Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 32 (2005) 205–211
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Purification and properties of D-hydantoin hydrolase and *N*-carbamoyl-D-amino acid amidohydrolase from *Flavobacterium* sp. AJ11199 and *Pasteurella* sp. AJ11221

Hiroyuki Nozaki*, Ikuo Kira, Kunihiko Watanabe, Kenzo Yokozeki

Aminoscience Laboratories, Ajinomoto Co., Ltd., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan

Received 20 October 2004; received in revised form 21 November 2004; accepted 3 December 2004
Available online 8 January 2005

Abstract

We characterized recombinant D-hydantoin hydrolase (DHHase) and *N*-carbamoyl-D-amino acid amidohydrolase (DCHase) from *Flavobacterium* sp. AJ11199 and *Pasteurella* sp. AJ11221. The DHHases from these two strains showed a wide range of hydrolytic activity for various 5-monosubstituted D-hydantoin compounds, including a very high level activity for D-hydantoin compounds corresponding to D-aromatic amino acids such as D-tryptophan D-phenylalanine and D-tyrosine. The DCHases, in turn, were capable of catalyzing the hydrolysis of various *N*-carbamoyl-D-amino acids (NCD-A.A.) corresponding to D-aliphatic and D-aromatic amino acids. The combination of these enzymes was found to be applicable for the production of various D-amino acids.

Keywords: D-Hydantoin hydrolase; *N*-Carbamoyl-D-amino acid amidohydrolase; *Flavobacterium*; *Pasteurella*

1. Introduction

D-Amino acids are extensively used as pharmaceutical intermediates. The hydantoin hydrolysis method is one of the most useful enzymatic processes for producing D-amino acids. The method is particularly efficient in producing D-*p*-hydroxyphenylglycine, an intermediate for amoxicillin, by combining D-hydantoin hydrolase (DHHase), *N*-carbamoyl-D-amino acid amidohydrolase (DCHase) and spontaneous racemization of the corresponding hydantoin [1–3]. In screening for microorganisms capable of hydrolyzing D-hydantoin compounds from natural sources, *Pseudomonas* sp. AJ11220 was found to be a potent producer of D-*p*-hydroxyphenylglycine from the corresponding hydantoin [3]. The same screening also revealed that *Flavobacterium* sp. AJ11199 [4] and *Pasteurella* sp. AJ11221 [5] were both producers of D-amino acid. In both strains, the genes encoding DHHase and DCHase were already cloned.

Earlier papers have described the properties of several types of DHHases [6–11] and DCHases [12–16], as well as methods to produce D-amino acids by successive reactions between them [17–20]. Very few reports [10–15], however, have investigated the kinetic properties of these enzymes for various substrates.

Meanwhile, hydantoin racemase has been purified from *Microbacterium liquefaciens* (formerly named *Flavobacterium liquefaciens*) AJ3912, a strain that produces L-amino acids from hydantoin compounds [21], and the hydantoin racemase gene of this strain has been cloned [22]. Various types of D-amino acid can be efficiently produced from the corresponding DL-form of 5-substituted hydantoins by combining DHHase and DCHase with hydantoin racemase, an enzyme capable of releasing a rate-limiting step of hydantoin racemization.

We report here the expression of DHHase genes and DCHase genes from *Flavobacterium* sp. AJ11199 and *Pas-*

* Corresponding author. Tel.: +81 44 244 7137; fax: +81 44 244 6581.
E-mail address: hiroyuki_nozaki@ajinomoto.com (H. Nozaki).

1381-1177/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2004.12.003

teurella sp. AJ11221 in *Escherichia coli*, the purification of the enzymes from the recombinant strains, and the comparative features of enzymes in relation to the production of D-amino acid.

2. Materials and methods

2.1. Materials

Hydantoin compounds and *N*-carbamoylamino acids were prepared from the corresponding amino acids with potassium cyanate as described previously [23]. Restriction enzymes, *Taq* DNA polymerase, and pUC19 were purchased from Takara Shuzo Co., Ltd (Kyoto, Japan). D-Amino acid oxidase was purchased from Sigma Chemical Co., Ltd. (Missouri, USA). Peroxidase was obtained from Toyobo Co., Ltd. (Osaka, Japan). *p*-Dimethylaminobenzaldehyde, 4-aminoantipyrine, and phenol were purchased from Nacalai Tesque (Kyoto, Japan). The pKK223-3, Gel Filtration LMW Calibration Kit, Gel Filtration HMW Calibration Kit and all chromatography columns for protein purification were from Amersham Bioscience Corp. (New Jersey, USA). All other chemicals were of the highest grade commercially available.

2.2. Plasmids

A pUC19 derivative expression vector was constructed as follows. The trp promoter was amplified from *E. coli* W3110 chromosomal DNA with Primer 1 (5′-GTA TCA CGA GGC CCT AGC TGT GGT GTC ATG GTC GGT GAT C-3′) and Primer 2 (5′-TTC GGG GAT TCC ATA TGA TAC CCT TTT TAC GTG AAC TTG C-3′), and the 0.5 kb-fragment was subcloned into pGEM-T Easy (Promega, WI, USA). The fragment obtained by digestion with *Eco*O109I-*Eco*RI was inserted into *Eco*O109I/*Eco*RI-digested pUC19. Next, the rrnB terminator obtained from *Hin*dIII/*Hin*cII-digested pKK223-3 was inserted into the *Hin*dIII/*Pvu*II site of the plasmid, resulting in pTrp1. The SD sequence was arranged by amplifying the promoter region with Primers 1 and 3 (5′-GGG GGG CAT ATG CGA CCT CCT TAT TAC GTG AAC TTG-3′), and then replacing the fragment with the corresponding region of pTrp1 to give the vector pTrp4.

DHHase genes and DCHase genes were amplified with oligonucleotide primers (DHHase (AJ11199), 5′-GGG AAT TCC ATA TGA CCC ATT ACG ATC TCG TCA TTC-3′ and 5′-CGG AAT TCT CAG GCC GTT TCC ACT TCG CC-3′; DCHase (AJ11199), 5′-GGA ATT CCA TAT GCC AGG AAA GAT CAT TCT CGC G-3′ and 5′-CGC GGA TCC TCA GAC GGC GGC GAT CAA CCC GTA TT-3′; DHHase (AJ11221), 5′-CGG GAA TTC CAT ATG AGC AGG AAG ATG ATT CTC GCC GT-3′ and 5′-CGC GGA TCC TCA GGC GAG CTT CGG GGA ACG CGC GT-3′; DCHase (AJ11221), 5′-CGG GAA TTC CAT ATG AGC AGG AAG ATG ATT CTC GCC GT-3′ and 5′-CGC GGA TCC CTA GCG GAC CTG TTC TTC CGC GAT GA-3′) from the chromosomal DNA of *Flavobacterium* sp. AJ11199 and *Pastereulla* sp. AJ11221 as templates, respectively. Each amplified fragment was inserted into the *Nde*I/*Eco*RI site of pTrp4, resulting in the plasmids pTrp11199H4, pTrp11199C4, pTrp11221H4, and pTrp11221C4, respectively.

2.3. Expression and purification

E. coli JM109 cells were transformed with the individual plasmids. A volume of 1 ml of preculture with LB medium containing ampicillin (100 μg ml^{-1}) was inoculated into 50 ml of M9-casamino acid medium containing ampicillin (100 μg ml^{-1}) [24].

All purification procedures were carried out at 4 °C or on ice.

For DHHase purification, cells from a 0.5 l culture were harvested by centrifugation (8000 × *g*) and washed twice with 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM $MnSO_4$ (buffer A). The cell suspension in buffer A was sonicated with an Insonator 201 (Kubota, Tokyo, Japan), and centrifuged at 12,000 × *g* for 20 min. The resultant supernatant was collected for use as the cell-free extract and applied to a HiLoad 16/10 Q sepharose HP column equilibrated with buffer A. After eluting the enzyme with a linear gradient of 0–1 M NaCl, the active fraction was collected, dialyzed against buffer A containing 1 M $(NH_4)_2SO_4$, applied to a HiLoad 16/10 phenyl-sepharose HP column equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$, and eluted with a linear gradient of 1–0 M $(NH_4)_2SO_4$. The active fractions were pooled and concentrated by CentriPrep YM-10 (Millipore, MA, USA), and the concentrated enzyme was loaded onto a HiLoad 26/60 Superdex 200 pg column equilibrated with buffer A.

To purify the DCHases, crude extract was prepared from a 1 l culture using essentially the same method as described above, but with the use of 25 mM Tris–HCl buffer (pH 8.0) containing 20% (v/v) glycerol and 1 mM dithiothreitol (DTT) (buffer B). The cell-free extract was applied to a HiLoad 26/10 Q sepharose HP column equilibrated with buffer B, the enzyme was eluted with a linear gradient of 0–1 M NaCl and the active fraction was collected and dialyzed against buffer B containing 1 M $(NH_4)_2SO_4$. The dialyzed enzyme was applied to a HiLoad 16/10 phenyl-sepharose HP column equilibrated with buffer B containing 1 M $(NH_4)_2SO_4$ and then eluted with a linear gradient of 1–0 M $(NH_4)_2SO_4$. The active fractions were pooled and dialyzed against buffer B.

2.4. Enzyme assay

The DHHase assay mixture was consisted of 50 μmol of Tris–HCl (pH 8.0), 5 μmol of D-5-benzylhydantoin, 1 μmol of $MnSO_4$, and an appropriate amount of enzyme in 1 ml. After incubation for 5–30 min at 37 °C, the reaction was stopped by diluted phosphoric acid (pH 3.0), the reaction mixture

was centrifuged at 16,000 × *g* for 10 min, and the supernatant was analyzed by a high-performance liquid chromatograph packed with CHIRALPAK WH (4.6 mm × 250 mm) (Daicel Chemical Industries, Ltd., Tokyo, Japan) with 5 mM $CuSO_4$: MeOH (100:5) as the mobile phase. One unit was defined as the amount of enzyme catalyzing the formation of 1 μmol of NCD-Phe per minute. For substrate specificity experiments, Inertsil Ph-3 (4.6 mm × 250 mm) (GL Science Inc., Tokyo, Japan) was also used. The detection was performed at 210 nm at 40 °C, and 50 mM sodium phosphate buffer (pH 2.8): acetonitrile (95:5) was used as the mobile phase. Alternatively, NCD-A.A. was determined spectrophotometrically at 438 nm by *p*-dimethylaminobenzaldehyde according to the method of Grifantini et al. [20]. The cyclization reaction mixture consisted of 50 μmol of MES-NaOH buffer (pH 6.0), 50 μmol of NCD-Phe, 1 μmol of MnSO4, and an appropriate amount of enzyme in 1 ml. The reaction was carried out at 37 °C and D-5-benzylhydantoin was analyzed by the HPLC method (CHIRALPAK WH) described above.

The DCHase assay was carried out according to the method of Ogawa et al. [12]. The reaction mixture contained 200 μmol of potassium phosphate buffer (pH 7.0), 50 μmol of NCD-Phe, and an appropriate amount of enzyme in 1 ml. The reaction was performed at 37 °C and analyzed by the HPLC method described above. One unit was defined as the amount of enzyme catalyzing the formation of 1 μmol of D-Phe per minute. For substrate specificity experiments, D-amino acids were analyzed by HPLC method or spectrophotometrically by D-amino oxidase method with peroxidase, 4-aminoantipyrine, and phenol [12].

2.5. Protein analysis

Protein concentrations were determined by the Bradford methodology [25] using bovine serum albumin as standard. SDS-PAGE was performed on a 10–20% polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) with the Precision Protein Marker (Bio-Rad Laboratories, CA, USA) as marker proteins. The native relative molecular mass was determined by a HiLoad 26/20 Superdex 200 pg column with Gel Filtration LMWcalibration Kit and Gel Filtration HMW Calibration Kit.

3. Results and discussion

3.1. Expression and purification

Inclusion bodies were observed in *E. coli* JM109 carrying pTrp11199H4 or pTrp11221H4 when the cells were cultivated above 35 °C. In contrast, no inclusion bodies were found to form at 32 °C in the medium containing 1 mM $MnSO_4$. Since DHHases were activated by manganese ion as described below, we speculated the addition of $MnSO_4$ probably assists in forming soluble enzymes in *E. coli*. The SDS-PAGE analysis of the crude extracts indicated that the DHHases accounted for 5–10% of the total soluble protein. The purification procedures and yields are summarized in Table 1. Both purified DHHases were judged to be homogeneous by SDS-PAGE (52 kDa (AJ11199) and 50 kDa (AJ11221)), and their native molecular masses were estimated to be approximately 105 kDa (AJ11199) and 100 kDa (AJ11221) by chromatography in a Superdex 200 pg column, respectively (Fig. 1A).

The expression of the DCHase gene was also successfully obtained from *E. coli* JM109 carrying pTrp11199C4 or pTrp11221C4. In the case of DCHase, however, the enzyme in the cell-free extract was unstable without DTT and glycerol. When DCHases were incubated with DTT and glycerol, 95% of the activities in the cell-free extract remained after 1 week storage at 4 °C. Noting that the cysteine residues play important roles in several types of DCHases [12–16,26], we concluded that the effect of the DTT was probably preventing the oxidation of the SH group in the cysteine residue. Through all the purification steps, the purified enzyme catalyzed the hydrolysis of NCD-Phe at rates of 1.04 μmol min^{-1} mg^{-1} (AJ11199) and 2.18 μmol min^{-1} mg^{-1} (AJ11221), respectively (Table 2). These purified enzymes were judged to be homogeneous by SDS-PAGE (37 and 38 kDa) (Fig. 1B), and the relative molecular masses were estimated to be 116 and 112 kDa by gel filtration, respectively.

3.2. Effect of temperature and pH

The activities of both DHHases remained constant under 50 °C, but they decreased to 89% (AJ11199) and 68%

Table 1
Purification of recombinant DHHases

Fraction	Total protein (mg)	Specific activity (U mg^{-1})	Total activity (U)	Yield (%)
AJ11199				
Cell-free	480	1.40	671	100.0
Q-sepharose	135	3.90	528	78.6
Phenyl-sepharose	18.0	27.6	497	74.0
Superdex 200 pg	6.64	29.0	193	28.7
AJ11221				
Cell-free	466.4	9.83	4580	100.0
Q-sepharose	123.8	24.0	2960	64.7
Phenyl-sepharose	27.0	72.0	1940	42.4
Superdex 200 pg	9.4	101	949	20.7

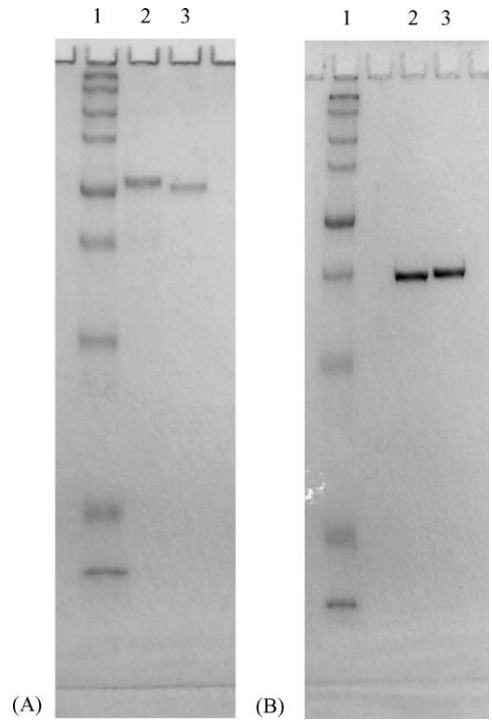

Fig. 1. SDS-PAGE of the recombinant DHHase and DCHase. (A) Lane 1, marker proteins: (from top) 250, 150, 100, 75, 50, 37, 25, 15 kDa; lane 2, purified DHHase from AJ11199; lane 3, purified DHHase from AJ11221. (B) Lane 1, marker proteins; lane 2, purified DCHase from AJ11199; lane 3, purified DCHase from AJ11221.

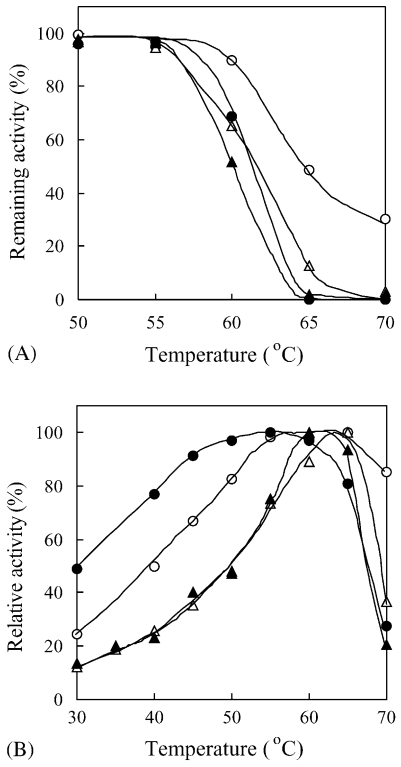

Fig. 2. Effect of temperature on DHHase and DCHase. (A) Thermostability. The remaining activities of DHHase from AJ11199 (◯) and AJ11221 (●) were assayed under a standard condition after incubation at indicated temperatures for 10 min with buffer A. The remaining activities of DCHases from AJ11199 (△) and AJ11221 (▲) were measured by the same method with buffer B. The remaining activity is expressed as the percentage of the activity before the incubation. (B) Temperature profile. The activities of DHHase from AJ11199 (◯) and AJ11221 (●) and the activities of DCHase from AJ11199 (△) and AJ11221 (▲) were assayed at various temperatures under standard condition, respectively. The relative activity was expressed as the percentage of the maximum activity under the experimental conditions.

(AJ11221) after the treatment at 60 °C for 10 min (Fig. 2A). Both the DHHases were stable in the range of pH 7.5–9.0. The optimal temperature and optimal pH were 50–60 °C and 8.5–9.5, respectively (Fig. 2B).

The activities of both of DCHases remained constant at 50 °C, but they decreased to 65% (AJ11199) and 51% (AJ11221) after the treatment at 60 °C for 10 min (Fig. 2A). Both of the DCHases were stable in the range of pH 6.5–7.0. The optimal temperature and optimal pH were 60–70 °C and 6.5–7.5, respectively (Fig. 2B).

3.3. *Effect of metal ions*

Table 3 shows the changes in DHHase and DCHase activities in response to metal ions (Table 3). DHHase from AJ11199 exhibited an enhanced activity in response to Fe^{2+}, Mg^{2+} and Mn^{2+}, versus a slightly inhibited activity in response to Ca^{2+}, Co^{2+} and Ni^{2+}. The addition of Mg^{2+} and Mn^{2+} also enhanced the activity of DHHase from AJ11221 (by 141% and 145%, respectively). In contrast, the addition of EDTA as a metal ion chelator produced no significant effect on these DHHases. An earlier study demonstrated an EDTA-induced inactivation of the DHHase from *Bacillus stearothermophillus* [7] followed by a manganese-ion-

Table 2
Purification of recombinant DCHases

Fraction	Total protein (mg)	Specific activity (U mg^{-1})	Total activity (U)	Yield (%)
AJ11199				
Cell-free	1200	0.410	493	100.0
Q-sepharose	412	0.761	315	63.9
Phenyl-sepharose	114	1.04	121	24.5
AJ11221				
Cell-free	906	0.390	355	100.0
Q-sepharose	256	1.22	313	88.1
Phenyl-sepharose	102	2.18	222	62.5

Table 3
Effect of metal ions and EDTA on DHHase and DCHase

	DHHase relative activity (%)[a]		DCHase relative activity (%)[b]	
	AJ11199	AJ11221	AJ11199	AJ11221
No addition	100	100	100	100
$CaCl_2$	73.4	84.8	97.5	104
$CoCl_2$	46.2	70.8	34.6	23.8
$CuCl_2$	ND	ND	4.78	0.47
$FeCl_2$	150	65.6	97.6	88.4
$MgCl_2$	149	141	105	101
$MnCl_2$	177	145	101	101
$NiCl_2$	54.3	80.8	64.7	44.8
$ZnCl_2$	ND	ND	3.23	0.46
EDTA[c]	105	116	105	109

[a] After the purified enzymes were dialyzed against 50 mM Tris–HCl buffer (pH 8.0), samples were incubated on ice for 30 min in the presence of the metal ions (1 mM). The activities were also measured with the metal ions (1 mM).
[b] The purified enzymes were dialyzed against buffer B.
[c] The final concentration of EDTA in incubation and enzyme assay was 10 mM.

induced recovery. Subsequent crystal studies characterized this DHHase as a metaloenzyme [27]. The enzyme from *Agrobacterium tumefaciens* [8], on the other hand, was not activated by magnesium ion. Three-dimensional analyses will elucidate why this divalent ion brings about different effects on the DHHases.

The DCHase activities were slightly inhibited by Co^{2+}, Ni^{2+}, strongly inhibited by Cu^{2+} and Zn^{2+}, and unaffected by Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and EDTA.

3.4. Substrate specificities and kinetic properties

A wide range of 5-monosubstituted D-hydantoin compounds for DHHase and *N*-carbamoyl compounds for DCHase were tested to investigate the substrate specificity of the enzyme. The V_{max} and K_m values were calculated from the double reciprocal of Lineweaver–Burk plots.

Table 4 shows the hydrolytic activities of DHHase from AJ11199 and AJ11221 on 5-monosubstituted D-hydantoin compounds. These DHHases can act on hydantoin and 5,6-dihydrouracil, but no activity was observed toward 5-monosubstituted L-hydantoin compounds. These enzymes have a high V_{max} value for D-hydantoins corresponding to D-aromatic amino acids such as D-phenylalanine, D-tryptophan, D-tyrosine and D-phenylglycine, and the highest V_{max}/K_m was detected using D-5-indorylmethylhydantoin as substrate in each case. Among D-hydantoin compounds corresponding to D-aliphatic amino acids, the D-5-*iso*-butylhydantoin exhibited effective hydrolysis, while the V_{max}/K_m of D-5-*tert*-butylhydantoin was low. In the case of D-5-carboxyethylhydantoin with a polar group, the activities were weak. Further, the hydrolytic activities for 5,6-dihydrouracil were lower than those for the 5-monosubstituted D-hydantoin compounds. DHHase from *Bacillus stearothermophilus* [7]

Table 4
The kinetic property of DHHase

Substrate	Assay[a]	AJ11199		AJ11221	
		V_{max} (U mg^{-1})	K_m (mM)	V_{max} (U mg^{-1})	K_m (mM)
hydantoin	A	23.1	16.6	2.34	2.98
D-5-carboxyethylhydantoin	A	2.6	23.0	73.5	285
D-5-methylsulfanylethylhydantoin	A	90.1	0.96	370	5.48
D-5-*iso*-propylhydantoin	A	109	19.0	175	20.2
D-5-*n*-propylhydantoin	A	86.2	4.60	178	7.88
D-5-*iso*-butylhydantoin	A	120	5.01	256	10.3
D-5-*n*-butylhydantoin	A	56.8	1.53	107	2.15
D-5-*t*-butylhydantoin	A	2.71	13.2	5.42	6.16
5,6-dihydrouracil	A	2.80	7.5	20.9	32.1
D-5-benzylhydantoin	B	137	17.2	294	7.59
D-5-*p*-hydroxybenzylhydantoin	B	132	2.84	455	3.27
D-5-indorylmethylhydantoin	B	135	1.14	555	1.44
D-5-phenylhydantoin	B	147	3.31	400	3.76

The activity was not determined for the following compounds: L-5-*iso*-propylhydantoin, L-5-*n*-propylhydantoin, L-5-*iso*-butylhydantoin, L-5-*n*-butylhydantoin, L-5-benzylhydantoin, L-5-indorylmethylhydantoin.
[a] A, *p*-dimethylaminobenzaldehyde method. B, HPLC method.

Table 5
The kinetic property of DCHases

substrate	Assay[a]	AJ11199		AJ11221	
		V_{max} (U mg^{-1})	K_m (mM)	V_{max} (U mg^{-1})	K_m (mM)
NCD-Ala	A	1.72	8.20	5.85	25.3
NCD -2-aminobutyric acid	A	2.00	2.90	4.49	4.12
NCD -norvaline	A	2.65	0.67	4.49	0.59
NCD -norleucine	A	1.03	0.33	5.55	1.40
NCD -Val	A	0.71	1.33	0.96	0.54
NCD -Leu	A	0.99	0.13	3.38	0.53
NCD -Met	A	1.16	0.22	5.50	0.99
NCD -Phe	B	0.96	0.51	2.30	1.20
NCD -Tyr	B	0.78	0.41	1.63	0.47
NCD -Trp	B	0.43	0.13	2.27	0.37
NCD -phenylglycine	B	2.31	5.30	7.89	19.4
NCD –*p*-hydroxyphenylglycine	B	1.71	2.60	4.42	4.13

The activity for the following compounds was less than 0.1 U mg^{-1} under the standard assay conditions: NCD-Lys, NCD-Cys, NCD-Glu and NCD-*tert*-Leu. The activity was not determined for the following compounds: NCD-Asp, *N*-carbamoyl-β-alanine, *N*-carbamoyl-sarcosine, *N*-carbamoyl-L-Ala, *N*-carbamoyl-L-2-aminobutyric acid, *N*-carbamoyl-L-norvaline, *N*-carbamoyl-L-norleucine, *N*-carbamoyl-L-Val, *N*-carbamoyl-L-Leu, *N*-carbamoyl-L-Phe, *N*-carbamoyl-L-Tyr, *N*-carbamoyl-L-Trp, *N*-carbamoyl-L-*p*-hydroxyphenylglycine.

[a] (A) D-amino acid oxidase method; (B) HPLC method.

has maximal activitity on hydantoin, and the enzyme from *Agrobacterium tumefaciens*[8], *Blastobacter* sp. [10], and *Pseudomonas striata* [11] showed the highest activity toward 5,6-dihydrouracil. The specificities of these DHHases are similar to each other, but partially different from DHHases previously reported [6–11].

DHHases also catalyzed the reversible reaction, enabling us to measure the dehydrative cyclization activity using NCD-Phe as the substrate. V_{max} and K_m was 1.0 U mg^{-1} and 1.5 mM (AJ11199), 160 U mg^{-1} and 7.8 mM (AJ11221), respectively. K_{eq} ([NCD-Phe]/[D-5-benzylhydantoin]) at pH 8.0 was 8.9 (AJ11199) and 9.4 (AJ11221), respectively.

Table 5 shows the hydrolytic activities of DCHase from AJ11199 and AJ11221 on NCD-A.A. No activity was detected toward *N*-carbamoyl-L-amino acids. The activities toward NCD-A.A. were detected on a wide range of substrates, but V_{max}/K_m for the NCD-A.A. with a short side chain (e.g., NCD-Ala and NCD-2-amino butyric acid) or a polar moiety (e.g., NCD-Glu, NCD-Asp and NCD-Lys) was lower. Further, the NCD-Val and NCD-*tert*-Leu were lower than the other NCD-A.A. These results suggest the branched chain at β-carbon position may cause the sterical hindrance at the active site. DCHase from *Blastobacter* sp. [13] and *Comamonas* sp. [12] showed maximal activity toward NCD-phenylglycine and NCD-Phe, respectively. Among the enzymes previously reported, DCHases in several substances exhibited specificities similar to the specificity of the DCHase from *Agrobacterium* sp. [15], but the enzyme has the highest activity on NCD-Met and can also act on NCD-Asp.

Among the substrates tested, the DCHase from AJ11199 showed the highest V_{max}/K_m on NCD-Leu (7.6 U mg^{-1} mM^{-1}), whereas the DCHase from AJ11221 showed the highest V_{max}/K_m on NCD-norvaline (7.6 U mg^{-1} mM^{-1}). Further, the DCHase from AJ11221 had a higher relative activity (V_{max}/K_m) on *N*-carbamoyl-D-aromatic amino acids than that from AJ11199.

4. Conclusion

DHHase and DCHase from *Flavobacterium* sp. AJ11199 and *Pasteurella* sp. AJ11221 were purified, characterized and compared with each other. These enzymes seemed to share several properties and to show good promise for the production of various kinds of D-amino acids.

References

[1] H. Yamada, S. Takahashi, Y. Kii, H. Kumagai, J. Ferment. Technol. 56 (1978) 484–491.
[2] R. Olivieri, E. Fascetti, L. Angelini, L. Degen, Enzyme Microb. Technol. 1 (1979) 201–204.
[3] K. Yokozeki, S. Nakamori, C. Eguchi, K. Yamada, K. Mitsugi, Agric. Biol. Chem. 51 (1987) 355–362.
[4] Japanese Patent, JP2003024073A2.
[5] Japanese Patent, JP2003024074A2.
[6] G.J. Kim, J.H. Park, D.C. Lee, H.S. Ro, H.S. Kim, Mol. Gen. Genet. 255 (1997) 152–156.
[7] S.G. Lee, D.C. Lee, S.P. Hong, M.H. Sung, H.S. Kim, Appl. Microbiol. Biotechnol. 43 (1995) 270–276.
[8] D.R. Durham, J.E. Weber, Biochem. Biophys. Res. Commun. 216 (1995) 1095–1100.
[9] G. Lapoite, S. Viau, D. Leblanc, N. Robert, A. Morin, Appl. Environ. Microbiol. 60 (1994) 888–895.
[10] C.L. Soong, J. Ogawa, M. Honda, S. Shimizu, Appl. Environ. Microbiol. 65 (1999) 1462–1495.
[11] S. Takahashi, Y. Kii, H. Kumagai, H. Yamada, J. Ferment. Technol. 56 (1978) 492–498.
[12] J. Ogawa, S. Shimizu, H. Yamada, Eur. J. Biochem. 212 (1993) 685–691.
[13] J. Ogawa, M.C.M. Chung, S. Hida, H. Yamada, S. Shimizu, J. Biotechnol. 38 (1994) 11–19.
[14] A. Louwrier, C.J. Knowles, Enzyme Micro. Technol. 19 (1996) 562–571.
[15] H. Nanba, Y. Ikenaka, Y. Yamada, K. Yajima, M. Takano, S. Takahashi, Biosci. Biotechnol. Biochem. 62 (1998) 875–881.
[16] Y. Ikenaka, H. Nanba, Y. Yamada, K. Yajima, M. Takano, S. Takahashi, Biosci. Biotechnol. Biochem. 62 (1998) 882–886.

[17] J.H. Park, G.J. Kim, H.S. Kim, Biotechnol. Prog. 16 (2002) 564–570.
[18] Y.P. Chen, C.J. Chiang, P.T. Chen, Biotechnol. Lett. (2000) 99–103.
[19] Y.P. Chao, H. Fu, T.E. Lo, P.T. Chen, J.J. Wang, Biotechnol. Prog. 15 (1999) 1039–1045.
[20] R. Grifantini, G. Galli, G. Carpani, C. Pratesi, G. Frascotti, G. Grandi, Microrbiology 144 (1998) 947–954.
[21] K. Yokozeki, K. Sano, C. Eguchi, K. Yamada, K. Mitsugi, Agric. Biol. Chem. 51 (1987) 363–369.
[22] Japanese Patent, JP2002330784A2.
[23] T. Suzuki, K. Igarashi, K. Hase, K. Tuzimura, Agric. Biol. Chem. 37 (1973) 411–416.
[24] S.H. Bass, D.G. Yansura, Mol. Biotechnol. 16 (2000) 253–260.
[25] M.M. Bradford, Anal. Biochem. 72 (1976) 248–251.
[26] R. Grifantini, C. Pratesi, G. Galli, G. Grandi, J. Biol. Chem. 271 (1996) 9326–9331.
[27] Y.H. Cheon, H.S. Kim, K.H. Han, J. Abendroth, K. Niefind, D. Schomburg, J. Wang, Y. Kim, Biochemistry 41 (2002) 9410–9417.