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ORIGINAL PAPER

Purification and properties of phenolic acid decarboxylase from *Candida guilliermondii*

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Abstract A heat-labile phenolic acid decarboxylase from *Candida guilliermondii* (an anamorph of *Pichia guilliermondii*) was purified to homogeneity by simple successive column chromatography within 3 days. The molecular mass was 20 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and 36 kDa by gel-filtration chromatography, suggesting that the purified enzyme is a homodimer. The optimal pH and temperature were approximately 6.0 and 25°C. Characteristically, more than 50% of the optimal activity was observed at 0°C, suggesting that this enzyme is cold-adapted. The enzyme converted *p*-coumaric acid, ferulic acid, and caffeic acid to corresponding products with high specific activities of approximately 600, 530, and 46 U/mg, respectively. The activity was stimulated by Mg^{2+} ions, whereas it was completely inhibited by Fe^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, 4-chloromericuribenzoate, *N*-bromosuccinimide, and diethyl pyrocarbonate. The enzyme was inducible and expressed inside the cells moderately by ferulic acid and *p*-coumaric acid and significantly by non-metabolizable 6-hydroxy-2-naphthoic acid.

Keywords Phenolic acid decarboxylase · *p*-Coumaric acid decarboxylase · Ferulic acid decarboxylase · *Candida guilliermondii* · *Pichia guilliermondii*

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Abbreviations

FA	Ferulic acid
PCA	*p*-Coumaric acid
CA	Caffeic acid
4-VG	4-Vinylguaiacol
4-VP	4-Vinylphenol
6H2N	6-Hydroxy-2-naphthoic acid
PAD	Phenolic acid decarboxylase
CgPAD	PAD from *C. guilliermondii*
PMSF	Phenylmethanesulfonyl fluoride
MES	2-Morpholinoethanesulfonic acid
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
HPLC	High-performance liquid chromatography
SD	Standard deviation
YNB	Yeast Nitrogen Base

Introduction

Ferulic acid (FA), a derivative of hydroxycinnamic acid, is found in cell walls, leaves, and seeds of plants such as rice, wheat, and oats, as well as in coffee, apple, artichoke, peanut, orange, and pineapple [31]. It occurs in them primarily in both its free form and as an ester linked to lignin and other polysaccharides. FA is a precursor of vanillin, one of the aromatic flavors used in the food, pharmaceutical, and cosmetic industries [32, 36]. In addition, owing to both its antioxidant and anti-inflammatory activities, FA has versatile functional and biological activities, such as protecting foods from oxidative spoilage [29] and whitening skin [28], and it has been shown to lower blood glucose level, blood pressure [1, 31], plasma

total cholesterol and low-density-lipoprotein cholesterol concentrations [25, 40], and inhibit tumor promotion [22].

Volatile phenols, including 4-vinylguaiacol (4-VG), 4-vinylphenol (4-VP), and ethylphenol, are frequently detected in beer, wine, and whiskey during brewing and fermentation. These phenolic compounds usually originate from the microbial decarboxylation of phenolic acids (hydroxycinnamic acids) present in the raw materials during fermentation [11, 38, 39, 46, 47] and in fruit juice production [14, 16]. They are valuable precursors in the biotransformation of flavors and fragrances [32] and are regarded as a good aroma and/or off-flavors in beers and wines [11, 34, 38, 39, 44, 47]. The microbial phenolic acid decarboxylases (PADs), which decarboxylate FA and/or *p*-coumaric acid (PCA) with concomitant production of 4-VG and/or 4-VP, respectively, are believed to be responsible for the detoxification of phenolic acids [7, 8, 10–12, 21, 39].

Phenolic acids inhibit the growth of microorganisms, including yeasts such as *Saccharomyces cerevisiae*, *Pichia anomala*, *Debaryomyces hansenii*, and *Candida guilliermondii* [2, 35, 41]. *C. guilliermondii* (an anamorph of *P. guilliermondii*) has been frequently isolated from grapes and musts as a contaminant [3, 13, 30]. *Candida* spp. decarboxylate FA, generating 4-VG as an off-flavor in improperly stored fruit juices [43] and as a characteristic flavor of soy sauce and miso [42]. However, little is known about the enzymatic properties of PADs from yeasts except for those of two species of *Brettanomyces* [15, 17]. The aim of this work was to purify and characterize a PAD from *C. guilliermondii* (CgPAD), which may be involved in the metabolism of phenolic acids by yeast.

Materials and methods

Materials

FA, caffeic acid (CA), 4-VG, and 6-hydroxy-2-naphthoic acid (6H2N) were purchased from Wako Pure Chemical (Osaka, Japan). PCA was from MP Biomedicals (Solon, OH), and 4-VP was from Sigma–Aldrich (Steinheim, Germany). All other chemicals used were of analytical grade.

Organism and culture conditions

The enzyme source was *C. guilliermondii* ATCC 9058. The growth medium was composed (w/v) of 1.0% glucose, 0.5% peptone (Bacto Peptone; Becton, Dickinson and Company, Sparks, MD), 0.2% yeast extract (Becton, Dickinson and Company), 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 1 mM 6H2N (pH 7.0). The yeast was grown at 25°C for 3 days, with shaking, in 200-ml aliquots of the medium placed in 2-l flasks. After collecting cells by centrifugation (3,000 $\times g$ for 5 min) at 4°C, the cell paste (17.7 g wet wt. from a 600-ml culture) was used as the starting material for purification of the enzyme. 6H2N was used as the pseudo-inducer because our preliminary experiments showed that it enhanced the expression of the enzyme in the cells much more than FA and PCA did.

Purification of CgPAD

Enzyme purification was done at a temperature not exceeding 4°C. The harvested 6H2N-induced cells were washed twice with physiological saline solution and then suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM each of phenylmethanesulfonyl fluoride (PMSF), $MgCl_2$, EDTA, and dithiothreitol. The yeast cells were disrupted six times for 50 s each with glass beads (0.5 mm in diameter) at 2,500 rpm in a homogenizer (Multi-Beads Shocker; Yasui Kikai, Osaka, Japan). After cell debris was removed by centrifugation (12,000 $\times g$, 15 min), the supernatant obtained was applied directly to a column of CM Toyopearl 650 M (2.5 cm × 24 cm; Tosoh, Tokyo, Japan) previously equilibrated with 20 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.0). The active fractions that passed through the column were combined, and the solution was immediately applied to a column of DEAE Toyopearl Fast Flow (2.5 cm × 25.5 cm; Tosoh) that had been equilibrated with the same buffer. The column was initially washed with 700 ml of 50 mM NaCl in MES buffer (pH 6.0), and proteins were eluted with a 600-ml linear gradient of 50–400 mM NaCl in the buffer. The active fractions were concentrated and exchanged with 50 mM phosphate buffer (pH 7.0) by ultrafiltration (Amicon Ultra-15; Millipore, Billerica, MA) to a small volume. The concentrate was then put on a column of Bio-Gel P-100 (2.5 cm × 45 cm; Bio-Rad, Hercules, CA) equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The gel chromatography was done by elution with the equilibration buffer, and the active fractions eluted were combined and concentrated by ultrafiltration. The retentate was used exclusively for further experiments as the final preparation of purified enzyme. The purified enzyme was stored at −20°C when necessary.

Estimation of molecular mass

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done with a 15% (w/v) acrylamide gel for determination of the subunit molecular mass using a PageRuler Unstained Protein Ladder (Thermo Fisher Scientific, Rockville, MD) as standard markers.

Proteins in the gel were stained with Coomassie Brilliant Blue R250. The molecular mass of the native form was estimated by gel-filtration chromatography using a column of Superdex75 10/300 GL (GE Healthcare & Bio-Sciences, Uppsala, Sweden) at a flow rate of 0.4 ml/min (L-7100 pump; Hitachi, Tokyo, Japan) with 50 mM phosphate buffer plus 0.15 M NaCl (pH 7.0). The column was calibrated with standard markers (Sigma–Aldrich), bovine serum albumin (66 kDa), chicken egg albumin (44 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa), using a UV detector operated at 220 nm (U-VIS L-7420; Hitachi).

Assay of CgPAD activity

The initial velocity of decarboxylation activity was measured at 25°C with a phenolic acid as substrate unless otherwise stated. The reaction mixture contained suitably diluted enzyme solution and a 5 mM substrate (neutralized with 1.0 N NaOH) in 0.1 M sodium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. The products formed were quantified by high-performance liquid chromatography (HPLC) on a system from Waters (Milford, MA) equipped with a 2487 Dual λ Absorbance Detector and a 2695 Separation Module. HPLC was done at 40°C on a packed column for reversed-phase chromatography (Cosmosil 5C18-MS-II, 4.6 mm × 150 mm; Nacalai Tesque, Tokyo, Japan) with acetonitrile/0.05% phosphoric acid (7:3, v/v) as the mobile phase at a flow rate of 0.6 ml/min. Ten µl of the sample were injected automatically, and the UV detector was operated at 260 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of 4-VG or 4-VP per min. Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific) with bovine serum albumin as the standard.

Induction experiments

Yeast Nitrogen Base (YNB; Invitrogen, Carlsbad, CA) broth was used to examine the inducibility of CgPAD. The carbon sources used were glucose, galactose, and sodium acetate at 0.5% (w/v) each, and inducer candidates were FA, PCA, and 6H2N each at 1.0 mM. Cultures were incubated at 25°C for 2 days, and the growth (A_{650}), pH of the spent media, and specific activities toward FA and PCA in the cell-free extracts were measured.

Results

Purification and physicochemical properties of CgPAD

A highly purified enzyme was obtained within 3 days by a simple purification procedure, as summarized in Table 1, with a high yield (19%). Approximately 87-fold purification to a specific activity of 531 U/mg was obtained when measured with FA as the substrate at 25°C in 0.1 M phosphate buffer at pH 6.0. The protein was homogeneous, and its molecular mass determined to be approximately 20 kDa, as judged by SDS–PAGE (Fig. 1a). Gel chromatography of the purified enzyme gave a molecular mass of approximately 36 kDa (Fig. 1b), suggesting that the enzyme is a dimer composed of two identical subunits. The absorption spectrum in the UV region exhibited a simple protein peak around at 280 nm, and the extinction coefficient at A_{280} (10 g/l; light path, 1 cm) was 20.5 in 50 mM phosphate buffer (pH 7.0) when the protein was quantified using bovine serum albumin as the reference standard.

Effects of metal ions and chemical reagents on activity

The enzyme reaction was carried out in the presence of various cations (5 mM each) at 25°C for 5 min with FA as the substrate. Fe^{2+}, Ni^{2+}, Cu^{2+}, and Hg^{2+} ions completely inhibited the reaction. Zn^{2+} ions caused 29% inhibition. Ca^{2+}, Mn^{2+}, Co^{2+}, Fe^{3+}, and Al^{3+} ions had either no effect or a slightly inhibitory effect. The decarboxylation activities toward FA and PCA gradually increased with the increase in the concentration of Mg^{2+} ions. The activation maxima of 180 and 153%, respectively, of the control activity were reached at around 10 mM without the cation. The cation-induced activation of CA decarboxylation fluctuated considerably, possibly due to the low activity. The effects of chemical reagents were examined by the

Table 1 Summary of a typical purification of CgPAD

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Cell-free extract	37.5	251	1,530	6.09	100	1.0
Toyopearl CM	103	128	1,170	9.12	76.5	1.5
DEAE Sepharose FF	37.0	9.81	323	32.9	21.1	5.4
Bio-Gel P-100	1.67	0.55	292	531	19.1	87.2

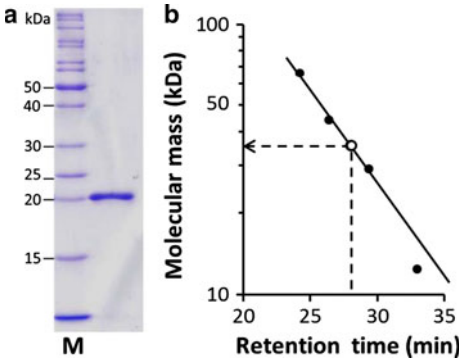

Fig. 1 Estimation of the molecular mass of the purified enzyme by SDS–PAGE (**a**) and gel-filtration chromatography (**b**). In **a** *M* denotes molecular mass markers (in kDa), and the amount of the purified enzyme analyzed was 1.0 μg

method of Igarashi et al. [23]. The activity was inhibited almost completely by 4-chloromercuribenzoate, *N*-bromosuccinimide, and diethyl pyrocarbonate, but not by *N*-ethylmaleimide and iodoacetate (0.5 mM) under the indicated conditions, as shown in Table 2. PMSF and EDTA had no effect on the activity.

Substrate specificity and kinetic parameters

The purified CgPAD was examined for its ability to decarboxylate various phenolic acids and their derivatives (5 mM each) under the standard assay conditions. It decarboxylated PCA, FA, and CA at relative ratios of approximately 100:89:8, as shown in Table 3. No reaction was observed with cinnamic acid, 2- and 3-hydroxycinnamic acids, *o*- and *m*-cinnamic acids, 3-(4-hydroxyphenyl)propionic acid, 2-naphthoic acid, and 6H2N. Among the active substrates, CgPAD tended to favor PCA over FA for catalysis, as judged by their K_m, k_{cat}, and catalytic efficiency (k_{cat}/K_m) values, which are shown in Table 4. The k_{cat} for PCA with 5 mM Mg^{2+} ions was approximately 1.4-fold greater than that without the cation. The K_m values were not affected by the presence of the cation. These results suggested that this cation enhanced the CgPAD activity.

Effects of pH and temperature on activity and stability

The pH ranges at which the purified enzyme was active and stable were determined using FA as the substrate. As shown in Fig. 2a, the maximal activity was observed at pH 6.1 when measured in various buffers at 50 mM. When Britton–Robinson buffers at different pH values were used, the optimal pH was 5.9. In the pH range between 5.5 and 7.5, the specific activity was 2- to 4-fold greater in various buffers than that in Britton–Robinson buffers. In the buffers, no detectable activity was observed at lower than pH 4.3 and higher than pH 8.8. To determine the pH stability, the enzyme was preincubated at 4°C for 3 h in 10 mM Britton–Robinson buffer and assayed at 25°C in 0.1 M phosphate buffer at pH 6.0. The enzyme was stable over the range between pH 6.5 and 8.5, and the activity was completely abolished at pHs lower than 3.5 and higher than 11.5 (data not shown).

Table 2 Effects of chemical reagents on the activity

Chemical reagent	Concentration (mM)	Relative activity[a] (%)
No additive	1.0	100 ± 11
N-Ethylmaleimide	1.0	96 ± 1.8
Diethyl pyrocarbonate	1.0	0
N-Bromosuccinimide[b]	0.1	4 ± 0.31
PMSF	1.0	94 ± 5.5
4-Chloromercuribenzoate[c]	0.5	7 ± 0.11
Iodoacetate[c]	0.5	84 ± 4.9
EDTA	1.0	99 ± 2.1

[a] The activity was measured after the enzyme had been treated with each chemical reagent for 20 min at the indicated pH and temperature in the suitable buffer. The treatment conditions were 25°C and at pH 6.0 in 10 mM phosphate buffer, and a 0.1-ml aliquot was used for the determination of the residual activity under the standard assay conditions. The values are the means of three experiments with standard deviation (SD) and shown as the percentages of the activity without additives, which is taken as 100%

[b] Treated for 20 min at 4°C and at pH 5.0 in 10 mM acetate buffer

[c] Treated for 20 min at 25°C and at pH 5.0 in 10 mM acetate buffer

Table 3 Substrate specificity of CgPAD

Substrate	Specific activity[a] (U/mg)	Relative activity (%)
p-Coumaric acid	600 ± 3.74	100
Caffeic acid	45.6 ± 2.47	7.6
Ferulic acid	531 ± 31.9	88.5

[a] Data represent the means of three experiments with SD

Table 4 Kinetic parameters of CgPAD

Substrate	Kinetic parameter[a]		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m[b] (s^{-1} mM^{-1})
FA	5.32 ± 0.288	114 ± 2.67	21.4 ± 1.44
PCA	2.66 ± 3.91	113 ± 11.1	42.5 ± 3.06
PCA + 5 mM Mg^{2+}	2.64 ± 0.581	158 ± 22.0	59.8 ± 5.61

[a] The values are the means of three experiments with SD and shown as the percentages of the activity without additives, which is taken as 100%

[b] The values were calculated assuming the native molecular mass as 36 kDa

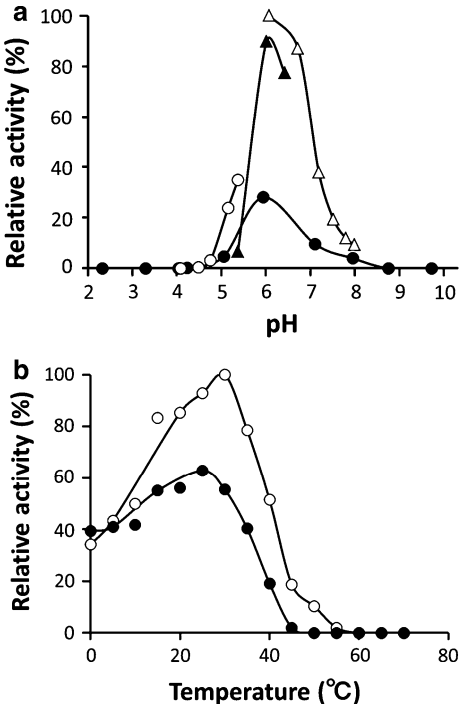

Fig. 2 Effects of pH and temperature on the activity of purified CgPAD. **a** The pH–activity curve was measured twice at 25°C with FA as substrate in various buffers at 50 mM (*open circles*, sodium acetate; *filled triangles*, MES; *open triangles*, sodium phosphate) and 50 mM Britton–Robinson universal buffers at different pH values (*filled circles*). The actual pH in each reaction mixture was previously measured at 25°C. The average values are expressed as percentages, taking the maximal activity as 100%. **b** The temperature–activity curves were measured at 25°C and at pH 6.0 in 50 mM phosphate buffer in the absence (*filled circles*) and presence of $MgCl_2$ (5 mM; *open circles*). The activities were measured twice at different temperatures under the standard conditions of enzyme assay using 0.1 M phosphate buffer (pH 6.0) with FA as substrate. The average values are expressed as percentages, taking the maximal activity as 100%

The decarboxylation activity was measured at various temperatures at pH 6.0 in 50 mM phosphate buffer in the absence or presence of 10 mM Mg^{2+} ions, as shown in Fig. 2b. The optimal temperature for activity with FA as the substrate was approximately 25°C, whereas that in the presence of Mg^{2+} ions was 25–30°C. The activity was stimulated by this divalent cation at the temperature range examined. Notably, even at 0°C, more than 50% of the activity at the optimal temperature was observed. The thermal stability of the enzyme was assessed at pH 6.0 in 50 mM phosphate buffer after heating for 20 min at various temperatures. The enzyme was stable up to 30°C (or more), and complete loss of activity was observed at 50°C, as shown in Table 5. At the temperatures examined, the ratios of remaining activity with and without 10 mM Mg^{2+} ions were essentially the same, indicating again that the cation stimulates activity, but causes no protection from thermal inactivation.

Table 5 Thermal stability at different temperatures

Temperature (°C)	Relative activity (%)[a]		
	− Mg^{2+}	+ Mg^{2+}	+ Mg^{2+}/− Mg^{2+}
Control	100 ± 11.4	135 ± 2.13	135 ± 2.13
25	107 ± 5.69	131 ± 1.18	122 ± 1.10
30	102 ± 1.56	140 ± 2.70	137 ± 2.60
40	67 ± 1.15	87 ± 8.92	130 ± 13.3
50	8 ± 0.26	7 ± 1.56	88 ± 19.6

[a] The enzyme was pretreated at the indicated temperatures for 20 min in the absence (−) or presence (+) of Mg^{2+} ions (5 mM) in 0.1 M phosphate buffer (pH 6.0). The residual activities were determined under the standard enzyme assay conditions. The values are the means of three experiments with SD, taking the control (not heated) as 100%

Inducibility of CgPAD

In order to study the inducibility of CgPAD, we initially examined the effects of 0.5% (w/v) carbon sources on the expression of the enzyme in the YNB broth plus 1 mM 6H2N instead of the complex medium used for enzyme purification. Under shaking conditions, the induction on glucose with 6H2N was approximately double those on galactose or sodium acetate after incubation at 25°C for 2 days. Then, we examined the effects of 1 mM each of 6H2N, FA, and PCA on the inducibility of CgPAD in YNB broth plus 0.5% glucose. As shown in Table 6, the addition of PCA or FA to the media induced FA and PCA activities at equal ratios. Unexpectedly, 6H2N was found to induce both activities at approximately 16- and 6-fold greater levels than FA and PCA, respectively. In addition, 6H2N-induced cells grown on glucose, galactose, and sodium acetate contained the decarboxylation activities toward PCA and FA at relative ratios of 1:3, 1:2, and 1:4, respectively. Notably, when glucose was used as a carbon source, the aerobic growth with PCA or FA doubled that without the additive, whereas that with 6H2N did not.

Bioconversions of FA and PCA under growing conditions

Candida guilliermondii ATCC 9058 was grown aerobically in YNB broth plus 0.5% (w/v) glucose with FA and PCA (each at 1 mM) under the same conditions as described above. The phenolic acids were converted almost stoichiometrically to 4-VG and 4-VP, respectively, within 24 h, as shown in Fig. 3. Further conversion of the two products did not occur even after 2 days. When anaerobiosis was attained in a flask, which was filled with the media up to the narrow neck, the amount of growth at A_{650} on 0.5% (w/v) glucose was faint and reached 0.2 after a 2-day and only 0.5 even after a 5 day-incubation.

Table 6 Inducibility of CgPAD under different growth conditions

Growth condition[a]	Growth (A_{650})	pH of medium	Specific activity toward (U/mg)[b]		Ratio of FA/PCA
			FA	PCA	
Glucose					
No additive	8.6	2.4	0.24	0.33	0.73
+ 1 mM FA	15	2.3	0.72	0.94	0.77
+ 1 mM PCA	17	2.1	0.97	1.31	0.74
+ 1 mM 6H2N	6.7	2.6	16.7	6.11	2.73
Galactose					
+ 1 mM 6H2N	5.4	2.6	9.83	4.58	2.15
Sodium acetate					
+ 1 mM 6H2N	2.7	5.1	8.54	2.18	3.89

[a] Each culture was grown with shaking in YNB broth plus 0.5% (w/v) carbon source at 25°C for 2 days

[b] The activities toward both substrates were measured twice, and the average values are shown

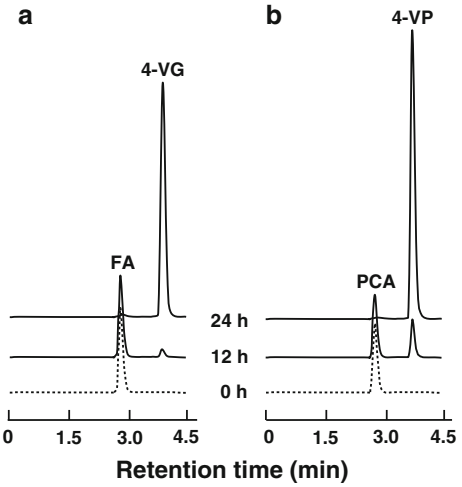

Fig. 3 Bioconversions of FA and PCA during the growth of *C. guilliermondii*. The yeast was grown with shaking at 25°C on 0.5% (w/v) glucose in YNB broth plus 1.0 mM each of FA and PCA. At intervals (12, 24, and 36 h), aliquots (10 µl) of each broth were withdrawn and subjected to HPLC with the uninoculated broth as reference (0 h, *dotted line*). **a** Elution profiles of HPLC when FA was added. Retention times of FA and 4-VG were 2.83 and 3.90 min, respectively. **b** Those when PCA was added. Retention times of PCA and 4-VP were 2.79 and 3.75 min, respectively. The amounts of 4-VG and 4-VP accumulated after 24 h were calculated to be approximately 1 mM for both. The HPLC profiles after 36 h were similar to those after 24 h

Discussion

In this study, we purified a substrate-inducible PAD from *C. guilliermondii* ATCC 9058 (CgPAD) to homogeneity by a simple purification procedure within 3 days. Our purification procedure does not require concentration of the enzyme solution prior to column chromatography on cation and anion exchangers equilibrated at an appropriate pH (6.0 in the case of CgPAD). The native CgPAD was a homodimer with two identical subunits, which is similar to those of yeasts such as *Brettanomyces anomalus* [15] and *Brettanomyces bruxellensis* [17] and bacteria such as *Bacillus subtilis* [8], *Bacillus pumilus* [12], and *Pseudomonas fluorescens* [21]. Exceptionally, the *p*-coumaric acid decarboxylase from *L. plantarum* LpCHL2 has a larger molecular mass of 93 kDa consisting of four identical 23.5-kDa subunits [7]. Characteristically, CgPAD was cold-adapted in that, even at 0°C, it exhibited more than 50% of the activity at the optimal temperature, which is similar to the *B. pumilus* enzyme [12]. CgPAD was heat-labile as in the cases of PADs from *B. pumilus* [12], *Lactobacillus brevis* [27], and *L. plantarum* [37]. The enzyme activity was abolished by *N*-bromosuccinimide and diethyl pyrocarbonate, suggesting that tryptophan and histidine residues contribute to the enzyme catalysis. It is generally accepted that tryptophan around an active site plays a role in substrate binding [9, 26] and histidine is essential for activity in some enzymes [24]. Heavy metal ions and 4-chloromercuribenzoate also inhibited the CgPAD activity completely. The contribution of cysteine residues to the catalysis is unclear because iodoacetate and *N*-ethylmaleimide exhibited either no or a moderately inhibitory effect, respectively.

CgPAD was active toward PCA, FA, and CA, but inactive toward cinnamic acid, 2- and 3-hydroxycinnamic acids, *o*- and *m*-cinnamic acids, and notably 3-(4-hydroxyphenyl)propionic acid, indicating that the *p*-hydroxycinnamic acid (4-hydroxycinnamic acid) derivatives serve as substrates for this enzyme. CgPAD decarboxylated PCA, FA, and CA at relative ratios of 100:89:8, the values of which are very different from those of the enzymes of yeasts *B. anomalus* (100:266:84) [15] and *B. bruxellensis* (100:120:80) [17]. Furthermore, the activity of CgPAD toward FA and/or PCA was stimulated by Mg^{2+} ions and their specific activities were much higher than those reported for these and other yeasts [10, 15, 17, 33] and bacteria [12, 27].

PADs are believed to be responsible for the detoxification of phenolic acids, and most of them are inducible in yeasts [10, 17, 18] and bacteria [4, 5, 7, 8, 12, 19, 45]. The genetic mechanism of PAD expression has been well explained by the PadR-mediated response to phenolic acids in bacteria such as *Pediococcus pentosaceus* [5], *L. plantarum* [19], and *B. subtilis* [45]. However, such a bacterial induction mechanism might not be applicable to eukaryotic *C. guilliermondii* ATCC 9058 because antimicrobial PCA and FA rather stimulated the growth rate of this yeast in YNB broth. The stimulation was reproducibly observed in the presence of both phenolic acids at concentrations from

0.1 to 2.0 mM, although they significantly retarded growth at 10 mM (data not shown).

The hyperinduction of PAD by 6H2N was first demonstrated by Hashidoko et al. [20] using Gram-negative *Klebsiella oxytoca*. It seems likely that 6H2N can induce PADs in other eukaryotes and also those in Gram-positive bacteria if 6H2N were not degraded by the organisms. In fact, 6H2N was not degraded by *C. guilliermondii* ATCC 9058 in YNB broth as judged by HPLC. In the case of *C. guilliermondii* ATCC 9058, both PCA and FA may induce CgPAD because the ratio of induced PCA and FA activities is comparable to that of the purified enzyme. However, the ratios of the activities toward PCA and FA in the 6H2N-induced cells varied from 1:2 to 1:4 depending on the carbon sources added. It is possible that 6H2N induces a PAD other than CgPAD, although we did not detect such activity during the course of purification. To resolve this inconsistency we are studying whether the 6H2N induces the other PAD or an unidentified mechanism occurs in *C. guilliermondii* ATCC 9058 cells.

When *C. guilliermondii* ATCC 9058 was grown aerobically in YNB broth, stoichiometric conversions of FA and PCA to 4-VG and 4-VP, respectively, were completed within 24 h. 4-Ethylguaiacol, 4-ethylphenol, and other derivatives from the products [6, 13, 17, 42] were not detectable even after 2 days. Therefore, the reaction with CgPAD or fermentation using this yeast would allow industrial production of 4-VG and 4-VP, which are precursors of flavors and fragrances [32, 36] and detected as the aroma of beers and wines [11, 34, 38, 39, 44, 47] and flavors of soy sauce and miso [42].

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