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Investigation of the Biosynthetic Process of Indigoidine

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INVESTIGATION OF THE BIOSYNTHETIC PROCESS OF INDIGOIDINE

by

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Approved:

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ABSTRACT

Investigation of the biosynthetic process of indigoidine

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Utah State University, 2019

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Indigoidine (5,5′-diamino-4,4′-dihydroxy-3,3′-diazadiphenoquinone-(2,2′)) is a natural product synthesized by a non-ribosomal peptide synthetase (NRPS). It features a unique bright blue color similar to industrial dye indigo. It has antioxidant and antimicrobial activities, and the responsible gene can be used as a reporter system for gene expression. Several bacterial strains have the potential to produce indigoidine. However, some hosts have silent indigoidine biosynthetic genes, while others have very limited production of indigoidine. Heterologous expression of the responsible synthetase gene will significantly benefit its large-scale production and industrial applications.

Previously, we cloned a putative indigoidine synthetase gene (*Sc-indC*) from *Streptomyces chromofuscus* ATCC 49982. Heterologous expression of the *Sc-indC* in *Escherichia coli* (*E. coli*) achieved indigoidine production of 20 g/L under optimized conditions. Exploring the biosynthetic mechanism of indigoidine will potentially open new doors for further improvement of the production of this natural dye.

As NRPSs, most indigoidine synthetases were predicted to have four functional domains: adenylation domain (A), thiolation (T), thioesterase (TE), and a special oxidation (Ox) integrated into the A domain, except that Sc-IndC has an additional C-terminal 4-oxalocrotonate tautomersase (4-OT) domain. It was proposed that the A domain activates the substrate L-glutamine (L-Gln), and transfers it to the T domain, where the TE domain catalyzes the cyclization to produce the intermediate pyro-glutamine (pyro-Gln). The intermediate compound was then released from T domain as a free molecule, and subsequently oxidized by the Ox domain, yielding 5-amino-3H-pyridine-2,6-one, which is finally dimerized to produce indigoidine. However, the proposed biosynthetic mechanism has not been confirmed and the sequence of the oxidation and cyclization still remains unknown.

Although Sc-IndC has been reconstituted in *E. coli* BAP1 with higher activity, we found it had relatively lower *in vitro* activity compared to BpsA. So, my study was mainly focused on the biosynthetic mechanism of indigoidine by BpsA and Sc-IndC. The corresponding genes *bpsA* and *Sc-indC* were derived from two different bacteria.

Firstly, I cloned and heterologously expressed *bpsA*, *Sc-indC*, and their gene fragments in *E. coli* BAP1, and then purified the recombinant proteins. Then, I chemically synthesized L-glutamine-SNAC, which mimics the thiolated glutamine in the biosynthetic process. Finally, I optimized the *in vitro* enzyme assay conditions to test the enzymatic activity of the purified intact enzymes and domains on the substrate L-glutamine, and the intermediates L-glutamine-SNAC and 3-

aminopiperidine-2,6-dione (pyro-Gln). The products were analyzed by LC-MS to reveal the biosynthetic process of indigoidine by BpsA and Sc-IndC. My results showed that in the presence of ATP, BpsA can convert substrate L-glutamine, the intermediates L-glutamine-SNAC and 3-aminopiperidine-2,6-dione to indigoidine.

PUBLIC ABSTRACT

Investigation of the biosynthetic process of indigoidine

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Indigoidine is a natural blue dye with antioxidant and antimicrobial activities. It has also been used as an indicator for gene expression based on its distinctive blue color. Similar to the industry blue dye indigo, indigoidine has a promising potential to be applied in industry as a blue dye. However, the indigoidine production level in the original microorganisms was very low. Heterologous expression of the responsible synthetase gene in *Escherichia coli* can facilitate the fast and large-scale production of indigoidine. Also, a good understating of the working mechanism of the synthetase is favorable for the industrial application.

In our previous study, a putative indigoidine synthetase gene (*Sc-indC*) has been heterologously expressed in *E. coli*, and 20 g/L of indigoidine was produced under optimized culture conditions. To further improve the production, we intended to explore the indigoidine biosynthetic process by studying the working mechanism of two indigoidine synthetases BpsA and Sc-IndC from two different bacteria.

Both BpsA and Sc-IndC were predicted to have similar domain architecture that consists of an adenylation domain (A) with an embedded oxidation (Ox)

domain, thiolation (T) domain, and thioesterase (TE) domain, except that Sc-IndC has an additional C-terminal. To explore the enzymatic mechanism, I dissected the gene between domains to get A-Ox-T/TE and A-Ox/T-TE, and co-expressed the dissected fragments to find out whether they can work in the combination of pieces as the intact enzymes do. On the other hand, I separately expressed the domains in *E. coli* BAP1 and purified the resulting proteins, and tested the *in vitro* activities on precursors individually or in different combinations. My results showed that the intact enzymes can convert glutamine, glutamine-SNAC and pyor-Gln to indigoidine in the presence of ATP. However, none of the dissected enzymes showed the ability to form the blue dye *in vitro* or *in vivo*.

DEDICATION

I would like to dedicate this work to my family, my appreciation to my parents and my fiancé for their altruistic love and tireless support.

Yi Chen

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A special thanks to my family for all the sacrifices they have made for me.

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ABBREVIATIONS

A	Adenylation
ATCC	American Type Culture Collection
bpsA	Blue-pigment indigoidine synthetase gene
BpsA	Blue-pigment indigoidine synthetase
DMSO	Dimethyl sulfoxide
HPLC	High performance liquid chromatography
indC	Indigoidine synthetase gene
IndC	Indigoidine synthetase
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
LC-MS	Liquid chromatography–mass spectrometry
L-Gln	L-glutamine
L-Gln-SNAC	L-glutamine-SNAC
NMR	Nuclear magnetic resonance
NRPS	Non-ribosomal peptide synthetase
ORF	Open reading frame
Ox	Oxidation
PCR	Polymerase chain reaction
PPTase	4′-Phosphopantetheinyl transferase
pyro-Gln	3-aminopiperidine-2,6-dione
T	Thiolation
TE	Thioesterase

CHAPTER I INTRODUCTION

1.1 Background

Introduction of indigo.

Indigo (indigotin, or 2,2'-Bis (2,3-dihydro-3-oxoindlyliden) features distinctive bright blue color and has been broadly used for textile dyeing, printing, food, and cosmetic applications [1]. Since the 19th century, the worldwide demand of blue jeans and denim cloth has been dramatically increased, accordingly the production of indigo also intensely amplified [1b, 2]. In 2011, 50,000 tons of indigo was produced, of which up to 95% was applied in the dying of denim cloth [3].

Originally indigo was a rare natural dye. It was extracted from the leaves of the plant genus *Indigofera*, specifically *Indigofera tinctoria* (or *I. sumatrana*), which was mainly distributed in tropics, and predominantly in India[1a, 1b, 4]. People who resided in less-warm areas, such as China, Japan, and Europe, also alternatively extracted blue dye from native plants such as *Strobilanthes cusiaw*, *Persicaria tinctoria*, *Polygonum tinctorum*, and *Isatis tinctoria*; another optional source was the sea snail mollusk [1a, 1b, 4]. However, the production of these blue-dyes were of low concentration and purity, and easily tinged by other dye. So the true indigo production was mainly originated from *I. tinctoria*, which was very limited and rare. Hence people traded it as a luxury product and referred it as blue gold [1b]. Though later broad plantation brought relatively higher production, the labor work was arduous and the production was highly environment-dependent. The supply was still far less than the growing demand. For centuries, the plant–

derived indigo was the only source of this blue dye. *Figure 1* shows the pathway of indigo in plant.

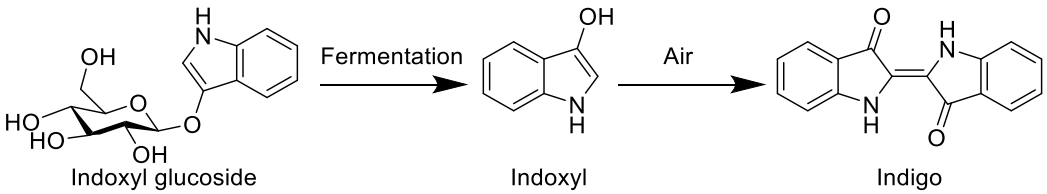


Figure 1 Chemical transformations occurred in the processing of the leaves in dyeing with indigo [1b].

In the 1860s, chemists started to develop strategies to chemically synthesize this compound. In 1870, the German chemist Adolf von Baeyer firstly synthesized indigo from isatin. Later on he developed several other methods, but all were in laboratory scale [5]. To achieve the industrious production, Heumann developed a strategy to heat the precursor *N*-(2-carboxyphenyl)glycine with sodium hydroxide, and subsequently decarboxylated the resulting product to form indoxyl, which was immediately oxidized as indigo dye in air. In the 1990s, Johannes Pfleger realized the commercial production in large scale. They produced the single unit indoxyl in one step through *N*-phenylglycine under harsh base condition[1b, 6]. Since then, almost all of the indigo dye has been produced by chemical synthesis [7]. Nowadays, thousands of tons of indigo is produced anniversary. However, mass chemical production unenviably brings serious environmental pollution and material waste.

Microbial production of indigo could be an eco-friendly and high-efficient strategy. It has been reported that microorganisms also produced indigo. In recent years, biosynthetic pathway has been developed in *E. coli* to "naturally" produce

indigo from indole [8]. However, the production has been in small scale and merely satisfies the huge industrial demand.

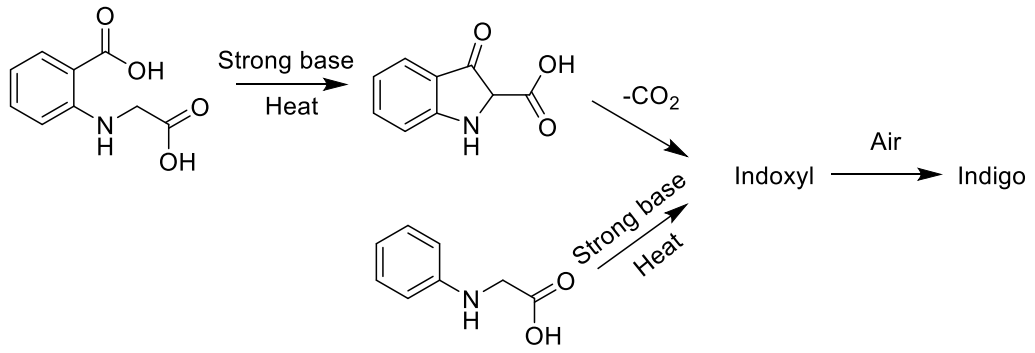


Figure 2 Chemical synthesis of indigo based on Heumann-Pfleger process.

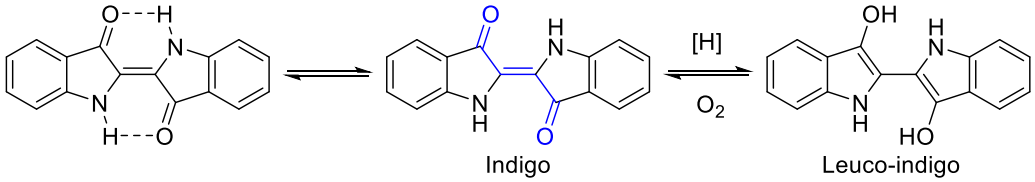
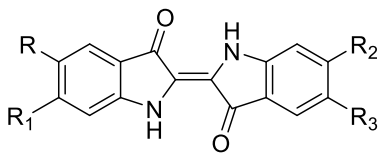


Figure 3 Transformation of indigo in the dyeing process.

Indigo has good solubility in DMSO, but is less soluble in water and other organic solvents, since it's easy for this molecule to form intra- and intermolecular hydrogen bond. A suppression on the formation of intramolecular hydrogen bond might help with the solubility. During the dyeing process with indigo, the low solubility makes the dyeing challenging. Usually reductant agent is required to convert indigo into a soluble form, which is leuco-indigo, also known as indigo white. The soluble indigo white physically attaches to the fabric, and returns to indigo after being oxidized in the air, turning the fabric from yellow-green to blue [9]. Dying pigment has been a big problem of the water

pollution, and the tough reducing condition of the dying with indigo make it more hazardous.



R, R_1, R_2, R_3 = H	Indigo
R_1, R_2 = H, R, R_3 = $NaSO_3$	Indigo carmine
R_1 = Br, R, R_2, R_3 = H	6-Bromoindigo
R_1, R_2 = Br, R, R_3 = H	6,6'-Dibromoindigo
R, R_1, R_2, R_3 = Br	5,7,5',7'-Tetrabromoindigo

Figure 4 Chemical structures for indigo and its colorant derivatives.

The chemical structure of indigo features a planar conjugation system of adjacent double bonds, which contributes to its distinctive blue color (λ_{max} = 613 nm). The modification of indigo molecule provides many different derivative colorants, such as indigo carmine, thioindigo, tyrian purple (6,6'-dibromoindigo. 6-bromoindigo), and ciba blue (5,7,5′,7′-tetrabromoindigo). Indigo carmine is the salt form of chemically synthesized indigo. It was listed as one of the rare blue colorant, FD&C Blue No. 2, applied in food and cosmetic industry[10].

Introduction of indigoidine.

Indigoidine (5,5′-diamino-4,4′-dihydroxy-3,3′-diazadiphenoquinone-(2,2′)) is a non-ribosomal peptide synthetase (NRPS)-associated natural product. It features a distinct blue color similar to indigo, thus named as indigodine. While chemically synthesized indigo might bring health risks, the naturally produced indigoidine has an advantage of antioxidant and anti-microbial activities [11] [12]. Owing to the highly oxidized and conjugated system in the chemical structure, indigoidine acts as scavenger for free radicals, hence is less stable in liquid, especially at high temperature, which makes it degradable and less-threatening to

the water system. These properties make indigoidine a promising excellent substitution of indigo as blue dye.

Due to its bright blue color, the responsible indigoidine synthetase gene has been introduced to both *E. coli* and mammalian cells to construct reporter system, which has been used as a visual indicator to high-throughput screen gene expression. This was the first report that NRP could be produced by engineered mammallian cells [13]. The indigoidine synthetase BpsA has also been developed as a reporter to screen PPTase and PPTase inhibitors [14], as well as PPTase-associated natural product biosynthetic genes [15].

Previous studies showed that the indigoidine synthetase were present in different strains (*Table 1*). However, this gene was silent in some of these strains, while other bacteria had very limited production of indigoidine. Through regulatory gene manipulation and heterologous expression, activation and enhancement of indigoidine production can be achieved. To activate the indigoidine NRPS, a 4'-phosphopantetheinyl transferase (PPTase) is required, which transforms the indigoidine synthetase to the holo form by adding the phosphopantetheinyl group from coenzyme A (CoA) to the T domain [13 16]. It was proved that the disruption of *farR2* gene could lead to delayed production of indigoidine in *Streptomyces lavendulae* FRI-5 [17], and the disruption of either *pecS* or *pecM* in *Streptomyces lavendulae* gene cluster could repress the indigoidine production [11 18]. By replacing the promotor of ORF with strong constitutive *rpsM* promoter, the silent *Sc-indC* gene in *Photorhabdus luminescens* was successfully activated to produce the blue pigment [16].

The indigoidine synthetase BpsA contains one single module, which is predicted to consist of an adenylation (A) domain, thiolation (T) domain, thioesterase (TE) domain, and a special oxidation (Ox) domain integrated in the A domain. Sc-IndC has the same domain organization except that it has an additional C-terminal 4-oxalocrotonate tautomersase (4-OT) domain. The native substrate L-glutamine (L-Gln) is activated by the A domain as amino acyl adenylates (acyl-adenylated L-Gln), and transferred to the activated T domain, where it is hydrolyzed and cyclized by the TE domain to form and release the intermediate 5-aminopiperidine-2,6-dione. The single molecule is further oxidized by the Ox domain, and dimerized in air to yield indigoidine [11, 19]. However, it is also possible that the oxidation happens when the substrate is linked to the T-domain [19]. In this case, T-tethered L-Gln is firstly oxidized by the Ox domain, and then cyclized and released from the enzyme by the TE domain [20].

Table 1 Strains possessing an indigoidine biosynthetic gene cluster.

Strains	Ref.
Arthrobacter atrocyaneus	[18]
Arthrobacter crystallopoietes	[21]
Arthrobacter polychromogenes	[22]
Corynebacterium insidiosum	[23]
Erwinia chrysanthemi	[11 18 24 25]
Phaeobacter sp.	[12]
Photorhabdus luminescens	[16]
Pseudomonas indigofera	[26]

Streptomyces lavendulae	[13 17 19]
Streptomyces aureofaciens	[27]
Streptomyces chromofuscus	[28]

1.2 Objectives

To be used in the industry, efficient production of the indigoidine blue dye is necessary [29]. In our previous study, a putative indigoidine synthetase gene (*Sc-indC*) was cloned from *Streptomyces chromofuscus* ATCC 49982 [28]. Heterologous expression of *Sc-indC* in *E. coli* achieved indigoidine production of 20 g/L under optimized conditions. Reportedly, fed-batch fermentation of an engineered fungi *Rhodosporidium toruloides* with *bpsA* also produced indigoidine reaching titers of 86.3 ±7.4 g/L[30]. Exploring the mechanisms of indigoidine synthesis would potentially open new doors to further improve the production or create new indigoidine derivatives. Though arduous studies have been done on NRPSs, the synthetic mechanism of indigoidine through such a simple mono-module NRPS is still unknown. To better understand the enzymatic mechanism of NRPSs, this study was aimed to explore the biosynthetic process of indigoidine by working on *bpsA* and *Sc-indC*.

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CHAPTER II MATERIALS AND METHODS

2.1 General Methods

Products were analyzed and purified on an Agilent (Santa Clara, CA, USA) 1200 HPLC instrument equipped with a UV detector and a C18-reversed phase HPLC column (4.6 × 250 mm, 50 μm KANTO Reagents, Japan). ESI-MS spectra were obtained on an Agilent 6130 quadrupole LC-MS. NMR spectra were recorded on a Bruker (Billerica, MA, USA) NMR instrument (500 MHz for ^{1}H NMR). The chemical shift (δ) values were given in parts per million (ppm). The coupling constants (*J* values) were reported in Hertz (Hz).

2.2 Strains, Media and Culture Conditions

Streptomyces chromofuscus ATCC 49982 and *Streptomyces lavendulae* ATCC 11924 were obtained from the American Type Culture Collection (ATCC). They were grown at 30℃ in YEME medium [1] for the extraction of genomic DNA. *E. coli* XL1-Blue was used for routine cloning and amplification; *E. coli* BAP1 was used for protein expression. They were grown at 37℃ on Luria–Bertani (LB) agar plates or in liquid LB medium supplemented with appropriate antibiotics (ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; streptomycin, 50 μg/ml). When OD_{600} value reached 0.4~0.6, the expression host *E. coli* BAP1 was induced with 200 μM of isopropyl-1-thio-β-D-galactopyranoside (IPTG), and incubated at 18℃ or 28℃ for enzyme expression.

2.3 Gene Manipulation and Plasmid Construction

Restriction enzymes, Phusion® High-Fidelity DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA).

The GeneJET™ Plasmid Miniprep Kit and GeneJET™ PCR Purification Kit were purchased from Fermentas (Glen Burnie, MD, USA) for plasmids extraction and gel purification. The CloneJET™ PCR Cloning Kit was purchased from Fermentas (Glen Burnie, MD, USA) for DNA cloning and sequencing. The pET28a and pCDFDuet-1 vectors were purchased from Novagen (Madison, WI, USA) for protein expression in *E. coli* BAP1. The *Sc-indC* gene [2] and *bpsA* gene [3] were cloned from the genome of *Streptomyces chromofuscus* ATCC 49982 and *S. lavendulae* ATCC 11924, respectively. The primers for PCR amplification were shown in *Table 2*. The PCR products were gel purified and ligated into the pJET1.2 cloning vector to yield pFC12 and pFC13, respectively (*Table 3*). The genes were confirmed by digestion check and DNA sequencing.

The genes were excised from pFC12 and pFC13 via NdeI/HindIII, and ligated to pET28a between the same sites to form pFC15 and pFC14 (*Table 3*). The *Sc-indC* and *bpsA* related gene fragments were amplified by PCR from pFC15 and pFC14 using corresponding primers (*Table 2*). Similarly, those genes were gel purified and ligated into the pJET1.2 for amplification (*Table 3*). After confirmed by digestion check and DNA sequencing, they were excised from pJET1.2-derived plasmids, and ligated to pET28a or pCDFDuet-1 (*Table 3*). The *Sc-indC-A-Ox*, *bpsA-TE*, and *bpsA-Ox* genes were amplified by PCR and purified with the PCR purification Kit. After digesting with appropriate sites, they were directly ligated to pET28a to yield pFC147, pFC146, and pFC148, respectively.

The primers were designed based on the *Sc-indC* and *bpsA* sequences and synthesized by Invitrogen (Carlsbad, CA, USA).

Table 2 Primers used for PCR amplification in this study.

Primer	Sequence (5′-3′)	Gene size	Product
Sc-indC-PacI-NdeI-5	TTAATTAACATATGAGCGTAGAGACCATCCCCTGCT	4,134 bp	*Sc-indC*
Sc-indC-HindIII-NheI-3	AAGCTTGCTAGCTTTCAGTAGTTGGGCGTCTTGC		
Sc-indC-PacI-NdeI-5	TTAATTAACATATGAGCGTAGAGACCATCCCCTGCT	3,084 bp	*Sc-indC-A-Ox-T*
Sc-indC-T-XhoI-HindIII-stop-3	AACTCGAGAAGCTTTCAGCCCCTGCCCTCGGGCTGGAG		
Sc-indC-PacI-NdeI-5	TTAATTAACATATGAGCGTAGAGACCATCCCCTGCT	2370 bp	*Sc-indC-A-Ox*
Sc-indC-Ox-long-XhoI-stop-3	AACTCGAGTCAGACCAGGCTGATACCGAA		
Sc-indC-TE-NdeI-5	AACATATGGAGGCCACGTCACCCCGGC	1110 bp	*Sc-indC-TE*
Sc-indC-HindIII-NheI-3	AAGCTTGCTAGCTTTCAGTAGTTGGGCGTCTTGC		
Sc-indC-T-NdeI-5	AACATATGCAGACCACTCAGGAAGCCCAACTGACC	1374 bp	*Sc-indC-T-TE*
Sc-indC-HindIII-NheI-3	AAGCTTGCTAGCTTTCAGTAGTTGGGCGTCTTGC		
Sc-indC-Ox-NdeI-5	AACATATGCGCGACGAACTCGGTACGAT	435 bp	*Sc-indC-Ox*
Sc-indC-Ox-XhoI-3	AACTCGAGGAAGCTGCCCAGGTAGTGGT		
Sc-indC-Ox-long-NdeI-5	AACATATGCTGGCCCACCCCGGCTGCC	873 bp	*Sc-indC-long Ox*
Sc-indC-Ox-long-XhoI-stop-3	AACTCGAGTCAGACCAGGCTGATACCGAA		
Sc-indC-TE-NdeI-5	AACATATGGAGGCCACGTCACCCCGGC	888 bp	*Sc-indC-TE-2*
Sc-indC-TE-XhoI-stop-3	AACTCGAGTCACACTCCGACCTCCTGGACC		
BpsA-NdeI-5	AACATATGACTCTTCAGGAGACCAGCGTGCTC	3849 bp	*BpsA*
11924-*BpsA*-HindIII-3	AAAAGCTTCTCGCCGAGCAGGTAGCGGATGTG		
BpsA-Ox-long-NdeI-5	AACATATGAAGGCGCAGCTGTCCAACCCGG	900 bp	*BpsA-long Ox*
BpsA-Ox-long-XhoI-stop-3	AACTCGAGTCAGTACTTCAGCCACTCCTCC		
BpsA-Ox-NdeI-5	AACATATGTACTCGCGCAAGGCGGCCGACC	450 bp	*BpsA-Ox*
BpsA-Ox-XhoI-stop-3	AACTCGAGTCAGTAGTAGTCCTCGTCGGCG		
BpsA-TE-NdeI-5	AACATATGCTGGAGCGCGAGGTCGCCCAG	828 bp	*BpsA-TE*
11924- *BpsA*-HindIII-3	AAAAGCTTCTCGCCGAGCAGGTAGCGGATGTG		

BpsA-TE-NcoI-5	AACCATGGTGCTGGAGCGCGAGGTCGCCCAG	828 bp	*BpsA-TE-2*
11924- *BpsA*-HindIII-3	AAAAGCTTCTCGCCGAGCAGGTAGCGGATGTG		
BpsA-T-BamHI-NdeI-5	AAGGATTCCATATGGCCGCCTCCGACCAGGTCAAC	327 bp	*BpsA-T*
BpsA-T-HindIII-EcoRI-3	TTGAATTCAAGCTTAGGCCTTGCCGGTCTCCGCGTGCAG		
BpsA-NdeI-5	AACATATGACTCTTCAGGAGACCAGCGTGCTC	3087 bp	*BpsA-A-Ox-T*
BpsA-T-HindIII-EcoRI-3	TTGAATTCAAGCTTAGGCCTTGCCGGTCTCCGCGTGCAG		
BpsA-T-BamHI-NdeI-5	AAGGATTCCATATGGCCGCCTCCGACCAGGTCAAC	1089 bp	*BpsA-T-TE*
11924-*BpsA*-HindIII-3	AAAAGCTTCTCGCCGAGCAGGTAGCGGATGTG		
BpsA-NdeI-5	AACATATGACTCTTCAGGAGACCAGCGTGCTC	2832 bp	*BpsA-A-Ox*
BpsA-A-Ox-HindIII-3	ATAAGCTTCTCCGTCTCCGTGCGCGGGGCGACGAAG		

Note: the enzyme digestion sites are underlined; “bp” refers to “base pair”.

Table 3 Plasmids constructed in this study.

Plasmid	Description	Ligation sites
pFC12	*Sc-indC* in pJET1.2	-
pFC13	*BpsA* in pJET1.2	-
pFC14	*BpsA* in pET28a	NdeI/HindIII
pFC15	*Sc-indC* in pET28a	NdeI/HindIII
pFC135	*Sc-indC-Ox* in pJET1.2	-
pFC136	*Sc-indC-Ox* in pET28a	NdeI/XhoI
pFC137	*Sc-indC-TE* in pJET1.2	-
pFC138	*Sc-indC-long Ox* in pJET1.2	-
pFC139	*Sc-indC-TE* in pET28a	NdeI/XhoI
pFC140	*Sc-indC-long Ox* in pET28a	NdeI/XhoI
pFC146	*BpsA-TE* in pET28a	NdeI/XhoI
pFC147	*Sc-indC-A-Ox* in pET28a	NdeI/XhoI
pFC148	*BpsA-Ox* in pET28a	NdeI/HindIII
pYC41	*BpsA-A-Ox* in pJET1.2	-
pYC42	*BpsA-A-Ox* in pET28a	NdeI/HindIII
pYC43	*BpsA-A-Ox-T* in pJET1.2	-
pYC44	*BpsA-T* in pJET1.2	-
pYC45	*BpsA-T-TE* in pJET1.2	-
pYC46	*BpsA-T-TE* in pCDFDuet-1	NdeI/XhoI
pYC47	*BpsA-TE* in pCDFDuet-1	NdeI/XhoI
pYC48	*BpsA-A-Ox-T* in pET28a	NdeI/HindIII
pYC49	*BpsA-T* in pET28a	NdeI/HindIII
pYC50	*Sc-indC-A-Ox* in pJET1.2	-
pYC51	*Sc-indC-T-TE* in pJET1.2	-
pYC52	*Sc-indC-TE* in pJET1.2	-
pYC53	*Sc-indC-A-Ox-T* in pJET1.2	-
pYC54	*BpsA-TE-2* in pJET1.2	-
pYC55	*Sc-indC-A-Ox* in pCDFDuet-1	NdeI/XhoI
pYC56	*Sc-indC-T-TE* in pET28a	NdeI/HindIII
pYC57	*Sc-indC-TE-2* in pET28a	NdeI/HindIII
pYC58	*Sc-indC-A-Ox-T* in pCDFDuet-1	NdeI/XhoI
pYC59	*BpsA-TE-2* in pCDFDuet-1	NcoI/HindIII

Table 4 *E. coli* BAP1 transformants and screening antibiotics.

Transformants	Description	Antibiotics	Protein/domains
1	pFC14	K	BpsA
2	pFC15	K	Sc-IndC
3	pFC136	K	Sc-IndC-Ox
4	pFC139	K	Sc-IndC-TE
5	pFC140	K	Sc-IndC-long Ox

6	pFC146	K	BpsA-TE
7	pFC147	K	IndC-A-Ox
8	pFC148	K	BpsA-Ox
9	pYC42	K	BpsA-A-Ox
10	pYC46	S	BpsA-T-TE
11	pYC42/pYC46	K + S	BpsA-A-Ox/BpsA-T-TE
12	pYC47/pYC48	K + S	BpsA-A-Ox-T/BpsA-TE
13	pYC46/pYC48	K + S	BpsA-A-Ox-T/BpsA-T-TE
14	pYC48	K	BpsA-A-Ox-T
15	pYC49	K	BpsA-T
16	pYC48/pYC59	K + S	BpsA-A-Ox/BpsA-T
17	pYC55/pYC56	K + S	Sc-IndC-A-Ox/ Sc-IndC-T-TE
18	pYC57/pYC58	K + S	Sc-IndC-TE/ Sc-IndC-A-Ox-T

Note: "K" refers to kanamycin, "S" refers to streptomyces.

2.4 Gene Expression in *E. coli* BAP1 and Protein Purification

The pET28a and pCDFDuet-1-derived plasmids were expressed or co-expressed in *E. coli* BAP1 (*Table 4*). Correct transformants 1-18 were selected on LB agar supplemented with corresponding antibiotics, and then grown in 50 ml of LB broth supplemented with appropriate antibiotics at 37℃ and 250 rpm. When the OD_{600} reached 0.4~0.6, IPTG was added at a final concentration of 200 μM to induce the expression at 18℃ or 28℃ for 18-20 hours.

2.5 SDS-PAGE Analysis of Protein Expression and Purification

After expression, the cells were collected by centrifugation at 2,100×*g* for 10 minutes and resuspended in 5 ml of lysis buffer (20 mM Tris–HCl, 500 mM NaCl, pH 7.9). After 5 minutes of ultrasonication (18 W, 45-second interval in every 15-second), the resulting lysates were centrifuged at 21,000×*g* for 30 minutes. The isoluble proteins were dissolved in 8 M urea. Both soluble and insoluble fractions (designated as sample S and sample IS) were analyzed by 10-12 % SDS-PAGE.

The soluble proteins were mixed with 200 μl of HisPur Ni-NTA Resin (Thermo Fisher Scientific, Waltham, MA, USA), and incubated at 4℃ for 5 hours, and then the mixture was loaded to the gravity-flow column to drain out, which was how we got sample F. The packed resin was washed with four resin-bed volumes of buffer A (50 mM Tris–HCl, 2 mM EDTA, pH 7.9) (sample A), 25 mM imidazole-buffer A (sample 25), and 50 mM imidazole-buffer A (sample 50) in a series, and the His-tagged proteins were eluted from the resin with two resin-bed volumes of 250 mM imidazole-buffer A (sample 250). Each flow-through was collected in a separate tube and subjected to SDS-PAGE to monitor the protein elution and purity. The target protein elution was condensed with centrifugal filter units (Sigma-Aldrich, St. Louis, MO, USA) at 2,100×*g*, and stored in the PB reaction buffer (50 mM Na_2HPO_4, 50 mM KH_2PO_4) with 50% v/v glycerol at -20℃.

2.6 Chemical Synthesis of Aminoacyl-N-acetylcysteamine Thioesters: L-glutamine-SNAC

We modified a previous method [4] and chemically synthesized L-glutamine-SNAC (L-Gln-SNAC) (see *Figure 5*) as a mimicking substrate for *in vitro* enzymatic assays. In step 2, the synthesized aminoacyl-SNAC and Boc-aminoacyl-SNAC were purified by HPLC with CH_3CN/H_2O/formic acid 5:95:0.1, and L-Gln-SNAC was purified by HPLC with CH_3CN/H_2O/formic acid 2:98:0.1.

Step 1. Synthesis of SNAC

- 1.14 g cysteamine hydrochloride
- 0.56 g Potassium hydroxide (KOH)
- 2.52 g Sodium bicarbonate ($NaHCO_3$)
- 50 ml double-distilled water (H_2O)
- Dropwise addition of 0.95 ml acetic anhydride

↓ Stir for 10 min at 25°C

Add HCl to adjust pH to 7.3

↓ Extracted with 150 ml ethyl acetate

Add $MgSO_4$ to dry

↓ Vacuum rotary evaporated

Obtain N-acetylcysteamine

Step 2. Synthesis of aminoacyl-SNAC

- 206.3 mg N,N'-Dicyclohexylcarbodiimide (DCC)
- 135.1 mg Hydroxybenzotriazole (HOBt)
- 15 ml Tetrahydrofuran (THF)
- 119.2 mg N-acetylcysteamine

↓ Stir for 45 min at 25°C

Add 69 mg Potassium carbonate (K_2CO_3)

- Stir for 3h at 24°C
- Filtered and rotary evaporated
- Extracted with 50 ml ethyl acetate
- Washed once with 50 ml10% $NaHCO_3/H_2O$

Organic phase dried with Na_2SO_4

- Vacuum rotary evaporated
- HPLC purification

Obtain Boc-aminoacyl-SNAC

↓

Add 15 ml Trifluoroacetic acid/Dichloromethane (TFA/CH_2Cl_2 1:1)

- Stand at 25°C for 1 hour
- Vacuum rotary evaporated

Twice taken up in CH_2Cl_2 and concentrated to remove TFA

- Vacuum rotary evaporated
- HPLC purification

Afford *L*-glutamine-SNAC

Figure 5 Protocol for chemical synthesis of L-glutamine-SNAC.

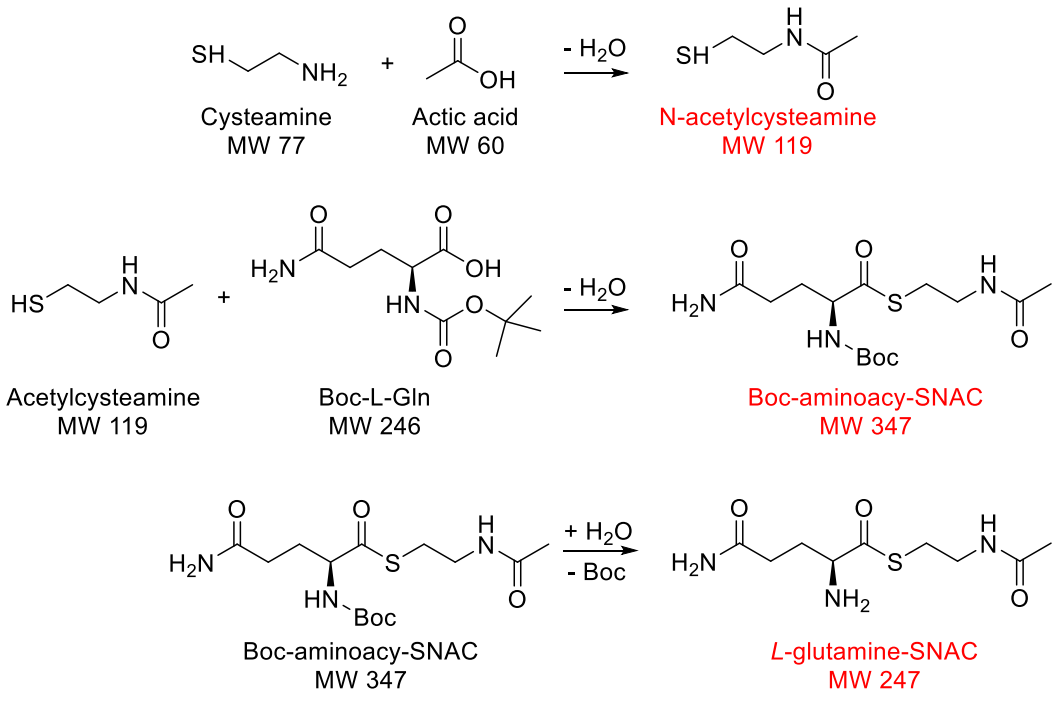


Figure 6 Schematic diagram for chemical synthesis of L-glutamine-SNAC.

2.7 Identification of the Substrate and Intermediates

Characterization of L-Gln: ^{1}H NMR (D_2O): δ 3.77 (1H, dd, *J* = 4.20, 7.99 Hz), 2.46 (2H, m), 2.14 (2H, dd, *J* = 6.98, 13.94 Hz).

Characterization of L-Gln-SNAC: ^{1}H NMR (D_2O): δ 4.36 (1H, dd, *J* = 5.45, 13.44 Hz), 2.87 (2H, m), 2.43 (1H, m), 2.22 (1H, m); ESI-MS: $[M+H]^+$ m/z 248.

Characterization of 3-aminopiperidine-2,6-dione (pyro-Gln): ^{1}H NMR (D_2O): δ 4.39 (1H, t, *J* = 6.28 Hz), 3.45 (2H, m), 3.27 (1H, dt, *J* = 6.35, 13.02 Hz), 3.16 (1H, m), 2.52 (2H, m), 2.31 (1H, td, *J* = 7.09, 14.13 Hz), 2.21 (1H, td, *J* = 7.20, 14.70 Hz), 1.98 (3H, s); ESI-MS: $[M+H]^+$ m/z 129.

2.8 *In vitro* Enzymatic Reactions

The *in vitro* enzymatic reaction system was prepared according to *Table 5*, and reacted at 28℃ for 2 hours. The substrate and intermediates referred to the native substrate L-Gln (Sigma-Aldrich, St. Louis, MO, USA), chemically synthesized mimicking substrate L-Gln-SNAC, and 3-aminopiperidine-2,6-dione (pyro-Gln) (Sigma-Aldrich, St. Louis, MO, USA).

Table 5 Protocol for *in vitro* enzyme assay.

Ingredients	Final concentration
ATP/$MgCl_2$/PB buffer	1 mM/1mM
Substrate/intermediates	1 mM
Enzyme	1–10 μM
PB Buffer, pH7.5	50 mM

2.9 Analysis of Products

The *in vivo* production of indigodine was checked visually or by GENESYS 20 ultraviolet spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The enzymatic reactions were quenched by adding equal volume of methanol. The products were centrifuged at 21,000×*g* for 20 minutes, and the supernatants or precipitates (dissolved with DMSO) were injected into the HPLC with a gradient mobile phase of CH_3CN/H_2O (0.1% formic acid) (0:100 – 90:10, 20 minutes) at 1 ml/minute.

2.10 References

1. Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A., Practical Streptomyces genetics. *The John Innes Foundation, Norwich* **2000**.

2. Yu, D.; Xu, F.; Valiente, J.; Wang, S.; Zhan, J., An Indigoidine Biosynthetic Gene Cluster from *Streptomyces chromofuscus* ATCC 49982 Contains an Unusual IndB Homologue. *Journal of Industrial Microbiology & Biotechnology* **2013,** *40* (1), 159-168.

3. Takahashi, H.; Kumagai, T.; Kitani, K.; Mori, M.; Matoba, Y.; Sugiyama, M., Cloning and Characterization of a Streptomyces Single Module Type Non-ribosomal Peptide Synthetase Catalyzing a Blue Pigment Synthesis. *The Journal of biological chemistry* **2007,** *282*, 9073-9081.

4. Ehmann, D. E.; Trauger, J. W.; Stachelhaus, T.; Walsh, C. T., Aminoacyl-SNACs as Small-Molecule Substrates for the Condensation Domains of Nonribosomal Peptide Synthetases. *Chemistry & Biology* **2000,** *7* (10), 765-772.

CHAPTER III RESULTS

3.1 PCR for Gene Fragments

Plasmid pFC14 and pFC15 were used as template for PCR amplification, and gene fragments *bpsA-A-Ox* (2832 bp), *bpsA-A-Ox-T* (3087 bp), *bpsA-T* (327 bp), *bpsA-T-TE* (1089 bp), *bpsA-TE* (828 bp), *Sc-indC-A-Ox* (2370 bp), *Sc-indC-A-Ox-T* (3084 bp), *Sc-indC-T-TE* (1374 bp), *Sc-indC-TE* (1110 bp) were obtained using corresponding primers listed in *Table 2*.

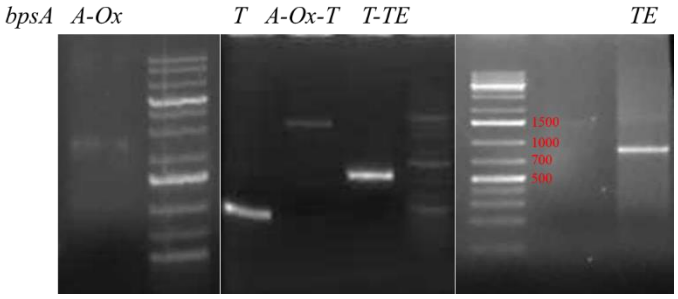


Figure 7 PCR result for dissected *bpsA* gene fragments.

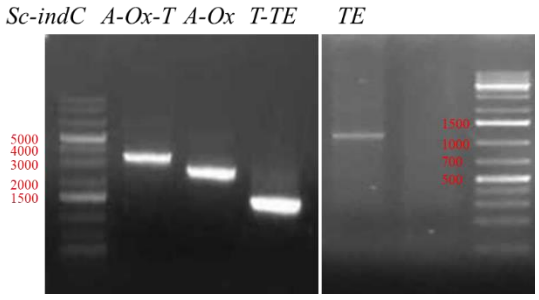


Figure 8 PCR result for dissected *Sc-indC* gene fragments.

3.2 Protein Expression and Purification

Expression plasmids pFC14, 15, 136, 139, 140, 146-148, pYC42, 46-49, 55-59 (*Table 3*) were constructed, and were transferred into *E. coli* BAP1 to get transformants 1-18 (*Table 4*) for protein expression in LB broth. Below are the SDS-PAGE analysis for protein expression and purification.

Expression of pFC14 (BpsA) (18℃).

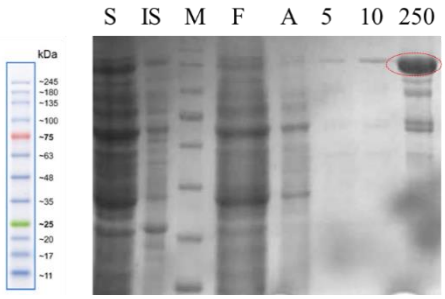


Figure 9 SDS-PAGE for the expression of pFC14 (BpsA).

Note: S-soluble, IS-insoluble, M-protein marker, F-flow through, A-buffer A elution, 5-5 mM imidazole/buffer A elution, 10-10 mM imidazole/buffer A elution, 250-250 mM imidazole/buffer A elution.

Expression (18℃) and purification of pYC49 (BpsA-T).

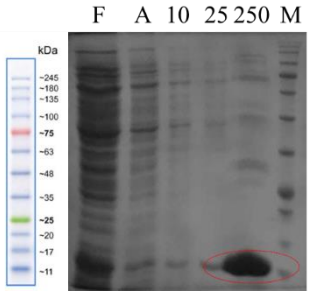


Figure 10 SDS-PAGE for the expression of pYC49 (BpsA-T).

Expression of pFC136 (Sc-IndC-Ox).

The plasmid pFC136 was transferred into various *E. coli* strains including *E. coli* BAP1, *E. coli* BL21(DE3), *E. coli* BL21(DE3)-pLysS, *E. coli* BL21(DE3)-RIL, and expressed at different temperatures (18 or 28℃). However, all showed that the target proteins were in the insoluble parts.

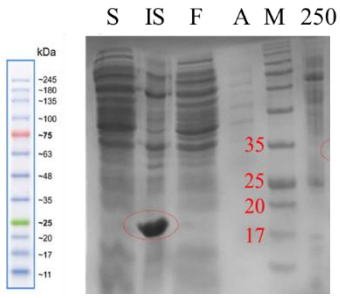


Figure 11 SDS-PAGE for the expression of pFC136 (Sc-IndC-Ox).

Expression (28℃) and purification of pFC139 ((Sc-IndC-TE).

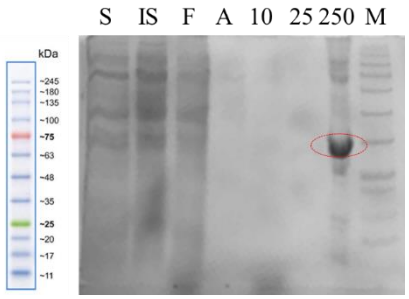


Figure 12 SDS-PAGE for the expression of pFC139 ((Sc-IndC-TE).

Expression (28℃) and purification of pFC146 (BpsA-TE).

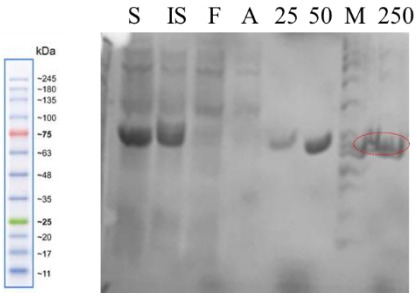


Figure 13 SDS-PAGE for the expression of pFC146 (BpsA-TE).

Expression (18℃) and purification of pFC147 (Sc-IndC-A-Ox).

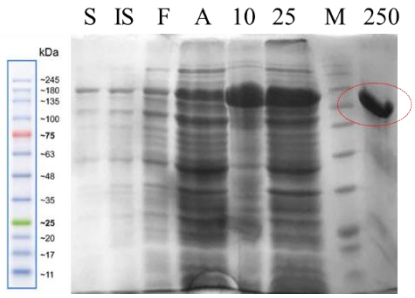


Figure 14 SDS-PAGE for the expression of pFC147 (Sc-IndC-A-Ox).

Expression (28℃) and purification of pFC148 (BpsA-Ox).

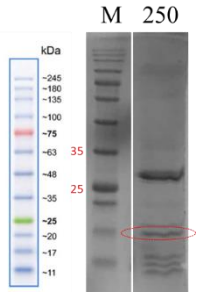


Figure 15 SDS-PAGE for the expression of pFC148 (BpsA-Ox).

Table 6 Expression/purification of proteins and dissected domains.

Plasmid	Protein/Domain	Expression/Purification
pFC14	BpsA	Purified
pFC15	Sc-IndC	Purified
pFC136	Sc-IndC-Ox	Expressed in insoluble
pFC139	Sc-IndC-TE	Purified
pFC146	BpsA-TE	Purified
pFC147	Sc-IndC-A-Ox	Purified
pFC148	BpsA-Ox	Purified
pYC49	BpsA-T	Purified

3.3 Chemical Synthesis of L-Glutamine-SNAC

The synthesis of SNAC provided 400 mg N-acetylcysteamine mixture, which was confirmed by LC-MS.

The product mixture of Boc-aminoacyl-SNAC was subjected to LC-MS to locate the target compound.

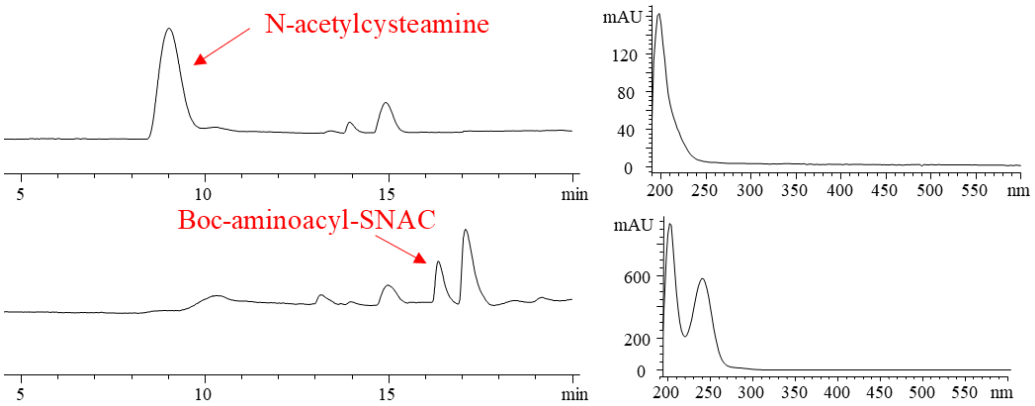


Figure 16 HPLC analysis of the synthesized samples of N-acetylcysteamine and Boc-aminoacyl-SNAC.

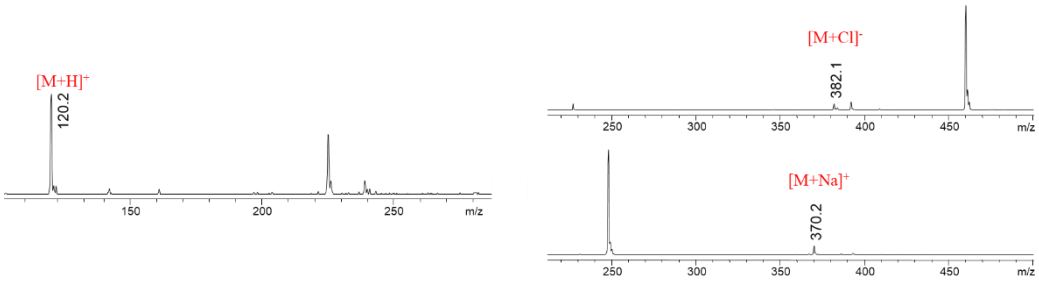


Figure 17 MS spectra of N-acetylcysteamine and Boc-aminoacyl-SNAC.

Boc-aminoacyl-SNAC was then purified with HPLC to afford the single compound for the next deprotecion reaction.

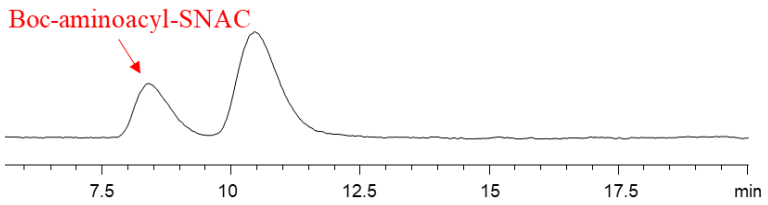


Figure 18 A typical HPLC trace of Boc-aminoacyl-SNAC purification.

After deprotection, the mixture was analyzed by LC-MS to locate the target compound L-Gln-SNAC, which was finally purified with HPLC to yield 5 mg of white powder.

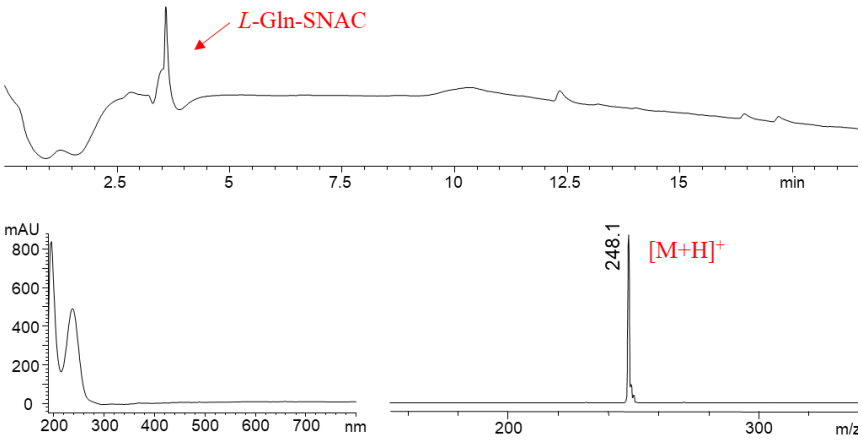


Figure 19 LC-MS analysis of aminoacyl-SNAC in the de-protected mixture. HPLC trace (top), UV spectrum (bottom left), MS spectrum (bottom right).

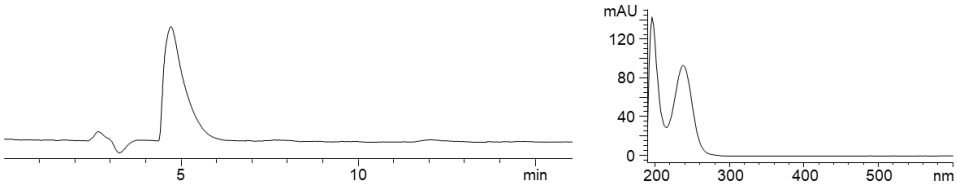


Figure 20 A representative HPLC trace of L-glutamine-SNAC purification.

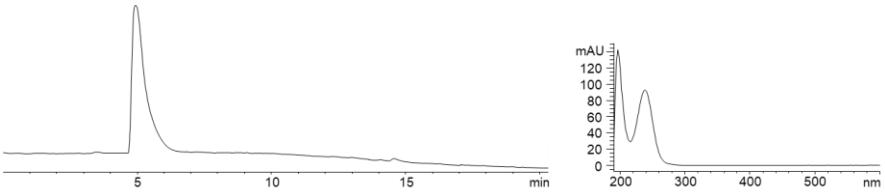
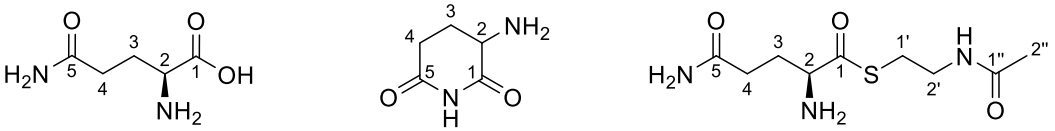


Figure 21 Purity check of L-glutamine-SNAC after purification.

3.4 Characterization of the Substrate and Intermediates



***L*-Glutamine** MW146.15 | **3-aminopiperidine-2,6-dione** MW 128.06 | ***L*-glutamine-SNAC** MW: 247.31

Figure 22 Chemical structures of the substrate and intermediates.

L-Gln, L-Gln-SNAC, and pyro-Gln were characterized by ^{1}H NMR (D_2O).

Table 7 ^{1}H NMR data (500 MHz) for the substrate and intermediates (in D_2O).

Position	L-Gln	L-Gln-SNAC	Pyro-Gln
2 or 3	3.77 (1H, dd, 8.0, 4.2)	4.36 (1H, dd, 13.4, 5.5)	4.39 (1H, t, 6.3)
2 or 3	2.14 (2H, m)	2.43 (1H, m), 2.22 (1H, m)	2.52 (2H, m)
4	2.46 (2H, m)	2.87 (2H, m)	2.31 (1H, td, 14.2, 7.1), 2.21 (1H, td, 14.2, 7.2)
1′ or 2′			3.27 (1H, td, 13.0, 6.4), 3.16 (1H, m)
1′ or 2′			3.45 (2H, m)
2″			1.98 (3H, s)

Characterization of L-glutamine.

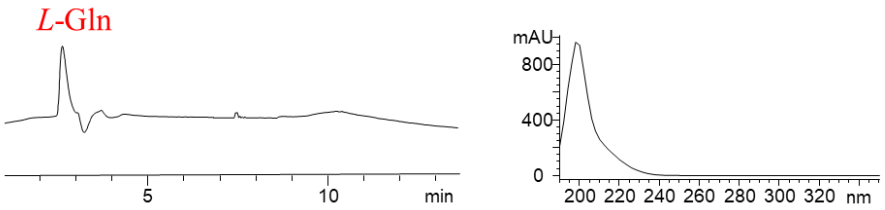


Figure 23 HPLC analysis of L-glutamine.

Characterization of L-glutamine-SNAC.

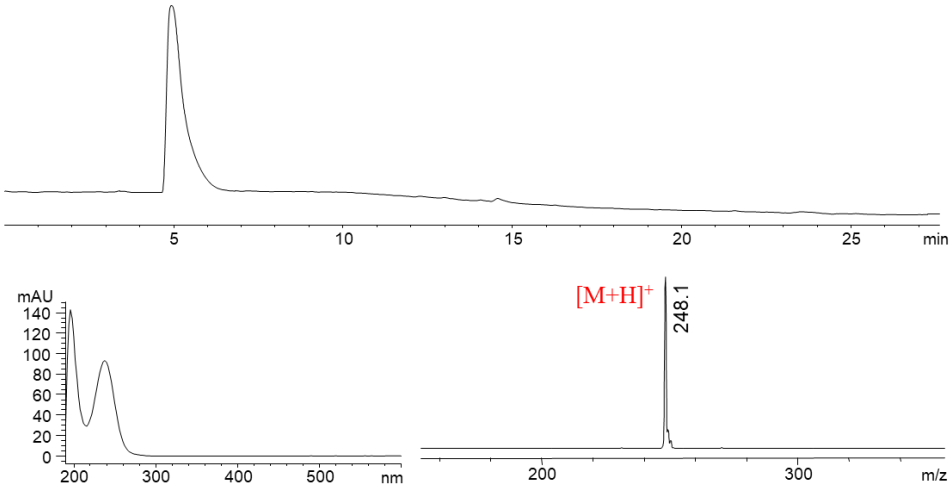


Figure 24 LC-MS analysis of purified L-glutamine-SNAC.

HPLC trace (top), UV spectrum (bottom left), MS spectrum (bottom right).

Characterization of 3-aminopiperidine-2,6-dione.

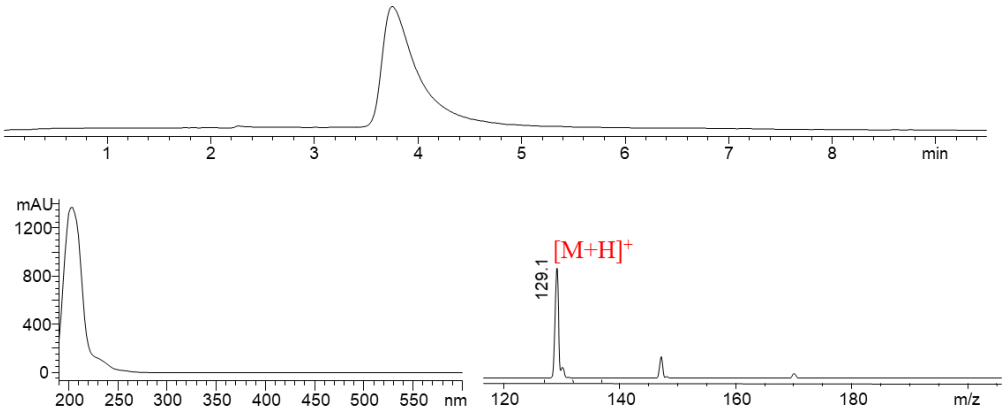


Figure 25 LC-MS analysis of 3-aminopiperidine-2,6-dione hydrochloride.

HPLC trace (top), UV spectrum (bottom left), MS spectrum (bottom right).

3.5 *In vitro* Enzyme Activity and Heterologous Co-expression

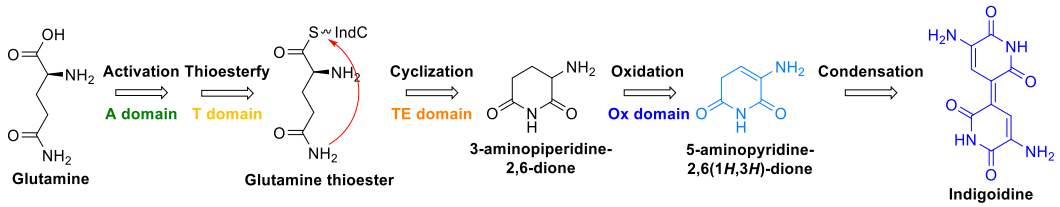


Figure 26 Proposed biosynthetic process of indigoidine.

Enzymatic activity of Sc-IndC and BpsA with L-glutamine, and the effect of reaction temperature (18℃ or 28℃).

It showed that BpsA had higher *in vitro* activity than Sc-IndC, and the effect of the two temperatures on the productivity was not significant (*Figure 27*). Hence the following reactions were conducted at 28℃.

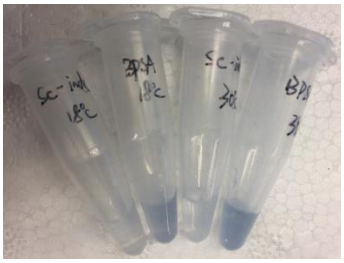


Figure 27 Enzymatic activity of Sc-IndC and BpsA with L-glutamine.

From left to right: Sc-IndC, 18℃; BpsA, 18℃; Sc-IndC, 28℃; BpsA, 28℃.

Enzymatic activity of BpsA with different amounts of L-glutamine and ATP.

The substrate L-Gln was tested at 1, 4, and 8 mM. However, no significant difference was observed, so 1 mM substrate was applied to the following enzymatic reaction system.

Similarly, ATP was tested in the reactions at 0.5, 1, 2, and 4 mM, respectively, and 1 mM ATP worked the best in this system. Higher concentrations of ATP might have inhibited the reaction.

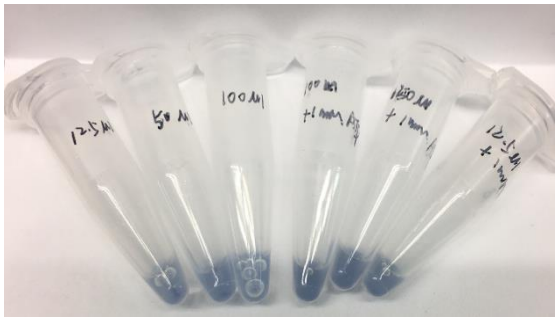


Figure 28 Enzymatic activity of BpsA with different amounts of L-glutamine and ATP.

From left to right: 0.5 mM ATP/1 mM L-Gln; 0.5 mM ATP/4 mM L-Gln; 0.5 mM ATP/8 mM L-Gln; 1 mM ATP/1 mM L-Gln; 1 mM ATP/4 mM L-Gln; 1 mM ATP/8 mM L-Gln.

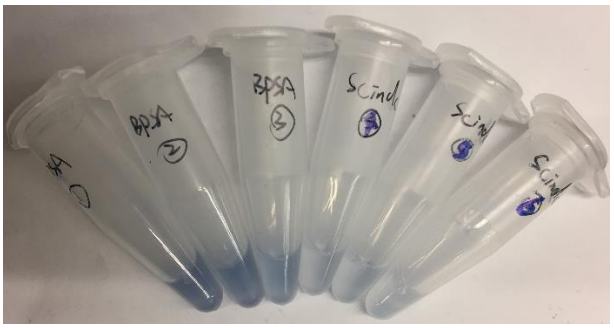


Figure 29 Enzymatic activity of Sc-IndC and BpsA with different amounts of ATP.

From left to right: BpsA/1 mM ATP; BpsA/2 mM ATP; BpsA/4 mM ATP; Sc-IndC/1 mM ATP; Sc-IndC/2 mM ATP; Sc-IndC/4 mM ATP.

Enzymatic activity of BpsA with L-glutamine, 3-aminopiperidine-2,6-dione hydrochloride, and L-glutamine-SNAC.

When BpsA was reacted with L-glutamine, 3-aminopiperidine-2,6-dione hydrochloride, and L-glutamine-SNAC, all the reactions turned blue. HPLC and UV analysis indicated that indigoidine was synthesized, which was further confirmed by MS analysis. These indicated that BpsA not only takes the substrate L-Gln, but also converts the intermediate pyro-Gln and the mimicking intermediate L-Gln-SNAC to the blue dye.

Based on the color intensity, we found that the blue color of the L-Gln group was deeper than that of pyro-Gln and L-Gln-SNAC. Thus, we supposed that though BpsA could convert all these three compounds to indigoidine, it had higher activity on the native substrate, L-Gln.

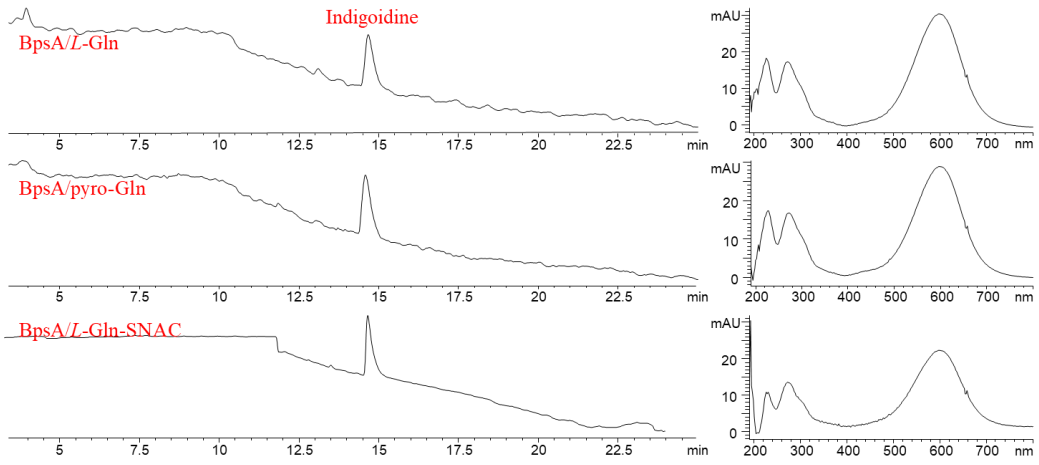


Figure 30 HPLC analysis of the enzymatic reactions of BpsA with L-glutamine, 3-aminopiperidine-2,6-dione hydrochloride, or L-glutamine-SNAC.

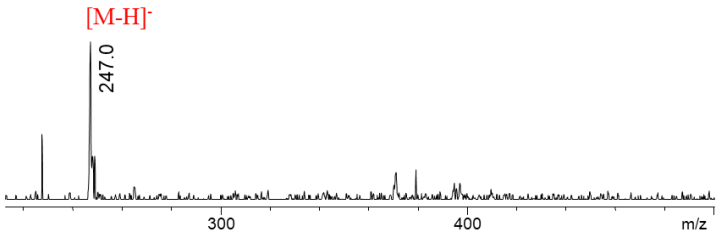


Figure 31 MS spectrum of indigoidine synthesized in the enzymatic reactions.

Interestingly, in the reaction buffer of BpsA with L-Gln-SNAC, except for the production of the blue pigment, the supernatant turned yellowish (as shown in *Figure 33*). LC-MS analysis showed that there was an extra peak of MW 358 produced (*Figure 34* and *Figure 35*). Further work needs to be done to find the structure of this compound.

Without ATP, neither Sc-IndC nor BpsA can convert these substrate/intermediates to indigoidine.

Table 8 Enzymatic reaction of BpsA with L-glutamine, 3-aminopiperidine-2,6-dione hydrochloride, and L-glutamine-SNAC.

Ingredients	Concentration	1	1B	1Δ	2	2B	2Δ
ATP	1 mM			0 mM			0 mM
$MgCl_2$	1 mM						
Substrate	1 mM	L-Gln	L-Gln	L-Gln	pyro-Gln	pyro-Gln	pyro-Gln
BpsA	15 ul		Inactive			Inactive	
PB, pH7.5	50 mM						
Result		**** / /	/ /	/ /	* / /	/ /	/ /
		3	3B	3Δ	4	4B	4Δ
ATP	1 mM			0 mM			0 mM
$MgCl_2$	1 mM						
Substrate	1 mM	L-Gln-SNAC	L-Gln-SNAC	L-Gln-SNAC	L-Gln-SNAC	L-Gln-SNAC	L-Gln-SNAC
BpsA	15 ul		Inactive			Inactive	
PB, pH7.5	50 mM						
Result		** ΔΔ	/ /	/ /	*** ΔΔΔ	/ /	/ /

Note:

"*" Represents there was blue precipitate produced, and the number of "*" indicates the color intensity.

"Δ" Represents the reaction buffer turned yellow, and the number of "Δ" indicates the color intensity.

"1Δ, 2Δ, 3Δ, 4Δ" Indicates there was no ATP added to the reaction buffer.

"/" Indicates no apparent color change was observed.

"Inactive" The BpsA was inactivated by heating at 98℃ for five minutes.

Figure 32 *In vitro* enzymatic reaction of BpsA with L-glutamine, 3-aminopiperidine-2,6-dione hydrochloride, and L-glutamine-SNAC.

Top: Picture for all the reaction groups. Bottom left: picture for Group 1-4.

Bottom right: picture for Group 1-4 after centrifuging at 21,000×*g*.

Enzymatic assay of dissected fragments of the indigoidine synthetase.

With or without ATP or FAD, the TE, Ox, and TE/ Ox domains didn't work on the precursors.

Table 9 *In vitro* enzymatic assay result for the domains′ functional characterization.

Enzyme assay	Enzymes	Color change	LC-MS
pFC139, L-Gln	Sc-indC-TE	/	/
pFC139, L-Gln-SNAC	Sc-indC-TE	/	/
pFC139, pyro-Gln	Sc-indC-TE	/	/
pFC140, L-Gln	Sc-IndC-LOx	/	/
pFC140, pyro-Gln	Sc-IndC-LOx	/	/
pFC146, L-Gln	BpsA-TE	/	/
pFC146, L-Gln-SNAC	BpsA-TE	/	/
pFC146, pyro-Gln	BpsA-TE	/	/
pFC146/pFC147, L-Gln	BpsA-TE, Sc-IndC-A-Ox	/	/
pFC146/pFC147, pyro-Gln	BpsA-TE, Sc-IndC-A-Ox	/	/
pFC147, L-Gln	Sc-IndC-A-Ox	/	/
pFC147, pyro-Gln	Sc-IndC-A-Ox	/	/
pFC148, L-Gln	BpsA-Ox	/	/
pFC148, L-Gln-SNAC	BpsA-Ox	/	/
pFC148, pyro-Gln	BpsA-Ox	/	/

Note: "/" indicates no possible intermediates or final products were detected.

Figure 33 *In vitro* reactions of BpsA or dissected fragments of BpsA/Sc-IndC with L-glutamine-SNAC.

From left to right: BpsA/L-Gln; BpsA/L-Gln-SNAC; BpsA-TE/L-Gln-SNAC; Sc-IndC-A-Ox/L-Gln-SNAC, blank control (without any enzyme).

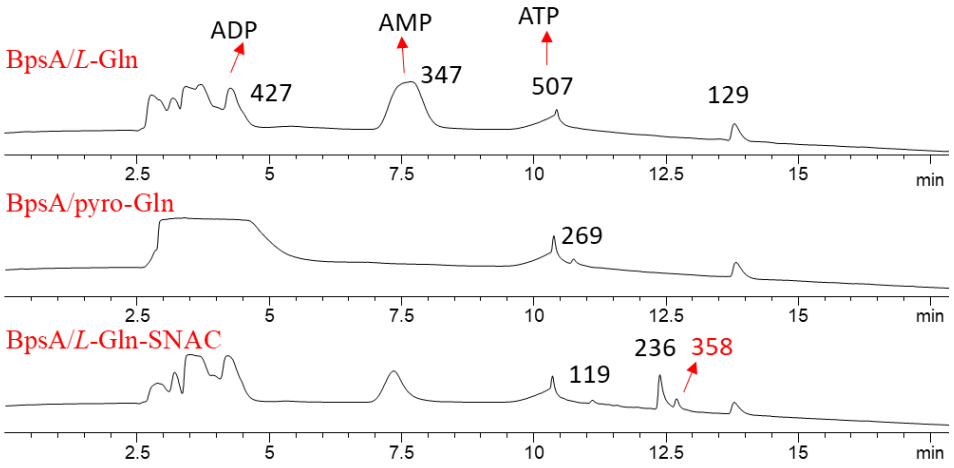


Figure 34 LC-MS analysis of the reaction products in the supernatant.

Possible molecule weights of the peaks were labeled based on the MS analysis.

From top to bottom: BpsA with L-Gln, pyro-Gln, and L-Gln-SNAC.

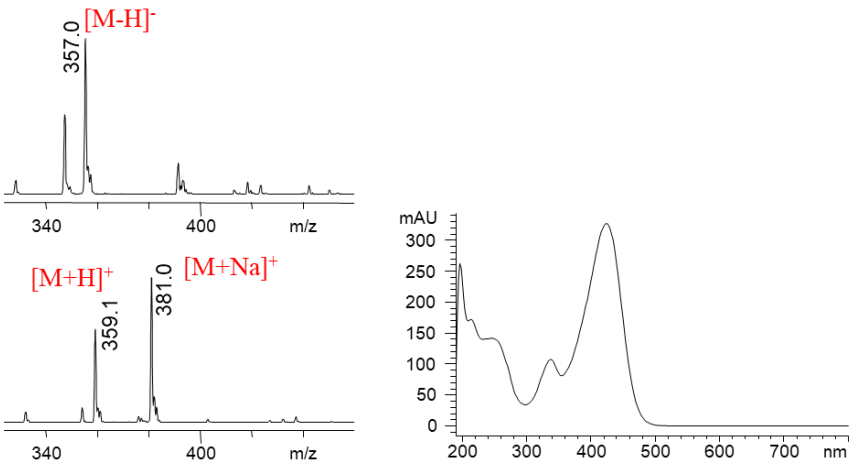


Figure 35 UV and MS spectra for the enzymatic reaction product of molecular weight 358.

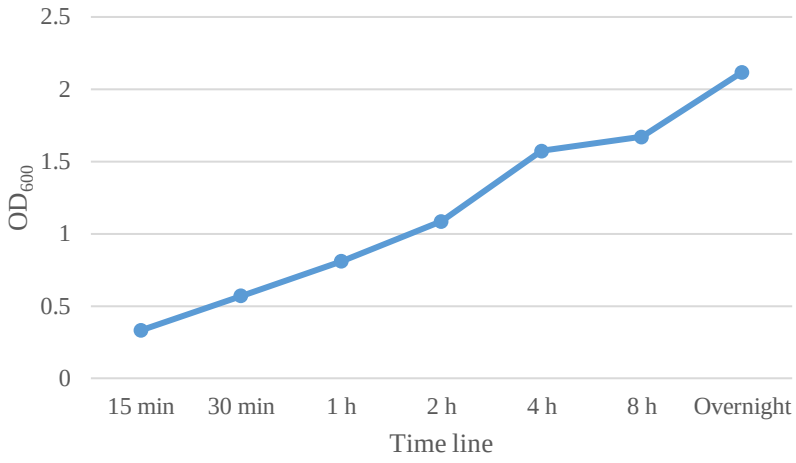


Figure 36 Time course analysis of the *in vitro* reaction of BpsA with L-glutamine.

The time course analysis of the *in vitro* reaction of BpsA with L-Gln showed that the indigoidine production increased with the reaction time.

LC-MS analysis (not shown in this paper) for the supernatant taken at t = 15 minute, 30 minute, 1 hour, and 2 hours showed similar HPLC traces, and no MS signals of expected intermediates pyro-Gln (MW128), oxidized pyro-Gln (126),

and indigoidine (248) were tracked, likely due to the extremely low amounts. Signals of ATP and its derivatives were found (ATP, ADP, and AMP).

Heterologous co-expression.

Heterologous co-expression of transformants 11-13, 16-18 (*Table 4*) in LB at 18℃ or 28℃ didn't get the blue dye, while the positive control *E. coli* BAP1/pFC15 turned deep blue. HPLC analysis of these co-expression experiments also didn't detect any indigoidine in the broth and cells.

CHAPTER IX DISCUSSION AND CONCLUSIONS

We live in a world full of pigments. Chemical synthesis has been a predominant source of pigments in industry, which not only brings serious environmental pollution, but also raises health concerns, especially from the applications in food, medical, and cosmetic industries. An alternative to traditional chemical method is biosynthesis, which is environmentally advantageous with regard to sustainability and less chemical hazards. Biosynthetic natural products have better safety profiles than those chemically synthesized for food and pharmaceutical applications. Hence natural colorants and dyes have been of great interest with the increasing attention to environment protection and human health.

Indigoidine is a bacteria-derived natural blue dye. It has the advantages of antioxidant and anti-microbial activities. Technically, the responsible biosynthetic genes are more compatible to be recombinated in engineering host models for large-scale production. Previously, we expressed *Sc-indC* in *E. coli* BAP1 and reached a production of ~20 g/L using L-glutamine in a batch fermentation. In another case, an engineered yeast with *bpsA* produced indigoidine reaching titers of ~86 g/L using glucose in a fed-batch fermentation. These indicate that indigoidine has great potential to be produced in mass production by biosynthetic approach. To further improve the production, this study dedicated to reveal the biosynthetic process of indigoidine by BpsA and Sc-IndC originated from two different strains.

In this study, both BpsA and Sc-IndC were functionally expressed in *E. coli* BAP1, which confirmed that their catalytic activity can reconstituted *in vitro*. This provided a great platform for the following study on the catalytic domains in these modular NRPSs.

By dissecting the indigoidine synthetic genes *Sc-indC* and *bpsA*, we established a library of biosynthetic bricks with four biosynthetic domains, including A, Ox, T, and TE domain. The dissected domains were co-expressed in *E. coli* BAP1 in different combinations, or expressed individually and purified for *in vitro* enzymatic assay to test their function in the biosynthesis of indigoidine.

The intermediate L-Gln-SNAC, an aminoacyl-N-acetylcysteamine thioester (aminoacyl-SNAC) was chemically synthesized and characterized. *In vitro* enzymatic assay showed that the intact BpsA enzyme could take it as a precursor to synthesize indigoidine.

BpsA, as an intact enzyme, with the presence of ATP, worked on the native substrate, L-Gln, the intermediate, pyro-Gln, and the mimicking intermediate, L-Gln-SNAC, to produce indigoidine. However, no predicted intermediates, according to the proposed biosynthetic pathway from the literature, were detected by LC-MS during the reaction process. Furthermore, in the reaction of BpsA with L-Gln-SNAC, there was a molecule of MW 358 produced, which might contribute to the emerged yellow color in the reaction buffer and might be a possible intermediate produced in the biosynthetic process of indigoidine.

When the indigoidine synthetases were split into domains and worked individually on the native substrate L-Gln or the intermediates, neither putative

intermediates nor the final product indigoidine was detected. Even when various domains were co-existed *in vitro*, no apparent activity was observed. For the co-expression of the combinations of dissected domains A-Ox/T-TE, A-Ox-T/TE, no blue dye was produced, which indicates no apparent activity of the dissected domains neither. It is possible that the *in vitro* reaction system or fermentation condition not benefit the enzymatic activities of the dissected domains. But we cannot exclude the possibility that the truncated and dissected enzymes might have less activity or maybe even inactive. In this case, the reaction methods need to be further optimized. Alternatively, gene deficit or site-directed mutagenesis, rather than dissection and co-expression methods, might be applied to study the biosynthetic mechanism of the indigoidine biosynthetic process.

This study provided important platforms and technical basis for further study on the biosynthetic mechanism of indigoidine. Further work needs to be done to reveal the biosynthetic process and improve the production of indigoidine.

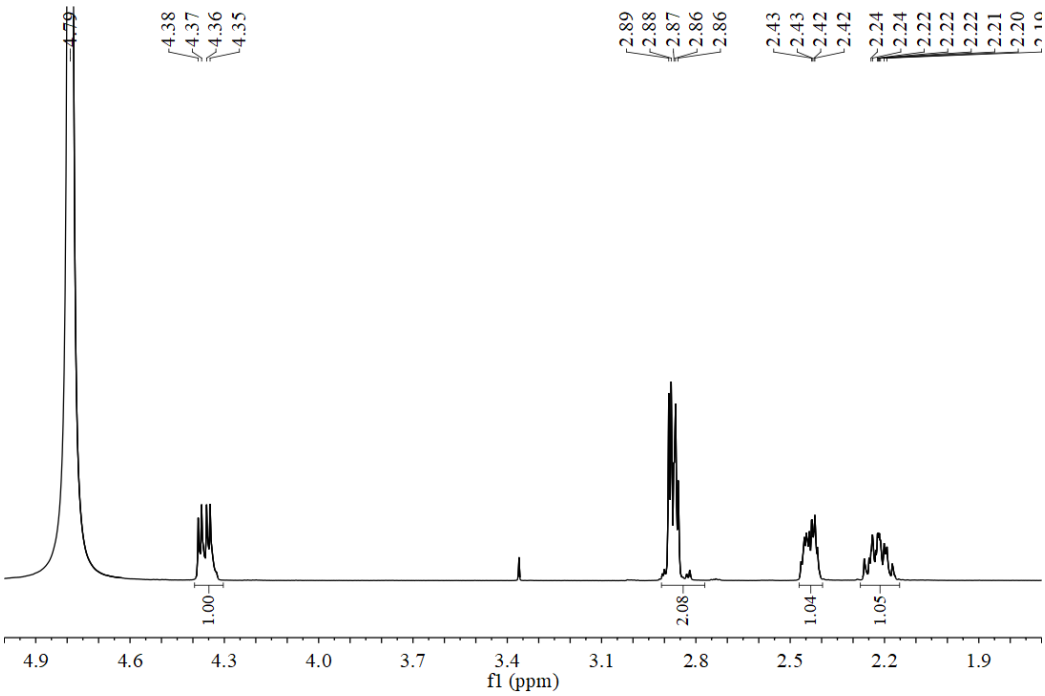


Figure 37 ^{1}H NMR spectrum of L-glutamine (in D_2O).

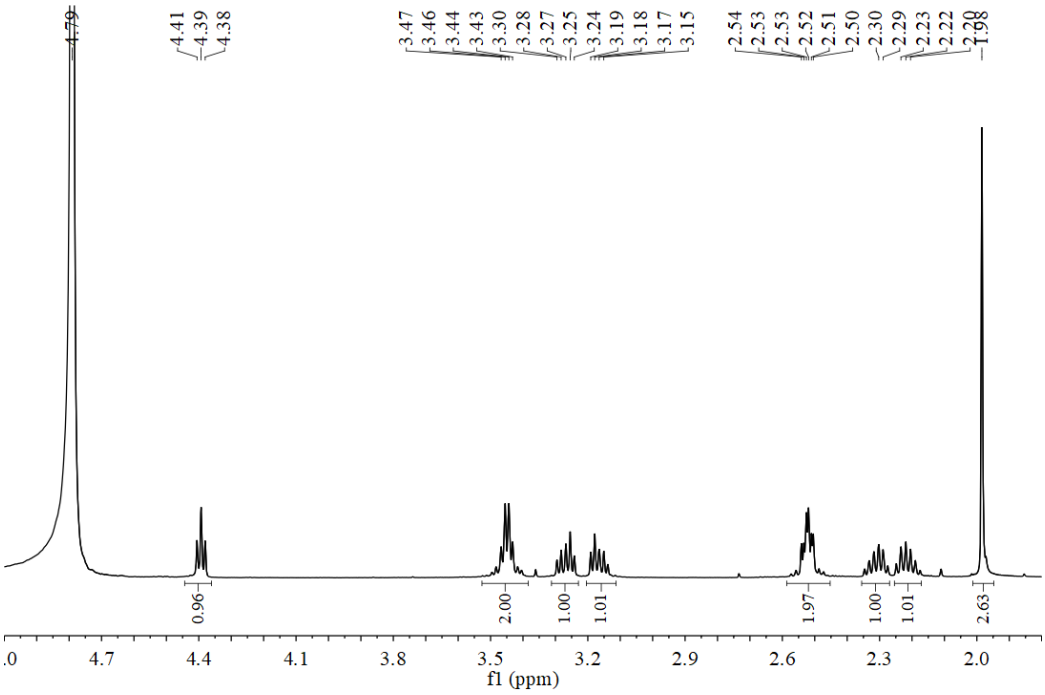


Figure 38 ^{1}H NMR spectrum of L-glutamine-SNAC (in D_2O).

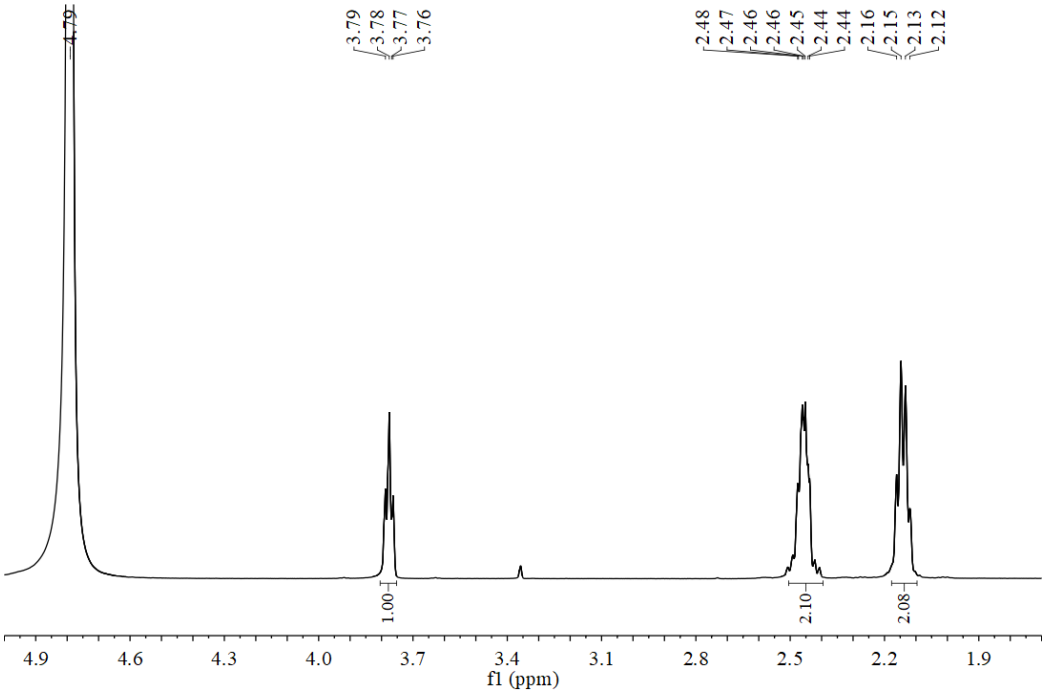


Figure 39 ^{1}H NMR spectrum of 3-aminopiperidine-2,6-dione (in D_2O).

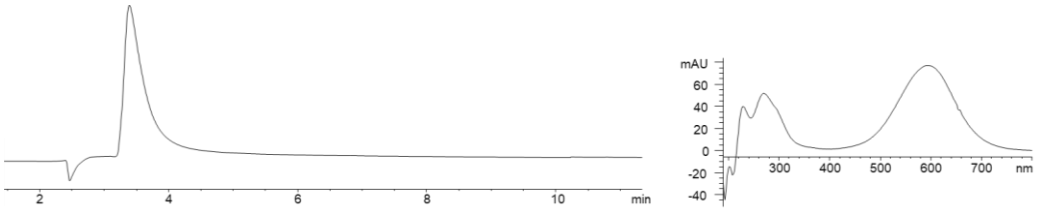


Figure 40 HPLC trace and UV spectrum of indigoidine.