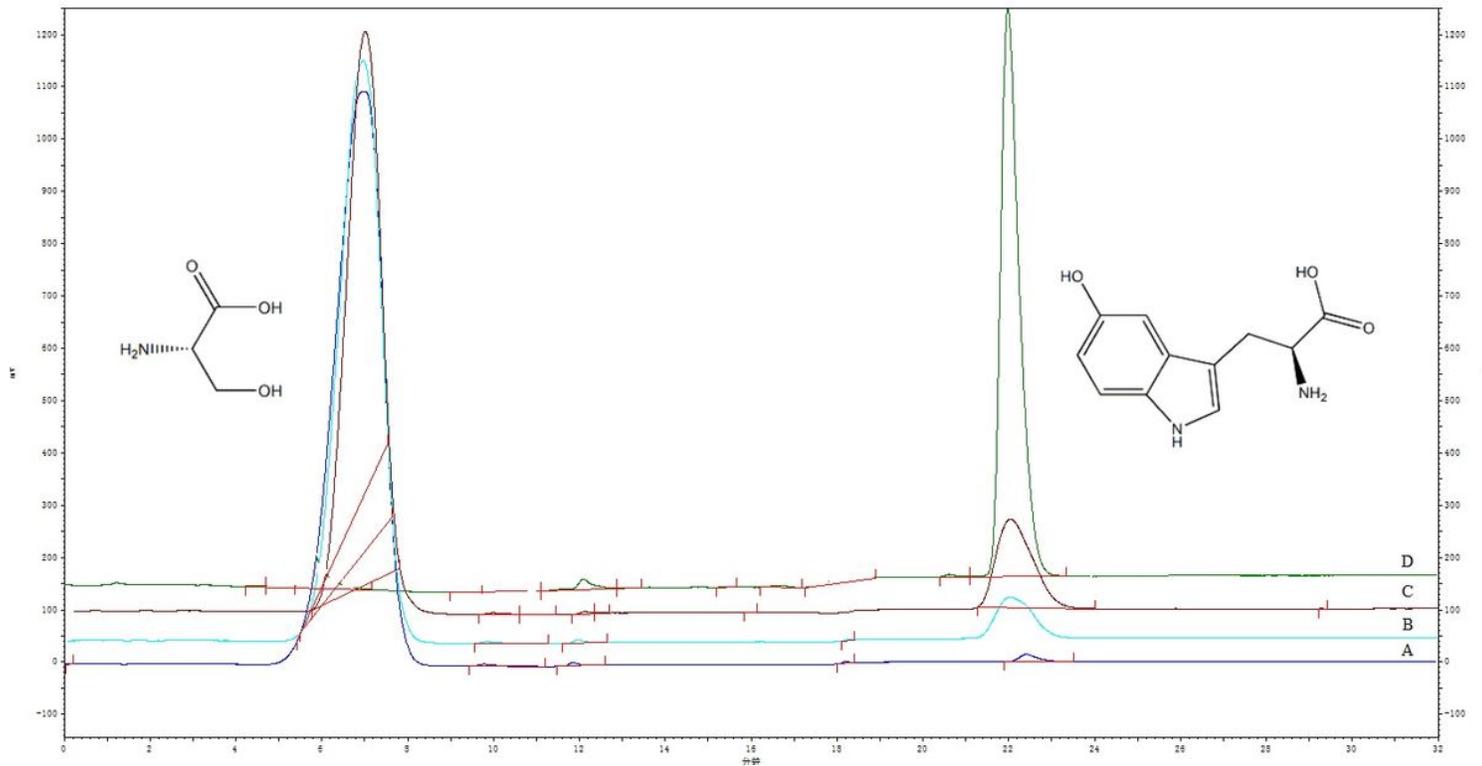


Figure 4

Synthesis of L-5-hydroxytryptophan by the mutated tryptophan synthase (V231A/K382G). (A: 1 h; B: 2 h; C: 4 h; D: 15 h). The reaction conditions for the mutated enzyme were an L-serine concentration of 100 mmol/L, a reaction temperature of 35°C, and a pH of 8.5.

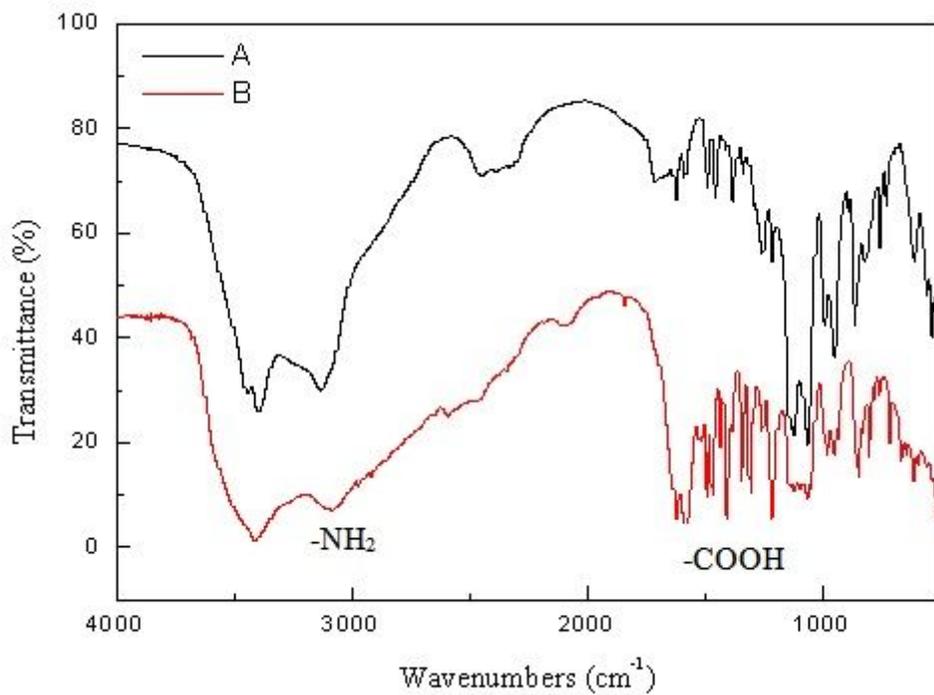
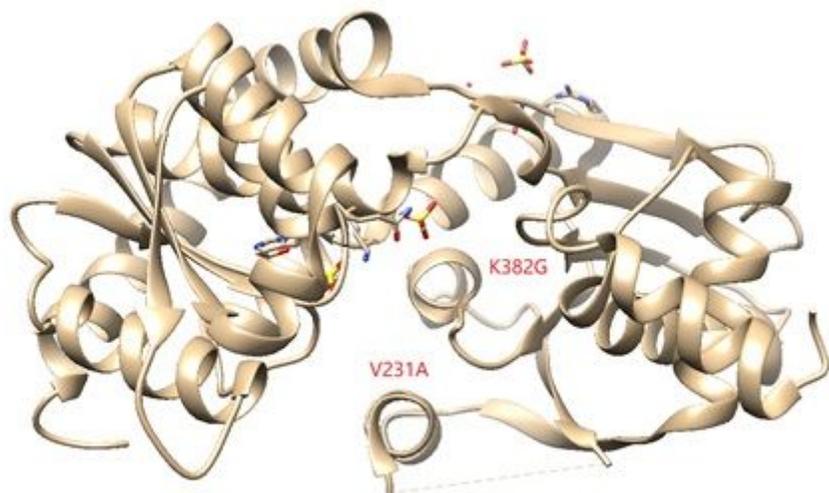


Figure 5

FTIR spectra of (B) L-5-hydroxytryptophan and (A) 5-hydroxyindole.



A



B

Figure 6

Relationship between the substrate channel and the binding sites of residues 231 and 382 in the mutant (A). The structure of the tryptophan synthase wild type (B).