

# Direct formation of chromopyrrolic acid from indole-3-pyruvic acid by StaD, a novel hemoprotein in indolocarbazole biosynthesis

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**Abstract**—Indole-3-pyruvic acid was transformed to chromopyrrolic acid by a novel heme-containing enzyme StaD responsible for staurosporine biosynthetic pathway in *Streptomyces* sp. TP-A0274.  
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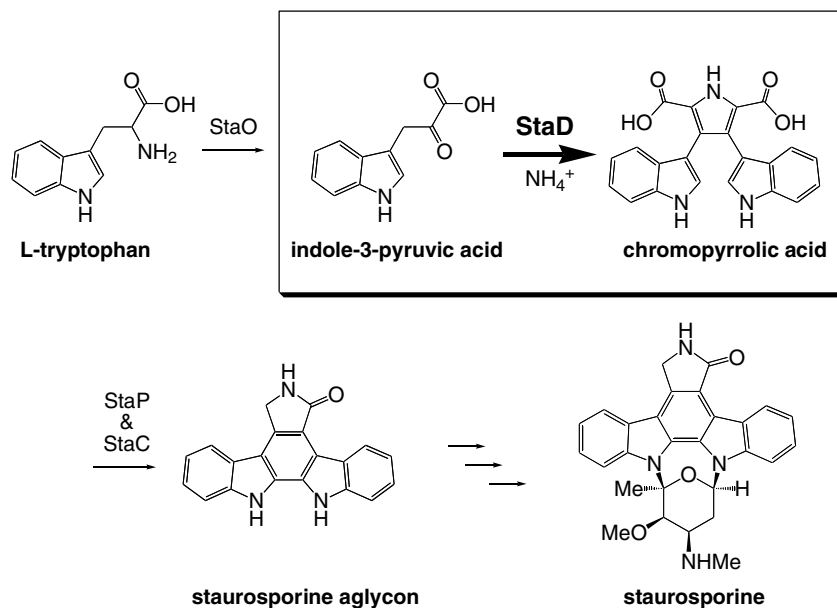
Indolocarbazole alkaloids are members of natural products from actinomycetes. Among them, staurosporine is the most well known and acts as a strong protein kinase inhibitor.<sup>1</sup> Recently, staurosporine and rebeccamycin biosynthetic gene clusters have been cloned and characterized.<sup>2–5</sup> The biosynthetic studies with gene disruption experiments revealed the indolocarbazole skeleton biosynthesis as follows. Two molecules of tryptophan are the precursor of indolocarbazole skeleton. One molecule would be oxidatively deaminated to indole-3-pyruvic acid, and the keto acid has been presumed to be used for the coupling reaction with L-tryptophan to yield chromopyrrolic acid. The sequentially oxidative steps convert chromopyrrolic acid to indolocarbazole skeleton.<sup>2,3</sup> Chromopyrrolic acid is the key intermediate in an indolocarbazole biosynthesis.<sup>2</sup> Characterization of staurosporine biosynthetic gene cluster isolated from *Streptomyces* sp. TP-A0274 suggested that StaD is responsible for the coupling reaction of two molecules of C3 unit containing indole moiety. Here, we functionally expressed the StaD protein in *Escherichia coli* and revealed that it is a tetrameric hemoprotein and catalyzes the coupling reaction with two molecules of indole-3-pyruvic acid and  $\text{NH}_4^+$  to yield chromopyrrolic acid (Scheme 1).

StaD protein was overexpressed in *E. coli* with the His<sub>6</sub>-tagged form at its C-terminus by use of an StaD expression vector, pETStaD<sup>6</sup> in which, the gene was expressed under the control of the T7 RNA polymerase promoter. The active form of StaD could be produced by coexpression with chaperon proteins, GroES and GroEL.<sup>7</sup> We purified StaD with Ni-Sepharose column (Amersham Biosciences Co.) and used for further enzymatic characterization.

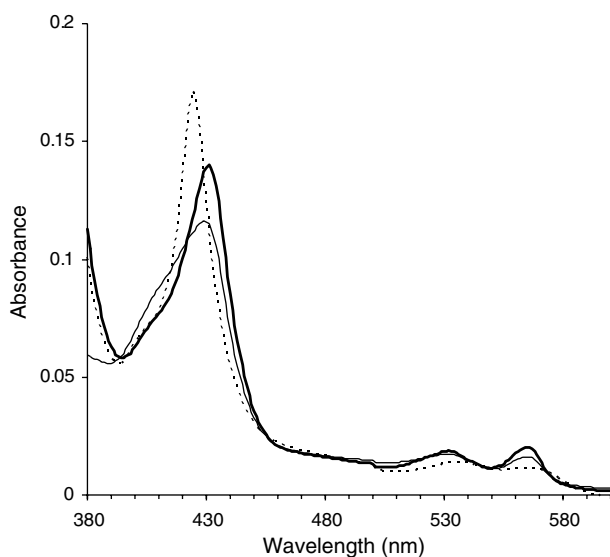
The molecular weight of the StaD was calculated to be 120.5 kDa by the deduced amino acid sequence. The molecular weight of the native enzyme was 420–530 kDa according to gel filtration chromatography by using Superdex 200 10/300 GL (Amersham Bioscience Co.), indicating that the native enzyme is tetramer. The purified StaD protein has a pale orange color, therefore, we suspected that StaD contains heme. Figure 1 shows the absorption spectrum of the enzyme (solid line). It has an absorption maxima (Soret) at 430 nm and smaller absorption bands around 520–580 nm, suggesting that the enzyme contains heme. Reduction of the enzyme with  $\text{Na}_2\text{S}_2\text{O}_4$  resulted in no shift change of the Soret peak (Fig. 2, thick line), indicating that the enzyme has a ferrous form. By bubbling CO gas (Fig. 1, dotted line), the Soret peak was shifted to 424 nm, suggesting that StaD is not a P450 enzyme. To examine whether the heme constitutes an integral part of the purified enzyme, heme-staining was carried out after native PAGE.<sup>8</sup> The gel was stained with 3,3-dimethoxybenzidine/ $\text{H}_2\text{O}_2$  and Western blotting by using His<sub>6</sub>-tag antibody for heme and protein,

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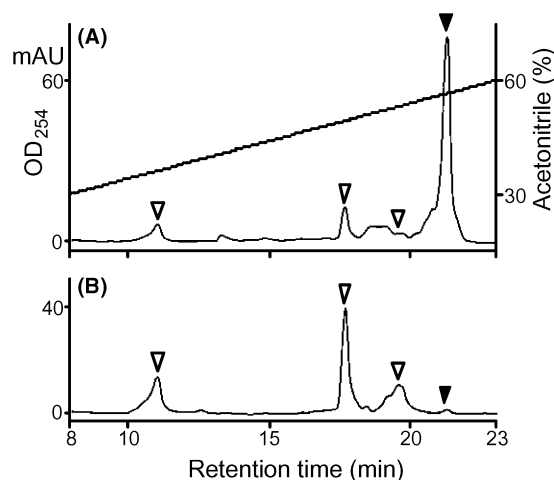
**Scheme 1.** Proposed biosynthetic pathway of staurosporine.



**Figure 1.** Electronic absorption spectra of nontreated (solid line),  $\text{Na}_2\text{S}_2\text{O}_4$ -treated (thick line), and CO-treated (dotted line) forms of StaD.

respectively. Both methods gave a single band, and the positions of the bands were the same (data not shown).

The reaction proceeded by incubation of indole-3-pyruvic acid with  $\text{NH}_4\text{Cl}$ , but did not by incubation of indole-3-pyruvic acid with L-tryptophan, as expected. Incubation of indole-3-pyruvic acid and  $\text{NH}_4^+$  with StaD<sup>9</sup> afforded a new reaction product having its retention time at 21.2 min on HPLC (Fig. 2A). The reaction product was purified<sup>10</sup> and its structure was determined using LC/MS and NMR<sup>11</sup> to be chromopyrrolic acid.<sup>12</sup> Surprisingly, a small amount of chromopyrrolic acid was spontaneously produced by simply incubation of indole-3-pyruvic acid with  $\text{NH}_4^+$  (Fig. 2B). We believe



**Figure 2.** HPLC analysis of the StaD enzyme reaction. Reaction condition was described in Ref. 9. The HPLC profile (A) is for the presence of StaD enzyme, and (B) is absence of the enzyme. Chromopyrrolic acid is indicated by filled arrow head, and indole-3-pyruvic acid is indicated by blank arrow heads. Presumably, because of the coexistence of keto-enol isomer of indole-3-pyruvic acid and its imine form, the substrates were detected as distinct peaks on HPLC analysis. HPLC analysis was performed with an HP1100 (Hewlett–Packard) system by using a COSMOSIL column (4.6 mm i.d.  $\times$  250 mm length, Nacalai Tesque). The temperature was 30  $^\circ\text{C}$ , and the flow rate was 0.7 ml/min. Acetonitrile–0.15%  $\text{KH}_2\text{PO}_4$  (pH 3.5) was used as the solvent, and detection was performed at 254 nm.

that StaD is a unique enzyme which acts as an enhancer for chromopyrrolic acid formation, because  $k_{\text{cat}}/k_{\text{uncat}}$  at 250  $\mu\text{M}$  indole-3-pyruvic acid concentration was 101.2. On the other hand, L-tryptophan did not act as a substrate. These results demonstrated that StaD encodes chromopyrrolic acid synthase accepting indole-3-pyruvic acid as a substrate.  $K_m$  value for indole-3-pyruvic acid was determined to be 235  $\mu\text{M}$  from Hanes–Woolf plots of the kinetic data.<sup>9</sup>

This report is the first example of the functional expression studies of StaD family proteins. To date, only two homologs of StaD have been reported. One is RebD (55% amino acids identity), which is presented in the rebeccamycin biosynthetic gene cluster in *Lechevarielia aerocolonigenes* ATCC39243, the other is VioB (37% identity), which is presented in the violacein biosynthetic gene cluster in *Chromobacterium violaceum*.<sup>13</sup> Proteins belonging to StaD family consist of about 1000 amino acids and the amino acid sequence database search revealed no significant conserved domains between StaD family and other proteins including hemoproteins. It is suggested that the family is a new type of hemoprotein that has a novel structure and function. In our previous report, we proposed that both L-tryptophan and indole-3-pyruvic acid are precursors for coupling reaction by StaD,<sup>2</sup> however, its functional expression elucidated that indole-3-pyruvic acid is solely required by StaD for the formation of chromopyrrolic acid in staurosporine biosynthesis. Detailed characterization of StaD including stoichiometry of the reaction, substrate specifics and reaction mechanisms are in progress.

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- Construction of pETStaD, StaD expression vector.* The GTG start codon of StaD in pSTAI<sup>2</sup> was changed into NdeI site by QuikChange site-directed mutagenesis kit (Stratagene) with the two following primers: StaD-Nde-sense, 5'-CAGGTCGAAGACGCTCATATGGACCGG-TCCGAGCGGC-3'; StaD-Nde-anti, 5'-GCCGCTCGG-ACCGGTCCATATGAGCGTCTTCGACCTG-3'. The mutated plasmid was digested with NdeI and HindIII, and the resulting fragment was inserted between the NdeI and HindIII sites of pET26b(+) (Novagen) to generate pETStaDN. The C-terminal of StaD is amplified by using PCR with the following primers: StaD-C-up, 5'-TGAT-GACGGCCTGATGCGGCCGCTGGCGG-3'; StaD-C-Xho, 5'-CCGGCTCGAGCGGCTCGGCGGGCGG-AAGTGGTCGGCCCAG-3'. The amplified 257 bp fragment was inserted between the NotI and XhoI sites of pETStaDN to generate pETStaD.
- Protein expression of the recombinant chromopyrrolic synthase.* When the recombinant *E. coli* BL21(DE3) cells containing pETStaD were cultured at 25 °C in LB medium, StaD protein was detected at the insoluble fraction of the cell extract in SDS-PAGE analysis, because of the formation of inclusion bodies. We tried co-translationally expression with some chaperone proteins to facilitate protein folding and reduce inclusion bodies, and the active-form of StaD could be produced by coexpression with the GroES and GroEL. The chaperone coexpression vector is pGro7, which was purchased from Takara Bio Inc. Overexpression of StaD was carried out in 2 × YT medium at 25 °C supplemented with kanamycin (50 µg/ml) and chloramphenicol (20 µg/ml). After reaching the cell growth to OD<sub>600</sub> = 0.6, 100 µM IPTG and L-arabinose (1 mg/ml) were added to induce StaD and chaperones, and grown for an additional 15 h.
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- The reaction conditions of StaD and K<sub>m</sub> value determination.* The purified StaD enzyme was incubated with 250 µM indole-3-pyruvic acid and 2.5 mM NH<sub>4</sub>Cl at 30 °C for 30 min in 100 mM sodium phosphate buffer (pH 7.0). After incubation, the StaD was inactivated by addition of isopropyl alcohol and the reaction product was subjected into HPLC. For K<sub>m</sub> value determination, various concentrations of indole-3-pyruvic acid (100–333 µM) and 25 mM NH<sub>4</sub>Cl were incubated at 30 °C for 5 min in 100 mM sodium phosphate buffer (pH 7.0). The amount of reaction product (chromopyrrolic acid) was estimated from the area of HPLC elution profile.
- Isolation of chromopyrrolic acid from the reaction mixture.* The reaction mixture (20 ml) was adjusted to pH 3.5 with HCl and extracted with an equal volume of ethyl acetate twice and concentrated under reduced pressure to give 4.9 mg of the crude product. After dissolving the residue in DMSO, it was purified by HPLC on a Shimadzu HPLC system (SPD-M10A) with a photodiode array detector (LC10-AT, Shimadzu) and COSMOSIL column (20 i.d. × 250 mm; Nacalai Tesque). The temperature was 30 °C, flow rate 20 ml/min, solvent 35% acetonitrile–0.1% CH<sub>3</sub>COOH (pH 3.5) detected at 254 nm. The collected fractions were combined and were lyophilized for NMR analysis.
- <sup>1</sup>H NMR δ (DMSO-*d*<sub>6</sub>, 400 MHz): 6.74 (t, *J* = 7.4 Hz, 2H), 6.91 (t, *J* = 7.4 Hz, 2H), 6.97 (d, *J* = 2.0 Hz, 2H), 7.05 (d, *J* = 8.1 Hz, 2H), 7.19 (d, *J* = 8.1 Hz, 2H), 10.76 (s, 2H); <sup>13</sup>C NMR δ (DMSO-*d*<sub>6</sub>, 100 MHz), 108.35, 110.99, 118.08, 119.51, 120.18, 123.70, 123.70, 124.91, 127.82, 135.43, 161.81, ESI-MS [M+H]<sup>+</sup>: *m/z* = 386.1.
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