ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 12 (2001) 15–25

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Comparative biochemistry of bacterial *N*-acyl-D-amino acid amidohydrolase

Mamoru Wakayama, Mitsuaki Moriguchi*

Department of Applied Chemistry, Faculty of Engineering, Oita University, Oita 870-1192, Japan

Received 2 July 1999; received in revised form 9 November 1999; accepted 9 November 1999

Abstract

N-acyl-D-amino acid amidohydrolases can be classified into three types based on substrate specificity. D-aminoacylase has been reported to occur in a very few bacteria such as *Pseudomonas*, *Streptomyces*, and *Alcaligenes*. *N*-acyl-D-aspartate amidohydrolase (D-AAase) has been reported in only *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6 (*Alcaligenes* A-6) while *N*-acyl-D-glutamate amidohydrolase (D-AGase) has been isolated in two stains of *Pseudomonas* sp. 5f-1 and *Alcaligenes* A-6. The physiological roles of these enzymes in these microbes are not clear. They are individually characteristic in their substrate specificities, inducer profiles, inhibitors, isoelectric points, metal dependency, and some physicochemical properties. The primary structures of all the three types of *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 were determined from their nucleotide sequences. Comparison of their primary structures revealed high homology (46–56%) between the different enzymes. The three enzymes showed 26–27% sequence homology with L-aminoacylases from *Bacillus stearothermophilus*, porcine, and human. Chemical modification and site-directed mutagenesis identified the histidyl residues essential for catalysis. The *Alcaligenes N*-acyl-D-amino acid amidohydrolases share significant sequence similarities with some members of the urease-related amidohydrolase superfamily proposed by Holm and Sander [L. Holm, C. Sander, Proteins: Structure, Function and Genetics 28 (1997) 72].

Keywords: *N*-acyl-D-amino acid; Amidohydrolase; *Alcaligenes*; Enzyme characterization; Primary structure

1. Introduction

N-acyl-D-amino acid amidohydrolase catalyzes the hydrolysis of *N*-acyl derivatives of various D-amino acids to D-amino acids and fatty acids (Fig. 1). This type of enzyme is classified into three types according to substrate specificity. D-aminoacylase catalyzes the hydrolysis of *N*-acyl derivatives of neutral D-amino acids. *N*-acyl-D-glutamate amidohydrolase (D-AGase) and *N*-acyl-D-aspartate amidohydrolase (D-AAase) are specific for *N*-acyl-D-glutamate and *N*-acyl-D-aspartate, respectively. Unlike L-aminoacylase (*N*-acyl-L-amino acid amidohydrolase EC 3.5.1.14) [1], the physiological roles of the *N*-acyl-D-amino acid amidohydrolases are currently unknown, but the enzyme is industrially important for producing D-amino acids from DL-amino acids. Several methods have been developed for the preparation of D-amino acids, e.g. optical resolution of the racemate [2], the use of hydantoinase [3,4], and the use of coenzyme pyridoxal 5′-phosphate-dependent enzymes [5,6]. The resolution of DL-amino acids has commercial importance and has been performed by physicochemical, chemical, and enzymatic methods. The

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

1381-1177/01/$ – see front matter
PII: S1381-1177(00)00199-5

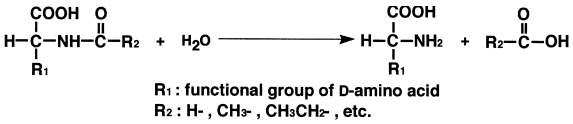

Fig. 1. Deacylation of *N*-acyl-D-amino acid amidohydrolase.

physicochemical and chemical methods which have been used, however, are not suitable for industrial resolution. At present, enzymatic methods are the most useful and convenient. One enzyme, L-aminoacylase has been immobilized on DEAE-Sephadex and is utilized in industry [7].

Little attention has been directed towards D-amino acids because they are not components of protein. However, it has been demonstrated that D-amino acids are important constituents of the natural peptide antibiotics [8] and neuropeptides (e.g. dermorphin) [9,10]. Some of them are important as industrial materials of semisynthetic antibiotics, bioactive peptides, and other physiologically active compounds. Recently, there has been renewed interest in D-amino acids, stemming from the fact that D-aspartate accumulates with age in the human tooth, eye lens, and brain [11–15]. It has been also reported that various free D-amino acids are present in Mammalia [16–21].

In this review, we summarize recent progress in the study of uncharacterized *N*-acyl-D-amino acid amidohydrolases from various microbes. The enzymatic properties of D-aminoacylase, D-AGase, and D-AAase from *Alcaligenes* A-6, and the molecular cloning and nucleotide sequencing of the genes encoding the three enzymes, are described.

2. Enzymatic characterization of *N*-acyl-D-amino acid amidohydrolases

2.1. D-*aminoacylase*

D-aminoacylase was first isolated from soil bacteria by Kameda et al. [22] and has been reported to occur in a very few bacteria including *Pseudomonas*, *Streptomyces*, and *Alcaligenes*. Two strains of *Pseudomonas* sp. 1158 [23,24] and AAA6029 [25], two strains of *Streptomyces*, *S. olivaceus* [26] and *S. tuirus* [27], and four strains of *Alcaligenes* (*A. denitrificans* subsp. *denitrificans* DA181 (*Alcaligenes* DA-181) [28], *A. faecalis* DA1 (*Alcaligenes* DA1) [29], *A. denitrificans* subsp. *xylosoxydans* MI-4 (*Alcaligenes* MI-4) [30], and *Alcaligenes* A-6 [31]) containing these enzymes have been isolated.

In general, D-aminoacylases are induced by *N*-acetyl-D-amino acids or D-amino acids. *N*-acetyl-D-valine and *N*-acetyl-D-leucine are good inducers for the enzymes from *Pseudomonas*, *Streptomyces*, and *Alcaligenes*. The ability of various hydrophobic D-amino acids and their acetyl derivatives to induce these D-aminoacylases was examined in *Alcaligenes* MI-4 and *Alcaligenes* A-6. Two new inducers have been found: the D-leucine analog, γ-methyl-D-leucine and its acetyl derivative [32]. Fig. 2 shows that induction increased with an increase in the carbon chain length of D-amino acids. The relationship between substrate reactivity and inducing ability was determined. For *Alcaligenes* A-6 D-aminoacylase, *N*-acetyl-γ-methyl-D-leucine, a poor substrate, was the best inducer [31], but for *Alcaligenes* MI-4 D-aminoacylase, γ-methyl-D-leucine, the product, was the best inducer [32] (Table 1). High-level production of enzyme in *Alcaligenes* A-6 was also observed when *N*-acetyl-D-*allo*-isoleucine, an inhibitor of enzyme activity and an inert substrate, was used [31]. Poor substrates (*N*-acetyl-D-valine for the enzyme from

Table 1
Comparison of inducers on the production of the D-aminoacylases from *Alcaligenes* A-6 and *Alcaligenes* MI-4

Inducer	Specific activity	
	Alcaligenes A-6[a]	*Alcaligenes* MI-4[b]
N-acetyl-γ-methyl-D-leucine	5.34	0.25
γ-Methyl-D-leucine	0.9	2.16
N-acetyl-*tert*-DL-leucine	0.65	0.02
tert-D-leucine	ND[c]	0.12
N-acetyl-D-leucine	2.52	0.12
D-leucine	1.78	0.09
N-acetyl-DL-leucine	2.43	0.30
DL-leucine	0.95	0.19
N-acetyl-DL-norleucine	0.06	0.05
DL-norleucine	ND[c]	0.38
N-acetyl-D-methionine	0.32	0.13
D-methionine	0.57	0.11
N-acetyl-D-valine	0.98	0.08
D-valine	0.84	0.11

[a] Expressed as μmol of D-leucine per min per mg of protein.
[b] Expressed as μmol of D-methionine per mg of protein.
[c] ND, not determined.

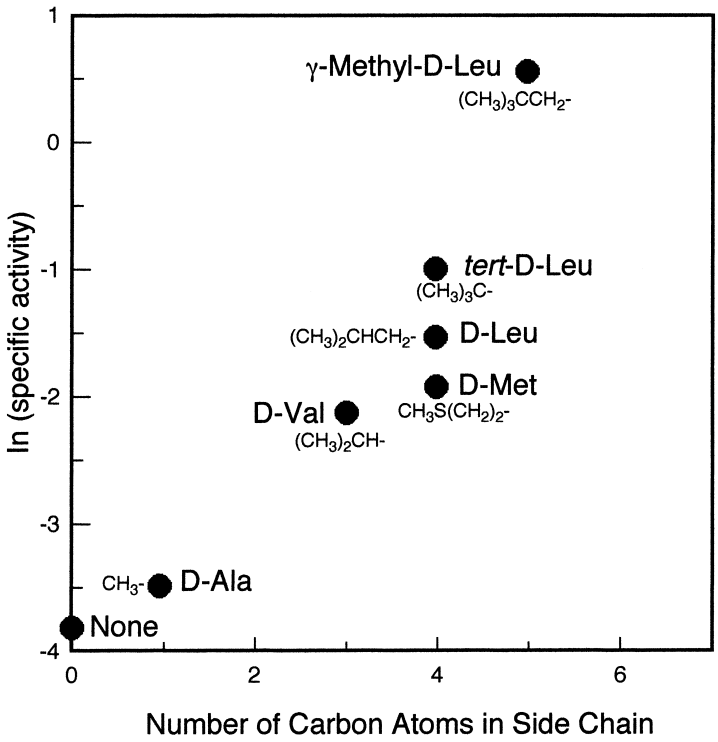

Fig. 2. Relationship between logarithm of specific activity and the number of carbon atoms in side chain of amino acid. The chemical formula of side chain (R-group) of the respective D-amino acid is given in the figure. D-Leu: D-leucine; D-Met: D-methionine; D-Val: D-valine; and D-Ala: D-alanine.

Alcaligenes DA181 [28] and *N*-acetyl-D-phenylglycine for the enzyme from *S. tuirus* [27]) were also strong inducers. The relationship between the structure and induction ability of these inducers is not clear. Enzyme induction is specific for the D-form of these compounds. The L-form repressed the induction of the D-aminoacylases of both *Alcaligenes* A-6 and *Alcaligenes* DA181 [31].

The enzymes from *Pseudomonas* sp. 1158 [23], *Pseudomonas* AAA6029 [25], *Alcaligenes* DA181 [33], *Alcaligenes* DA1 [29], *Alcaligenes* MI-4 [34], *Alcaligenes* A-6 [31] and *S. olivaceus* [26] are monomeric, with molecular masses of 100, 45, 58, 55, 51, 58 and 45 kDa, respectively. The isoelectric points were determined to be 4.95, 4.4, 5.4, and 5.2 for the enzymes from *Pseudomonas* sp. 1158 [23], *Alcaligenes* DA181, *Alcaligenes* DA1, and *Alcaligenes* A-6, respectively.

The D-aminoacylases from *Alcaligenes* [29,31,33, 34], *Pseudomonas* [24,25], and *Streptomyces* [26,27] have high stereospecificity and broad substrate specificity. Although the relative activities towards substrates differed among the respective enzymes, *N*-acetyl-D-methionine, *N*-acetyl-D-leucine, and *N*-acetyl-D-phenylalanine were the preferred substrates for all of the enzymes. *N*-acetyl derivatives of D-tryptophan, D-norleucine, D-alanine, D-valine, D-tyrosine, D-asparagine and D-phenylglycine also functioned as substrates for these enzymes. *Alcaligenes* MI-4 enzyme was only slightly active toward the *N*-acetyl derivatives of D-alanine and D-valine, but when the *N*-chloroacetyl derivatives of these amino acids were used, these substrates were considerably hydrolyzed [34]. On further examination of the acyl moiety specificities of the *Alcaligenes* A-6 and *Alcaligenes* MI-4 enzymes, the activities towards formyl and chloroacetyl groups were higher than those toward the acetyl group [31,34]. Thus, when the preferred chloroacetyl group is used as *N*-acyl derivatives in the case of substrates with low reactivity, an

efficient optical resolution of the DL-amino acid is obtained. L-aminoacylases from *Bacillus stearothermophilus* [35] and human kidney [36] hydrolyzed *N*-acetyl-L-glutamate, in addition to *N*-acyl derivatives of neutral L-amino acids. For L-aminoacylase from human kidney, *N*-acetyl-L-methionine was the best substrate, followed by *N*-acetyl-L-glutamate (relative activity of about 20%), *N*-acetyl-L-leucine, *N*-acetyl-L-alanine, and *N*-acetyl-L-valine [36]. *N*-acetyl-L-glutamate (relative activity of 1.5% toward *N*-acetyl-L-methionine) was only slightly hydrolyzed by *B. stearothermophilus* L-aminoacylase [35]. *Aspergillus* and *B. thermoglucosidus* L-aminoacylases were inert toward *N*-acetyl-L-glutamate [37,38]. On the other hand, D-aminoacylase did not act on *N*-acetyl-D-glutamate. The hydrolysis rate of the optically inactive substrate, *N*-acetylglycine, by L-aminoacylase from *Pseudomonas* sp. 1158 was 1.6-fold higher than that of *N*-acetyl-L-methionine [24]. The D-aminoacylase from *Pseudomonas* sp. 1158 had 30% relative activity for *N*-acetylglycine compared to *N*-acetyl-D-methionine [24], whereas the D-aminoacylases from *Pseudomonas* AAA 6029 [25], and *Alcaligenes* A-6 (Wakayama et al., unpublished result) and MI-4 [34], were inert (relative activity of 0 or less than 1% for *N*-acetylglycine). D-aminoacylase and L-aminoacylase both slowly hydrolyzed some peptides. Dipeptidase from *B. stearothermophilus* also acted on a variety of *N*-acyl-L-amino acids with rates of 3–30% of those for dipeptidase [39]. D-peptidase S from *Nocardia orientalis* had D-aminoacylase activity in addition to dipepitase activity. *N*-acetyl derivatives of D-phenylalanine, D-tyrosine, D-tryptophan, and D-leucine were hydrolyzed at the rates of 33, 22, 22 and 14% of that for D-leucyl-D-leucine [40].

The L-aminoacylases from *Aspergillus oryzae* [27], pig kidney [41], and *B. stearothermophilus* [35] are activated by Co^{2+}, and contain Zn^{2+} as a prosthetic metal. Activation of D-aminoacylases by metal ions was not observed [24,25,31,33,34]. Co^{2+} in the D-aminoacylase of *S. olivaceus* was thought to act to protect the enzyme from denaturation, although the enzyme's metal content has not been reported [26]. *Alcaligenes* DA181 D-aminoacylase contained about 2.1 g atom of Zn per mole of enzyme. The inactivation of this enzyme by EDTA was reversed fully by Co^{2+} and partially by Zn^{2+}. The enzyme activity from *Alcaligenes* MI-4 was not affected by metal ions (Ba^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, and Ca^{2+} at 1 mM each), but was inhibited 44% by Ni^{2+}, and was completely inhibited by Cu^{2+} and Hg^{2+}. 1,10-Phenanthroline strongly inhibited *Alcaligenes* MI-4 D-aminoacylase (residual activity 10%, 1.0 mM). Moreover, the substrate, *N*-acetyl-D-methionine (5 mM), protected the enzyme from inactivation (40% protection) by 1,10-phenanthroline (1 mM) (Wakayama et al., unpublished result). The enzyme from *Alcaligenes* A-6 was inhibited 20–30% by Ni^{2+}, Co^{2+}, and Fe^{3+} (1 mM), and 92 and 90% by Zn^{2+} and Cu^{2+} (1 mM), respectively [31]. Furthermore, enzyme activity was completely lost after treatment with 5 mM EDTA for 50 min. However, addition of Zn^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} (0.2 mM) to apoenzyme restored 48, 40, 30 and 19% of the enzyme activity, respectively. The enzyme contained about 2.3 g·atom of Zn per mole [42].

D-aminoacylase from *Alcaligenes* MI-4 was completely inactivated by iodoacetate (10 mM), phenylglyoxal (10 mM), 2,3-butanedione (5 mM) and dietyl pyrocarbonate (DEPC) (5 mM), suggesting that reactive cysteine, arginine and histidine are required for catalysis (Wakayama et al., unpublished data).

2.2. N-acyl-D-glutamate amidohydrolase

D-AGase was first discovered in cell-free extracts from *Alcaligenes* A-6 and *Pseudomonas* sp. 5f-1 [43,44]. Though its physiological role in *Alcaligenes* and *Pseudomonas* is not clear, it can be utilized for the resolution of DL-glutamate. To date, enzymes hydrolyzing *N*-acyl-L-glutamate have been found in *Pseudomonas* spp. [45–48], and *Streptomyces coelicolor* [49]. One of them, *N*-formyl-L-glutamate amidohydrolase (L-FGase) from *P. putida*, catalyzes the terminal reaction in the five-step pathway of histidine metabolism [45–49] and is induced by *N*-formyl-L-glutamate and urocanate, the first product in the histidine pathway [47]. The other, *N*-acetyl-L-glutamate amidohydrolase (L-AGase) from *P. aeruginosa*, is believed to be involved in the arginine biosynthetic pathway [46], and is induced by *N*-acetyl-L-glutamate and L-glutamate, but not by L-ornithine, an intermediate in arginine biosynthesis [46]. D-AGase from *Alcaligenes* A-6 was induced by *N*-acetyl-D-glutamate, but not by *N*-acetyl-L-glutamate, *N*-acetyl-D-aspartate, *N*-acetyl-L-aspartate, D-glutamate, L-glutamate, D-aspartate, L-aspartate, or the *N*-acetyl derivatives of

neutral D- or L-amino acids [43]. Whereas, D-AGase from *Pseudomonas* sp. 5f-1 was induced by the D-isomers of *N*-acetyl-glutamate, glutamate, aspartate, and asparagine, but not by *N*-acetyl-D-(or L)-aspartate, *N*-acetyl-L-glutamate, L-aspartate, or L-asparagine [44]. The D-AGases from *Alcaligenes* A-6 and *Pseudomonas* sp. 5f-1 differed in inducer specificity as described above.

Alcaligenes A-6 produced two D-AGases (I and II) which were separated on a Mono Q HR 5/5 column [49]. The two D-AGases have been purified to homogeneity. The final specific activities of D-AGases I and II were 1120 and 1000 U/mg, respectively. The isoelectric points of D-AGase I and D-AGase II from *Alcaligenes* A-6, and the D-AGase from *Pseudomonas* sp. 5f-1 were 5.5, 5.1 and 8.8, respectively. The *Alcaligenes* A-6 D-AGases (I and II), and the *Pseudomonas* sp. 5f-1 D-AGase were monomers with molecular masses of 59, 59, and 55 kDa, respectively. The *Alcaligenes* A-6 D-AGases (I and II) were highly specific to *N*-acyl derivatives of D-glutamate, and also hydrolyzed the dipeptide, glycyl-D-glutamate. *N*-acetyl derivatives of L-glutamate and D- or L-aspartate, and of neutral D-amino acids such as alanine, methionine, leucine, phenylalanine, and tryptophan, were inert towards D-AGases I and II [50]. The *Pseudomonas* sp. 5f-1 D-AGase was active towards various *N*-acyl derivatives of D-glutamate and the dipeptide, glycyl-D-glutamate. *N*-acetyl-L-glutamate and *N*-acetyl-D- or *N*-acetyl-L-derivatives of aspartate, alanine, phenylalanine, valine, tryptophan, and methionine did not act as substrates [44]. Although the two D-AGases are highly specific for *N*-acyl-D-glutamate, they differ in acyl moiety specificity. The D-AGase activity from *Alcaligenes* A-6 decreased with increasing *N*-acyl chain length; formyl (relative activity of 100%), chloroacetyl (91%), acetyl (22%), propionyl (5%), and butyryl (1.7%) [50], whereas the D-AGase activity from *Pseudomonas* sp. 5f-1 decreased in the order of formyl (relative activity of 100%), acetyl (58%), propionyl (21%), butyryl (16%), and chloroacetyl (7.5%) [44].

L-AGase from *P. aeruginosa* was a dimer with an apparent molecular mass of 90 kDa. The isoelectric point of L-AGase was 5.2. In addition to *N*-acetyl-L-glutamate, *N*-acetyl-L-glutamine, *N*-acetyl-L-methionine, *N*-acetylglycine, and *N*-acetyl-L-alanine were substrates of the enzyme, with rates of about 20, 18, 16, and 10% of *N*-acetyl-L-glutamate. The enzyme showed no peptidase activity. The order of reactivity of L-AGase with the various *N*-acyl moieties of L-glutamate was acetyl (relative activity of 100%) > formyl (88%) > fluoroacetyl (42%) > propionyl (22%) > butyryl (4%) [46].

The molecular mass of L-FGase from *P. putida* was 50 kDa, this enzyme was a mononer. *N*-formyl-L-glutamate was a substrate for L-FGase, but *N*-acetyl-L-glutamate was not [47].

The role of metal ions in D-AGase has been characterized in detail by use of the enzyme from *Pseudomonas* sp. 5f-1. *Pseudomonas* sp. 5f-1 enzyme was a zinc-metalloenzyme which contained 2.06–2.61 g atom of Zn per mole of enzyme. Dialysis of EDTA-treated enzyme removed the metal to give the apoenzyme, which was devoid of enzyme activity, and the restoration of the activity by the addition of Zn^{2+} indicates that Zn^{2+} is essential for catalysis. Co^{2+} is able to substitute for Zn^{2+} to restore activity, as reported for other zinc-metalloenzymes [51]. Thermostability increased about 10°C in the presence of Zn^{2+} or Co^{2+}. Furthermore, when the enzyme was purified in the presence of 0.05 mM Zn^{2+} (added as stabilizer), its final specific activity was three-fold greater than that in the absence of Zn^{2+} [52]. These results suggest that Zn^{2+} has a role in maintaining the stability of the enzyme. The catalytic activities of L-AGase and L-FGase depended on Co^{2+}, although their metal content was not determined [46,47].

Pseudomonas sp. 5f-1 D-AGase was inhibited by DEPC, phenylglyoxal, phenylmethylsulfonyl fluoride, and tosyl-L-phenylalanine chloromethyl ketone (1 mM each, 86, 46, 40 and 39% inhibition, respectively). It was shown that the inactivation of the enzyme by DEPC is due to the modification of a histidine residue. The substrate, *N*-acetyl-D-glutamate, and a competitive inhibitor, sodium α-ketoglutarate, protected the enzyme against the inactivation by DEPC, suggesting the presence of an essential histidine residue at or near the active site of the enzyme [53].

2.3. *N*-acyl-D-aspartate amidohydrolase

We found D-AAase in a cell-free extract of *Alcaligenes* A-6 [43]. The enzyme was a monomer with a molecular mass of 56 kDa, and an isoelectric point of 4.8. This pI value is low compared to that of the

Pseudomonas sp. 5f-1 D-AGase, which has a pI of 8.8 [44], but close to that of *Alcaligenes* A-6 D-AGase (pI 5.5) [50]. D-AAase was induced by *N*-acetyl-D-aspartate, but not by *N*-acetyl-D-glutamate, *N*-acetyl-L-glutamate, *N*-acetyl-L-aspartate, D-glutamate, L-glutamate, D-aspartate, L-aspartate, and *N*-acetyl derivatives of neutral D- or L-amino acids [43]. D-AAase was specific for *N*-acyl derivatives of D-aspartate. No measurable activities were obtained with *N*-carbobenzoxy-D-aspartate, *N*-acetyl-L-aspartate, *N*-acetyl-D-glutamate, and *N*-acyl derivatives of neutral D-amino acids. Enzyme activity decreased with increasing size of the *N*-substituent of D-aspartate: formyl (relative activity of 100%) > acetyl (36%) > propionyl (8.7%) > butyryl (4.3%). Similar specificity for *N*-acyl chain length was observed in D-AGase from *Alcaligenes* A-6. Both D-AAase and D-AGase had a very high structural specificity for the amino acid moiety and low specificity toward the acyl moiety, but D-aminoacylase had low structural specificity toward both moieties. Enzyme activity was completely inhibited by Cu^{2+}, Ni^{2+}, and Hg^{2+} (each at 2 mM) and EDTA (10 mM), and was 37, 59, 61, 73 and 80% with Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, and Co^{2+} (each at 2 mM), respectively. *N*-ethylmaleimide, iodoacetate, dithiothreitol, and ρ-chloromercuribenzoate (each at 2 mM) inhibited enzyme activity 74, 76, 85 and 88%, respectively [54]. The properties of D-AAase are similar to those of the D-AGases from *Alcaligenes* A-6 [50] and *Pseudomonas* sp. 5f-1 [44] with respect to molecular mass, subunit composition, pH optima, and inhibitors. The feature that distinguishes D-AAase from D-AGase is substrate specificity.

N-acyl-L-aspartate amidohydrolase (L-AAase) (aspartoacylase, EC 3.5.1.15), which has an almost absolute specificity for *N*-acyl-L-aspartate, has been characterized from hog kidney [55], and bovine brain [56]. L-AAase from bovine brain was a 58 kDa monomer. Enzyme activity was stimulated 20–40% by divalent cations such as Zn^{2+} (0.001 mM), Mn^{2+} (0.01 mM), Mg^{2+} (1.0 mM), and Ca^{2+} (1.0 mM). Metal chelators (5 mM) such as EGTA and EDTA did not have any effect on the enzyme activity. Sulfhydryl agents such as dithiothreitol and 2-mercaptoethanol stabilized the enzyme [56].

The properties of the *N*-acyl-D-amino acid amidohydrolases are summarized in Table 2.

3. Primary structure of *N*-acyl-D-amino acid amidohydrolases

3.1. Cloning and expression of N-acyl-D-amino acid amidohydrolase genes

As described in previous sections, the enzymatic and physicochemical properties of *N*-acyl-D-amino acid amidohydrolase from *Pseudomonas*, *Streptomyces*, and *Alcaligenes* have been reported, but no information on the structures of these enzymes, including primary structure, has been obtained. The genes of three *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 were cloned in *Escherichia coli* JM109 [57–59]. The transformants which contained the *N*-acyl-D-amino acid amidohydrolase genes were isolated by activity staining using D-amino acid oxidase from pig kidney and D-aspartate oxidase from *Fusarium sacchari* ver. *elongatum* [60] with *o*-dianisidine. The complete nucleotide sequences of the genes were determined by the dideoxy terminator method [61]. The coding region of D-aminoacylase comprised 1452 nucleotides starting with ATG and terminating in TGA, and an amino acid sequence of 484 amino acids was deduced from the nucleotide sequence. The molecular weight of D-aminoacylase was calculated to be 51,918 on the basis of the nucleotide sequence. The open reading frame of D-AAase consisted of 1494 base pairs, and encoded a protein of 498 amino acids with a molecular weight of 53,581. The open reading frame of D-GAase was comprised of 1464 bp, and encoded a protein of 488 amino acids with a molecular weight of 51,490. The codon usage of the three genes was biased towards codons with cytidine or guanosine in the third position (G + C content, 70.5–72.3%). The three enzymes are inducibly produced by the addition of their substrates, the substrate derivatives, or inhibitors in *Alcaligenes* A-6 cells [31,50,54]. None of the recombinant enzymes were, however, inducibly produced in *E. coli* cells by the addition of their substrates to the medium, but all were inducibly produced by the addition of isopropyl-β-thiogalactopyranoside. But, the expression level of the three enzymes in *E. coli* was low, and at almost the same level as those found in *Alcaligenes* A-6 (0.2–1.0 U/mg). The sequences of putative promotors and ribosome-binding sites of the mRNA encoding the enzymes have relatively weak homology

Table 2
Some properties of *N*-acyl-D-amino acid amidohydrolases[a]

Source name	*Alcaligenes xylosoxydans* A-6			*Pseudomonas* sp. 5f-1	*Alcaligenes* denitrificans Ml-4	*Alcaligenes faecalis* DA1	*Alcaligenes* denitrificans DA181
	D-AGase	D-Aaase	D-ANase	D-AGase	D-ANase	D-ANase	D-ANase
Substrate	*N*-Ac-D-Glu	*N*-Ac-D-Asp	*N*-Ac-D-Leu	*N*-Ac-D-Glu	*N*-Ac-D-Leu	*N*-Ac-D-Met	*N*-Ac-D-Met
Inducer	*N*-Ac-D-Glu	*N*-Ac-D-Asp	*N*-Ac-D-Leu	D-Asp	γ-Me-D-Leu	*N*-Ac-D-Leu	*N*-Ac-D-Val
Molecular weight	59,000	54,000	52,000	55,000	51,000	55,000	58,000
Op. pH	7.5	7.5	7.0	7.0	7.8	8.0	7.5
Stable pH	7.0	8.0	7.5	9.0	7.8	5.0–11.0	6.0–11.0
Op. Temperature (°C)	55	50	50	45	50	45	45
Stable temperature (°C)	50	45	40	48	40	40	55
K_m (mM)	0.13	2.5	9.8	6.7	14.1	1.0	0.48
V_{max} (U/mg)	1100	175	990	662	264	580	2380
Inhibitor	Co^{2+}, Cu^{2+}, EDTA	Cu^{2+}, Zn^{2+}, EDTA	Cu^{2+}, Zn^{2+}, EDTA	Cu^{2+}, Zn^{2+}, EDTA	Cu^{2+}, Hg^{2+}	Zn^{2+}, EDTA	EDTA
pl	5.0	4.8	5.2	8.8	–	5.4	4.4
N-terminal amino acid	MQEKLDLVCE	TDRSTLDDAP	SQSDSQPFDL	AGNTPVALNL	AQSDSQPFDL	–	SQPDATPFDY

[a] D-AGase: *N*-acyl-D-glutamate amidohydrolase; D-AAase: *N*-acyl-D-aspartate amidohydrolase; D-ANase: D-aminoacylase; Ac: acetyl; D-Glu: D-glutamate; D-Asp: D-aspartate; D-Leu: D-leucine; D-Met: D-methionine; D-Val: D-valine; γ-Me: γ-methyl; –: not determined.

with the canonical sequences. This might not allow for efficient expression of the genes of *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 in *E. coli* cells. To achieve a higher level of expression of these genes in *E. coli* cells, high-expression plasmids were constructed using pKK223-3 or pET23-d(+) as expression vectors, and introducing an appropriate Shine–Dalgarno (SD) sequence upstream of the initiation codon (ATG). The high expression plasmid containing the D-aminoacylase gene was designated pKNSD2. A cell-free extract of a transformant haboring pKNSD2 exhibited a specific activity of 162 U/mg, which is over 150-fold higher than that of the parent strain [62]. A relatively high expression level of D-AAase in *E. coli* cells was achieved using the pET system. The host *E. coli*, BL21(DE3)pLysS, was transformed by a pET derivative containing the D-AAase gene, pETAD1. This transformant exhibited a specific activity of 8.3 U/mg, a value which is 32 times greater than that produced by *Alcaligenes* A-6 [58]. In the case of D-AGase, a plasmid allowing for relatively high expression levels, pKGSD2, was constructed by introducing the canonical SD sequence (AAGGAG) and using the tac promotor of pKK-223-3. A transformant haboring pKGSD2 exhibited a specific activity of 22.0 U/mg, 22 times greater than that of *Alcaligenes* A-6 (Wakayama et al., unpublished data).

3.2. Comparison of primary structures of N-acyl-D-amino acid amidohydrolases

The deduced amino acid sequences of the three *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 showed good homology (46–56%), and some local regions were highly conserved (Fig. 3) [59]. These results strongly suggest that these enzymes have evolved from a common ancestral gene. Aligned hydropathy profiles, which are of value in predicting secondary structural features, of the three *N*-acyl-D-amino acid amidohyrolases, with some gaps, are compared in Fig. 4 [63]. The profiles as a whole resemble each other. There are some small segments which show prominent opposite hydropathy in D-AAase and D-AGase; from residue numbers 45–60, 105–130, 460–485, and 500–510. The sequences of these segments may be responsible for the discrimination between *N*-acyl-D-aspartate and *N*-acyl-

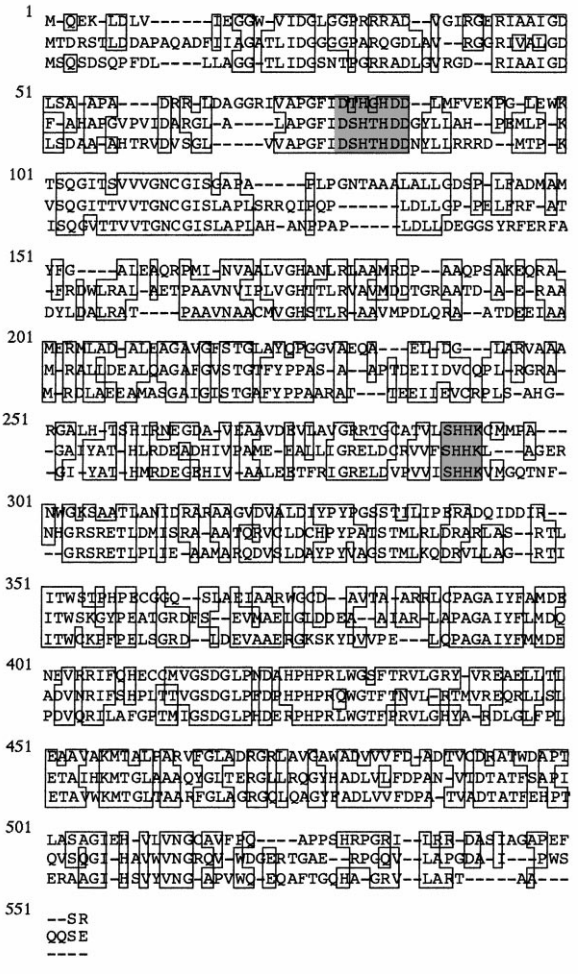

Fig. 3. Comparison of the amino acid sequences of *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6. Dashes indicate gaps introduced into the sequences so that the higher homology may be obtained. Amino acids that are conserved in all or two of three enzymes are boxed. Upper lane: D-Agase; middle lane: D-Aaase; lower lane: D-aminoacylase.

D-glutamate. Comparing the profile of D-aminoacylase with that of D-AAase and D-AGase, however, there are four regions that show distinct opposite hydropathy; i.e. 120–145, 325–340, 360–380, and 480–500. These four sites may be related to the recognition of the difference between neutral amino acids and acidic amino acids. The α-helical and β-sheet regions of these three enzymes were calculated by analyzing the amino acid sequences with Chou and Fasman's method [64]; D-AAase (α, 47%; β, 34%), D-GAase (α, 57%; β,

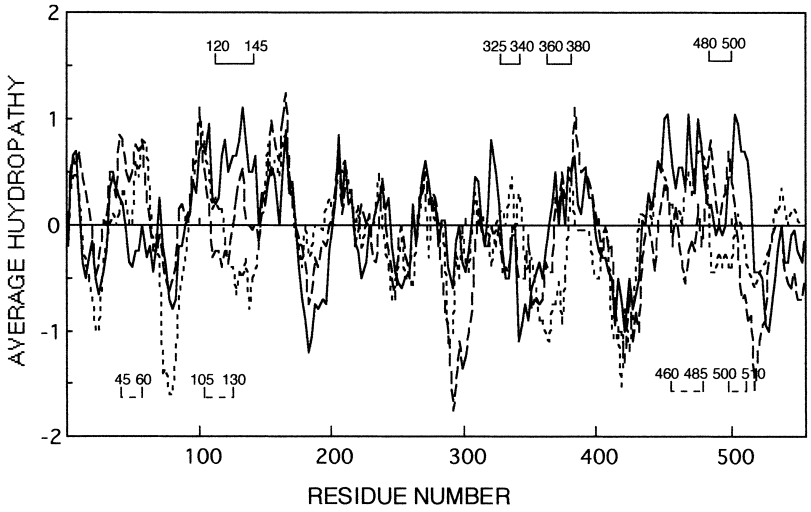

Fig. 4. Comparison of hydropathy profiles of *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6. Consecutive hydropathy averages are plotted for a five-residue window advancing from the *N*- to *C*-terminus. Relative hydrophilicity and hydrophobicity were recorded in the range +2.0 to −2.0 for each of three sequences (––––, D-AGase; –– ––, D-Aaase; – – –, D-aminoacylase), which had been aligned by introducing gaps (see Fig. 3).

24%), D-aminoacylase (α, 53%; β, 27%). These results suggest that the three enzymes have similar secondary structure arrangements. The *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 showed sequence homology with L-aminoacylase (26–27%) from *B. stearothermophilus*, porcine, and human [35,65,66] and with *N*-carbamyl-L-amino acid amidohydrolase (26–27%) from *Pseudomonas* sp. strain NS671 [67]. Moreover, interestingly, a consensus pentapeptide (–Gly–X–Ser–X–Gly–), which is conserved in lipase, esterase, and serine protease [68], is also conserved in D-aminoacylase, D-AAase, and D-AGase at nearly equivalent positions (Fig. 3). A sequence analogous to this motif is present in other acyl-group releasing enzymes; i.e. LQSFG (L-aminoacylase from *B. stearothermophilus*) [35], VTSTG (L-aminoacylase from porcine and human) [65,66], GASLF (amido-peptidase T from *Thermus aquaticus* YT-1) [69], GGSHG (acyl-peptide hydrolase from rat) [70], and GLSGG (cephalosporin acylase from *Pseudomonas* sp.) [71]. It is of interest to know whether this motif is important for structure and function. As mentioned in a previous section, it has been suggested that at least one histidine residue is necessary for the catalytic event in D-AGase from *Pseudomonas* sp. 5f-1. A chemical modification study using DEPC has revealed that the three *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 also require at least one histidine residue for catalysis [42]. Alignment of the primary structures of the three enzymes from *Alcaligenes* A-6 has revealed that eight conserved histidine residues exist and among them, and that three enzymes show significant similarity or identity in amino acids up- and down-stream of the histidine residues in both the Asp–X_1–His_a–X_2–His_b–Asp–Asp and Ser–His_c–His_d–Lys sequences. The conserved histidine residues described above were individually replaced by asparagine (Asn) or isoleucine (Ile) via site-directed mutagenesis, and the properties of Asn- and Ile-mutant enzymes were investigated. His_a and His_c appears to be essential for the catalytic event, and His_b appears to be necessary for maintaining or forming the higher-order structure of the enzyme. The D-aminoacylase from *Alcaligenes* A-6 has been reported to be a Zn enzyme [42]. Some Zn-dependent enzymes such as carbonic anhydrase [72], β-lactamase [73], and dihydroorotase [74], require histidyl residues as Zn-binding ligands, and the His–X–His sequence, which has been suggested to be one of zinc-binding motifs in some metal enzymes, is conserved in the three enzymes of *Alcaligenes* A-6 and Zn-dependent

enzymes described above. These results suggest that the His–X–His sequence is involved in Zn binding in *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6. However, there has been no report on Zn-binding ligands of *N*-acyl-D-amino acid amidohydrolases from any genera. As mentioned above, mutation studies suggested that the His_b residue of Asp–X_1–His_a–X_2–His_b–Asp–Asp is involved in the formation or maintenance of the higher-order structure of the *N*-acyl-D-amino acid amidohydrolase from *Alcaligenes* A-6. It has been reported that L-aminoacylase from porcine kidney requires Zn ion to maintain the catalytically active conformation at the active site [75], and the intrinsic Zn ion of thermostable L-aminoacylase from *B. stearothermophilus* has a structural role [76]. In the *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6, the His_b residue mentioned above might participate in Zn binding. Recently, the three-dimensional (3D) structure of urease (Ni enzyme) from *Klebiella aerogenes* has been revealed by X-ray crystallographic analysis [77]. Holm and Sander proposed striking similarities of enzyme architecture including the active-site between urease and the amidohydrolases involved in nucleotide metabolism such as dihydroorotases, allantoinases, and hydantoinases, etc., based on a computer analysis of the signature patterns conserved in the 3D structures of these enzymes [78]. They categorized these enzymes in the urease-related amidohydrolase superfamily. The urease-related amidohydrolases began to diverge from a common ancestor that had similar structure and biochemical function at a very early evolutionary stage. On the other hand, the three closely related *N*-acyl-D-amino acid amidohydrolases (D-aminoacylase, D-AAase, and D-AGase) from *Alcaligenes* A-6 exhibited the significant sequence similarities with the members of the superfamily, but are unrelated with the sequences of *Bacillus* and animal aminoacylases. Though as yet no information on the active-site structures of these aminoacylases has been available, the *Alcaligenes* aminoacylases might represent convergent evolution of similar enzyme activity from the different structural origin [78]. In any event, information on the reaction mechanism, i.e. essential amino acid residues for catalysis, and Zn-binding ligands, in both *N*-acyl-D-amino acid amidohydrolase and L-aminoacylase will be obtained by X-ray crystallographic analysis.

References

[1] M.W. Anders, W. Dekant, in: J. Thomas (Ed.), Advances in Pharmacology, Vol. 27, Academic Press, New York, 1994, p. 31.
[2] G.C. Barrett, in: G.C. Barrett (Ed.), Chemistry and Biochemistry of the Amino Acids, Champman & Hall, London, 1985, p. 338.
[3] S. Takahashi, Y. Kii, H. Kumagai, H. Yamada, J. Ferment. Technol. 56 (1978) 492.
[4] H. Yamada, S. Takahashi, Y. Kii, H. Kumagai, J. Ferment. Technol. 56 (1978) 484.
[5] H. Nagasawa, H. Hosono, H. Yamano, H. Ohkishi, Y. tani, H. Yamada, Agric. Biol. Chem. 47 (1983) 861.
[6] H. Tanaka, N. Yamada, N. Esaki, K. Soda, Agric. Biol. Chem. 49 (1985) 2525.
[7] T. Tosa, T. Mori, N. Fuse, I. Chibata, Biotech. Bioeng. 9 (1967) 603.
[8] J.S. Davies, in: B. Weinstein (Ed.), Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 4, Marcel Dekker, New York, Basel, 1980, p. 1.
[9] G. Kreil, in: C.C. Richardson (Ed.), Annual Review of Biochemistry, Vol. 66, Annual Reviews INC, CA, 1997, p. 337.
[10] C.L. Bevins, M. Zasloff, in: C.C. Richardson (Ed.), Annual Review of Biochemistry, Vol. 59, Annual Reviews INC, CA, 1990, p. 395.
[11] P.M. Helfman, J.L. Bada, Proc. Natl. Acad. Sci. U. S. A. 72 (1975) 2891.
[12] P.M. Helfman, J.L. Bada, Nature 262 (1976) 279.
[13] P.M. Masters, J.L. Bada, J.S. Zigler Jr., Nature 268 (1997) 71.
[14] P.M. Masters, J.L. Bada, J.S. Zigler Jr., Proc, Natl. Acad. Sci. U. S. A. 75 (1978) 1204.
[15] W.H. Garner, A. Spector, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 3618.
[16] D.S. Dunlop, A. Neidle, D. McHale, A. Lajtha, Biochem. Biophys. Res. Commun. 141 (1986) 27.
[17] A. Neidle, D.S. Dunlop, Life Sci. 46 (1990) 1517.
[18] H. Nagasaki, R. Yamada, R. Konno, Y. Yasumura, A. Iwashima, Experientia 46 (1990) 468.
[19] A. Hashimoto, T. Nishikawa, T. Oka, T. Takahashi, J. Neurochem. 60 (1993) 783.
[20] A. D'Aniello, G. D'Onofrio, M. Pischetola, G. D'Aniello, A. Vetene, L. Petrucelli, G. Fisher, J. Biol. Chem. 268 (1993) 26941.
[21] Y. Kera, H. Aoyama, H. Matsumura, A. Hasegawa, N. Nagasaki, R. Yamada, Biochimica et Biophysica Acta 1243 (1995) 282.
[22] Y. Kameda, E. Toyoura, H. Yamazoe, Y. Kimura, Y. Yasuda, Nature 170 (1952) 888.
[23] T. Kubo, T. Ishikura, Y. Fukagawa, J. Antibiot. 33 (1980) 550.
[24] K. Kubo, T. Ishikura, Y. Fukagawa, J. Antibiot. 33 (1980) 556.
[25] Y. Kameda, H. Hase, S. Kanamoto, Y. Kita, Chem. Pharm. Bull. Tokyo 26 (1978) 2698.
[26] M. Sugie, H. Suzuki, Agric. Biol. Chem. 42 (1978) 107.

[27] M. Sugie, H. Suzuki, Agric. Biol. Chem. 42 (1980) 1089.
[28] Y.C. Tsai, C.P. Tseng, K.M. Hsiao, L.Y. Chen, Appl. Environ. Microbiol. 54 (1988) 984.
[29] Y.B. Yang, C.S. Lin, C.P. Tseng, Y.J. Wang, Y.C. Tsai, Appl. Environ. Microbiol. 57 (1991) 1259.
[30] M. Moriguchi, K. Ideta, Appl. Environ. Microbiol. 54 (1988) 2767.
[31] M. Moriguchi, K. Sakai, Y. Miyamoto, M. Wakayama, Biosci. Biotech. Biochem. 57 (1993) 1149.
[32] K. Sakai, T. Obata, S. Takano, M. Moriguchi, Agric. Biol. Chem. 53 (1989) 2285.
[33] Y.B. Yang, K.M. Hsaio, H. Li, H. Yano, A. Tsugita, Y.C. Tsai, Biosci. Biotech. Biochem. 56 (1992) 1392.
[34] K. Sakai, T. Obata, K. Ideta, M. Moriguchi, J. Ferment. Bioeng. 71 (1991) 79.
[35] V. Sakanyan, L. Desmarez, C. Legrain, D. Charlier, I. Mett, A. Kochikyan, A. Savchenko, A. Boyen, P. Falmagne, A. Pierard, N. Glandsdorff, Appl. Environ. Microbiol. 59 (1993) 3878.
[36] S. Pittelkow, H. Lindner, K.H. Röhm, Protein Expression Purif. 12 (1998) 3878.
[37] I. Gentzen, H.G. Löffler, F. Schneider, Z. Naturforsch 35C (1980) 544.
[38] H.Y. Cho, K. Tanizawa, H. Tanaka, K. Soda, Agric. Biol. Chem. 51 (1987) 2793.
[39] H.Y. Cho, K. Tanizawa, H. Tanaka, K. Soda, J. Biochem. 103 (1988) 622.
[40] M. Sugie, H. Suzuki, N. Tomizuka, Agric. Biol. Chem. 50 (1986) 1397.
[41] W. Kördel, F. Schneider, Z. Naturfosch 32C (1977) 342.
[42] M. Wakayama, H. Yada, S. Kanda, S. Hayashi, Y. Yatsuda, K. Sakai, M. Moriguchi, Biosci. Biotech. Biochem. 64 (2000) 1.
[43] K. Sakai, K. Imamura, M. Goto, I. Hirashiki, M. Moriguchi, Agric. Biol. Chem. 54 (1990) 841.
[44] K. Sakai, K. Oshima, M. Moriguchi, Appl. Environ. Microbiol. 57 (1991) 2540.
[45] J.G. Coote, T. Leisinger, Biochem. J. 132 (1973) 423.
[46] H. Früh, T. Leisinger, J. Gen. Microbiol. 125 (1981) 1.
[47] L. Hu, L.M. Mulfinger, A.T. Phillips, J. Bacteriol. 169 (1987) 4696.
[48] T.G. Lessie, F.C. Neidhardt, J. Bacteriol. 93 (1967) 1800.
[49] K.L. Kendrick, M.L. Wheelis, J. Gen. Microbiol. 128 (1982) 2029.
[50] K. Sakai, K. Imamura, Y. Sonoda, H. Kido, M. Moriguchi, FEBS Lett. 289 (1991) 44.
[51] B.L. Vallee, A. Galdes, Adv. Enzymol. Relat. Areas, Mol. Biol. 56 (1984) 284.
[52] M. Wakayama, Y. Miura, K. Oshima, K. Sakai, M. Moriguchi, Biosci. Biotech. Biochem. 59 (1995) 1489.
[53] M. Wakayama, T. Tsutsumi, H. Yada, K. Sakai, M. Moriguchi, Biosci. Biotech. Biochem. 60 (1996) 650.
[54] M. Moriguchi, K. Sakai, Y. Katsuno, T. Maki, M. Wakayama, Biosci. Biotech. Biochem. 57 (1993) 1145.
[55] S.M. Birnbaum, in: S.P. Colowick, N.O. Kaplan (Eds.), Method in Enzymology, Vol. 2, Academic Press, New York, 1955, p. 115.
[56] R. Kaul, J. Casanova, A.B. Johnson, P. Tang, R. Matalon, J. Neurochem. 56 (1991) 129.
[57] M. Wakayama, Y. Katsuno, S. Hayashi, Y. Miyamoto, K. Sakai, M. Moriguchi, Biosci. Biotech. Biochem. 59 (1995) 2115.
[58] M. Wakayama, E. Watanabe, Y. Takenaka, Y. Miyamoto, Y. Tau, K. Sakai, M. Moriguchi, J. Ferment. Bioeng. 80 (1995) 311.
[59] M. Wakayama, T. Ashika, Y. Miyamoto, T. Yoshikawa, Y. Sonoda, K. Sakai, M. Moriguchi, J. Biochem. 118 (1995) 204.
[60] M. Wakayama, S. Nakashima, K. Sakai, M. Moriguchi, J. Ferment. Bioeng. 78 (1994) 377.
[61] F. Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 5463.
[62] M. Wakayama, S. Hayashi, Y. Yatsuda, Y. Katsuno, K. Sakai, M. Moriguchi, Protein Expression Purif. 7 (1996) 395.
[63] J. Kyte, R.F. Doolitle, J. Mol. Biol. 157 (1982) 105.
[64] P.Y. Chou, G.D. Fasman, Adv. Enzymol. Relat. Areas Mol. Biol. 47 (1978) 45.
[65] M. Mitta, H. Ohnogi, A. Yamamoto, I. Kato, F. Sakiyama, S. Tsunasawa, J. Biochem. 112 (1992) 737.
[66] M. Mitta, I. Kato, S. Tsunasawa, Biochim. Biophys. Acta 1174 (1993) 201.
[67] K. Watabe, T. Ishikawa, Y. Mukohara, H. Nakamura, J. Bacteriol. 174 (1992) 962.
[68] S. Brenner, Nature 334 (1988) 528.
[69] H. Motoshima, N. Azuma, S. Kaminogawa, M. Ono, E. Minagawa, H. Matsuzawa, T. Ohta, K. Yamauchi, Agric. Biol. Chem. 54 (1990) 2385.
[70] K. Kobayashi, L.W. Lin, J.E. Yeadon, L.B. Klickstein, J.A. Smith, J. Biol. Chem. 264 (1989) 8892.
[71] A. Matsuda, K. Toma, K. Komatsu, J. Bacteriol. 169 (1987) 5821.
[72] K.K. Kannan, B. Notstrand, K. Fridborg, S. Lovgren, A. Ohlsson, M. Petef, Proc. Nat. Acad. Sci. U. S. A. 72 (1975) 51.
[73] B.J. Sutton, P.J. Artymiuk, A.E. Cordero-Borboa, C. Little, D.C. Phillips, S.G. Waley, Biochem. J. 248 (1987) 181.
[74] D.C. Brown, K.D. Collins, J. Biol. Chem. 266 (1991) 1597.
[75] Z.Y. Tang, J.Y. Yu, Q. Zhou, B.A. He, Z.F. Wang, H.M. Zhou, J. Biochem. 118 (1995) 706.
[76] H.M. Weiss, G.J. Palm, K.H. Rohm, Biol. Chem. Hoppe–Seyler 376 (1995) 643.
[77] S.J. Lippard, Science 268 (1995) 996.
[78] L. Holm, C. Sander, Protein: Struct. Function Genet. 28 (1997) 72.