Microbial transformations of hydantoin-related compounds

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ABBREVIATIONS

DEAE-	Diethylaminoethyl-
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
MES	2-(*N*-Morpholino)ethanesulfonic acid
MOPS	3-(*N*-Morpholino)propanesulfonic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Pi	Inorganic phosphate
SDS	Sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)aminomethane
C-D-HPG	*N*-Carbamoyl-D-*p*-hydroxyphenylglycine
C-D-Phe	*N*-Carbamoyl-D-phenylalanine
DL-HPG-hyd	DL-5-(*p*-Hydroxyphenyl)hydantoin
D-HPG	D-*p*-Hydroxyphenylglycine
HPLC	High-performance liquid chromatography
PAGE	Polyacrylamide gel electrophoresis
TLC	Thin-layer chromatography
K_m	Michaelis constant
M_r	Relative molecular mass
V_{max}	Maximum velocity
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
dATP	Deoxyadenosine-5'-triphosphate
CTP	Cytidine-5'-triphosphate
dCTP	Deoxycytidine-5'-triphosphate
GTP	Guanosine-5'-triphosphate

dGTP	Deoxyguanosine-5'-triphosphate
ITP	Inosine-5'-triphosphate
dITP	Deoxyinosine-5'-triphosphate
dTTP	Thymidine-5'-triphosphate
UTP	Uridine-5'-triphosphate
dUTP	Deoxyuridine-5'-triphosphate
XTP	Xanthosine-5'-triphosphate

INTRODUCTION

The term "hydantoin" was originally used as the systematic name for imidazolidine-2,4-dione or 2,4-diketotetrahydroimidazole, but it is now also used as the group name for a family of different hydantoin derivatives. The known naturally occurring derivatives are allantoin, L-5-carboxymethylhydantoin, L-5-carboxyethylhydantoin and *N*-methylhydantoin. They are the intermediates or the by-products of purine nucleotide [1-3], orotate [4,5], histidine [6] and creatinine [7,8] degradation, respectively. Other than these, synthetic hydantoins are commonly used in organic synthesis and their physiological activities have been characterized. The most important members of this family are the 5-monosubstituted hydantoins, which are used as precursors for the chemical synthesis of DL-α-amino acids [9]. The other compounds of interest are 5,5-disubstituted hydantoins, which are of pharmacological interest as hypnotic and narcotic agents [10].

Hydantoins are metabolized by an enzyme identified the presence of enzyme responsible for the metabolism was long been known in many plants and animals [11]. The enzyme was partially purified in 1949 and it was shown to catalyze the hydrolytic ring-opening reaction of hydantoin to hydantoic acid [12]. Thereafter, Wallach and Grisolia [13] found that the animal enzyme was identical to dihydropyrimidinase (EC 3.5.2.2) which catalyzes the transformation of dihydropyrimidines into *N*-carbamoyl-β-amino acids.

These hydantoin-cleaving reactions were applied during the 1970s, to produce the D-amino acids, D-phenylglycine, D-*p*-hydroxyphenylglycine and D-thienylglycine, as side chains for semisynthetic penicillins and cephalosporins. Based upon the investigations of Dudley *et al.* [14,15] into the metabolism of the racemic forms of *N*-substituted 5-phenylhydantoins, which were postulated to be D-selective, Cecere *et al.* [16] found in 1975 that dihydropyrimidinase from calf liver could be used to produce several *N*-carbamoyl-D-amino acids from the corresponding DL-5-monosubstituted hydantoins. In 1978, Yamada and co-workers showed that microbial cells are good

catalysts for this reaction [17-21]. Olivieli *et al.* then found a bacterium which could hydrolyze *N*-carbamoyl-D-amino acids [22]. Several bacteria are now known to have both DL-5-substituted hydantoin-hydrolyzing activity to *N*-carbamoyl-D-amino acids, and *N*-carbamoyl-D-amino acids-hydrolyzing activity to D-amino acids [22-24]. With these bacteria, D-amino acids can be directly obtained from the corresponding DL-5-monosubstituted hydantoins.

Similar reactions transform DL-5-monosubstituted hydantoins to L-amino acids. Yokozeki *et al.* analyzed the reaction mechanism of L-tryptophan production from DL-5-indolylmethylhydantoin by *Flavobacterium* sp. [25], and found that non-stereospecific hydrolysis of hydantoin derivative and the L-selective hydrolysis of racemic *N*-carbamoylamino acid were involved in this transformation. The same results were obtained by Syldatk *et al.* who studied L-arylalkylamino acid production from corresponding DL-5-monosubstituted hydantoins in *Arthrobacter* sp. [26]. Yamashiro *et al.* found the L-selective hydrolysis of a hydantoin derivative in L-valine production from DL-5-isopropylhydantoin by *Bacillus* sp. [27]. and that ATP was absolutely required for this amidohydrolytic reaction.

The same ATP-dependent hydantoin hydrolysis was found in the microbial transformation of creatinine [28]. *N*-Methylhydantoin, a deimidated product of creatinine, is hydrolyzed with the concomitant hydrolysis of ATP to ADP. This reaction should be a promising tool for the clinical analysis of creatinine in blood and urine, which is an indicator of renal dysfunction [29].

Despite the practical importance of these hydantoin-transforming reactions, the enzymes involved in these reactions have never been studied in detail except for the D-5-monosubstituted hydantoin-hydrolyzing enzymes, which are called D-specific hydantoinase. Because D-specific hydantoinase is identical to dihydropyrimidinase [18,30], it was proposed that the hydrolysis of the *N*-carbamoyl-D-amino acid was catalyzed by β-ureidopropionase (EC 3.1.5.6), which is involved in the pyrimidine degradation pathway, and that the D-amino acid was produced from DL-5-monosubstituted hydantoin by the action of series of enzymes involved in the pyrimidine

degradation pathway [23]. However, this contention has remained moot because of the lack of details about *N*-carbamoyl-D-amino acid-hydrolyzing and pyrimidine-degrading enzymes. Furthermore, the physiological functions of the other hydantoin-tranforming enzymes are not yet known at all.

These unsolved problems prompted the author to study the enzymes which are involved in microbial transformations of hydantoin-related compounds. In this thesis, I have attempted the purification and characterization of the enzymes involved in D- or L-amino acid production from DL-5-monosubstituted hydantoin, in pyrimidine transformation and in creatinine transformation. These results may be valuable for elucidating the physiological roles of these enzymes, and for broadening the applications of these enzymes.

Chapter I describes the evaluation of pyrimidine- and hydantoin-degrading activities in aerobic bacteria. The findings pose questions regarding the notion that D-amino acid production from DL-5-monosubstituted hydantoin involves the series of enzymes involved in reductive pyrimidine degradation [23,31,32], and suggest the existence of specific enzymes for hydantoin degradation.

Chapter II describes the analysis of microbial *N*-carbamoylamino acid amidohydrolases involved in hydantoin- and pyrimidine-transformations. The enzymes responsible for *N*-carbamoyl-D-amino acid hydrolysis, namely *N*-carbamoyl-D-amino acid amidohydrolases, were purified from *Comamonas* sp. and *Blastobacter* sp., and were characterized. β-Ureidopropionase and the *N*-carbamoyl-L-amino acid-hydrolyzing enzyme, *N*-carbamoyl-L-amino acid amidohydrolase, were also purified from *Pseudomonas putida* and *Alcaligenes xylosoxidans*, respectively, and characterized. These results revealed differences among these enzymes and suggested specific roles for each enzyme in each metabolic pathway.

Chapter III describes the analysis of microbial cyclic ureide-hydrolyzing enzymes involved in hydantoin, pyrimidine and creatinine transformation. First, two different dihydropyrimidine- and hydantoin-hydrolyzing enzymes in *Blastobacter* sp are described. One is a D-specific hydantoinase and the other is a novel enzyme which

should be called imidase. Next, the purification and characterization of dihydroorotase (EC 3.5.2.3) from *P. putida* is described. This enzyme is involved in pyrimidine biosynthesis, and is regulated by the intermediates of pyrimidine metabolism. The purification and characterization of an ATP-dependent enzyme, *N*-methylhydantoin amidohydrolase, from *P. putida*, which is involved in the creatinine transformation is described, and the nucleoside-triphosphatase activity of this enzyme is discussed. These indicate suggest the diversity of cyclic ureide-hydrolyzing enzymes in microorganisms.

CHAPTER I

Evaluation of Pyrimidine- and Hydantoin-Transforming Enzyme Activities in Aerobic Bacteria[a]

As mentioned in the introduction to this thesis, it is well known that DL-5-monosubstituted hydantoins are converted to D-amino acids via *N*-carbamoyl-D-amino acids by some aerobic bacteria through the pathway shown in Fig. 1A [23,31,32]. These aerobic bacteria and their enzymes are applied to the practical production of D-amino acids such as D-*p*-hydroxyphenylglycine (D-HPG) [21]. However, the basic and biochemical aspects of these enzymes remain to be explained. Takahashi *et al.* revealed that, in *Pseudomonas putida* IFO 12996, DL-5-monosubstituted hydantoins are hydrolyzed to *N*-carbamoyl-D-amino acids by dihydropyrimidinase, which catalyzes the amide ring-opening step of the reductive degradation of pyrimidine bases (Fig. 1B) [18]. The same results were obtained for other *Pseudomonas* species [33,34], *Comamonas* species [34], *Bacillus* species [21], *Arthrobacter* species [23], *Agrobacterium* species [33], and rat liver [35].

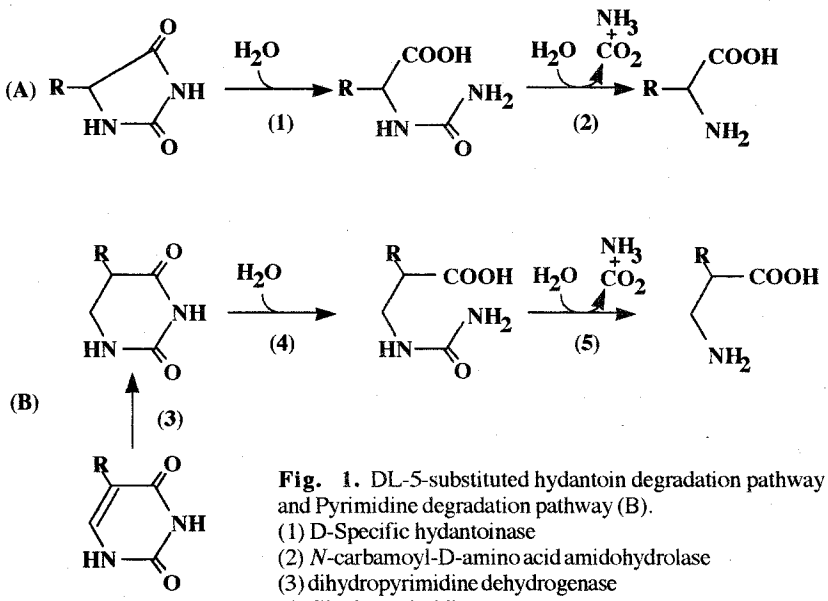


Fig. 1. DL-5-substituted hydantoin degradation pathway (A) and Pyrimidine degradation pathway (B).
(1) D-Specific hydantoinase
(2) *N*-carbamoyl-D-amino acid amidohydrolase
(3) dihydropyrimidine dehydrogenase
(4) dihydropyrimidinase
(5) β-ureidopropionase

From these results, it is believed that D-amino acid production from DL-5-monosubstituted hydantoins involves the action of the series of enzymes involved in the pyrimidine degradation pathway [23,31,32]. However, there have been no reports on the pyrimidine degradation by hydantoin-degrading aerobic bacteria, and few studies have examined reductive pyrimidine base degradation by aerobic bacteria [36,37].

In this chpater, I describe the investigation of the enzyme activities involved in reductive pyrimidine base degradation in hydantoin-transforming bacteria, and discuss the relation between the enzymes concerned in the pyrimidine and hydantoin degradation.

MATERIALS AND METHODS

Microorganisms and cultivation. Recently, LaPointe *et al.* revealed the distribution of dihydropyrimidine- and hydantoin-hydrolyzing enzyme genes in various *Pseudomonas* species belonging to the rRNA homology group I of pseudomonads [34]. I selected *P. putida* IFO 12996 [18] as a representative of this kind of microorganism. The microorganisms, which have *N*-carbamoyl-D-amino acid-hydrolyzing activity, are not so much. I used in this study *Comamonas* sp. E222c, and *Blastobacter* sp. A17p-4 which were found to have the activity in our laboratory.

The basal medium contained 1 g KH_2PO_4, 1 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 3 g yeast extract (Oriental Yeast Co., Japan), 3 g meat extract (Mikuni Co., Japan), 10 g glycerol and 2 g Polypepton (Daigo Nutritional, Japan) in 1 l tap water, pH 7.0. Each strain was inoculated into a test tube containing 5 ml of medium, and then incubated for 16-24 h at 28 °C with shaking. The culture was transferred to a 2-l shake flask containing 500 ml of the same medium, and then incubated until the late exponential phase for 24 h (*P. putida* and *Comamonas* sp.) or for 72 h (*Blastobacter* sp.) at 28°C with shaking.

Preparation of cell-free extract. Cells were harvested by centrifugation at 14,000 x *g*, for 20 min, and then washed twice with 0.85% (w/v) NaCl and suspended

in 20 mM Tris/HCl buffer, pH 7.4. The cell suspensions (50% (w/v)) were ultrasonically disrupted on ice for the time which realizes the best recovery of each activity (30 min for *P. putida* and *Comamonas* sp., and 60 min for *Blastobacter* sp.; 9 kHz, Insonator model 201M; Kubota, Japan), and then centrifuged at 14,000 x *g*, for 60 min. The resulting supernatants were used as cell-free extracts. Protein was measured by the Bradford method [38].

Enzyme assays. Enzyme assays were performed at 30°C. Dihydropyrimidine dehydrogenase activity was measured using an assay mixture (2.5 ml) containing 200 mM Tris/HCl buffer (pH 7.4), 0.64 mM NADH or NADPH, 0.2 mM uracil or thymine, and cell-free extract. The oxidation of NAD(P)H to NAD(P) was followed at 340 nm over a 10 min period. Specific activity was expressed as nmol dihydropyrimidine formed per min per mg protein.

Dihydropyrimidinase activity was assayed over a 30 min period for cell-free extracts or a 4 h period for cell suspensions using an assay mixture (1 ml) containing 100 mM Tris/HCl buffer (pH 7.4), 5 mM dihydrouracil or dihydrothymine, and cell-free extract or cell suspension. Enzyme activity was determined by measuring the total amount of *N*-carbamoyl-β-amino acids and β-amino acids produced, as described below. Specific activity was expressed as nmol *N*-carbamoyl-β-amino acids plus β-amino acids formed per min per mg protein or wet cells.

β-Ureidopropionase activity was assayed over a 30 min period for cell-free extracts or a 4 h period for cell suspensions using an assay mixture (1 ml) containing 100 mM Tris/HCl buffer (pH 7.4), 10 mM β-ureidopropionate or β-ureidoisobutyrate, and cell-free extract or cell suspension. Enzyme activity was determined by measuring the amount of β-amino acids produced. Specific activity was expressed as nmol β-amino acids formed per min per mg protein or wet cells.

D-Specific hydantoinase activity was assayed over a 5 h period using an assay mixture (1 ml) containing 100 mM Tris/HCl buffer (pH 7.4), 50 mM DL-5-(*p*-hydroxyphenyl)hydantoin (DL-HPG-hyd), and cell-free extract or cell suspension. The

air in the reaction mixture had been replaced with nitrogen gas for prevention against the oxidation of the substrate. Enzyme activity was determined by measuring the total amount of *N*-carbamoyl-D-*p*-hydroxyphenylglycine (C-D-HPG) and D-HPG produced, as described below. Specific activity was expressed as nmol C-D-HPG plus D-HPG formed per min per mg protein or wet cells.

N-Carbamoyl-D-amino acid amidohydrolase activity was assayed over a 5 h period using an assay mixture (1 ml) containing 100 mM Tris/HCl buffer (pH 7.4), 50 mM C-D-HPG, and cell-free extract or cell suspension. The air in the reaction mixture had been replaced with nitrogen gas for prevention against the oxidation of the substrate. Enzyme activity was determined by measuring the amount of D-HPG produced. Specific activity was expressed as nmol D-HPG formed per min per mg protein or wet cell.

Specific activity of each enzyme was the average of 2 separate determinations that were reproducible within ±10%.

Analytical methods. Quantitative determination of *N*-carbamoyl-β-amino acids were carried out colorimetrically according to the methods of Takahashi *et al.* [18]. Quantitative determination of β-amino acids were performed with an amino acid analyzer (Model K-101; Kyowa Seimitu, Japan). Quantitative determination of C-D-HPG and D-HPG were performed with Shimadzu LC-6A (Japan) high-performance liquid chromatography (HPLC) at 254 nm using a Cosmosil $5C_{18}$ packed column (4.6 x 250 mm; Nakalai Tesque, Japan) at a flow rate of 1.0 $ml \cdot min^{-1}$, using $H_2O/CH_3CN/H_3PO_4$ (95/5/0.01, by vol.)

RESULTS AND DISCUSSION

Table 1 summarizes the enzyme activities involved in hydantoin degradation, namely D-specific hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase, in the cell-free extracts of the three bacteria, *P. putida*, *Comamonas* sp. and *Blastobacter* sp.

These two enzyme activities do not always coexist in one bacterium. *P. putida* has D-specific hydantoinase activity but not *N*-carbamoyl-D-amino acid amidohydrolase activity. *Comamonas* sp. has *N*-carbamoyl-D-amino acid amidohydrolase activity but not D-specific hydantoinase activity. *Blastobacter* sp. has both activities. The same tendencies were observed with each bacterium with different substrate concentrations, and with other DL-5-monosubstituted hydantoins or other *N*-carbamoyl-D-amino acids tested.

With the cell-free extracts of these bacteria, the enzyme activities involved in reductive pyrimidine degradation, namely dihydropyrimidine dehydrogenase, dihydropyrimidinase and β-ureidopropionase, were assayed with both uracil or thymine degradation intermediates as substrates (Table 1).

Table 1. Hydantoin- and pyrimidine-degrading enzyme activities of aerobic bacteria

Enzyme	Substrate	Specific activity (nmol/min/mg protein)		
		P. putida	*Comamonas* sp.	*Blastobacter* sp.
D-Specific hydantoinase	DL-HPG-hyd	0.365	n.d.	2.07
N-Carbamoyl-D-amino acid amidohydrolase	C-D-HPG	n.d.	3.12	15.5
Dihydropyrimidine dehydrogenase	U	1.00	1.21	n.d.
	T	1.00	1.21	n.d.
Dihydropyrimidinase	DU	11.2	n.d.	2.02
	DT	14.4	n.d.	0.0950
β-Ureidopropionase	β-UP	8.84	1.20	2.99
	β-UIB	5.45	1.74	2.12

DL-HPG-hyd, DL-5-(*p*-hydroxyphenyl)hydantoin; C-D-HPG, *N*-carbamoyl-D-*p*-hydroxy-phenylglycine; U, uracil; T, thymine; DU, dihydrouracil; DT, dihydrothymine; β-UP, β-ureidopropionate; β-UIB, β-ureidoisobutyrate.
n.d., not detected.

Only *P. putida* possesses all three enzyme activities, and these three enzymes of *P. putida* react on uracil and thymine degradation intermediates with almost the same activity. The dihydropyrimidine dehydrogenase activity of *P. putida* was detectable with

NADPH, but not with NADH because of the rapid decomposition of NADH by the cell-free extracts. *Comamonas* sp. clearly showed NADH dependent dihydropyrimidine dehydrogenase activity and β-ureidopropionase activities. No dihydropyrimidine dehydrogenase activity was detected with NADPH. These two enzymes of *Comamonas* sp. show almost the same activities toward uracil and thymine degradation intermediates. However, *Comamonas* sp. did not show dihydropyrimidinase activity. *Blastobacter* sp. shows dihydropyrimidinase and β-ureidopropionase activities but not dihydropyrimidine dehydrogenase activity. Dihydropyrimidinase of *Blastobacter* sp. hydrolyzed dihydrouracil more efficiently than dihydrothymine, while β-ureidopropionase of *Blastobacter* sp. hydrolyzed β-ureidopropionate and β-ureidoisobutyrate at almost the same rates.

From the results in Table 1, the following points as to the existence of hydantoin- and pyrimidine-degrading enzymes in these bacteria are obvious. (I) In *P. putida*, amide ring-opening enzymes, i.e., D-specific hydantoinase and dihydropyrimidinase, coexist. As for *N*-carbamoylamino acid-hydrolyzing enzymes, β-ureidopropionase activity exists in *P. putida*, while *N*-carbamoyl-D-amino acid amidohydrolase activity does not. (II) *Comamonas* sp. has both *N*-carbamoyl-D-amino acid amidohydrolase and β-ureidopropionase activities, while it has neither D-specific hydantoinase nor dihydropyrimidinase activity. (III) In *Blastobacter* sp., both two amide ring-opening enzyme activities and two *N*-carbamoylamino acid-hydrolyzing activities coexist. Since the two amide ring-opening activities in *P. putida* was proved to be catalyzed by one enzyme, dihydropyrimidinase [18], it is suspected that all other coexisting amide ring-opening activities or *N*-carbamoylamino acid-hydrolyzing activities are catalyzed by identical enzymes involved in reductive pyrimidine degradation. However, the *N*-carbamoylamino acid-hydrolyzing activity in *P. putida* suggests that the β-ureidopropionase of this bacterium does not show *N*-carbamoyl-D-amino acid amidohydrolase activity.

To clarify the relation between pyrimidine-degrading enzymes and hydantoin-degrading enzymes, we analyzed each enzyme activity in these three bacteria grown in

basal liquid media containing 0.15% (w/v) of various pyrimidine- and hydantoin-related compounds (Table 2).

Table 2. Effects of pyrimidine and hydantoin related compounds on enzyme formation

Additives in medium	None	DU	β-UP	DL-HPG-hyd	C-D-HPG
Strain / Enzyme	Activity(x 10^{-2} nmol/min/mg wet cells)				
P. putida					
Dihydropyrimidinase (DPase)	74.6	75.3	67.4	81.7	80.4
D-Specific hydantoinase (Hydase)	3.99	4.45	3.99	4.70	4.25
Ratio of DPase / Hydase	18.7	16.9	16.9	17.4	18.9
β-Ureidopropionase (UPase)	13.3	20.5	28.5	15.9	10.4
N-Carbamoyl-D-amino acid amidohydrolase (DCase)	n.d.	n.d.	n.d.	n.d.	n.d.
Comamonas sp.					
Dihydropyrimidinase (DPase)	n.d.	n.d.	n.d.	n.d.	n.d.
D-Specific hydantoinase (Hydase)	n.d.	n.d.	n.d.	n.d.	n.d.
β-Ureidopropionase (UPase)	85.3	42.9	225	90.1	195
N-Carbamoyl-D-amino acid amidohydrolase (DCase)	31.3	20.3	43.7	26.9	38.4
Ratio of UPase / DCase	2.73	2.11	5.15	3.35	5.08
Blastobacter sp.					
Dihydropyrimidinase (DPase)	29.5	49.7	201	29.0	20.5
D-Specific hydantoinase (Hydase)	7.34	4.21	62.1	9.08	6.43
Ratio of DPase / Hydase	4.02	11.8	3.24	3.19	3.19
β-Ureidopropionase (UPase)	11.4	14.6	57.5	tr	tr
N-Carbamoyl-D-amino acid amidohydrolase (DCase)	1.68	8.80	33.3	2.22	1.89
Ratio of UPase / DCase	6.79	1.66	1.73	$< 10^{-2}$	$< 10^{-2}$

DU, dihydrouracil; β-UP, β-ureidopropionate; DL-HPG-hyd, DL-5-(*p*-hydroxyphenyl)hydantoin; C-D-HPG, *N*-carbamoyl-D-*p*-hydroxyphenylglycine.
n.d., not detected; tr, trace means less than the activity of 10^{-4} nmol/min/mg wet cells.

In the case of *Comamonas* sp. and *Blastobacter* sp., β-ureidopropionate enhanced both the amide ring-opening activites and *N*-carbamoylamino acid-hydrolyzing activities. *Blastobacter* sp. grown in DL-HPG-hyd or C-D-HPG containing medium

showed a trace amount of β-ureidopropionase activity (less than 10^{-4} nmol·min^{-1}·mg^{-1} wet cells), while it showed a detectable amount of *N*-carbamoyl-D-amino acid amidohydrolase activity (more than 10^{-2} nmol·min^{-1}·mg^{-1} wet cells). Table 2 also shows the ratios of coexisting amide ring-opening activities and those of coexisting *N*-carbamoylamino acid-hydrolyzing activities. Except for the amide ring-opening activities in *P. putida*, the ratios of corresponding enzyme activities changed remarkably depending on the additives in the media. These results suggest that the coexisting amide ring-opening activities in *Blastobacter* sp., and the coexisting *N*-carbamoylamino acid-hydrolyzing activities in *Comamonas* sp. and *Blastobacter* sp. are catalyzed by more than two different enzymes under different expressional regulation. These findings pose questions as to the conjecture that the D-amino acid production from DL-5-monosubstituted hydantoin involves the action of the series of enzymes involved in reductive pyrimidine degradation [23,31,32], and also suggest the existence of specific enzymes for hydantoin degradation [39].

SUMMARY

The enzyme activities responsible for the reductive pyrimidine base degradation by aerobic bacteria, which produce hydantoin-degrading enzymes, were investigated. *Pseudomonas putida* IFO 12996, which is a D-specific hydantoinase producer, has dihydropyrimidinase activity, and *Comamonas* sp. E222c and *Blastobacter* sp. A17p-4, which are *N*-carbamoyl-D-amino acid amidohydrolase producers, have β-ureidopropionase activity. *Blastobacter* sp. also possesses both D-specific hydantoinase and dihydropyrimidinase activities. Thus, two amide ring-opening activities and/or two *N*-carbamoylamino acid-hydrolyzing activities coexist in these bacteria. However, the differences of the induction levels of each enzyme activities for the several pyrimidine- and hydantoin-related compounds suggest that these corresponding amide ring-opening or *N*-carbamoylamino acid-hydrolyzing activities are not always catalyzed by the same enzymes.

CHAPTER II

Analysis of Microbial *N*-Carbamoylamino acid amidohydrolases

Section 1. *N*-Carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp. E222c: purification and Characterization[b]

Some microorganisms hydrolyze 5-substituted hydantoins to *N*-carbamoylamino acids and successively hydrolyze *N*-carbamoylamino acids to amino acids. Two hydrolases are involved in this process, the former is hydantoinase and the latter is *N*-carbamoylamino acid amidohydrolase. As for the hydrolysis of *N*-carbamoylamino acids, both L- and D- specific reactions have been observed. For example, L-specific activities have been detected in several microorganisms [26,27,40,41], and the enzyme had been purified from *Arthrobacter* sp. DSM 3747 and characterized [41]. D-Specific reactions were observed in crude extracts of some microorganisms [22,23,31,32] and the enzyme was separated from D-specific hydantoinase in crude extracts of *Pseudomonas* sp. AJ-11220 [32]. However, it was not further purified, nor further characterized in detail.

From the fact that D-specific hydantoinase is identical with dihydropyrimidinase [18,30], it has been proposed that *N*-carbamoyl-D-amino acid amidohydrolase is identical with β-ureidopropionase. This enzyme splits the carbamoyl moiety of β-ureidopropionate and is involved in the pyrimidine degradation pathway [23]. However, in previous chapter, I posed questions as to this proposition and suggested the exsitence of specific enzymes for hydantoin degradation.

In this section, I describe the purification and characterization of a *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp. E222c, and compare the characteristics of the purified enzyme to those of other *N*-carbamoylamide amidohydrolases such as β-ureidopropionase [42-44], ureidosuccinase (EC 3.5.1.7)

[45], *N*-carbamoylsarcosine amidohydrolase (EC 3.5.1.59) [46] and citrullinase (EC 3.5.1.20) [47]. I also discuss the relationship of the *N*-carbamoyl-D-amino acid amidohydrolase to the pyrimidine degradation pathway.

MATERIALS AND METHODS

Materials. *N*-Carbamoyl derivatives of D-amino acids were prepared enzymatically [18]. Those of other amino acids were gifts from the Kaneka Corporation (Japan). Standard proteins for high performance gel permeation chromatography, for SDS-polyacylamide gel electrophoresis (SDS/PAGE), and for analytical gel filtration on Sephadex G-150 were purchased from the Oriental Yeast Co., Daiichi Pure Chemicals Co. (Japan) and Boehringer Mannheim (Germany), respectively. All other chemicals used were of analytical grade and commercially available.

Microorganism and Cultivation. *Comamonas* sp. was inoculated into a test tube (16 x 165 mm) containing 5 ml of the basal medium described in chapter I supplemented with 0.15% (w/v) β-ureidopropionate, then incubated for 15 h at 28°C with shaking. The culture was transferred to a 2-l shake flask containing 500 ml of the same medium, then incubated for 27 h at 28°C with shaking.

Enzyme assay. To purify the enzyme, the assay mixture contained 200 mM potassium phosphate buffer (pH 7.0), 25 mM *N*-carbamoyl-D-*p*-hydroxyphenylglycine (C-D-HPG) and the enzyme in a total volume of 100 μl. After 5-60 min incubation at 30°C, the reaction was stopped by the addition of 100 μl ethanol. The D-*p*-hydroxyphenylglycine (D-HPG) produced was analyzed by high-performance liquid chromatography (HPLC) as described in chapter I. One unit of the enzyme was defined as the amount of enzyme that catalyzes the formation of D-HPG at the rate of 1 $\mu mol \cdot min^{-1}$ under the assay conditions mentioned above. The standard enzyme assay mixture contained 200 mM potassium phosphate buffer (pH 7.0), 10 mM *N*-carbamoyl-

D-phenylalanine (C-D-Phe) and the enzyme in a total volume of 500 μl. The reaction proceeded at 30°C for 5-20 min and was stopped with 500 μl of ethanol. The amount of D-phenylalanine or ammonia formed was determined as described below.

Analysis of C-D-Phe, D-phenylalanine, ammonia and carbon dioxide. C-D-Phe and D-phenylalanine were quantified by HPLC or enzymatically. HPLC elution proceeded on a Cosmosil $5C_{18}$ packed column (4.6 x 250 mm; Nakalai Tesque (Japan)) with CH_3OH/H_2O, 50/50 (by vol.), pH 2.5 at a flow rate of 1.0 $ml \cdot min^{-1}$ and was monitored at 254 nm. The enzymatic assay mixture contained in 500 μl was as follows: 200 mM potassium phosphate buffer (pH 7.0), 1.5 mM 4-aminoantipyrine, 2.1 mM phenol, 2.25 U peroxidase from horse radish (Boehringer Mannheim) [48], 0.375 U D-amino acid oxidase from porcine kidney (Boehringer Mannheim) [49] and a suitable amount of the supernatant of reaction mixture. After 60 min at 37°C, the increase in absorbance at 500 nm was determined.

Ammonia was estimated with glutamate dehydrogenase [50] or by the indophenol method [51].

Carbon dioxide was measured by the conventional manometric method at 37°C with air as the gas phase [52].

Stereochemical analysis of amino acids. The ratio of D- and L-amino acids was estimated by thin-layer chromatography (TLC) and HPLC. TLC analysis was performed on CHIRALPLATE (Macherey-Nagel (Germany)) with the developing solvent, $CH_3OH/H_2O/CH_3CN$, 1/1/4 (by vol.). Amino acids were detected with a ninhydrin spray. HPLC analysis was performed on a MCI GEL CRS packed column 10W (4.6 x 50 mm; Mitsubishi Kasei (Japan)) at 254 nm eluted with 2 mM $CuSO_4$ at a flow rate of 1.0 $ml \cdot min^{-1}$.

Analytical methods for the *N*-carbamoyl-D-amino acid amidohydrolase. The relative molecular mass of the enzyme was determined by

analytical gel filtration on a calibrated column (1.5 x 80 cm) packed with Sephadex G-150, eluted with 10 mM potassium phosphate buffer (pH 7.0) containing 200 mM NaCl and 1 mM dithiothreitol. It was also determined on a GS-520 column (0.76 x 50 cm; Asahi Kasei (Japan)) eluted with same buffer. Determination of protein concentration, SDS/PAGE on a 12.5% polyacrylamide gel, isoelectric focusing at a pH range of 3.5-11.5, estimation of the amino acid composition of the purified enzyme and the COOH-terminal amino acid analysis were performed as described previously [53]. The NH_2-terminal amino acid sequence analysis was performed as described previously [54].

Enzyme purification. All procedures were carried out at 0-5°C. Potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and centrifugation at 14,000 x *g* for 30 min were usually used throughout the enzyme purification procedure.

Step 1: *Comamonas* sp. cells (33 g wet cells) from 4 l culture broth suspended in 10 mM buffer (100 ml), were ultrasonically disrupted for 2 h (19 kHz, Insonator model 201M; Kubota (Japan)).

Step 2: After centrifugation, the resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained at 20-40% saturation was collected by centrifugation and dissolved in 10 mM buffer. The resultant enzyme solution (55 ml) was dialyzed against 5 l of the same buffer for 17 h.

Step 3: The dialyzed solution (71 ml) was applied to a DEAE-Sephacel column (3.6 x 30 cm) equilibrated with 10 mM buffer. The enzyme was eluted with a linear gradient of NaCl (0-1.0 M in the buffer 800 ml). The fractions containing enzyme activity were combined (115 ml).

Step 4: The NaCl concentration was adjusted to 4 M, then the enzyme solution was placed on a phenyl-Sepharose CL-4B column (1.8 x 10 cm) equilibrated with 10 mM buffer containing 4 M NaCl. The enzyme was eluted by lowering the ionic strength of NaCl linearly from 4 to 0 M in 10 mM buffer (140 ml), then washed with 5 mM buffer containing 50% (v/v) ethylene glycol. The active fractions were combined (51 ml) then concentrated to about 4 ml with an Amicon membrane filter apparatus (Amicon Co.

(U.S.A.)) equipped with a YM-10 membrane.

Step 5: The enzyme (4 ml) was applied to a Sephadex G-150 column (1.5 x 80 cm) equilibrated with 10 mM buffer containing 0.2 M NaCl and eluted with the same buffer. The active fractions were collected (21 ml) then dialyzed against 10 mM buffer (3 l) for 12 h.

Step 6: The enzyme solution (22 ml) was applied to a MonoQ HR 5/5 column equilibrated with 10 mM buffer. The enzyme was eluted by increasing the ionic strength of NaCl linearly over the range 0-1.0 M, in the same buffer. The active fractions were collected (4.0 ml) and used for to characterize the enzyme.

RESULTS

Enzyme purification

Various *N*-carbamoyl compounds, hydantoin derivatives and D-amino acids were tested for the ability to enhance *N*-carbamoyl-D-amino acid amidohydrolase formation. When urea, β-ureidopropionate, D-phenylglycine, and *N*-carbamoyl-DL-methionine, (0.15% (w/v)), were added to the basal medium, each showed good activity. With β-ureidopropionate, both specific and total activities of the enzyme doubled when compared to those without supplementation. Cell-free extracts prepared from cells of *Comamonas* sp. cultured in β-ureidopropionate supplemented medium were used as the enzyme source.

Table 1. Purification of the *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp.

Step	Total protein (mg)	Total activity (U)	Specific activity (10^{-3} U/mg)	Yield (%)
1) Cell-free extract	4750	13.9	2.93	100
2) Ammonium sulfate	2390	12.5	5.24	89.9
3) DEAE-Sephacel	294	10.2	34.8	73.4
4) Phenyl-Sepharose CL-4B	36.2	6.52	180	46.8
5) Sephadex G-150	22.9	5.13	224	36.7
6) Mono Q HR 5/5	14.4	4.95	344	36.0

Through the procedures described in the materials and methods, the enzyme was purified about 119 fold, with a yield of 36% (Table 1). The purified enzyme catalyzed the hydrolysis of C-D-HPG to D-HPG at a rate of 0.34 μmol·min^{-1}·mg^{-1} under the assay conditions for the enzyme purification (see materials and methods).

Criteria for purity

The purified enzyme (preparation at the MonoQ HR 5/5 column chromatography step) gave one band on SDS/PAGE (Fig. 1A). Ampholytic electrofocusing yielded only one absorption peak for protein (pI 4.0), which coincided with that of C-D-HPG hydrolyzing activity. Further evidence for the purity of the enzyme preparation was the result of high performance gel permeation liquid chromatography, which gave a reasonably symmetrical single protein peak.

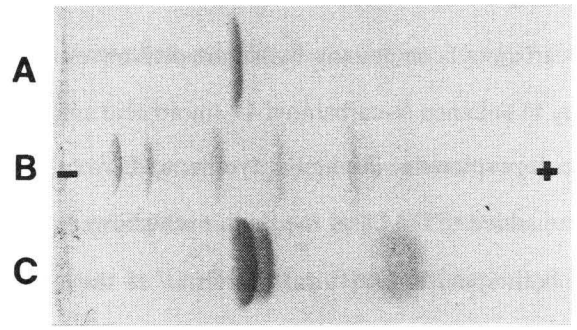


Fig. 1. SDS/PAGE of the *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp.
A, purified enzyme, 10 μg. B, standards: (from left) phosphorylase *b* (M_r = 97,400), bovine serum albmin (66,267), aldolase (42,400), carbonic anhydrase (30,000) and soybean trypsin inhibitor (20,100). C, purified enzyme decomposed under preservation without N_2, 10μg. The gel was stained with Coomassie Brilliant Blue R-250 and destained in ethanol/acetic acid/water, 3/1/6 (by vol.).

Molecular mass, amino acid composition and subunit structure

On a calibrated column of Sephadex G-150, the relative molecular mass of the enzyme was estimated to be about 111,000, and that determined by high performance gel permeation chromatography on GS-520 was about 117,000. SDS/PAGE showed that the relative molecular mass of the material was about 40,000 (Fig. 1A).

The enzyme contained all the common amino acids, and the minimum value with

the nearest integral numbers of all amino acids found, was 40,564 (Table 2), which agrees well with the value determined on SDS/PAGE.

Table 2. Amino acid composition of the *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp.

Amino acid	Number of residues per subunit	
	Found	Integral
Aspartic acid or asparagine	34.26	34
Threonine	12.84	13
Serine	11.10	11
Glutamic acid or glutamine	38.22	38
Proline	20.88	21
Glycine	34.80	35
Alanine	34.80	35
Methionine	13.20	13
Isoleucine	16.62	17
Valine	22.20	22
Leucine	27.30	27
Tyrosine	13.02	13
Tryptophan	6.00	6
Phenylalanine	16.80	17
Lysine	11.88	12
Histidine	15.18	15
Arginine	25.38	25
Half-cystine	8.04	8

There was no significant differences among the amino acid residues between the 20-h hydrolyzate and the 40- or 70-h hydrolyzates; data are from the 20-h hydrolyzate.

Furthermore, this coincided with the fact that the amount of phenylalanine, as the COOH-teminal amino acid, was 1.08 mol/40,600 g of the enzyme preparation, and leucine was released successively on amino acid analysis of the enzyme hydrolyzate with carboxypeptidase Y. It is evident that the enzyme has a single COOH-terminal amino acid, phenylalanine, which implies that it is composed of identical subunits. These results pertaining to the relative molecular mass of the native enzyme and its subunit imply that the enzyme is composed of three identical subunits. Such enzymes are so rare [55], that further evidence may be necessary to confirm this implication.

The enzyme shows an absorption value of 1.10 for 1 mg/ml and a 1-cm light path at 280 nm.

The enzyme showed no absorption in the region of 350-750 nm. Only one peak,

at 280 nm, appeared in the region of 250-350 nm.

NH_2-Terminal amino acid sequence

Automated Edman degradation with a pulsed liquid-phase sequencer revealed that the amino terminal amino acid sequence is Ser-Arg-Ile-Val-Asn-Tyr-Ala-Ala-Ala-Gln-Leu-Gly-Pro-Ile-Gln-Arg-Ala-Asp-Ser-Arg-Ala-Asp-Val-Met-Glu-Arg-Leu-Leu-Ala-His-. When this sequence was compared with those of proteins stored in the protein sequence data base, no apparent homology was found with other types of amidohydrolases.

Stoichiometry

The stoichiometry of the reaction catalyzed by the enzyme was investigated with a Gilson respirometer (Gilson Medical Electronics (U.S.A)) at 37°C. C-D-Phe (10 μmol) and 500 μmol of potassium phosphate buffer (pH 7.0) in 2.5 ml were placed within the main compartment of the reaction flask, and 0.03 units of enzyme (0.5 ml) in the side arm. The reaction was started by transferring the enzyme solution to the main compartment and terminated after 10 min by adding ethanol (3.0 ml) to the reaction mixture. The products were 3.18 μmol of phenylalanine, 3.15 μmol of ammonia, and 3.16 μmol of carbon dioxide. The amount of C-D-Phe remaining unhydrolyzed in the reaction mixture was about 6.25 μmol. Thus, the enzyme catalyzed the hydrolysis of C-D-Phe to produce phenylalanine, ammonia and carbon dioxide stoichiometrically. Phenylalanine produced by the purified enzyme was proven to be D-enantiomer by HPLC. As shown in Fig. 2, the purified enzyme hydrolyzed C-D-Phe and produced D-phenylalanine, but did not hydrolyze L-enantiomer. With racemic mixture as substrate, only C-D-Phe in the racemic substrate was hydrolyzed and D-phenylalanine was produced. These results revealed the strict specificity of the enzyme for the D-enantiomer.

Attempts to demonstrate the reversibility of the *N*-carbamoyl-D-amino acid amidohydrolase were consistently unsuccessful.

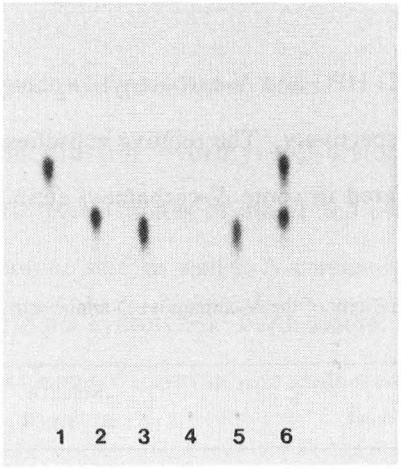


Fig. 2. Chromatogram of *N*-carbamoyl-D-amino acid amidohydrolase reaction products from *N*-carbamoyl-D-,-L- or -DL-phenylalanine (C-D-Phe, C-L-Phe, or C-DL-Phe).
The reaction mixture comprised in 100 μl, 0.5% (w/v) C-D-Phe, C-L-Phe, or C-DL-Phe, 200 mM potassium phosphate buffer (pH 7.0) and the enzyme. Reactions proceeded at 30°C for 1 h. Samples of 2.5 μl were separated by TLC as described under materials and methods. Lane 1), 0.5% (w/v) L-phenylalanine in H_2O (Rf value is 0.59); 2), 0.5% (w/v) D-ph-enylalanine in H_2O (Rf value is 0.49); 3), reaction mixture with C-D-Phe; 4), reaction mixture with C-LPhe; 5), reaction mixture with C-DL-Phe; 6), 1% (w/v) DL-phenylalanine in H_2O.

Substrate specificity

A broad range of the *N*-carbamoyl compounds was tested to investigate the substrate specificity of the enzyme (Table 3). The enzyme showed strict specificity toward *N*-carbamoyl-D-amino acids. *N*-Carbamoyl-L-amino acids were not hydrolyzed, and only the D-enantiomer in the racemic substrate was hydrolyzed to D-amino acid by the enzyme.

N-Carbamoylamino acids having a hydrophobic side chain, namely *N*-carbamoyl derivatives of D-phenylalanine, DL-methionine, DL-norleucine and DL-tryptophan, were efficently hydrolyzed. On the other hand, *N*-carbamylamino acids having polar group or short chain alkyl group were weakly reactive. Normal hyperbolic kinetics were observed with all compounds tested and the K_m values obtained from Lineweaver-Burk plots suggested that the enzyme has high affinity for *N*-carbamoyl aliphatic amino acids. The K_m values for aliphatic amino acid derivatives were lower than those for aromatic amino acid derivatives, i.e., the K_m values for *N*-carbamoyl-D-valine, *N*-carbamoyl-D-leucine, *N*-carbamoyl-DL-norleucine were 1.0 mM, 3.6 mM and 4.8 mM, respectively, and

those for C-D-Phe, CD-HPG and *N*-carbamoyl-D-phenylglycine were 19.7 mM, 13.1 mM and 26.9 mM, respectively. The relative activities for the D-enantiomer and the racemic substrate differed in some *N*-carbamoyl amino acids, especially in aromatic amino acid derivatives.

Table 3. Substrate specificity of the *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp.

Compound	Relative activity (%)	K_m (mM)
N-Carbamoyl-		
D-alanine	23	12
D-valine	10	1.0
D-leucine	28	3.6
D-serine	7.2	24
D-phenylalanine	100	20
D-phenylglycine	24	27
D-*p*-hydroxyphenylglycine	47	13
DL-alanine	20	-
DL-α-amino-*n*-butyric acid	14	-
DL-valine	8.4	-
DL-norvaline	6.3	-
DL-norleucine	90	4.8
DL-methionine	92	7.5
DL-serine	6.7	-
DL-threonine	3.0	-
DL-phenylalanine	59	-
DL-tryptophan	55	-
DL-phenylglycine	14	-
DL-*p*-hydroxyphenylglycine	39	-

Each compound (10 mM) was incubated under the standard enzyme assay conditions. For *N*- and *O*-carbamoyl compounds, ammonia release was assayed. For *N*-acetyl and *N*-formyl compounds, the corresponding amino acid was assayed. The following compounds did not serve as substrates under the standard assay conditions, *N*-carbamoylglycine, *N*-carbamoyl-L-alanine, *N*-carbamoyl-L-valine, *N*-carbamoyl-L-leucine, *N*-carbamoyl-L-isoleucine, *N*-carbamoyl-L-serine, *N*-carbamoyl-L-glutamate, *N*-carbamoyl-L-arginine, *N*-carbamoyl-L-asparagine, *N*-carbamoyl-L-phenylalanine, *N*-carbamoyl-L-tryptophan, *N*-carbamoyl-L-tyrosine, *N*-carbamoyl-L-histidine, DL-α-aminoisobutyric acid, *RS*-α-methyl-*N*-carbamoylphenyl-glycine and *RS*-α-methyl-*N*-carbamoyl-3,4-dioxymethylene-phenylalaine, *N*-acetylglycine, *N*-acetyl-DL-glutamate, *N*-acetyl-DL-norleucine, *N*-acetyl-DL-methionine, *N*-acetyl-DL-histidine, *N*-acetyl-DL-alanine, *N*-acetyl-D-phenylalanine, *N*-acetyl-DL-tryptophan, *N*-acetyl-DL-valine, ε-*N*-acetyl-DL-lysine, *N*-formyl-D-phenylalanine, *N*-formyl-DL-methionine, *N*-formyl-DL-tryptophan, *N*-formyl-DL-alanine, *N*-formyl-DL-leucine, β-ureidopropinate, DL-ureidosuccinate, *N*-carbamoylsarcosine, DL-citrulline, allantoin, allantoic acid, *S*-carbamoyl-cysteine, albizziin, phenylurea, benzylurea, *m*-hydroxyphenylurea, methylurea, carbamic acid *n*-butylester, carbamic acid benzylester and urea.
-; not determined

Disubstituted *N*-carbamoyl amino acids at the α-carbon such as *RS*-α-methyl-*N*-carbamoylphenylglycine and *RS*-α-methyl-*N*-carbamoyl-3,4-dioxymethylenephenyl-

alanine were not hydrolyzed by the purified enzyme.

The purified enzyme did not hydrolyze β-ureidopropionate and DL-ureidosuccinate, which are the intermediates of uracil and orotic acid degradation, respectively. Allanotin and allantoic acid, as well as *N*-carbamoyl compounds involved in purine degradation, were also not hydrolyzed. Furthermore, *N*-carbamoylsarcosine, DL-citrulline, *N*-acetylamino acids and *N*-formylamino acids were not substrates for the enzyme.

Inhibition

The various compounds were examined as to thier inhibitory effects on the enzyme activity, with C-D-Phe (5 mM) as substrate. Cu^{2+}, Zn^{2+}, Cd^{2+}, Ag^{+} and Hg^{2+} caused considerable inhibition by 96.0, 97.6, 98.1, 88.6 and 99.0% at 2 mM, respectively. Co^{2+} and Ni^{2+} also caused 30.5 and 66.8% inhibition at 2 mM, respectively. Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Be^{2+} and monovalent ions did not have significant effects on the enzyme activity. Fe^{2+}, Fe^{3+}, Al^{3+}, Sn^{2+} and Pb^{2+} slightly enhanced the enzyme activity to about 110% of the standard activity. Some sulfhydryl reagents such as *N*-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoate), *p*-chloromercuribenzoate and iodoacetate inhibited the reaction by 86.8, 94.6, 100 and 89.1% at 1 mM, respectively, suggesting that sulfhydryl groups of the enzyme are involved in its activity. The enzyme was not sensitive to metal ion chelators such as EDTA, 4,5-dihydroxy-1,3-benzenesulfonate, *o*-phenanthroline, 8-hydroxyquinoline and α,α'-dipyridyl at 2 mM. No inhibition occured in the presence of NaCN, NaF, NaN_3, semicarbazide, phenylhydrazine, $NH_2OH \cdot HCl$, diisopropyl phosphorofluoridate, phenylmethanesulfonyl fluoridate, 2-mercaptoethanal or dithiothreitol.

Effects of non-reactive substrate-like compounds

In the presence of various substrate like compounds, the C-D-Phe (5 mM) hydrolyzing activity of the enzyme was estimated. With the existence of *N*-carbamoyl-L-amino acid derivatives of phenylalanine, leucine or alanine at 5 mM, no inhibition was

observed. *N*-Carbamoyl-L-phenylalanine at concentrations of 10 and 20 mM, also showed no inhibition. Thus, the D-enantiomer specific hydrolysis catalyzed by the enzyme was not affected by the L-enantiomer.

D-Phenylalanine, which is a product of the reaction, caused slight inhibition by 12.0% at 5 mM. Ammonium ions did not significantly inhibit at 5-50 mM (ammonium sulfate 2.5-25 mM). However, about 20% loss of activity was observed at 100 mM (ammonium sulfate 50 mM).

L-Phenylalanine, L-alanine and *N*-acetyl derivatives of D-phenylalanine and DL-methionine at 5 mM had no effects on the enzyme activity. These results appear to reflect the strict specificity of the enzyme toward *N*-carbamoyl-D-amino acids.

Stability of the enzyme

About 50% of the activity was lost after storage for 8 days in 10 mM potassium phosphate buffer containing 1 mM dithiothreitol at 4°C. The activity completely disappeared after 3 weeks. However, when preserved under a N_2 atmosphere, about 50% activity remained after 8 weeks, so the inactivation during preservation seems to due to oxidation. Fig. 1C shows the SDS/PAGE results of purified enzyme preserved without N_2 gas and inactivated during preservation. A new band, with a relative molecular mass of about 34,000, was generated. The NH_2-terminal amino acid sequence of the decomposed enzyme having molecular mass of about 34,000 was identical for up to 30 residues with that of original purified enzyme. The breakdown, probably caused by oxidation, seemed to occur in the COOH-terminal region.

After the enzyme had been incubated for 20 min in buffers of various pH at 30°C, the enzyme activity was assayed under the standard conditions. The enzyme was most stable from pH 7-9 (Fig. 3). After the enzyme had been incubated for 20 min in 10 mM potassium phosphate buffer (pH 7.0) at various temperatures, its activity was assayed under the standard conditions. The enzyme was found to be stable below 40°C (Fig. 3).

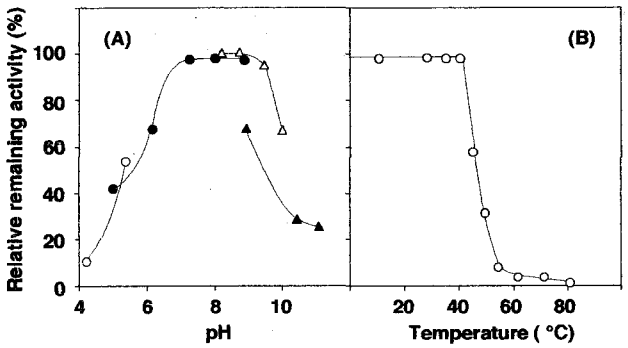


Fig. 3. Effects of pH and temperature on the stability of the *N*-carbamoyl-D-amino acid amidohydrolase.
The effect of pH on the enzyme stability (A) and that of temperature (B) are shown. (A) 5 x 10^{-3} units of *N*-carbamoyl-D-amino acid amidohydrolase was incubated at 30°C for 20 min in 70 mM buffer / acetic acid/HCl buffer (○) for pH 4.2-5.9; in potassium phosphate buffer (●) at pH 5.0-8.8; in Tris/HCl buffer (△) at pH 8.1-9.9; in glycine/NaOH buffer (▲)□ at pH 8.9-11.1. The remaining activity was measured under the standard assay conditions. (B)The remaining activity was assayed under the standard enzyme assay conditions after the enzyme (5 x 10^{-3} units) had been placed at indicated temperatures for 20 min with 400mM potassium phosphate buffer (pH 7.0). The relative remaining activity is expressed as the percentage of the maximum remaining activity attained under the experimental conditions.

Effects of pH and temperature on the enzyme activity

The enzyme showed maximum activity at pH 8-9 with C-D-Phe as substrate (Fig. 4). The enzyme was active under alkaline conditions. The activity was measured at various temperatures as shown in Fig. 4. The initial velocity of the hydrolysis increased with increasing temperature, reaching the maximum at 40°C.

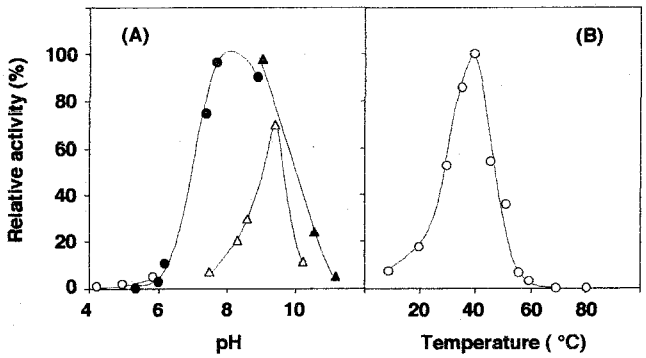


Fig. 4. Effects of pH and temperature on the activity of the *N*-carbamoyl-D-amino acid amidohydrolase.
The effects of pH (A) and temperature (B) on the activity are shown. (A) The enzyme activity was assayed under the standard enzyme assay conditions except that the following buffers (200 mM) were used: acetic acid/HCl buffer (○) at pH 4.2-5.9, potassium phosphate buffer (●) at pH 5.0-8.8, Tris/HCl buffer (△) at pH 7.6-9.9 and glycine/NaOH buffer (▲) at pH 8.9-11.1. (B) Assays were performed at various temperatures under the standard enzyme assay conditions. The relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions.

DISCUSSION

N-Carbamoyl-D-amino acid amidohydrolase was purified to homogeneity from extracts of *Comamonas* sp. This bacterium showed no D-specific hydantoinase or dihydropyrimidinase activity as described in chapter I, and is the first time that *N*-carbamoyl-D-amino acid amidohydrolase activity has been shown to exist independently of dihydropyrimidinase and D-specific hydantoinase activities. Yokozeki *et al.* partially purified *N*-carbamoyl-D-amino acid amidohydrolase from *Pseudomonas* sp. AJ-11220, and investigated some properties of the enzyme [32]. The partially purified enzyme of *Pseudomonas* has an optimum pH around neutral, as well as the crude preparations from *Arthrobacter crystalopoietes* AM 2 [23] and *Agrobacterium* sp. IP-I 671 [31], and it hydrolyzed various *N*-carbamoyl-D-amino acids and β-ureidopropionate. On the other hand, the purified enzyme from *Comamonas* sp. had an optimum pH at alkaline pH, 8-9, and though it hydrolyzed various *N*-carbamoyl-D-amino acids, but it did not affect β-ureidopropionate. It has been reported that the *N*-carbamoyl-D-amino acid hydrolyzing activity in the crude extracts of *Agrobacterium radiobacter* NRRL B 11291 [22] and *Agrobacterium* sp. IP-I 671 [31] are inhibited by ammonia, and the activity of *A. radiobacter* NRRL B 11291 is inhibited by *N*-carbamoyl-L-amino acids. However, the *Comamonas* enzyme was not significantly affected either by ammonia or by *N*-carbamoyl-L-amino acids.

N-Carbamoyl-D-amino acid amidohydrolase of *Comamonas* sp. has some differences from other *N*-carbamoylamide hydrolyzing enzymes. The *Comamonas* enzyme showed no activity towared β-ureidopropionate, suggesting that it is not the β-ureidopropionase involved in the pyrimidine degradation pathway. The *Comamonas* enzyme also differed from β-ureidopropionase in relative molecular mass. β-Ureidopropionase is much larger (150,000-200,000) [42-44] than the *Comamonas* enzyme (120,000). Furthermore, the β-ureidopropionase of *Clostridium uracilium* [42] is not affected by sulfhydryl reagents, whereas the *Comamonas* enzyme displayed

sensitivity to sulfhydryl reagents.

The fact that DL-ureidosuccinate is not hydrolyzed by the *N*-carbamoyl-D-amino acid amidohydrolase of the *Comamonas* sp. suggests that the enzyme is not the ureidosuccinase involved in pyrimidine metabolism. The *N*-carbamoyl-D-amino acid amidohydrolase of the *Comamonas* sp. does not show any similarity to the ureidosuccinase [45] in that the former has no metal ion requirement whereas the latter requires divalent metal ions for its reaction. The *Comamonas* enzyme was not affected by the presence of 2 mM EDTA.

N-Carbamoylsarcosine amidohydrolase form *Pseudomonas putida* [46] hydrolyzes several *N*-carbamoyl-D-amino acids, but the *Comamonas* enzyme does not hydrolyze *N*-carbamoylsarcosine, suggesting that it is not *N*-carbamoylsarcosine amidohydrolase. Allantoin and allantoic acid, intermediates in purine catabolism, were not hydrolyzed by the *Comamonas* enzyme. Furthermore, DL-citrulline was not hydrolyzed by the enzyme, confirming that it is not citrullinase. These results indicate that the *Comamonas* enzyme is a novel *N*-carbamoylamide amidohydrolase sepcifically acting on *N*-carbamoyl-D-amino acids.

Some microorganisms produce D-amino acids from the corresponding DL-5-substituted hydantoins via *N*-carbamoyl-D-amino acids [23,30,32]. The first step in this reaction is catalyzed by D-specific hydantoinase, which is identical with dihydropyrimidinase, so it was proposed that the D-amino acid was produced from DL-5-substituted hydantoin by the action of a series of enzymes involved in the pyrimidine degradation pathway [23], and that the hydrolysis of the *N*-carbamoyl-D-amino acid was catalyzed by β-ureidopropionase. However, considering the present results, *N*-carbamoyl-D-amino acid amidohydrolase of *Comamonas* sp. appears not to be involved in the pyrimidine degradation pathway elucidated so far. The biological functions of the *Comamonas* enzyme, *N*-carbamoyl-D-amino acid amidohydrolase should therefore be of considerable interest.

SUMMARY

N-Carbamoyl-D-amino acid amidohydrolase was purified 119-fold, with 36% overall recovery from a cell-free extract of *Comamonas* sp. E222c. The purified enzyme was homogeneous as judged by SDS/PAGE. The relative molecular mass of the native enzyme and that of the subunit were 120,000 and 40,000, respectively. The purified enzyme hydrolyzed various *N*-carbamoyl-D-amino acids to D-amino acids, ammonia and carbon dioxide. *N*-Carbamoyl-D-amino acids having hydrophobic groups served as good substrates for the enzyme. The K_m and V_{max} values for C-D-Phe were 19.7 mM and 13.1 units·mg^{-1}, respectively, and those for C-D-HPG were 13.1 mM and 0.56 units·mg^{-1}, respectively. The enzyme strictly recognized the configuration of the substrate and only the D-enantiomer of the *N*-carbamoylamino acid was hydrolyzed. The enzyme activity was not significantly affected by *N*-carbamoyl-L-amino acids and ammonia. The enzyme was sensitive to thiol reagents and did not require metal ions for its activity. The enzyme did not hydrolyze β-ureidopropionate or β-ureidosuccinate suggesting that the enzyme is different from the *N*-carbamoylamide hydrolyzing enzymes involved in the pyrimidine degradation pathway. The enzyme did not hydrolyze allantoin and allantoic acid which are intermediates in purine degradation, *N*-carbamoylsarcosine and citrulline; suggesting that it is a novel *N*-carbamoylamide amidohydrolase.

Section 2. *N*-Carbamoyl-D-amino acid amidohydrolase from *Blastobacter* sp. A17p-4: purification and characterization[c]

In the previous section, I described the purification and properties of *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp. E222c, which showed no D-specific hydantoinase or dihydropyrimidinase activity. The results suggest that the *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp., which exists independently of D-specific hydantoinase and dihydropyrimidinase, is distinct from β-ureidopropionase. However, it is not clear whether this suggestion is common or particular in the *N*-carbamoyl-D-amino acid amidohydrolase which exists independently of D-specific hydantoinase and dihydropyrimidinase.

In this section, I describe the purification and characterization of a *N*-carbamoyl-D-amino acid amidohydrolase coexisting with D-specific hydantoinase and dihydropyrimidinase from *Blastobacter* sp. A17p-4, and discuss the possibility that 5-monosubstituted hydantoin and pyrimidine are not degraded by the same series of enzymes.

MATERIALS AND METHODS

Microorganism and Cultivation. *Blastobacter* sp. was inoculated into a test tube (16 x 165 mm) containing 5 ml of the basal medium described in chapter I supplemented with 0.15% (w/v) uracil and 0.03% (w/v) $FeSO_4 \cdot 7H_2O$,, then incubated for 4 days at 28°C with shaking. The culture was transferred to a 2-l shake flask containing 300 ml of the same medium, then incubated for 7 days at 28°C with shaking. The cells were harvested by centrifugation and washed with 0.85% NaCl.

Enzyme assay. The assay mixture for enzyme purification contained, in 100 μl, 200 mM potassium phosphate (pH 7.0), 10 mM *N*-carbamoyl-D-*p*-hydroxyphenylglycine (C-D-HPG) and the enzyme. After 5-60 min incubation at 30°C,

the reaction was stopped by the addition of 100 μl of ethanol. The D-*p*-hydroxyphenylglycine (D-HPG) produced was analyzed as described in chapter I. One unit of the enzyme was defined as the amount of enzyme that catalyzes the formation of D-HPG at the rate of 1 μmol·min^{-1} under the assay conditions mentioned above. The standard enzyme assay was carried out with 5 mM *N*-carbamoyl-D-phenylalanine (C-D-Phe) as substrate under the conditions mentioned above, and the amount of D-phenylalanine or ammonia formed was determined as described in section 1.

Analytical methods for the *N*-carbamoyl-D-amino acid amidohydrolase. The relative molecular mass of the enzyme was determined by analytical gel filtration on a calibrated column (1.5 x 80 cm) packed with Sephadex G-150, eluted with 10 mM potassium phosphate pH 7.0 containing 200 mM NaCl and 1 mM dithiothreitol. It was also determined by High-performance gel-permeation liquid chromatography on a GS-520 column (0.76 x 50 cm; Asahi Kasei (Japan)) eluted with the same buffer. Determination of protein concentration, SDS-polyacrylamide gel electrophoresis (SDS/PAGE) on a 12.5% polyacrylamide gel, isoelectric focusing at a pH range of 3.5-11.5, amino acid analysis and NH_2-erminal amino acid sequencing of the purified enzyme were performed as described previously [53].

Enzyme purification. Purification procedures were carried out at 0-5°C. 10 mM potassium phosphate (pH 7.0) containing 1 mM dithiothreitol is referred to as buffer.

Step 1: *Blastobacter* sp. cells (63 g wet cells obtained from 4.8 l medium) suspended in 120 ml buffer, were disrupted with 0.25 mm diameter glass beads (Dyno-Mill KDL, Switzerland) at 5°C for 20 min.

Step 2: After centrifugation the resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained at 20-40% saturation was collected, and dissolved in buffer. The resultant enzyme solution (41 ml) was dialyzed against 5 l of the same buffer for 12 h.

Step 3: The dialyzed solution was applied to a DEAE-Sephacel column (3.5 x 10 cm) equilibrated with buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in 500 ml buffer. The fractions containing enzyme activity were combined (49 ml).

Step 4: The NaCl concentration of the pooled fractions was adjusted to 4 M, then the enzyme solution was placed on a phenyl-Sepharose CL-4B column (1.8 x 12 cm) equilibrated with buffer containing 4 M NaCl. The enzyme was eluted by lowering the ionic strength of NaCl linearly from 4 to 0 M in 90 ml buffer. The active fractions were combined (32 ml) and then concentrated to about 3 ml with an Amicon membrane filter apparatus (Amicon Co. (U.S.A.)) fitted with a YM-10 membrane.

Step 5: The enzyme (3 ml) was applied to a Sephadex G-150 column (1.5 x 80 cm) equilibrated with buffer containing 0.2 M NaCl and eluted with the same buffer. The active fractions were collected (10 ml), dialyzed against 500 ml buffer for 12 h.

Step 5: The enzyme solution was applied to a Mono Q HR 5/5 column equilibrated with buffer. The enzyme was eluted by increasing the ionic strength of NaCl linearly over the range 0-1.0 M in 60 ml buffer. The active fractions were collected (2.0 ml) and used to characterize the enzyme.

RESULTS

Enzyme purification

Through the procedures described in the materials and methods, the enzyme was purified about 37-fold with a yield of 2.3 % (Table 1). The purified enzyme gave one band on SDS/PAGE (Fig. 1). Electrofocusing yielded only one absorption peak for protein (pI 5.3), which coincided with that of the C-D-HPG hydrolyzing activity.

Table 1. Purification of the *N*-carbamoyl-D-amino acid amidohydrolase from *Blastobacter* sp.

Step	Total protein (mg)	Total activity (U)	Specific activity (10^{-3} U/mg)	Yield (%)
1) Cell-free extract	4660	52.0	11.2	100
2) Ammonium sulfate	797	30.2	37.9	58.1
3) DEAE-Sephacel	284	18.3	64.4	35.2
4) Phenyl-Sepharose CL-4B	89.6	7.60	84.8	14.6
5) Sephadex G-150	15.4	4.16	270	8.00
6) Mono Q HR 5/5	2.93	1.21	410	2.33

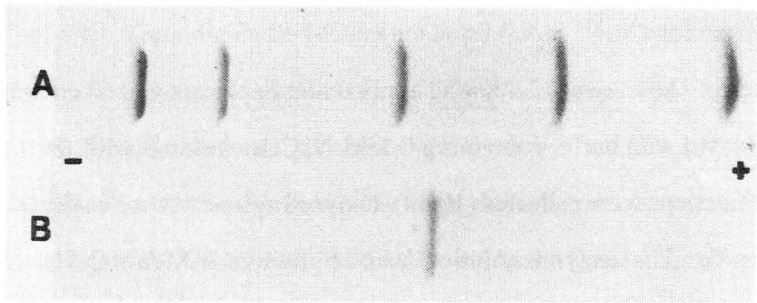


Fig. 1. SDS/PAGE of the *N*-carbamoyl-D-amino acid amidohydrolase from *Blastobacter* sp.
A, standards: (from left) phosphorylase *b* (M_r = 97,400), bovine serum albmin (66,267), aldolase (42,400), carbonic anhydrase (30,000) and soybean trypsin inhibitor (20,100). B, purified enzyme, 10 μg. The gel was stained with Coomassie Brilliant Blue R-250 and destained in ethanol/acetic acid/water, 3/1/6 (by vol.).

Substrate specificity and kinetic properties

The action of the enzyme on a variety of *N*-carbamoylamino acids and related compounds are shown in Table 2. The enzyme was specific for *N*-carbamoyl-D-amino acids. *N*-Carbamoyl-D-amino acids having an aromatic group or long alkyl chain were efficiently hydrolyzed by the purified enzyme. Normal hyperbolic kinetics were observed with all compounds tested and the K_m values obtained from Lineweaver-Burk plots were in the range of 0.3 to 5 mM for the substrates tested. The V_{max} values for C-D-HPG and C-D-Phe were 0.69 and 0.70 units·mg^{-1} protein, respectively.

The purified enzyme did not act on *N*-acetylamino acids and *N*-formylamino acids. *N*-Carbamoylsarcosine, a potent substrate for *N*-carbamoylsarcosine

amidohydrolase [46], was inert as a substrate. β-Ureidopropionate and DL-ureidosuccinate, which are substrates for β-ureidopropionase [42-44,47] and ureidosuccinase [45], respectively, and are intermediates of pyrimidine metabolism, were also inert as substrates. Allantoin and allantoic acid, which are intermediates of purine metabolism, were not hydrolyzed.

Table 2. Substrate specificity of the *N*-carbamoyl-D-amino acid amidohydrolase from *Blastobacter* sp.

Compound	Relative activity (%)	K_m (mM)
N-Carbamoyl-		
D-alanine	29	4.0
D-valine	55	0.41
D-leucine	60	0.36
D-serine	10	2.4
D-phenylalanine	100	0.50
D-phenylglycine	170	0.88
D-*p*-hydroxyphenylglycine	130	1.7
DL-alanine	27	-
DL-α-amino-*n*-butyric acid	48	-
DL-valine	50	-
DL-norvaline	26	-
DL-norleucine	92	0.79
DL-methionine	84	0.71
DL-serine	3.0	-
DL-threonine	18	-
DL-phenylalanine	46	-
DL-tryptophan	18	-
DL-phenylglycine	100	-
DL-*p*-hydroxyphenylglycine	130	-

Each compound (10 mM) was incubated under the standard enzyme assay conditions. For *N*- and *O*-carbamoyl compounds, ammonia release was assayed. For *N*-acetyl and *N*-formyl compounds, the corresponding amino acid was assayed. The following compounds did not serve as substrates under the standard assay conditions, *N*-carbamoylglycine, *N*-carbamoyl-L-alanine, *N*-carbamoyl-L-leucine, *N*-carbamoyl-L-serine, *N*-carbamoyl-L-glutamate, *N*-carbamoyl-L-phenylalanine, *N*-carbamoyl-L-tryptophan, *N*-carbamoyl-α-aminoisobutyrate, *RS*-α-methyl-*N*-carbamoylphenylglycine and *RS*-α-methyl-*N*-carbamoyl-3,4-dioxymethylenephenylalanine, *N*-acetyl-DL-norleucine, *N*-acetyl-DL-methionine, *N*-acetyl-D-phenylalanine, *N*-acetyl-DL-tryptophan, *N*-formyl-D-phenylalanine, *N*-formyl-DL-methionine, *N*-formyl-DL-tryptophan, *N*-formyl-DL-alanine, *N*-formyl-DL-leucine, β-ureidopropionate, DL-ureidosuccinate, *N*-carbamoylsarcosine, DL-citrulline, allantoin, allantoic acid, *N*-carbamoyl-cysteine, phenylurea, benzylurea, carbamic acid benzylester and urea.
-; not determined.

Physical properties

C-D-Phe hydrolyzing activities measured as a function of temperature from 20 to 80°C showed that the activity was highest at 55°C for 20 min reaction (Fig 2A). Upon

incubation for 20 min at varying temperatures, the enzyme was found to be stable up to 50°C (Fig. 2B). The enzyme maintained maximum C-D-Phe hydrolyzing activity at pH 8.0-9.0 for 20 min at 30°C. When the enzyme was incubated at 40°C for 40 min with buffers of various pHs, more than 90% of the activity was retained between pH 6.0 and pH 9.0.

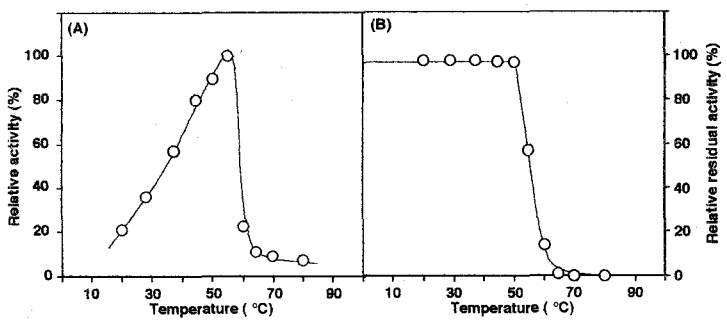


Fig. 2. The effect of temperature on the activity (A) and stability (B) of the purified *N*-carbamoyl-D-amino acid amidohydrolase from *Blastobacter* sp. (A) Assays were performed at various temperatures for 20 min under the standard enzyme assay conditions. The relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions. (B) The enzyme (5×10^{-3} units) in 50 μl of 400 mM potassium phosphate, pH 7.0, was incubated for 20 min at various temperatures. The remaining activity was assayed under the standard enzyme assay conditions. The relative residual activity is expressed as the percentage of the maximum residual activity attained under the experimental conditions.

The relative molecular mass of the enzyme was calculated to be about 120,000 and 111,000 based on gel filtration on a Sephadex G-150 column and high-performance gel permeation chromatography on a GS-520 column, respectively. The relative molecular mass of the subunit of the enzyme was estimated to be about 40,000 by SDS/PAGE (Fig. 1). These results showed that the enzyme is composed of three identical subunits.

The integral numbers of amino acid residues per subunit were revealed as follows: Asx (29), Thr (20), Ser (12), Glx (41), Pro (18), Gly (33), Ala (34), Met (12), Ile (16), Val (24), Leu (29), Tyr (12), Trp (7), Phe (16), Lys (17), His (14), Arg (19) and half-cystine (5). The NH_2-terminal amino acid sequence determined by Edman degradation is as follows: Ala-Arg-Lys-Leu-Asn-Leu-Ala-Val-Ala-Gln-Leu-Gly-Pro-Ile-Ala-Arg-Ala-Glu-Thr-Arg-Asp-Gln-Val-Val-Ala-Arg-Leu-Met-Glu-Met-. The purified

enzyme showed an absorption maximum at 278 nm and no absorption in the visible region.

Inhibition

The enzyme activity was inhibited by heavy metal ions (2 mM) and sulfhydryl reagents (1 mM). Co^{2+}, Zn^{2+}, $NaAsO_2$ and *N*-ethylmaleimide inhibited the activity by 20 to 50%, and Cu^{2+}, Ag^{+}, Cd^{2+}, Hg^{2+}, 5,5'-dithiobis(2-nitrobenzoate), *p*-chloromercuribenzoate and iodoacetate inhibited by 90 to 100%. The other divalent and monovalent metal ions (2 mM), metal ion chelators (2 mM), carbonyl reagents and serine protease inhibitors (1 mM) did not affect the enzyme activity.

When the C-D-Phe (substrate) concentration was 5 mM, the enzyme was inhibited by the opposite enantiomer *N*-carbamoyl-L-phenylalanine at 2.5, 5.0, 10.0 and 20.0 mM by 48, 67, 78 and 96%, respectively. D-Phenylalanine, one of the products of the reaction, inhibited the reaction by 28% at 5 mM, while NH_4^{+}, the other product, inhibited the enzyme by 6, 10 and 20% at 20, 50 and 100 mM, respectively.

DISCUSSION

The physiological and biochemical aspects of *N*-carbamoyl-D-amino acid amidohydrolase is still unclear. An *N*-carbamoyl-D-amino acid amidohydrolase coexisting with D-specific hydantoinase has been supposed to be identical with β-ureidopropionase [23] because D-specific hydantoinase is revealed to be identical with dihydropyrimidinase [18,56]. However, *N*-carbamoyl-D-amino acid amidohydrolase coexisting with D-specific hydantoinase has not yet been purified to homogeneity and not characterized. In previous section, I described that the *N*-carbamoyl-D-amino acid amidohydrolase, which does not coexist with D-specific hydantoinase and dihydropyrimidinase, is different from β-ureidopropionase in that it does not hydrolyze β-ureidopropionate. For comparison, I purified to homogeneity a *N*-carbamoyl-D-amino acid amidohydrolase coexisting with D-specific hydantoinase in *Blastobacter* sp. for the

first time.

The characterization of the *Blastobacter* enzyme revealed that the enzyme is also different from β-ureidopropionase and has some similarities with the enzyme of *Comamonas* sp. The native molecular mass of both *N*-carbamoyl-D-amino acid amidohydrolases from *Blastobacter* and *Comamonas* were about 120,000, and those of subunits of each enzyme were about 40,000. They consisted of three identical subunits. The amino terminal amino acid sequences of both enzymes showed 50% homology with each other, and the amino acid compositions of both enzymes resemble to each other, except for the slight differences in the contents of the hydrophilic amino acids. Both enzymes do not hydrolyze β-ureidopropionate and DL-ureidosuccinate, which are substrates for β-ureidopropionase [42-44,47] and ureidosuccinase [45], respectively, and are intermediates of pyrimidine metabolism, while they specifically hydrolyze *N*-carbamoyl-D-amino acids. The K_m values of the *Blastobacter* enzyme toward *N*-carbamoyl-D-amino acids are considerably lower than those of the *Comamonas* enzyme. The former was in the range of 0.3 to 5 mM (Table 2), while the latter was between 1 and 27 mM (see section 1).

As described above, the *N*-carbamoyl-D-amino acid amidohydrolase coexisting with D-specific hydantoinase in *Blastobacter* sp. is different from β-ureidopropionase, and is specific for *N*-carbamoyl-D-amino acids. Recently, a D-specific hydantoinase that dose not exhibit dihydropyrimidinase activity has been purified from an *Agrobacterium* sp. [39]. These findings pose questions as to the conjecture that the D-amino acid production from DL-5-substituted hydantoin involves the action of a series of enzymes involved in pyrimidine degradation, and also suggest the existence of specific enzymes for hydantoin degradation.

SUMMARY

The *N*-carbamoyl-D-amino acid amidohydrolase of *Blastobacter* sp. A17p-4, which coexist with D-specific hydantoinase and dihydropyrimidinase, was purified to

homogeneity and characterized. It has a relative molecular mass of about 120,000 and consists of three identical subunits with a relative molecular mass of about 40,000. *N*-Carbamoyl-D-amino acids having hydrophobic groups served as good substrates for the enzyme. It has been suggested that D-amino acid production from DL-5-substituted hydantoin involves the action of a series of enzymes involved in pyrimidine degradation, namely amide-ring opening enzyme, dihydropyrimidinase, and *N*-carbamoylamide hydrolyzing enzyme, β-ureidopropionase. However, the purified enzyme did not hydrolyze β-ureidopropionate; suggesting that the *N*-carbamoyl-D-amino acid amidohydrolase coexisting with D-specific hydantoinase, probably dihydropyrimidinase, in *Blastobacter* sp. A17p-4 is different from β-ureidopropionase.

Section 3. β-Ureidopropionase from *Pseudomonas putida* IFO 12996: purification and characterization[d]

There are two known degradation pathways for pyrimidine. These pathways involve the reductive and oxidative catabolism of pyrimidine bases, respectively. The reductive pathway, which appears prevalent in mammals, plants and microorganisms, involves three enzymatic steps, uracil or thymine being converted to β-alanine or β-aminoisobutyrate via dihydrouracil or dihydrothymine, as well as carbon dioxide and ammonia. The *N*-carbamoylamide hydrolyzing step of this pathway is catalyzed by β-ureidopropionase. β-Ureidopropionase activity has been detected in various organisms, such as in rat liver [42,57-59], mouse liver [60], *Euglena glacilis* [44], and *Clostridium uracilicum* [43]. Recently, the enzyme from rat liver was highly purified and characterized [61,62]. However, there have been no detailed reports on an enzyme from aerobic bacteria.

Takahashi *et al.* studied the DL-5-substituted hydantoin metabolism in an aerobic bacterium, *Pseudomonas putida* IFO 12996, and revealed that DL-5-substituted hydantoins are hydrolyzed to *N*-carbamoyl-D-amino acids by dihydropyrimidinase, which catalyzes the amide-ring opening step of the reductive degradation pathway for pyrimidine [18]. The product *N*-carbamoyl-D-amino acids are further hydrolyzed to D-amino acids in some bacteria and this reaction has been assumed to be catalyzed by an enzyme of the pyrimidine degrading pathway, β-ureidopropionase [23,31,32]. However, I revealed, in previous section, that an *N*-carbamoyl-D-amino acid-hydrolyzing enzyme is specific for *N*-carbamoyl-D-amino acids and does not react on β-ureidopropionate at all. This suggests that hydantoin and pyrimidine are not always degraded by the same series of enzymes. Because of these findings, the character of the β-ureidopropionases from aerobic bacteria has become a focus of research interest.

In this section, I describe purification and properties of the β-ureidopropionase from an aerobic bacterium, *P. putida* IFO12996. The specific features of the enzyme are quite different from those of the β-ureidopropionases from other sources, such as

mammals, protozoa and anaerobic bacteria, and also differ from those of other *N*-carbamoylamide-hydrolyzing enzymes, especially *N*-carbamoyl-D-amino acid hydrolyzing enzymes. This confirms the differences between pyrimidine-degrading enzymes and hydantoin-degrading enzymes in aerobic bacteria.

MATERIALS AND METHODS

Microorganism and cultivation. *P. putida* was used as the enzyme source. The medium consisted of 1 g KH_2PO_4, 1 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 3 g yeast extract (Oriental Yeast Co., Japan), 3 g meat extract (Mikuni Co., Japan), 10 g glycerol, 2 g Polypepton (Daigo Nutritional, Japan), and 2 g hydantoin in 1 l tap water, pH 6.4. A subculture was performed aerobically in a test tube (16 x 165 mm) containing 5 ml of the medium at 20°C for 16 h. The subculture was transferred to a 2-l shaking flask containing 500 ml of the medium and then incubated for 24 h at 28°C with shaking.

Enzyme assay. The activity of β-ureidopropionase was assayed at 30°C by measuring the production of β-alanine or ammonia from β-ureidopropionate. The standard assay mixture comprised 15 μmol of Tris/HCl, pH 7.4, 0.6 μmol of $CoCl_2 \cdot 6H_2O$, 3 μmol of β-ureidopropionate and the enzyme in a total volume 0.3 ml. The reaction was terminated by adding 0.3 ml of ethanol to the reaction mixture. The supernatant obtained by centrifugation was analyzed for β-alanine or ammonia as described below. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of β-alanine at the rate of 1 $\mu mol \cdot min^{-1}$ under the standard assay conditions.

The substrate specificity was examined under the assay conditions mentioned above except that the substrate concentrations were changed.

Amino acid and ammonia analysis. Quantitative analysis of amino acids was performed with an amino acid analyzer (Model K-101; Kyowa Seimitu, Japan) or

by high-performance liquid chromatography (HPLC) on a reverse-phase packed column (ODS-M, 4 x 150 mm; Shimadzu Techno-Research, Inc., Japan) with fluorescence detection (excitation, 340 nm; emission, 455 nm) after derivatization with *o*-phthalaldehyde [63], elution being performed with methanol/50 mM KH_2PO_4 (42/68, by vol.) at the flow rate of 1.0 ml·min^{-1}.

Ammonia was determined with a commercial kit (Boehringer Mannheim, Germany), based on the glutamate dehydrogenase reaction [50].

Stereochemical analysis of amino acids. The ratio of D- and L-amino acids was estimated by HPLC. Alanine and serine were diastereomerically derivatized with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate [64,65], and then analyzed on a reverse-phase packed column (Cosmosil 5C18, 4.6 x 250 mm; Nacalai Tesque, Japan) with UV detection at 254 nm, elution being performed with methanol/water (45/55, by vol.), pH 2.5, for alanine or methanol/water (30/60, by vol.), pH 2.5, for serine, at the flow rate of 1.0 ml·min^{-1}. 2-Aminovaleric acid and α-amino-*n*-butyrate were directly assayed on a MCI GEL CRS 10W packed column (4.6 x 50 mm; Mitsubishi Kasei, Japan) with UV detection at 254 nm, elution being performed with 1 mM $CuSO_4$ for 2-Aminovaleric acid or 0.5 mM $CuSO_4$ for α-amino-*n*-butyrate, at the flow rate of 1 ml·min^{-1}.

Analytical methods for β-ureidopropionase. The relative molecular mass of the enzyme was determined by HPLC on a GS-520 column (7.6 x 500 mm; Asahi Kasei, Japan) or G-3000SW column (7.5 x 600 mm; Tosoh, Japan), at the flow rate of 0.3 ml·min^{-1}, with an elution buffer consisting of 20 mM Tris/HCl, pH 7.4, containing 0.2 M NaCl. Determination of the protein concentration, SDS-polyacrylamide gel electrophoresis (PAGE) on a 12.5% polyacrylamide gel, and estimation of the amino acid composition of the purified enzyme were performed as described previously [53]. NH_2-terminal amino acid sequence analysis was performed as described previously [54].

Enzyme purification. All procedures were carried out at 0 - 5°C. Tris/HCl, pH 7.4, containing 1 mM $CoCl_2 \cdot 6H_2O$ and 5% (by vol.) ethylene glycol was used as the buffer.

Step 1: *P. putida* cells (150 g wet cells obtained from 20 l culture broth) suspended in 150 ml of 20 mM buffer were disrupted with 0.25 mm diameter glass beads (Dyno-Mill KDL, Switzerland) at 5°C for 10 min. The disrupted cell suspension was passed through a glass filter and then centrifuged at 14,000 x *g* for 60 min. The supernatant (640 ml) obtained was used as the cell-free extract.

Step 2: The cell-free extract was fractionated with solid ammonium sulfate (20 to 40% saturation). The active precipitate was dissolved in 20 mM buffer (115 ml) and then dialyzed against 5 l of the same buffer for 12 h.

Step 3: DEAE-Sephacel resin (400 ml) equilibrated with 20 mM buffer was added to the dialyzed active fraction, and then the enzyme was adsorbed to the resin with stirring for 1.5 h. After washing the resin with 20 mM buffer containing 0.2 M NaCl (1000 ml), the enzyme was eluted with 20 mM buffer containing 0.3 M NaCl (1000 ml). The resin suspension was passed through a glass filter and the active solution was retained (990 ml).

Step 4: The active solution was concentrated to 100 ml with an Amicon membrane filter apparatus (Amicon Co., USA) equipped with a YM-30 membrane. After adjusting the NaCl concentration to 4 M with solid NaCl, the enzyme solution was applied to a phenyl-Sepharose CL-4B column (1.5 x 20 cm) equilibrated with 20 mM buffer containing 4 M NaCl. After washing the column with the same buffer, the enzyme was eluted with 20 mM buffer (100 ml).

Step 5: The active fractions were combined and the NaCl concentration was adjusted to 4 M. The enzyme solution was applied to a phenyl-Sepharose CL-4B column (1.5 x 20 cm) equilibrated with 20 mM buffer containing 4 M NaCl. The enzyme was eluted with a linear gradient of NaCl and then ethylene glycol (4 to 0 M and 5 to 50% (by vol.), respectively) in 20 mM buffer. The active fractions were combined

(118 ml), concentrated to 10 ml by ultrafiltration, and then dialyzed against 500 ml of 20 mM buffer for 12 h.

Step 6: The dialyzed solution was applied to a MonoQ HR 5/5 column equilibrated with 20 mM buffer. The enzyme was eluted by increasing the ionic strength of NaCl linearly over the range of 0 - 1 M in 20 mM buffer. The active fractions were collected (4 ml) and concentrated to 1 ml by ultrafiltration.

Step 7: The active solution (200 ml) was applied to a Superose 12 HR 10/30 column equilibrated with 20 mM buffer containing 0.2 M NaCl and then eluted with the same buffer. This operation was performed five times, and the eluted active fractions were collected (5 ml) and then used for characterization of the enzyme.

RESULTS

Enzyme purification

The enzyme was purified about 60-fold, with a 1.6% yield, from the cell-free extract (Table 1). The purified enzyme was found to be homogeneous on SDS-PAGE (Fig. 1), and was eluted as a single peak on high-performance gel-permeation liquid chromatography on a GS-520 column (Fig. 2A).

Table 1. Purification of the β-ureidopropionase from *P. putida*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
1) Cell-free extract	13800	411	0.0299	100
2) Ammonium sulfate	4260	149	0.0351	36.2
3) DEAE-Sephacel	663	91.5	0.138	22.2
4) Phenyl-Sepharose CL-4B (1st)	309	84.2	0.273	20.5
5) Phenyl-Sepharose CL-4B (2nd)	39.1	32.6	0.835	7.93
6) MonoQ HR 5/5	7.20	6.56	0.911	1.59
7) Superose 12 HR 10/30	3.60	6.41	1.78	1.56

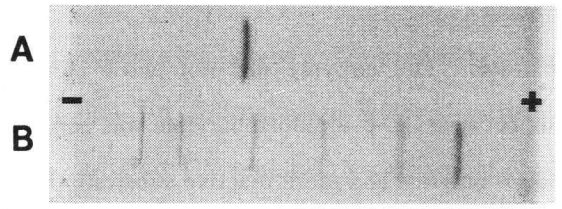


Fig. 2. SDS/PAGE of the β-ureidopropionase from *P. putida*.
A, Purified enzyme. B, standards: (from left) phosphorylase *b* (M_r = 97,400), bovine serum albumin (66,267), aldolase (42,400), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). The gel was stained with Coomassie brilliant blue R-250 and destained in ethanol/acetic acid/water (3/1/6, by vol.).

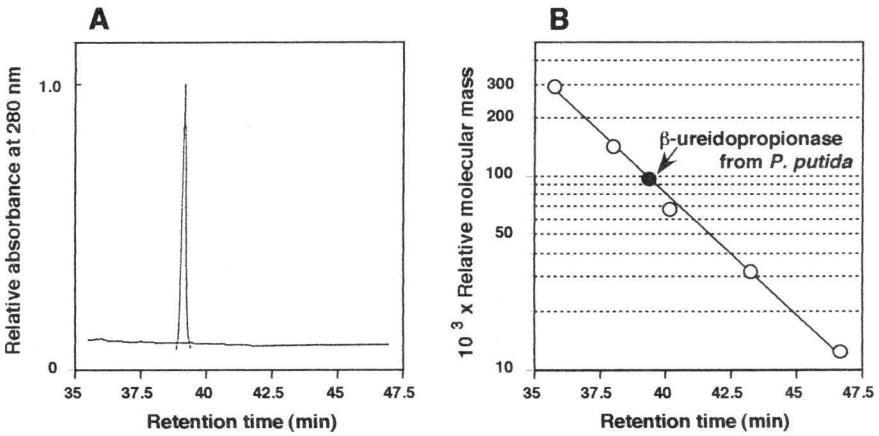


Fig. 3. High-performance gel-permeation chromatography of the β-ureidopropionase from *P. putida*.
The elution profile of the enzyme (A) and determination of the relative molecular mass of the enzyme (B) are shown. The protein standards (○), in order of decreasing molecular mass were: glutamate dehydrogenase (M_r = 290,000), lactate dehydrogenase (142,000), enolase (67,000), adenylate kinase (32,000), and cytochrome *c* (12,400). The ultraviolet absorption at 280 nm is expressed as the relative absorbance. Relative absorbance represents the percentage of the full-scale deflection on the recorder expressed from 0 to 1.0 on the ordinate.

Substrate specificity and kinetic properties

Normal hyperbolic kinetics were observed with all compounds tested, and the K_m, V_{max} and V_{max}/K_m values for several preferred substrates, calculated from double-reciprocal plots, are shown in Table 2. While β-ureidopropionase from mammals and anaerobic bacteria showed strict specificity for *N*-carbamoyl-β-amino acids, the enzyme from *P. putida* was found to act on a variety of *N*-carbamoyl-α-, β- and γ-amino acids. β-Ureidoisobutyrate, an intermediate of thymine metabolism, served

as a good substrate. The enzyme did not show stereospecificity toward β-ureidoisobutyrate, because DL-β-ureidoisobutyrate was completely hydrolyzed by the enzyme. γ-Ureido-*n*-butyrate is a more reactive substrate than β-ureidopropionate; its V_{max} value is higher by about 5-fold than that of β-ureidopropionate. *N*-Carbamoyl-α-amino acids with small substituents, such as *N*-carbamoylglycine, *N*-carbamoyl-L-alanine, *N*-carbamoyl-DL-α-amino-*n*-butyrate, *N*-carbamoyl-DL-2-aminovaleric acid and *N*-carbamoyl-L-serine, served as good substrates. In addition to *N*-carbamoylamino acids, *N*-formyl- and *N*-acetyl-DL-alanine also served as substrates, but the rates were lower than that for *N*-carbamoyl-L-alanine. The K_m values for *N*-carbamoyl-α-amino acids with small substituents were lower than those for *N*-carbamoyl-β- and *N*-carbamoyl-γ-amino acids. However, the V_{max}/K_m value of β-ureidopropionate is higher than that of *N*-carbamoyl-α-amino acids; suggesting that β-ureidopropionate is a suitable substrate of the enzyme. On the other hand, *N*-carbamoyl-α-amino acids with bulky substituents and *N*-carbamoyl-α-D-amino acids were not hydrolyzed by the enzyme.

Table 2. Substrate specificity of the β-ureidopropionase from *P. putida*

Compound	Substrate concentration (mM)	Relative activity (%)	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m
β-Ureidopropionate	10	100	3.7	4.1	1.1
DL-β-Ureidoisobutyrate	10	43	4.5	1.0	0.22
γ-Ureido-*n*-butyrate	10	290	12	19	1.7
N-Carbamoylglycine	10	17	0.68	0.091	0.13
N-Carbamoyl-L-alanine	10	120	1.6	1.0	0.64
N-Carbamoyl-DL-α-amino-*n*-butyrate	10	31	2.8	1.1	0.38
N-Carbamoyl-DL-2-aminovaleric acid	5	8.9	42	1.1	0.027
N-Carbamoyl-L-serine	10	34	75	3.8	0.050
N-Carbamoyl-DL-threonine	5	0.97	-	-	-
N-Carbamoyl-DL-aspartate	10	0.14	-	-	-
N-Carbamoyl-L-asparagine	5	1.6	-	-	-
N-Carbamoyl-L-glutamate	10	0.29	-	-	-
N-Formyl-DL-alanine	10	75	7.7	0.84	0.11
N-Acetyl-DL-alanine	10	6.3	8.8	0.067	0.0077

-; not determined.

The following compounds were judged to be inactive as subtrates, the velocity being less than 0.03% that for β-ureidopropionate: *N*-carbamoyl-D-alanine, *N*-carbamoyl-D-valine, *N*-carbamoyl-D-leucine, *N*-carbamoyl-D-phenylalanine, *N*-carbamoyl-D-*p*-hydroxyphenylglycine, *N*-carbamoyl-L-leucine, *N*-carbamoyl-L-isoleucine, *N*-carbamoyl-L-arginine, *N*-carbamoyl-L-phenylalanine, *N*-carbamoyl-L-tryptophan, *N*-carbamoyl-L-tyrosine, *N*-carbamoyl-DL-2-aminohexanoic acid, *N*-carbamoyl-α-aminoisobutyrate, *S*-carbamoyl-L-cysteine, DL-β-ureido-*n*-butyrate, *N*-carbamoylsarcosine, allantoic acid, allantoin and citrulline.

Stereospecificity

The stereochemistry of the reaction products derived from racemates was investigated. Alanine formed from *N*-carbamoyl-DL-alanine, *N*-formyl-DL-alanine and *N*-acetyl-DL-alanine, and serine, α-amino-*n*-butyrate and 2-aminovaleric acid produced from the corresponding *N*-carbamoyl-DL-amino acids were all revealed to be L-enantiomers by HPLC analysis. Typical chromatograms of serine and 2-aminovaleric acid are shown in Fig. 3. These results suggest that β-ureidopropionase of *P. putida* exhibits *N*-carbamoyl-α-L-amino acid amidohydrolase activity.

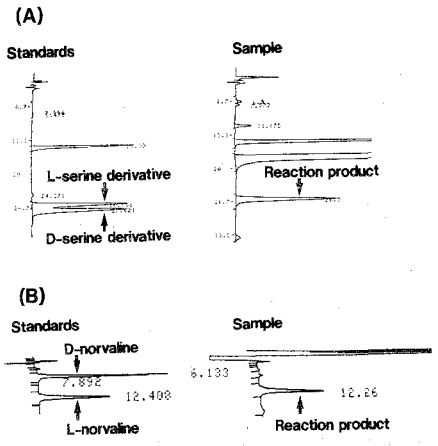


Fig. 4. Stereochemical analysis of serine and norvaline produced by the β-ureidopropionase of *P. putida* with the corresponding racemic *N*-carbamoylamino acids as substrates.
HPLC analysis of serine (A) and 2-aminovaleric acid (B) produced by the enzyme are shown. Serine, derivatized with tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate, and 2-aminovaleric acid were assayed as described under materials and methods.

Inhibition

Various inhibitors, at 2 mM unless otherwise noted, were examined as to their inhibitory effects on the enzyme activity with 10 mM β-ureidopropionate as the substrate. Sulfhydryl reagents such as *p*-chloromercuribenzoate, *N*-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoate) caused considerable inhibition, 100%, 95% and 100%, respectively. Carbonyl reagents such as phenylhydrazine and semicarbazide inhibited the reaction by 49% and 78%, respectively. The activity of the enzyme was not lost in the presence of the following reagents: NaCN, NaF, NaN_3, NH_2OH, Na_2AsO_2, iodoacetate, diisopropyl phosphorofluoridate, phenylmethanesulfonyl fluoride and tosylphenylalanylchloro-methylketone (0.2 mM).

In the presence of various compounds, the enzyme activity was estimated with 5 mM β-ureidopropionate as the substrate. Metabolic intermediates of uracil and thymine, uracil (1 mM), dihydrouracil (5 mM), thymine (1 mM) and dihydrothymine (5 mM), did not affect the enzyme activity. The reaction products, β-alanine (2 mM) and L-alanine (5 mM), caused 84% and 75% inhibition, respectively. However, β-aminoisobutyrate (5 mM), $(NH_4)_2SO_4$ (10 mM), and $NaHCO_3$ (10 mM), did not affect the enzyme activity. Sodium propionate (5 mM), which is an inhibitor of the β-ureidopropionase from rat liver [61], inhibited the reaction by 58%, and was a competitive inhibitor of the β-ureidopropionate hydrolysis (K_i = 4.69 mM). Substrate-like compounds such as *N*-carbamoyl-L-leucine (2.5 mM), *N*-carbamoyl-L-phenylalanine (5 mM), and *N*-carbamoyl-D-alanine (5 mM) inhibited the reaction by 38%, 40% and 44%, respectively. *N*-Carbamoyl-D-alanine was a competitive inhibitor of the *N*-carbamoyl-L-alanine hydrolysis (K_i = 3.84 mM).

Effects of metal ions on the enzyme activity

Metal ion chelators such as EDTA, 8-hydroxyquinoline, *o*-phenanthroline and 2,2'-dipyridyl caused considerable inhibition at 2 mM, 100%, 100%, 93% and 70%, respectively. The enzyme showed no activity in the presence of 1 mM EDTA, and the addition of some divalent metal ions such as Mn^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} restored the

enzyme activity. 93% and 51% of the initial activity was observed when 6 mM Co^{2+} and Ni^{2+} were added, respectively. On exhaustive dialysis against 20 mM Tris/HCl, pH 7.4, the enzyme also lost activity, the latter decreasing to 4.6% of the initial level. The addition of 2 mM Co^{2+} and Mn^{2+} to the dialyzed enzyme restored the activity to 55% and 20% of the initial level. However, further recovery of the activity was not attained on increasing the concentration of Co^{2+} further. On the other hand, metal ions such as Cu^{2+}, Ag^{+} and Hg^{2+} caused considerable inhibition, 65%, 80% and 100%, respectively, at 2 mM. Zn^{2+}, Sn^{2+}, Pb^{2+} and Cd^{2+} were also rather inhibitory (20% - 40% inhibition at 2 mM). The enzyme was not affected by Li^{+}, Na^{+}, K^{+}, Rb^{+}, Cs^{+}, Be^{2+}, Fe^{3+} or Al^{3+} at 2 mM. The absorption spectrum of the enzyme showed a maximum at 275 nm, but it did not show any absorption in the visible region of 320 - 700 nm.

Relative molecular mass and subunit structure

The relative molecular mass of the enzyme was calculated to be about 95,000 by high-performance gel-permeation liquid chromatography on GS-520 column (Fig. 2B). It was also calculated to be 94,000 by high-performance gel-permeation liquid chromatography on G-3000SW column. The relative molecular mass of the subunit was estimated to be about 45,000 by SDS-PAGE (Fig. 1). The native enzyme probably consists of two subunits identical in molecular weight.

NH_2-terminal amino acid sequence and amino acid composition

An NH_2-terminal sequence of twenty amino acid residues was found for the enzyme protein on automated Edman degradation: Met-Thr-Pro-Ala-Gln-Gln-Val-Leu-Gln-Ser-Thr-Gln-His-His-Ile-Asp-Ser-Thr-Arg-Leu-. When this sequence was compared with those of proteins stored in the protein sequence data base, no apparent similarity was found with other types of amidohydrolases. The amino acid composition of the enzyme was also investigated. The number of residues found per subunit are as follows: Asp & Asn (33), Thr (22), Ser (16), Glu & Gln (44), Pro (20), Gly (44), Ala

(53), Met (10), Ile (18), Val (28), Leu (40), Tyr (5), Trp (13), Phe (13), Lys (9), His (14), Arg (26) and Half-cystine (7).

Effects of pH and temperature

The enzyme activity and stability were assayed in 0.1 M potassium phosphate, 0.1 M Tris/HCl and 0.1 M borate/NaOH buffer systems at pH values in the ranges of 6.1 - 8.5, 7.7 - 9.8, and 9.0 - 10.7, respectively. Under the standard assay conditions, the pH optimum for β-ureidopropionate hydrolysis was 7.5 - 8.2. When the enzyme was incubated at 30°C for 30 min with the buffers mentioned above, more than 80% of the initial activity was retained at pH 6.1 to pH 8.6.

The initial velocity of the hydrolysis increased with increasing temperature, reaching a maximum at 60°C. 80% of the initial activity remained after incubation at 65°C for 30 min at pH 7.4, but about 80% of the activity was lost on incubation at 70°C.

DISCUSSION

The β-ureidopropionase from *P. putida* is quite different from the enzymes reported previously from mammals [61,62], protozoa [44], and anaerobic bacteria [43]. The first and most distinct difference is its broad substrate specificity not only towards *N*-carbamoyl-β-amino acids, but also *N*-carbamoyl-γ-amino acids and *N*-carbamoyl-α-amino acids, with L-enantioselectivity. While the enzymes from other sources exhibit strict substrate specificity toward β-ureidopropionate and β-ureidoisobutyrate. Only the rat liver enzyme hydrolyzes *N*-carbamoylglycine and *N*-carbamoyl-DL-alanine, but very slowly (less than 5% of the activity toward β-ureidopropionate) [62]. The second difference is its metal ion dependence. The enzymes from rat liver [62], mouse liver [60], and *C. uracilicum* [43] do not require metal ions for their activity. Only the stimulatory effect of Mg^{2+} on the activity of the enzyme from *E. gracilis* has been reported [44]. The β-ureidopropionase of *P. putida* also requires reduced sulfhydryl groups for its activity, while the enzyme of an anaerobic bacterium, *C. uracilicum*, does

not [43]. The third difference is its molecular mass. The relative molecular mass of the enzyme from *P. putida* is about 95,000, and it consists of two identical subunits of a relative molecular mass of about 45,000. The enzymes from other sources have much high relative molecular masses, i.e., 150,000 to 330,000. The rat liver enzyme, of which the relative molecular mass is about 240,000, consists of six identical subunits of a relative molecular mass of about 42,000, and the amino acid composition of the subunit has been reported [61]. However, the amino acid composition of the *P. putida* enzyme is quite different from that of the rat liver enzyme, and rather similar to that of *N*-carbamoyl-D-amino acid amidehydrolase of *Comamonas* sp (see section 1). There is no similarity between the NH_2-terminal amino acid sequence of the *P. putida* enzyme and that of the rat liver enzyme predicted from its cDNA [66].

The β-ureidopropionase of *P. putida* also differs from already known *N*-carbamoylamide amidohydrolases, such as citrullinase [47] and *N*-carbamoylsarcosine amidohydrolase [46], in that it does not hydrolyze the substrates for the above *N*-carbamoylamide amidohydrolases, i.e., citrulline and *N*-carbamoylsarcosine, respectively. The β-ureidopropionase of *P. putida* exhibits *N*-carbamoyl-α-L-amino acid amidohydrolase activity. *N*-Carbamoyl-α-L-amino acid hydrolyzing activity has been found in some microorganisms [26,28,32,40,45,67-69], but the enzymes catalyzing the reactions have not been purified except for the ureidosuccinase from *C. oroticum* [45], which exhibits specificity for *N*-carbamoyl-L-aspartate. *N*-Carbamoyl-DL-aspartate is hydrolyzed by the β-ureidopropionase of *P. putida*, but the reaction rate is very low (less than 0.2% of that of β-ureidopropionate hydrolysis) (Table 2). Therefore, the β-ureidopropionase of *P. putida* is also different from ureidosuccinase.

Some microorganisms produce D-amino acids from the corresponding DL-5-substituted hydantoins via *N*-carbamoyl-α-D-amino acids [23,31,32]. It was proposed that the D-amino acid production is the action of a series of enzymes involved in the pyrimidine degradation pathway [23]. In this section, I described the properties of the β-ureidopropionase from *P. putida*, which hydrolyzes *N*-carbamoyl-α-L-amino acids but not *N*-carbamoyl-α-D-amino acids. These findings imply that the *N*-carbamoylamide

hydrolyzing steps of the hydantoin and pyrimidine degradation pathways may be catalyzed by respective enzymes with different substrate specificities and stereospecificities.

SUMMARY

β-Ureidopropionase of aerobic bacterial origin was first purified to homogeneity from *Pseudomonas putida* IFO 12996. It shows a broad substrate specificity; not only β-ureidopropionate (K_m = 3.74 mM, V_{max} = 4.12 units·mg^{-1}), but also γ-ureido-*n*-butyrate (K_m = 11.6 mM, V_{max} = 19.4 units·mg^{-1}), and several *N*-carbamoyl-α-amino acids such as *N*-carbamoylglycine (K_m = 0.68 mM, V_{max} = 0.0914 units·mg^{-1}), *N*-carbamoyl-L-alanine (K_m = 1.56 mM, V_{max} = 1.00 units·mg^{-1}), *N*-carbamoyl-L-serine (K_m = 75.1 mM, V_{max} = 3.78 units·mg^{-1}), and *N*-carbamoyl-DL-a-amino-*n*-butyrate (K_m = 2.81 mM, V_{max} = 1.08 units·mg^{-1}) are hydrolyzed. The hydrolysis of *N*-carbamoyl-α-amino acids is strictly L-enantiomer specific. *N*-Formyl and *N*-acetyl-L-alanine are also hydrolyzed by the enzyme, but the rate of hydrolysis is lower than that for *N*-carbamoyl-L-alanine. The enzyme requires a divalent metal ion, such as Co^{2+}, Ni^{2+} or Mn^{2+}, for the activity, and is significantly affected by sulfhydryl reagents. The enzyme consists of two polypeptide chains with an identical relative molecular mass of 45,000. The broad substrate specificity and metal ion dependence of the enzyme show that the β-ureidopropinase of this aerobic bacterium is quite different from those of mammals and anaerobic bacteria.

Section 4. *N*-Carbamoyl-L-amino acid amidohydrolase from *Alcaligenes xylosoxidans* A35: purification and characterization[e]

In section 1 and 2, I described that *N*-carbamoyl-D-amino acid amidohydrolase is distinct from β-ureidopropionase in *Comamonas* sp. and in *Blastobacter* sp. Furthermore, in section 3, I indicated that β-ureidopropionase from *Pseudomonas putida* showed *N*-carbamoyl-L-amino acid-hydrolyzing activity.

N-Carbamoyl-L-amino acid-hydrolyzing activities have been found in microorganisms which metabolize DL-5-monosubstituted hydantoins to L-amino acids [26,40,67,69,70]. However, the enzyme that hydrolyzes *N*-carbamoyl-L-amino acid has scarcely been purified because of its instability and it has not been characterized in detail.

In this section, I describe the purification and characterization of *N*-carbamoyl-L-amino acid amidohydrolase from the enzyme producer, *Alcaligenes xylosoxidans* A35, which assimilates *N*-carbamoyl-L-amino acids.

MATERIALS AND METHODS

Microorganism and cultivation. *A. xylosoxidans* A35 was used as the enzyme source. The medium consisted of 1 g KH_2PO_4, 1 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 1 g yeast extract (Oriental Yeast Co., Japan), 10 g glycerol, 1 g NH_4Cl, and 2 g *N*-carbamoyl-L-leucine in 1 l tap water, pH 7.0. The organism was pre-cultured aerobically in test tubes (16 x 165 mm) containing 5 ml of the medium at 28°C for 22 h. The subcultures were transferred to a 2-l shaking flask containing 500 ml of the medium, then incubated for 25 h at 28°C with shaking.

Enzyme assay. The activity of *N*-carbamoyl-L-amino acid amidohydrolase was assayed at 30°C by measuring the production of L-valine or ammonia from *N*-carbamoyl-L-valine. The standard assay mixture comprised 15 μmol of Tris/HCl, pH

7.4, 0.6 μmol of $NiCl_2 \cdot 6H_2O$, 3 μmol of *N*-carbamoyl-L-valine and the enzyme in a total volume 0.3 ml. The reaction was terminated by adding 0.3 ml of ethanol to the reaction mixture. The supernatant obtained by centrifugation, at 9,000 x *g* for 10 min, was analyzed for produced amino acid or ammonia as described in section 3. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of L-valine at the rate of 1 $\mu mol \cdot min^{-1}$ under the standard assay conditions.

Analytical methods for *N*-carbamoyl-L-amino acid amidohydrolase. The relative molecular mass of the enzyme was determined by high-performance gel-permeation liquid chromatography on a GS-520 column (7.6 x 500 mm; Asahi Kasei, Japan) or a G-3000SW column (7.5 x 600 mm; Tosoh, Japan), at a flow rate of 0.3 $ml \cdot min^{-1}$, with an elution buffer consisting of 20 mM Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol and 0.2 M NaCl. Determination of the protein concentration, SDS-polyacrylamide gel electrophoresis (SDS/PAGE) on a 12.5% polyacrylamide gel, NH_2-terminal amino acid sequence and estimation of the amino acid composition of the purified enzyme were performed as described previously [53,54].

Enzyme purification. All procedures were performed between 0-5°C. The buffer was 20 mM Tris/HCl, pH 7.4, containing 10 mM 2-mercaptoethanol and 10% (by vol.) glycerol.

Step 1: *A. xylosoxidans* cells (35 g wet cells obtained from 8.5 l culture broth) suspended in 40 ml buffer were disrupted with 0.25-mm diameter glass beads (Dyno-Mill KDL, Switzerland) at 5°C for 10 min. The disrupted cell suspension was passed through a glass filter, then centrifuged at 14,000 x *g* for 60 min. The supernatant (400 ml) was used as the cell-free extract.

Step 2: The cell-free extract was dialyzed against 5 l buffer for 12 h, and applied to a DEAE-Sephacel column (5.5 x 20 cm) equilibrated with buffer. After washing the column with the buffer containing 0.08 M NaCl (2 l), the enzyme was eluted with a

linear gradient of 0.08-0.25 M NaCl in 1 l buffer. The fractions containing enzyme activity were combined (140 ml).

Step 3: The pooled enzyme was fractionated with solid ammonium sulfate (35 to 55% saturation). The active precipitate was dissolved in buffer (11.5 ml).

Step 4: After being adjusted the NaCl concentration to 4 M with solid NaCl, the enzyme was applied to a phenyl-Sepharose CL-4B column (1.0 x 15 cm) equilibrated with buffer containing 4 M NaCl. After washing the column with the same buffer (100 ml), the enzyme was eluted with a linear gradient of NaCl and ethylene glycol (4 to 0 M and 0 to 50% (by vol.), respectively) in 600 ml buffer. The active fractions were combined (51 ml).

Step 5: The enzyme solution was concentrated to 7 ml by ultrafiltration through a YM-10 membrane (Amicon Co., USA), then dialyzed against 500 ml buffer for 12 h. The dialyzate was applied to a MonoQ HR 5/5 column equilibrated with buffer. After washing the column with the same buffer (20 ml), the enzyme was eluted by increasing the ionic strength of NaCl linearly over the range of 0-0.7 M in 20 ml buffer. The active fractions were collected (2 ml) and concentrated to 1 ml by ultrafiltration.

The active solution (200 μl) was applied to a Superose 12 HR 10/30 column equilibrated with 20 mM Tris/HCl, pH 7.4, containing 10 mM 2-mercaptoethanol and 0.2 M NaCl, then eluted with the same buffer. This procedure was repeated five times, and the eluted active fractions were collected (5 ml). The purified enzyme was stabilized with 500 μl of glycerol, then characterized.

RESULTS

Stabilization and purification

During storage in 20 mM Tris/HCl buffer pH 7.4 at 4°C, the enzyme activity of the cell-free extract decreased to 45 and 25% of the initial activity after 24 and 72 h, respectively. However, the activity of the cell-free extract was stabilized by adding 10

mM 2-mercaptoethanol and 10% (by vol.) glycerol into the buffer. About 70% of the initial activity was retained after 20 days in the improved buffer at 4°C (Fig. 1).

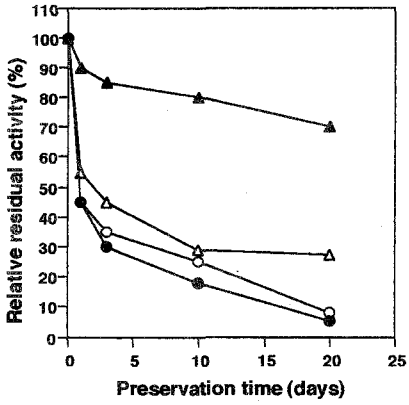


Fig. 1. Effects of chemicals on the enzyme stability.
Reactions proceeded as described in materials and methods with the cell-free extracts preserved in the buffers: (●), 20 mM Tris/HCl, pH 7.4; (○), 20 mM Tris/HCl, pH 7.4, containing 10% (by vol.) glycerol; (▲), 20 mM Tris/HCl, pH 7.4, containing 10% (by vol.) glycerol and 10 mM 2-mercaptoethanol; (△), 20 mM Tris/HCl, pH 7.4, containing 10% (by vol.) glycerol and 2 mM Ni^{2+}. The relative residual activity is expressed as the percentage of the initial activity of the cell-free extract.

Using the improved buffer, the enzyme was purified about 15-fold, with a 0.34% yield, from the cell-free extract (Table 1). SDS/PAGE showed that the purified enzyme was homogeneous (Fig. 2).

Table 1. Purification of the *N*-carbamoyl-L-amino acid amidohydrolase from *A. xylosoxidans*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
1) Cell-free extract	3830	317	0.0830
2) DEAE-Sephacel	710	142	0.200
3) Ammonium sulfate	123	56.1	0.456
4) Phenyl-Sepharose CL-4B	23.9	13.1	0.549
5) MonoQ HR 5/5	3.13	2.53	0.809
6) Superose 12 HR 10/30	0.893	1.09	1.22

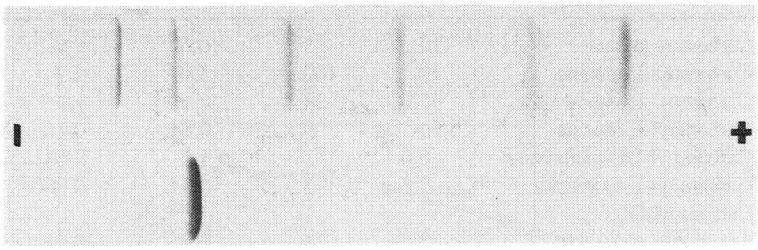


Fig. 2. SDS/PAGE of the *N*-carbamoyl-L-amino acid amidohydrolase from *A. xylosoxidans*.
A, Standards: (from left) phosphorylase *b* (M_r = 97,400), bovine serum albumin (66,267), aldolase (42,400), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). B, Purified enzyme. The gel was stained with Coomassie brilliant blue R-250 and destained in ethanol/acetic acid/water (3/1/6, by vol.).

Substrate specificity and kinetic properties

The purified enzyme hydrolyzed various *N*-carbamoylamino acids with strict L enantio-selectivity. In addition to these, *N*-formylamino acids were hydrolyzed quite quickly rates, and *N*-acetylamino acids were also hydrolyzed, though very slowly. Normal hyperbolic kinetics were observed with all the compounds, and the K_m, V_{max} and V_{max}/K_m values for several hydrolyzed substrates, calculated from double-reciprocal plots, are shown in Table 2. The K_m values for long chain aliphatic and aromatic *N*-carbamoyl-L-amino acids were lower than those of short chain aliphatic *N*-carbamoyl-L-amino acids. However, V_{max} values for short chain *N*-carbamoyl-L-amino acids such as *N*-carbamoylglycine and *N*-carbamoyl-L-alanine were higher than those of the other substrates. Considering from V_{max}/K_m values, *N*-carbamoyl-L-valine seems to be the most favored substrate for the enzyme among the compounds tested.

Table 2. Substrate specificity of the *N*-carbamoyl-L-amino acid amidohydrolase from *A. xylosoxidans*

Compound	Substrate concentration (mM)	Relative activity (%)	K_m (mM)	V_{max} (mmol/min/mg)	V_{max}/K_m
N-Carbamoylglycine	10	75	6.6	1.9	0.28
N-Carbamoyl-L-alanine	10	100	3.0	4.7	1.6
N-Carbamoyl-L-valine	5.0	28	0.40	1.4	3.4
N-Carbamoyl-L-leucine	10	9.1	0.86	0.16	0.19
N-Carbamoyl-DL-methionine	5.0	12	3.2	0.23	0.072
N-Carbamoyl-L-isoleucine	2.5	5.3	0.31	0.080	0.26
N-Carbamoyl-DL-2-aminohexanoic acid	2.5	24	0.85	0.95	1.1
N-Carbamoyl-L-phenylalanine	10	4.5	0.92	0.070	0.076
N-Carbamoyl-DL-serine	10	19	10	0.81	0.081
N-Carbamoyl-DL-threonine	5.0	8.8	-	-	-
N-Carbamoyl-L-asparagine	5.0	64	1.5	1.2	0.80
N-Formyl-DL-alanine	10	13	16	0.47	0.029
N-Formyl-DL-leucine	10	5.2	-	-	-
N-Formyl-DL-methionine	10	5.4	-	-	-
N-Acetyl-L-phenylalanine	10	0.74	-	-	-
N-Acetyl-DL-2-aminohexanoic acid	10	0.060	-	-	-

As for the relative activity, the rate of hydrolysis with *N*-carbamoyl-L-alanine was taken as 100%. The following compounds did not serve as substrates under the standard assay conditions: *N*-carbamoyl-L-glutamate, *N*-carbamoyl-L-arginine, *N*-carbamoyl-L-tyrosine, *N*-carbamoyl-L-tryptophan, *N*-carbamoyl-D-alanine, *N*-carbamoyl-D-leucine, *N*-carbamoyl-D-phenylglycine, *N*-carbamoyl-D-*p*-hydroxyphenylglycine, *N*-carbamoyl-α,α'-dimethylglycine, *N*-formyl-DL-tryptophan, *N*-formyl-D-phenylalanine, *N*-acetyl-DL-alanine, *N*-acetyl-L-glutamine, *N*-acetyl-L-glutamate, *N*-acetyl-L-cysteine, *N*-acetyl-DL-valine, *N*-acetyl-DL-tryptophan, *N*-acetyl-DL-methionine, *N*-acetyl-DL-histidine, *N*-acetylglycine, β-ureidopropionate, β-ureidoisobutyrate, γ-ureido-*n*-butyrate, DL-ureidosuccinate, *N*-carbamoylsarcosine, allantoin, allantoic acid, DL-citrulline.
-; not determined.

Effects of metal ion chelators and metal ions

Metal ion chelators such as EDTA (10 mM), 8-hydroxyquinoline (2 mM), *o*-phenanthroline (10 mM) and 2,2'-dipyridyl (2.5 mM) caused inhibition by 22, 65, 47 and 26%, respectively. The purified enzyme was dialyzed against to the buffer with chelator, i.e., 20 mM Tris/HCl, pH 7.4, containing 10 mM 2-mercaptoethanol and 2 mM 8-hydroxyquinoline, for 12 h at 4°C, and then, 8-hydroxyquinoline was removed by dialysis against to the buffer without chelator, i.e., 20 mM Tris/HCl, pH 7.4, containing 10 mM 2-mercaptoethanol, for 12 h at 4°C. After these treatment, the enzyme showed

only 38% of the initial activity, and it was activated by Mn^{2+}, Co^{2+} or Ni^{2+}, and the other divalent metal ions tested were inert. At the optimum concentration, Mn^{2+} (0.3 mM), Ni^{2+} (0.3 mM) and Co^{2+} (0.5 mM) greatly enhanced the activity to 360, 200 and 195% of the 8-hydroxyquinoline treated enzyme activity (Fig. 3).

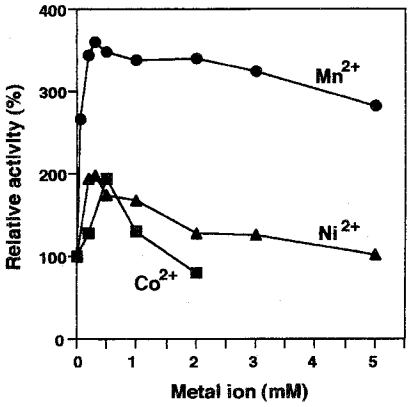


Fig. 3. Effects of Mn^{2+}, Ni^{2+} and Co^{2+} ions on the activity of the 8-hydroxyquinoline treated *N*-carbamoyl-L-amino acid amidohydrolase from *A. xylosoxidans*.
The purified enzyme was dialyzed against 20 mM Tris/HCl, pH 7.4, containing 10 mM 2-mercaptoethanol and 2 mM 8-hydroxyquinoline for 12 h at 4°C, and dialyzed against 20 mM Tris/HCl, pH 7.4, containing 10 mM 2-mercaptoethanol for 12 h at 4°C. The enzyme activity was then determined in the presence of divalent metal ions under the standard assay conditions. The relative activity is expressed as the percentage of the specific activity of 8-hydroxyquinoline treated enzyme without divalent metal ions (0.464 μmol/min/mg). Symbols: ●, Mn^{2+}; ▲, Ni^{2+}; ■, Co^{2+}.

Inhibition

The inhibitory effects of various compounds were tested upon the enzyme dialyzed against 20 mM Tris/HCl buffer containing 10% (by vol.) glycerol for 12 h. Sulfhydryl reagents (2 mM) such as iodoacetate, *p*-chloromercuribenzoate, *N*-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoate) caused inhibition by 43, 100, 100 and 100%, respectively. Heavy metal ions (2 mM) such as Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} also inhibited by 85, 85, 86 and 100%, respectively. Carbonyl reagents such as $NaAsO_2$ (5 mM) and phenylhydrazine (2 mM) inhibited the enzyme activity by 20 and 48%, respectively. Serine protease inhibitors such as diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride also inhibited the activity at high concentrations (5 mM)

by 18 and 31%, respectively. Sodium propionate (10 mM), which is an inhibitor of β-ureidopropionase [61], inhibited the reaction by 20%, and competitively inhibited the *N*-carbamoyl-L-alanine hydrolysis (K_i = 5.66 mM). The activity of the enzyme was not lost in the presence of 5 mM NaCN, NaF, NaN_3, NH_2OH, semicarbazide, $(NH_4)_2SO_4$, Na_2CO_3, *N*-carbamoyl-D-valine, *N*-carbamoyl-D-alanine or L-alanine.

Relative molecular mass and subunit structure

The relative molecular mass of the enzyme was measured to be about 134,000, by means of high-performance gel-permeation liquid chromatography on GS-520. It was also calculated to be 137,000 by high-performance gel-permeation liquid chromatography on G-3000SW. The relative molecular mass of the subunit was estimated to be 65,000 by SDS/PAGE (Fig. 2). The native enzyme probably consists of two subunits identical in molecular mass. The absorption spectrum of the enzyme showed a maximum at 275 nm, but it did not show any absorption in the visible region of 320 - 700 nm.

NH_2-terminal amino acid sequence and amino acid composition

An NH_2-terminal sequence of twenty amino acid residues was found for the enzyme by means of automated Edman degradation: Ala-His-Thr-Leu-Ala-Gln-Leu-Asn-Ala-Ala-Ala-Pro-Ala-Asp-Ala-Ala-Ala-Met-Leu-Asp-Gly-Leu-Tyr-Gly-Arg-Ala-Pro-. The NH_2-terminal amino acid sequence showed no similarity with the sequences of the enzymes from *Pseudomonas* sp. [71] and *Bacillus stearothermophilus* [72], or that of β-ureidopropionase from *P. putida* (see section 3). It was rather similar to those of *N*-carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp. (see section 1) and *Blastobacter* sp.(see section 2) (Fig. 4).

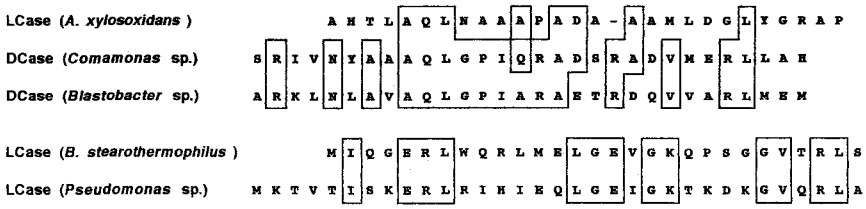


Fig. 4. Comparison of the NH_2-terminal amino acid sequences of *N*-carbamoyl-L-amino acid amidohydrolases (LCase) from *A. xylosoxidans*, *B. stearothermophilus* [72] and *Pseudomonas* sp. [71], and *N*-carbamoyl-D-amino acid amidohydrolases (DCase) form *Comamonas* sp. (see section 1) and *Blastobacter* sp. (see section 2).
Insertions (-) have been introduced to maximize the homology. Identical amino acid residues are enclosed in boxes.

The amino acid composition of the enzyme was also investigated. The number of residues found per subunit are as follows: Asp & Asn (59), Thr (25), Ser (23), Glu & Gln (67), Pro (31), Gly (53), Ala (82), Met (14), Ile (18), Val (35), Leu (65), Tyr (12), Trp (5), Phe (15), Lys (8), His (18), Arg(55) and Half-cystine (4).

Effects of pH and temperature

pH Optimum and stability were examined in 0.1 M sodium acetate, 0.1 M potassium phosphate, 0.1 M Tris/HCl and 0.1 M borate/NaOH buffer systems at pH values ranging from 4.5-6.5, 6.1-8.5, 7.7-9.8, and 9.0-10.7, respectively. Under the standard assay conditions, the pH optimum for *N*-carbamoyl-L-valine hydrolysis was 8.0-8.3. When the enzyme was incubated at 30°C for 30 min with the buffers described above, more than 80% of the initial activity was retained at pH 6.0 to pH 9.5.

The initial velocity of the hydrolysis increased with increasing temperature, reaching a maximum at 35°C. All of the initial activity remained after incubation at 30°C for 30 min at pH 7.4, but about 30% of the activity was lost after an incubation at 35°C for 30 min.

DISCUSSION

N-Carbamoyl-L-amino acid amidohydrolase activities have been found in several microorganisms. These enzymes are so unstable that only the *N*-carbamoyl-L-amino acid amidohydrolase from *Arthrobacter* spec. DSM 3747, which is specific for aromatic *N*-carbamoyl-L-amino acids [41], has been purified homogeneity. We investigated the conditions required to stabilize the enzyme activity, and purified to homogeneity, an enzyme with broad substrate specificity from *A. xylosoxidans.*

The *N*-carbamoyl-L-amino acid amidohydrolases were classified into two groups. The members of the one showed broad substrate specificities toward aliphatic and aromatic *N*-carbamoyl-L-amino acids [27,67,70], and those of the other showed narrow specificities toward aromatic *N*-carbamoyl-L-amino acids [26,25]. The enzyme from *A. xylosoxidans* belongs to the former group and has not been purified to homogeneity.

β-Ureidopropionase from *P. putida* hydrolyzed short chain *N*-carbamoyl-L-amino acids, but not long chain aliphatic and aromatic *N*-carbamoyl-L-amino acids (see section 3). Furthermore, *N*-carbamoyl-L-amino acid amidohydrolase from *A. xylosoxidans* did not hydrolyze β-ureidopropionate; suggesting that the enzyme is distinct from β-ureidopropionase in function. However, *N*-carbamoyl-L-amino acid amidohydrolase from *A. xylosoxidans* resembles β-ureidopropionase in that either is homodimer enzyme an in that either needs divalent metal ion for activity.

The *A. xylosoxidans* enzyme is also different from other *N*-carbamoylamino acid amidohydrolases such as ureidosuccinase [45], *N*-carbamoylsarcosine amidohydrolase [46] and *N*-carbamoyl-D-amino acid amidohydrolase (see section 1 and 2), in that it cannot hydrolyze DL-ureidosuccinate, *N*-carbamoylsarcosine and *N*-carbamoyl-D-amino acids. These results suggest that the enzyme from *A. xylosoxidans* is a novel amidohydrolase acting on *N*-carbamoyl-L-amino acids with broad substrate specificity.

SUMMARY

N-Carbamoyl-L-amino acid amidohydrolase was purified to homogeneity for the first time from *Alcaligenes xylosoxidans* A35. The enzyme showed high affinity toward *N*-carbamoyl-L-amino acids with long chain aliphatic or aromatic substituents, and hydrolyzed those with short chain substituents quite well. The enzyme hydrolyzed *N*-formyl and *N*-acetylamino acids quickly and very slowly, respectively. The enzyme did not hydrolyze β-ureidopropionate and ureidosuccinate. The relative molecular mass of the native enzyme was about 135,000 and the enzyme consisted of two identical polypeptide chains. The enzyme activity was significantly inhibited by sulfhydryl reagents and required the following divalent metal ions for activity: Mn^{2+}, Ni^{2+} and Co^{2+}.

CHAPTER III

Analysis of Microbial Cyclic Ureide-Hydrolyzing Enzymes

Section 1. Diversity of cyclic ureide-hydrolyzing enzymes in *Blastobacter* sp. A17p-4*f*

The microbial transformation of cyclic ureide compounds became more interesting along with the growing interest of industry in producing optically active amino acids [73]. The transformation of hydantions, five-membered cyclic ureides, comprises two reactions: ring opening hydrolysis of cyclic ureides and subsequent hydrolysis of the resultant *N*-carbamoylamino acids to amino acids. In chapter II, I analyzed *N*-carbamoylamino acid-hydrolyzing enzymes, and revealed that there are many kinds of enzymes with different stereospecificities and regiospecificities. These results suggest that diverse enzymes may also be involved in catalysis of the upstream reaction, cyclic ureide hydrolysis. In Chapter I, I analyzed the cyclic ureide compound-, dihydropyrimidine- and hydantoin-hydrolyzing activities in various microorganisms, and found that more than two enzymes are involved in cyclic ureide hydrolysis in *Blastobacter* sp. A17p-4 . In this section, I describes the occurrence of two cyclic ureide-hydrolyzing enzymes with different substrate specificities in *Blastobacter* sp.

MATERIALS AND METHODS

Microorganism and cultivation. *Blastobacter* sp. was grown aerobically in a 2-l flask containing 300 ml of medium described in section 2, chapter II, at 28°C for 5 days.

Preparation of cell-free extract. *Blastobacter* sp. cells (7 g wet weight from 3 l culture) were harvested by centrifugation (10,000 x *g* at 4°C) and suspended in

30 ml of buffer (20 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM dithiothreitol), and then disrupted with 0.25-0.50 diameter glass beads (Dyno-Mill KDL, Switzerland) at 5°C for 30 min. The disrupted cell suspension was centrifuged at 14,000 x *g* for 60 min at 4°C, and the resultant supernatant was used as the cell-free extract.

Enzyme assay. Cyclic ureide-hydrolyzing activity was analyzed with dihydrouracil, a typical substrate for dihydropyrimidinase, as the substrate. The reaction mixture comprised 10 μmol Tris/HCl (pH 7.5), 2 μmol dihydrouracil and an appropriate amount of enzyme, in a total volume of 100 μl. The reactions were carried out at 30°C for 30 min and stopped with 10 μl of 15% (by vol.) perchloric acid. After adding 90 μl of 500 mM potassium phosphate buffer (pH 7.0), the mixtures were centrifuged at 10,000 x *g* for 10 min, and then the supernatants were analyzed as to the decrease in dihydrouracil and the increase in β-ureidopropionate, a product, by Shimadzu LC-6A high-performance liquid-chromatography (HPLC) at 210 nm, on a Cosmosil $5C_{18}$-packed column (4.6 x 250 mm; Nacalai Tesque) at the flow rate of 1.0 $ml \cdot min^{-1}$, with 250 mM KH_2PO_4 as the eluent. One unit of enzyme is defined as the amount of enzyme that catalyzes the consumption of the substrate or the formation of the product at the rate of 1 $\mu mol \cdot min^{-1}$ under the above assay conditions. Protein was measured by the method of Bradford [38].

Enzyme purification. The cyclic ureide-hydrolyzing enzymes were partially purified. The cell-free extract (total protein, 1510 mg; total activity, 90.0 units) was dialyzed against buffer and then applied to a DEAE-Sephacel column (2.5 x 10 cm) eqilibrated with the buffer. After washing the column with the buffer, the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in 500 ml buffer. The fractions containing enzyme activity were combined (total protein, 271 mg; total activity, 79.4 units). After adjusting the NaCl concentration to 4 M with solid NaCl, the enzyme solution was applied to a phenyl-Sepharose CL-4B column (1.5 x 10 cm) equilibrated with the buffer containing 4 M NaCl. After washing the column with the same buffer,

the enzyme was eluted with a linear gradient of 4 to 0 M NaCl in 250 ml buffer, and then washed with buffer without NaCl. The fractions containing activity were combined and used for characterization.

RESULTS

Partial purification of cyclic ureide-hydrolyzing enzymes

On the phenyl-Sepharose CL-4B column chromatography, two dihydrouracil-hydrolyzing fractions were obtained as shown in Fig. 1 (fraction I: total protein, 47.2 mg; total activity, 42.8 units, fraction II: total protein, 64.0 mg; total activity, 25.6 units).

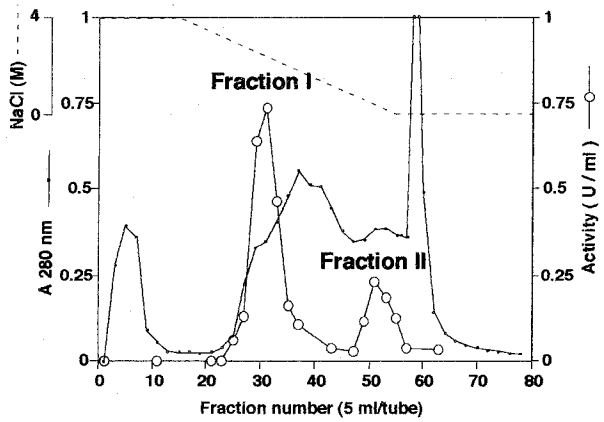


Fig. 1. Separation of the two cyclic ureide-hydrolyzing enzymes on phenyl-Sepharose CL-4B column chromatography.

Substrate specificity of cyclic ureide-hydrolyzing enzymes

Fraction I hydrolyzed cyclic imide compounds such as glutarimide and succinimide, other than cyclic ureide compounds such as dihydrouracil and hydantoin (Table 1). The reaction product derived from succinimide was identified as succinamic acid and not succinate by HPLC analysis (*Rt* values for succinate, succinamic acid and the reaction product: 4.1, 3.5 and 3.5 min, respectively). Thus, fraction I catalyzed the hydrolysis of cyclic imides to monoamidated dicarboxyrates stoichiometrically.

Fraction II hydrolyzed dihydropyrimidines and 5-substituted hydantoins to the corresponding *N*-carbamoylamino acids, but not cyclic imides (Table 1). The hydrolysis of 5-substituted hydantoins was D-stereospecific, because the products, *N*-carbamoyl amino acids, were hydrolyzed by *N*-carbamoyl-D-amino acid amidohydrolase (see section 1 and 2, chapter II) but not by *N*-carbamoyl-L-amino acid amidohydrolase (see section 4, chapter II).

Table 1. Substrate specificities of the two cyclic ureide-hydrolyzing enzymes (fractions I and II)

Substrate	Concentration (mM)	Relative activity (%) Fraction I	Fraction II
Dihydrouracil	10	100	100
Dihydrothymine	10	-	72
Hydantoin	10	110	140
DL-5-Methylhydantoin	10	-	53
DL-5-Methylthioethylhydantoin[a]	2.5	-	12
DL-5-Phenylhydantoin[a]	2.5	-	20
DL-5-(*p*-Hydroxyphenyl)hydantoin[a]	5	-	4.0
Glutarimide	10	630	-
Succinimide	10	530	-

Assays were carried out as described in the text except that the test compounds were used as substrates at the indicated concentrations. The following compounds were not hydrolyzed by either fraction: *N*-methylhydantoin, creatinine, DL-pyroglutamate, DL-pyroglutamate methyl ester, DL-5-carboxyethylhydantoin, L-5-caboxymethylhydantoin, L-5-isopropylhydantoin and dihydro-L-orotate.
-; not hydrolyzed.
[a]Reaction products were measured by HPLC as described in chapter I.

Effects of pH on the activity and stability of cyclic ureide-hydrolyzing enzymes

The pH optima of fraction I for dihydrouracil hydrolysis and β-ureidopropionate cyclization were pH 7.5 and 6.5, respectively, and those of fraction II were above pH 10.0 and 5.0, respectively. The activity profiles as to pH were remarkably different from each other, while stability plofiles as to pH showed no apparent differences (Fig. 2). This may be due to a difference in the reaction mechanisms in the fractions. However, further analysis is necessary to confirm this.

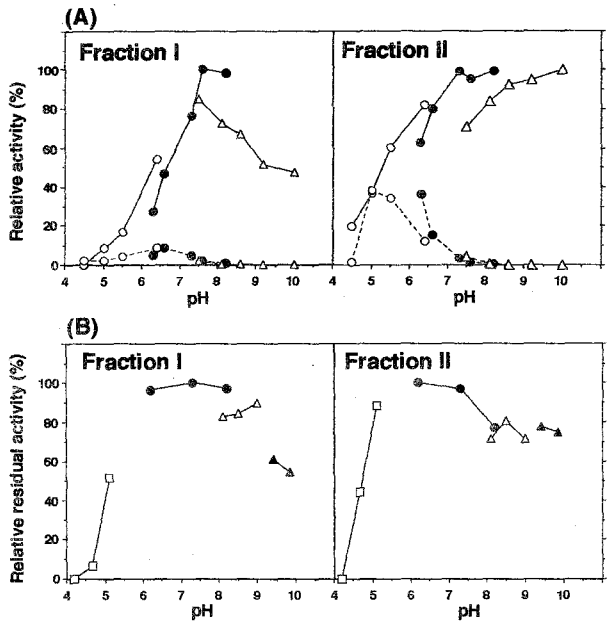


Fig. 2. Effects of pH on the activity (A) and stability (B) of the two cyclic ureide-hydrolyzing enzymes.
(A) The enzyme activity was assayed with dihydrouracil (solid lines) or β-ureidopropionate (broken lines) as the substrate under the conditions described in the text except that the following 100 mM buffers were used: MES/NaOH, pH 4.5-6.4 (○); MOPS/NaOH, pH 6.2-8.2 (●); and Tris/HCl, pH 7.5-10.0 (△). The relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions. (B) Enzyme solutions were incubated at 30°C for 30 min in 30 mM buffers: sodium acetate/acetate, pH 4.2-5.1 (□); MOPS/NaOH, pH 6.2-8.2 (●); Tris/HCl, pH 8.1-9.0 (△); and $NaHCO_3/Na_2CO_3$, pH 9.4-10.0 (▲). The remaining activity was assayed with dihydrouracil as the substrate under the conditions described in the text, and is expressed as a percentage of the maximum remaining activity attained under the experimental conditions.

Enzyme formation

Differences were also found in the effects of various compounds on appearance of each activity (Table 2). The specific activity of fraction II was increased by the addition of dihydrothymine and thymine to the culture medium, and decreased by the addition of DL-5-methylhydantoin, succinimide and glutarimide, while the specific activity of fraction I was not significantly influenced by these compounds. These results suggest that these two activities are under different expressional regulation.

Table 2. Effects of various compounds on the appearance of each cyclic ureide-hydrolyzing activity

Compound	Specific activity ($x10^{-4}$ units/mg wet cells) Fraction I	Fraction II
None	5.1	1.5
Dihydrouracil	5.8	2.1
Dihydrothymine	6.1	8.0
Hydantoin	5.4	2.7
DL-5-Methylhydantoin	5.6	0.29
DL-5-Methylthioethylhydantoin	5.9	1.1
DL-5-Phenylhydantoin	4.0	1.9
Uracil	5.1	3.4
Thymine	7.4	5.7
Succinimide	5.2	0.25
Glutarimide	5.7	0.12

Cultivations were carried out as described in the text except that the test compounds (1.5 g/l) were used instead of uracil. The activities of fractions I and II were measured with succinimide or DL-5-methylhydantoin as the substrate, respectively. The reaction mixture comprised 100 μmol Tris/HCl (pH 7.5), 20 μmol substrate and cells from 2.5 ml culture broth. The reaction and assay methods were the same as those for the enzyme reaction described in the text.

DISCUSSION

All the results described here constitute good evidence for the occurrence of two distinct cyclic ureide-hydrolyzing enzymes in *Blastobacter* sp. One is a novel enzyme, which should be named an imidase. The only enzyme which has previously been reported to hydrolyze cyclic imides, is the dihydropyrimidinase from rat liver [74]. However, the present imidase is distinct from rat liver dihydropyrimidinase in that the imidase does not hydrolyze compounds with some substituents on their ring structures such as dihydrothymine and 5-substituted hydantoins, and in that the imidase hydrolyzes cyclic imides more preferably than cyclic ureides. The other one hydrolyzes 5-substituted hydantoins D-stereospecifically and shows dihydropyrimidinase activity. This kind of enzyme, i.e., D-specific hydantoinase, has been reported in *Pseudomonas* [18], *Bacillus* [21], and *Agrobacterium* [75]. However, activity toward cyclic imide compounds was first investigated in this report and revealed to be inert. Rat liver dihydropyrimidinase also hydrolyzes 5-substituted hydantoins D-stereospecifically [76],

but is different from fraction II in that it hydrolyzes cyclic imides well. These results indicate the possibility that the functions of eukaryotic dihydropyrimidinases are due to two distinct enzymes in prokaryotes. More detailed analysis will be of interest as to the evolution and reaction mechanisms of these enzymes.

SUMMARY

Two cyclic ureide compound-hydrolyzing enzymes were found in *Blastobacter* sp. A17p-4, and partially purified. One hydrolyzed 5-substituted hydantoins D-stereospecifically and showed dihydropyrimidinase activity (Fig. 3). The other was a novel enzyme which should be called an imidase. The imidase preferably hydrolyzed cyclic imide compounds such as glutarimide and succinimide more than cyclic ureide compounds, and produced monoamidated dicarboxyrates (Fig. 3).

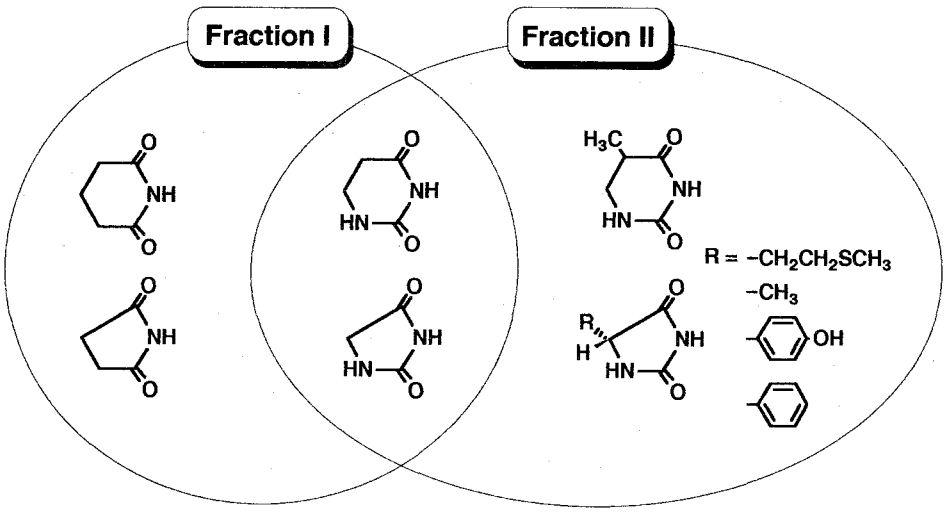
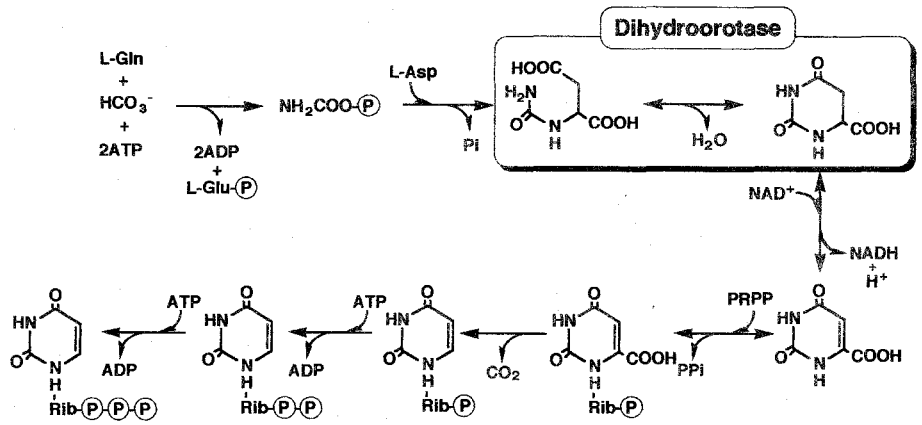


Fig. 3. Substrate specificity of the two cyclic ureide-hydrolyzing enzymes (fraction I and II)

Section 2. Dihydroorotase from *Pseudomonas putida* IFO 12996: purification and characterization[g]

Dihydroorotase (EC 3.5.2.3) catalyzes the reversible cyclization of L-ureidosuccinate to dihydro-L-orotate, the third step in pyrimidine biosynthesis (Scheme 1). Dihydroorotase plays the central role in pyrimidine biosynthesis in nucleotide and nucleic acid metabolism in all organisms, however, there is a paucity of information regarding prokaryotic dihydroorotases. Prokaryotic dihydroorotases have only been purified only from *Escherichia coli* [77,78] and *Clostridium oroticum* [79], and have been revealed to be simple monofunctional proteins, while eukaryotic dihydroorotases are parts of complex multifunctional proteins [80].



Scheme 1. Pathway of pyrimidine biosynthesis.

I described the enzymes involved in the pyrimidine catabolism in a potent bacterium, *Pseudomonas putida* IFO 12996 [17], especially dihydropyrimidinase (see chapter I) and β-ureidopropionase (see chapter I and section 3, chapter II). Dihydropyrimidinase catalyzes a similar reaction to dihydroorotase, i.e., reversible cyclization of *N*-carbamoyl-β-amino acids to dihydropyrimidines. Given the information provided by comparative studies on the function, structure and evolution of these enzymes, it is of interest and important to analyze the dihydroorotase from *P. putida*. It

is also of interest to characterize a dihydroorotase from a bacterium with high pyrimidine-transforming activity, in respect of the regulation of pyrimidine biosynthesis and catabolism.

In this section, I describes the purification and characterization of the dihydroorotase from *P. putida* and discuss the possible role of the enzyme in regulation of pyrimidine biosynthesis.

MATERIALS AND METHODS

Chemicals. 5-Substituted hydantoins were kind gifts from Kanegafuchi Chemical Industries Co. (Japan). Dihydro-L-orotate methyl ester and DL-pyroglutamate methyl ester were synthesized through condensation of acetyl chloride with the respective acids. All other chemicals used in this work were of analytical grade and commercially available.

Microorganism and cultivation. *P. putida* was used as the enzyme source. The medium described in section 3, chapter II was used. The organism was pre-cultured aerobically in a test tube (16 x 165 mm) containing 5 ml of the medium at 28°C for 24 h. Each subculture was transferred to a 2-l shaking flask containing 500 ml of the medium, followed by incubation for 24 h at 28°C with shaking.

Enzyme assay. Enzyme activity was determined from changes in the absorbance at 230 nm (ε = 1170 $M^{-1} \cdot cm^{-1}$ [77]) due to the dihydropyrimidine ring system. For routine rate measurements of dihydro-L-orotate synthesis, the reaction mixture comprised 50 μmol of sodium acetate/acetate buffer (pH 6.0), 0.5 μmol of DL-ureidosuccinate and the enzyme, in a total volume of 0.5 ml. For dihydro-L-orotate hydrolysis, the reaction mixture comprised 50 μmol of Tris/HCl buffer (pH 7.5), 0.5 μmol of dihydro-L-orotate and the enzyme, in a total volume of 0.5 ml. The reactions were carried out at 30°C and initiated by the addition of the substrate.

Enzyme activity was also determined by high-performance liquid chromatography (HPLC) essentially as described above, except that the substrate concentrations were changed to 5 μmol for DL-ureidosuccinate and 5 μmol for dihydro-L-orotate, in a total volume of 0.5 ml. The reactions were carried out at 30°C for 30-60 min and stopped by the addition of 0.5 ml ethanol. The supernatant obtained on centrifugation was analyzed by HPLC as described in chapter I.

One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the production of 1 μmol of dihydro-L-orotate per min under the conditions given for dihydro-L-orotate synthesis.

Analytical methods for dihydroorotase. The relative molecular mass of the enzyme was determined by high-performance gel-permeation liquid chromatography on a GS-520 column (7.6 x 500 mm; Asahi Kasei, Japan) with an elution buffer consisting of 20 mM Tris/HCl buffer (pH 7.4), 0.1 mM dithiothreitol and 0.2 M NaCl, at the flow rate of 0.3 $ml \cdot min^{-1}$. Protein concentration determination, SDS-polyacrylamide gel electrophoresis (SDS/PAGE) on a 12.5% polyacrylamide gel and NH_2-terminal amino acid sequence analysis were performed as described previously [53,54].

Enzyme purification. All procedures were performed between 0-5°C. The buffer was 20 mM Tris/HCl (pH 7.4) containing 0.1 mM dithiothreitol.

Step 1: *P. putida* cells (160 g wet cells obtained from 20 l culture broth) suspended in 160 ml buffer were disrupted with 0.25 - 0.50 mm diameter glass beads (Dyno-Mill KDL, Switzerland) at 5°C for 10 min. The disrupted cell suspension was passed through a glass filter and then centrifuged at 14,000 x *g* for 60 min. The supernatant (500 ml) was used as the cell-free extract.

Step 2: The cell-free extract was fractionated with solid ammonium sulfate. The precipitate obtained at 20-80% saturation was collected by centrifugation and dissolved in

buffer. The resultant enzyme solution (210 ml) was dialyzed against 10 l buffer for 12 h.

Step 3: The dialyzed solution was applied to a DEAE-Sephacel column (3.6 x 30 cm) equilibrated with buffer. After washing the column with the same buffer, the enzyme was eluted with a linear gradient of 0 to 1 M NaCl in 2 l buffer. The fractions containing enzyme activity were combined (190 ml).

Step 4: After adjusting the NaCl concentration to 4 M with solid NaCl, the enzyme was applied to a phenyl-Sepharose CL-4B column (3.2 x 22 cm) equilibrated with buffer containing 4 M NaCl. After washing the column with the same buffer, the enzyme was eluted with a linear gradient of NaCl and then ethylene glycol (4 to 0 M and 0 to 10% (by vol.), respectively) in 500 ml buffer. The active fractions were combined (122 ml) and dialyzed against 5 l buffer for 12 h.

Step 5: The dialyzed solution was applied to a DEAE-Sephacel column (1.6 x 15 cm) equilibrated with buffer. After washing the column with the buffer, the enzyme was eluted with a linear gradient of 0 to 0.3 M NaCl in 400 ml buffer. The fractions containing enzyme activity were combined (50 ml).

Step 6: After adjusting the NaCl concentration to 4 M with solid NaCl, the enzyme was applied to a butyl-Toyopeal 650M (Tosoh, Japan) column (1.6 x 10 cm) equilibrated with buffer containing 4 M NaCl. The enzyme was eluted with the same buffer and the active fractions were combined (100 ml). The enzyme solution was dialyzed against 5 l buffer for 12 h and then concentrated to 5 ml by ultrafiltration through a YM-10 membrane (Amicon Co., USA).

Step 7: The concentrated enzyme solution was applied to a Yellow H-E3G (Wako Pure Chemical Industries, Japan) column (1.0 x 3.5 cm) equilibrated with buffer. After washing the column with the same buffer, the enzyme was eluted by increasing the NaCl concentration from 0 to 0.5 M in the buffer (20 ml) in 0.05 M steps . The active fractions were combined (8 ml) and concentrated to 0.2 ml by ultrafiltration with a Centricon-10 concentrator (Amicon Co.).

Step 8: The concentrated enzyme solution was applied to a Superose 12 HR10/30 column equilibrated with buffer containing 0.2 M NaCl and eluted with same buffer. The active fractions were collected (5 ml) and used for the characterization.

RESULTS

Purification of dihydroorotase from *P. putida*

Dihydroorotase was purified approximately 950-fold, with a 5.4% yield, from the cell-free extract (Table 1). Yellow H-E3G column chromatography, a kind of dye-protein affinity chromatography, was effective for the purification. The purified enzyme was found to be homogeneous on SDS/PAGE (Fig. 1), and was eluted as a single peak on high-performance gel-permeation liquid chromatography on a GS-520 column.

Table 1. Purification of dihydroorotase from *P. puitda*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
1) Cell-free extract	19400	371	0.0191
2) Ammonium sulfate	17000	357	0.0210
3) DEAE-Sephacel	10500	276	0.0263
4) Phenyl-Sepharose CL-4B	1380	127	0.0920
5) DEAE-Sephacel	380	84.5	0.222
6) Butyl-Toyopearl 650M	126	43.8	0.348
7) Yellow H-E3G	3.06	29.4	9.60
8) Superose 12 HR 10/30	1.10	20.0	18.2

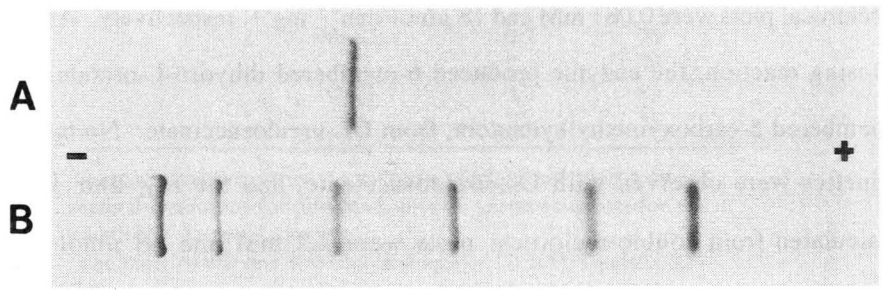


Fig. 1. SDS-PAGE of dihydroorotase from *P. putida*..
A, Purified dihydroorotase. B, Standards: (from left) phosphorylase *b* (97,400), bovine serum albumin (66,267), aldolase (42,400), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). The gel was stained with Coomassie brilliant blue R-250 and destained in ethanol/acetic acid/water (3/1/6, by vol.).

Relative molecular mass and subunit structure

The relative molecular mass of the enzyme was determined to be about 82,000 by means of high-performance gel-permeation liquid chromatography. The relative molecular mass of the subunit was estimated to be 41,000 by SDS/PAGE (Fig. 1). The native enzyme probably consists of two subunits identical in molecular mass. NH_2-terminal amino acid sequence analysis by automated Edman degradation revealed only 0.5 mol% of the expected free NH_2-terminus in the purified enzyme. We thus concluded that the purified dihydroorotase has a blocked NH_2-terminus.

Substrate specificity and kinetic properties

Among many cyclic amide compounds tested, only dihydro-L-orotate and dihydro-L-orotate methyl ester were hydrolyzed by the purified dihydroorotase. The rate of dihydro-L-orotate methyl ester-hydrolysis was approximately 70% of that of dihydro-L-orotate-hydrolysis. The following compounds were judged to be inactive as substrates for the hydrolytic reaction of the enzyme: dihydrouracil, dihydrothymine, allantoin, hydantoin, L-5-carboxymethylhydantoin, DL-5-carboxyethylhydantoin, DL-5-methylhydantoin, L-5-isopropylhydantoin, DL-5-methylthioethylhydantoin, DL-5-phenylhydantoin, DL-5-(*p*-hydroxyphenyl)hydantoin, L-5-benzylhydantoin, *N*-methylhydantoin, creatinine, barbiturate, succinimide, glutarimide, DL-pyroglutamate, DL-pyroglutamate methyl ester and pyrrolidone. Normal hyperbolic kinetics were observed with dihydro-L-orotate, and the K_m and V_{max} values calculated from double-reciprocal plots were 0.081 mM and 18 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. As for the ring closing reaction, the enzyme produced 6-membered dihydro-L-orotate, but not 5-membered 5-carboxymethylhydantoin, from DL-ureidosuccinate. Normal hyperbolic kinetics were observed with DL-ureidosuccinate, and the K_m and V_{max} values calculated from double-reciprocal plots were 2.2 mM and 68 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively.

Effects of metal ions and metal ion chelators

The effects of metal ions on the purified enzyme were investigated. Among various metal ions tested, only $ZnCl_2$ at low concentration (0.4 mM) enhanced the dihydro-L-orotate-synthesizing activity, i.e., to 113% of the initial level. However, $ZnCl_2$ at high concentration (4 mM) inhibited the enzyme activity by 33%. The enzyme activity was completely inhibited by $FeCl_3$ (2 mM), Ag_2SO_4 (2 mM), $HgCl_2$ (0.2 mM), $PbCl_2$ (2 mM), and $SnCl_2$ (2 mM). $CuSO_4$ (0.4 mM) and $FeSO_4$ (0.4 mM) also caused considerable inhibition, by 73 and 92%, respectively.

Metal ion chelators such as EDTA, 8-hydroxyquinoline, *o*-phenanthroline and 2,2'-dipyridyl caused inhibition (Table 3). The purified enzyme was dialyzed against 20 mM Tris/HCl (pH 7.4) containing 0.1 mM dithiothreitol and 2 mM EDTA for 20 h at 4°C, and then EDTA was removed by dialysis against 20mM Tris/HCl (pH 7.4) containing 0.1 mM dithiothreitol for 20 h at 4°C. After these treatments, the enzyme showed only 32% of the initial activity, and it was re-activated by the addition of $ZnCl_2$, $CoCl_2$, $NiCl_2$, $MnCl_2$ or $CaCl_2$ (Table 2).

Table 2. Effects of EDTA and metal ions

Sample	Metal ion	Concentration (mM)	Relative activity (%)
Native enzyme	-	-	100
EDTA-treated enzyme[a]	-	-	32
	$ZnCl_2$	0.2	110
		2	95
	$CoCl_2$	0.2	75
		2	87
	$NiCl_2$	0.2	65
		2	70
	$MnCl_2$	0.2	75
		2	78
	$CaCl_2$	0.2	83
		2	78

The reactions were carried out under the standard spectrophotometric method conditions for dihydro-L-orotate synthesis except for the addition of the metal ions.

[a]The purified enzyme was dialyzed against 20 mM Tris/HCl (pH 7.4) containing 0.1 mM dithiothreitol and 2 mM EDTA for 20 h at 4°C, and then EDTA was removed by dialysis against 20 mM Tris/HCl (pH 7.4) containing 0.1 mM dithiothreitol for 20 h at 4°C.

$ZnCl_2$ at low concentration (0.2 mM) most effectively restored the activity of the EDTA-treated enzyme, i.e., to 110% of the initial level. However, $ZnCl_2$ at high concentration (2 mM) was less effective (Table 2).

Inhibition

Various inhibitors were examined as to their inhibitory effects on the dihydro-L-orotate synthesis activity (Table 3). Sulfhydryl reagents such as iodoacetate, *p*-chloromercuribenzoate and 5,5'-dithiobis-(2-nitrobenzoate) caused considerable inhibition, by 100, 66 and 100%, respectively. Carbonyl reagents such as phenylhydrazine and semicarbazide inhibited the reaction by 76 and 43%, respectively. A serine protease inhibitor, diisopropyl fluorophosphate, also inhibited it by 56%.

Table 3. Effects of inhibitors, substrate-like compounds and pyrimidine-metabolism intermediates

Compound	Concentration (mM)	Relative activity (%)
None	-	100
EDTA[a]	2	70
8-Hydroxyquinoline[b]	2	86
o-Phenanthroline[b]	2	66
2,2'-Dipyridyl[b]	2	85
Iodoacetate[b]	2	0
p-Chloromercuribenzoate[b]	2	34
N-Ethylmaleimide[b]	2	84
5,5'-Dithiobis-(2-nitrobenzoate)[b]	2	0
$NH_2OH \cdot HCl$[a]	2	85
Phenylhydrazine[b]	2	24
Semicarbazide[a]	2	57
Diisopropyl fluorophosphate[a]	2	44
N-Carbamoylglycine[a]	1	77
N-Carbamoyl-L-glutamate[a]	1	90
N-Carbamoyl-L-serine[a]	1	92
N-Carbamoyl-L-asparagine[a]	1	72
N-Carbamoylsarcosine[a]	1	82
DL-5-Methylhydantoin[a]	1	87
Dihydrouracil[a]	1	84
Orotate[a]	0.2	80
	0.4	63

The reactions were carried out under the standard spectrophotometric method (a) or HPLC method (b) conditions for dihydro-L-orotate synthesis except for the addition of the test compounds.

In the presence of various substrate-like compounds and pyrimidine metabolism intermediates, the dihydro-L-orotate synthesis activity was estimated (Table 3). Among the *N*-carbamoylamino acids tested, *N*-carbamoylglycine, *N*-carbamoyl-L-glutamate, *N*-carbamoyl-L-serine, *N*-carbamoyl-L-asparagine and *N*-carbamoylsarcosine affected the reaction. *N*-Carbamoylglycine inhibited the dihydro-L-orotate synthesis competitively, with an inhibition constant (K_i) of 2.7 mM (Fig. 2). Among the cyclic amide compounds tested, DL-5-methylhydantoin and pyrimidine intermediates such as dihydrouracil and orotate affected the reaction. Dihydrouracil and orotate inhibited the dihydro-L-orotate synthesis noncompetitively with inhibition constants (K_i) of 3.4 and 0.75 mM, respectively (Fig. 2). The following compounds, at 1 mM, did not affect the reaction: *N*-carbamoyl-L-alanine, *N*-carbamoyl-DL-threonine, β-ureidopropionate, β-ureidoisobutyrate, γ-ureido-*n*-butyrate, allantoic acid, allantoin, dihydrothymine, hydantoin, L-5-carboxymethylhydantoin and DL-5-carboxyethylhydantoin.

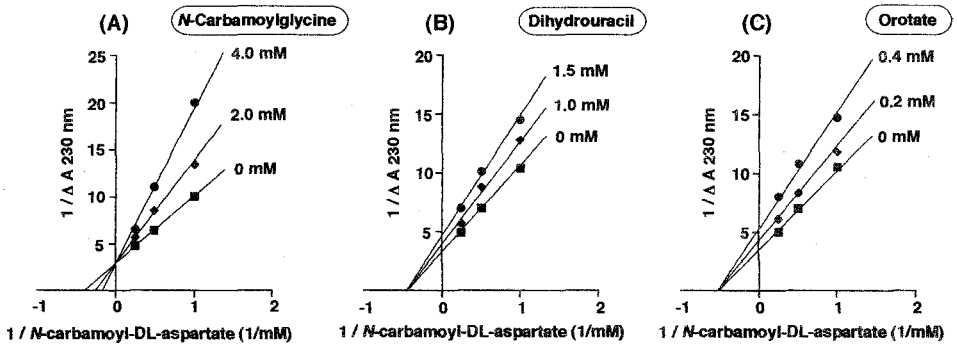


Fig. 2. Inhibition of dihydroorotase by *N*-carbamoylglycine (A), dihydrouracil (B), and orotate (C). The reactions were carried out under the standard spectrophotometric assay conditions for dihydro-L-orotate synthesis except for the addition of the test compounds.

Effects of pH and temperature

The enzyme activity and stability were assayed in 50 mM sodium acetate/acetate, 50 mM potassium phosphate, 50 mM Tris/HCl and 50 mM $NaCO_3$/NaOH buffer systems at pH 3.0-5.5, 6.0-7.4, 7.4-9.0, and 9.5-10.5, respectively. Under the standard assay conditions, the pH optima for dihydro-L-orotate synthesis and dihydro-L-

orotate hydrolysis were pH 4.5-5.0 and pH 8.5-9.0, respectively (Fig. 3). When the enzyme was incubated at 30°C for 30 min with the buffers mentioned above, more than 80% of the initial activity was retained at pH 5.5 to 11.0.

The initial velocity of the dihydro-L-orotate synthesis increased with increasing temperature, reaching a maximum at 60°C. 85% of the initial activity remained after incubation at 55°C for 30 min at pH 7.4, but approximately 65% activity was lost on incubation at 60°C.

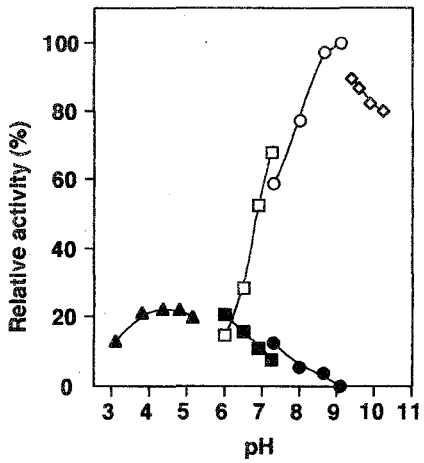


Fig. 3. Effects of pH on dihydro-L-orotate synthesis (closed symbols) and dihydro-L-orotate hydrolysis (open symbols).
The buffers (50 mM) used for the assay were sodium acetate/acetate (pH 3.0-5.5), potassium phosphate (pH 6.0-7.4), Tris/HCl (pH 7.4-9.0), and NaCO3/NaOH (pH 9.5-10.5). The reactions were carried out under the spectrophotometric assay conditions.

DISCUSSION

Two cyclic ureide compound-hydrolyzing enzymes, dihydropyrimidinase [18] and dihydroorotase (in this section) were purified from *P. putida*, a potent pyrimidine-transforming bacterium. As for structure, the relative molecular masses of the subunits of these enzymes are similar (41,000 for dihydroorotase and 49,000 for dihydropyrimidinase). However, the relative molecular masses of the native enzymes are quite different (82,000 for dihydroorotase and 190,000 for dihydropyrimidinase). Dihydroorotase consists of two identical subunits, while dihydropyrimidinase consists of four identical subunits. As for function, dihydroorotase is specific to 6-membered

dihydropyrimidines, while dihydropyrimidinase shows broad specificity to not only 6-membered dihydropyrimidines but also 5-membered hydantoin derivatives. Dihydroorotase only hydrolyzes dihydro-L-orotate and its methyl ester. This is the first report that dihydroorotase hydrolyzes dihydro-L-orotate methyl ester. A free carbonyl group seems not to be necessary for the substrate of the dihydroorotase. All above results suggest that dihydroorotase and dihydropyrimidinase are distinct from each other. However, since the reactions catalyzed by the two enzymes are similar, there is a possibility that the active sites of these enzymes are derived from the same ancestor protein [34].

There are some similarities between dihydroorotase from *P. putida*, and those from *C. oroticum* [79] and *E. coli* [78,81]. All these dihydroorotases consist of two identical subunits of molecular masses of 40,000-55,000, and exhibit higher affinity for dihydro-L-orotate than for DL-ureidosuccinate. Dihydroorotase from *P. putida* is activated by Zn^{2+} and inhibited by metal ion chelators, like the enzyme from *C. oroticum* [79], while the enzyme from *E. coli* [81] is not affected by Zn^{2+} or metal ion chelators. However, Zn^{2+} at high concentration is rather inhibitory for the enzyme from *P. putida*. Since the dihydroorotases from *C. oroticum* [82,83] and *E. coli* [84,85] were reported to contain zinc, the enzyme from *P. putida* may also contain zinc. Further analysis is needed to prove this. The dihydroorotase from *P. putida* is inhibited by sulfhydryl reagents and competitively inhibited by *N*-carbamoylamino acids, like the enzyme from *E. coli* [81]. However, the dihydroorotase from *P. putida* is distinct from the enzyme from *E. coli* in that the enzyme from *P. putida* is noncompetitively inhibited by pyrimidine metabolism intermediates such as orotate and dihydrouracil. The inhibition by orotate suggests the possibility that the dihydroorotase from *P. putida* is regulated by the products of pyrimidine biosynthesis, orotate, and plays some role in the control of pyrimidine biosynthesis. The inhibition by dihydrouracil also suggests the possibility that there are some interactions between pyrimidine biosynthesis and degradation. It is not clear whether these phenomena are common or restricted to bacteria with high pyrimidine-transforming activity such as *P. putida*. However, these results may be clues

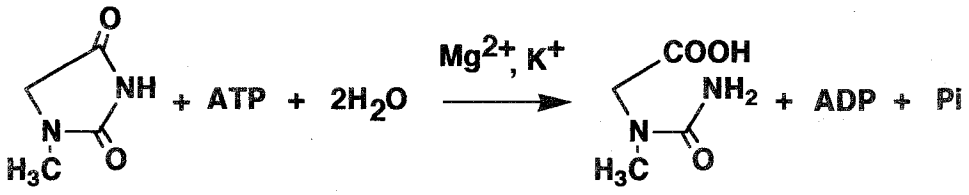
for proving the possible role of the dihydroorotase in the regulation of pyrimidine biosynthesis.

SUMMARY

Dihydroorotase was purified to homogeneity from *Pseudomonas putida* IFO 12996, which exhibits high pyrimidine-transforming activity. The relative molecular mass of the native enzyme was 82,000 and the enzyme consisted of two identical subunits with a relative molecular mass of 41,000. The enzyme only hydrolyzed dihydro-L-orotate and its methyl ester, and the reactions were reversible. The K_m and V_{max} values for dihydro-L-orotate hydrolysis (at pH 7.4) were 0.081 mM and 18 μmol·min^{-1}·mg^{-1}, respectively; and those for DL-ureidosuccinate (at pH 6.0) were 2.2 mM and 68 μmol·min^{-1}·mg^{-1}, respectively. The enzyme was inhibited by metal ion chelators and activated by Zn^{2+}. However, excessive Zn^{2+} was rather inhibitory. The enzyme was inhibited by sulfhydryl reagents, and competitively inhibited by *N*-carbamoylamino acids such as *N*-carbamoylglycine, with a K_i value of 2.7 mM. The enzyme was also inhibited noncompetitively by pyrimidine-metabolism intermediates such as dihydrouracil and orotate, with K_i value of 3.4 and 0.75 mM, respectively; suggesting that the enzyme activity is regulated by pyrimidine-metabolism intermediates and that dihydroorotase plays some role in the control of pyrimidine biosynthesis.

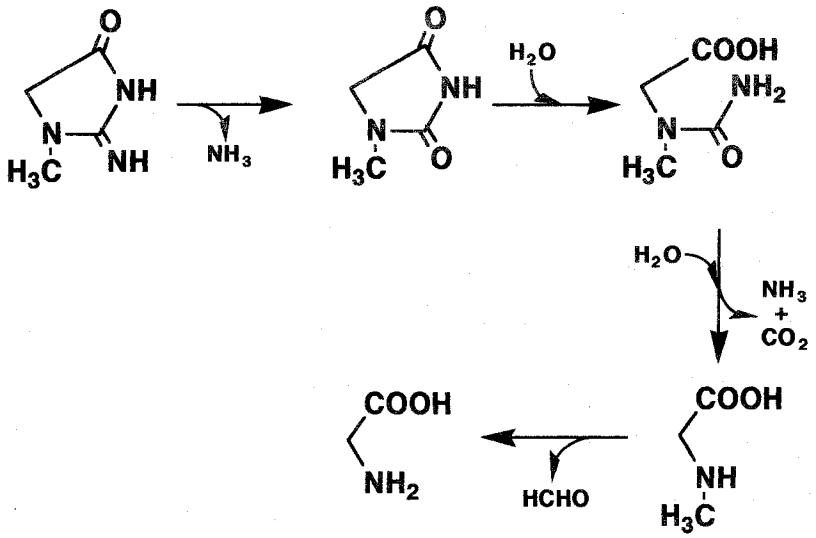
Section 3. *N*-Methylhydantoin amidohydrolase from *Pseudomonas putida* 77: purification and characterization[h]

An ATP-dependent amidohydrolase, *N*-methylhydantoin amidohydrolase, which catalyzes the reaction presented in Scheme 1, was first found in *Pseudomonas putida* 77 by Yamada *et al.* [28,29].



Scheme 1. *N*-Methylhydantoin amidohydrolase reaction.

The enzyme catalyzes the second step reaction in the degradation route from creatinine to glycine, *via* *N*-methylhydantoin, *N*-carbamoylsarcosine, and sarcosine as successive intermediates [7,8,28,29,46,86-89] (Scheme 2).



Scheme 2. Pathway of microbail creatinine degradation.

N-Methylhydantoin amidohydrolase resembles dihydropyrimidinase, which is widely distributed from microorganisms to mammals [16,18,76,90-93], in that both enzymes hydrolyze hydantoin compounds. Different from *N*-methylhydantoin amidohydrolase, however, dihydropyrimidinase does not require ATP for the hydrolysis of its substrates.

Enzymes that catalyze this type of ATP-dependent amidohydrolysis reaction comprise 5-oxoprolinase (EC 3.5.2.9) [94-98], urea amidolyase [99.100], L-isomer specific hydantoinase [27] and nonstereospecific hydantoinase [67], other than *N*-methylhydantoin amidehydrolase. Through extensive studies by Meister and coworkers, it has been shown that rat kidney 5-oxoprolinase is involved in the metabolism of glutathione [101], and that phosphorylation of the substrate is involved in the reaction mechanism [102-104]. The rat kidney 5-oxoprolinase catalyzes the uncoupled hydrolysis of ATP in the presence of several structural analogs of 5-oxo-L-proline, such as L-2-imidazolidone-4-carboxylate, dihydroorotate, *etc.* [95,98,105], and is considered to be composed of two subunits identical in molecular mass [98]. On the other hand, the bacterial 5-oxoprolinase easily separates into two components on purification and the overall reaction only proceeding after remixing of the components [106]. Urea amidolyase from *Saccharomyces cerevisiae* catalyzes successive two reactions; the first of which is an ATP, Mg^{2+} and K^{+} dependent carboxylation of urea to give allophanate, and the second is a hydrolysis of allophanate to carbon dioxide and ammonia [100]. These reactions are catalyzed by a polypeptide of M_r = 200,000, and the native enzyme exists as homo-dimer of this polypeptide [100]. There is a few data on the enzyme protein of L-isomer specific hydantoinase [27] and nonstereospecific hydantoinase [67].

In this section, I describe the purification and characterization of the *N*-methylhydantoin amidohydrolase from *P. puitda.* I also compare the enzyme with other known enzymes that require ATP for the hydrolysis of amide compounds or utilize hydantoin compounds as substrates. The results indicate that *N*-methylhydantoin amidohydrolase differs from the others in its physical properties and substrate specificity.

MATERIALS AND METHODS

Materials. *N*-Carbamoylsarcosine, and *N*-carbamoyl-L- and D-alanine were prepared from the corresponding amino acids and potassium cyanate, respectively, according to the method of Nyc and Mitchell [107]. *N*-Methylhydantoin was obtained from Aldrich Chemicals, USA. All other hydantoin compounds were kind gifts from Kanegafuchi Chemical Co., Japan. *N*-Carbamoylsarcosine amidohydrolase and sarcosine oxidase were prepared as described by Kim *et al.* [46,88,89]. Standard proteins for high-performance gel-permeation liquid chromatography, SDS-polyacrylamide gel electrophoresis (SDS/PAGE), and analytical gel filtration on a Sephacryl S-300 column were purchased from Oriental Yeast Co., Japan, Pharmacia, Sweden, and Boeringer Mannheim, Germany, respectively. All other chemicals used in this work were of the purest commercial grade available.

Microorganism and cultivation. *P. putida* 77 (AKU 875; Faculty of Agriculture, Kyoto University, Kyoto, Japan) [7,8] was cultivated aerobically at 28°C for 36 h in a medium containing 1% glycerol, 0.1% yeast extract, 0.1% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.4% *N*-methylhydantoin [8].

Enzyme assays. Standard assay: The reaction mixture (0.1 ml) contained 50 mM Tris/HCl buffer (pH 8.0), 10 mM *N*-methylhydantoin, 2.5 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, and the enzyme. After 5-30 min incubation at 37°C, the reaction was stopped by immersing the reaction tubes in boiling water for 2 min. After filtration through a SJHV 004 NS filter (Nihon Millipore Kogyo, Japan), the filtrate was assayed for the formation of *N*-carbamoylsarcosine and ADP or the degradation of *N*-methylhydantoin and ATP by high performance liquid chromatography (HPLC) on a Zorbax SAX column (Dupont Co., USA) [28].

ADP formation: ADP was assayed enzymatically with pyruvate kinase (from rabbit muscle, Boehringer Mannheim) and lactate dehydrogenase (from rabbit muscle, Boehringer Mannheim) according to the procedure described by Kim *et al.* [28].

N-Carbamoylsarcosine assay: The reaction mixture used for the standard assay was also used for the analysis of *N*-carbamoylsarcosine. The assay mixture (0.3 ml) contained 10-50 μl of a sample solution, 50 mM Tris/HCl buffer (pH 8.0), 1.5 mM phenol, 1.5 mM 4-aminoantipyrine, and 0.3 units each of *N*-carbamoylsarcosine amidohydrolase, sarcosine oxidase and peroxidase (from horseradish, Boehringer Mannheim). After 30 min at 37°C, the increase in absorbance at 505 nm due to H_2O_2 generation, which was the result of *N*-carbamoylsarcosine hydrolysis to sarcosine and successive oxidation of sarcosine, was determined. A molar extinction coefficient of 17,140 $M^{-1} \cdot cm^{-1}$ for quinoneimine was used.

One unit of *N*-methylhydantoin amidohydrolase was defined as the amount catalyzing the formation of 1 μmol of *N*-carbamoylsarcosine or ADP, or the consumption of 1 μmol of *N*-methylhydantoin or ATP per min under the standard assay conditions.

Separation of subunit polypeptides. The purified enzyme (0.5 mg) was dissociated with 10% SDS and 10% 2-mercaptoethanol at 95°C for 10 min and then electrophoresed on a 8.5% SDS-polyacrylamide slab gel. Tris/bicine was used as the buffer system. Small gel slices from both sides and the center of the gel were stained with Coomassie brilliant blue. The zones corresponding to the subunit bands on the remaining gel were cut out and then crushed with a teflon homogenizer in a small volume of distilled water. Each homogenate was suspended in distilled water (30 ml) and then shaken for 40 h at 20°C to extract the proteins. After filtration of the gels followed by washing with 30 ml of distilled water, the combined filtrate was dialyzed against 500 ml of distilled water and then lyophilized. After confirming its purity by SDS/PAGE, the polypeptide in the lyophilizate was subjected to amino acid analysis and NH_2-terminal amino acid sequence.

Analytical methods for *N*-methylhydantoin amidohydrolase. The relative molecular mass of the enzyme was determined by analytical gel filtration on a calibrated column (1.5 x 100 cm) packed with Sephacryl S-300, eluted with purification buffer described below containing 0.2 M KCl. It was also determined by high-performance gel-permeation liquid chromatography on GS-520 column (0.76 x 50 cm; Asahi Kasei, Japan) and on TSK G-4000SW column (0.75 x 60 cm; Tosoh, Japan) eluted with same buffer. Protein concentrations were determined as described previously [53] or by measuring the absorbance at 280 nm. An absorbance value 0.65 for 1.0 $mg \cdot ml^{-1}$ and 1-cm light path, as determined by absorbance and dry-weight measurements, was used. Polyacrylamide gel electrophoresis (PAGE) on 5% disc gel, SDS/PAGE on 12.5% slab gel and isoelectric focusing of the native enzyme at pH range 3.5-10.0 were performed as described previously [53]. Estimation of amino acid composition, NH_2-terminal amino acid sequence and the COOH-terminal amino acid analysis with carboxypeptidase Y (Oriental Yeast Co.,) of the native enzyme and subunit protein were performed as described previously [53,54].

Analytical ultracentrifugation. Analytical ultracentrifugation was carried out with a Spinco model E ultracentrifuge at 20°C. The purity of the enzyme and its sedimentation coefficient were determined with a phase plate as a Schlieren diagram. The relative molecular mass of the enzyme was determined by the ultracentrifugal equilibrium method of Van Holde and Baldwin [108] with Rayleigh interference optics. Multicell operations were carried out for 4 samples with different initial concentrations, ranging from 1.12 to 2.23 $mg \cdot ml^{-1}$, using an An-G rotor and double cells of different side-wedge angles. The rotor was centrifuged at 4609 rpm for 20 h, and the interference patterns were photographed at intervals of 30 min. The relationship between the enzyme concentration and the fringe shift was determined using a synthetic boundary cell. The calculations were carried out as described by Schachman [109].

Purification of *N*-methylhydantoin amidohydrolase. The enzyme was partially purified from wet cells (45 g) of *P. putida* obtained from 5 liters of culture, by essentially the same procedures, i.e., sonication, ammonium sulfate fractionation, and DEAE-Sephacel and phenyl-Sepharose CL-4B column chromatographies, as described by Kim *et al.* [28], except that 10 mM Tris/HCl buffer (pH 7.4) containing 2 mM *N*-methylhydantoin and 0.1 mM DTT was used as purification buffer throughout and that the phenyl-Sepharose step was carried out on a 1.8 x 15 cm column equilibrated with the above buffer containing 3 M KCl. Elution was performed successively with 100 ml of the buffer containing 3 M KCl, 500 ml of the buffer for linear gradient elution (3 to 0 M KCl) and then 100 ml of the buffer without KCl, at a flow rate of 27 $ml \cdot h^{-1}$. The active fractions were combined and then concentrated to about 3 ml with an Amicon membrane filter (Amicon Co., USA) equipped with a YM 30 membrane. The enzyme concentrate was subjected to gel filtration on a Sephacryl S-300 column (2.1 x 85 cm) equilibrated with the purification buffer containing an additional 0.2 M KCl, the enzyme being eluted with the equilibrium buffer at a flow rate of 10 $ml \cdot h^{-1}$. The active fractions were collected, dialyzed 3 times against 1.5 liters of the purification buffer and then stored at 4°C. After removing *N*-methylhydantoin in the enzyme solution by filtration on a Sephadex G-25 column (0.9 x 2.8 cm), the enzyme was immediately subjected to characterization.

RESULTS

Induction of *N*-methylhydantoin amidohydrolase

Various amides, pyrimidines and *N*-carbamoyl compounds were examined for their induction activity of the enzyme. When *P. putida* was cultured in the medium described in materials and methods except that various compounds (0.4% (w/v)) were added instead of *N*-methylhydantoin, the enzyme activity was found only with creatinine. This suggest the specific role of the enzyme in the creatinine metabolism.

The specific activity of the cells obtained by creatinine containing media was 73% of that obtained by *N*-methylhydantoin containing media.

Stabilizing effects of chemicals on the enzyme activity

Without *N*-methylhydantoin in the purification buffer, the enzyme significantly lost its activity. As for the various amide compounds, pyrimidines, metal ions and ATP, stabilizing effects on the enzyme activity were examined. After the cell-free extracts were dialyzed against to the purification buffer containing test compounds (2 mM) instead of *N*-methylhydantoin at 4°C for 20 h, the remaining activities were assayed. Other than *N*-methylhydantoin, only 2-pyrrolidone showed stabilizing effect, which was 43% of that of *N*-methylhydantoin (2 mM). At 5 mM the stabilizing effect of 2-pyrrolidone increased to 80% of that of *N*-methylhydantoin which kept same effect over 2 mM.

Purification of *N*-methylhydantoin amidohydrolase

The enzyme was purified about 70-fold to homogeneity, with an about 62% overall recovery (Table 1). On Sephacryl S-300 column chromatography, *N*-methylhydantoin amidohydrolysis and ATP hydrolysis activities were co-detected, with overlapping activity profiles, as a major symmetrical protein peak, and the *N*-methylhydantoin amidohydrolysis/ATP hydrolysis activity ratio remained at near unity for all the active fractions. In the *N*-methylhydantoin (2 mM) containing buffer, the purified enzyme could be stored for at least 6 months at 0-8°C without noticeable loss of the activities toward *N*-methylhydantoin and ATP.

Table 1. Purification of *N*-methylhydantoin amidohydrolase from *P. putida*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification -fold
1) Cell-free extract	3840	273	0.0710	100	1
2) Ammonium sulfate	1750	247	0.141	90	1.99
3) DEAE-Sephacel	310	236	0.761	86	10.7
4) Phenyl-Sepharose CL-4B	46.4	210	4.53	77	63.8
5) Sephacryl S-300	33.1	167	5.10	62	71.8

Crystallization of the enzyme

The enzyme can be crystallized by the addition of solid ammonium sulfate, little by little, to the concentrated enzyme solution (about 10 $mg \cdot ml^{-1}$) at the Sephacryl S-300 step. Fine needle-shaped crystals appeared at about 30% ammonium sulfate saturation after standing in a refrigerator for 2 days (Fig. 1). The specific activity of the crystalline enzyme was 5.03 $units \cdot mg^{-1}$, which was essentially the same as that of the purified enzyme at the Sephacryl S-300 step.

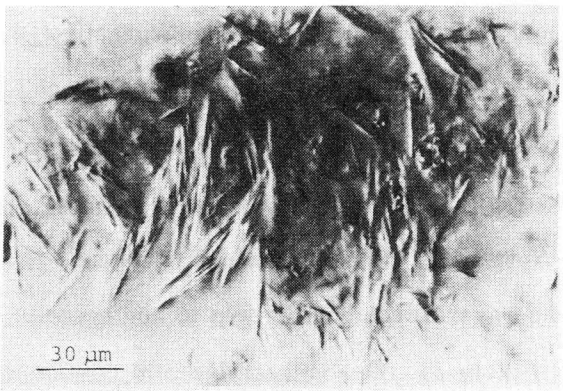


Fig. 1. Microphotograph of crystals of the *N*-methylhydantoin amidohydrolase from *P. putida*

Criteria for purity

The purified enzyme preparation gave a single band on PAGE (Fig. 2a). It sedimented as a single symmetrical peak in 0.2 M potassium phosphate buffer (pH 7.0) containing 2 mM *N*-methylhydantoin and 0.1 mM DTT on analytical ultracentrifugation. Ampholytic electrofocusing yielded only one absorption peak of protein (pI = 4.15), which coincided with the *N*-methylhydantoin amidohydrolase activity. Further evidence for the purity of the enzyme preparation comprised the results of high-performance gel-permeation liquid chromatography on TSK G-4000SW and Asahipak GS-520 columns, which gave a quite symmetrical single protein peak.

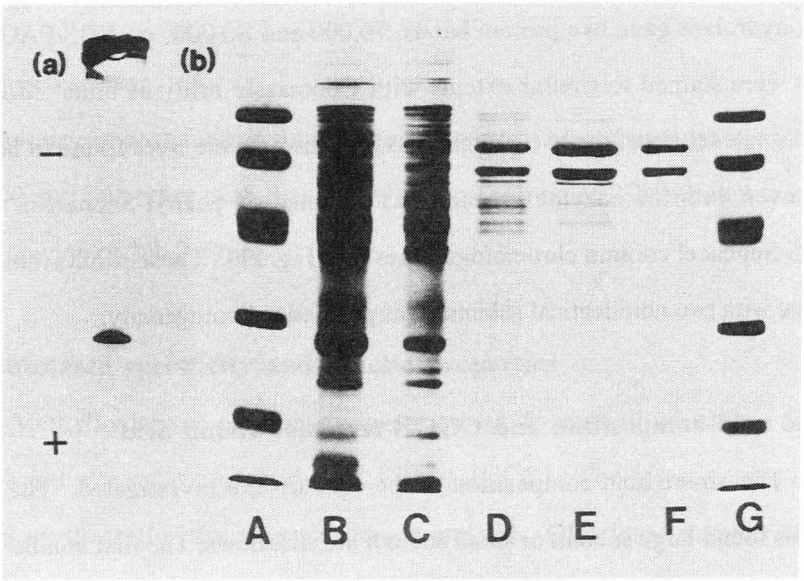


Fig. 2. PAGE of samples at each step of the purification of the *N*-methylhydantoin amidohydrolase in the absence (a) and the presence (b) of SDS.
(a) The purified enzyme (about 5 μg) at the Sephacryl S-300 step was electrophoresed. (b) A and G, standards; B, cell-free extract, 80 μg; C, ammonium sulfate, 42 μg; D, DEAE-Sephacel, 13 μg; E, phenyl-Sepharose CL-4B, 20 μg; F, Sephacryl S-300, 12 μg. The standards and their molecular masses were as follows; phosphorylase *b*, 97,400; bovine serum albumin, 66,267; ovalbumin, 42,400; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; and lactoalbumin, 14,400. In both cases the gel was stained for protein with Coomassie Brilliant Blue G-250 and then destained in ethanol/acetic acid/water (3:1:6, by vol.). The purification steps were shown in Table 1.

Molecular mass and subunit structure

The sedimentation coefficient ($s_{20,w}$) of the enzyme dissolved in 0.2 M potassium phosphate buffer (pH 7.0) containing 2 mM *N*-methylhydantoin and 0.1 mM DTT was determined to be 9.9 S. A relative molecular mass of 300,000 ± 10,000 was determined by the sedimentation equilibrium method for the enzyme dissolved in 0.2 M potassium phosphate buffer (pH 7.0) containing 2 mM *N*-methylhydantoin and 0.1 mM DTT, assuming a partial specific volume of 0.74 $cm^3 \cdot ml^{-1}$. On a calibrated column of Sephacryl S-300, the relative molecular mass of the enzyme was estimated to be 300,000, and those determined by high-performance gel-permeation liquid

chromatography on TSK G-4000SW and Asahipak GS-520 columns were 325,000 and 290,000, respectively. As shown in Fig. 2b, the purified *N*-methylhydantoin amidohydrolase gave two protein bands, 70,000 and 80,000, on SDS/PAGE; the two bands were stained to similar extents with Coomassie brilliant blue. Moreover, the staining intensities of the two protein bands of the enzyme were found to be nearly the same even with the enzyme preparations obtained on phenyl-Sepharose CL-4B and DEAE-Sephacel column chromatographies (see Fig. 2b). These results indicate that an enzyme with two nonidentical subunits was purified to homogeneity.

Amino acid composition and COOH-terminal amino acid

The amino acid composition of the subunits are investigated. The number of residues found/large subunit or small subunit are as follows: The first number is for large subunit and the second is for small subunit, Asp & Asn (85, 77), Thr (45, 34), Ser (50, 50), Glu & Gln (94, 78), Pro (46, 38), Gly (83, 74), Ala (68, 67), Met (9, 10), Ile (46, 33), Val (54, 45), Leu (67, 52), Tyr (12, 19), Trp (10, 10), Phe (19, 22), Lys (23, 17), His (20, 16), Arg, (46, 38), Half-cystine (8, 6). The minimum values with the nearest integral numbers of all amino acids found were calculated to be 74,692 and 86,825 for the small subunit and the large subunit, respectively. The amino acids released on carboxypeptidase Y digestion of both large and small subunits were leucine. It is evident that each subunit has the same single COOH-terminal amino acid, leucine. These results suggest that the enzyme is a tetramer of two identical small subunits (M_r = 70,000) and two identical large subunits (M_r = 80,000).

NH_2-Terminal amino acid sequences of the subunits

NH_2-Terminal amino acid sequences of twenty-two and thirty amino acid residues were found for large and small subunits, respectively, on automated Edman degradation. That for large subunit is Ala-Arg-Ile-Gly-Val-Asp-Val-Gly-Gly-Thr-Asn-Thr-Asp-Leu-Ile-Leu-Glu-Thr-Thr-Glu-Arg-Asp-. That for small subunit is Thr-Thr-

Gln-Leu-Val-Asp-Pro-Ile-Thr-Leu-Gln-Val-Ile-Ser-Gly-Ala-Leu-Lys-Thr-Ile-Ala-Glu-Glu-Met-Gly-His-Val-Leu-Tyr-Arg-.

Spectral properties

The enzyme contained no substance showing any absorption in the visible region of 350-600 nm. No other peak than one with a maximum at 278 nm appeared in the ultraviolet region of 250-340 nm.

Amide substrate specificity and kinetic properties

N-Methylhydantoin amidohydrolase requires *N*-methylhydantoin for ATP hydrolysis and *vice versa*, as reported by Kim *et al.* [28]. The effectiveness of other amides as substrates for the enzyme were examined. Of a large number of amide compounds tested, amide compounds listed in Table 2, other than *N*-methylhydantoin, were newly found to cause detectable hydrolysis of ATP. Among these substrates, hydantoin compounds (i.e., *N*-methylhydantoin, hydantoin, and DL-5-methylhydantoin), glutarimide and succinimide were hydrolyzed to the corresponding carbamoyl compounds upon coupled hydrolysis of ATP. With hydantoin compounds, stoichiometry between amide bond hydrolysis and ATP hydrolysis were observed under the tested conditions. However, the amount of hydrolyzed glutarimide and succinimide did not correspond to the amount of hydrolyzed ATP. While the other amide compounds, 2-pyrrolidone, 2-oxazolidone, δ-valerolactam, 2,4-thiazolidinedione, 2-imidazolidone, L- and DL-5-oxoproline methyl ester, dihydrouracil, dihydrothymine, uracil and thymine were not hydrolyzed, but rapid hydrolysis of ATP occured in the presence of these substrates. Normal hyperbolic kinetic was observed in the initial verocity of the ATP hydrolysis by the enzyme, in the pressence of fixed excess amount of ATP (2.5 mM) and variety amount of amide substrates. Lineweaver-Burk treatment of the data yielded that the apparent K_m value of *N*-methylhydantoin for ATP hydrolysis was 32 μM and the V_{max} value of *N*-methylhydantoin-dependent ATP hydrolysis was 9.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. The set of slopes obtained from Lineweaver-Burk plot

for *N*-methylhydantoin-dependent ATP hydrolysis at several fixed ATP concentration, from 50 to 200 μM, were found to be nearly constant, which excludes the possibility of a sequential-randam bi-bi mechanism for the reaction (data not shown). The low K_m values of amide substrates for ATP hydrolysis were also found with glutarimide, dihydrouracil, δ-valerolactam and succinimide (Table 2).

Table 2. Substrate specificity of *N*-methylhydantoin amidohydrolase for amide compounds and pyrimidines

Substrate	Products found[a]		K_m[b] (mM)	V_{max}[b] (μmol/min/mg)
	Carbamoyl amino acid (μmol/ml)	ADP[c] (μmol/ml)		
N-Methylhydantoin	4.03[d]	4.33	0.032	9.0
Hydantoin	1.15[e]	1.20	1.7	3.6
DL-5-Methylhydantoin	0.93[e]	1.02	2.0	3.7
Glutarimide	1.91[d]	4.34	0.070	2.2
Succinimide	2.51[d]	4.38	0.13	2.2
2-Pyrrolidone	n.h.[d]	3.87	0.75	4.7
2-Oxazolidone	n.h.[d]	1.20	18	2.1
δ-Valerolactam	n.h.[d]	2.22	0.11	2.4
2,4-Thiazolidinedione	n.h.[d]	2.92	10	3.8
2-Imidazolidone	n.h.[d]	1.95	40	3.1
L-5-Oxoproline methyl ester	n.h.[d]	0.55	n.d	n.d.
DL-5-Oxoproline methyl ester	n.h.[d]	0.74	n.d.	n.d.
Dihydrouracil	n.h.[d]	3.87	0.083	5.8
Dihydrothymine	n.h.[d]	2.92	2.0	4.3
Uracil	n.h.[d]	3.17	6.6	8.2
Thymine	n.h.[d]	1.31	16	2.8

Neither amidohydrolysis nor ATP hydrolysis was observed on incubation with the following amide compounds at 5 mM; 2-amino-4,6-dihydropyrimidine, cytosine, orotic acid, L- and DL-dihydroorotic acid, L- and DL-dihydroorotic acid methyl ester, adenine, guanine, xanthine, uridine, cytidine, thymidine, adenosine, guanosine, xanthosine, inosine, UMP, CMP, IMP, GMP, XMP, AMP, thioadenine, dTMP, D-, L- and DL-5-oxoproline, 2-imidazolidone-4-carboxylic acid, creatinine, *N-trans*-cinnamoylimidazole, L- and D-1-amino-4-caprolactam, nocodazole, uric acid, barbituric acid, L- and DL-citrulline, alloxazine, alloxane, nicotinamide, urea, D- and L-5-benzylhydantoin, DL-5-phenylhydantoin, DL-5-(*p*-hydroxyphenyl)hydantoin, DL-5-(*p*-methylphenyl)hydantoin, DL-5-(*p*-methoxyphenyl)hydantoin, DL-5-carboxymethylhydantoin, DL-5-(2-thienyl)hydantoin, DL-5-carboxyethylhydantoin, 5,5-dimethylhydantoin and L-5-isopropylhydantoin.

[a]Reactions were carried out under the standard assay conditions for 30 min except that the indicated amide compounds (5 mM) were used as substrates.

[b]In these studies, initial velocities of the amide substrate-dependent formation of ADP were determined by means of the coupled assay with pyruvate kinase and lactate dehydrogenase. The reactions were carried out with fixed amount of ATP (2.5 mM) and variety amounts of amide substrates for determination of K_m and V_{max} values.

[c]The amide substrate-dependent formation of ADP were determined by HPLC on a Zorbax SAX column as described in materials and methods.

[d]The formation of carbamoyl amino acids and the consumption of amide substrates were determined by HPLC on a Zorbax SAX column as described in materials and methods or a Cosmosil $5C_{18}$ column (4.6 x 100 mm; Nacalai Tesque, Japan) [113].

[e]The formation of carbamoyl amino acids were determined by chemical methods [110].

n.h. = not hydrolyzed.

n.d. = not determined.

Stereospecificty toward 5-methylhydantoin

Since the rate of ATP hydrolysis with L-5-methylhydantoin was found to be about 7-fold higher than that with the D-isomer, when monitored by means of the coupled assay with pyruvate kinase and lactate dehydrogenase, the enzyme was seemed to show stereospecificity toward 5-substituted hydantoin. Table 3 shows the results of more detailed analysis of the stereospecificity of the enzyme reaction.

Table 3. Stereospecificity of the *N*-methylhydantoin amidohydrolase reaction for 5-methylhydantoin

		Compound found on reaction (μmol/ml)			
Substrate	pH	5-Methyl-hydantoin	ATP	Carbamoyl-alanine	ADP
L-5-Methyl-hydantoin	6	0.70	0.73	1.81 (0)	1.75
	7	0.50	0.52	1.95 (0)	1.99
	8	0.31	0.33	2.11 (0)	2.20
	9	0.35	0.39	2.00 (0)	2.11
D-5-Methyl-hydantoin	6	2.51	2.47	0.01 (0)	0.02
	7	2.43	2.40	0.03 (0)	0.08
	8	2.11	2.08	0.35 (0)	0.40
	9	1.85	1.87	0.63 (0)	0.71

The enzyme (0.5 units) was incubated with ATP (2.5 mM) and the L- or D-form of 5-methylhydantoin (2.5 mM) at the indicated pHs for 30 min in a total volume of 1.0 ml. Other conditions were the same as for the standard assay. The buffers (100 mM) used were Pipes/NaOH and Tris/HCl for pH 6 and 7, and for pH 8 and 9, respectively. 5-Methylhydantoin, ATP, and ADP were determined by HPLC on a Zorbax SAX column. *N*-Carbamoylalanine was determined chemically [110]. The values for *N*-carbamoylalanine in parentheses were obtained using *N*-carbamoylsarcosine amidohydrolase which only hydrolyzes the D-isomer of *N*-carbamoylalanine [46]. The reaction mixture for the hydrolysis contained 0.1 ml of a sample solution containing 5-50 nmol of *N*-carbamoylalanine, 5 μmol of Tris/HCl buffer (pH 7.4), and 0.3 units of *N*-carbamoylsarcosine amidohydrolase. After incubation at 37°C for 30 min, the mixture was analyzed for D-alanine with an amino acid analyzer.

The amounts of *N*-carbamoylalanine (or ADP) found after the reaction with the D-isomer were about 70 and 3 times smaller than those with the L-isomer at pH 7 and 9, respectively, when assayed by the chemical method [110]. Furthermore, the reaction with the D-isomer was accelerated under alkaline pH conditions, whereas no significant change in the product formation or the reaction rate was observed with the L-isomer. *N*-Carbamoylalanine was not detected on assaying enzymatically with *N*-

carbamoylsarcosine amidohydrolase, which hydrolyzes only the D-isomer of *N*-carbamoylalanine [46], in any of the tubes examined. This indicates that the *N*-carbamoylalanine formed was the L-isomer, regardless of the optical form of the substrate. Dudley and Bius reported that spontaneous racemization of optically active 5-monosubstituted hydantoin compounds takes place under mild alkaline conditions [90,111]. We also observed that either L- or D-isomers easily undergo racemization in reaction mixtures of pH 8 and 9 at 37°C in the absence of the enzyme. All of these results led to the conclusion that the *N*-methylhydantoin amidohydrolase only hydrolyzes the L-isomer of 5-methylhydantoin, and that the apparent activity with the D-isomer was due to spontaneous racemization of the substrate.

Requirement of cations for the enzyme reaction

N-Methylhydantoin amidohydrolase requires Mg^{2+} and K^+ besides ATP for the amidohydrolysis of *N*-methylhydantoin [28]. To investigate the cation requirement of the enzyme activity, ammonium and several metal chlorides were tested. NH_4^+, Rb^+ and Cs^+ (Fig. 3), and Mn^{2+} (42% of that with Mg^{2+} at 10 mM) and Co^{2+} (23% of that with Mg^{2+} at 10 mM) could substitute for K^+ and Mg^{2+} as monovalent and divalent cations, respectively. In every case, stoichiometry of the coupled hydrolysis of *N*-methylhydantoin and ATP was observed.

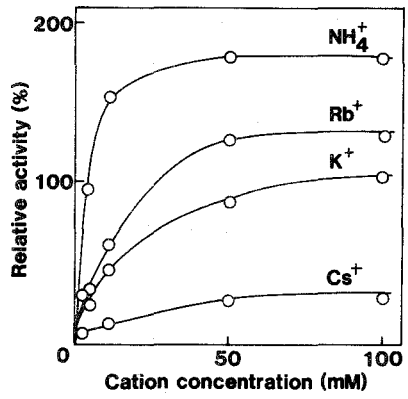


Fig. 3. Effect of the monovalent cation concentration on the activity of the *N*-methylhydantoin amidohydrolase.
The enzyme activity was assayed under the standard assay conditions with 30 min incubation, except that different monovalent cations (chloride salts) at the indicated concentrations were used. Relative activity is expressed as the percentage of the activity obtained at 100 mM KCl (5.12 units/mg).

These cations were also effective as to uncoupled hydrolysis of ATP in the presence of dihydrouracil instead of *N*-methylhydantoin. Neither *N*-methylhydantoin hydrolysis nor ATP hydrolysis was observed on incubation with the following monovalent (at 10 and 100 mM) or divalent (at 10 mM) cations; Li^+, Na^+, $(CH_3)_3NH^+$, Zn^{2+}, Cu^{2+}, Fe^{2+}, Be^{2+}, Ba^{2+}, Ca^{2+}, Pb^{2+}, Sn^{2+}, Ni^{2+}, and Cd^{2+}.

Effects of inhibitors and metal ions

Various inhibitors, substrate analogs, and metal ions were tested as to their inhibitory effect on the enzyme activity with *N*-methylhydantoin and ATP as substrates. The enzyme activity was measured under the standard assay conditions except for the addition of each tested compound or metal salt. The enzyme activity was completely inhibited by sulfhydryl reagents such as *p*-chloromercuribenzoate (at 0.1 mM), 5,5'-dithio-bis(2-nitrobenzoate) (at 0.1 mM), *N*-ethylmaleimide (at 1 mM), $HgCl_2$ (at 0.1 mM), and $AgNO_3$ (at 1 mM). The inhibition with 1 mM semicarbazide, hydroxylamine, phenylhydrazine, and 8-hydroxyquinoline was 33, 100, 100, and 85%, respectively. $ZnCl_2$, $CuSO_4$, $CdCl_2$, and $FeSO_4$ at 1 mM were also inhibitory (100, 87, 100, and 35% inhibition, respectively). The activity of the enzyme did not change on incubation with the following reagents (1 mM): NaCN, NaF, sodium azide, Na_2AsO_2, EDTA, *o*-phenanthroline, 2,2'-dipyridyl, iodoacetate, iodoacetamide, $BeCl_2$, and $CaCl_2$.

Effects of pH and temperature on the enzyme stability

As reported by Kim *et al.*, the enzyme catalyzed the amidohydrolysis of *N*-methylhydantoin coupled with ATP hydrolysis over the pH range of 6-10 and the temperature range of 20-50°C [28]. Essentially the same activity profiles were observed when ATP hydrolysis was assayed under the uncoupled conditions with dihydrouracil (data not shown). A significant reduction in enzyme activity toward either *N*-methylhydantoin or ATP was not observed when the enzyme was incubated at 50°C for 40 min. More than 60% of the initial activity remained at 60°C under the same conditions. No loss of activity was observed between pH 5.5 and 9.5 on incubation of

the enzyme at 37°C for 30 min in the same buffer system described by Kim *et al.* [28]. In all cases, stoichiometry between *N*-methylhydantoin hydrolysis and ATP hydrolysis was observed.

DISCUSSION

N-Methylhydantoin amidohydrolase catalyzes the coupled hydrolysis of *N*-methylhydantoin and ATP. The enzyme is not a constitutive enzyme but a inducible one. The enzyme is induced only by *N*-methylhydantoin and creatinine. These results suggest that the phygiological function of the enzyme is assimilation of creatinine. The enzyme is unusual in that it requires ATP hydrolysis for amide bound hydrolysis. The reason why the enzyme requires energy for the hydrolysis of amide bonds is unclear. However, it is expected that the equilibium of the *N*-methylhydantoin-*N*-carbamoylsarcosine reaction so much favors cyclization that energy is required for ring opening reaction, like 5-oxoproline-glutamate reaction catalyzed by 5-oxoprolinase [94]. Besides *N*-methylhydantoin, the enzyme hydrolyzes hydantoin, L-5-methylhydantoin, glutarimide and succinimide upon coupled hydrolysis of ATP. Moreover, 2-pyrrolidone, 2-oxazolidone, δ-valerolactam, 2,4-thiazolidinedione, 2-imidazolidone, L- and DL-5-oxoproline methyl ester and four pyrimidines, dihydrouracil, dihydrothymine, uracil and thymine stimulated the ATP hydrolysis to a great extent without undergoing amidohydrolysis themselves. It is interesting that the enzyme showed ATP-hydrolyzing activity in the presence of some amide compounds without the hydrolysis of amide compounds. This function, ATP hydrolysis, seems to not have important physiological role, considering for the induction profile of the enzyme. However, there is a possibility that the enzyme controls the cellular ATP level during the assimilation of creatinine. The present finding that several widely-distributed pyrimidine compounds, i.e., dihydrouracil, dihydrothymine, uracil, and thymine, caused rapid hydrolysis of ATP without undergoing amidohydrolysis themselves, together with the observation that dihydroorotate stimulates ATP hydrolysis by 5-oxoprolinase [95], may have implications

with respect to the homeostasis of a cell at the ATP level, especially in relation to pyrimidine metabolism.

N-Methylhydantoin amidohydrolase seems to resemble 5-oxoprolinase [94-98, 101-106] and urea amidolyase [99,100], regardless of their different substrate specificities for amide compounds. 5-Oxoprolinase also catalyzes the rapid hydrolysis of ATP in the presence of L-2-imidazolidone-4-carboxylate and dihydroorotate, which are not hydrolyzed by the enzyme. With regard to cation requirements, both 5-oxoprolinase and urea amidolyase require K^+ or NH_4^+, and Mg^{2+} or Mn^{2+}, as does *N*-methylhydantoin amidohydrolase. However, no compound that stimulates ATP hydrolysis without undergoing hydrolysis itself has been reported for urea amidolyase.

It is noteworthy that naturally-occurring pyrimidine compounds, especially dihydropyrimidines, markedly stimulated ATP hydrolysis by *N*-methylhydantoin amidohydrolase, because the amidohydrolysis of various hydantoin compounds is generally catalyzed by dihydropyrimidinase, which is involved in the pyrimidine metabolism in various species, from bacteria to mammals [16,18,76,90-93], and *N*-methylhydantoin is a derivative of hydantoin. Highly purified dihydropyrimidinases from calf and rabbit liver [76] appear to be composed of two nonidentical subunits; they might be comparable to *N*-methylhydantoin amidohydrolase in terms of the subunit structure. However, dihydropyrimidinase from rat liver, which was recently purified to homogeneity, composed of four identical subunits in molecular mass [112]. Different from *N*-methylhydantoin amidohydrolase, dihydropyrimidinases rapidly catalyze hydrolysis of various hydantoin and dihydropyrimidine compounds in the absence of ATP. The stereospecificity for 5-methylhydantoin is also different; the present enzyme hydrolyzed only the L-isomer, whereas dihydropyrimidinases hydrolyze the D-isomer but not the L-isomer [18,90].

Recently, L-isomer specific hydantoin amidohydrolase and nonstereospecific hydantoin amidohydrolase were found in *Bacillus brevis* AJ-12299 [27] and *Pseudomonas* sp. NS671 [67], respectively. These enzymes also required ATP, Mg^{2+}, and K^+ for amide substrate hydrolysis, but detail characterization of these enzyme

proteines still not performed. However, DNA fragment containing the gene of nonstereospecific hydantoin amidohydrolase of *Pseudomonas* sp. was cloned and analyzed [71]. The enzyme consists of two different subunits, and the large subunit and the small subunit of the enzyme are encoded in different open reading frames being closely spaced, and are translationally coupled. Surprisingly, the NH_2-terminal sequences of large subunits of nonstereospecific hydantoin amidohydrolase and *N*-methylhydantoin amidohydrolase are very similar with each other, and those of small subunits are also similar with each other (Fig. 4). However, these two enzymes distinct from each other in their substrate specificity; the enzyme from *Pseudomonas* sp. dose not hydrolyze *N*-methylhydantoin. Other than nonstereospecific hydantoin amidohydrolase, there are no protein showing homology with *N*-methylhydantoin amidohydrolase in the protein data base, NBRF and SWISS, and in the DNA data base, EMBL and GenBank. It requires further studies to prove that these functionally resembling enzymes such as *N*-methylhydantoin amidohydrolase, 5-oxoprolinase, L-isomer specific hydantoinase and nonstereospecific hydantoin amidohydrolase, form a structural family or not.

Another dihydropyrimidine amindohydrolase, dihydroorotase, hydrolyzes the L-isomer of dihydroorotate specifically, while *N*-methylhydantoin amidohydrolase does not. The dihydroorotases from *Escherichia coli* (M_r = 80,900) [78] and *Clostridium oroticum* (M_r = 110,000) [79] can also be easily distinguished from *N*-methylhydantoin amidohydrolase in that the formers are fairly small enzymes with two identical subunits.

All these observations indicate that *N*-methylhydantoin amidohydrolase is a novel enzyme, different from other known enzymes.

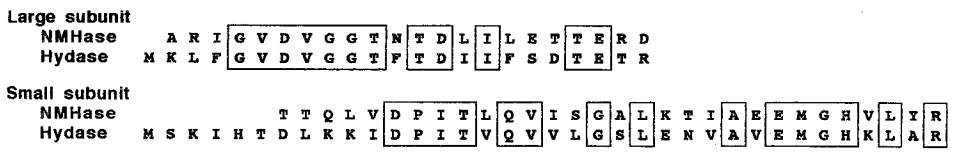


Fig. 4. Comparison of the NH_2-terminal amino acid sequences of *N*-methylhydantoin amidohydrolase (NMHase) from *P. putida* and nonstereospecific hydantoin amidohydrolase (Hydase) form *Pseudomonas* sp. NS671 [40]. Identical amino acid residues are enclosed in boxes.

SUMMARY

N-Methylhydantoin amidohydrolase, an ATP-dependent amidohydrolase involved in microbial degradation of creatinine, was purified to homogeneity from *Pseudomonas putida* 77. The enzyme has a relative molecular mass of 300,000. It is a tetramer of two identical small subunits (M_r = 70,000) and two identical large subunits (M_r = 80,000). The enzyme requires ATP for the amidohydrolysis of *N*-methylhydantoin and *vice versa*. Mg^{2+}, Mn^{2+} or Co^{2+}, and K^+, NH_4^+, Rb^+ or Cs^+ were absolutely required concomitantly for the enzyme activity as divalent and monovalent cations, respectively. The K_m and V_{max} values for *N*-methylhydantoin were 32 μM and 9.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. The hydrolysis of amide compounds and coupled hydrolysis of ATP were observed with hydantoin, L-5-methylhydantoin, glutarimide and succinimide besides *N*-methylhydantoin. 2-Pyrrolidone, 2-oxazolidone, δ-valerolactam, 2,4,-thiazolidinedione, 2-imidazolidone, D- and DL-5-oxoproline methyl ester, and naturally-occurring pyrimidine compounds, i.e., dihydrouracil, dihydrothymine, uracil, and thymine, effectively stimulated ATP hydrolysis by the enzyme without undergoing detectable hydrolysis of themselves.

Section 4. Nucleoside-triphosphatase activity of *N*-methylhydantoin amidohydrolase[i]

In previous section, I mentioned the properties of *N*-methylhydantoin amidohydrolase, which catalyzes the coupled hydrolysis of *N*-methylhydantoin and ATP to yield *N*-carbamoylsarcosine, ADP and Pi. The hydrolysis of ATP by this enzyme is stimulated by some cyclic amide compounds without undergoing detectable amide hydrolysis. This result suggests the possibility that the enzyme shows nucleoside-triphosphatase activity.

In this section, I describe an unique nucleoside triphosphate-hydrolyzing activity of this enzyme, *N*-methylhydantoin amidohydrolase. Besides ATP, various kinds of nucleoside triphosphates can substitute and be hydrolyzed to yield their corresponding nucleoside diphosphates not only in the presence but also in the absence of amide compounds.

MATERIALS AND METHODS

Materials. The sodium salts of ATP, ADP, CTP, UTP, ITP, dCTP, dGTP, dUTP and dTTP were obtained from Yamasa Shoyu (Japan). Those of XTP and dITP were from Sigma (USA) and Boehringer (Germany), respectively. *N*-Methylhydantoin was purchased from Aldrich Chemical Co. (USA). Both pyruvate kinase and lactate dehydrogenase from rabbit muscle were products of Boehringer. All other chemicals used in this work and homogeneously purified *N*-methylhydantoin amidohydrolase from *Pseudomonas putida* 77 were prepared as described in section 3, chapter III.

Enzyme assays. The reaction mixture (0.1 ml) for end-point assay contained 50 mM Tris/HCl buffer (pH 8.5), 10 mM amide substrate, 6.25 mM nucleoside triphosphate, 100 mM KCl, 10 mM $MgCl_2$ and the enzyme. After 30 min incubation at 37°C, the reaction was stopped by immersing the reaction tubes in boiling water for 2

min. After filtration, the filtrate was assayed for the formation of corresponding *N*-carbamoyl compound and nucleoside triphosphate by high-performance liquid chromatography HPLC on a Zorbax SAX column (Dupont Co., USA) as described previously [28].

For the determination of the initial rate of nucleoside triphosphate hydrolysis to nucleoside diphosphate, enzymatic assay with pyruvate kinase and lactate dehydrogenase was performed [114]. The reaction mixture (0.8 ml) contained 50 mM Tris/HCl buffer (pH 8.5),. 10 mM amide substrate, 1.25 mM nucleoside triphosphate, 100 mM KCl, 10 mM $MgCl_2$, 0.25 mM NADH, 5 mM phosphoenolpyruvate, 5 units·ml^{-1} each of pyruvate kinase and lactate dehydrogenase and the enzyme. The reaction was carried out at 37°C and the decrease in 340 nm was monitored. Control experiments established that pyruvate kinase and lactate dehydrogenase were not rate limiting for any of the nucleotides used. One unit of *N*-methylhydantoin amidohydrolase was defined as the amount catalyzing the formation of 1 μmol of ADP per min under the standard assay conditions.

RESULTS

Nucleoside-triphosphatase activity of *N*-methylhydantoin amidohydrolase

As reported in previous section, *N*-methylhydantoin amidohydrolase catalyzes the cleavage of *N*-methylhydantoin to *N*-carbamoylsarcosine; this reaction is coupled with the cleavage of ATP to ADP and Pi. Besides ATP, some other nucleoside triphosphates were found to be effective in inducing *N*-methylhydantoin hydrolysis upon their hydrolysis (Table 1). However, it was found that the hydrolysis of all tested nucleoside triphosphates, other than ATP, substantially exceed that of the *N*-methylhydantoin, if effective. In the presence of *N*-methylhydantoin, the hydrolysis rates for ATP and dATP were much higher than those of any other nucleoside triphosphates tested.

Table 1. Hydrolysis of various nucleoside triphosphates by *N*-methylhydantoin amidohydrolase in the presence and absence of amide substrat

Substrate NTP[d]	Amide	Products found[a] NDP[e] (μmol/ml)	CA[f]	Relative[b,c] rate of NTP hydrolysis(%)	K_m[b] (μM)	V_{max}[b] (μmol/min/mg)
ATP	NMH[g]	4.8	4.7	100	7.7	3.1
CTP		2.4	0.71	37	110	1.3
UTP		1.5	0.50	22	150	1.0
GTP		1.0	0.40	28	400	2.5
ITP		1.7	0.63	27	330	1.3
XTP		1.5	0.70	28	330	1.3
dATP		3.8	1.6	130	40	4.2
dCTP		0.95	0.23	5	220	0.3
dUTP		0.25	0.05	11	100	0.27
dGTP		0.75	0.20	17	1000	1.7
dITP		0.75	0.40	11	500	0.80
dTTP		nt[i]	nt	5	300	0.18
ATP	DHU[h]	4.7	0	130	14	5.5
CTP		3.1	0	74	250	5.0
UTP		3.7	0	89	120	3.9
GTP		2.5	0	89	250	4.2
ITP		3.2	0	98	250	4.2
XTP		3.0	0	67	140	3.1
dATP		3.9	0	148	40	6.2
dCTP		2.25	0	8	200	0.35
dUTP		0.70	0	28	110	0.70
dGTP		1.4	0	41	3300	2.1
dITP		1.7	0	28	670	2.5
dTTP		nt	nt	6	100	0.80
ATP	-	0	-	0	-	-
CTP		0.80	-	33	42	2.4
UTP		1.7	-	42	25	2.7
GTP		1.2	-	67	40	4.6
ITP		0.50	-	75	41	5.5
XTP		2.1	-	50	47	3.3
dATP		0	-	0	-	-
dCTP		0.90	-	4	910	1.8
dUTP		0.90	-	17	29	3.1
dGTP		0.80	-	33	26	3.3
dITP		1.1	-	19	66	0.54
dTTP		nt	-	nt	nt	nt

[a]Reactions were carried out under the end-point assay conditions. [b]In these studies, initial rate of nucleoside triphosphate hydrolysis to nucleoside diphosphate was determined with pyruvate kinase and lactate dehydrogenase asssy. For determination of relative rates, reactions were carried out with nucleoside triphosphate (1.25 mM) and amide substrate (10 mM). For determination of K_m and V_{max} values, reaction were carried out with fixed amount of amide substrate (10 mM) and variety amounts of nucleoside triphosphates.

[c]Relative activity is expressed as the percentage of the activity calculated from the decrease in absorbance at 340 nm obtained with ATP in the presence of *N*-methylhydantoin.

[d]NTP, nucleoside triphosphate.

[e]NDP, nucleoside diphosphate.

[f]CA, carbamoyl amino acid.

[g]NMH, *N*-methylhydantoin.

[h]DHU, dihydrouracil.

[i]nt, not tested.

The enzyme also could hydrolyze various kinds of nucleoside triphosphates in the presence of amide substrate, which was not hydrolyzed by the enzyme, such as dihydrouracil (Table 1). On incubation with dihydrouracil, all tested nucleoside triphosphates were hydrolyzed, but dihydrouracil itself again remained unhydrolyzed in every case tested. The relative hydrolysis rates for CTP, UTP, GTP, ITP, XTP, dUTP, dGTP and dITP were 2 to 4 fold higher than those with *N*-methylhydantoin.

Surprisingly, various kinds of nucleoside triphosphates, other than ATP and dATP, were well hydrolyzed by the enzyme in the absence of amide substrate (Table 1). However, in the absence of amide substrate, neither ATP nor dATP were hydrolyzed by the enzyme. Among various kinds of nucleoside triphosphates tested, the enzyme showed the highest hydrolysis rate with ITP in the absence of amide substrate. The relative hydrolysis rates for UTP, GTP, ITP, XTP, dUTP, dGTP and dITP in the absence of amide substrate were higher than those in the presence of *N*-methylhydantoin, but lower than those in the presence of dihydrouracil. In contrast to this, those of CTP and dCTP were the lowest in the absence of amide substrate.

Normal hyperbolic kinetics were observed in the hydrolysis of every nucleoside triphosphate tested by the enzyme. Analysis of the data according to the Lineweaver-Burk plots yielded K_m and V_{max} values as shown in Table 1. All nucleoside triphosphates tested, other than dCTP, gave lowest K_m values in the absence of amide substrate. Most of V_{max} values obtained from various nucleoside triphosphates tested were higher in the reaction with dihydrouracil, unhydrolyzed amide substrates, and without amide substrate than those with *N*-methylhydantoin, hydrolyzed amide substrate.

Requirement of cations for nucleoside triphosphate-hydrolyzing activities

N-Methylhydantoin amidohydrolase absolutely requires Mg^{2+} and K^+ besides *N*-methylhydantoin for the hydrolysis of ATP [28]. In the presence of dihydrouracil, unhydrolyzed amide substrate, Mg^{2+} and K^+ requirements for ATP-hydrolyzing activity of *N*-methylhydantoin amidohydrolase was investigated. As shown in Table 2, Mg^{2+}

and K^+ were required for full activity, and the ATP hydrolyzing activity were 80 and 4% in the reaction containing only K^+ or Mg^{2+}, respectively. In the absence of these cations, the enzyme shows almost no ATP hydrolyzing activity in the presence of dihydrouracil.

In the absence of amide substrate, Mg^{2+} and K^+ requirements for some nucleoside triphosphate-hydrolyzing activities of *N*-methylhydantoin amidohydrolase was also investigated (Table 2). It was found that all of the nucleoside triphosphate-hydrolyzing activities tested, Mg^{2+} and K^+ were required for their full activities. The nucleoside triphosphate-hydrolyzing activities were about 50 and 30% in the reaction containing only K^+ or Mg^{2+}, respectively, compared with those containing both K^+ and Mg^{2+}. In the absence of these cations, the enzyme shows almost no nucleoside triphosphate-hydrolyzing activity.

Table 2. Cation requirement of nucleoside triphosphate-hydrolyzing activity of *N*-methylhydantoin amidohydrolase

NTP[a]	Amide	Relative rate of NTP hydrolysis (%)			
		Complete	$-Mg^{2+}$	$-K^+$	$-Mg^{2+}$,$-K^+$
ATP	DHU[b]	100	40	4	tr[c]
ITP	-	100	59	35	tr
dITP	-	100	62	29	tr
CTP	-	100	42	36	tr
dCTP	-	100	62	33	tr

Each reaction was carried out under the end-point assay conditions except for the omission of indicated compounds. Activities were determined by monitoring formation of nucleoside diphosphates by high performance liquid chromatography. The values obtained from complete assay conditions in each nucleoside triphosphate used were set as 100%.
[a]NTP, nucleoside triphosphate.
[b]DHU, dihydrouracil.
[c]tr, less than 1%.

Effects of pH and temperature on ATP- and CTP-hydrolyzing activities in the presence and absence of *N*-methylhydantoin

The enzyme catalyzed the hydrolysis of CTP over the temperature range of 37 to 70°C in the presence and absence of *N*-methylhydantoin, whereas ATP hydrolysis was

observed only in the presence of *N*-methylhydantoin with apparent maxima at 50°C (Fig. 1). The highest activity of CTP hydrolysis in the absence of *N*-methylhydantoin was found at 55°C, and that with *N*-methylhydantoin at 50°C (Fig. 1). No loss of these enzyme activities were observed when the enzyme was incubated at a temperature up to 50°C for 30 min. In every case, about 70 and 20% of the initial activity remained at 60 and 70°C, respectively, under the same conditions. Any significant reduction in both ATP- and CTP-hydrolyzing activities were not observed between pH 7.4 and 9.0 after incubating the enzyme at 45°C for 30 min, but 25 and 30% of the initial activity were lost at pH 5.0 and 10.6, respectively. No significant difference in temperature-stability profiles and pH-stability profiles between these nucleoside triphosphate-hydrolyzing activities was found, suggesting that these activities are due to the same enzyme protein.

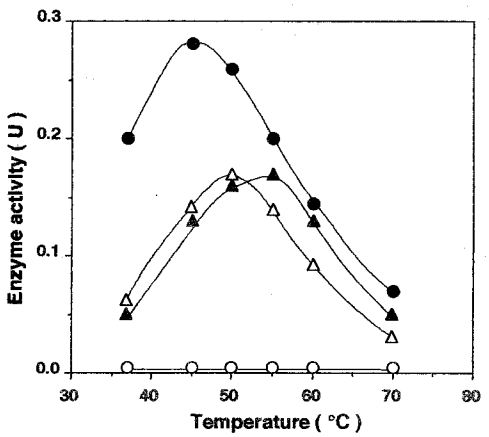


Fig. 1. Effect of temperature on ATP and CTP hydrolysis activities in the presence and absence of *N*-methylhydantoin.
Each reaction was carried out under the end-point assay conditions with (closed symbols) and without (open symbols) *N*-methylhydantoin (10 mM) for 30 min at indicated temperature. Circle and triangle symbols indicate ATP-hydrolyzing and CTP-hydrolyzing activities, respectively.

DISCUSSION

N-Methylhydantoin amidohydrolase catalyzes the coupled hydrolysis of *N*-methylhydantoin and ATP to yield *N*-carbamoylsarcosine, ADP and Pi [28]. For *N*-methylhydantoin hydrolysis the enzyme can utilize several nucleoside triphosphates

besides ATP to a greater extent than urea amidolyase (EC 6.3.4.6) and 5-oxoprolinase (EC 3.5.2.9), both of which are ATP-dependent amidohydrolase and require only ATP and dATP for active hydrolysis of their amide substrates [115,116].

N-Methylhydantoin amidohydrolase also hydrolyzed various nucleoside triphosphates not only in the presence of amide substrate but also in the absence of the amide substrate. 5-Oxoprolinase was also found to hydrolyze various kinds of nucleoside triphosphates to the corresponding nucleoside diphosphates in the absence of 5-oxo-L-proline, its natural amide substrate. With attempt to clarify the mechanism of catalytic reaction of 5-oxoprolinase, Meister and his co-workers [101,103,104,106] found that the binding of both ATP and 5-oxoproline to the enzyme induces a conformational change that brings the substrates into a juxtaposition that facilitates the reaction. In respect to this, *N*-methylhydantoin amidohydrolase seems to change its conformation in the presence of amide substrate and nucleoside triphosphate. Considered from the K_m values observed, it is likely that the binding of amide substrates such as *N*-methylhydantoin and dihydrouracil to the enzyme induces the conformational change in which the binding of various nucleoside triphosphates, other than ATP and dATP, to the enzyme are inhibited. In contrast to these, the binding of amide substrate to the enzyme might lead to the suitable conformation for ATP and dATP. Without this conformation, both ATP and dATP might be unable to bind to the enzyme. On the other hand, the ability of the enzyme to catalyze amide substrate-independent nucleoside triphosphate-hydrolyzing reaction for various kinds of nucleoside triphosphates except ATP and dATP indicate that each substrate is able to bind to the enzyme in the absence of amide substrate.

SUMMARY

N-Methylhydantoin amidohydrolase that catalyzes the coupled hydrolysis of *N*-methylhydantoin and ATP to yield *N*-carbamoylsarcosine, ADP and Pi, was found to hydrolyze several nucleoside triphosphates not only in the presence but also in the

absence of amide substrates. Amide substrates, such as *N*-methylhydantoin and dihydrouracil, seems to be absolutely necessary for hydrolysis of ATP and dATP. However, *N*-methylhydantoin led to inhibitory effects for the hydrolysis of nucleoside triphosphates other than ATP and dATP. The kinetic data suggest that the presence of amide substrate brings change on the affinity of the enzyme toward nucleoside triphosphates.

CONCLUSION

This thesis described the microbial transformations of hydantoin-related compounds and the enzymes involved in these transformations. The enzymes analyzed herein are very useful for the synthesis of optically pure amino acids and for the clinical assay for creatinine. The results described in each chapter can be summarized as follows:

CHAPTER I

Through screening potent hydantoin-transforming microorganisms, *Pseudomonas putida* IFO 12996, which is a D-specific hydantoinase producer, *Comamonas* sp. E222c and *Blastobacter* sp. A17p-4, which are *N*-carbamoyl-D-amino acid amidohydrolase producers, were selected. To determine whether or not hydantoin degradation is identical with pyrimidine degradation, the author investigated the enzyme activities responsible for reductive pyrimidine degradation in these bacteria, and showed that *P. putida* has dihydropyrimidinase activity and that *Comamonas* sp. and *Blastobacter* sp. have β-ureidopropionase activity. Thus, two cyclic ureide-hydrolyzing activities and/or two *N*-carbamoylamino acid hydrolyzing activities involved in hydantoin and pyrimidine transformations coexist in these bacteria. However, differences in the induction levels of each enzyme activities for the several pyrimidine- and hydantoin-related compounds suggest that these corresponding cyclic ureide-hydrolyzing activities or *N*-carbamoylamino acid-hydrolyzing activities are not always catalyzed by the same enzymes.

CHAPTER II

This chapter described the diversity of *N*-carbamoylamino acid-hydrolyzing enzymes.

N-Carbamoyl-D-amino acid amidohydrolases, which are involved in the microbial transformation of DL-5-monosubstituted hydantoins to D-amino acids, were

purified to apparent homogeneity from *Comamonas* sp. and *Blastobacter* sp. Both enzymes had a relative molecular mass of about 120,000 and consisted of three identical subunits with a relative molecular mass of about 40,000. Both enzymes strictly recognized the configuration of the substrate and only the D-enantiomer of the *N*-carbamoylamino acids was hydrolyzed to D-amino acids, ammonia and carbon dioxide. *N*-Carbamoyl-D-amino acids having hydrophobic groups served as good substrates for both enzymes. Neither hydrolyzed β-ureidopropionate, suggesting that *N*-carbamoyl-D-amino acid amidohydrolase differs from β-ureidopropionase, which is involved in pyrimidine degradation (Section 1 and 2).

β-Ureidopropionase of aerobic bacterial origin was purified to homogeneity from *P. putida* IFO 12996. It shows broad substrate specificity for not only *N*-carbamoyl-β-amino acids, but also *N*-carbamoyl-γ-amino acids, and several *N*-carbamoyl-α-amino acids are hydrolyzed. The hydrolysis of *N*-carbamoyl-α-amino acids was strictly L-enantiomer specific. The enzyme required a divalent metal ion, such as Co^{2+}, Ni^{2+} or Mn^{2+}, for the activity, and it was significantly affected by sulfhydryl reagents. The enzyme consisted of two polypeptide chains with an identical relative molecular mass of 45,000. The broad substrate specificity and metal ion dependence of the enzyme showed that the β-ureidopropionase of this aerobic bacterium is quite different from those of mammals and anaerobic bacteria. The enzyme did not hydrolyze *N*-carbamoyl-D-amino acid; suggesting that it is not involved in hydantoin transformation (Section 3).

N-Carbamoyl-L-amino acid amidohydrolase, which is involved in microbial transformation of DL-5-monosubstituted hydantoins to L-amino acids, was purified to homogeneity from *Alcaligenes xylosoxidans* A35. The enzyme had high affinity toward *N*-carbamoyl-L-amino acids with long chain aliphatic or aromatic substituents, and hydrolyzed those with short chain substituents quite well. The relative molecular mass of the native enzyme was about 135,000 and the enzyme consisted of two identical polypeptide chains. The enzyme activity was significantly inhibited by sulfhydryl reagents and required the following divalent metal ions for activity: Mn^{2+}, Ni^{2+} and Co^{2+}. The enzyme did not hydrolyze *N*-carbamoyl-D-amino acids and β-

ureidopropionate; suggesting that it is distinct from *N*-carbamoyl-D-amino acid amidohydrolase and β-ureidopropionase (Section 4).

Thus, the author revealed a variety of *N*-carbamoylamino acid-hydrolyzing enzymes with different stereo and substrate specificities. These enzymes can be used as potent catalysts in the preparation of optically pure amino acids.

CHAPTER III

The diversity of the cyclic ureide-hydrolyzing enzymes was described.

Two cyclic ureide compound-hydrolyzing enzymes were found in *Blastobacter* sp. and partially purified. One hydrolyzed 5-substituted hydantoins D-stereospecifically and showed dihydropyrimidinase activity. The other was a novel enzyme which should be called an imidase. This imidase preferably hydrolyzed cyclic imide compounds such as glutarimide and succinimide more than cyclic ureide compounds, and produced monoamidated dicarboxyrates (Section 1).

Dihydroorotase was purified to homogeneity from *P. putida* IFO 12996. The relative molecular mass of the native enzyme was 82,000 and the enzyme consisted of two identical subunits with a relative molecular mass of 41,000. The enzyme only hydrolyzed dihydro-L-orotate and its methyl ester, and the reactions were reversible. The enzyme was inhibited by metal ion chelators and activated by Zn^{2+}. However, excess Zn^{2+} was rather inhibitory. The enzyme was inhibited by sulfhydryl reagents, and competitively inhibited by *N*-carbamoylamino acids such as *N*-carbamoylglycine. The enzyme was also inhibited noncompetitively by pyrimidine-metabolism intermediates such as dihydrouracil and orotate, respectively, suggesting that the enzyme activity is regulated by pyrimidine-metabolism intermediates and that dihydroorotase plays a role in the control of pyrimidine biosynthesis (Section 2).

Thus, the author showed that many kinds of cyclic ureide-hydrolyzing enzymes are involved in pyrimidine transformation, and that these enzymes are useful for the asymmetric synthesis of amino acids from cyclic ureide compounds.

N-Methylhydantoin amidohydrolase, an ATP-dependent amidohydrolase involved in microbial degradation of creatinine, was purified to homogeneity from *Pseudomonas putida* 77. The enzyme has a relative molecular mass of 300,000. It is a tetramer of two identical small subunits (M_r = 70,000) and two identical large subunits (M_r = 80,000). The enzyme requires ATP for the amidohydrolysis of *N*-methylhydantoin and *vice versa*. Mg^{2+}, Mn^{2+} or Co^{2+}, and K^+, NH_4^+, Rb^+ or Cs^+ were absolutely required concomitantly for the enzyme activity as divalent and monovalent cations, respectively. The enzyme hydrolyzed amide compounds coupled with ATP hydrolysis when hydantoin, L-5-methylhydantoin, glutarimide and succinimide, as well as *N*-methylhydantoin were used as substrates. ATP hydrolysis by the enzyme was stimulated by some amide compounds without undergoing detectable self-hydrolysis (Section 3).

N-Methylhydantoin amidohydrolase hydrolyzed several nucleoside triphosphates not only in the presence, but also in the absence of amide substrates. Amide substrates, such as *N*-methylhydantoin and dihydrouracil, seemed to be absolutely necessary for the hydrolysis of ATP and dATP. However, *N*-methylhydantoin inhibited the hydrolysis of nucleoside triphosphates other than ATP and dATP. The kinetic data suggested that the presence of amide substrates changes the affinity of the enzyme for nucleoside triphosphates (Section 4).

N-Methylhydantoin amidohydrolase was specific for *N*-methylhydantoin, a intermediate of microbial creatinine transformation, and the reaction was irreversible, so the enzyme can be a useful tool for clinical assays of creatinine.

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