Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 23 (2003) 71–85
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Review

Production of D-amino acids by *N*-acyl-D-amino acid amidohydrolase and its structure and function

Mamoru Wakayama [a], Kazuaki Yoshimune [c], Yoshihiko Hirose [b], Mitsuaki Moriguchi [c,*]

[a] *Department of Bioscience and Biotechnology, Faculty of Science and Engineering, Ritsumeikan University, Noji, Kusatsu, Shiga 525-8577, Japan*
[b] *Amano Enzyme Inc., Gifu R&D Center, Sue 4-179-35, Kakamigahara, Gifu 509-0108, Japan*
[c] *Department of Applied Chemistry, Faculty of Engineering, Oita University, Dannoharu 700, Oita 870-1192, Japan*

Received 6 February 2003; accepted 7 May 2003

Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

D-Amino acids have been widely used as synthetic materials for various compounds such as pharmaceuticals and agrochemicals. The manufacture of D-amino acids by fermentation is difficult, and enzymatic methods are mainly employed. At present, the optical resolution method using *N*-acyl-D-amino acid amidohydrolase is the most useful and convenient. In this review, the application of *N*-acyl-D-amino acid amidohydrolase to the production of D-amino acids and recent progress in the study of structure–function relationships from the standpoint of improving this enzyme for industrial application are discussed.

Keywords: D-Amino acids; Optical resolution enzymes for D-amino acids; *N*-Acyl-D-amino acid amidohydrolase

1. Introduction

Recent interest in producing chiral compounds for pharmaceuticals, agrochemicals, and chemicals stems from the fact that not all stereoisomers of a compound are active. In addition, certain isomers may have antagonistic or toxic effects. Chiral compounds are produced in two ways, by direct organic asymmetric synthesis and by the enzymatic resolution of racemates. A method of asymmetric synthesis has yet to be developed for supply of most chemicals. In general, therefore, organic synthesis followed by resolution has been used. At present, enzymatic chiral resolutions have been reported for lipase, protease, aminoacylase, amidase and hydantoinase. The resolution using lipases involves hydrolysis, esterification, and transesterification [1]. A resolution process using the esterase activity of protease has also been developed [2]. L-Aminoacylase (*N*-acyl-L-amino acid amidohydrolase, EC 3.5.1.14), a representative optical resolution enzyme, is the first to be used industrially [3].

In recent years, much attention has been paid to using *N*-acyl-D-amino acid amidohydrolase for the

* Corresponding author. Tel.: +81-97-554-7891; fax: +81-97-554-7890.
E-mail address: mmorigu@cc.oita-u.ac.jp (M. Moriguchi).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1381-1177(03)00074-2

production of D-amino acids. In this review, the present circumstances in terms of the application of *N*-acyl-D-amino acid amidohydrolase to D-amino acid production and recent progress in the study of the structure–function relationship of this enzyme are discussed.

2. Occurrence of D-amino acids

Among amino acids, the L-form is by far the more predominant enantiomer found in nature. However, recently, the sensitivity and efficiency of separative determination of D- and L-amino acids have improved dramatically due to the development of new derivatization reagents of amino acids, new chiral columns and new protocols for analysis by GC [4], GC/MS [5] and HPLC [6]. As a result, an increasing number of studies on the physiological role of D-amino acids have been reported. D-Amino acids occur in plants, microbes and higher organisms. In the living body, D-amino acids can be found in the bound form as well as the free form. But the former has received most attention physiologically as a component of peptides or proteins. D-Amino acid-containing peptides are especially important as pharmaceuticals due to their strong antibacterial activity. These contain D-aspartic acid (bacitracin and mycobacillin), D-glutamic acid (bacitracin), D-cysteine (malformin), D-leucine (circulin and malformin), D-ornithine (bacitracin), D-phenylalanine (bacitracin, fungisporin, gramicidin, polymyxin, and tyrocidine), and D-valine (actinomycin, fungisporin and valinomycin). The amphibian skin peptides dermorphin, which are the most potent morphine-like agonist, contain D-alanine [7]. These physiologically active D-amino acid-containing peptides are shown in Fig. 1. Attention has also been paid to D-amino acids as indicators of aging. D-Aspartic acid has been found in tooth [8], eye lens [9], and white matter of brain [10] in the aged human. In addition, the occurrence of D-aspartic acid and D-serine in amyloid protein related to Alzheimer disease has been reported [11], and the roles of D-amino acids in some diseases have been investigated [12,13]. One possibility is that D-serine, D-serine ethylester and D-cycloserine will serve in the treatment of psychoneurosis diseases such as schizophrenia and dementia [14].

3. The production of D-amino acids by *N*-acyl-D-amino acid amidohydrolase

3.1. Source of enzymes used for producing D-amino acids

D-Amino acids are important chiral intermediates for pharmaceuticals, agrochemicals and food additives. Since fermentation is not an efficient method of producing D-amino acids, increasing demand has made enzymatic production an interesting research subject. There are many enzymes which are used for the production of D-amino acids: *N*-acyl-D-amino acid amidohydrolase [15], D-hydantoinase [16], *N*-carbamoyl-D-amino acid amidohydrolase (D-*N*-carbamoylase) [17], D-amino acid amidase [18], proteases [19], amino acid oxidases [20], and D-amino acid aminotransferase [21] (Fig. 2). When amino acid oxidase is used, one enantiomer of the DL-amino acid is destroyed, and the other can be recovered. Therefore, the procedure is not a resolution. Amino acid aminotransferase can be used for the synthesis of chiral amino acids from the corresponding α-keto acids.

N-Acyl-D-amino acid amidohydrolase catalyzes the hydrolysis of *N*-acyl-D-amino acid to D-amino acid and fatty acid [15]. It has been proposed that L-aminoacylase participates in the hydrolysis of terminal *N*-acylpeptide, in xenobiotic detoxication and bioactivation, and in the interorgan processing of xenobiotic-derived amino acid conjugates [22]. Unlike L-aminoacylase, the role of the *N*-acyl-D-amino acid amidohydrolase is unknown. However, *N*-acyl-D-amino acid amidohydrolase is used industrially for optical resolution production of D-amino acids from DL-amino acids (Fig. 3). *N*-Acyl-D-amino acid amidohydrolase (D-aminoacylase) that acts on *N*-acyl derivatives of various neutral D-amino acids (Fig. 4) has been reported from *Alcaligenes* [15,23–31], *Pseudomonas* [32–37], *Streptomyces* [38,39], *Sebekia* [40], *Variovorax* [41]. D-Aminoacylase from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6 (*Alcaligenes* A-6) has been produced and sold by Amano Enzyme Co. as a commercial enzyme (D-aminoacylase “Amano”) for the manufacture of neutral D-amino acids [42] (Fig. 3). *N*-Acyl-D-glutamic acid amidohydrolase [43] and *N*-acyl-D-aspartic acid amidohydrolase [44] are also useful in the manufacture of D-glutamic acid and D-aspartic acid, respectively. Table 1 shows the

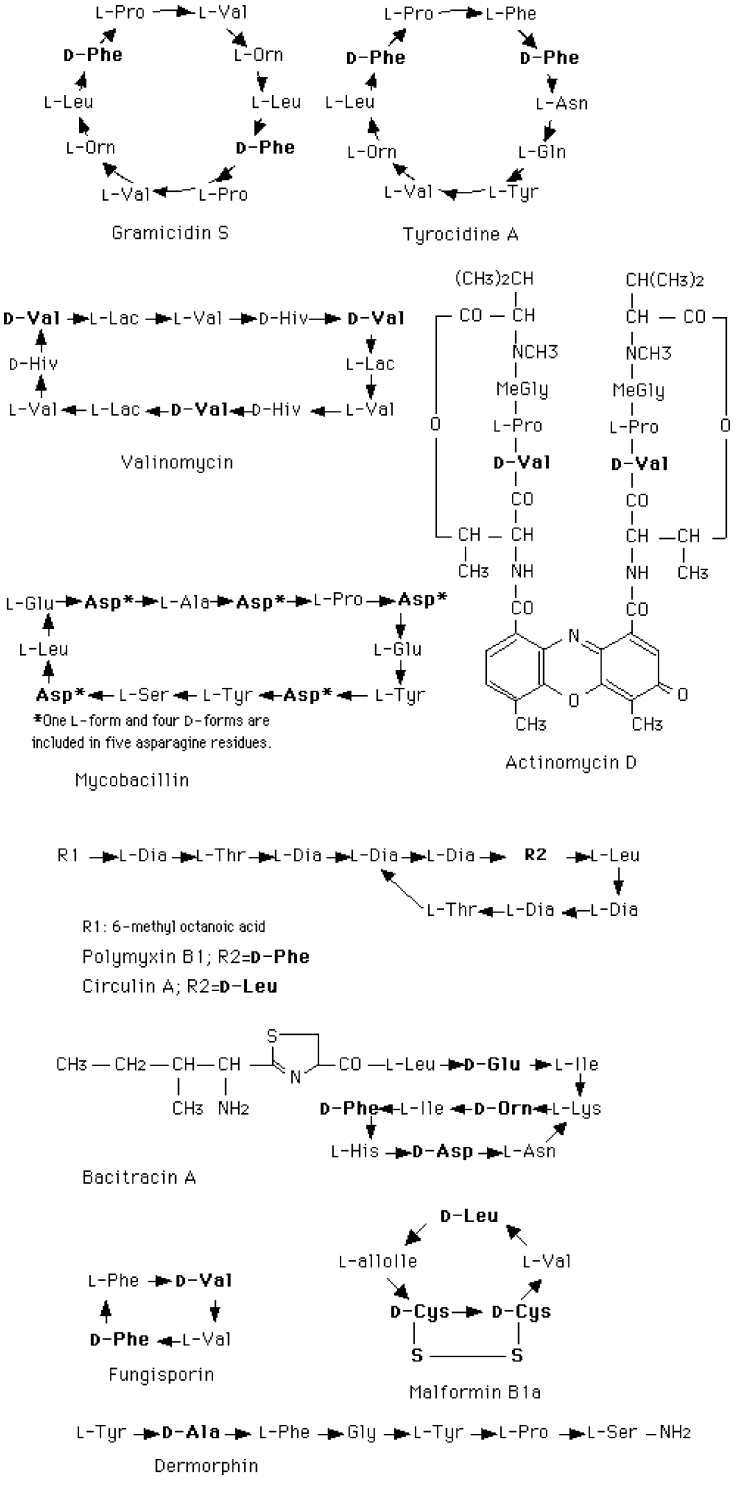


Fig. 1. D-Amino acid-containing bioactive peptide. Dia: α,γ-diaminobutyric acid; Hiv: 3-hydroxyisovaleic acid; Lac: lactic acid; Orn: ornithine.

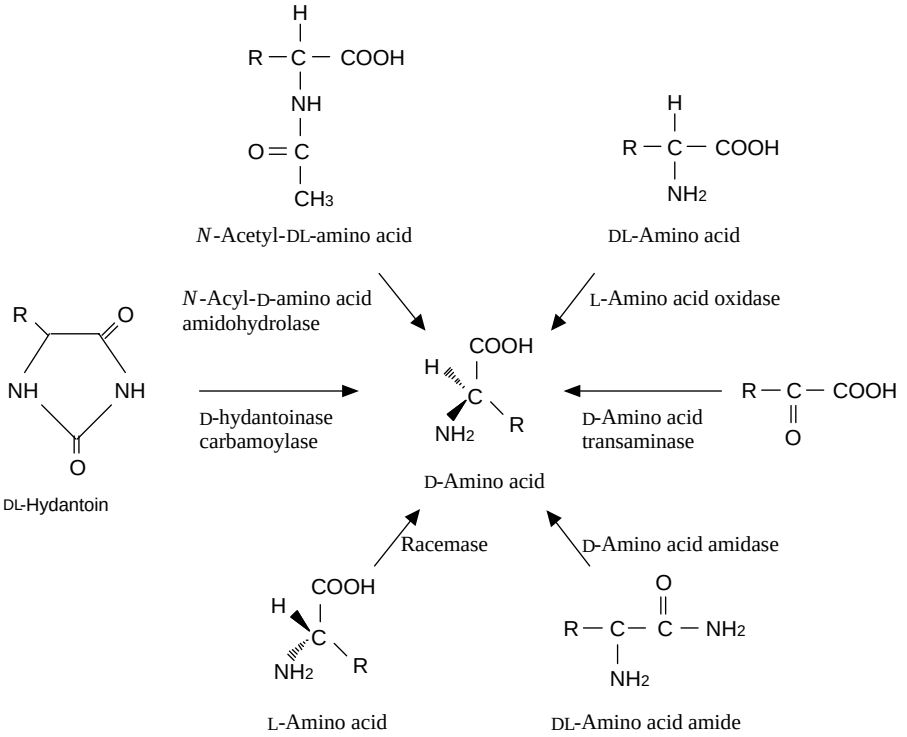


Fig. 2. Several routes for the synthesis of D-amino acid.

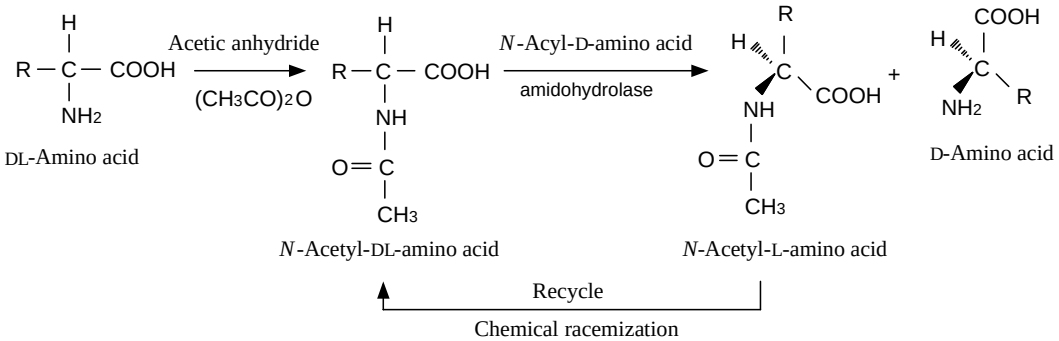
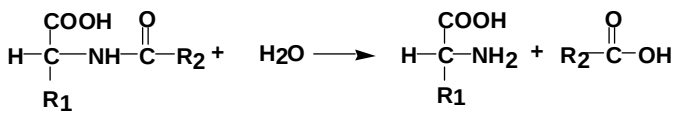


Fig. 3. Commercial process for the production of D-amino acid by *N*-acyl-D-amino acid amidohydrolase.



R2 : H-, ClCH2-, CH3-, CH3CH2-, CH3(CH2)2-

Fig. 4. Deacylation catalyzed by *N*-acyl-D-amino acid amidohydrolase.

Table 1
Summary for D-amino acids produced using *N*-acyl-D-amino acid arnidohydrolases from various microbial sources

	Relative activity (%)												
	Alcaligenes xylosoxydans A-6			*Pseudomonas* sp. 5f-1 D-AGase	*Alcaligenes denitrificans* MI-4 D-ANase	*Alcaligenes faecalis* DA1 D-ANase	*Alcaligenes denitrificans* DA181 D-ANase	*Pseudomonas* sp. 1158 D-ANase	*Pseudomonas* AAA6029 D-ANase	*Streptomyces olivaceus* D-ANase	*Streptomyces tuirus* D-ANase	*Trichoderma harzianum* Rifai D-ANase	*Variovorax paradoxus* D-ANase
	D-AGase	D-AAase	D-ANase										
D-Alanine	–	–	–	–	1	14	25	96	3	7	9	24	53
D-Alloisoleucine	–	–	–	–	1	–	12	–	–	–	–	11	–
D-Arginine	–	–	–	–	–	–	–	–	–	1	1	–	
D-Asparagine	–	–	–	–	–	8	17	33	–	–	2	–	19
D-Aspartic acid	–	100	–	–	–	–	–	–	–	–	–	–	–
D-Glutamic acid	100	–	–	100	–	–	–	–	–	–	6	–	
D-Leucine	–	–	100	–	17	52	60	35	23	100	100	54	84
D-Methionine	–	–	67	–	100	100	100	100	30	47	60	96	100
D-Norleucine	–	–	74	–	38	–	–	–	–	–	–	–	–
D-Phenylalanine	–	–	87	–	80	65	81	39	100	76	89	100	24
D-Phenylglycine	–	–	–	–	–	3	5	–	–	10	9	–	–
D-Tryptophan	–	–	–	–	5	14	33	40	–	7	10	37	5
D-Tyrosine	–	–	–	–	–	–	–	–	–	32	46	–	4
D-Valine	–	–	8	–	1	6	6	18	–	27	28	43	18

D-AGase: *N*-acyl-D-glutamate amidohydrolase; D-AAase: *N*-acyl-D-aspartate amidohydrolase; D-ANase: D-aminoacylase.

possibility of producing various D-amino acids using D-aminoacylases from various sources.

3.2. Commercial production of D-aminoacylase

The manufacture of D-amino acids by fermentation is difficult, and enzymatic methods are mainly employed. Optical resolution with L-aminoacylase has been used by the industry for some time, and it is regularly applied commercially in enzyme reactions. This section describes an improved method for large-scale expression of D-aminoacylase.

3.2.1. Improvement of producer bacteria for commercial production

The gene encoding D-aminoacylase from *Alcaligenes* A-6 was cloned into pKK223-3. This new construct, pKNSD2, was used for recombinant bacterial expression of D-aminoacylase [45]. Since D-aminoacylase has two zinc ions coordinated in the catalytic site [46], we investigated whether the addition of zinc to the growth medium could increase D-aminoacylase yield. Not surprisingly, supplementation of zinc did increase enzyme yield. A concentration of 2 mM zinc was found to be optimum (Table 2) [42]. However, no effect of zinc ion on D-aminoacylase production was observed on strain *Alcaligenes* A-6.

Table 2
Effect of a zinc ion concentration on the D-aminoacylase productivity

Zinc ion concentration (mM)	*Alcaligenes* A-6 (U ml^{-1})	TG1/pKNSD2 (U ml^{-1})
0	0.29	21.8
0.2	0.12	58.9
2.0	0.29	109

One unit of D-aminoacylase activity was defined as the amount of enzyme which catalyzes the formation of 1 μmol of D-amino acid per 30 min.

3.2.2. Commercial production of D-aminoacylase

Since this is a bacterial intracellular enzyme and the production of bacterial cells is an index of productivity, increasing the production of bacterial cells would directly impact enzyme yield. Various materials were investigated, and the optimum culture conditions for the production of D-aminoacylase were established [42]. The production was found to be increased to 40 g l^{-1} wet weight.

Sufficient recovery of the enzyme from the lysed cells is important in the purification process. Cell lysis by enzymes is practical for the commercial process. For cell wall lysis enzymes, for example, lytic enzymes and lysozymes of our company can be used. In such cases, the addition of surfactants, such as Triton-X, markedly increased the efficiency of enzyme extraction. In the actual purification process, after the lysis of bacterial cells using enzymes, almost pure D-aminoacylase was obtained by the standard method shown in Fig. 5. The final form of the enzyme was freeze-dried. We successfully obtained 10 U g^{-1} or more of freeze-dried product as activity on *N*-acetyl-D-methionine. The yield for enzyme extraction from the bacteria is predicted to be about 75%.

3.2.3. Enzyme properties

The optimum temperature and pH for D-aminoacylase were found to be 45 °C and pH 8.0, respectively. However, the enzyme was also shown to be stable and active in solution at 50 °C or lower and at a pH of 5 or higher. The reactivity to each amino acid (substrate specificity), which is the most important property, is shown in Table 3. Commenting on substrate specificity is difficult, but the enzyme does not generally react with sterically hindered amino acids.

A concrete example of a reaction in the process of producing D-phenylalanine from *N*-acetyl-DL-phenylalanine is described. D-Aminoacylase was added to a 0.5 M *N*-acetyl-DL-phenylalanine solution and adjusted to 18 U ml^{-1}. At pH 7.5 and 30 °C, 47.5

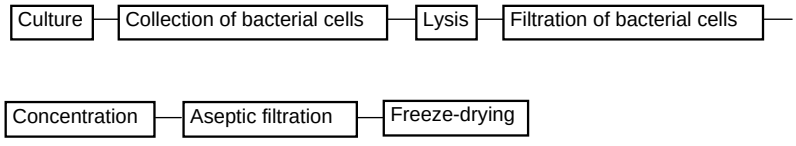
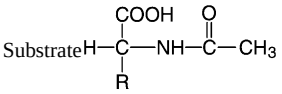
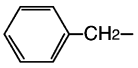
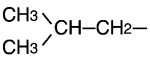
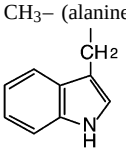
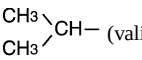
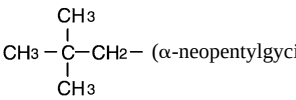
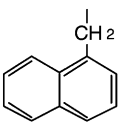


Fig. 5. Purification process of D-aminoacylase.

Table 3
Substrate specificity of D-aminoacylase

Substrate 	Relative activity (%)
CH_3–S–CH_2–CH_2– (methionine)	100
 (phenylalanine)	82
 (leucine)	70
CH_3– (alanine)	12
 (tryptophan)	4
 (valine)	3
CH_2OH– (serine)	2
 (α-neopentylglycine)	60
CH_3–CH_2– (aminobutyric acid)	70
 (1-naphthyalanine)	0

and 49.5% of D-phenylalanine were obtained after 17 and 41 h, respectively. The product was quantitated using an HPLC system (Fig. 6). The optical purity of D-phenylalanine was 100%. The residual *N*-acetyl-L-phenylalanine can be repeatedly used as a substrate in the reaction after racemization by the standard method under alkaline or acidic conditions.

This enzyme was markedly inhibited: 98, 75, 72, and 60%, by 2 mM copper, iron, zinc, and nickel, respectively. This inhibition was dependent on the type of substrate amino acid, for instance, it was observed in the case of resolution of *N*-acetyl-DL-phenylalanine, but not in the case of *N*-acetyl-DL-methionine. Although the enzyme is a zinc protein [46], an excess of zinc ion inhibited the activity of the enzyme. One reason for this inhibition is that excessive zinc ion concentrations may block essential SH groups of the enzyme [47–49].

To test the safety of the enzyme and commercial product, a toxicity study of D-aminoacylase was performed. In a single oral dose toxicity study in rats, the LD_{50} value was estimated to be higher than 2000 mg kg^{-1} for males and females, showing that the enzyme is safe for normal use.

3.2.4. D-Amino acid business

Hydantoinase and amidase are used in the commercial production of D-amino acids. The use of D-aminoacylase is a third alternative. Pharmaceutical intermediates derived from D-amino acids are shown in Fig. 7, and the application for various compounds is much expected. Pharmaceutical drugs derived from D-amino acids are shown in Fig. 8 and they are now under development. There are compounds possessing D-amino acids in their backbone, which are frequently used as materials for pharmaceuticals and agricultural chemicals. Highly useful D-amino acids at present include D-phenylalanine, D-serine, D-valine, and D-tryptophan. D-*p*-Hydroxyphenylglycine used for the side chain of antibiotics is a typical non-natural amino acid.

Although predicting future trends is difficult, the market in this field may expand. Pharmaceuticals in the early phase of clinical study include many products with D-amino acids. For example, Tadalafil (Ciatis) being developed as a therapeutic drug for astasia by Eli Lily uses D-tryptophan. Part of the structure of this drug is shown in Fig. 8.

4. Structure and function of *N*-acyl-D-amino acid amidohydrolase

4.1. Prediction of active center structure of *N*-acyl-D-amino acid amidohydrolase

As described in the previous section, for D-amino acid production, the following enzymes have been studied, i.e. D-aminoacylases from *Alcaligenes* A-6, *Alcaligenes denitrificans* DA181 (*Alcaligenes* DA181) [29,30], and *Alcaligenes faecalis* DA1 (*Alcaligenes* DA1) [31]; *N*-acyl-D-glutamic acid amidohydrolases from *Pseudomonas* sp. 5f-1 [35] and *Alcaligenes* A-6; and *N*-acyl-D-aspartic acid amidohydrolase from *Alcaligenes* A-6. In recent years, the D-aminoacylases from *Sebekia benihana* [40], and *Variovorax paradoxus* [41] have been reported. However, studies on the structure of the active site using chemical modification

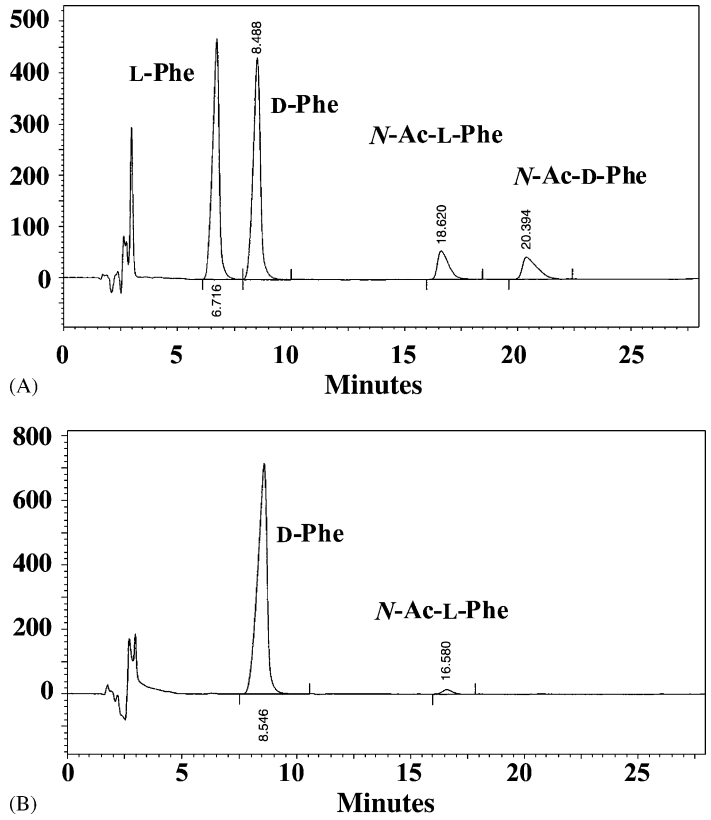


Fig. 6. HPLC chromatogram: (A) standard; (B) reaction mixture. The HPLC column was a SUMICHIRAL OA-5000. The mobile phase was 2 mM copper sulfate:isopropanol (85:15) at a flow rate of 0.8 ml min^{-1} with UV detection at 254 nm.

have been reported only for three enyzymes, the D-aminoacylases from *Alcaligenes* A-6 [46] and *Alcaligenes* DA1 [50], and *N*-acyl-D-glutamic acid amidohydrolase from *Pseudomonas* sp. 5f-1 [37]. Various chemical modification reagents are used for investigating the active site of an enzyme. The effects of these reagents on the enzymatic reaction are summarized in Table 4. The activity of D-aminoacylase from *Alcaligenes* A-6 and *N*-acyl-D-glutamic acid amidohydrolase from *Pseudomonas* sp. 5f-1 largely decreased in the presence of diethylpyrocarbonate and phenylglyoxal. While D-aminoacylase from *Alcaligenes* DA1 was strongly inhibited by PCMB (over 95% inhibition), 39% inhibition of the activity by PCMB was observed in *N*-acyl-D-glutamic acid amidohydrolases from *Alcaligenes* A-6. Diethylpyrocarbonate and phenylglyoxal were found to be inactivate both D-aminoacylase from *Alcaligenes* A-6 and *N*-acyl-D-glutamic acid amidohydrolase from *Pseudomonas* sp. 5f-1, strongly suggesting that histidine and arginine residues are important for hydrolytic catalysis at the enzyme catalytic site. On the other hand, *N*-acetylimidazole only affects *Alcaligenes* A-6 D-aminoacylase while *p*-chloromercuribenzoic acid specifically inhibits *Alcaligenes* DA1 D-aminoacylase. This suggests that tyrosine and cysteine residues are not necessarily conserved in the catalytic sites.

The primary structure of *N*-acyl-D-glutamic acid amidohydrolase from *Alcaligenes* A-6 was for the first time determined as a *N*-acyl-D-amino acid amidohydrolase, followed by *N*-acyl-D-aspartic acid amidohydrolase and D-aminoacylase from *Alcaligenes* A-6. As mentioned in the previous sections, these enzymes produced by *Alcaligenes* A-6, which definitely differ from each other in substrate specificity, are expected to be useful for D-amino acid production. So, for

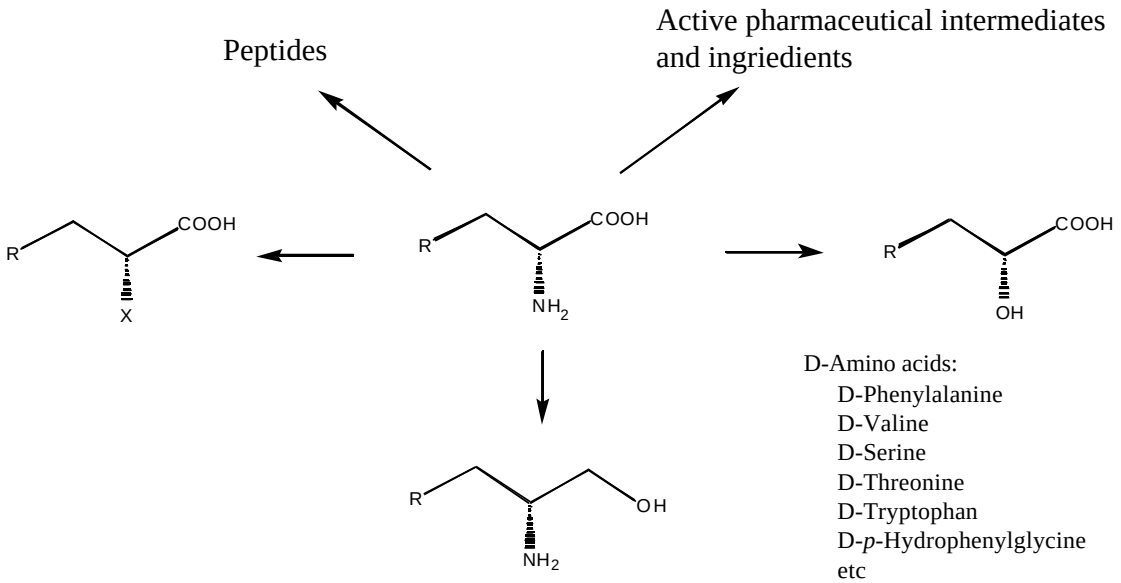


Fig. 7. Pharmaceutical intermediates derived from D-amino acids.

Therapeutic drugs using D-amino acids

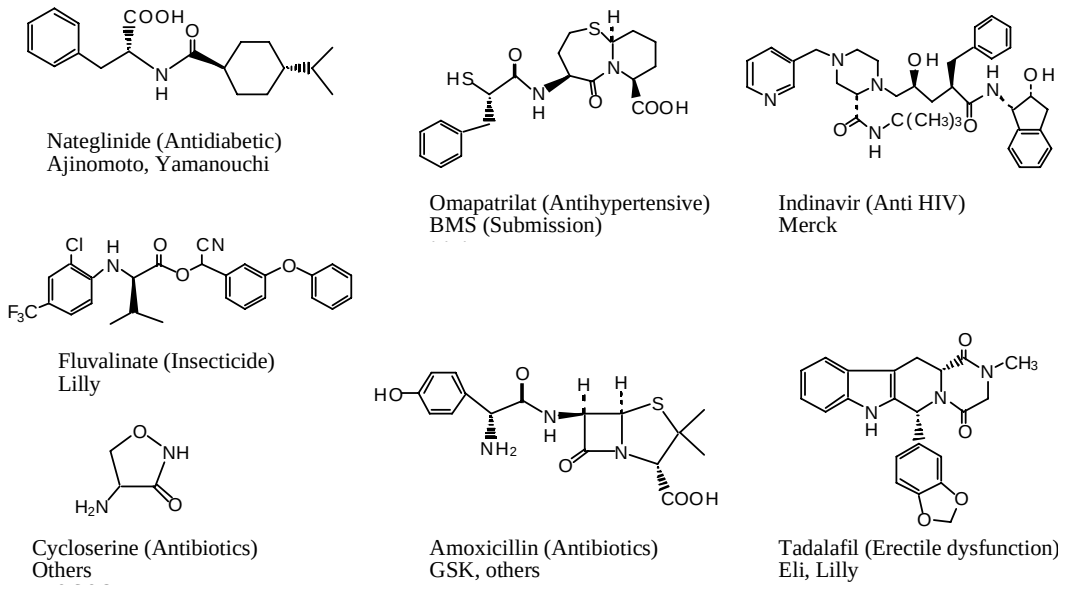


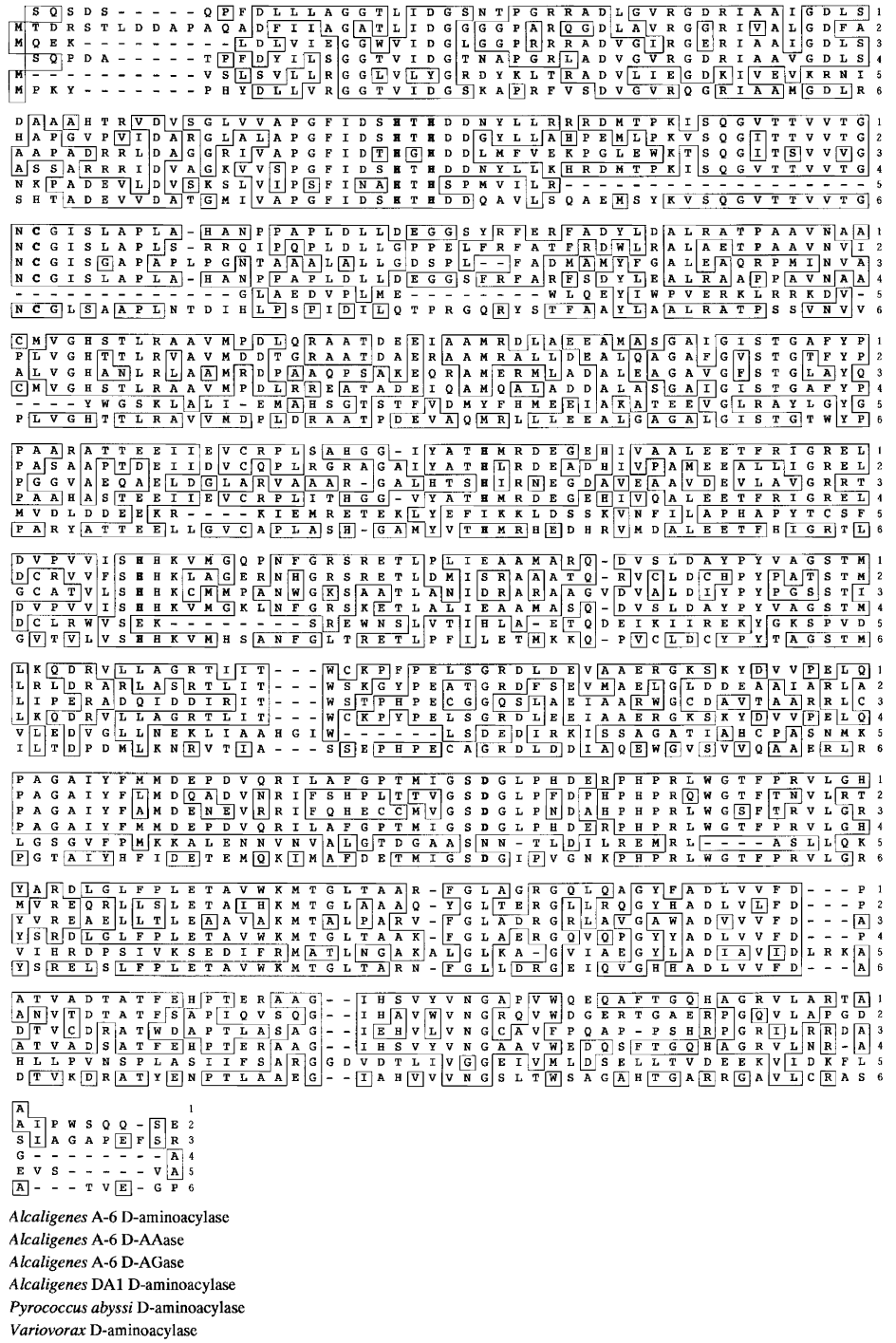
Fig. 8. Therapeutic drugs using D-amino acids.

Table 4
Effects of various site-specific reagents on the enzyme activity

Reagents (1 mM)	Residual activity (%)		
	Alcaligenes A-6 D-ANase	*Pseudomonas* sp. 5f-1 D-AGase	*Alcaligenes faecalis* DA1 D-ANase
No addition	100	100	100
N-Acetylimidazole	12	79	n.d.
4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride	83	78	n.d.
3-Bromopyruvate	99	98	n.d.
p-Chloromercuribenzoic acid	n.d.	82	n.d.
Diethyl pyrocarbonate	7.1	14	n.d.
Diisopropyl fluorophosphate	99	85	n.d.
5′,5′-Dithiobis(2-nitrobenzoic acid)	84	90	65
L-*trans*-Epoxysuccinyl-leucylamido-(4-guanidino)butane	n.d.	n.d.	96
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide	84	106	n.d.
N-Ethylmaleimide	90	96	74
Iodoacetate	117	94	n.d.
Phenylglyoxal	45	54	n.d.
Phenylmethylsulfonyl fluoride	n.d.	60	n.d.
Tosyl-L-lysine chloromethyl ketone	n.d.	70	n.d.
Tosyl-L-phenylalanine chloromethyl ketone	n.d.	61	n.d.
2,4,6-Trinitrobenzene-1-sulfonate	60	87	n.d.

D-ANase: D-aminoacylase; D-AGase: *N*-acyl-D-glutamate amidohydrolase; n.d.: not determined.

the purpose of constructing a high-expression system and understanding the structure–function relationship of these enzymes, the genes of these enzymes were cloned and their nucleotide sequences were determined [43,44,51]. Recently, D-aminoacylase genes from *Alcaligenes* DA1 and *V. paradoxus* have been cloned and their primary structures have been also clarified [41,50]. Genomic projects of *Pyrococcus abyssi*, *Mycobacterium tuberculosis*, *Streptomyces coelicolor*, and *Caulobacter crescentus* have revealed that these microorganisms have homologous proteins with D-aminoacylases from *Alcaligenes* A-6, *Alcaligenes* DA1 and *V. paradoxus* (refer to the EMBL/GenBank/DDBJ databases). An alignment of the amino acid sequences of these enzymes is shown in Fig. 9. These enzymes share high sequence identity and similarity. Lin et al. have pointed out that at least seven conserved regions exist among them [41]. A comparison of the primary structures revealed the existence of highly conserved histidine residues implied by chemical modification, and strongly impressed the importance of histidine residues in these enzymes. In particular, histidine residues in both conserved regions 1 and 3, compared to the active site of urease from *Klebsiella aerogenes* [52] which is recognized to have structural similarity with *N*-acyl-D-amino acid amidohydrolase, were assumed to be of importance in catalysis. The amino acid residues involved in the essential functions of hydrolytic catalysis and substrate recognition have been investigated by site-directed mutagenesis [46,50]. According to chemical modification studies, and alignments of amino acid sequences, histidine and arginine residues were selected as targets for an amino acid substitution. In zinc dependent enzymes, histidine, cysteine, and aspartic acid residues are most frequently involved in metal binding. Thus, cysteine and aspartic acid were selected as good candidates for mutational analysis. Some mutant enzymes of D-aminoacylase from *Alcaligenes* A-6 have been constructed by replacing histidine residues of conserved regions 1 and 3 with asparagine or isoleucine and then characterized (Table 5). Both the H67N and H67I mutants scarcely showed any enzyme activity. Both the H69N and H69I mutants were overproduced in the insoluble fraction [46]. The activity of the H250N mutant strikingly decreased and the Zn content of this mutant was less than half of that of wild-type enzyme. These results suggested that the H67 residue might be essential for catalysis and the H250 residue might be responsible for Zn binding. Both H69N and H69I mutants were overproduced in insoluble fraction [46]. We hypothesized that the H69



1 *Alcaligenes* A-6 D-aminoacylase
2 *Alcaligenes* A-6 D-AAase
3 *Alcaligenes* A-6 D-AGase
4 *Alcaligenes* DA1 D-aminoacylase
5 *Pyrococcus abyssi* D-aminoacylase
6 *Variovorax* D-aminoacylase

Fig. 9. An alignment of the amino acid sequences of D-aminoacylases. The conserved amino acid residues are boxed. The amino acid residues that locate in the active site of D-aminoacylase from *Alcaligenes denitrificans* DA181 are in bold type.

Table 5
Kinetic parameters of the wild-type and mutated enzymes

	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,mM^{-1}$)	Zinc content (g atom mol^{-1})
Alcaligenes A-6				
Wild-type	1.93	7.35×10^4	3.81×10^4	2.3
H67N	1.44	0.88	0.61	2.2
H250N	11.6	1.78×10	1.53	0.7
H251N	0.61	3.56×10^4	5.83×10^4	n.d.[a]
Alcaligenes DA-1				
Wild-type	0.63	2.76×10^5	4.38×10^5	1.43
C144A	0.82	2.85×10^5	3.46×10^5	1.43
C207A	0.53	1.94×10^5	3.69×10^5	1.44
C308A	0.95	1.94×10^5	2.04×10^5	1.43
C96A	n.d.	n.d.	n.d.	0.23
D65A	n.d.	n.d.	n.d.	1.27
H220A	n.d.	n.d.	n.d.	0.69
D366A	n.d.	n.d.	n.d.	1.42

[a] Not determined.

mutant might lose the Zn-binding ability, and this could affect the enzyme stability. Hsu et al. predicted the structural fold of D-aminoacylase from *Alcaligenes* DA1 using the program 3D-PSSM [53] on the basis of the crystal structures of *K. aerogenes* urease [52] and *E. coli* dihydroorotase [54]. According to the predicted model, they proposed that H67, H69, C96, and D366 are the ligands for the first zinc ion, and C96, H220, and H250 are the ligands for the second zinc ion. To test this model, the identified residues including C144, C207, C308, C96, D65, H220, and D366 were subjected to alanine substitution mutation analysis. The kinetic properties of the mutant enzymes are also shown in Table 5. Hsu et al. proposed that C96, D366, and H220 should newly join the members of Zn ligands and C96 is responsible for the binding of two Zn ions. Hsu et al. explained that this was the first example of a cysteine residue that might coordinate with zinc ions in the α/β-barrel amidohydrolase superfamily. In that regard, D-aminoacylase from *Alcaligenes* DA1 might be considered as a novel subset of the amidohydrolase superfamily.

4.2. Basic understanding of structure–function relationship of N-acyl-D-amino acid amidohydrolase

In recent years, the recognition that the computational prediction methods of protein 3D structure can provide the 3D structure highly reflecting its real 3D structure has been received in public, and many researchers have studied protein structure and developed medicines using computational analysis program of protein 3D structure. Since this method was considered to be helpful for understanding structure–function relationships and improving an enzyme, a prediction of the 3D structure of *N*-acyl-D-amino acid amidohydrolase from *Alcaligenes*A-6 strain has been made using programs developed recently. As mentioned in the previous section, Hsu et al. have predicted the 3D structure of D-aminoacylase from *Alcaligenes* DA1 using the program 3D-PSSM on the basis of 3D structures of both urease from *K. aerogenes* and dihydroorotase from *E. coli* to clarify the role of the amino acid residues composing the active site [50]. Hsu et al. proposed that C96 and H220 should newly join in the members of Zn ligands and C96 is responsible for the binding of two Zn ions, and D366 might be serve as a general base in the enzyme catalysis. On the other hand, the authors indicated that the D-aminoacylase from *Alcaligenes* A-6 has a similar 3D structure to urease from *K. aerogenes* on the basis of the following results; D-aminoacylase from *Alcaligenes* A-6 showed high homology (48%) with D-aminoacylase from *P. abysii* in the amino acid sequence and D-aminoacylase from *P. abysii* exhibited high structural similarity with urease from *K. aerogenes* by PSI-BLAST ($E < 3 \times 10^{-4}$) [55]. We constructed a 3D structure model of D-aminoacylase

from *Alcaligenes* A-6 using the program 3D-PSM or FFAS on the basis of the crystal structure of urease from *K. aerogenes* (unpublished data). The predicted structures indicated that C96, D366, H67, H69, H220, and H250 were located in the cavity of the enzyme and that these amino acid residues could play important roles in catalytic events.

Quite recently, Tsai and his colleagues reported the results of the X-ray crystallographic analysis of D-aminoacylase from *Alcaligenes* DA1 [56,57]. They provided detailed information on the 3D structure. Their analysis revealed that the enzyme not only can be classified in the amidohydrolase superfamily having an α/β barrel structure-like urease [52], dihydroorotase [54], adenosine deaminase [58], and cytosine deaminase [59], but also belongs to a novel subset as Hsu et al. have proposed from the results of a site-specific mutagenesis study [50]. The crystal structure of the enzyme revealed that the amino acid residues, H67, H69, H220, H250, C96, and D366, are predicted to be located in the active site by sequence homology analysis, site-specific mutagenesis, and structural modeling, actually exist in the active pocket, and suggested hypothetical roles for them in direct catalysis or zinc ion binding. Characteristic of this enzyme is that two zinc ions bind to the binuclear metal center with different affinities and are bridged by a thiolate ligand of cysteine (C96) instead of a carboxylate ligand of glutamate or carboxylated lysine. Also, the enzyme is a mononuclear zinc enzyme containing a binuclear active site. Here, we have to touch upon the catalytic mechanism of D-aminoacylase from *Alcaligenes* DA1 proposed on the basis of the 3D structure. Liaw et al. have proposed a catalytic mechanism by constructing a 3D structure model coupled with the modeled substrate, *N*-acetyl-D-methionine. In this proposed mechanism, D366 acts to abstract the proton from the water molecule, and the tightly bound zinc ion, coordinated by C96, H220, and H250, supports the formation of a tetrahedral intermediate.

Finally, we would like to give some consideration to ways of improving the enzymatic properties to make it more suitable for industrial use on the basis of the results described above. As the amino acid residues responsible for the abstraction of protons from water molecules in the hydrolytic process, H67 and D366 should be nominated. According to the crystal structure of D-aminoacylase from *Alcaligenes* DA1, C96, H69, H220 and H250 as well as H67 could be considered as residues essential to zinc ion binding. To change catalytic property, it seems to be interesting to make the mutant enzyme substituted D366 to glutamic acid. If D366 may serve as a general base at the active center, on the basis of X-ray crystallographic analysis of D-aminoacylase from *Alcaligenes* DA1, substitution of glutamic acid for D366 changes the length between carboxylic group and amido bond of substrate. This change might bring about the changes of substrate preference or catalytic ability of the enzyme. Or focusing on the ability for zinc ion binding, mutant enzymes in which C96 is substituted with lysine, glutamic acid, aspartic acid, threonine, and serine should be investigated for their interaction with other metal ions, Co^{2+} and Ni^{2+}. The activity of D-aminoacylase from *Alcaligenes* DA181 was almost completely inhibited by Zn^{2+} at a concentration of 1 mM, but the same concentration of Co^{2+} scarcely inhibited the activity. Moreover, EDTA-inactivated enzyme could be reactivated to full activity by the addition of 1.0–3.0 mM Co^{2+}, while the addition of 0.1–1.0 mM Zn^{2+} to EDTA-inhibited enzyme produced at maximum less 1/2 recovery than Co^{2+} [30]. These activation/inhibition profiles of Zn^{2+} and Co^{2+} in *Alcaligenes* DA181 D-aminoacylase are similar to the profiles seen for *Alcaligenes* A-6 D-aminoacylase [46]. D-Aminoacylase, which acts on only *N*-acyl derivatives of neutral D-amino acids such as methionine, leucine, and tryptophan, does not hydrolyze *N*-acyl derivatives of acidic or basic D-amino acids except D-arginine. *N*-Acyl-D-glutamic acid amidohydrolase and *N*-acyl-D-aspartic acid amidohydrolase are the only enzymes reported to catalyze the hydrolysis of *N*-acyl derivatives of acidic D-amino acids. Tsai et al. indicated that L298 of D-aminoacylase from *Alcaligenes* DA1 is substituted with arginine in *N*-acyl-D-glutamic aicd amidohydrolase and *N*-acyl-D-aspartic acid amidohydrolase from *Alcaligenes* A-6. It may be possible to construct a D-aminoacylase with broad substrate specificity able to act on *N*-acyl derivatives of acidic D-amino acids by substituting L298 of D-aminoacylase from *Alcaligenes* DA1 with arginine, histidine, or lysine. The information on the amino acid residues responsible for the specificity of the side chain and acyl moiety has been unclear. C96, H69, and H250 which are considered as indispensable to zinc ion binding should be of course

considered to be important for enzyme stability. We examined the roles of seven arginine residues, R26, R152, R296, R302, R354, R377, and R391, which are conserved in *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6, by constructing various mutant enzymes. A search for single substitutions to lysine among conserved arginine residues in D-aminoacylase identified R26K, R152K, R296K, and R302K as mutations that increase partitioning into inclusion bodies. It is possible that these arginine residues may have a role in maintaining the local protein structure and are critical for the soluble expression of D-aminoacylase. In contrast, no mutants with substitutions within the carboxyterminal segment were found to increase partitioning into inclusion bodies. We concluded that arginine residues positioned between the *N*-terminus and central region could play an important role in facilitating folding of D-aminoacylase or stabilizing the final structure (unpublished data). Obtaining a better understanding of the precise structural role of the arginine residues in the enzyme could facilitate improvements in stability.

Further study on the active site using mutagenesis will provide a detailed mechanism of substrate recognition, and analysis of crystals with inhibitors is important for confirming the catalytic mechanism of the enzyme. At this time, a 3D structural analysis based on X-ray crystallography has been reported for D-aminoacylase, one of the *N*-acyl-D-amino acid amidohydrolases, which acts on only *N*-acyl derivatives of neutral D-amino acids such as methionine, leucine, and tryptophan. The 3D structural analysis of *N*-acyl-D-aspartic acid amidohydrolase and *N*-acyl-D-glutamic acid amidohydrolase acting on *N*-acyl derivatives of D-aspartic and D-glutamic acids, respectively, from *Alcaligenes* A-6 should be also performed to obtain a good understanding of substrate specificity.

References

[1] R. Kazlauskas, U. Bornscheuer, in: H.J. Rehm, G. Reed, A. Puhler, P.J.W. Stadler, D.R. Kelly (Eds.), Biotechnology, vol. 8a, Wiley-VCH, Weinheim, 1998, p. 37.
[2] Sepracor. Pat. WO 88/07582 (PCT/US/01098).
[3] T. Tosa, T. Moei, N. Fuse, I. Chibata, Biotech. Bioeng. 9 (1967) 603.
[4] M.H. Engel, P.E. Hare, in: G.C. Barrett (Ed.), Chemistry and Biochemistry of the Amino Acids, Chapman & Hall, London, 1985, p. 462.
[5] R.A.W. Johnstone, M.E. Rose, in: G.C. Barrett (Ed.), Chemistry and Biochemistry of the Amino Acids, Chapman & Hall, London, 1985, p. 480.
[6] D. Perrett, in: G.C. Barrett (Ed.), Chemistry and Biochemistry of the Amino Acids, Chapman & Hall, London, 1985, p. 426.
[7] A. Mor, A. Delfour, P. Nicolas, J. Biol. Chem. 266 (1991) 6264.
[8] P.M. Helfman, J.L. Bada, Proc. Natl. Acad. Sci. U.S.A. 72 (1975) 2891.
[9] P.M. Masters, J.L. Bada, J.S. Zigler, Nature 268 (1977) 71.
[10] E.H. Man, M.E. Sandhouse, J. Burg, G.H. Fisher, Science 220 (1983) 1407.
[11] R. Shapira, G.E. Austin, S.S. Mirra, J. Neurochem. 50 (1998) 69.
[12] G.H. Fisher, A. D'Aniello, A. Vetere, L. Padula, G.P. Cusano, E.H. Man, Brain Res. Bull. 26 (1991) 983.
[13] G.H. Fisher, N. Lorenzo, H. Abe, E. Fujita, W.H. Frey, C. Emory, M.M. DiFiore, A. D'Aniello, Amino Acids 15 (1998) 263.
[14] K. Saigoh, K. Matsui, K. Takahashi, Brain Res. 808 (1998) 42.
[15] M. Wakayama, M. Moriguchi, J. Mol. Catal. B: Enzym. 12 (2001) 15.
[16] H. Yamada, S. Takahashi, Y. Kii, H. Kumagai, J. Ferment. Technol. 56 (1978) 484.
[17] Y. Ikenaka, H. Nanba, K. Yajima, Y. Yamada, M. Takano, S. Takahashi, Biosci. Biotechnol. Biochem. 63 (1999) 91.
[18] H. Komeda, Y. Asano, Eur. J. Biochem. 267 (2000) 2028.
[19] J.L. Abernnethy, E. Albano, J. Comyns, J. Org. Chem. 36 (1971) 1580.
[20] W. Walter, R.F. Becker, Liebigs, Ann. Chem. 755 (1972) 145.
[21] N. Nakajima, K. Tanizawa, H. Tanaka, K. Soda, J. Biotechnol. 8 (1988) 243.
[22] M.W. Anders, W. Dekant, in: J. Thomas (Ed.), Advances in Pharmacology, vol. 27, Academic Press, New York, 1994, p. 431.
[23] M. Moriguchi, K. Ideta, Appl. Environ. Microbiol. 54 (1988) 2767.
[24] K. Sakai, T. Obata, K. Ideta, M. Moriguchi, J. Ferment. Bioeng. 71 (1991) 79.
[25] M. Moriguchi, K. Sakai, Y. Miyamoto, M. Wakayama, Biosci. Biotechnol. Biochem. 57 (1993) 1149.
[26] K. Sakai, K. Imamura, M. Goto, I. Hirashiki, M. Moriguchi, Agric. Biol. Chem. 54 (1990) 841.
[27] K. Sakai, K. Imamura, Y. Sonoda, H. Kido, M. Moriguchi, FEBS Lett. 289 (1991) 44.
[28] M. Moriguchi, K. Sakai, Y. Katsuno, T. Maki, M. Wakayama, Biosci. Biotechnol. Biochem. 57 (1993) 1145.
[29] Y.C. Tsai, C.P. Tseng, K.M. Hsiano, L.Y. Chen, Appl. Environ. Microbiol. 54 (1988) 984.
[30] Y.B. Yang, K.M. Hsaio, H. Li, H. Yano, A. Tsugita, Y.C. Tsai, Biosci. Biotechnol. Biochem. 56 (1992) 1392.

[31] Y.B. Yang, C.S. Lin, C.P. Tseng, Y.J. Wang, Y.C. Tsai, Appl. Environ. Microbiol. 51 (1991) 1259.
[32] T. Kubo, T. Ishikura, Y. Kukagawa, J. Antibiot. 33 (1980) 550.
[33] K. Kubo, T. Ishikura, Y. Fukagawa, J. Antibiot. 33 (1980) 556.
[34] Y. Kameda, H. Hase, S. Kanamoto, Y. Kita, Chem. Pharm. Bull. Tokyo 26 (1978) 2698.
[35] K. Sakai, K. Oshima, M. Moriguchi, Appl. Environ. Microbiol. 57 (1991) 2540.
[36] M. Wakayama, Y. Miura, K. Oshima, K. Sakai, M. Moriguchi, Biosci. Biotechnol. Biochem. 59 (1995) 1489.
[37] M. Wakayama, T. Tsutsumi, H. Yada, K. Sakai, M. Moriguchi, Biosci. Biotechnol. Biochem. 60 (1996) 650.
[38] M. Sugie, H. Suzuki, Agric. Biol. Chem. 42 (1978) 107.
[39] M. Sugie, H. Suzuki, Agric. Biol. Chem. 42 (1980) 1089.
[40] S. Tokuyama, US Patent 6 030 823 (2000).
[41] P.H. Lin, S.C. Su, Y.C. Tsai, C.Y. Lee, Eur. J. Biochem. 269 (2002) 4868.
[42] K. Takeuchi, Y. Koide. Y. Hirose, M. Moriguch, K. Isobe, Pat. WO 00/78926.
[43] M. Wakayama, T. Ashika, Y. Miyamoto, T. Yoshikawa, Y. Sakai, K. Sonoda, M. Moriguchi, J. Biochem. 118 (1995) 204.
[44] M. Wakayama, E. Watanabe, Y. Takenaka, Y. Miyamoto, Y. Tau, K. Sakai, M. Moriguchi, J. Ferment. Bioeng. 80 (1995) 311.
[45] M. Wakayama, S. Hayashi, Y. Yatsuda, Y. Katsuno, K. Sakai, M. Moriguchi, Protein Expression Purif. 7 (1996) 395.
[46] M. Wakayama, H. Yada, S. Kanda, S. Hayashi, Y. Yatsuda, K. Sakai, M. Moriguchi, Biosci. Biotechnol. Biochem. 64 (2000) 1.
[47] W. Kondel, F. Schneider, Z. Naturfosch 32c (1977) 337.
[48] W. Kondel, F. Schneider, Z. Naturfosch 32c (1977) 342.
[49] J.E. Coleman, in: C.C. Richardson (Ed.), Annual. Review of Biochemistry, vol. 61, Annual Reviews Inc., CA, 1992, p. 897.
[50] C.S. Hsu, W.L. Lai, W.W. Chang, S.H. Liaw, Y.C. Tsai, Protein Sci. 11 (2002) 2545.
[51] M. Wakayama, Y. Katsuno, S. Hayashi, Y. Miyamoto, K. Sakai, M. Moriguchi, Biosci. Biotechnol. Biochem. 59 (1995) 2115.
[52] E. Jabri, M.B. Carr, R.P. Hausinger, P.A. Karplus, Science 268 (1995) 998.
[53] L.A. Kelley, R.M. MacCallum, M.J.E. Sternber, J. Mol. Biol. 299 (2000) 499.
[54] J.B. Thoden, G.N. Phillips, T.M. Neal, F.M. Raushel, H.M. Holden, Biochemistry 40 (2001) 6989.
[55] M. Wakayama, M. Moriguchi, M. Ota, K. Nishikawa, Kagaku to Seibutsu 40 (2002) 452 (in Japanese).
[56] C.S. Hsu, S.J. Chen, Y.C. Tsai, T.W. Lin, S.H. Liaw, A.H.J. Wang, Acta Cryst. D58 (2002) 1482.
[57] S.H. Liaw, S.J. Chen, T.P. Ko, C.S. Hsu, C.J. Chen, A.H.J. Wang, Y.C. Tsai, J. Biol. Chem. 278 (2003) 4957.
[58] D.K. Wilson, F.B. Rudolph, F.A. Quiocho, Science 252 (1991) 1278.
[59] G.C. Ireton, G. McDermott, M.E. Black, B.L. Stoddard, J. Mol. Biol. 315 (2002) 687.