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Paenidase, a Novel D-Aspartyl Endopeptidase from *Paenibacillus* sp. B38: Purification and Substrate Specificity

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We discovered and characterized a novel type D-aspartyl endopeptidase (DAEP) produced extracellarly by *Paenibacillus* sp. B38. This bacterial DAEP of M_r 34,798 (named paenidase) appeared to be converted into a smaller form of M_r 34,169 by the proteolytic removal of 5 amino acid residues from the N-terminal. The intact and modified forms of the enzyme displayed essentially the same enzymatic properties. The enzyme specifically hydrolyzed succinyl-D-aspartic acid α-(*p*-nitroanilide) and succinyl-D-aspartic acid α-(4-methylcoumaryl-7-amide) to generate *p*-nitroaniline and 7-amino-4-methylcoumarin, and internally cleaved a synthetic peptide (D-A-E-F-R-H-[D-Asp]-G-S-Y) of the [D-Asp]^7 amyloid β (Aβ) protein between [D-Asp]^7-G^8. Either was totally inert to the normal Aβ peptide sequence containing L-Asp, instead of D-Asp at the 7th position. Thus, paenidase is the first DAEP from a microorganism that specifically recognizes an internal D-Asp residue to cleave [D-Asp]-X peptide bonds.

Key words: D-amino acid, D-aspartic acid, endopeptidase, protease, substrate specificity.

Abbreviations: Aβ, amyloid β; DAEP, D-aspartyl endopeptidase; Suc, succinyl; iDAEP, (Benzyl-L-arginine-L-histidine-D-aspart-1-yl) chloromethane; *p*NA, *p*-nitroanilide; MALDI-TOF/MS, matrix-assisted laser desorption/ionozation time-of-flight mass spectrometer; MCA, 4-methylcoumaryl-7-amide.

Various kinds of proteinases have been found in microorganisms including basidiomycetes. Some proteinases show high specificity for a particular residue *e.g.* the lysyl endopeptidase from *Achromobacter lyticus* M497-1 (*1*), prolyl aminopeptidases from *Grifola frondosa* (*2*), *Aeromonas sorbia* (*3*), and *Bacillus coagulans* (*4*), pyroglutamate aminopeptidase from *Bacillus amyloliquefaciens* (*5*), and an alcohol resistant metalloproteinase from *Vibrio* sp. T1800 (*6*). Microbial enzymes that recognize D-amino acids have also been described (*7–15*). D-Amino acid oxidases from *Rodotorular gracilis* (*8*) and yeast (*9*) catalyze the oxidation of D-amino acids, D-amino acid peptidases (*10–12*) cleave N-terminal D-amino acids, and D-amino acid amidase (*13–15*) catalyzes the hydrolysis of D-amino acid amide to yield D-amino acid and ammonia.

The accumulation of D-Asp in proteins during aging has been implicated in the pathogenesis of several diseases (*16–23*). For instance, increases in D-Asp in amyloid β (Aβ) and tau proteins may cause Alzheimer's disease (*16*, *17*). The failure to degrade D-Asp containing proteins causes an accumulation of abnormal proteins in the tissues. In mammalian tissues, D-aspartyl endopeptidase (DAEP) may serves as a scavenger of D-Asp-containing Aβ and tau proteins by specifically degrading such abnormal proteins (*24*). The mammalian DAEP is located in the inner mitochondrial membranes and forms a proteosome like multi-complex structure with a molecular mass of 600 kDa (*24*). To our knowledge, no DAEP from a microorganism has been reported to date. In the present study, we isolated a DAEP-producing microorganism from soil. DAEPs (named paenidases) were purified from the culture medium of *Paenibacillus* sp. B38 and some properties of the purified enzymes were characterized

MATERIALS AND METHODS

Materials—A novel chromogenic substrate for DAEP, succinyl (Suc)-[D-Asp]-*p*-nitroanilide (*p*NA), was designed and custom-synthesized by the Peptide Institute (Osaka, Japan). Peptidyl-4-methylcoumaryl-7-amides (MCAs), Ala-*p*NA, Leu-*p*NA, Suc-Ala-Ala-Ala-*p*NA, Suc-Ala-Pro-Ala-*p*NA, leupeptin, pepstatin, antipain, amastatin, and iDAEP [(Benzyl-L-arginine-L-histidine-D-aspart-1-yl) chloromethane] (*24*) were obtained from the Peptide Institute. Other peptidyl-*p*NAs, except D-Asp-*p*NA, were obtained from Bachem (Bubendorf, Switzerland). Normal Aβ peptide (DAEFRHDSGY) was custom-synthesized by Funakoshi (Tokyo, Japan). [D-Asp]^7 Aβ peptide (DAEFRH-[D-Asp]-SGY) and D-Asp-*p*NA were custom-synthesized by Biologica (Nagoya, Japan). BCA and Micro BCA protein assay kits were obtained from Pierce (Rockford, IL, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA) and Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Screening of Microorganisms—DAEP activities of the culture media of microorganisms were determined using Suc-[D-Asp]-*p*NA as a substrate. An enzyme solution (20 μl) was mixed with 80 μl of 1 mM Suc-[D-Asp]-*p*NA in 50 mM Tris-HCl, pH 8.0, 0.02% Tween 20, 0.02% NaN_3, and incubated at 37°C for 24 h. After incubation, the reaction was terminated by the addition of 400 μl of 10% acetic acid, and then the absorbance at 405 nm was measured.

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Peptidyl-MCA Hydrolyzing Activity—DAEP activities were also determined using Suc-[D-Asp]-MCA as a substrate. An enzyme solution (20 μl) was mixed with 80 μl of 0.625 mM Suc-[D-Asp]-MCA in 50 mM Tris-HCl, pH 8.0, 0.02% Tween 20, 0.02% NaN_3, 5% DMSO, and incubated at 37°C for 30 min. After incubation, the reaction was terminated by the addition of 400 μl of 10% acetic acid, and then the fluorescence of the liberated 7-amino-4-methylcoumarin was measured at an emission wavelength of 460 nm upon excitation at 380 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol 7-amino-4-methylcoumarin per min. Other peptidyl-MCAs were also used for the determination of substrate specificities.

Purification of Paenidases—*Paenibacillus* sp. B38 was aerobically grown at 30°C for 6 days in a medium containing 1% beef extract, 1% polypeptone, 1% glucose, and 0.3% NaCl, pH 7.0. Paenidases in the culture supernatant were fractionated with 0–80% saturation of ammonium sulfate and dialyzed against 50 mM Tris-HCl, pH 8.0. The dialyzed sample was centrifuged at 20,000 × *g*, for 30 min to remove insoluble materials. DEAE-Sepharose FF (50 ml, Amersham Biosciences, Uppsala, Sweden) was added to the supernatant and the mixture was stirred for 30 min to adsorb the enzymes. The adsorbed proteins were eluted with 200 ml of Tris-HCl, pH 8.0, containing 0.2 M NaCl. The eluate was concentrated to 40 ml with an Amicon PM-10 membrane and applied to a column of Sephacryl S-100 HR (5 × 80 cm, Amersham Biosciences) previously equilibrated with 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.02% NaN_3. Fractions containing paenidase activity were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0. The dialyzed sample was applied to a column of DEAE-Sepharose FF (1.5 × 8.0 cm) equilibrated with the same buffer, and paenidases were eluted by a linear gradient of NaCl (0–0.2 M, 400 ml). Fractions containing paenidase activity were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0. The sample was applied to an FPLC equipped with a Mono Q HR 5/5 column (0.5 × 5 cm, Amersham Biosciences) equilibrated with the same buffer, and paenidase activity was eluted with 30 ml of 0 to 0.2 M NaCl linear gradient at a flow rate of 1 ml/min. Paenidase activity eluted from the Mono Q column as two peaks (paenidases I and II). Fractions containing paenidases I and II were collected separately and dialyzed against 50 mM Tris-HCl, pH 8.0. The dialyzed paenidases I and II were further purified by Mono Q FPLC as above.

Protein concentrations were determined with BCA and Micro BCA protein assay kits using bovine serum albumin as a standard. SDS-PAGE was performed by the method of Laemmli (*25*) using 10–20% polyacrylamide gel (E-T1020L, ATTO, Tokyo, Japan). After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/MS)—The masses of purified enzymes were measured with a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA, USA) using sinapinic acid (Nakalai Tesque, Kyoto, Japan) as the matrix.

Hydrolysis of Synthetic Aβ peptides by Paenidase—The reaction mixture was composed of 100 μl normal Aβ peptide (DAEFRHDSGY) or [D-Asp]^7 Aβ peptide (DAEFRH-[D-Asp]-SGY) (each 10 nmol) in 50 mM Tris-HCl, pH 8.0, 0.02% Tween 20, 0.02% NaN_3 and 20 μl of Paenidase I (40 mU), and was incubated at 37°C for 24 h. Then the reaction mixture (10 μl) was applied to a high performance liquid chromatography reversed-phase column (ODS-L80 column, YMC, Tokyo, Japan), and eluted with a linear gradient of 0–100% acetonitrile in H_2O.

N-Terminal Amino Acid Sequence of Paenidase Hydrolyzed Aβ peptides—The amino acid sequences of the peptide fragments derived from D-Asp^7 Aβ peptide were determined with a PPSQ-10 amino acid sequencer (Shimadzu, Kyoto, Japan).

RESULTS

Isolation of Bacteria—About 400 bacterial strains isolated from soil in Akita Prefecture, Japan were tested for the ability to produce Suc-[D-Asp]-*p*NA endopeptidase. Most of the bacteria showed no enzymatic activity. But one bacterium, B38 strain, was found to produce the enzyme. This was classified as genus *Paenibacillus* on the basis of its physicochemical properties and 16s rRNA sequence.

Purification of Paenidase—Two forms paenidases (I and II) were purified from the culture supernatant of *Paenibacillus* sp. B38. A summary of the purification is shown in Table 1. From 1,250 ml of culture supernatant, 43 μg of paenidase I and 65 μg of paenidase II were

Table 1. **Summary of the purification of paenidase from the culture medium of *Paenibacillus* sp. B38.**

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Culture medium	1,500	5,090	0.29	1	100
Ammonium sulfate	1,320	737	1.79	6.2	88.0
DEAE batch	1,034	419	2.47	8.8	68.9
Sephacryl S-100 HR	733	4.2	17.5	60.3	48.9
DEAE-Sepharose FF	583	2.5	233	803	38.9
1st Mono Q					
P-I*[1]	129	0.159	811	2,800	8.6
P-II*[2]	222	0.144	1,540	5,310	14.8
2nd Mono Q					
P-I*[1]	102	0.043	2,370	8,170	6.8
P-II*[2]	187	0.065	2,880	9,930	12.5

Paenidase activity was measured using Suc-[D-Asp]-MCA as a substrate. *[1]P-I, paenidase I; *[2]P-II, paenidase II.

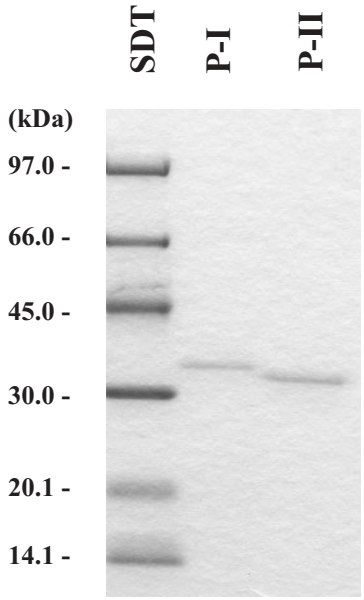

Fig. 1. **SDS-PAGE of the purified paenidases I and II.** SDS-PAGE was carried out in a 10–20% polyacrylamide gel that was subsequently stained with Coomassie Brilliant Blue R-250. STD, protein size marker; P-I, paenidase I (0.5 μg); P-II, paenidase II (0.5 μg).

obtained with yield of 6.8 and 12.5%, respectively. The purified paenidases showed single protein bands on SDS-PAGE with molecular weights of 34,000 (paenidase I) and 33,000 (paenidase II) (Fig. 1). The purified paenidases also showed single protein peaks after native gel filtration through Superdex 75 FPLC with an apparent molecular weight of 35,000. The molecular weights of the enzymes were also determined by MALDI-TOF/MS to be 34,798 for paenidase I and 34,169 for paenidase II.

Effects of pH on Paenidase Activity—Optimum pH of paenidases I and II were around pH 8.0 when Suc-[D-Asp]-MCA was used as a substrate (Fig. 2A). When Suc-[D-Asp]-*p*NA was used as a substrate, the enzymes showed same optimum pH (data not shown). Stability of the purified enzymes was examined by incubating the enzymes at pH 8.0 and at various temperatures for 30 min (Fig. 2B). Paenidases I and II were stable below 30°C, but lost the activity as the temperature increased, almost losing activity at 70°C.

Effects of Proteinase Inhibitors on Paenidase Activity—The enzyme activities were inactivated by relatively high concentrations of pepstatin and iDAEP, but not by PMSF, DFP, SBTI, leupeptin, NEM, IAA, PCMB, antipain, amastatin, EDTA, or 1,10-phenanthroline (Table 2).

Effects of Metal Ions on Paenidase Activity—Table 3 shows the effects of divalent cations on paenidase activities. Zn^{2+} strongly inhibited and Co^{2+} and Mn^{2+} showed a weak inhibitory effect. Ca^{2+} and Mg^{2+} had no effect on the enzyme activities.

Substrate Specificity of Paenidases—The substrate specificities of paenidases I and II are also shown in Tables 4 and 5. Paenidases I and II specifically cleaved Suc-[D-Asp]-*p*NA or Suc-[D-Asp]-MCA; neither cleaved other peptidyl-*p*NA and -MCA substrates.

Kinetic Parameters—Kinetic parameters of the enzymes were determined using Suc-[D-Asp]-*p*NA and

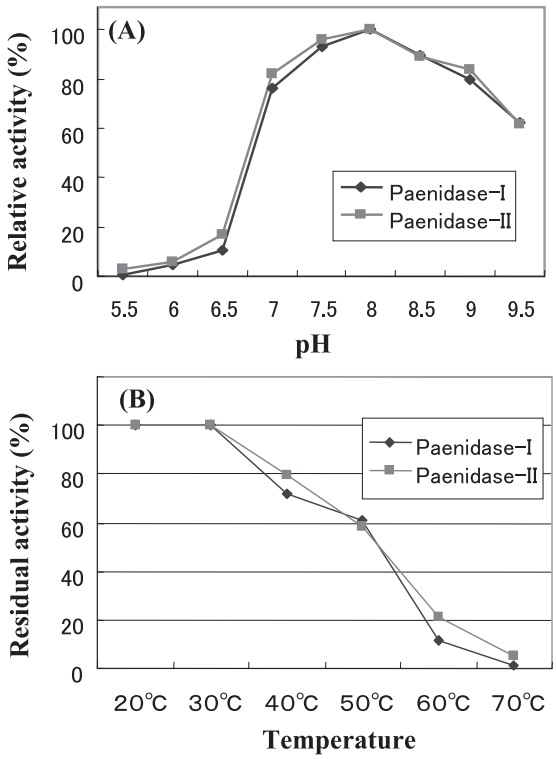

Fig. 2. **Effects of pH and thermal stability.** (A) Effects of pH on paenidase activity: The enzyme activity at various pHs was measured in 50 mM MES-Tris-HEPPSO buffer using Suc-[D-Asp]-MCA as a substrate. (B) Thermal stability: Paenidases I and II (20 U/ml in 50 mM Tris-HCl, pH 8.0, 0.02% Tween 20, 0.02% NaN_3) were incubated at each temperature for 30 min and the remaining activity was measured.

Table 2. **Effects of protease inhibitors on paenidase activity.**

Inhibitor	Concentration	Relative activity (%)	
		Paenidase I	Paenidase II
none		100	100
PMSF*[1]	0.1 mM	90.6	98.9
DFP*[2]	0.1 mM	106	100
SBTI*[3]	0.1 mg/ml	101	103
Leupeptin	0.1 mM	95.4	101
NEM*[4]	0.1 mM	91.4	91.3
IAA*[5]	0.1 mM	100	100
PCMB*[6]	0.1 mM	131	138
Pepstatin	10 μg/ml	46.6	51.6
Antipain	10 μg/ml	112	112
Amastatin	10 μg/ml	100	100
EDTA	1.0 mM	101	100
1,10-Phenanthroline	1.0 mM	101	100
i-DAEP*[7]	1.0 mM	32.8	32.1

Suc-[D-Asp]-MCA was used as a substrate. *[1]PMSF, phenylmethylsulfonyl fluoride; *[2]DFP, diisopropylfluorophosphate; *[3]SBTI, soybean trypsin inhibitor; *[4]NEM, *N*-ethylmaleimide; *[5]IAA, iodoacetic acid; *[6]PCMB, *p*-chrolomercuri benzoic acid; *[7]i-DAEP, Bz-Arg-His-[D-Asp]-CH_2Cl.

Suc-[D-Asp]-MCA (Table 6). Paenidases I and II had nearly the same K_m, k_{cat}, and k_{cat}/K_m values with both substrates.

Hydrolysis of Synthetic Aβ Peptide by Paenidase—To understand further the ability to hydrolyze of D-Asp

Table 3. **Effects of metal ions on paenidase activity.**

Metals	Concentration	Relative activity (%)	
		Paenidase I	Paenidase II
none		100	100
$CaCl_2$	1.0 mM	98.1	97.1
$CoCl_2$	1.0 mM	68.5	54.3
$MgCl_2$	1.0 mM	119	101
$MnCl_2$	1.0 mM	68.5	40.0
$ZnCl_2$	0.05 mM	38.2	40.3
	0.1 mM	31.6	31.1
	0.25 mM	2.5	2.5
	0.5 mM	0.5	0.4
	1.0 mM	0	0

Suc-[D-Asp]-MCA was used as a substrate.

Table 4. **Substrate specificity of paenidases.**

Substrate*[1]	Relative activity (%)	
	Paenidase I	Paenidase II
1. Suc-[D-Asp]-*p*NA	100	100
2. Ac-[L-Asp]-*p*NA	<0.5	<0.5
3. D-Asp-*p*NA	<0.5	<0.5
4. D-Ala-pNA	<0.5	<0.5
5. D-Leu-*p*NA	<0.5	<0.5
6. D-Phe-*p*NA	<0.5	<0.5
7. L-Asp-*p*NA	<0.5	<0.5
8. L-Ala-*p*NA	<0.5	<0.5
9. L-Leu-*p*NA	<0.5	<0.5
10. L-Phe-*p*NA	<0.5	<0.5
11. L-Arg-*p*NA	<0.5	<0.5
12. L-Glu-*p*NA	<0.5	<0.5
13. Gly-*p*NA	<0.5	<0.5
14. L-His-*p*NA	<0.5	<0.5
15. L-Ile-*p*NA	<0.5	<0.5
16. L-Lys-*p*NA	<0.5	<0.5
17. L-Met-*p*NA	<0.5	<0.5
18. L-Pro-*p*NA	<0.5	<0.5
19. L-Val-*p*NA	<0.5	<0.5
20. L-Pyr-*p*NA	<0.5	<0.5
21. Suc-AAA-*p*NA*[2]	<0.5	<0.5
22. Suc-APA-*p*NA*[3]	<0.5	<0.5

*[1]Final substrate concentration was 1 mM. *[2]Suc-AAA-*p*NA, Suc-L-Ala-L-Ala-L-Ala-*p*NA. *[3]Suc-APA-*p*NA, Suc-L-Ala-L-Pro-L-Ala-*p*NA.

Table 5. **Substrate specificity of paenidases.**

Substrates	Proteinase/ peptidases	Relative activity (%)	
		Paenidase I	Paenidase II
Suc-[D-Asp]-MCA	DAEP	100	100
Arg-MCA	Cathepsin H	<0.1	<0.1
Bz-Arg-MCA	Trypsin	<0.1	<0.1
Boc-Gln-Ala-Arg-MCA	Trypsin	<0.1	<0.1
Pro-Phe-Arg-MCA	Kallikrein/ Proteasome	<0.1	<0.1
Ac-Tyr-Val-Ala-Asp-MCA	Caspase-1	0.7	0.4
Ac-Asp-Glu-Val-Asp-MCA	Caspase-3/7/8	1.8	1.2
Ac-Val-Glu-Ile-Asp-MCA	Caspase-6	0.5	0.3

Final substrate concentration was 0.5 mM.

Table 6. **Kinetic parameters of paenidases.**

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Paenidase I			
Suc-[D-Asp]-*p*NA	1.03	4.7	4.6
Suc-[D-Asp]-MCA	1.25	1.93	1.54
Paenidase II			
Suc-[D-Asp]-*p*NA	1.26	6.6	5.2
Suc-[D-Asp]-MCA	1.00	1.66	1.66

containing peptides, we used synthetic Aβ peptides consisting of 10 amino acid residues. When paenidase I was incubated with the D-Asp-containing peptide at 37°C for 24 h, the peptide was specifically hydrolyzed on the COOH side of [D-Asp][7] (Fig. 3B). On the other hand, a normal Aβ peptide containing L-Asp instead of the D-isomer was not hydrolyzed by paenidase I (Fig. 3, C and D). Paenidase II also cleaved the D-Asp–containing Aβ peptide but not the normal Aβ peptide (data not shown).

DISCUSSION

We discovered a novel DAEP-producing microorganism from soil. The bacterium was identified as *Paenibacillus* sp. B38 strain and the DAEP was named paenidase (*Paenibacillus* D-aspartyl endopeptidase). Paenidases I and II were purified from the culture supernatant of the bacterium by 4 steps of column chromatography (Table 1). The purified paenidases I and II showed single protein bands on SDS-PAGE with molecular weights of 34,000 and 33,000. The molecular weights of the enzymes were also determined to be 34,798 (paenidase I) and 34,169 (paenidase II) by MALDI-TOF/MS. The difference in molecular weight between paenidase I and II is 629. The purified preparations showed single protein peaks on native gel filtration of Superdex 75 with the apparent molecular weights of 35,000, indicating that the enzymes exit as monomers. The N-terminal amino acid sequences of paenidase I and II were determined to be NH_2-Thr-Ile-Arg-Ile-Gln-Thr-Asp-Ala-Val-Thr-Lys-Tyr-Gly-Lys-Glu-Asp-Ala-Ala-Ile-Asp- and NH_2-Thr-Asp-Ala-Val-Thr-, respectively. The N-terminal sequence analyses of the enzymes showed that paenidase II lacks 5 of the residues present in the N-terminal sequence of paenidase I. The amino acid composition of the N-terminal 5 amino acids of paenidase I is Arg (1), Ile (2), Gln (1), and Thr (1), with a calculated molecular weight of 630, in good agreement with the MALDI-TOF/MS data. Thus, paenidase II appears to be an N-terminal hydrolyzed derivative of paenidase I. The sequence of the amino-terminal 20 amino acid residues of paenidase I was compared to other proteins using the Swiss port protein database, but we could not find homologous proteins. These results indicate that the purified paenidases are novel enzymes.

Paenidases I and II showed high activity over a relatively wide range of pH, with more than 80% activity remaining between pH 7.0 and pH 9.0; however, the enzymes were inactive below pH 5.5 (Fig. 2A). The enzymes are not very temperature tolerant; the enzyme

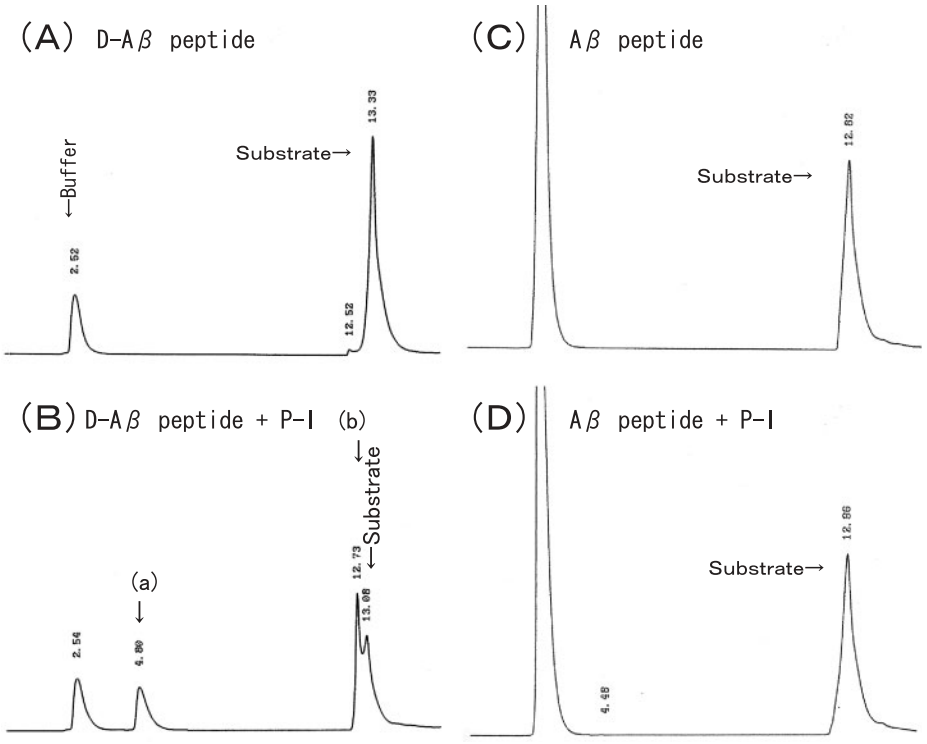

Fig. 3. **Reversed-phase HPLC of synthesized Aβ peptides hydrolyzed by paenidase.** (A) The synthesized [D-Asp][7] Aβ peptide, DAEFRH[D-Asp]SGY. (B) The reaction mixture after incubation of the synthesized [D-Asp][7] Aβ peptide with paenidase I at 37°C for 24 h. The N-terminal analyses of peaks (a) and (b) resulted in peak (a) Ser-Gly-Tyr and peak (b) Asp-Ala-Glu-Phe-Arg-His-X. (C) The synthesized normal Aβ peptide, DAEFRHDSGY. (D) The reaction mixture after incubation of the synthesized normal Aβ peptide with paenidase I at 37°C for 24 h.

activities decreased about 25% even after incubation for 30 min at 40°C (Fig. 2B).

The effects of various proteinase inhibitors on paenidase activity were examined (Table 2). The enzyme activity was not inhibited by serine-, cysteine-, or metal-proteinase inhibitors. The enzyme was inhibited by the aspartic proteinase inhibitor pepstatin and mammalian DAEP-specific inhibitor iDAEP. Hence, the enzyme appears to be an aspartic proteinase with a neutral pH optimum, like renin (*26–28*). At present, full sequence data for the enzyme are not available beyond the 20 residues of the N-terminal amino acid sequence. Further physicochemical and enzymatic studies are necessary to identify the active site residues of the enzymes.

Paenidase showed specific activity toward Suc-[D-Asp]-*p*NA and Suc-[D-Asp]-MCA (Tables 4 and 5). Other substrates used in this study were not cleaved by the enzymes. Paenidase I also specifically cleaved DAEFRH[D-Asp]SGY, an abnormal Aβ peptide at [D-Asp][7] (Fig. 3, A and B). The enzyme did not completely cleave the substrate after 24 h incubation, and may have been inactivated at 37°C as indicated in Fig. 2B. On the other hand, the enzyme did not hydrolyze the normal Aβ peptide (Fig. 3, C and D). These results indicate that paenidases specifically recognize internal D-Asp residues and hydrolyze them on the COOH side. Recently, DAEP in mammalian tissues has been reported (*24*). The mammalian DAEP has nearly the same enzymatic properties as those of paenidases. However, the enzyme is a membrane protein and is included in a proteasome of large molecule size (600 kDa) with a subunit structure. At present, there is no information about the primary structure of mammalian DAEP. Detailed studies of paenidase will provide useful information for understanding the physiological roles of DAEP in mammals. We are now cloning the paenidase gene from *Paenibacillus* sp. B38 in order to conduct a detailed characterization of the enzyme.

Nucleotide sequence data (*Paenibacillus* sp. B38 16s rRNA) is available in the DDBJ/EMBL/GeneBank databases under the accession number AB213458. DNA sequencing analysis was conducted with the CREST-Akita Plant Molecular Science Satellite Laboratory in Life Research Support Center in Akita Prefectural University.

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