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Screening and characterization of a novel reversible 4-hydroxyisophthalic acid decarboxylase from *Cystobasidium slooffiae* HTK3

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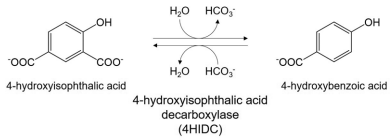
ABSTRACT

Owing to carboxylation activity, reversible decarboxylases can use CO_2 as a C1-building block to produce useful carboxylic acids. Although many reversible decarboxylases can synthesize aromatic monocarboxylic acids, only a few reversible decarboxylases have been reported to date that catalyze the synthesis of aromatic dicarboxylic acids. In the present study, a reversible 4-hydroxyisophthalic acid decarboxylase was identified in *Cystobasidium slooffiae* HTK3. Furthermore, recombinant 4-hydroxyisophthalic acid decarboxylase was prepared, characterized, and used for 4-hydroxyisophthalic acid production from 4-hydroxybenzoic acid.

Graphical Abstract



Reversible decarboxylase that can synthesize 4-hydroxyisophthalic acid obtained from an isolated strain *Cystobasidium slooffiae* HTK3.

Keywords: aromatic dicarboxylic acid, microbial screening, reversible decarboxylase

In modern society, the production of functional chemicals is dependent on CO_2-emitting fossil resources. In recent years, finding ways to reduce CO_2 emission and to achieve effective CO_2 utilization have received increased attention, with the use of reversible decarboxylases for chemical production gaining main focus (Payer, Faber and Glueck 2019). Reversible decarboxylases can catalyze both decarboxylation and carboxylation, enabling the use of CO_2 as a C1-building block for carboxylic acid production. Compared with other CO_2 fixation methods, such as the Kolbe–Schmitt reaction (Lindsey and Jeskey 1957), reversible decarboxylases can produce carboxylic acids with high regioselectivity under mild conditions, such as ambient temperature and ordinary pressure.

Reversible decarboxylases can be categorized into 3 classes (Payer, Faber and Glueck 2019). The amidohydrolase superfamily catalyzes *ortho*-carboxylation of phenols, synthesizing

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the corresponding carboxylic acids with salicylic acid moieties, such as resorcylic acid (Ishii *et al.* 2004; Yoshida, Fukuhara and Oikawa 2004), whereas the cofactor-independent phenolic acid decarboxylases catalyze the side-chain carboxylation of *para*-hydroxystyrenes, synthesizing the corresponding carboxylic acids with *p*-coumaric acid moieties, such as ferulic acid (Wuensch *et al.* 2015). Lastly, the recently discovered prenylated flavin mononucleotide-dependent decarboxylases catalyze the *para*-carboxylation of phenols and (de)carboxylation of electron-rich heterocyclic and acrylic acid derivatives, including 2,5-furandicarboxylic acid (Payne *et al.* 2019). Although many reversible decarboxylases can synthesize aromatic monocarboxylic acids, few decarboxylases are known to be capable of synthesizing aromatic dicarboxylic acids. Many aromatic dicarboxylic acids are produced by conventional petrochemical processes. For example, terephthalic acid is produced by air oxidation of *p*-xylene using metal catalysts at high temperature and pressure (Gavriilidis *et al.* 2016). Thus, novel reversible decarboxylases applicable for the synthesis of aromatic dicarboxylic acids are required.

4-Hydroxyisophthalic acid (4HIPA), an aromatic dicarboxylic acid, has been reported to exert strong antipyretic and analgesic effects with low toxicity, similar to acetylsalicylic acid (Chesher *et al.* 1955). In addition, recent studies have shown that 4HIPA provides cytoprotection against oxidative stress, maintains intracellular glutathione levels, scavenges reactive oxygen species, and inhibits lipid peroxidation (Srivastava, Jagan Mohan Rao and Shivanandappa 2012).

In the current study, *Cystobasidium slooffiae* HTK3 cells, harboring carboxylase activity toward 4-hydroxybenzoic acid (4HBA), were used to identify and characterize a reversible 4HIPA decarboxylase (4HIDC). These findings may contribute to an expansion in the number of aromatic dicarboxylic acids that can be synthesized by enzymatic carboxylation.

Materials and methods

Screening a microorganism with reversible 4HIDC activity

To isolate a microorganism with 4HIDC activity, 4HIPA-degrading microorganisms in soil samples were cultured using 4HIPA as the sole carbon source. The screening medium contained the following (L^{-1}): 0.91 g of 4HIPA, 1.6 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of $NaNO_3$, 0.5 g of $(NH_4)_2SO_4$, 0.24 g of $MgSO_4$, 2 mL of metal solution, and 1 mL of vitamin solution. Metal solution contained the following (L^{-1}): 1.5 g of $FeCl_2 \cdot 4H_2O$, 0.19 g of $CoCl_2 \cdot 6H_2O$, 0.10 g of $MnCl_2 \cdot 6H_2O$, 0.024 g of $NiCl_2 \cdot 6H_2O$, 0.070 g of $CuCl_2 \cdot 2H_2O$, 0.070 g of $ZnCl_2$, 11 g of $CaCl_2$, 0.060 g of H_3BO_3, 0.036 g of $Na_2MoO_4 \cdot 2H_2O$, and 0.030 g of KI. Vitamin solution contained the following (L^{-1}): 2.0 g of *myo*-inositol, 1.0 g of thiamine, 0.5 g of calcium pantothenate, 0.5 g of niacin, 0.5 g of pyridoxine hydrochloride, 0.5 g of *p*-aminobenzoic acid, 0.2 g of biotin, and 0.02 g of cyanocobalamin. A small portion of soil sample was suspended in 1 mL of 0.9% (w/v) NaCl, and 50 µL of the suspension was inoculated in screening medium (5 mL) and incubated at 30 °C with reciprocal shaking at 120 rpm for 5 days. 4HIPA-degrading activity in well-grown samples was evaluated by performing a Fe^{3+} colorimetric reaction. In this assay, *ortho*-hydroxy aromatic acids are chelated with Fe^{3+}, resulting in a purplish brown-colored complex (Kino *et al.* 2020). In the presence of $FeCl_3$, 4HIPA exhibited a purplish brown color, although its decarboxylated counterpart, 4HBA, did not (Figure S1). The colorimetric reaction was performed by adding 50 µL of 50 mM $FeCl_3$ to 50 µL of the medium. Next, 50 µL of the culture medium with 4HIPA-degrading activity was subcultured to a freshly prepared screening medium (5 mL). After repeating this procedure 3 times, the culture was spread on Luria–Bertani (LB) medium solidified with 1.0% (w/v) agar to isolate the candidate strains as a single colony.

The candidate strains were cultured in LB medium (5 mL) at 30 °C with reciprocal shaking at 120 rpm for 24 h. To induce 4HIPA-degrading activity, the culture solution was inoculated in the same volume of the screening medium and further incubated at 30 °C with reciprocal shaking at 120 rpm for 3 days. Cells were then harvested by centrifugation (20 000 × *g*, 4 °C, 10 min), washed with 0.9% (w/v) NaCl, and again subjected to centrifugation (20 000 × *g*, 4 °C, 10 min). Cells were resuspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.0). Next, reversible 4HIPA decarboxylation activity was examined. Decarboxylation reactions were performed in a solution (500 µL) containing 50 mM HEPES-NaOH (pH 7.0), 5 mM 4HIPA, 1% (v/v) dimethyl sulfoxide (DMSO), and whole cells (OD_{660}: 8.0). Carboxylation reactions were performed in a solution (500 µL) containing 50 mM HEPES-NaOH (pH 7.0), 5 mM 4HBA, 1% (v/v) DMSO, 3 M $KHCO_3$, and whole cells (OD_{660} : 80). The reaction mixtures were incubated at 30 °C for 24 h with vigorous shaking, following which the cells were removed by centrifugation (20 000 × *g*, 4 °C, 30 min). The reaction products were analyzed using high-performance liquid chromatography (HPLC). An aliquot of the reaction mixture was applied to a C_{18} column (TSKgel ODS-100V column; 4.6 mm i.d. × 150 mm; Tosoh, Tokyo, Japan), and the compounds were separated using a mixture containing 65% potassium phosphate (50 mM, pH 2.7), 30% acetonitrile, and 5% methanol as the mobile phase. The flow rate was 1 mL min^{-1}, and the column temperature was set at 40 °C. The compounds were detected using an ultraviolet detector (A_{254}). The concentrations of 4HIPA and 4HBA were determined using a standard curve generated with defined concentrations of the compounds. The microorganism with reversible 4HIDC activity was analyzed morphologically and genetically by Techno Suruga Laboratory Co. (Shizuoka, Japan).

Purification of the 4HIDC protein

C. slooffiae HTK3 cells were cultured in induction medium (1.2 L) with a jar fermenter at 30 °C for 24 h. The induction medium contained the following (L^{-1}): 0.1 g of 4HIPA, 2.21 g of K_2HPO_4, 0.99 g of KH_2PO_4, 0.85 g of $NaNO_3$, 0.66 g of $(NH_4)_2SO_4$, 0.60 g of $MgSO_4$, 2 mL of metal solution, and 1 mL of vitamin solution. The stirring speed was 280 rpm, and the airflow rate was 1 L day^{-1}. Cells were harvested by centrifugation (5000 × *g*, 4 °C, 10 min), washed with 0.9% NaCl, and collected by centrifugation (5000 × *g*, 4 °C, 10 min). Cells obtained from 14 L of the medium were resuspended in 10 mL (g cell)$^{-1}$ of 50 mM HEPES-NaOH (pH 7.0), disrupted by sonication using an ultrasonic disruptor (UD-200; TOMY; Tokyo, Japan; output: 3, duty: 50, 60 min), and centrifuged (20 000 × *g*, 4 °C, 30 min) to remove the insoluble fractions.

To purify the 4HIDC protein, the soluble proteins were loaded onto an anion exchange column, the TOYOPEARL DEAE 650S (Tosoh), and the proteins were eluted with a linear gradient of KCl (0-0.35 M) in 50 mM HEPES-NaOH (pH 7.0). 4HIDC activity in the fractions was evaluated as 4HIPA-degrading activity by colorimetric reaction, as described above. 4HIPA (2 mM) was added to a small portion of the fractions, incubated overnight at 30 °C, and mixed with $FeCl_3$ (50 mM). Fractions with 4HIDC activity were collected and the buffer was exchanged with 50 mM HEPES-NaOH (pH 7.0) containing 1.0 M $(NH_4)_2SO_4$ by ultrafiltration using

Amicon-Ultra (MWCO 30 k; Merck, Darmstadt, Germany). The protein solution was applied to a hydrophobic interaction column, HiLoad 16/10 Phenyl Sepharose HP (Cytiva, Marlborough, MA, USA), and the proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ (0.8-0.1 M) in 50 mM HEPES-NaOH (pH 7.0). The concentration of $(NH_4)_2SO_4$ in the fractions with 4HIPA-degrading activity was reduced by ultrafiltration with 50 mM HEPES-NaOH (pH 7.0) using Amicon-Ultra. The fractions were applied to an anion exchange column, HiLoad 16/10 Q Sepharose FF (Cytiva), and proteins were eluted with a linear gradient of KCl (0-0.6 M) in 50 mM HEPES-NaOH (pH 7.0). The buffer of the protein solution was exchanged with 50 mM HEPES-NaOH (pH 7.0) containing 1.0 M $(NH_4)_2SO_4$ by ultrafiltration using Amicon-Ultra and applied to a hydrophobic interaction column, HiPrep 16/10 Butyl FF (Cytiva). The proteins were then eluted with a linear gradient of $(NH_4)_2SO_4$ (1.0-0.34 M) in 50 mM HEPES-NaOH (pH 7.0). Fractions exhibiting 4HIDC activity were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted onto a P plus membrane (ATTO, Tokyo, Japan), and stained with Coomassie Brilliant Blue. The main band in the purified protein fraction was located at approximately 40 kDa (Figure S3), and we hypothesized that this protein was correlated with 4HIPA-degrading activity. This band was excised from the membrane, and its N-terminal amino acid sequence was determined by Hokkaido System Science (Sapporo, Japan).

Cloning of the 4HIDC-coding gene

Total RNA was isolated from *C. slooffiae* HTK3 cells using ISOGEN II (Nippon Gene, Toyama, Japan) and polyadenylated using *Escherichia coli* poly(A) polymerase (New England Biolabs, Ipswich, MA, USA). Double-stranded cDNA was synthesized from RNA using the PrimeScript Double Strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan).

To amplify the gene encoding 4HIDC, a forward primer was designed from the N-terminal amino acid sequence of the purified protein (5′-CCATATGMGIGGIAARATHATHYTI GARGARGCITGGAA-3′), whereas the reverse primer contained the oligo $(dT)_{18}$ sequence (5′-ATCTAGAGGTACCGGA TCCTTTTTTTTTTTTTTTTTT-3′). After amplification using this primer set, a single deoxyadenosine (dA) overhang was added to the 3′ end of the amplified DNA fragment using dA-overhang reaction Mix (Nippon Gene). Next, the DNA fragment was inserted into the pANT vector (Nippon Gene) using the TA-Enhancer Cloning Kit (Nippon Gene), resulting in the pANT-4HIDC vector.

The plasmid for 4HIDC expression in *E. coli* was constructed as follows: a specific primer set was designed based on the sequence of pANT-4HIDC (forward primer: 5′-GTG CCGCGCGGCAGCCATATGCGGGGGAAGATCATTCTGGAAGAGGC GTGGAA-3′, reverse primer, 5′-GTGGTGGTGGTGGTGCTCGA GCTAATACTGCCCAATACCAAAGACGTGTCCCTGGA-3′), and the 4HIDC-coding gene was amplified using pANT-4HIDC as a template. The amplified DNA fragment was inserted into the pET28a(+) vector (Merck) after digestion with *Nde*I and *Xho*I using the In-Fusion HD Cloning Kit (Takara Bio), resulting in the generation of the pET-4HIDC vector.

Preparation of the recombinant 4HIDC protein

E. coli BL21 (DE3) cells were transformed with the expression plasmid pET-4HIDC and grown at 37 °C with rotary shaking at 120 rpm for 3 h in LB medium (200 mL) containing 50 μg mL^{-1} kanamycin. Gene expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside, and the cells were further cultured at 16 °C with rotary shaking at 120 rpm for 40 h. Next, the cells were harvested by centrifugation (5000 × *g*, 4 °C, 10 min), washed with 0.9% (w/v) NaCl, and collected by centrifugation (5000 × *g*, 4 °C, 10 min). Cells were resuspended in 10 mL (g cell)$^{-1}$ of 20 mM sodium phosphate (pH 7.4) in a solution containing 500 mM NaCl and 20 mM imidazole and disrupted by sonication using the UD-200 ultrasonic disruptor (Output: 3, Duty: 50, 60 min). After centrifugation (20 000 × *g*, 4 °C, 30 min), the supernatant was applied to a Ni^{2+} column, the His GraviTrap (Cytiva), and the 4HIDC protein was eluted with 20 mM sodium phosphate (pH 7.4), containing 500 mM NaCl and 500 mM imidazole. The buffer was exchanged with 50 mM HEPES-NaOH (pH 7.0), containing 500 mM NaCl or 0-2.5 M $KHCO_3$ using the PD-10 desalting column (Cytiva).

Next, recombinant protein activity was confirmed. Decarboxylation reaction was carried out in a solution (300 μL) containing 50 mM HEPES-NaOH (pH 7.0), 5 mM 4HIPA, 1% (v/v) DMSO, and 45 μg of the recombinant 4HIDC protein, and it was performed at 30 °C for 16 h. To examine the substrate specificity of the recombinant protein, salicylic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 4-nitrosalicylic acid, 5-nitrosalicylic acid, 3-methoxysalicylic acid, 4-methoxysalicylic acid, 6-methoxysalicylic acid, 4-methylsalicylic acid, 5-methylsalicylic acid, 2,4-dihydroxy-6-methylbenzoic acid, 2,6-dihydroxy-4-methylbenzoic acid, vanillic acid, 5-sulfosalicylic acid, 5-formylsalicylic acid, 1-hydroxy-2-naphthoic acid, 2-hydroxy-1-naphthoic acid, 3-hydroxy-2-naphthoic acid, or 6-hydroxy-2-naphthoic acid was added to the reaction mixture, instead of 4HIPA. Carboxylation reactions were performed in a solution (300 μL) containing 50 mM HEPES-NaOH (pH 7.0), 5 mM 4HBA, 1% (v/v) DMSO, 3 M $KHCO_3$, and 270 μg of the recombinant 4HIDC protein. The reaction mixtures were incubated at 30 °C for 24 h, and the reaction products were analyzed using HPLC and liquid chromatography-mass spectrometry (LC-MS). For HPLC analysis, the reaction was terminated by adding 300 μL of 5 M HCl and 300 μL of DMSO, and the proteins were removed by centrifugation (20 000 × *g*, 4 °C, 30 min). HPLC analysis was performed as described above, but 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) were used as the mobile phases. Mobile phase B was increased from 10% to 100% for 10 min, held at 100% for 5 min, decreased to 10% for 0.1 min, and held at 10% for 4.9 min. For the LC-MS analysis, the reaction was terminated by adding 300 μL of 5 M HCl and 300 μL of ethyl acetate, and the proteins were removed by centrifugation (20 000 × *g*, 4 °C, 30 min). The upper layer was collected, air-dried, resuspended in methanol, and subjected to LC-MS analysis.

Biochemical analysis of 4HIDC

Decarboxylation activity was measured using standard methods. The reaction mixture (400 μL) contained 100 mM HEPES-NaOH (pH 7.5), 25 mM NaCl, 5 mM 4HIPA, 5% (v/v) DMSO, and 10 μg of purified 4HIDC. After preincubation at 30 °C for 5 min, 4HIPA and DMSO were added to initiate the reaction. The reaction was carried out for 2, 4, 6, 8, or 10 min and terminated by adding 400 μL of 5 M HCl and 400 μL of DMSO, and the proteins were removed by centrifugation (20 000 × *g*, 4 °C, 20 min). Analysis of the reaction products was performed by HPLC, as described above. To examine the effects of pH, the following buffers were used instead of HEPES-NaOH (pH 7.5): acetate-NaOH (pH 4.0-5.5), 2-morpholinoethanesulfonic acid (MES)-NaOH (pH 5.5 to 7.0), HEPES-NaOH (pH 7.0-8.0),

N-[Tris(hydroxymethyl)methyl]glycine (Tricine)-NaOH (pH 8.0-9.0), and *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES)-NaOH (pH 9.0-10.0). To examine the effects of temperature, the reaction was performed at various temperatures (10-50 °C). To examine thermostability, purified 4HIDC (500 μg mL^{-1}) in 50 mM HEPES-NaOH (pH 7.5) containing 500 mM NaCl was incubated for various periods at 20, 30, or 40 °C. After rapid cooling on ice, decarboxylation activity was measured using the standard method (described above). To examine the effects of substrate concentration, 4HIPA concentration was varied from 0 to 5 mM.

Next, carboxylation activity was measured using standard methods. The reaction mixture (400 μL) contained 100 mM HEPES-NaOH (pH 7.5), 600 mM $KHCO_3$, 5 mM 4HBA, 5% (v/v) DMSO, and 40-160 μg of purified 4HIDC. After preincubation at 30 °C for 5 min, the reaction was initiated by adding 4HBA and DMSO and carried out for 2, 4, 6, 8, or 10 min. To terminate the reaction, 300 μL of the reaction mixture was added to 300 μL of 5 M HCl, followed by the addition of 300 μL of DMSO. Then, the proteins were removed by centrifugation (20 000 × *g*, 4 °C, 20 min). The reaction products were analyzed using HPLC, as described above. The effect of $KHCO_3$ concentration on carboxylation activity was examined by varying the concentration from 0 to 2500 mM in the presence of a constant concentration of 4HBA (5 mM). The effect of 4HBA concentration on the activity was examined by varying the concentration from 0 to 40 mM in the presence of a constant concentration of $KHCO_3$ (600 mM).

Results

Microorganism with 4HIDC activity

Since we hypothesized that microorganisms with 4HIDC activity could assimilate 4HIPA, we cultured microorganisms in soil samples using 4HIPA as the sole carbon source. We tested 48 soil samples collected from the Kanto region of Japan and observed microbial growth in several samples. A microorganism with 4HIPA-degrading activity was isolated, and its 4HIDC activity was examined. When cells were incubated with 4HIPA, we observed a decrease in 4HIPA levels but without increased 4HBA production, which was likely due to further degradation of 4HBA (Figure S2A). Contrastingly, when cells were incubated with 4HBA and $KHCO_3$, 4HIPA production was observed (Figure S2B). The isolated microorganism was an ellipsoidal-ovoid yeast, and its 26S ribosomal DNA-D1/D2 sequence was 99%-100% identical to several strains of *C. slooffiae*. Based on these results, we named the microorganism as *C. slooffiae* HTK3.

Protein with 4HIDC activity in *C. slooffiae* HTK3

To identify the 4HIDC protein, proteins with 4HIPA-degrading activity were purified from cell-free extracts of *C. slooffiae* HTK3. After each purification step, by examining 4HIPA-degrading activity using a colorimetric reaction with Fe^{3+}, fractions with observable activity were collected and further purified as described in the Materials and Methods (Figure S3). A protein weighing approximately 40 kDa was suggested to be correlated with 4HIPA-degrading activity. Based on the N-terminal amino acid sequence of the protein, the degenerated primer was constructed and used to amplify the gene encoding the corresponding protein. The amplified DNA fragment was inserted into the pANT vector, and sequence analysis was performed to reveal the complete amino acid sequence of the protein. The molecular size was estimated to be 41.5 kDa, which was identical to that of the purified protein (Figure S3).

The gene encoding 4HIDC was subcloned into the pET28a(+) vector, resulting in the expression plasmid pET-4HIDC. Using pET-4HIDC, the recombinant protein was produced in *E. coli* and purified by Ni^{2+} affinity chromatography (Figure S4). 4HIDC activity of the recombinant protein was examined using HPLC and LC-MS, which confirmed 4HBA generation from 4HIPA, as well as 4HIPA formation in the presence of 4HBA and $KHCO_3$ (Figure S5). We examined substrate specificity using various aromatic carboxylic acids and found that the recombinant protein did not show decarboxylation activity toward the carboxylic acids.

Enzymatic properties of the recombinant 4HIDC protein

Next, the effects of pH and temperature on the decarboxylation activity of recombinant 4HIDC were evaluated. The highest levels of decarboxylation activity were observed at pH 6.5-8.0 °C (Figure 1a) and 30-40 °C (Figure 1b). The half-life of this protein at 20, 30, and 40 °C was of 40, 11, and 2.4 h, respectively (Figure 1c). The effects of 4HIPA concentration on decarboxylation activity were also examined by measuring the activity with varying concentrations of 4HIPA. The decarboxylation reaction followed Michaelis-Menten kinetics, with a K_m value of 0.18 ± 0.02 mM, and a V_{max} value of 0.86 ± 0.02 μmol min^{-1} mg^{-1} (Figure 2a). The effect of $KHCO_3$ concentration on carboxylation activity was also examined (Figure 2b), and strong substrate inhibition was observed. The equation $v = V_{max}[S]/(K_s+[S]+[S]^2/K_i)$ did not fit the data well, where v is the initial velocity, V_{max} is the maximum velocity, [S] is $KHCO_3$ concentration, K_s is the dissociation constant of the substrate concentration, and K_i is the dissociation constant of the inhibitory substrate. The effects of 4HBA concentration on the carboxylation reaction were also examined (Figure 2c). Although a kinetic parameter could not be calculated due to precipitation in the reaction mixture at 4HBA concentrations higher than 40 mM, an increase in catalytic activity was observed for higher 4HBA concentrations, up to 40 mM. In addition, a time-dependent increase in 4HIPA concentration was observed in the presence of 500 mM $KHCO_3$, with 2 mM 4HIPA being produced from 25 mM 4HBA (Figure 3).

Discussion

In this study, *C. slooffiae* HTK3, harboring a novel reversible decarboxylase that catalyzes the synthesis of the aromatic dicarboxylic acid, 4HIPA, was isolated from soil samples (Figure 4). The 4HIDC protein consists of 366 amino acids, the sequence of which suggests that 4HIDC is a member of the amidohydrolase superfamily (Payer, Faber and Glueck 2019). However, 4HIDC exhibited low sequence similarity (14%-40% identity) with several other experimentally investigated decarboxylases belonging to the amidohydrolase superfamily, such as 2,3-dihydroxybenzoic acid decarboxylases from *Aspergillus niger* and *Fusarium oxysporum* (An23DHBDC (Santha *et al.* 1995) and Fo23DHBDC (Zhang *et al.* 2018), respectively), γ-resorcylate decarboxylase from *Rhizobium* sp. MTP-10005 and *Rhizobium radiobacter* (Rhi26DHBDC (Yoshida, Fukuhara and Oikawa 2004) and Rr26DHBDC (Ishii *et al.* 2004), respectively), salicylic acid decarboxylase from *Trichosporon moniliiforme* (SDC) (Kirimura *et al.* 2010), 5-carboxyvanillate decarboxylases from *Sphingomonas paucimobilis* SYK-6 (LigW (Peng *et al.* 2002) and LigW2 (Peng *et al.* 2005)), 2-hydroxy-1-naphthoic acid decarboxylase from *Burkholderia* sp. BC1 (2H1NDC) (Pal Chowdhury *et al.* 2016), α-amino-β-carboxymuconic-ε-semialdehyde decarboxylase from

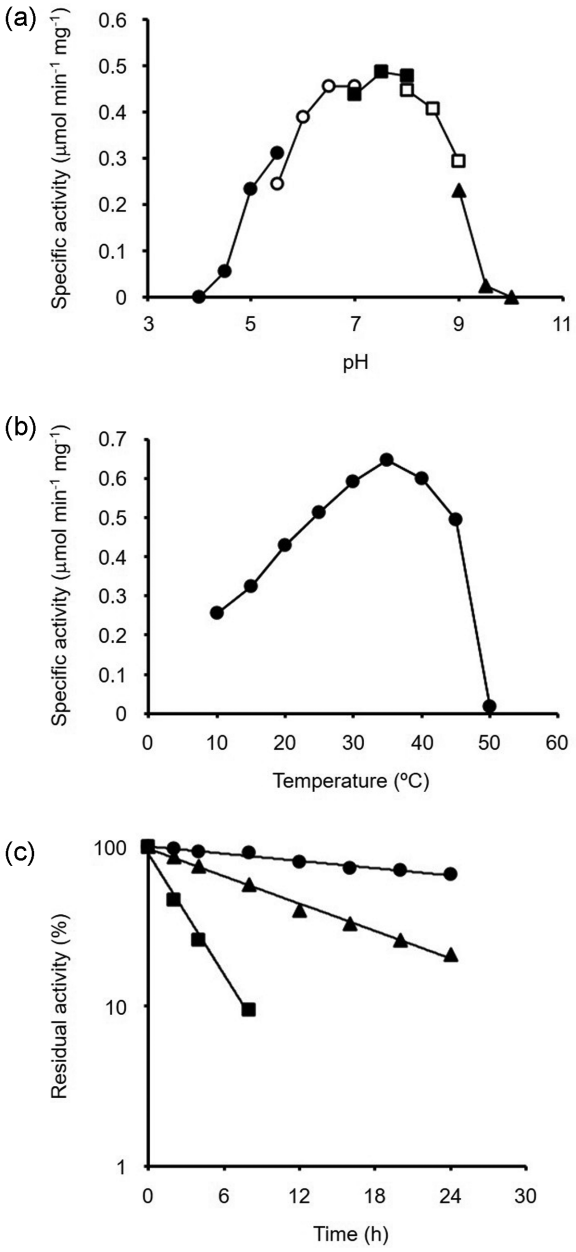

Figure 1. Effects of pH and temperature on the decarboxylase activity of 4HIDC. (a) Effects of pH on the decarboxylase activity. Symbols: closed circles, acetate-NaOH (pH 4.0-5.5); open circles, MES-NaOH (pH 5.5-7.0); closed squares, HEPES-NaOH (pH 7.0-8.0); open squares, Tricine-NaOH (pH 8.0-9.0); closed triangles, CHES-NaOH (pH 9.0-10.0). (b) Effects of temperature on the decarboxylase activity. (c) Thermostability of 4HIDC. The decarboxylase activity was measured after incubation at 20 °C (circles), 30 °C (triangles), and 40 °C (squares).

Pseudomonas fluorescens (ACMSDC) (Li *et al.* 2006), isoorotate decarboxylases from *Neurospora crassa*, *Cordyceps militaris*, and *Metarhizium anisopliae* (NcIDCase, CmIDCase, and MaIDCase, respectively) (Xu *et al.* 2013), 3,6-dihydroxypicolinic acid decarboxylase from *Alcaligenes faecalis* JQ135 (36DHPDC) (Qiu *et al.* 2019), and orsellinic acid decarboxylase from *Arthrobacter* sp. K8 (OAD) (Kino *et al.* 2020) (Figure S6). Although LigW and LigW2 have been reported to decarboxylate the aromatic dicarboxylic acid, 5-carboxyvanillate, they share only 26% and 27% identity with 4HIDC, respectively (Peng *et al.* 2002, 2005).

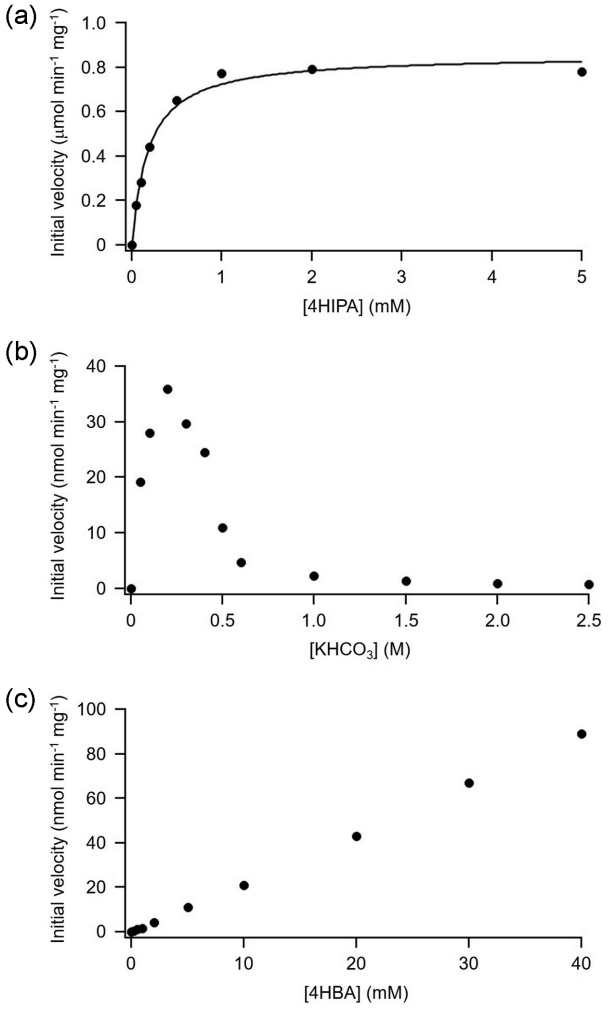

Figure 2. Effects of substrate concentration on 4HIDC activity. (a) Effects of 4HIPA concentration on the decarboxylase activity. (b) Effects of $KHCO_3$ concentration on the carboxylase activity in the presence of 5 mM 4HBA. (c) Effects of 4HBA concentration on the carboxylase activity in the presence of 600 mM $KHCO_3$.

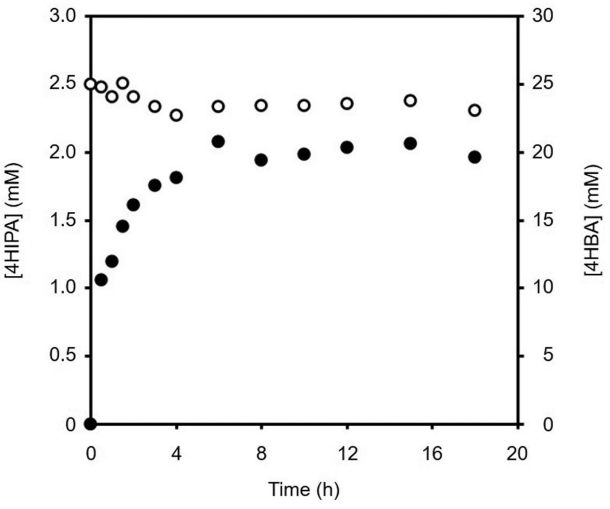

Figure 3. 4HIPA production by the recombinant 4HIDC protein. The carboxylase reaction was performed in the presence of 25 mM 4HBA and 500 mM $KHCO_3$. Symbols: closed circles, 4HIPA; open circles, 4HBA.

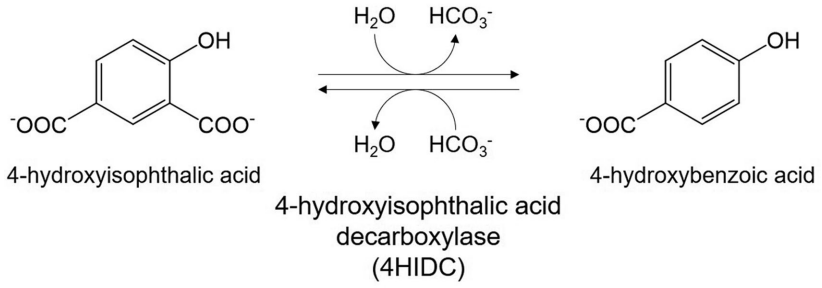

Figure 4. Reversible decarboxylation catalyzed by 4HIDC.

Rr26DHBDC and SDC catalyze the reversible decarboxylation reaction on aromatic monocarboxylic acids, and their carboxylation activities increase depending on $KHCO_3$ concentration up to 2.5 M (Ishii *et al.* 2004; Kirimura *et al.* 2010). In the current study, 4HIDC catalyzed the reversible decarboxylation reaction on the aromatic dicarboxylic acid 4HIPA. However, in the case of 4HIPA production, substrate inhibition by $KHCO_3$ was observed (Figure 2b). Reversible aromatic monocarboxylic acid decarboxylases recognize aromatic compounds without a carboxy group as substrates. Nevertheless, 4HIDC recognizes 4HBA, which contains 1 carboxy group. Although further study is necessary to elucidate the reason for the $KHCO_3$ substrate inhibition, based on the properties of the substrates, it is possible that the bicarbonate ion can bind to amino acid residues that recognize the carboxy group, resulting in the inhibition of 4HBA incorporation into 4HIDC.

In conclusion, this study describes the features of a novel reversible decarboxylase, 4HIDC, which can synthesize an aromatic dicarboxylic acid, 4HIPA, by carboxylation. The substrate of the carboxylation reaction, 4HBA, was reported to be produced by carboxylation of phenol by 4-hydroxybenzoate decarboxylase (4HBDC) (Matsui *et al.* 2006). This finding suggests that 4HIPA could be produced from phenol by a coupling reaction using 4HIDC and 4HBDC. Future study will focus on increasing the efficiency of aromatic dicarboxylic acid production by 4HIDC. Although higher levels of products are predicted to be produced from higher amounts of substrates, the carboxylation reaction by 4HIDC was inhibited by $KHCO_3$. Therefore, elucidating the mechanism underlying substrate inhibition by $KHCO_3$ is necessary to achieve production that is more efficient.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The strain *C. slooffiae* HTK3 was deposited in the NITE Biological Resource Center (Chiba, Japan) under the collection number NBRC 114281. The nucleotide sequence of the 4HIDC-coding gene from *C. slooffiae* HTK3 was deposited in the DNA Data Bank of Japan under the accession number LC583748.

Author contribution

R.A., T.Y., and K.K. designed the study. R.A., T.Y., and H.N. performed the experiments. R.A. wrote the manuscript with assistance from all authors. K.K. supervised the study.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

Chesher GB, Collier HOJ, Robinson FA *et al.* Analgesic and antipyretic activities of 4-hydroxyisophthalic acid. *Nature* 1955;**175**:206.

Gavriilidis A, Constantinou A, Hellgardt K *et al.* Aerobic oxidations in flow: opportunities for the fine chemicals and pharmaceuticals industries. *React Chem Eng* 2016;**1**:595-612.

Ishii Y, Narimatsu Y, Iwasaki Y *et al.* Reversible and nonoxidative gamma-resorcylic acid decarboxylase: characterization and gene cloning of a novel enzyme catalyzing carboxylation of resorcinol, 1,3-dihydroxybenzene, from *Rhizobium radiobacter*. *Biochem Biophys Res Commun* 2004;**324**:611-20.

Kino K, Hirokawa Y, Gawasawa R *et al.* Screening, gene cloning, and characterization of orsellinic acid decarboxylase from *Arthrobacter* sp. K8 for regio-selective carboxylation of resorcinol derivatives. *J Biotechnol* 2020;**323**:128-35.

Kirimura K, Gunji H, Wakayama R *et al.* Enzymatic Kolbe–Schmitt reaction to form salicylic acid from phenol: enzymatic characterization and gene identification of a novel enzyme, *Trichosporon moniliiforme* salicylic acid decarboxylase. *Biochem Biophys Res Commun* 2010;**394**:279-84.

Li TF, Iwaki H, Fu R *et al.* alpha-amino-beta-carboxymuconic-epsilon-semialdehyde decarboxylase (ACMSD) is a new member of the amidohydrolase superfamily. *Biochemistry* 2006;**45**:6628-34.

Lindsey AS, Jeskey H. The Kolbe–Schmitt reaction. *Chem Rev* 1957;**57**:583-620.

Matsui T, Yoshida T, Hayashi T *et al.* Purification, characterization, and gene cloning of 4-hydroxybenzoate decarboxylase of *Enterobacter cloacae* P240. *Arch Microbiol* 2006;**186**:21-9.

Pal Chowdhury P, Basu S, Dutta A *et al.* Functional characterization of a novel member of the amidohydrolase 2 protein family, 2-hydroxy-1-naphthoic acid nonoxidative decarboxylase from *Burkholderia* sp. Strain BC1. *J Bacteriol* 2016;**198**:1755-63.

Payer SE, Faber K, Glueck SM Non-oxidative enzymatic (de)carboxylation of (hetero)aromatics and acrylic acid derivatives. *Adv Synth Catal* 2019;**361**;2402-20.

Payne KAP, Marshall SA, Fisher K *et al*. Enzymatic carboxylation of 2-furoic acid yields 2,5-furandicarboxylic acid (FDCA). *ACS Catalysis* 2019;**9**;2854-65.

Peng X, Masai E, Kasai D *et al*. A second 5-carboxyvanillate decarboxylase gene, ligW2, is important for lignin-related biphenyl catabolism in *Sphingomonas paucimobilis* SYK-6. *Appl Environ Microbiol* 2005;**71**;5014-21.

Peng X, Masai E, Kitayama H *et al*. Characterization of the 5-carboxyvanillate decarboxylase gene and its role in lignin-related biphenyl catabolism in *Sphingomonas paucimobilis* SYK-6. *Appl Environ Microbiol* 2002;**68**; 4407-15.

Qiu JG, Zhang YT, Yao SG *et al*. Novel 3,6-dihydroxypicolinic acid decarboxylase-mediated picolinic acid catabolism in *Alcaligenes faecalis* JQ135. *J Bacteriol* 2019;**201**, DOI: 10.1128/JB.00665-18.

Santha R, Savithri HS, Rao NA *et al*. 2,3-Dihydroxybenzoic acid decarboxylase from *Aspergillus niger*—a novel decarboxylase. *Eur J Biochem* 1995;**230**:104-10.

Srivastava A, Jagan Mohan Rao L, Shivanandappa T. A novel cytoprotective antioxidant: 4-hydroxyisophthalic acid. *Food Chem* 2012;**132**:1959-65.

Wuensch C, Pavkov-Keller T, Steinkellner G *et al*. Regioselective enzymatic beta-carboxylation of para-hydroxy-styrene derivatives catalyzed by phenolic acid decarboxylases. *Adv Synth Catal* 2015;**357**:1909-18.

Xu S, Li W, Zhu J *et al*. Crystal structures of isoorotate decarboxylases reveal a novel catalytic mechanism of 5-carboxyl-uracil decarboxylation and shed light on the search for DNA decarboxylase. *Cell Res* 2013;**23**:1296-309.

Yoshida M, Fukuhara N, Oikawa T Thermophilic, reversible gamma-resorcylate decarboxylase from *Rhizobium* sp. strain MTP-10005: purification, molecular characterization, and expression. *J Bacteriol* 2004;**186**:6855-63.

Zhang X, Ren J, Yao P *et al*. Biochemical characterization and substrate profiling of a reversible 2,3-dihydroxybenzoic acid decarboxylase for biocatalytic Kolbe–Schmitt reaction. *Enzyme Microb Technol* 2018;**113**:37-43.