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A new D-aminoacylase from *Defluvibacter* sp. A 131-3

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Abstract

A new D-aminoacylase was found from *Defluvibacter* sp. A 131-3. It was purified to homogeneity with an overall yield of 37%. This enzyme was specific to *N*-acyl-D-amino acids, and the activity on *N*-acetyl-D-valine was higher than that of the other *N*-acyl-D-amino acids. The enzyme activity was inhibited with $CoCl_2$, $NiCl_2$, $MnCl_2$, and $ZnCl_2$. Optimum pH and temperature were 8.0 and 37 °C, respectively. This enzyme is monomeric with a molecular mass of 56 kDa and an isoelectric point of 5.3. The NH_2-terminal sequence similarity was less than 30% of the previously reported D-aminoacylase. This enzyme has great potential for production of D-valine.

Keywords: D-aminoacylase; D-valine; *Defluvibacter*

1. Introduction

N-Acyl-D-amino acid amidohydrolase (EC.3.5.1.81, D-aminoacylase), which catalyzes enantioselective hydrolysis of *N*-acyl-D-amino acids, has long been recognized as a potential biocatalyst for D-amino acid production. It was found from 3 genera, *Pseudomonas* [1], *Streptomyces* [2,3], and *Alcaligenes* [4–7], more than 10 years ago. Recently, D-amino acids have become increasingly important as raw materials for valuable bioactive compounds in pharmaceuticals, and two strains from *Stenotrophomonas* [8] and *Variovorax* [9] were newly found to be producers of D-aminoacylase. All these enzymes catalyzed specific hydrolysis of *N*-acetyl derivatives of D-leucine, D-phenylalanine, D-methionine, and D-valine, but the reaction rate for *N*-acetyl-D-valine was much slower than that of other amino acid derivatives. Thus, when D-valine was produced using D-aminoacylases, from known bacterial strains, a large amount of D-aminoacylase and a long time reaction were required. These conditions posed great drawbacks for D-valine production on an industrial scale. Therefore, we screened for a microorganism capable of producing a D-aminoacylase with high activity on *N*-acetyl-D-valine. The present paper describes the identification of an isolated strain and some remarkable properties of a new D-aminoacylase produced by the isolated strain.

2. Materials and methods

2.1. Isolation of microorganism

After the cultivation was enriched three-times in an *N*-acetyl-DL-valine medium, pH 8.5, consisting of 0.2% *N*-acetyl-DL-valine, 0.1% Na_2HPO_4, 0.2% KH_2PO_4, 0.2% NH_4NO_3, and 0.05% $MgSO_4{\cdot}7H_2O$, the microorganisms were isolated on an agar plate with *N*-acetyl-DL-valine medium. The isolates were then incubated in 5 ml of the *N*-acetyl-DL-valine medium in a test tube (ϕ1.6 cm × 18 cm) at 30 °C for 3 days and the *N*-acetyl-D-valine concentration of the culture broth and D-aminoacylase activity of cells were assayed by HPLC and spectrophotometric methods, respectively. The strain producing D-aminoacylase with high activity on *N*-acetyl-D-valine was, finally, selected by comparing D-aminoacylase activity on *N*-acetyl-D-valine to that of *N*-acetyl derivatives of D-leucine, D-methionine, D-phenylalanine, and D-tryptophane.

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2.2. Identification of isolated strain

The strain was cultivated on a medium of Nutrient agar (Oxoid, UK) at 30 °C for 3 days. The DNA for a 16S rDNA sequencing analysis was prepared using a PrepMan method (Applied Biosystems, CA, USA), and the 16S rDNA sequence was determined using a MicroSeq Full Gene 16S rDNA Kit (Applied Biosystems, CA, USA). The sequences of the other bacteria used for alignment and for calculating the homology levels were obtained from the MicroSeq Bacterial Full Gene Library v.0001 (Applied Biosystems, CA, USA). MicroSeq Microbial Identification System Software V.1.4.1 (Applied Biosystems, CA, USA) was used to align the sequences, and the phylogenetic distance was calculated using the neighbor-joining method. The sequence alignment and calculation of the homology levels were also carried out by the BLAST program [10] using the Gen Bank database. A morphological characterization of the isolated strain was performed using a medium of nutrient agar. The physiological characterization was performed according to the method of Barrow and Feltham [11], and oxidase activity was assayed with the Cytochrome Oxidase test strip (Nissui Co., Tokyo, Japan).

2.3. Cultivation of microorganism

The isolated strain was cultured in the *N*-acetyl-DL-valine medium, pH 8.5 (200 ml/500 ml shaking culture flask) at 30 °C for 3 days. The culture (300 ml) was then inoculated into the second medium (20 l/30 l jar fermentor), which was prepared adding 0.05% NaCl, 0.1% yeast extract and 0.1% polypeptone to the *N*-acetyl-DL-valine medium. Cultivation was done at 30 °C for 27 h under the aeration of 10 l/min. After the cells were harvested by centrifugation at 10,000 rpm for 10 min, they were washed with distilled water and stored at −20 °C, until use.

2.4. Purification of enzyme

All operations were done at 4–10 °C. The buffer used was Tris–HCl, pH 8.0, unless otherwise stated.

2.4.1. Preparation of cell-free extract

Cells (110 g wet weight) from 20 l of culture broth were suspended in 300 ml of 20 mM buffer, and disrupted by sonication (Nihonseiki Seisakusho, Tokyo, Japan) for 60 min. One-third of the supernatant obtained by centrifugation at 10,000 rpm for 20 min was used for the purification of D-aminoacylase.

2.4.2. Toyopearl SuperQ-650 column chromatography

The supernatant solution (120 ml) was dialyzed against a 20 mM buffer containing 0.1 M NaCl, and precipitates formed were discarded by centrifugation. The supernatant solution was applied to a Toyopearl SuperQ-650 column (4.4 cm × 37.5 cm) equilibrated with 20 mM buffer containing 0.1 M NaCl, and the column was washed with 1.5 l of the same buffer. Then, the adsorbed enzyme was eluted by a linear gradient of 20 mM buffer containing 0.1 M NaCl and 20 mM buffer containing 0.3 M NaCl (5.7 l each). The active fractions were then combined and dialyzed against a 5 mM potassium phosphate buffer, pH 7.2.

2.4.3. Hydroxyapatite column chromatography

The dialyzed enzyme solution was applied to a Hydroxyapatite column (2.2 cm × 20 cm) equilibrated with 5 mM potassium phosphate buffer, pH 7.2. After the column was washed with 350 ml of 5 mM potassium phosphate buffer, pH 7.2, the adsorbed enzyme was eluted by a linear gradient of 5 mM potassium phosphate buffer, pH 7.2 and 0.2 M potassium phosphate buffer, pH 7.2 (750 ml each). The active fractions were combined and concentrated by ultrafiltration.

2.4.4. Gel filtration on Superdex 200

The concentrated enzyme solution was applied to a Superdex 200 column (2.2 cm × 66 cm) equilibrated with 20 mM buffer containing 0.3 M NaCl. The active fractions were combined and concentrated by ultrafiltration.

2.5. Assay of enzyme activity

Enzyme activity was measured by the spectrophotometric or HPLC methods.

2.5.1. Spectrophotometric method

Enzyme activity was assayed by measuring the formation rate of hydrogen peroxide in the combination of D-amino acid oxidase and peroxide as follows. The reaction mixture (0.90 ml) was composed of 10 mM *N*-acetyl-D-valine and color reagent consisting of 0.031 mg of 4-aminoantipyrine, 0.166 mg of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate, 0.1 unit of D-amino acid oxidase and 1.5 units of peroxidase/ml of 0.1 M potassium phosphate, pH 8.0. The reaction was started by the addition of 100 μl of enzyme solution, and the reaction mixture was incubated at 37 °C for 30 min The absorbance change was assayed at 555 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole hydrogen peroxide/min by the oxidation of D-valine generated from *N*-acetyl-D-valine.

2.5.2. HPLC method

Ten mM of *N*-acetyl-D-valine adjusting to pH 7.8–8.0 with 5 M NaOH was incubated with the enzyme at 37 °C for 30 min, and the concentration of D-valine formed was measured by HPLC with an Inertsil ODS-2 column (GL Science, Tokyo, Japan) at a flow rate of 0.5 ml/min at 30 °C using 0.015% 1-pentasulfonic acid sodium, pH 2.5/acetonitrile (80:20) as solvent. Detection was carried out at 230 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole D-valine/min.

2.6. Resolution of *N*-acetyl-DL-valine

Ten ml of reaction mixture containing 1.5 g of *N*-acetyl-DL-valine, which was adjusted to pH 7.8–8.0 with 5 M NaOH, was incubated with 4.4 units of D-aminoacylase at 30 °C for 3 days. Resolved D-valine was assayed by HPLC with a SUMICHIRAL OA-5000 column (Sumika Chemical Analysis Service, Osaka, Japan) at a flow rate of 0.8 ml/min at 40 °C using a solvent of 2 mM copper (II) sulfate in water/acetonitrile (90:10). Detection was carried out at 230 nm.

2.7. Protein and molecular mass measurement

Protein was measured with a BCA protein assay kit (Pierce Biotechnology, Rockford, USA). Molecular mass was estimated by gel filtration on Superdex 200 HR 10/30 column and by SDS-PAGE according to the method of Laemmli [12].

2.8. Isoelectric point

The isoelectric point was determined using an IPG Tube Gel "DAIICHI" 4–10 and 2D-Protein p*I* Marker "DAIICHI" (Daiichi Pure Chemicals, Tokyo, Japan).

3. Results

3.1. Screening of strain producing D-aminoacylase with high activity on *N*-acetyl-D-valine

In the first step, 34 strains with high D-aminoacylase activity on *N*-acetyl-D-valine were selected from more than 250 strains isolated by the method described above. Substrate specificity of each enzyme was then assayed by HPLC. The strain producing D-aminoacylase with the highest activity on *N*-acetyl-D-valine was selected and used in the following studies.

3.2. Identification of isolated strain

The isolated strain was identified by phylogenetic analysis and biochemical characteristics. The 16S rDNA sequence analysis with the MicroSeq bacterial full gene library showed high similarity to the *Labrys monachus*, but the percentage of DNA similarity was less than 97% (95.47%). The 16S rDNA sequence analysis by the BLAST program using the Gen Bank database showed the highest similarity to the strain *Defluvibacter lusatiensis* (99.9%) [13]. The biological traits of this strain showed that the cell was a rod (0.6–0.7 μm × 1.5–2.0 μm), motile, Gram-negative, and nonspore-forming. This strain was strictly aerobic, methophilic (grew at 37 °C, but not at 45 °C), catalase-positive, oxidase-positive, and non-fermentative. It assimilated the following compounds as a sole carbon source: glucose, *N*-acetyl-D-glucosamine, potassium gluconate, and DL-malic acid. These biological characteristics are the same as those of the genus *Defluvibacter*. Thus, the isolated strain might belong to the genus *Defluvibacter* and locate within the same cluster as *Defluvibacter lusatiensis*. However, at present only one strain is reported as the genus *Defluvibacter*, and the isolated strain could not compare to other strains of the genus *Defluvibacter*. Therefore, the isolated strain was named *Defluvibacter* sp. A131-3.

3.3. Enzyme production

The newly isolated strain, *Defluvibacter* sp. A131-3, was cultured in a medium containing glucose, sucrose, *N*-acetyl-DL-valine, *N*-acetyl-D-valine, *N*-acetyl-L-valine, *N*-acetyl-DL-leucine, *N*-acetyl-D-leucine or *N*-acetyl-DL-methionine as a sole carbon source at 30 °C for 3 days. The strain grew well in all media tested, and D-aminoacylase activity on *N*-acetyl-D-valine was obtained from all cells cultured in the above media. However, high concentration of D-valine was obtained by the reaction with the cells from the medium containing *N*-acetyl derivatives of D-valine or D-leucine (Table 1). Therefore, the medium containing *N*-acetyl-DL-valine was used for production of D-aminoacylase.

3.4. Purification and molecular mass

The purification procedure is summarized in Table 2. Approximately 70-fold purification was achieved with an overall yield of 37%. The purified enzyme showed a single protein band on SDS-PAGE (data not shown). The molecular masses of the native and denatured enzymes were estimated to be approximately 56 kDa on a Superdex 200 column and SDS-PAGE (data not shown). These results indicate that this D-aminoacylase might be a monomeric enzyme.

Table 1
Effects of carbon source on D-aminoacylase production by *Defluvibacter* sp. A131-3

Carbon source	Growth (OD_{660})	D-Valine formed (%)
Glucose	1.64	66.6
Sucrose	0.76	50.7
N-Acetyl-DL-valine	1.20	90.8
N-Acetyl-DL-leucine	1.18	89.9
N-Acetyl-DL-methionine	0.70	57.0
N-Acetyl-DL-phenylalanine	0.74	66.6
N-Acetyl-DL-tryptophane	0.72	40.6
N-Acetyl-D-valine	1.20	92.7
N-Acetyl-D-leucine	1.26	90.6
N-Acetyl-L-valine	0.98	67.9

The strain was cultured at 30 °C for 3 days in the medium containing 0.1% Na_2HPO_4, 0.2% KH_2PO_4, 0.2% NH_4NO_3, 0.05% $MgSO_4 \cdot 7\ H_2O$, 0.05% NaCl, 0.1% yeast extract, 0.1% polypeptone, and 0.2% of indicated compounds. The same cell amounts harvested were incubated with 15% *N*-acetyl-DL-valine at pH 8.0 for 3 days, and the concentration of D-valine resolved was determined by HPLC with SUMICHIRAL OA-5000 column.

Table 2
Summary of purification of D-aminoacylase from *Defluvibacter* sp. A131-3

Step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Recovery (%)	Purification (fold)
Cell-free extract	6020	4600	1.3	100	1
Super Q	2950	47	62.8	49	48
Hydroxyapatite	2490	27	92.2	41	71
Superdex 200	2220	24	92.5	37	71

Enzyme activity was assayed by spectrophotometric method. Specific activity was expressed as units/mg of protein.

3.5. Substrate specificity and kinetic parameter

This enzyme hydrolyzed *N*-acetyl-D-amino acids, but not *N*-acetyl-L-amino acids. In the *N*-acetyl-D-amino acids, this enzyme preferentially hydrolyzed *N*-acetyl derivatives of aliphatic D-amino acids, such as *N*-acetyl-D-valine, *N*-acetyl-D-leucine, and *N*-acetyl-D-methionine. In *N*-acetyl derivatives of aliphatic D-amino acids, *N*-acetyl-D-valine was the preferred substrate, and the apparent K_m value for *N*-acetyl-D-valine was estimated to be 1.8 mM. *N*-Acetyl derivatives of aromatic D-amino acids such as *N*-acetyl-D-phenylalanine and *N*-acetyl-D-tyrosine were also hydrolyzed, but their reaction rates were slower than those of *N*-acetyl derivatives of aliphatic D-amino acids (Table 3).

3.6. Effects of pH and temperature

After 20 mM *N*-acetyl-D-valine was incubated with enzyme at 30 °C for 10 min in 20 mM citrate; potassium phosphate; boric acid; diethylbarbital buffer, pH 6.0–11.0, the reaction was terminated by boiling for 3 min Then, D-valine formed in this period was assayed at pH 8.0 by the spectrophotometric method for enzyme activity with D-amino acid oxidase. The maximum reaction rate was obtained at pH 8.0 (Fig. 1A). The pH stability of this enzyme was investigated by incubating at 30 °C for 24 h between pH 6.0 and 11. The residual enzyme activity was assayed under standard assay conditions of the spectrophotometric method. More than 80% of the enzyme activity remained between pH 7.5 and 10 (Fig. 1A). The effect of temperature on enzyme activity was assayed by the spectrophotometric method at various reaction temperatures. The maximum reaction rate was obtained at around 37 °C (Fig. 1B). The heat stability of the enzyme was obtained by incubating with 0.1 M Tris–HCl, pH 8.5, at 0–50 °C for 24 h. Approximately 90% of the enzyme activity remained at 30 °C, while almost of the enzyme activity was lost at 50 °C (Fig. 1B).

Table 3
Substrate specificity of D-aminoacylase from *Defluvibacter* sp. A131-3

Substrate (50 mM)	Relative activity (%)
N-Acetyl-D-valine	100
N-Acetyl-D-leucine	73
N-Acetyl-D-methionine	13
N-Acetyl-D-phenylalanine	5
N-Acetyl-D-tyrosine	1
N-Acetyl-D-tryptophane	0.2
N-Acetyl-D-glutamic acid	0

Inert: *N*-acetyl-L-valine, *N*-acetyl-L-leucine, *N*-acetyl-L-methionine, *N*-acetyl-L-phenylalanine, *N*-acetyl-L-tyrosine, *N*-acetyl-L-tryptophane, *N*-acetyl-L-glutamic acid.

3.7. Effects of compounds on enzyme activity

Effects of compounds on enzyme activity were assayed by the HPLC method, after 1 mM metals or 5 mM chemicals were incubated in the reaction mixture containing 0.5 M *N*-acetyl-D-valine and 1.1 unit of enzyme at 30 °C for 24 h. The enzyme activity was strongly inhibited by $CoCl_2$, $NiCl_2$, $MnCl_2$, and $ZnCl_2$ among the 10 kinds of metals tested. Among 7 chemicals, *o*-phenanthroline, 2-mercaptoethanol, L-cysteine, and dithiothreitol inhibited enzyme activity, but other reagents had no significant influence (Table 4). The inhibition of enzyme activity with *o*-phenanthroline was recovered by addition of 0.1 mM Zn^{2+}, but not by 0.1 mM Co^{2+} (data not shown).

3.8. Isoelectric point

A single protein band was observed at pH 5.3 by electrophoresis.

Table 4
Effects of chemicals and metals on D-aminoacylase activity

Chemical or metal	Relative act (%)
None	100
EDTA	112
o-Phenanthroline	53
Iodoacetoamide	107
N-Ethylmaleimide	100
2-Mercaptoethanol	47
L-Cysteine	78
Dithiothritol	38
$CaCl_2$	99
$MgCl_2$	90
$CuSO_4$	90
$FeCl_2$	88
$MnCl_2$	48
$ZnCl_2$	31
$CoCl_2$	27
$NiCl_2$	20

Enzyme activity was assayed by HPLC method, after 5 mM chemicals or 1 mM metals were incubated with 1.1 unit of enzyme and 0.5 M *N*-acetyl-D-valine at 30 °C for 24 h.

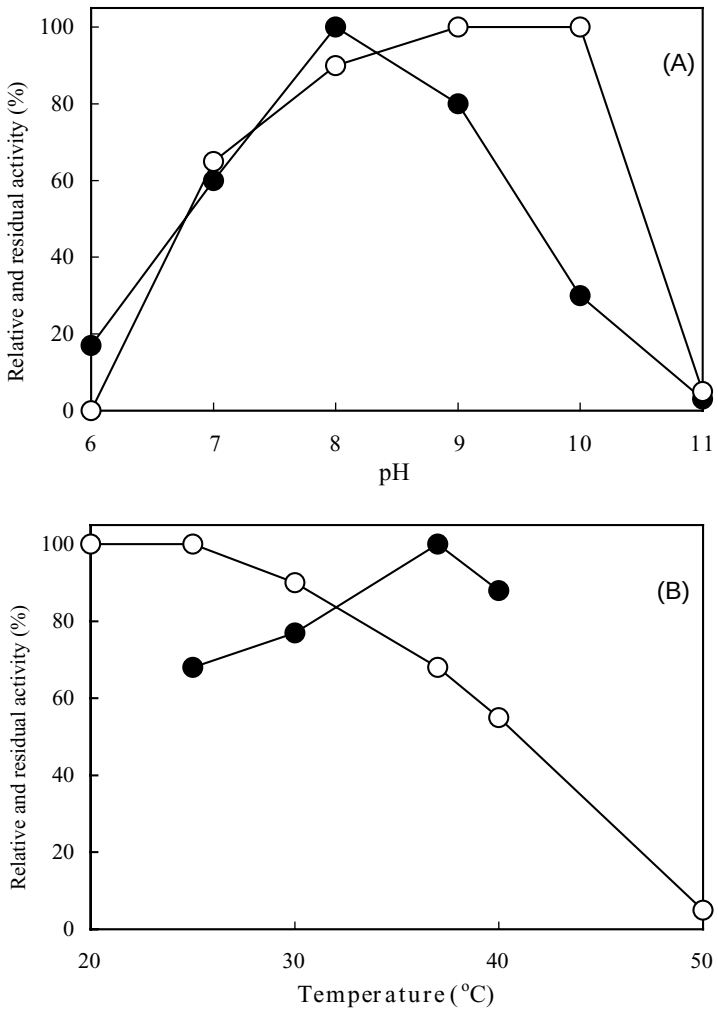

Fig. 1. Effects of pH and temperature on activity and stability of D-aminoacylase from *Defluvibacter* sp. A131-3. (A) Effects of pH: the reaction rate (closed circles) was assayed by the method described in the text. The pH stability (open circles) was assayed by the spectrophotometric method, after heating at 30 °C for 24 h at the indicated pH. The percentage of residual activity was obtained by the ratio to the activity without heating. (B) Effects of temperature: The reaction rate (closed circles) was assayed under the standard assay conditions of spectrophotometric method except for the reaction temperature. The heat stability (open circles) was assayed under the standard assay conditions of spectrophotometric method, after heating in 0.1 M Tris–HCl, pH 8.5, for 24 h at the indicated temperature. The percentage of residual activity was obtained by the ratio to the activity without heating.

3.9. NH_2-terminal amino acid sequence

The NH_2-terminal sequence of the intact protein was found to be MAKSFDLVIRNGRVVDPETGH, which exhibited low similarity with those of other D-aminoacylases reported previously (Fig. 2).

3.10. Resolution of *N*-acetyl-DL-valine

Resolution of *N*-acetyl-DL-valine was investigated using 15% *N*-acetyl-DL-valine. When 1.5 g of *N*-acetyl-DL-valine was incubated with 4.4 units of D-aminoacylase in 10 ml of reaction mixture of pH 8.0 at 30 °C for 3 days, more than

***Defluvibacter* sp.A 131**				M	A	K	S	F	D	L	V	I	R	N	G	R	V	V	D	P	E	T	G	H	This study
***Alcaligenes denitrificans* MI-4**	A	Q	S	D	S	Q	P	F	D	L	X	T	A	G	G	T	L	I	D	G	X	N	T	P	4
***Alcaligenes xylosoxydans* A-6**	S	Q	S	D	S	Q	P	F	D	L	L	R	A	G	G	T	L	I	D	G	S	N	T	P	7
***Alcaligenes denitrificans* DA 181**	S	Q	P	D	A	T	P	F	D	Y	I	L	S	G	G	T	V	I	D	X	T	N	X	P	5
Variovorax paradoxus		M	P	K	Y	P	H	Y	D	L	L	V	R	G	G	T	V	I	D	G	S	K	A	P	9

Fig. 2. Alignment of the NH_2-terminal amino acid sequence of D-aminoacylase from *Defluvibacter* sp. A131-3 and related sequences. Identical amino acid residues are enclosed in boxes.

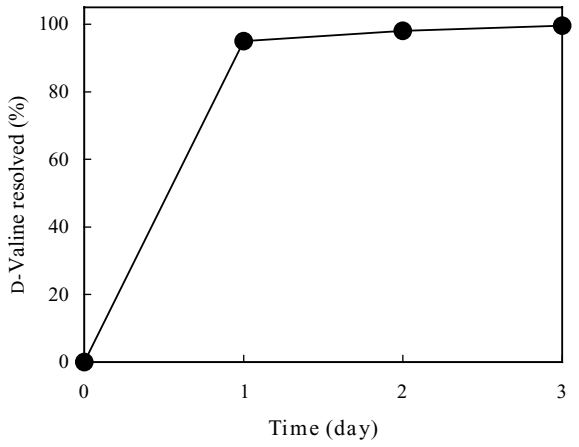

Fig. 3. Resolution of *N*-acetyl-DL-valine. *N*-Acetyl-DL-valine (1.5 g) was incubated with 4.4 units of D-aminoacylase in 10 ml of reaction mixture under standard reaction conditions.

90% D-valine was produced by one day and approximately 100% D-valine was produced by three days reaction, while L-valine was not formed (Fig. 3).

4. Discussion

It is known that D-amino acids are able to be produced by the enzymatic resolution of *N*-acyl-DL-amino acids. However, the present D-aminoacylases were not used for producing a wide range of D-amino acids because of their limited substrate specificity. Until now, D-aminoacylase has been obtained from five genera; *Pseudomonas* [1], *Streptomyces* [2,3], *Alcaligenes* [4–7], *Stenotrophomonas* [8], and *Variovorax* [9]. All these enzymes hydrolyzed *N*-acetyl derivatives of D-leucine, D-phenylalanine, D-methionine, and D-valine, but the reaction rate for *N*-acetyl-D-valine was much slower than that of other amino acid derivatives (Table 5). Thus, when D-valine was produced using the present D-aminoacylases, a large amount of D-aminoacylase and a long time reaction were required, presenting obstacles to the production of D-valine on an industrial scale. Therefore, we screened for a microorganism capable of producing D-aminoacylase with a high reaction rate for *N*-acetyl-D-valine, and isolated *Defluvibacter* sp. A 131-3 using a medium containing *N*-acetyl-DL-valine. The D-aminoacylase from *Defluvibacter* sp. A 131-3 was specific to *N*-acetyl-D-amino acids, and hydrolyzed *N*-acetyl derivatives of D-valine, D-leucine, D-phenylalanine, and D-methionine. In the reaction of *N*-acetyl-D-amino acids, the reaction rate for *N*-acetyl-D-valine was much faster than that of other *N*-acetyl-D-amino acids, and specific activity for *N*-acetyl-D-valine was higher than that of the previously reported D-aminoacylases. In addition, D-valine was efficiently resolved from *N*-acetyl-DL-valine. Thus, it was concluded that D-aminoacylase from a new isolated strain, *Defluvibacter* sp. A 131-3, has great potential for production of D-valine on

Table 5
Properties of D-aminoacylase from *Defluvibacter* sp. A131-3 and other microorganisms

	Defluvibacter sp. A 131-3	*Pseudomonas* sp. 6029	*Streptomyces olivaceus*	*Streptomyces tuirus*	*Alcaligenes* MI4	*Alcaligenes* DA181	*Alcaligenes* DA1	*Alcaligenes* A-6	*Variovorax paradoxus*
Substrate specificity									
N-Acetyl-D-valine	100	0	32	28	1	ND	4	8	18
N-Acetyl-D-leucine	73	23	100	100	17	45	47	100	84
N-Acetyl-D-methionine	13	30	74	60	100	100	100	68	100
N-Acetyl-D-phenylalanine	11	100	53	89	80	85	60	87	24
N-Acetyl-D-tyrosine	1	ND	18	46	ND	ND	ND	ND	4
N-Acetyl-D-tryptophane	0.2	ND	11	10	5	ND	10	ND	5
Molecular mass									
Gel filtration (kDa)	56	45	45	ND	47	58	55	58	52.8
SDS-PAGE (kDa)	56	ND	ND	ND	51	ND	ND	52	ND
Isoelectric point	5.3	ND	ND	ND	5.4	4.4	ND	5.2	5.12
Opt-temperature (°C)	37	ND	50	ND	50	45	45	50	50
Opt-pH	8.5	6.8	7.0	ND	7.8	7.5	8.0	7.0	7.5
pH-stability	7.5–10	7–9	6–10	ND	ND	6–11	5–11	7.4–8.3	6–8
Reference	This report	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[9]

Values of substrate specificity are relative activity against highest substrate. ND: no data.

an industrial scale. This enzyme was classified into a group of *N*-acyl-D-amino acid amidohydrolase (D-aminoacylase), but its reaction rate for *N*-acetyl derivatives of D-amino acids was different from that of the other D-aminoacylases. In addition, the NH_2-terminal sequence of this enzyme was also different from those of other D-aminoacylases reported to date [4,5,7,9]. We intend to elucidate the primary structure of this D-aminoacylase in more detail in a future study.

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