J. Microbiol. Biotechnol. (2000), **10**(5), 573–579

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

New Enzymes Acting on Peptides Containing D-Amino Acids: Their Properties and Application

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Received: March 29, 2000
Accepted: August 19, 2000

Abstract Knowledge on the enzymes acting on D-amino-acid-containing peptides appears to be somewhat limited when compared with those acting on peptides composed of L-amino acids. Less than ten D-stereospecific enzymes are hitherto known. This review describes about several novel D-stereospecific peptidases and amidases of microbial origin, including D-aminopeptidase (E.C. 3.4.11.19), alkaline D-peptidase, and D-amino acid amidase, which are applied to the synthesis of D-amino acids and/or D-amino acid derivatives.

Key words: D-Amino acid, enzymatic production, D-aminopeptidase, alkaline D-peptidase, D-amino acid amidase

D-Stereospecific enzymes acting on D-amino-acid-containing peptides or amides include DD-hydrolyzing enzymes involved in cell wall synthesis (DD-carboxypeptidase), D-aminopeptidase (DAP, E.C. 3.4.11.19) [2, 3, 6, 8, 11, 12], alkaline D-peptidase (ADP) (9,10,29), DD-dipeptidase involved in vancomycin resistance [41, 51], non-stereospecific dipeptidase (peptidyl-D-amino acid hydrolase, E.C. 3.4.13.17) [17], membrane dipeptidase (renal membrane peptidase, E.C. 3.4.13.19) [18, 49], D-aminoacylase [16, 37, 45, 47, 48, 51, 54], β-lactamase (EC 3.5.2.6) [21, 23], and D-amino acid amidase [1, 29, 40, 52]. The latter three enzymes (D-aminoacylase, β-lactamase, and D-amino acid amidase) may be excluded from the group of D-stereospecific hydrolases acting on D-amino-acid-containing peptides or amides, since they act on amino acid derivatives or cyclic amides (lactam) rather than peptides. The stereospecificity of bacterial enzymes is rather strict, while that of non-stereospecific dipeptidase and membrane dipeptidase of animal origin is loose: in some cases, racemic peptides are utilized. The active sites of the enzymes, for D-aminoacylase, class B β-lactamase, and DD-dipeptidase involved in vancomycin resistance, are Zn^{2+}-dependent. Class A, C, and D β-lactamases [39], DD-hydrolyzing enzymes and PBP involved in cell wall synthesis, and the newly found DAP, ADP, and DAA are all serine enzymes, which are structurally related and can be categorized as penicillin-recognizing enzymes [29], although DAA is not sensitive to penicillin. On the stereochemistry and mode of action of the enzymes, most of them appear to recognize the D-configuration of one amino acid, and some act on the acyl group of the amino group (D-aminoacylase), or hydrolyze the carboxy terminus amide bond (DD-carboxypeptidase, DAP, ADP, and DAA). The configuration of the amino acid at the C-termini of the peptides varies: DD-carboxypeptidase and DD-dipeptidase involved in vancomycin resistance require DD stereochemistry on the peptides, while DAP and ADP utilize peptides containing either D- or L-amino acids, with a preference for D-amino acids. ADP is an endopeptidase acting on substrates whose *N*-terminal groups are masked, and this feature resembles that of β-lactamase which hydrolyzes the β-lactam ring. Some of the properties of these enzymes are briefly summarized in Table 1.

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PRODUCTION AND USAGE OF D-AMINO ACIDS

Currently, most L-amino acids are produced fermentatively, whereas almost all D-amino acids are obtained by enzymatic methods. A fermentative preparation has only been reported for D-Ala [42]. D-Amino acids are important starting materials for various pharmaceuticals, herbicides, and food additives. *p*-Hydroxy-D-phenylglycine is one of the most important intermediates for the preparation of semi-synthetic β-lactam antibiotics [53]. D-Ala is an important component of synthetic sweeteners such as alitame [25]. The synthesis of biologically active peptide hormones or antibiotics with D-amino acids instead of their L-counterparts can lead to metabolically stable and long acting products.

More and more new enzymatic methods of synthesizing D-amino acids or related peptides are expected to be

Table 1. Enzymes acting on peptides containing D-amino acid.

Enzyme	Origin	Active site	Sensitivity to Penicillin	Stereochemistry*	Literature
DD-Carboxypeptidase	Bacteria	Ser	Yes	D-D (C-terminus)	22
D-Aminoacylase	*Alcaligenes*	Zn^{2+}	No	Acyl-D	37,47,48,54
	Pseudomonas				
	Streptomyces				
D-Aminopeptidase	*Ochrobactrum*	Ser	Yes	D-DL (*N-terminus*)	2,11
Alkaline D-peptidase	*Bacillus*	Ser	Yes	DL-D-DL (Endo)	9,12
D-Amino acid amidase	*Ochrobactrum*	Ser	No	D-Amide	1,30
DD-Dipeptidase (VanX)	*Enterococcus*	Zn^{2+}	No	DL-D	33,41,51
Non-stereospecific dipeptidase	Animal		No	Nonspecific	17
Membrane dipeptidase	Animal	Zn^{2+}	Yes	Nonspecific	31

*Underlines indicate the site where the substrates are recognized.

developed. Recent examples include the optical resolution of *N*-acylamino acids [46], the asymmetric hydrolysis of hydantoins [53], and the employment of four enzymes, D-amino acid aminotransferase, alanine racemase, alanine dehydrogenase, and formate dehydrogenase with α-keto acid, as a starting material [4, 53]. This paper proposes that the use of D-stereospecific peptide hydrolases acting on the C-terminus of amino acid amides could be an alternative to the optical resolution of *N*-acylamino acids by acylases, as shown in Fig. 1. Amino acid amides can be prepared by the hydration of amino nitrile, which is synthesized from aldehyde and cyanide by the Strecker method [26]. As a result, D-amino acids can be prepared in three steps, which is faster than the D-amino acylase route, which requires four steps [24]. As shown in Fig. 2, when the D-aminoacyl-enzyme complex formed by a D-stereospecific amide hydrolase is deacylated by a nucleophile with a stronger nucleophilicity than water, a peptide bond with the D-amino acid is formed, whereafter D-Ala-*N*-alkyl amide can be synthesized by reacting amine with racemic alanine amide or ester. Under appropriate conditions, the use of a D-stereospecific enzyme results in the D-oligomers adroitly avoiding the complicating protection and deprotection steps which might be required with the conventional method. This is a major advantage over the similar methods using L-specific proteolytic enzymes like subtilisin [34] or α-chymotrypsin [50] in organic solvents to relax the stereospecificity.

NEW ENZYMES ACTING ON D-AMINO ACID-CONTAINING PEPTIDES

D-Aminopeptidase (E.C. 3.4.11.19)

The screening for a new enzyme catalyzing the synthesis of D-Ala-*N*-alkyl amides, similar to the unique structure of the artificial sweetener alitame (L-aspartyl-D-alanine thiethane amide), led to the discovery of D-aminopeptidase [2]. Asano *et al.* isolated *Ochrobactrum anthropi* sp. C1-38 using an enrichment culture technique from soil samples. The enzyme acted on D-Ala amide (relative velocity: 100%, K_m value: 0.65 mM), Gly amide (44%, 22.3 mM), D-α-aminobutyric acid amide (30%, 18.3 mM), D-Ser amide (29%, 27.0 mM), D-Ala 3-aminopentane amide (32%, 2.27 mM), D-Ala-*p*-nitroanilide (96%, 0.51 mM), D-$AlaOCH_3$ (75%), the dimer (21%), trimer (92%, 0.57 mM) and tetramer (89%, 0.32 mM) of D-Ala, D-Ala-Gly (95%, 0.98 mM), D-Ala-L-Ala-L-Ala (100%, 0.65 mM), and D-Ala-Gly-Gly (45%,

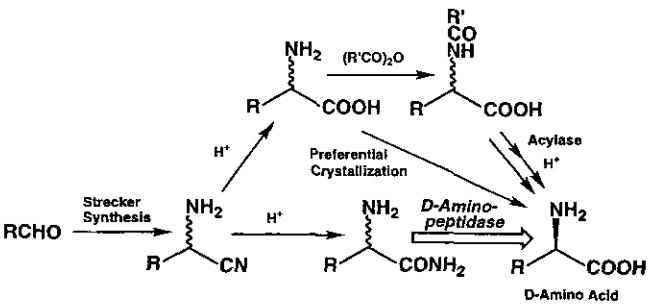

Fig. 1. Synthesis of D-amino acid from aldehyde.

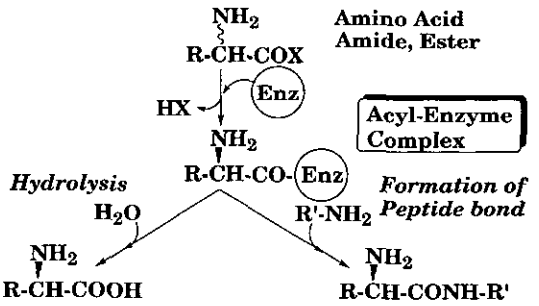

Fig. 2. Formation of acyl-enzyme complex.

Table 2. Kinetic parameters for D-aminopeptidase from *Ochrobactrum anthropi* sp. C1-38 [2,11,12].

Substrate	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg · mM)
D-Ala amide	0.65	600.0	923.00
Gly amide	22.30	365.0	16.3.00
D-α-Aminobutyric acid amide	18.00	576.0	32.00
D-Ser amide	27.00	22.0	0.81
D-Thr amide	100.00	60.3	0.60
$(D\text{-Ala})_2$	10.20	326.0	32.00
$(D\text{-Ala})_3$	0.57	866.0	1,520.00
$(D\text{-Ala})_4$	0.32	702.0	2,190.00
D-Ala-Gly	0.98	1,000.0	1,020.00
D-Ala-Gly-Gly	0.37	799.0	2,160.00
D-Ala-L-Ala	1.03	312.0	303.00
D-Ala-L-Ala-L-Ala	0.65	730.0	1,120.00
D-Ala-*p*-nitroanilide	0.51	696.0	1,370.00
D-Ala *n*-butylamide	0.73	670.0	918.00
D-Ala-3-aminopentane amide	2.27	288.0	127.00

0.37 mM), etc. It is an intracellular enzyme expressed constitutively. As shown in Table 2, the V_{max}/K_m values decrease markedly when their substituents of D-amino acid amides become larger, yet peptides are better substrates than the D-amino acid amides; for example, D-Ala tetramer is the best substrate. Aminopeptidases release the amino-terminal residue from peptide substrates. The stereochemistry of the second amino acid from the amino-terminus does not appear to be important; a D-L peptide bond is hydrolyzed to a lesser extent than a D-D bond. D-Aminopeptidase (DAP) from *O. anthropi* acts on peptides containing amino-terminal D-residues. Their primary structure displays a strong similarity to carboxypeptidase DD and β-lactamase [8], and, thus, DAP can be considered as a penicillin-recognizing enzyme [22]. A significant inhibition is caused by β-lactam compounds although they are not suitable as a substrate. With a relative molecular weight of 122,000, DAP is composed of two identical subunits (M_r= 59,000). The active center bears the essential amino acid sequence SXXK, which is a conserved motif in penicillin-recognizing enzymes. By generating several mutant enzymes at the active center, it has been shown that Ser 62 is responsible for the nucleophilic attack on the carbon of the peptide bond to be hydrolyzed in a substrate or the β-lactam carbonyl carbon in an antibiotic molecule. Taking into account its high stereospecificity and rather narrow substrate specificity, together with its acceptance of only low molecular weight D-amino acid derivatives, suggests several conceivable physiological roles [2]. It can hydrolyze peptidoglycan fragments composed of D-Ala and Gly [43], the dipeptide D-Ala-D-Ala, which is a product of the D-alanyl-D-alanine ligase [38], or the degradation product of naturally occurring D-amino acid derivatives as synthesized in rice plants [35].

Recently, two aminopeptidases, DmpA and DmpB, were further isolated from *O. anthropi* LMG7991 [15, 19, 20], both of which are active on D-Ala-*p*-nitroanilide. DmpB was purified to 90% homogeneity and its amino-terminal sequence exhibited a 60% homology with the DAP enzyme from *O. anthropi* C1-38 [3]. The second enzyme, DmpA, hydrolyzes *p*-nitroanilide derivatives of Gly and D- and L-Ala with a preference to the D-substrate.

Application to Synthesis of D-Amino Acids and Their Derivatives

Stereospecific hydrolysis of the D-component of a DL-amino acid amide mixture to yield D-amino acid was achieved by the enzyme. An expression plasmid of DAP [pC138DP (4.5 kb)] was constructed. The amount of DAP in the cell-free extract of *E. coli* JM109/pC138DP was elevated up to 28,800 U/l culture, which is about 3,600-fold higher than that of the wild-type, *O. anthropi* C1-38. It is calculated that about 30% of the enzyme is comprised of a total extractable cellular protein. The intact cells of an *E. coli* transformant were used as a catalyst for the D-stereospecific hydrolysis of several racemic amino acid amides HCl [5]. A complete hydrolysis of D-Ala amide was achieved in a short time (4.5 h) from 5.0 M of racemic Ala amide HCl using the *E. coli* transformant cells. The concentration of D-Ala reached up to 220 g/l. The unbroken cells or the cell-free extracts catalyzed the synthesis of D-α-amino butyric acid, D-Met, D-norVal, and D-norLeu from their corresponding amides in a similar manner with a yield of 100, 97, 86, and 100%, respectively.

The enzyme also catalyzed only D-stereospecific aminolysis of DL-$AlaOCH_3$ to yield D-Ala *N*-alkyl amide in organic solvents [3, 27]. The enzyme was immobilized by urethane prepolymer PU-623, and was incubated in water-saturated organic solvents, as shown in Table 3. D-Ala 3-aminopentane amide was quantitatively synthesized from D-$AlaOCH_3$ and 3-aminopentane in 1 h in solvents such as benzene, butyl acetate, and 1,1,1-trichloroethane. Under this aminolysis reaction, the catalytic center activity (k_{cat}) was calculated to be as high as 7,700 min^{-1}, which is several hundred to ten-thousand times greater than that in α-chymotrypsin- or subtilisin- catalyzed peptide formations, respectively. D-Ala 3-aminopentane amide was synthesized with a 46% yield from DL-$AlaOCH_3$, while no product was formed from L-$AlaOCH_3$. The aminolysis reaction also proceeded well with a high yield with D-Ala amide. The substrate specificity for the acyl donor, part of this aminolysis reaction, was also reflected in the hydrolytic reaction. However, the substrate specificity for the nucleophile part differed: no formation of D-Ala polymer was observed when only D-$AlaOCH_3$ was incubated with the immobilized

enzyme, yet the D-Ala *N*-alkyl amides of *n*-butylamine, benzylamine, and neopentylamine were synthesized with high yields. This nucleophile specificity would depend on the nucleophilicity of the amines employed. The synthesis of D-Ala oligopeptides from D-AlaOCH$_3$-HCl has been demonstrated with the use of immobilized DAP from *O. anthropi* in nonaqueous media. In this case, addition of D-AlaOCH$_3$, but no other nuclophile, resulted in the formation of D-Ala oligomers. The ester moiety appeared to be hydrolyzed during the reaction. D-Ala dimer and trimer were obtained with 56% and 6% yields, respectively, when 250 mM of the substrate was incubated for 3 h with urethane-prepolymer immobilized DAP (1.5 U/ml) and 3 equivalents of triethylamine in water-saturated toluene [3, 28].

Mutant DAPs from *O. anthropi* with increased thermal stability were obtained by random mutagenesis. One of the mutants, no. 65, was derived from *E. coli* cells transformed with DNA treated with sodium nitrite. The remaining activity of the purified mutant enzyme no. 65 after heat treatment at 52°C for 10 min was 20% that of the untreated mutant enzyme no. 65, whereas the native enzyme showed only 5% of the untreated native enzyme activity after the same treatment. The gene for the mutant enzyme no. 65 was sequenced and it was found that Gly155 and Gly279 in the native enzyme were replaced by Ser and Asp, respectively. Five mutants carrying one or two mutations were generated from the native gene by site-specific mutagenesis. The enhancement of the thermal stability of mutant enzyme no. 65 was attributed to the substitution of Gly155 with Ser [7].

Application to Prodrugs

For a study to improve the effectiveness of the anticancer drug methotrexate, DAP was considered as a potential candidate for antibody-directed enzyme prodrug therapy (ADEPT). Since most drugs are not only selective for tumor tissue, but also harmful for normal tissue, studies have been undertaken to develop inactive prodrugs that are only activated at the tumor site. Using the ADEPT strategy, an antibody conjugated with enzyme can be located on the tumor surface [44].

In an effort to improve the selectivity of the anticancer drug, methotrexate (MTX), a series of potential prodrugs were synthesized in which the 2-amino group is acylated with various amino acids (as well as L-pyroglutamic acid). Such derivatives were anticipated to yield MTX by appropriate aminopeptidases localized (overexpressed naturally or targeted as anti-tumor antibody conjugates) in the vicinity of the tumor. L-Leucyl, L-valyl, L-isoleucyl, D-alanyl, and L-pyroglutamyl derivatives were all assessed as to their suitability as prodrugs. The L-leucyl, L-valyl, and L-isoleucyl derivatives were readily hydrolyzed to MTX by aminopeptidase M (EC 3.4.11.2), while the L-pyroglutamyl and D-alanyl compounds were activated by pyroglutamate aminopeptidase (EC 3.4.19.3) (from *Bacillus amyloliquefaciens*) and DAP (from *Ochrobactrum anthropi*), respectively.

D-Amino Acid Amidase

Increasing attention has been paid to D-amino acid amidases since they can be used as a catalyst in the stereospecific production of D-amino acids by hydrolysis of D-amino acid amides.

A D-stereospecific amino acid amidase (DAA) accepting a broader range of substrates was isolated from *Ochrobactrum anthropi* SV3 [1]. This enzyme catalyzes the stereospecific hydrolysis of D-amino acid amides yielding D-amino acids and ammonia. Its major substrates were D-Phe amide (relative activity: 100%), D-Tyr amide (97%), D-Leu amide (46%) and D-Ala amide (33%). Their K_m values were calculated to be 0.089, 0.18, 0.057, and 0.54 mM, respectively. The enzyme prefers D-amino acid amides with a free α-amino group. Its M_r was estimated to be about 38,000. The maximal activity was displayed at pH 7.5 to 8.0. Neither chelating nor sulfhydryl reagents had any effect on the enzyme activity. Serine protease inhibitors, such as phenylmethane sulfonyl fluoride and diisopropyl fluorophosphates inhibit an 80–95% of the enzyme activity. In a recent study, the DAA-encoding gene was cloned and sequenced showing the active site motifs typical for PBP and β-lactamases such as SXXK, YXN, and HXG [30]. The conservation of these identical residues suggests that DAA can be classified as a penicillin-recognizing enzyme [22]. The SXXK motif was also found in DAP.

D-Ala amide amidase producer *Arthrobacter* sp. NJ-26 was isolated to prepare D-Ala from DL-Ala amide [40]. The strain possessed an amide hydrolase with a high D-stereospecificity towards alanine amide and glycine amide. A low activity for the L-Ala amide was detected, with a relative velocity of only 0.67% (K_m, 26.1 mM) of that toward the D-Ala amide (K_m, 4.19 mM). The reactivity with other amide substrates was very weak. After purification near to homogeneity (about 260-fold), the relative molecular mass was calculated to be approximately 67,000 and 51,000 Da by gel filtration and SDS-PAGE, respectively. Neither a requirement for metal ions nor for cofactors was detected. There was no evidence of hydrolyzing dipeptides or amino acid esters. After optimization of the culture conditions, 10 g/l of wet cells in a total volume of 2 l kinetically resolved 210 g/l (2.4 M) of DL-Ala amide to give 105 g/l of D-Ala with an optical purity of more than 99%.

Alkaline D-Peptidase (D-Stereospecific Peptide Hydrolase, EC 3.4.11.-)

Asano and coworkers screened for D-peptide-degrading microorganisms with synthetic (D-Phe)$_4$ as the substrate, which led to the isolation of the strain *Bacillus cereus* DF4-

B. From the culture broth, an extracellular D-stereospecific endopeptidase, alkaline D-peptidase (ADP), was isolated and purified to homogeneity [9, 10]. The enzyme was strictly stereospecific towards oligopeptides composed of D-Phe such as $(D\text{-Phe})_4$, (relative activity: 100%, K_m value: 0.398 mM), $(D\text{-Phe})_3$ (90%, 0.127 mM), $(D\text{-Phe})_6$ (1.8%) forming $(D\text{-Phe})_2$ and D-Phe. An examination of its mode of action revealed that it is a D-stereospecific dipeptidyl endopeptidase, hydrolyzing the second amide bond from the first amino-terminus. Maximal activity was displayed at about pH 10.3 and, accordingly, the enzyme was named as alkaline D-peptidase. A weak β-lactamase activity towards ampicillin (8.9%, 73.1 mM) and penicillin G (9.7%, 48.9 mM) was detected, yet neither DD-carboxypeptidase nor DAP activities were found. It is composed of a single peptide chain of M_r of 37,952. The primary structure similar to several other enzymes were found, including DD-carboxypeptidase from *Streptomyces* R61 (35.0% identical over 346 amino acids), PBPs, and class C β-lactamases. The typical sequence Ser-X-X-Lys is conserved in all of these enzymes, therefore, ADP can be categorized as one of the "Penicillin-recognizing enzymes" [22]. Recently, Komeda *et al.* sequenced the *adp* gene and found that three similar genes are tandemly located in the *B. cereus* genome (H. Komeda and Y. Asano, unpublished results).

Application to D-Phe Oligopeptide Synthesis

Kinetically controlled peptide synthesis was performed with ADP [28]. By placing the PCR-amplified *adp* under the promoter of pKK223-3, the expression plasmid pKADP was constructed and then transformed into *E. coli* JM109. In an alkaline aqueous medium (pH 11.5) in the presence of DMSO and $MgSO_4$, the enzyme catalyzed the polymerization of D-PheOCH$_3$ HCl. The formation of $(D\text{-Phe})_2$, $(D\text{-Phe})_3$, and $(D\text{-Phe})_4$ was confirmed and quantified by HPLC. Maximum yields of $(D\text{-Phe})_2$ and $(D\text{-Phe})_3$ of 12.5% and 6.5%, respectively, were obtained with a substrate concentration of 50 mM. At higher concentrations, the yield decreased, probably due to the substrate inhibition of ADP. Studies on the time course of oligomerization led to the production of $(D\text{-Phe})_2$ and trimer $(D\text{-Phe})_3$ with 25.4% and 8.6% yield, respectively, when 50 mM of the substrate was incubated for 8 h with ADP (2.0 U/ml and 0.4 U/ml, respectively) in 100 mM triethylamine-HCl (pH 11.5).

DD-Dipeptidase Involved in Vancomycin Resistance

The glycopeptide antibiotic, vancomycin, acts against Gram-positive bacteria, and, thus, is a powerful tool in the treatment of infections caused by strains such as *Staphylococcus aureus* or *Enterococcus faecalis*. Vancomycin is the most recently found antibiotic which eliminate multidrug-resistant strains such as MRSA, however, lately an increasing number of clinically resistant bacteria have appeared. Interestingly, vancomycin binds to the D-Ala-D-Ala moiety of peptidoglycan precursors, thereby blocking the subsequent transpeptidation step.

For resistance against vancomycin, the expression of five genes was found to be necessary in Transposon Tn1546. The *vanX* gene product is a D-Ala-D-Ala hydrolyzing DD-peptidase located in the cytoplasm. In an examination of the substrate specificity, VanX, L-Ala-D-Ala, and D-Ala-Gly were accepted as further substrates [33, 41]. VanX is dependent for divalent metal [51]. A recent mutagenesis study on VanX from *E. faecium* identified a putative enzyme ligand for zinc coordination, which involves residues His116, Asp123, and His184 [36].

Concluding Remarks

As D-amino acid-hydrolyzing enzymes are attractive biocatalysts, the development of methods for their application as well as the discovery of new enzymes is of great interest. Asano *et al.* [13] synthesized $(D\text{-Asp})_8$ and $(D\text{-Glu})_8$ with amide bonds between the 1-carboxylic acid and the 2-amino group in order to screen for new microbial enzyme which degrade unnatural D-amino acid peptides. Microorganisms from soil acclimated to a medium containing the oligopeptides were successfully isolated. The octamers were consumed by the microorganisms. It is expected that knowledge on new enzymes and related biochemistry will be developed further [14, 32].

Acknowledgments

This work was supported in part by Grants-in Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, and the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Korea.

Abbreviations: ADP, alkaline D-peptidase; DAP, D-aminopeptidase; DAA, D-stereospecific amino acid amidase; PBP, Penicillin-binding protein

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