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Production of *p*-Aminosalicylic Acid through Enzymatic Kolbe–Schmitt Reaction Catalyzed by Reversible Salicylic Acid Decarboxylase

Kohtaro Kirimura,* Satomi Yanaso, Sachiyo Kosaka, Keiko Koyama, Takasumi Hattori, and Yoshitaka Ishii
Department of Applied Chemistry, Faculty of Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555

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A reversible salicylic acid decarboxylase (Sdc), found in the yeast *Trichosporon moniliiforme* WU-0401, is applicable for the production of *p*-aminosalicylic acid (PAS) from *m*-aminophenol (*m*-AP). For the high-yield production of PAS, used as an antituberculous agent, we developed F195Y, a genetically engineered Sdc mutant. We succeeded in selectively producing PAS from *m*-AP through an enzymatic Kolbe–Schmitt reaction in aqueous solution by using recombinant *Escherichia coli* cells expressing the gene encoding F195Y. We found that 70 mM PAS was produced at 30 °C in 15 h with a conversion yield of 70% (mol/mol).

The Kolbe–Schmitt reaction is a well-known method for synthesizing aromatic hydroxycarboxylic acids via a carboxylation reaction of phenol salts of alkali metal at high temperature and pressure.[1] Various salicylic acid derivatives are industrially produced by this method for use as medicines, herbicides, and industrial products. However, the Kolbe–Schmitt reaction generates a large amount of by-products, which then have to be separated; moreover, the high reaction temperature and pressure have a negative impact on the environment.

The biological carboxylation of phenolic compounds using enzymes is expected to be a novel ecological alternative to the Kolbe–Schmitt reaction because regioselective and ecological carboxylation might be possible under environmentally benign conditions. Several decarboxylases such as 3,4-dihydroxybenzoate decarboxylase,[2] γ-resorcylic acid decarboxylase,[3–5] and pyrrole-2-carboxylate decarboxylase[6] have been found to act as non-oxidative decarboxylases, which reversibly catalyze the carboxylation of aromatics into aromatic carboxylic acids. Further, we previously isolated *Trichosporon moniliiforme* WU-0401 as a salicylic acid-degrading yeast, and found that both whole cells and cell-free extract of *T. moniliiforme* WU-0401 could convert salicylic acid to phenol.[7] Moreover, we discovered a novel enzyme salicylic acid decarboxylase (Sdc) in *T. moniliiforme* WU-0401, which reversibly catalyzes the regioselective carboxylation of phenol to salicylic acid.[8] We have already reported the purification, characterization, gene cloning, and gene expression of Sdc in a previous paper.[8] In the course of study, we confirmed that recombinant *Escherichia coli* strongly expressing the gene (*sdc*) encoding Sdc can be used as an efficient and convenient biocatalyst for the selective production of salicylic acid from phenol via a whole cell reaction.[8]

p-Aminosalicylic acid (PAS) is an important derivative of salicylic acid and has widely been used as an antituberculous agent. In a previous paper, we found that Sdc catalyze the regioselective carboxylation of *m*-aminophenol (*m*-AP) to form PAS and the decarboxylation of PAS to form *m*-AP, that is reversible conversion as shown in Figure 1.[8] Moreover, we have recently succeeded in generating a mutant enzyme, F195Y, more suitable for production of PAS, by site-directed mutagenesis toward Sdc (unpublished data).

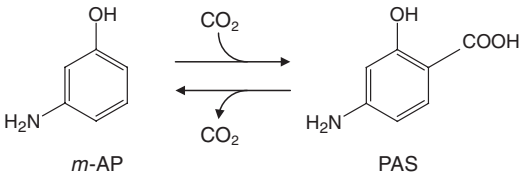

Figure 1. Enzymatic reversible conversion of *m*-aminophenol (*m*-AP) to *p*-aminosalicylic acid (PAS).

In this report, we describe the production of PAS through the enzymatic Kolbe–Schmitt reaction using Sdc and F195Y through the whole cell reaction of recombinant *E. coli* cells. To the best of our knowledge, this is the first report on the application of enzymes in the Kolbe–Schmitt reaction for selective and high-yield production of PAS in aqueous solution under environmentally benign conditions, i.e., at room temperature and atmospheric pressure.

First, we carried out the enzymatic Kolbe–Schmitt reaction through a whole cell reaction with recombinant *E. coli* cells expressing *sdc* and then identified the product obtained from the carboxylation reaction of *m*-AP substrate. The *sdc* gene was inserted into pET-21a (Novagen, WI, USA) to generate pSdc as described previously.[8] A whole cell reaction with recombinant *E. coli* BL21(DE3) harboring pSdc was then performed with *m*-AP and $KHCO_3$. High-performance liquid chromatography (HPLC) analysis revealed a unique product in the whole cell reaction mixture with a retention time of 10.7 min, and liquid chromatography-mass spectrometry (LC-MS) analysis revealed that the protonated molecule of this product was m/z 154.1 $(M + H)^+$ (Figure 2A). Both of these findings are identical to those of an authentic PAS sample. Based on the chemical shifts of authentic PAS, the signals in ^{1}H NMR and ^{13}C NMR are assigned as shown in Figure 2B and Figure 2C.[9] Based on the chemical shifts of authentic *m*-AP, the signal at δ 7.0 (dd) in ^{1}H NMR was assigned to the proton at C5 of *m*-AP, signals in the range of δ 6.25–6.31 were assigned to protons at C2 and C6. A distinctive signal of estimated chemical shift at δ 7.18 (dd) in 2-amino-6-hydroxybenzoic acid, as a regioisomer of PAS, was detected in ^{1}H NMR, but a distinctive signal of estimated chemical shift at δ 136.2 was not detected in ^{13}C NMR. A distinctive signal of estimated chemical shift at δ 164.5 of 2-amino-4-hydroxybenzoic acid was not detected in ^{13}C NMR. Other signals of estimated chemical shifts of 2-amino-6-hydroxybenzoic acid, 2-amino-4-hydroxybenzoic acid, and 3-amino-5-hydroxybenzoic acid cannot be assigned because these signals are similar to those of PAS and *m*-AP. These results

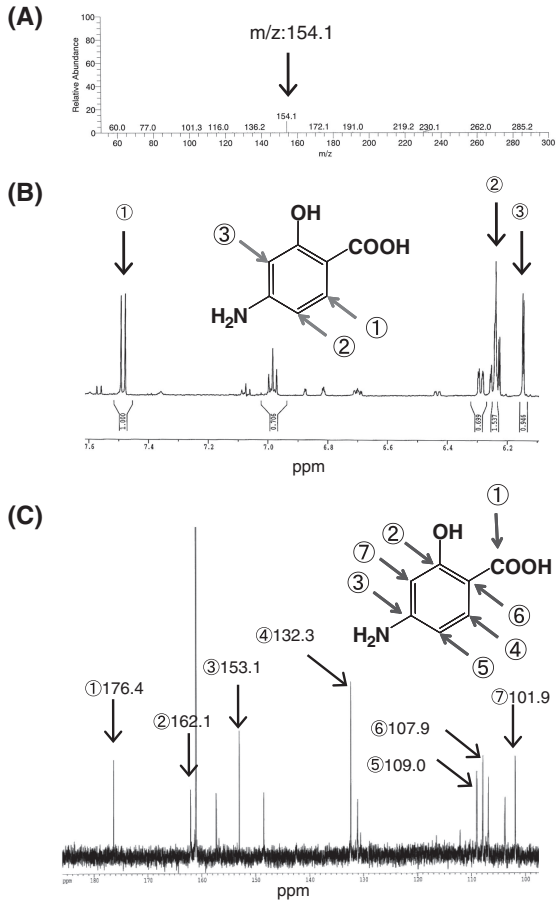

Figure 2. Liquid chromatography-mass spectrometry (LC-MS) (A), proton nuclear magnetic resonance (^{1}H NMR) (B), and carbon-13 nuclear magnetic resonance (^{13}C NMR) (C) analyses of the product *p*-aminosalicylic acid (PAS) obtained from *m*-aminophenol (*m*-AP) after carboxylation reaction.

clearly support our assumption that Sdc catalyzes the regioselective carboxylation of *m*-AP to PAS.

We investigated the optimal conditions for PAS production, that is, carboxylation of *m*-AP to PAS, by the whole cell reaction of recombinant *E. coli* BL21(DE3) harboring pSdc. The recombinant *E. coli* cells were prepared as described previously.[7] The optimal substrate (*m*-AP) concentration and optimal temperature for carboxylation were 100 mM and 30 °C, respectively (details not shown). Under these conditions, the time course of PAS production by whole cells of recombinant *E. coli* was investigated.[10] By the whole cell reaction, 70 mM (10.7 g L^{-1}) PAS was produced in 24 h with a conversion yield of 70% (mol/mol), as shown in Figure 4.

In a previous paper, the optimal substrate (phenol) concentration and optimal temperature for carboxylation of phenol to salicylic acid were given as 30 mM and 30 °C, respectively.[8] In this previous study, for salicylic acid production by the whole cell reaction of recombinant *E. coli* cells harboring pSdc, the concentration of salicylic acid had reached its maximum, 10.6 mM, after 9 h and then remained constant.[8] However, in the PAS production in this report, the optimal substrate (*m*-AP) concentration was 100 mM, and the concentration of PAS reached 70 mM after 24 h and then remained constant. Therefore, the optimal substrate (*m*-AP) concentration for PAS production is higher than that (phenol, 30 mM) for salicylic acid production; moreover, the reaction for PAS production from *m*-AP continued for 24 h, which is longer than that for salicylic acid production from phenol (9 h).[8] The difference in results may be due to the harmful effects of the substrates (phenol and *m*-AP) on Sdc and *E. coli* cells.

Table 1. The kinetic constants of wild-type Sdc and F195 mutant

Enzyme	Carboxylation of *m*-AP		
	K_m/mM	k_{cat}/s^{-1}	(k_{cat}/K_m)/mM^{-1} s^{-1}
Wild type	6.0×10	2.0×10^{-1}	3.3×10^{-3}
F195Y	2.8×10	4.8×10^{-1}	1.8×10^{-2}

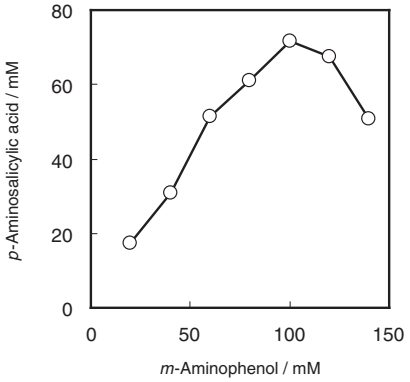

Figure 3. Effects of substrate concentration on the carboxylase activity of F195Y, the Sdc mutant enzyme, for production of *p*-aminosalicylic acid (PAS) from *m*-aminophenol (*m*-AP).

We have recently developed several mutant enzymes from Sdc by genetic engineering and site-directed mutagenesis, and have revealed that among them 1 mutant enzyme, F195Y, in which Phe195 of Sdc was replaced with tyrosine (Tyr), is suitable for PAS production (unpublished data). The kinetic parameters of wild-type Sdc and the mutant F195Y for carboxylation of *m*-AP were determined. The Michaelis constant (K_m) values show that F195Y has a higher affinity for *m*-AP than the wild-type Sdc, as shown in Table 1. The higher affinity of F195Y may be because of the formation of a hydrogen bond between the amino group of *m*-AP and the hydroxy group of Tyr (details not shown). Because the catalytic constant $(k_{cat})/K_m$ value of F195Y is 5-times greater than that of the wild-type Sdc, we carried out PAS production using F195Y, an Sdc mutant enzyme.

The expression plasmid pSdc–F195Y was developed by site-directed mutagenesis of the *sdc* gene in pSdc. For PAS production using the F195Y mutant, only the optimal substrate concentration was determined, because the optimal temperature for PAS production by wild-type Sdc was similar to that for salicylic acid production, as mentioned above. The optimal substrate (*m*-AP) concentration for PAS production using the whole cells of recombinant *E. coli* BL21(DE3) harboring pSdc–F195Y was found to be 100 mM, as shown in Figure 3. This result is similar to that obtained from the whole cell reaction of recombinant *E. coli* BL21(DE3) harboring pSdc, which contained the gene encoding wild-type Sdc.

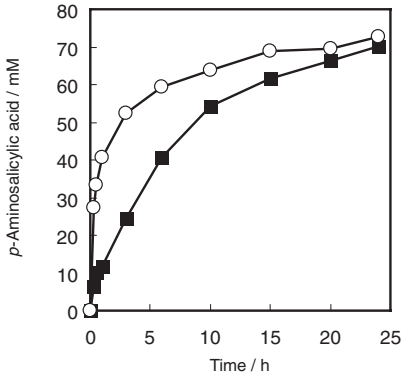

Figure 4. *p*-Aminosalicylic acid (PAS) production by whole cell reaction of recombinant *E. coli* BL21(DE3) harboring pSdc or pSdc–F195Y under optimal conditions. Symbols: closed square, wild-type Sdc; open circle, F195Y mutant.

The time course of PAS production using the whole cells of recombinant *E. coli* BL21(DE3) harboring pSdc–F195Y was investigated as mentioned above. As shown in Figure 4, 27 mM PAS was produced in 15 min through the carboxylation of *m*-AP, and this value is 4.2-times greater than the PAS production by wild-type Sdc, and 70 mM (10.7 g L^{-1}) PAS was produced in 15 h. The reaction time for PAS production by the carboxylation of the same amount of *m*-AP (70 mM) was drastically shortened from 24 h for the reaction using the whole cells of recombinant *E. coli* BL21(DE3) expressing wild-type Sdc to 10 h for the reaction using the F195Y mutant. Although the final concentration of PAS produced in the reaction with F195Y mutant was the same as that with the wild-type Sdc, the specific activity of the F195Y mutant toward *m*-AP on carboxylation increased and the production time of PAS was improved.

It was reported that 3,4-dihydroxybenzoate decarboxylase and γ-resorcylic acid decarboxylase reversibly catalyze the carboxylation of phenolic compounds into aromatic hydroxycarboxylic acids. The study showed that 0.01 mM 3,4-dihydroxybenzoate was produced (0.17% molar conversion yield) from 6 mM catechol in 45 min by 3,4-dihydroxybenzoate decarboxylase.[2] For γ-resorcylic acid decarboxylase, 26 mM γ-resorcylic acid (37% molar conversion yield) was produced from 70 mM resorcinol, respectively, in 16 h by whole cell reaction.[11] As compared to other enzymes, Sdc and the F195Y mutant are more effective and useful enzymes for PAS production through the enzymatic Kolbe–Schmitt reaction.

As for the Kolbe–Schmitt reaction, Morinaga et al. reported that PAS was produced as a major product with a conversion yield of 65.2% under 0.5 MPa CO_2 at 150 °C for 3 h, and that 4-amino-6-hydroxyisophthalic acid was generated as a major by-product with a conversion yield of 0.2%.[12] On the other hand, as for our enzymatic reaction in this report, PAS was produced with a conversion yield of 70% without any by-products at room temperature and atmospheric pressure.

In summary, we confirmed that PAS was produced from the carboxylation of *m*-AP by reversible Sdc, that is, an enzymatic Kolbe–Schmitt reaction in aqueous solution. Moreover, we succeeded in obtaining a high yield, 70 mM (10.7 g L^{-1}) PAS in 15 h, through a whole cell reaction with recombinant *E. coli* cells expressing the gene encoding the F195Y mutant. To the best of our knowledge, this is the first report describing the enzymatic Kolbe–Schmitt reaction in aqueous solution using Sdc and its mutant enzyme for production of PAS under environmentally benign conditions.

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9 The product obtained from *m*-AP was separated and purified using liquid–liquid extraction techniques with organic solvents, and it was then subjected to proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR) analyses. Chemical shift was estimated by Chem Draw Ultra 8.0.
10 Details of whole cell reaction and HPLC analysis methods: A reaction mixture containing the recombinant *E. coli* cells ($O.D._{660} = 30$), 100 mM *m*-AP, and 2 M $KHCO_3$ in 500 μL of 50 mM K_2HPO_4–KH_2PO_4 buffer (pH 6.0) was incubated at 30 °C. After the reaction, the amounts of PAS and *m*-AP were determined by HPLC (type LC10ADvp; Shimadzu, Kyoto, Japan) equipped with a TSKgel-ODS-100V column (Tosoh, Tokyo, Japan). The mobile phase was 50 mM ammonium formate–formic acid buffer (pH 4.5)/acetonitrile (97/3, v/v), and the flow rate was 0.5 mL min^{-1}. Compounds were spectrophotometrically detected at 270 nm.
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