Studies on Microbial Metabolism of a Botanical Aromatic Aldehyde

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Shiori DOI

Studies on Microbial Metabolism of a Botanical Aromatic Aldehyde

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Contents

General introduction

Generally, higher plant species secrete carbohydrates, amino acids, vitamins and other nutrients produced by themselves. Therefore, there are large numbers of microorganisms around plants, especially plant roots, in order to obtain nutrients. Some microorganisms in the plant rhizosphere can convert nutrients composed of carbon, nitrogen, etc. into useful compounds for plants, and others can degrade toxic compounds against plants into nontoxic ones, resulting in symbiosis between plants and microorganisms. On the other hand, plant pathogenic microorganisms are able to infect host plant cells or grow by use of compounds produced by plants. Although plants defend themselves immediately against microbial infections through a defense mechanism, resulting in the prevention of infection in most cases, some microorganisms have the abilitiy to avoid the plant defense responses. As a result, there are an enormous number of plant-pathogen interactions that remain to be elucidated.

Aromatic aldehydes are some of the components of essential oils, and are produced as secondary metabolites by aromatic plants. In the essential oils, which are volatile, natural, complex compounds characterized by a strong odour, there are numerous compounds for example, terpenes, terpenoides and aromatic compounds. It is commonly assumed that essential oils play an important role in the protection of plants as antibacterials, antivirals, antifungals and insecticides, and also against herbivores by reducing their appetite for such plants. They also may attract some insects, facilitatory dispersion of pollen and seeds, or repel undesirable ones (Bakkali *et al*, 2008). However, little is known regarding the role of botanical volatile aromatic aldehydes in the essential oils derived from higher plants (Bakkali *et al*, 2008; Muhlemann *et al*, 2014), and findings

regarding the biological metabolism of aromatic aldehyde compounds are particularly limited (Ding *et al*, 2015). Here, the author focused on a floral volatilic secondary metabolite, piperonal, produced by the *Heliotropium* genus**.** Piperonal is known as a component of the essential oil of the heliotrope flower, and is an aldehyde frequently used in perfumes, cosmetics and flavoring agents, such as cinnamaldehyde and vanillin included in cinnamon spice and vanilla essence, respectively. However, the metabolic pathway of piperonal is unkown. In present article, the author report the isolation of microorganisms with piperonal-converting ability from soil, the purification and characterization of a piperonal-converting enzyme, and the cloning of its gene. The author also demonstrates that an O_2-dependent enzyme is involved in piperonal metabolism.

Chapter I

Discovery of piperonal-converting enzyme involved in the metabolism of a botanical aromatic aldehyde

Section I

Screening of piperonal-catabolizing microorganisms from soil

Introduction

For a long time, human beings have ingested primary metabolites produced by plants as nutrients, and have also utilized various secondary ones as potential drugs. It has been reported that there are almost 250 thousand plants including higher ones on earth. Therefore, there is an enormous diverse range of specific secondary metabolites depending on the species or group. Degradation of various kinds of secondary metabolites produced by higher plants has been reported. For example, caffeine, a kind of alkaloid present in coffee, is degraded by several enzymes derived from microorganisms in nature (Yu *et al*, 2008; Yu *et al*, 2009; Dash *et al*, 2006). Although a lot of microorganisms showing degradation ability as to plant secondary metabolites have been isolated, studies on enzymes involved in the degradation pathways have been limited.

Piperonal is a naturally occurring aromatic aldehyde, a secondary metabolite produced by higher plants, and well-known as a volatilic compound frequently used in perfumes, cosmetics and flavoring agents (Santos *et al,* 2004). To the best of our knowledge, there has been neither isolation of microorganisms nor elucidation of the metabolic pathway involved in piperonal metabolism. Furthermore, the enzymes, and their genes, involved in the degradation of piperonal remain unclear.

In this section, the author describes screening of piperonal-catabolizing microorganisms from soil and identification of the one shows highest activity as *Burkholderia* sp. CT39-3.

Materials and Methods

Materials

Piperonal was purchased from Nacalai Tesque Co., Inc. (Kyoto, Japan).

Isolation of piperonal-converting bacteria

Piperonal-converting bacteria were isolated from soil samples as follows. A spoonful of a soil sample was added to a test tube containing 10 ml of a screening medium (pH 7.5) consisting of 0.1% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) K_2HPO_4, 0.05 g KH_2PO_4, 0.2 g NaCl, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, and 0.01% (w/v) piperonal (final concentration). Then, 100 μl of the first subculture was inoculated into a test tube containing 10 ml of the screening medium containing 0.02% (w/v) piperonal (final concentration). The second subculture was inoculated into a test tube containing 10 ml of the screening medium containing 0.03 % (w/v) piperonal (final concentration) in the same way as for the second subculture. All subcultures were carried out at 28°C for 1 week with reciprocal shaking in a test tube containing 10 ml of the screening medium with stepwise increases in the piperonal concentration. After one month's further cultivation, microorganisms were spread on agar plates and isolated.

Assaying of piperonal-converting abilities of the isolated strains

Each of the isolated strains was inoculated into a test tube containing 10 ml of medium and then incubated at 28°C for 1 week with reciprocal shaking. Then, the cells were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (KPB) (pH 7.5), and

suspended in 10 mM KPB (pH 7.5).

The piperonal-converting abilities of the isolated strains were assayed by means of the resting cell reaction. The reaction mixture (1 ml) was composed of 10 mmol of KBP (pH 7.5), 2 mmol of piperonal, and an appropriate amount of cell suspension. The reaction was carried out at 25°C for 1 h, and stopped by adding an equal volume of acetonitrile to the reaction mixture and then rapidly removing the cells by centrifugation at 0-4°C. The residual amount of piperonal in the reaction mixture was determined by HPLC with a Shimadzu LC-10A system (Kyoto, Japan) equipped with a Cosmosil 5C_{18}-AR-II column (reversed-phase, 4.6 × 150 mm; Nacalai Tesque) and the diode array detector (SPD-M10Avp) of the original system. The following solvent system was used: acetonitrile/H_2O, 50:50 (v/v), at the flow rate 1.0 ml/min and 40°C. The absorbance was measured at 254 nm. One unit of piperonal-converting enzyme activity was defined as the amount of the enzyme that catalyzed the conversion of 1 μmol piperonal/min under the above conditions.

Genome sequencing of strain CT39-3

Genomic DNA was prepared from *Burkholderia* sp. strain CT39-3 as follows: the strain was cultured at 28°C for 24 h in 500 ml of 2× YT medium with reciprocal shaking. Cells were harvested by centrifugation, washed with 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA and 100 mM NaCl, and then suspended in 15 ml of the same buffer. The suspension was incubated with 7 mg/ml of lysozyme at 37°C for 3 h, and then 1 ml of 0.25 mM EDTA (pH 8.0), 0.5 ml of 10% (w/v) SDS and 4 mg of proteinase K (Wako Pure Chemical Industries, Ltd.) were added to the solution, followed by incubation at room temperature for 3 h. 5.3 ml of 5 M NaCl was added to the solution. DNA was purified by extraction of the lysate with phenol/chloroform/isoamylalcohol

(25/24/1; v/v/v) and then precipitation with isopropanol. The solution was subjected to equilibrium centrifugation in a CsCl-ethidium bromide gradient, and the fraction containing genomic DNA was pooled, extracted with *n*-butanol to remove ethidium bromide, and then precipitated with ethanol. The draft genome sequencing of strain CT39-3 was performed using an Illumina Hiseq platform (Hokkaido System Science Co., Ltd., Sapporo, Japan). The annotation of the contigs was performed by the Microbial Genome Annotation Pipeline (http://www.migap.org).

Results & Discussion

Isolation and identification of a piperonal-converting microorganism

After about one month from the start of the screening, using the acclimatization culture method described under "*Isolation of piperonal-converting bacteria",* the author isolated 77 microorganisms with piperonal-converting ability from the soil. From among them, the microorganism (CT39-3) showing the highest activity was selected, as described under "*Assaying of piperonal-converting abilities of the isolated strains".*

Morphologically, strain CT39-3 is a Gram-negative rod, nonendospore forming, and motile. Its physiological characteristics are as follows: nitrate reduction, positive; indole production, negative; acid production; glucose, negative; arginine dihydrolase, negative; urease, negative; esculine hydrolysis, negative; gelatin liquefaction, negative; β-galactosidase, negative; cytochrome oxidase, negative; starch hydrolysis, negative; lecithinase, negative; hipprate hydrolysis, negative; growth on sole carbon sources, positive with dextrose, L-arabinose, D-mannol, D-mannitol, L-rhamnose, L-malic acid, phenylacetic acid and *N*-acetylglucosamine, and negative with D-ribose, D-xylose, adipic acid, capric acid and gluconic acid. The 16S rRNA gene sequence of the isolated microorganism was used as a query to search for homologous sequences in the Ribosomal Database Project (RDP) (Cole *et al*, 2013). As a result, the nucleotide sequence of its 16S rRNA gene was found to show 98.6% similarity to that of the closest type strain, *Burkholderia grimmiae* $R27^T$, and 98.2% similarity to that of *Burkholderia zhejiangensis* $OP\text{-}1^T$ (Tian *et al*, 2013). Phylogenetic analysis revealed that the strain is closely related to the genus *Burkholderia* (see Fig. 1). Based on these characteristics together with its 16S rRNA sequence, strain CT39-3 was identified as

Burkholderia sp.

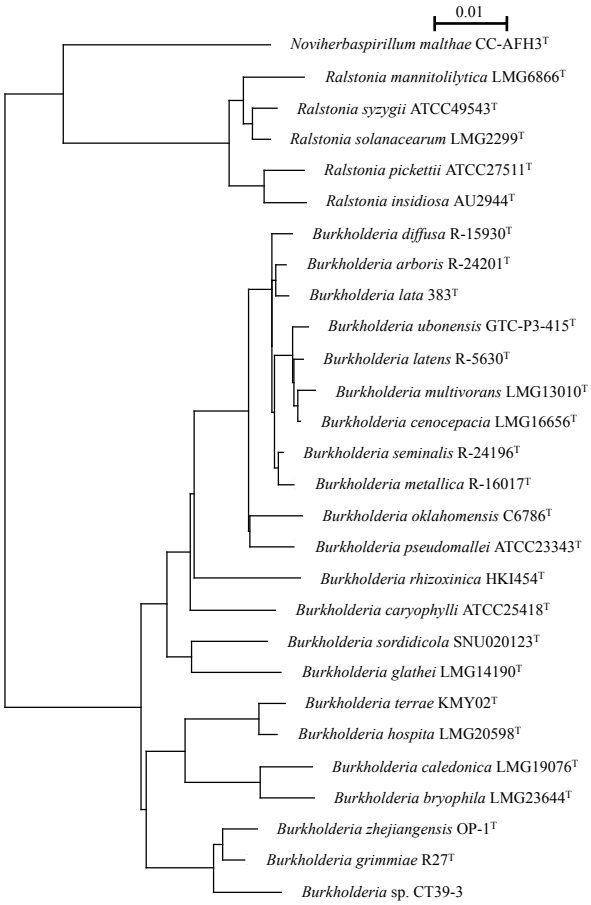

FIGURE 1. Phylogenetic tree of 16S rRNA genes from *Burkholderia* sp. CT39-3 and its relatives. The scale bar represents 1% dissimilar sequences.

Section II

Purification of piperonal-converting enzyme from *Burkholderia* sp. CT39-3

Introduction

In the previous section, the author isolated many piperonal-catabolizing microorganisms and chose one, CT39-3, which was identified as *Burkholderia* sp., from soil. Although this strain showed the highest activity as to the piperonal conversion among the isolated microorganisms, the amount of the cells cultured in the screening medium was extremely small, and the piperonal-converting activity was low in the cells. Thus, the author established the optimum culture conditions and purified the enzyme from a large amount of cells (108 L) cultured under these conditions.

Materials and Methods

Materials

Resource Q, Superose 12 HR10/30, HisTrap HP and an LMW calibration kit were obtained from GE Healthcare-Life Sciences (Buckinghamshire, UK). TOYOPEARL Butyl-650M and TSKgel BioAssistQ were purchased from Tosoh Co., Ltd. (Tokyo, Japan). Ceramic Hydroxyapatite Type I was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Standard proteins for high-performance gel filtration chromatography were purchased from Oriental Yeast (Tokyo, Japan). All other biochemicals were standard commercial preparations.

Culture conditions for Burkholderia sp. CT39-3

Burkholderia sp. CT39-3 was inoculated into a subculture medium (pH 7.5) comprising 0.05% (w/v) KH_2PO_4, 0.05% (w/v) K_2HPO_4, 0.2% (w/v) NaCl, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.5% (w/v) glycerol, 0.05% (w/v) malt extract, and 0.03% (w/v) piperonal (all final concentrations), and then incubated at 28°C for 96 h. The subculture was carried out at 28°C for 72 h with reciprocal shaking in a test tube containing 10 ml of the same medium. Then, 5 ml of the subculture was inoculated into a 2-l shaking flask containing 500 ml of the above-mentioned medium, followed by incubation at 28°C with reciprocal shaking. After 96 h incubation, the cells were harvested by centrifugation at 10,400 × *g* at 4°C and then washed twice with 10 mM KPB (pH 7.5).

The culture medium for investigating the function of piperonal-converting oxidase was the screening medium containing 0.03, 0.07 or 0.10 % (w/v) piperonal, or glucose as a sole carbon

source. *Burkholderia* sp. CT39-3 was inoculated (final conc., OD_{600} = 0.1), and cells were incubated at 28°C and 220 r.p.m.

Purification of a piperonal-converting enzyme

All purification procedures were performed at 0-4°C. KPB (pH 7.5) was used throughout the purification. Centrifugation was carried out for 30 min at 13,000 × g.

Step 1. Preparation of a Cell-free Extract. Washed cells from 108 liters of culture were resuspended in 78 ml of 100 mM buffer and then disrupted by sonication at 200 W for 30 min with an Insonator model 201M (Kubota, Tokyo, Japan). The cell debris was removed by centrifugation.

Step 2. Ammonium Sulfate Fractionation. The resulting supernatant solution was fractionated with ammonium sulfate (40-60% saturation), followed by dialysis against 10 mM buffer.

Step 3. TOYOPEARL Butyl-650M Column Chromatography. The enzyme solution from step 2 was brought to 3 M KCl, and then placed on a TOYOPEARL Butyl-650M column (2.6 × 22 cm; Tosoh Co., Ltd.) equilibrated with 10 mM buffer containing 3 M KCl. The enzyme was eluted by lowering the concentration of KCl (3 to 1.2 M saturation) in 240 ml of the same buffer. The active fractions were pooled and then dialyzed against 10 mM buffer.

Step 4. Ceramic Hydroxyapatite Type I Column Chromatography. The enzyme solution from step 3 was applied to a Ceramic Hydroxyapatite Type I column (5 ml; Bio-Rad Laboratories) equilibrated with 10 mM buffer. Protein was eluted from the column with 40 ml of the same buffer, the concentration of KPB being increased linearly from 10 mM to 300 mM. The active

fractions were collected and then dialyzed against 10 mM buffer.

Step 5. Resource Q Column Chromatography. The enzyme solution from step 4 was applied to a Resource Q column (6 ml; GE Healthcare UK Ltd.) equilibrated with 10 mM buffer. Protein was eluted from the column with 35 ml of the same buffer, the concentration of KCl being increased linearly from 0 to 0.6 M. The active fractions were pooled and then dialyzed against 10 mM buffer.

Step 6. TSKgel BioAssist Q Column Chromatography. The enzyme solution from step 5 was applied to a Bioassist Q column (1 ml; Tosoh Co., Ltd.) equilibrated with 10 mM buffer. Protein was eluted from the column with 60 ml of the same buffer, the concentration of KCl being increased linearly from 0 to 0.5 M. The active fractions were pooled and then dialyzed against 10 mM buffer.

Enzyme assays

Piperonal-converting activity was measured by means of the following two assay systems. All of the reactions were performed under linear conditions as to protein and time.

The standard assay A mixture comprised 100 mM KPB (pH 7.5), 2 mM piperonal, and an appropriate amount of enzyme, in a total volume of 200 μl. The reaction was started by the addition of piperonal and carried out at 25°C. The reaction was stopped by adding 200 μl of 99% (v/v) acetonitrile and 1% (v/v) formic acid to the reaction mixture, and a supernatant was obtained by centrifugation (15,000 × *g*, 3 min). The amount of piperonylic acid formed was determined by HPLC, which was performed with the same system as used for the measurement of piperonylic acid under "Section I *Assaying of piperonal-converting abilities of the isolated strains*". This assay

was used to routinely measure piperonal-converting activity, unless otherwise noted.

In the case of standard assay B, enzyme activity was assayed by measuring the decrease in O_2 with an oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK), which monitors the O_2 concentration. The reaction mixture was composed of 2 mM aldehyde, 100 mM KPB (pH 7.5), and an appropriate amount of enzyme (10 μl), in a final volume of 1 ml. The reaction was initiated by injecting the enzyme solution into an electrode cuvette and carried out at 25°C.

One unit of piperonal-converting activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of piperonylic acid per min and the consumption of 1 μmol of O_2 per min under the standard assay A and B conditions, respectively. Specific activity is expressed as units per milligram of protein. The protein concentrations were determined with a Nakalai Tesque protein assay kit using BSA as the standard, according to the method of Bradford (Bradford *et al*, 1976).

Molecular mass determination

The purified enzyme sample was applied to a Superose 12 HR10/30 column (GE Healthcare UK Ltd.), attached to an ÄKTA purifier, and then eluted with 10 mM KPB containing 0.15 M KCl at the flow rate of 0.5 ml/min. The absorbance of the effluent was recorded at 280 nm. The molecular mass of the enzyme was calculated from the mobilities of the standard proteins, glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome *c* (12.4 kDa).

Analytical methods

Protein concentrations were determined with a Nacalai Tesque Co., Inc. protein assay kit,

with bovine serum albumin as the standard. SDS-PAGE was performed in a 12% polyacrylamide slab gel according to Laemmli (Laemmli *et al*, 1970). The gel was stained with Coomassie brilliant blue R-250. The relative molecular mass of the enzyme was calculated from the mobilities of the marker proteins, phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and a-lactalbumin (14.4 kDa).

UV-Vis spectra were obtained with a Shimadzu UV-1700 spectrophotometer (Kyoto, Japan) at room temperature. Enzymes were dialyzed against 100 mM KPB (pH 7.5) and then prepared at 2.2 mg/ml.

The N-terminal amino acid sequences were determined with samples electroblotted onto a polyvinylidene difluoride (PVDF) membrane after SDS-PAGE using a Procise protein sequencer (Applied Biosystems).

Results & Discussion

Optimum culture conditions for formation of the piperonal-converting enzyme

To increase the enzyme activity in the cells, the author investigated several sets of culture conditions for strain CT39-3. A subculture of strain CT39-3 was carried out at 28°C for 72 h with reciprocal shaking in a test tube containing 10 ml of the screening medium. Next, 0.1 ml of the cell suspension was inoculated into a test tube containing 10 ml of one of the various media to be examined in each experiment. Each cultivation was carried out at 28°C for 72 h with reciprocal shaking.

First, the effects of various carbon sources were examined. Of different carbon sources, 0.2% (w/v) was added to the medium in the absence or presence of 0.03, 0.05, 0.1, or 0.15% (w/v) piperonal as follows: glucose, maltose, mannitol, glycerol, sucrose, lactose, and galactose. Among the tested culture media, the highest specific activity was observed in the medium containing 0.03% (w/v) piperonal in the presence of glycerol. Second, the effects of various nitrogen sources were examined. The following compounds were tested as nitrogen sources: casamino acid, malt extract, meat extract, tryptone, beef extract, NH_4Cl, NH_4HPO_4, NZ amine, peptone, yeast extract, bonito extract, $(NH_4)_2SO_4$, and urea. Each of the various compounds was added, to the final concentration of 0.2% (w/v), to the medium instead of $(NH_4)_2SO_4$. The replacement of $(NH_4)_2SO_4$ with malt extract increased the enzyme activity 1.5-fold compared with that in the medium with $(NH_4)_2SO_4$. Thus, malt extract was selected as the nitrogen source for the following experiments. Next, various concentrations of glycerol and malt extract were added to the medium. The best

concentrations were 0.5% (w/v) for glycerol and 0.05% (w/v) for malt extract, respectively, being 1.8-fold compared with that in the initial medium. Based on the above results, a medium containing 0.03% (w/v) piperonal, 0.5% (w/v) glycerol, 0.05% (w/v) malt extract, 0.05% (w/v) KH_2PO_4, 0.05% (w/v) K_2HPO_4, 0.2% (w/v) NaCl, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, and 0.001% (w/v) $FeSO_4 \cdot 7H_2O$ (pH 7.5) was found to be the most suitable medium for the preparation of cells exhibiting high piperonal-converting activity.

To increase the piperonal-converting ability, various culture conditions were examined (Table 1). Strain CT39-3 was inoculated into a 2-l shaking flask containing various volumes of the above-mentioned medium, followed by incubation at 28°C. As for culture volume conditions, higher specific activities were found in cells grown in the 0.5 L of the medium. Specific activity gradually increased until 96 h. Although a further significant increase in the enzyme activity was not obtained during incubation for longer than 96 h, the maximum activity was observed at 144 h cultivation. To obtain a large amount of cells, repeated large-scale cultures using a 2-l shaking flask was required. Therefore, 96 h incubation in a flask containing 0.5 l culture medium were taken as the optimum conditions. Under these conditions, the level of piperonal-converting activity in a cell-free extract was 4.39 units/mg; i.e., the piperonal-converting activity was increased 4.8-fold compared with that under the initial conditions.

Purification of the piperonal-converting enzyme

Through the purification steps described under "*Material and Methods"*, the piperonal-converting enzyme was purified 26-fold, with a yield of 0.044%. The purified enzyme showed specific activity of 54.7 units/mg (Table 2). The purity of the enzyme was

confirmed by elution of the protein as a single peak corresponding to 165 kDa on gel filtration chromatography (Fig. 2). The purified enzyme was bright yellow in color, and gave three bands corresponding to molecular masses of 20 kDa, 40 kDa, and 80 kDa on SDS-PAGE (Fig. 3). These findings indicate that the piperonal-converting enzyme consists of three subunits of these sizes.

Culture volume (*L*)	Specific activity (*units/mg*)													
	Cultivation time (*h*)													
	12	24	36	48	60	72	84	96	108	102	132	144	156	168
0.1	N.D.	0.15	0.64	2.05	2.30	2.77	2.63	3.18	3.17	3.97	3.02	3.07	3.47	4.16
0.5	N.D.	N.D.	0.49	1.76	2.67	3.06	3.70	4.39	4.46	4.50	4.91	5.02	4.94	4.63
1.0	N.D.	N.D.	0.30	1.22	2.08	2.52	2.61	3.07	3.35	3.06	3.57	3.74	4.03	3.85

Table 1. *Piperonal-converting enzyme activity of Burkholderia sp. CT39-3 under various conditions.*

N.D., not detected.

Step	Total protein	Total activity	Specific activity	Yield	Fold
	mg	*units*	*units/mg*	*%*	
Cell free extract	174	371	2.13	100	1.1
$(NH_4)_2SO_4$ (40%-60%)	75.4	173	2.30	46.6	1.1
Butyl-Toyopearl 650M	7.27	54.0	7.43	14.6	3.5
Ceramic Hydroxyapatite Type I	1.42	12.1	8.54	3.26	4.0
Resource Q	0.30	9.09	30.3	2.45	14
TSKgel Bioassist Q	0.0003	0.164	54.7	0.044	26

Table 2. *Purification of the piperonal-converting enzyme.*

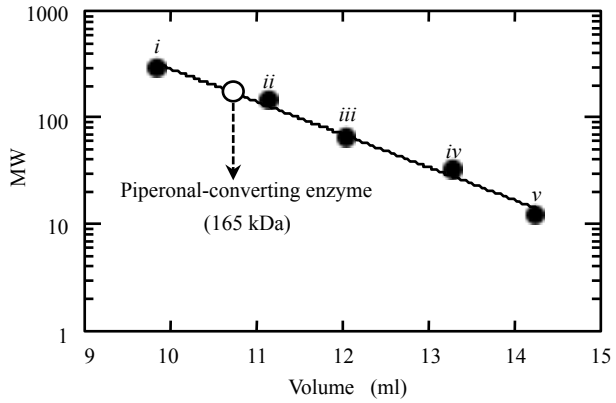

FIGURE 2. Molecular mass determination of the purified piperonal-converting enzyme. Marker proteins used for gel filtration: *(i)* glutamate dehydrogenase (yeast) (290 kDa); *(ii)* lactate dehydrogenase (pig heart) (142 kDa); *(iii)* enolase (yeast) (67 kDa); *(iv)* myokinase (yeast) (32 kDa); and *(v)* cytochrome c (horse heart) (12.4 kDa). The molecular mass determined is shown by an open circle.

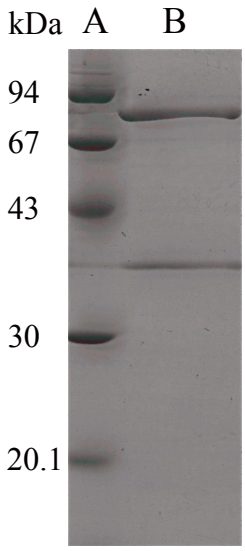

FIGURE 3. SDS-PAGE of the purified piperonal-converting enzyme.

Protein bands were detected by staining with Coomassie brilliant blue. *Lane A*, marker proteins. *Lane B*, purified piperonal-converting enzyme.

Chapter II

Gene cloning and characterization of piperonal-converting enzyme

Section I

Gene cloning of piperonal-converting enzyme

Introduction

In the previous chapter, the author purified the enzyme from a large amount of cells (108 L) cultured under optimum culture condition, through several chromatography steps. But the smallest subunit of the purified enzyme tends to be unstable. In order to obtain the stable enzyme briefly, the author constructed the N-terminal His・tag fusion expression system of the enzyme. Then the author was able to overexpress the recombinant enzyme, and purified it to characterize in detail.

Materials and Methods

Materials

Escherichia coli SCS110 (Agilent Technologikes) was used as the host for pBBR1MCS2 (Kovach *et al*, 1995) plasmids. *E. coli* transformants were grown in 2× YT medium containing 50 μg/ml kanamycin (Sambrook *et al*, 1989), unless otherwise noted.

Preparation of Burkholderia sp. CT39-3 competent cells

The subculture was carried out at 28°C with reciprocal shaking in a test tube containing 5 ml of the 2× YT medium until the OD_{600} reached ~ 0.5 . Then, the subculture was diluted to an OD_{600} of 0.01 in 50 ml of SOB medium (Sambrook *et al*, 1989) containing 10 mM $MgSO_4$, 10 mM $MgCl_2$, and 0.3% (w/v) glycine, followed by incubation at 28°C with reciprocal shaking until the OD_{600} reached 0.05. After incubation, the cells were harvested by centrifugation at 8,000 × *g* at 4°C. The pellet from a 100 ml culture was resuspended in 20 ml ice-cold FSB (pH 7.5) (Sambrook *et al*, 1989), incubated in an ice bath for 10 min, and then spun down three times. Then, the cells were washed twice with ice-cold 0.5 M sucrose and the suspension was centrifuged. After washing with 0.5 M sucrose, the cells were resuspended in 200 μl of ice-cold 0.5 M sucrose containing 10% (w/v) glycerol. Aliquots (100 μl) were stored at – 80°C until use (Hayashi *et al*, 2000; Dubarry *et al*, 2010).

Electroporation

Cells were thawed in ice, and transferred to a sterile, pre-chilled cuvette (interelectrode distance of 0.2 cm). Plasmid DNA (15 mg) and Ocr (Type One Restriction Inhibitor; Epicentre, Madison, WI, USA) were added to the cuvette, followed by mixing by inversion. Then, the cuvette was placed in a Gene Pulser Xcell (Bio-Rad Labolatories), and the cells were subjected to a pulse of 2.5 kV and 50 mF. Immediately after pulse application, the cell suspension was diluted with 1 ml of SOC medium (Sambrook *et al*, 1989). The cells were then transferred to a tube and incubated at 28°C for at least 4 h without any antibiotic. After incubation, aliquots were spread-plated on 2× YT medium containing 50 μg /ml kanamycin.

Expression and purification of the recombinant piperonal-converting enzyme

DNA fragments containing the coding regions (*pceSML*) of the enzyme (*piperonal-converting enzyme)* were amplified by PCR with genomic DNA extracted from the CT39-3 strain as a template. The following two oligonucleotide primers were used: sense primer, 5'-CATATCGAAGGTCGTCATATGCCACAACGTCACATCCCCCAATCG-3' containing an *Nde*I recognition site (underlined); and antisense primer, 5'-GCTCTAGAACTAGTGGGATCCTTACAGCAAGTCCTCGATCATCACCGGC-3' containing a *Bam*HI recognition site (underlined). Also, DNA fragments containing the His・tag coding sequence were amplified by PCR with pET-16b (Novagen) as a template. The following two oligonucleotide primers were used: sense primer, 5'-AAGGGAACAAAAGCTGGTACCGGGGAATTGTGAGCGGATAACAATTCCCC-3' containing a *Kpn*I recognition site (underlined); and antisense primer, 5'-CATATGACGACCTTCGATATGGCCGCTGC-3' containing an *Nde*I recognition site

(underlined). Both amplified DNA fragments were inserted into the *Kpn*I and *Bam*HI sites of pBBR1MCS2 using an In-Fusion® HD Cloning Kit (Clontech® Laboratories, Inc., Mountainview, CA, USA), and checked by DNA sequencing. The resulting plasmid, pBBR-His-*pceSML*, expresses piperonal-converting oxidase as an N-terminal His・tag fusion with PceS. In this construct, the *pceSML genes* were under the control of the *lac* promoter.

Burkholderia sp. CT39-3 was transformed with pBBR-His-*pceSML*, and the recombinant cells were used for the overproduction and purification of a recombinant piperonal-converting enzyme. The transformed cells were incubated with reciprocal shaking at 28°C in 100 ml of 2× YT medium containing 50 μg/ml kanamycin. After overnight cultivation, 10 ml of the culture was inoculated into 1 liter of the same medium. IPTG was then added to a final concentration of 1 mM to induce the *lac* promoter, followed by incubation with shaking at 28°C for 72 h.

All purification procedures were performed at 0-4°C. KPB (pH 7.5) was used throughout the purification. Centrifugation was carried out for 30 min at 13,000 × *g*.

Step 1. Preparation of a Cell-free Extract. Washed cells from 0.5 liters of a culture were resuspended in 50 ml of 100 mM buffer and then disrupted by sonication at 190 W for 30 min with an Insonator model 201M. The cell debris was removed by centrifugation.

Step 2. HisTrap HP Column Chromatography. The resulting supernatant solution was applied to a HisTrap HP column (1 ml; GE Healthcare UK Ltd.), attached to an ÄKTA purifier (GE Healthcare UK Ltd.) and equilibrated with 100 mM buffer. Protein was eluted from the column with 20 ml of the same buffer, the concentration of imidazole being increased linearly from 0 to 1 M. The active fractions were pooled and the homogeneity of the purified protein was confirmed by SDS-PAGE.

Identification of the enzyme flavin cofactor

For HPLC-based identification of the flavin cofactor of the enzyme, the enzyme was precipitated by the addition of 5% perchloric acid, sedimented at 10,000 × *g*, and then analyzed using a Shimadzu LC-10A HPLC system (Kyoto, Japan) with a Cosmosil $5C_{18}$-AR-II column (reversed-phase, 4.6 × 150 mm; Nacalai Tesque Co., Inc.) at 35°C. Isocratic separation was performed at a flow rate of 1.0 ml/min using methanol/0.2% KH_2PO_4 (20:80, all by volume) as the mobile phase. The absorbance was measured at 260 nm for flavins. Flavin-adenine-dinucleotide (FAD) and flavin-mononucleotide (FMN) were used as standards.

Analysis of cytidine monophosphate in MCD cofactor

Cytidine monophosphate (CMP) was released from MCD by 15 min incubation after the addition of 5% (v/v) sulfuric acid. The reaction product was sedimented at 10,000 × *g* and then separated by HPLC using a TSKgel Amide80 column (reversed-phase, 4.6 × 150 mm; Nacalai Tesque Co., Inc.) equilibrated in 30% 10 mM ammonium acetate and 70% acetonitrile at an isocratic flow rate of 1 ml/min at 35°C. CMP was quantified relative to standard CMP solutions (Neumann *et al*, 2009).

Metal analysis

All glassware was exhaustively rinsed with distilled water before use. Prior to analysis, the enzyme was dialyzed against 10 mM KPB (pH 7.5). The enzyme sample containing 1.1 mg PceSLM/ml was analyzed with an inductivity coupled radiofrequency plasma spectrometer,

Shimadzu ICPS-8100 (27.120 MHz; Kyoto, Japan). The metal contents of the enzyme sample were determined from the calibration curves for standard solutions.

Results

Cloning of the gene encoding the piperonal-converting enzyme

To identify the gene encoding the piperonal-converting enzyme, the N-terminal amino acid sequence of each of the 20 kDa, 40 kDa, and 80 kDa subunits of the purified enzyme was determined, as described under "*Material and Methods"*. A local BLAST search was run on the draft genome database for strain CT39-3 constructed in-house, using the respective amino acid sequences. As a result, three ORFs (*piperonal-converting enzyme*) coding for the 20 kDa, 40 kDa, and 80 kDa subunits were found, which were located adjacently in the same transcription direction (Fig. 5). The gene (named *pceS*) encoding the 20 kDa subunit consists of 561 nucleotides and codes for a protein of 186 amino acids with a calculated molecular mass of 19,576 Da. The gene (nemed *pceM*) encoding the 40 kDa subunit consists of 1,008 nucleotides and codes for a protein of 335 amino acids with a calculated molecular mass of 35,948 Da. The gene (nemed *pceL*) encoding the 80 kDa subunit consists of 2,226 nucleotides and codes for a protein of 741 amino acids with a calculated molecular mass of 78,956 Da. Each of the calculated molecular masses was consistent with that of each subunit of the purified enzyme determined on SDS-PAGE. The predicted promoter sequences, -35 (TCGCCG) and -10 (CTGCACAAT), in the region upstream of the ATG initiation codon of *pceS* indicated that three genes (*pceSML*) may be transcribed as one operon from that promoter.

A search with the BLAST program revealed that the deduced amino acid sequence of PceSML: the deduced amino acid sequence of PceS exhibits 71% similarity with 2Fe-2S iron-sulfur cluster binding domain-containig protein (fer2 superfamily and Fer2-2 superfamily) and CoxS (S

subunit of carbon monoxide dehydrogenase); the deduced amino acid sequence of PceM exhibits 77% similarity with molybdopterin dehydrogenase FAD-binding (FAD-binding-4 superfamily) and CoxM (M subunit of carbon monoxide dehydrogenase)； and the deduced amino acid sequence of PceL exhibits 77% similarity with those of aldehyde oxidase, xanthine dehydrogenase containing molybdopterin and CoxL (L subunit of carbon monoxide dehydrogenase) (Dobbek *et al*, 1999).

Accession codes

The nucleotide sequence data for the 16S rRNA gene and the piperonal-converting enzyme (piperonal-converting oxidase) gene cluster reported in this paper appear in the DDBJ/GenBank database under accession numbers LC088725 and LC088726, respectively.

Expression and purification of the recombinant piperonal-converting enzyme

In order to overproduce the piperonal-converting enzyme, an expression plasmid (pBBR-His-*pceSML*) was constructed and introduced into *Burkholderia* sp. CT39-3. Cell-free extracts prepared from the strain CT39-3 transformant carrying pBBR-His-*pceSML,* which was cultured at 28°C, rapidly converted piperonal (as a substrate) into piperonylic acid. The author analyzed cell-free extracts by SDS-PAGE, and detected remarkable protein bands at the 20 kDa, 40 kDa, and 80 kDa positions corresponding to three subunits of the enzyme purified from *Burkholderia* sp. CT39-3, respectively. On the other hand, these remarkable three bands were not detected for cell-free extracts prepared from strain CT39-3 carrying no expression plasmid. Therefore, overproduction of the piperonal-converting enzyme in the active form was attained.

The piperonal-converting enzyme produced in the recombinant cells was purified to

homogeneity through the HisTrap HP column chromatography described under "*Material and Methods"*. The purified enzyme gave three bands on SDS-PAGE (Fig. 6). The specific activity of the purified enzyme was found to be 62.2 units/mg, this value being almost the same as that of the enzyme purified from *Burkholderia* sp. CT39-3 (54.7 units/mg). These data indicated that the piperonal-converting enzyme purified from *Burkholderia* sp. CT39-3 carrying pBBR-His-*pceSML* is identical to that from strain CT39-3. Since the purified enzyme could not be obtained in a large amount from *Burkholderia* sp. CT39-3 and detailed analyses required a large amount of the enzyme, the author used the recombinant piperonal-converting enzyme in the following experiments.

From linear Lineweaver-Burk plots, the apparent K_m and V_{max} values of the purified enzyme were found to be 0.019 ± 0.002 mM and 65.21±4.92 units/mg, respectively

Cofactor analysis of the piperonal-converting enzyme

Purified PceSML was brownish yellow in solution and exhibited homology with molybdo-flavoenzymes, e.g., xanthine oxidase. Thus, the existence of molybdopterin (MPT), flavin and [2Fe-2S] cluster as cofactors in PceSML was expected.

First, in order to clarify whether the flavin cofactor is flavin-adenine-dinucleotide (FAD) or flavin-mononucleotide (FMN), an equal amount of 10% $HClO_4$ was added to the enzyme to release the flavin cofactor from PceSML and then the supernatant containing flavin was analyzed by HPLC. As a result, FAD was identified as the flavin cofactor (Fig. 6A), and its content was calculated to be 0.88 mol/mol of PceSML.

In order to identify the nucleotide bound to MPT in the enzyme by HPLC, purified PceSML was incubated for 15 min in the presence of sulfuric acid, which can release the nucleotide of the

molybdopterin dinucleotide cofactor, as described under *"Analysis of cytidine monophosphate in MCD cofactor"* (Neumann *et al*, 2009). As a result, cytidine monophosphate (CMP) was detected (Fig. 6B), the content of which was 0.61 mol/mol of PceSML. Quantitative analysis of metals (Mo and Fe) in the purified PceSML with an ICP emission spectroscope (ICPS-8100) revealed that PceSML contained Mo (0.55 mol/mol of PceSML). The content of CMP included in MPT corresponded well to that of Mo in PceSML. Furthermore, the enzyme contained Fe (5.2 mol/mol of PceSML), suggesting that there was enough Fe to comprise the two [2Fe-2S] clusters per enzyme. On the basis of these results, the piperonal-converting enzyme would contain FAD, molybdopterin cytosine dinucleotide cofactor (MCD), and [2Fe-2S] cluster in a ratio of 1:1:2 per PceSML.

UV-visible spectral analysis of the piperonal-converting enzyme

The purified enzyme exhibited an absorption spectrum with maxima at 320, 360, 420, 450, 470, and 560 nm (Fig. 6C), this being similar to the absorption spectra of other molybdo-flavoenzymes (Neumann *et al*, 2009). These peaks were suggested to be derived from the cofactors. The piperonal-converting enzyme gave cofactor absorption peaks at the following wavelengths: flavin absorption maxima at 386 nm and around 450 nm, and [2Fe-2S] center absorption around 325, 420, 465, and 550 nm (Uchida *et al*, 2003). The spectrum of PceSML on the addition of sodium dithionite (Fig. 6C) showed that the enzyme was completely reduced. In order to obtain a spectrum with the addition of substrate, benzaldehyde was used instead of piperonal, because piperonal exhibited its own absorption peaks at around 300 nm that prevented the measurement of a UV-visible spectrum. The resultant spectrum was different from those in the absence and presence of sodium dithionite, indicating that PceSML was partially reduced on the

addition of a substrate. During the reduction of PceSML with either benzaldehyde or sodium dithionite, a peculiar spectrum derived from the formed flavin semiquinone was not observed.

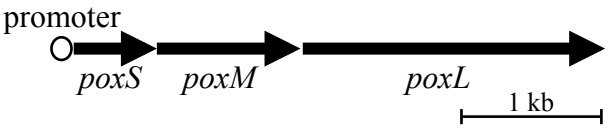

FIGURE 4. The gene organization of the piperonal-converting enzyme.

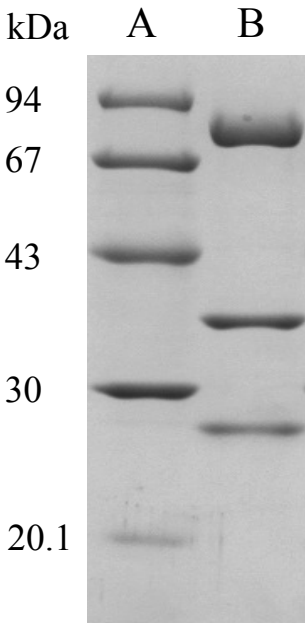

FIGURE 5. SDS-PAGE of the purified recombinant piperonal-converting enzyme.

Protein bands were detected by staining with Coomassie brilliant blue. *Lane A,* marker proteins. *Lane B*, purified recombinant piperonal-converting enzyme.

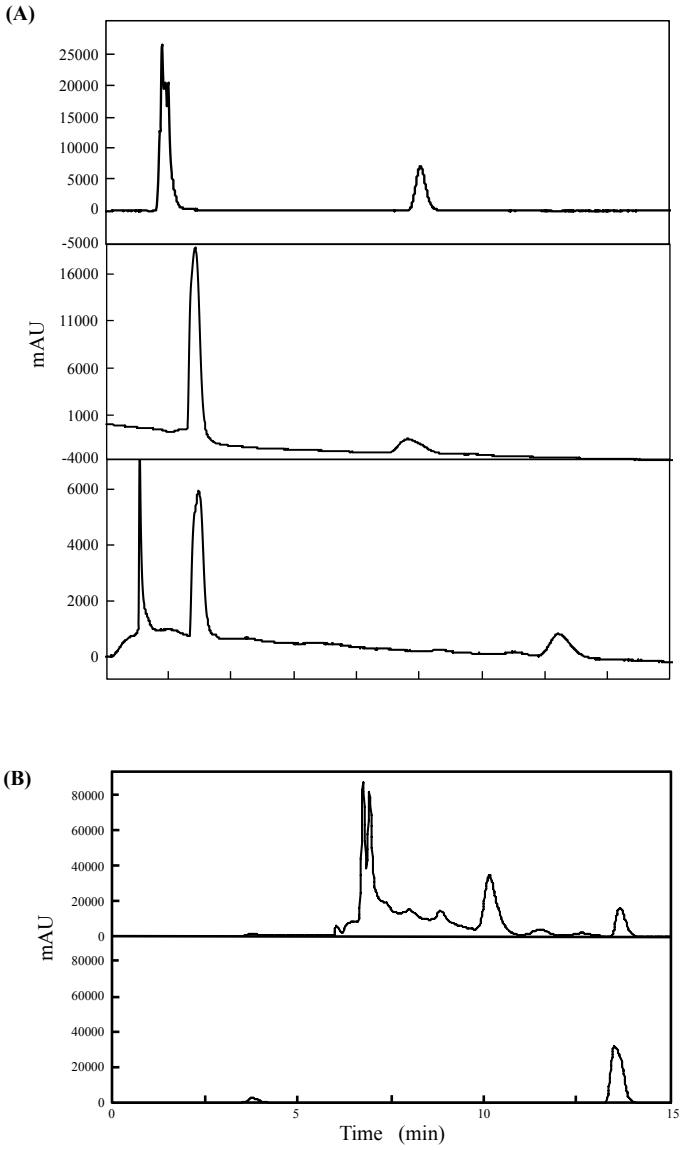
(A)
25000
20000
15000
10000
5000
0
-5000
16000
11000
6000
1000
-4000
6000
4000
2000
0
mAU
(B)
80000
60000
40000
20000
0
80000
60000
40000
20000
0
mAU
0
5
10
15
Time (min)
(C)
1.8
1.2
0.6
0
Absorbance
250
350
450
550
650
Wavelength (nm)

FIGURE 6. Cofactor analyses of the purified piperonal-converting enzyme.

(A) HPLC elution profiles of the reaction mixture containing the purified enzyme and $HClO_4$ (top), authentic FAD (middle), and authentic FMN (bottom). (B) HPLC elution profiles of the reaction mixture containing the purified enzyme and H_2SO_4 (top), and authentic CMP (bottom). (C) Absorption spectra of the piperonal-converting enzyme. The purified enzyme (solid line); the enzyme incubated with 2 mM benzaldehyde (dotted line); and the enzyme reduced with 10 mM sodium dithionite (dashed line). The concentration of enzyme protein was 2.0 mg/ml (250–700 nm) in 100 mM KPB (pH 7.5) and 1 mM EDTA. The same buffer was examined as a control. Absorption spectra were measured at room temperature.

Discussion

As for other aldehyde oxidoreductases, one using ferredoxin as an electron acceptor, which is involved in the detoxification of cinnamaldehyde, vanillin and benzaldehyde, has been reported in *E. coli* (Neumann *et al*, 2009). The enzyme is composed of three subunits (PaoA, PaoB and PaoC), and PaoA contains a Tat leader peptide that allows export of the active heterotrimer enzyme into the periplasmic space (Axel et al, 2010). On the other hand, the deduced amino acid sequences of the *pceS, pceM* and *pceL* genes all do not possess any signal sequences, indicating that the enzyme in strain CT39-3 is localized in the cytosol, i.e., not in the periplasm. It is interesting that bacterial aldehyde oxidoreductases belonging to the same xanthine oxidoreductase family are localized in the different spaces but play similar physiological roles.

Members of the molybdoenzyme family in bacteria need post-translational maturation processes, such as Fe-S cluster biosynthesis, FAD-binding, and incorporation of

molybdopterin(Zhang *et al*, 2010). For a final holoenzyme, molecular chaperones are involved (Neumann *et al* 2011). In bacterial genomes, it has been reported that structural genes encoding members of the aldehyde oxidoreductase family are clustered with those of the corresponding molecular chaperones. In *E. coli,* the gene cluster for the periplasmic aldehyde oxidoreductase consists of *paoABCD*. PaoA binding two distinct [2Fe-2S] clusters, PaoB binding FAD, and PaoC containing molybdepterin cytosine dinucleotide cofactor (MCD) are subunits of the trimeric aldehyde oxidoreductase, respectively. The product of *paoD*, which is located just downstream of the structural genes, is predicted to be involved in formation of MCD and its insertion into PaoC, because expression of *paoABC* without *paoD* results in the formation of apo-PaoABC, in which MCD is not included (Neumann *et al*, 2011). The gene cluster for another aldehyde oxidoreductase derived from *Metylobacillus* sp. KY4400 consists of *orf12345* (Yasuhara *et al*, 2005). The products of ORF1, ORF2 and ORF3 contain the [2Fe-2S] clusters, FAD and MCD, respectively. The deduced amino acid sequence of ORF5 located downstream of the structural genes shows sequence similarity to PaoD, and might be involved in maturation of MCD and its insertion into the product of ORF3. The deduced amino acid sequence of ORF4 exhibits weak sequence similarity only to some unknown proteins, thus, the function of *orf4* is unclear (Yasuhara *et al*, 2005). As for piperonal-converting oxidase (PceSML), PceS, which shows sequence similarity to the subunit containing the [2Fe-2S] clusters for aldehyde oxidoreductases, exhibits the characteristic absorption spectrum (peaks at 311, 319, 420, 470, and 550 nm) of the [2Fe-2S] centers. PceM shows sequence similarity to the subunit containing FAD, and had an absorption spectrum exhibiting a cofactor absorption peaks derived from the FAD absorption maxima at 360 nm and 450 nm. PceL shows sequence similarity to the subunit containing molybdopterin.

Considering the above all together, piperonal-converting oxidase is suggested to contain the [2Fe-2S] clusters, FAD and molybdopterin. Like for other aldehyde oxidoreductases, post-translational maturation of piperonal-converting oxidase seems to be suggested. In strain CT39-3, the structural genes *pceS, pceM* and *pceL* are clusterd, however, no genes are located between the promoter and *pceSML,* and another promoter for other gene(s) is located just downstream of *pceSML*. This gene organization indicates that the gene coding for the molecular chaperon for PceSML does not exist in either the upstream or downstream region. A search of the draft genome of strain CT39-3 revealed that some ORFs homologous to the *paoD* gene exist in a quite different locus of *pceSML*. One of them could be involved in the post-translational maturation of PceSML in the same manner as for other aldehyde oxidoreductases. Because these ORFs are not clustered with *pceSML*, the author constructed an expression plasmid harboring only *the pceSML* genes and used strain CT39-3 as a host in order to overproduce piperonal-converting oxidase.

Section II

Characterization of the piperonal-converting enzyme

Introduction

In Chapter II section I, the author succeeded to obtain enough amount of purified piperonal-converting enzyme to characterize enzymological properties. Further study was expected to elucidate enzymological properties of piperonal-converting enzyme for a better understanding of piperonal metabolism at the protein level.

This section describes the characterization of piperonal-converting enzyme purified from the strain CT39-3 transformant carrying an expression plasmid (pBBR-His-*pceSML*).

Material and Methods

Stoichiometry

The reaction mixture comprised 0.5 mM piperonal, 0.01 mg enzyme, and 100 mM KPB (pH 7.5), in a final volume of 200 μL. The reaction was started by the addition of piperonal and carried out at 25°C. The reaction was stopped by the addition of an equal volume of 0.2 M HCl. Piperonal and piperonynlic acid were analyzed by HPLC according under the "standard assay A" conditions. H_2O_2 was analyzed by HPLC with a Shimadzu LC-10Avp system equipped with a Shoudex SUGER KS-801 column (8.0 i.d. × 300 mm) under the following conditions: column temperature, 40°C; isocratic elution; mobile-phase solvent (2 mM Na_2SO_4, 20 nM EDTA); flow rate, 0.75 ml/min; and electrochemical detection, ECD700S (Eicom, Kyoto, Japan). O_2 was analyzed with an oxygen electrode with the same system as used for the measurement of O_2 under the "Chapter I Section II standard assay B" conditions.

Results

Identification of the reaction product

The product derived on piperonal conversion was analyzed by HPLC, First, the reaction was carried out for 30 min at 25°C using an excess of the partially purified enzyme in standard mixture A described under *"*Chapter I SectionII *Enzyme assays"*. In this case, only one peak with the consumption of piperonal was detected at 254 nm with the HPLC method. The retention time and UV-vis absorption spectrum of the reaction product were found to agree with those of authentic piperonylic acid (2.4 min). Thus, one of the reaction products was identified as piperonylic acid, but the other product remained unknown.

Identification of another substrate and another reaction product, and stoichiometry

Based on the identification of one of the reaction products as piperonylic acid and the results of the homology search with the piperonal-converting enzyme, another substrate and the other reaction product were suggested to be O_2 and H_2O_2, respectively. First, consumption of O_2 was analyzed with an oxygen electrode using the "standard assay B" conditions. A significant decrease of O_2 in the reaction mixture was demonstrated, demonstrating that another substrate was O_2. Next, production of H_2O_2 was analyzed by HPLC, and an increase in H_2O_2 in the reaction mixture was detected. Thus, another reaction product was identified as H_2O_2.

Using the purified enzyme, the stoichiometry of consumption of the substrates and formation of the reaction products during the enzyme reaction was examined in a reaction

mixture consisting of 100 mM KPB (pH 7.5), 0.5 mM piperonal and 0.01 mg/ml enzyme. The reaction was carried out at 25˚C. The amounts of residual piperonal, residual O_2, formed piperonylic acid and formed H_2O_2 in the reaction mixture were determined to be 451, 231, 12 and 9 μmol, respectively, when the initial amounts of piperonal and O_2 as the substrates were 462 and 243 μmol, respectively (Fig. 7). The results demonstrated that piperonylic acid and H_2O_2 were formed stoichiometrically with the consumption of piperonal and O_2 during the enzyme reaction. Together with the results of the homology search, these findings demonstrated that the enzyme involved in piperonal-conversion is piperonal-converting oxidase.

Substrate specificity

The following compounds were examined as to substrate specificity: piperonal, formaldehyde, acetaldehyde, crotonaldehyde, butylaldehyde, isobutylaldehyde, valeraldehyde, isovaleraldehyde, 1-hexalal, banzaldehyde, eugenol, salicylaldehyde, 4-hydroxybanzaldehyde, 2-hydroxybenzaldehyde, cinnamaldehyde, protocatechualdehyde, and vanilin. The level of O_2 consumption during the enzymatic reaction was determined with an oxygen electrode using the "Chapter I Section II standard assay B" conditions given under *Enzyme assays* above. Among the tested aldehydes, the enzyme showed high activity with almost all the aldehyde compounds regardless of whether they were aromatic or aliphatic ones. On the other hand, formaldehyde and acetaldehyde were inert. When salicylaldehyde was used as a substrate, the specific activity was about half (54%) (Table 3).

Effects of temperature and pH on the stability and activity of the enzyme

The effects of temperature and pH on the enzyme stability and activity were examined. The stability of the enzyme was investigated at various temperatures. After the enzyme had been preincubated for 30 min in 10 mM KPB (pH 7.5), an aliquot of the enzyme solution was taken and then the enzyme activity was assayed under the "Chapter I Section II standard assay A" conditions. The enzyme was most stable at from 10 to 62°C (Fig. 8C). The optimal reaction temperature appeared to be 40°C (Fig. 8A). At higher than 50°C, the specific activity gradually decreased. As the reaction temperature became higher, the concentration of O_2 dissolved in the reaction mixture decreased under the assay conditions used. It was suggested that the concentration of O_2 dissolved in the reaction mixture might have affected the specific activity, which became lower depending on the reaction temperature at higher than 50°C.

The stability of the enzyme was examined at various pH values. After the enzyme had been incubated at 25°C for 30 min in the 0.1 M Britton-Robinson buffer (pH 2.2–11.9) (Britton *et al*, 1931), an aliquot of the enzyme solution was taken and then the enzyme activity was assayed under the standard assay A conditions. The enzyme was most stable in the pH range of 4.6 to 11.9, with 80% of the initial activity being retained even at pH 11.9 (Fig. 8D). The enzyme exhibited maximum activity at pH 4.8 (Fig. 8B).

Table 3. *Substrate specificity.*

The reaction was carried out as described under "*Material and Methods*".

N.D., not detected.

Substrate	Relative activity
	%
Piperonal	100
Acetaldehyde	1.6
Propionaldehyde	104
Crotonaldehyde	94
Butyraldehyde	105
Isobutyraldehyde	82
Valeraldehyde	112
Isovaleraldehyde	110
1-Hexanal	117
Benzaldehyde	98
Salicylaldehyde	54
2-Hydroxybenzaldehyde	105
4-Hydroxybenzaldehyde	103
Cinnamaldehyde	112
Protocatechualdehyde	92
Vanillin	104
Xanthine	N.D.

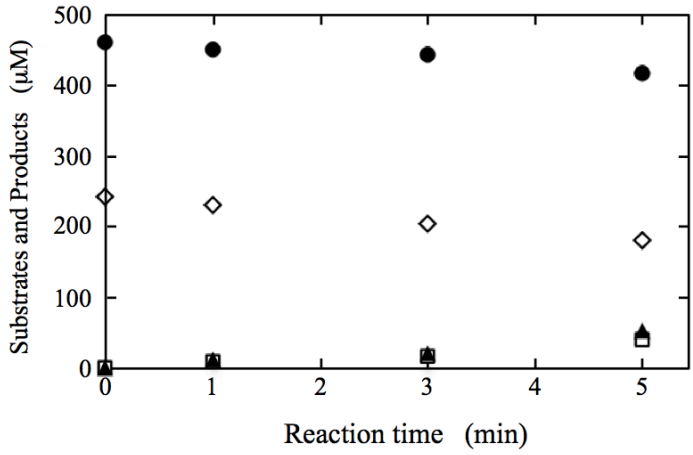

FIGURE 7. Stoichiometry analysis of the piperonal-converting reaction.

●, piperonal; ◇, O_2; ▲, piperonylic acid; □, H_2O_2. The methods for the analysis of reactants and products are described under *"Material and Methods"*. All data points represent the mean values ± S.D. for three experiments.

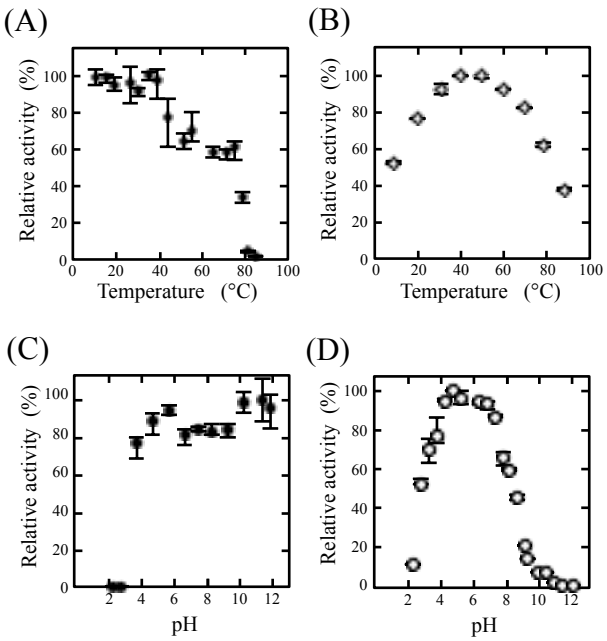

FIGURE 8. Effects of temperature and pH on the activity of piperonal-converting oxidase.

(A) Reactions were carried out at various temperatures. (B) Reactions were carried out in Britton-Robinson buffer (0.1 M) at different pH values. (C) The enzyme was preincubated at various temperatures for 30 min in 0.1 M KPB (pH 7.5), an aliquot of each enzyme solution was taken, and then the enzyme activity was assayed under the "Chapter II standard assay A" conditions. (D) The enzyme was preincubated at various pH values at 25°C for 30 min in Britton-Robinson buffer at a concentration of 0.1 M, an aliquot of each enzyme solution was taken, and then the enzyme activity was assayed under the "Chapter II standard assay A" conditions. The relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions used.

Discussion

In this section, the author found that piperonal-converting enzyme has been act on piperonal in the presence of O_2, leading to formation of piperonylic acid and H_2O_2. The enzyme also has been found to utilize only molecular oxygen, i.e., not NAD or NADP, as an electron acceptor. These findings indicate that the enzyme is piperonal-converting oxidase not piperonal dehydrogenase. The author, for the first time, clarified the initial step of piperonal metabolism and identified the enzyme involved in the metabolism: piperonal-converting oxidase.

In general, oxidoreductases that convert an aldehyde to the corresponding acid (including piperonal-converting oxidase) utilize an electron acceptor for the reaction. For instance, aldehyde dehydrogenases (EC 1.2.1.3, 1.2.1.4, and 1.2.1.5) use NAD and/or NADP as a coenzyme. *E. coli* periplasmic aldehyde oxidoreductase uses ferredoxin as an electron acceptor. Previously, an aldehyde oxidoreductase from a sulfate-reducing bacterium of genus *Desulfovivrio* using 2,6-dichlorophenol-indophenol as an electron acceptor has been isolated and characterized (Yasuhara *et al*, 2002). Furthermore, bacterial aldehyde oxidases using molecular oxygen, O_2, as an electron acceptor have already been reported (Yasuhara *et al*, 2002; Yamada et al, 2015). For these enzymes, the production of H_2O_2 is quantified by means of coupling methods to measure the enzyme activity, however, the consumption of O_2 as an electron acceptor has never been measured. In the present study, the author determined the levels of O_2 consumption with an oxygen electrode and H_2O_2 production by HPLC. To the best of our knowledge, our enzyme is the first aldehyde oxidase to have its stoichiometry measured.

Chapter III

Physiological role of piperonal-converting oxidase in *Burkholderia* sp. CT39-3

Introduction

Aldehyde oxidoreductase is a member of the xanthine oxidoreductase family, which consists of xanthine oxidoreductase, ferredoxin oxidoreductase, carbon monoxide dehydrogenase, quinoline 2-oxidoreductase, and so on. These enzymes are very important due to their primordial roles in bacterial cells. The overall amino acid sequence similarity between aldehyde oxidoreductases and xanthine oxidoreductases is approximately 50%, clearly indicating that both types of enzyme originated from a common ancestral precursor (Neumann *et al*, 2011). The biochemical and physiological functions of xanthine oxidoreductases were revealed to be in purine catabolism: conversion of hypoxanthine to xanthine and subsequent conversion of xanthine to uric acid. On the other hand, those of aldehyde oxidoreductases, which exist in the organs of man, archaea, bacteria and so on, have remained largely unclear (Axel *et al*, 2010). Also, the physiological role of aldehyde oxidases belonging to the aldehyde oxidoreductase family is not yet understood in detail, but they might have some significant roles in bacteria. In order to determine the physiological role of piperonal-converting oxidase, the author cultivated *Burkholderia* sp. CT39-3 in medium containing glucose or piperonal as the sole carbon source.

Material and Methods

Growth experiments

The culture medium for investigating the role of the piperonal-converting oxidase was composed of 0.1% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) K_2HPO_4, 0.05 g KH_2PO_4, 0.2 g NaCl, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, and 0.03, 0.07 or 0.10 % (w/v) piperonal or glucose (final concentration) (pH 7.5). Unless otherwise stated, piperonal and glucose were used as the sole carbon source, respectively.

Results

Growth of Burkholderia sp. CT39-3 in the minimal media containing different carbon sources

To determine the function of the piperonal-converting enzyme in vivo, *Burkholderia* sp. CT39-3 was grown in several minimal culture media. *Burkholderia* sp. CT39-3 exhibited full growth in the medium containing glucose as the sole carbon source (Fig. 9A), piperonal-converting enzyme activity being observed. On the other hand, even on replacement of glucose with 0.03% (w/v) piperonal, *Burkholderia* sp. CT39-3 grew (Fig. 9A). During the lag growth phase, the amount of piperonal decreased stoichiometrically with an increase in the amount of piperonylic acid in the culture medium. Then, the amounts of piperonal and piperonylic acid in the medium decreased with the growth of strain CT39-3. Finally, both piperonal and pipernonylic acid in the medium were completely consumed in the stationary phase (Fig. 9B). As shown in Fig. 5A, however, the bacterial growth became substantially delayed as the concentration of piperonal increased (0.07%) and was completely inhibited on the addition of 0.10 % piperonal. The piperonal-converting enzyme activity was also detected in the cells grown in the medium containing 0.03% or 0.07% piperonal.

Moreover, strain CT39-3 was grown in the medium containing piperonylic acid as a sole carbon source in the same way to as above-mentioned. Piperonylic acid in the medium decreased during the logarithmic growth phase and was completely consumed in the stationary phase (Fig. 9C).

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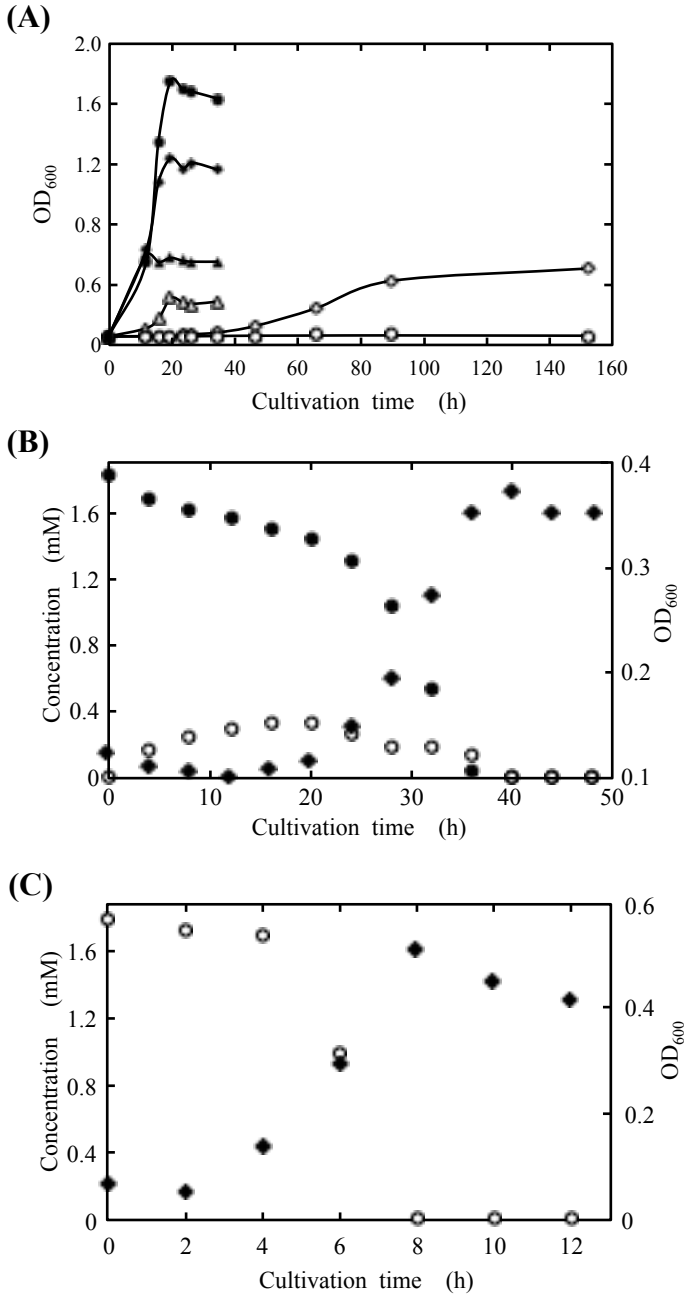

FIGURE 9. Growth of *Burkholderia* sp. CT39-3 in the minimal media containing different carbon sources.

(A) Cell growth of *Burkholderia* sp. CT39-3 cultured with 0.03% piperonal (△), 0.07% piperonal (◇), 0.10% piperonal (○), 0.03% glucose (▲), 0.07% glucose (◆), or 0.10% glucose (●). (B)

Cell growth of *Burkholderia* sp. CT39-3 (◆), concentration of piperonal in a medium (●), and concentration of piperonylic acid in a medium (○). (C) Cell growth of *Burkholderia* sp. CT39-3 (◆), concentration of piperonylic acid in a medium (○).

Discussion

CT39-3 grew well as the concentration of glucose in the medium became higher. On the contrary, CT39-3 grew slowly in the medium containing a low amount of piperonal [0.03% (w/v)]. The growth of CT39-3 was increasingly inhibited as the concentration of piperonal in the medium became higher (Fig. 9). Interestingly, the piperonal-converting oxidase acivity was observed under all conditions tested, even in the presence of glucose [0.026, 0.227 and 0.044 units/mg for 0.10, 0.07 and 0.03% (w/v) piperonal, respectively; and 0.043, 0.077 and 0.119 units/mg for 0.10, 0.07 and 0.03% (w/v) glucose, respectively]. Moreover, the concentration of piperonal did not affect the activity, namely the formation of piperonal-converting oxidase. These findings suggest that the enzyme is not inducible but is expressed constitutively. That the bacterial growth depended on the concentration of piperonal suggests that a high level [0.1% (w/v)] of piperonal is toxic for strain CT39-3. A lower amount of piperonal contributed as a carbon source and restored growth of strain CT39-3. Moreover, analysis of the substrate specificity of piperonal-converting oxidase showed broad substrate specificity with various aldehydes, whereas purines were not oxidized by piperonal-converting oxidase. These findings indicate that piperonal-converting oxidase is not a xanthine oxidase involved in purine catabolism, and that the physiological role of piperonal-converting oxidase is the utilization of various aldehyde compounds and/or their detoxification into less toxic acids. In particular, the apparent K_m value for piperonal is extremely low, this being reasonable if the purified enzyme is considered to be involved in the utilization and/or detoxification of piperonal *in vivo*. Some aromatic aldehydes, for example, vanillin and cinnamaldehyde, have been shown to have antimicrobial properties at high doses (Neumann *et al*,

2009). Thus, it had been reported that aromatic aldehydes play important roles due to their antimicrobial actions in plants (Bakkali *et al*, 2008; Muhlemann *et al*, 2014). Some *Burkholderia* species have been reported to be phytopathogenic bacteria, and to exist on the surface of plants or in soil (Ham *et al*, 2011; Seo *et al*, 2015). Considering that strain CT39-3 was identified as *Burkholderia* sp., PceSML would play an important role in the defense mechanism against the antimicrobial compounds derived from plant species, opening new avenues for further investigation of the relationship between plants and microorganisms.

Conclusion

Piperonal, an aromatic aldehyde, is a floral volatilic secondary metabolite and is known as a component of the essential oil of the heliotrope flower, commonly used as in perfumes, cosmetics and flavoring agents. Degradation of various kinds of secondary metabolites from higher plants has been reported. However, little is known regarding metabolism of aromatic aldehyde compounds, and findings regarding the roles of botanical volatile aromatic aldehydes in higher plants are limited. In particular, there has been neither isolation of microorganisms nor elucidation of the metabolic pathway involved in piperonal metabolism.

Here, the author, for the first time, isolated piperonal-converting microorganisms from soil, and identified the one (strain CT39-3) exhibiting the highest activity as *Burkholderia* sp. The author succeeded in purifying the piperonal-converting enzyme involved in the initial step of piperonal metabolism. This piperonal-converting enzyme has been found to act on piperonal in the presence of O_2, leading to formation of piperonylic acid and H_2O_2, clarifying the initial piperonal degradation pathway (piperonal → piperonylic acid). Gene cloning of the enzyme (*pceSML*) and a homology search revealed that the enzyme belongs to the xanthine oxidase family, which comprise molybdoenzymes containing a molybdopterin cytosine dinucleotide cofactor. Together with the results of the homology search, these findings demonstrated that the enzyme involved in piperonal-conversion is piperonal-converting oxidase. The author also found that piperonal-converting oxidase is invoved in not only intracellular detoxification of aldehyde compounds but assimilation of piperonal as well.

Analysis of the substrate specificity of PceSML showed a broad substrate specificity for

various aldehydes (Table 3). The specific activities shown in Table 3 are high, therefore, almost all aldehydes are rapidly converted to the corresponding carboxylic acids without the addition of any coenzyme (e.g., NAD or NADP) to the reaction mixture. It is obvious that an O_2-dependent aldehyde oxidase is significantly advantageous from an economical standpoint to produce carboxylic acids from aldehydes, rather than NAD(P)-dependent aldehyde oxidoreductases, because it does not require any expensive cofactors. The smallest subunit (PceS) of the purified enzyme tends to be unstable under aerobic conditions, because it is suggested to contain Fe-S clusters (Khoroshilova *et al*, 1997). However, the enzyme works stably during the resting cell reaction. Moreover, a recombinant piperonal-converting oxidase containing an N-terminal His・tag fusion to PceS is more stable than the enzyme purified from strain CT39-3. Therefore, it is possible to produce carboxylic acids effectively in the reaction mixture using the resting cells or recombinant piperonal-converting oxidase, which is easy to purify. Piperonal-converting oxidase exhibits great potential as to the bioconversion of various carboxylic acids from the corresponding aldehyde compounds in the future.

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