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A Novel α-Amino-Acid Esterase from *Bacillus mycoides* Capable of Forming Peptides of DD- and DL-Configurations

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A novel α-amino-acid esterase possessing some properties favorable for the synthesis of D-amino acid–containing peptides has been purified from the culture broth of *Bacillus mycoides*. The enzyme consisted of 4 subunits of 39 kDa, had an isoelectric point of 7.0, and showed its maximum activity at around 47°C and pH 7.6. The enzyme activity was strongly depressed by phenylmethanesulfonyl fluoride, but not by penicillin G or ampicillin, suggesting that the protein is a serine enzyme lacking penicillin-binding ability. The enzyme hydrolyzed a variety of D- and L-amino acid methyl esters with concomitant formation of homooligomers from D-Phe, D-Trp, D-Tyr, and D-Asp(OCH_3) methyl esters, but it did not act on the D- or L-amino acid amides tested. Incubation of a mixture of Ac-D-Phe-OMe and D-/L-Leu-NH_2 with the enzyme yielded Ac-D-Phe-D-/L-Leu-NH_2 together with Ac-D-Phe-OH, the hydrolysate of the carboxyl component. To its credit, the enzyme failed to hydrolyze casein as well as peptides including diastereomers of diphenylalanine and dialanine, indicating that the enzyme would not cause secondary hydrolysis of once-formed peptides. These observations indicate the potential utility of the newly isolated enzyme for the synthesis of D-amino acid–containing peptides.

Key words: α-amino-acid esterase, α-amino acid ester hydrolase, *Bacillus mycoides*, D-amino acid–containing peptide, enzymatic peptide synthesis.

With the increasing number of biologically important peptides found in living organisms, much attention has been directed to enzymatic peptide synthesis conducted under mild conditions (*1–7*). The advantages of enzymatic peptide synthesis over the chemical approach include (a) general freedom from racemization, (b) minimum need for side chain protection, and (c) recovery of catalytic activity through enzyme immobilization. With all its advantages, the methodology has not been fully exploited for possible synthesis of biologically active peptides. This is because (a) the substrates of proteases or peptidases exclusively used as biocatalysts are generally limited to L-amino acids, and hence peptides containing unusual amino acids cannot be prepared by means of this strategy, and (b) the amidase activity of these enzymes often causes secondary hydrolysis of once-formed peptides. Also, it is well-known that peptidyl antibiotics, hormones, neural transmitters, and prodrugs used in chemotherapy often contain a single D-amino acid residue (*8–14*). This residue is a key determinant in the overall structure of the molecule, and is a requisite for biological activity. The growing demand for these D-amino acid–containing peptides in optically pure forms has prompted manipulation of the reaction conditions for kinetically controlled peptide synthesis using conventional proteases (*15–18*). Although sophisticated, the proposed approaches need very high concentrations of organic solvents in the reaction system, sometimes anhydrous environments, to alter the stereoselectivity of the enzymes. Since proteases are generally unstable in such an environment, these approaches are not always applicable to practical peptide synthesis.

Recently, several D-amino acid–specific peptidases were isolated and characterized (*19–25*). Though some of them were found to be applicable to the formation of peptides of DD-configuration, there have been no examples of the synthesis of peptides of DL-configuration by use of these enzymes (*26–28*). In light of this situation we attempted to find a novel enzyme that can easily form peptides of DD- or DL-configuration, but lacks amidase activity. This is the first report of a unique α-amino-acid esterase from *Bacillus mycoides* that meets the above-mentioned demands.

MATERIALS AND METHODS

Materials—Peptone and casein were purchased from Nissui Pharmaceuticals (Tokyo) and Merck (Darmstadt, Germany), respectively. Phenylmethanesulfonyl fluoride (PMSF), trichloroacetic acid (TCA), and Coomassie Brilliant Blue solution (Quick CBB) were obtained from Wako Pure Chemical Industries (Osaka). The protein assay kit was from Bio-Rad (Hercules, USA). Amino acids [D-/L-Ala, D-/L-Asp, D-/L-Asp(OCH_3), D-/L-Glu, D-/L-Glu(OCH_3), D-/L-Leu, D-/L-Met, D-/L-Phe, Ac-D-/L-Phe, D-/L-Trp, and D-/L-Tyr], their methyl esters, amides of D-/L-Ala, L-Arg, L-Asp, D-/L-Asn, D-/L-Gln, D-/L-Glu, L-His, L-Ile, D-/L-Leu, L-Lys, L-Met, D-/L-Phe, D-/L-Pro, L-Ser, L-Thr, L-Trp, L-Tyr, L-Val, and Gly, and diastereomers of diphenylalanine and dialanine were

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obtained from Bachem (Bubendorf, Switzerland). Amides of D-Arg, D-Asn, D-His, D-Ile, D-Lys, D-Met, D-Ser, D-Thr, D-Trp, D-Tyr, and D-Val were prepared by the method of Wang and McMurray (*29*). Fatty acid methyl esters were products of Tokyo Kasei (Tokyo). DEAE-Toyopearl 650M was a product of Tosoh (Tokyo). The Superdex 200 column (1 × 30 cm), Pharmalyte 3-10 (carrier ampholytes), and molecular mass standards for gel filtration and SDS-PAGE were products of Amersham Pharmacia Biotech (Uppsala, Sweden). A Daisopak SP-120-5-ODS-BP column (6 × 150 mm) and Cadenza CD-C18 column (4.6 × 75 mm) were obtained from Daiso (Osaka) and Imtakt (Kyoto), respectively. All other reagents were of analytical grade.

Enzyme Assay—Enzyme activity was routinely assayed at pH 7.0 with 200 mM D-phenylalanine methyl ester as the substrate. A mixture of 95 μl of the substrate and 5 μl of the enzyme solution was incubated for 5 min at 25°C, and the reaction was stopped by the addition of 5 μl of twice-distilled 2 N HCl. The amount of methanol released during the incubation was determined by injecting 5 μl of the sample into the Daisopak SP-120-5-ODS-BP column kept at 40°C, which was connected to a Hitachi HPLC system (Hitachi, Tokyo) equipped with a L-7100 pump and a L-7490 differential refractometer. A mobile phase of 100 mM phosphate (pH 3.8) was used at a flow rate of 0.7 ml/min. One unit of esterase activity was defined as the activity producing 1 μmol of methanol per minute under the above assay conditions.

Screening and Cultivation of Microorganisms—Every microorganism isolated from soil samples was aerobically cultivated in 1 ml medium composed of 2% peptone, 1% corn steep liquor, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7.0, for 3 days at 26°C. The culture supernatant was incubated with 0.3 M D-Phe-OCH_3 for 24 h at pH 7.0 and 26°C. A few of these cultures caused precipitation of D-phenylalanine oligomers, and the one exhibiting the highest activity was selected as the source of the desired enzyme. The microorganism taxonomically identified as *B. mycoides* was cultivated in a shaking flask containing 150 ml of the above-mentioned medium for 4 days at 26°C.

Purification of α-Amino-Acid Esterase—All operations during the enzyme purification were carried out at 15°C unless otherwise stated. The culture broth of *B. mycoides* was centrifuged at 10,000 ×*g* for 20 min. To the supernatant was added solid ammonium sulfate to 70% saturation. The resulting precipitate was allowed to settle overnight and then recovered by filtration through Celite. The crude enzyme was dissolved in a minimal amount of water, dialyzed against 5 mM Tris-HCl, pH 8.0, containing 0.5% Tween 80 (buffer A), and then placed on a DEAE Toyopearl 650M column (4.5 × 20 cm) equilibrated with the same buffer. After the column had been washed with 500 ml of buffer A, proteins were eluted with a linear gradient of 0–600 mM NaCl at a flow rate of 20 ml/h. The active fractions were collected and concentrated 10-fold by ultrafiltration with a membrane of a nominal molecular mass cutoff of 10 kDa. The concentrated enzyme solution was then subjected to sucrose-density gradient electrofocusing in a 110 ml volume apparatus with Pharmalyte 3-10 as the carrier ampholytes. The electrophoresis was performed at 450 V for 40 h at 2°C, and proteins were collected with a fraction collector at 1 ml per tube. The active fractions were pooled, concentrated and then dialyzed against buffer A. The dialyzed sample was then subjected to preparative polyacrylamide gel electrophoresis with a NA-1800 apparatus (Nihon Eido, Tokyo) according to the method of Davis (*30*). A column containing a 7.5% separation gel (34 × 55 mm) and a 3% stacking gel (12 mm) was run at a constant voltage of 100 V, and proteins were continuously collected in numbered fractions at 0.5 ml per tube.

Determination of the Molecular Mass of the Enzyme—The molecular mass of the native enzyme was estimated by gel filtration on a Superdex 200 column using 50 mM phosphate, pH 7.0, containing 200 mM NaCl and 0.5% Tween 80. Alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) were used for calibration. The molecular mass of the denatured enzyme was estimated by SDS-PAGE according to the method of Laemmli on a 9% polyacrylamide gel (*31*). The marker proteins used were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactoalbumin (14.4 kDa). After the electrophoresis, the gel was stained with Quick CBB.

Protein Determination—The protein concentrations of the samples were determined with a Bio-Rad protein assay kit with bovine serum albumin as the standard. The chromatographic elution patterns were monitored in terms of the absorbance at 280 nm.

Inhibitory Assay—An enzyme solution was mixed with each inhibitor, and then the mixture was incubated for 1 h at 25°C in 50 mM phosphate, pH 7.0, before the enzyme assay with 200 mM D-Phe-OCH_3 as the substrate. Each assay was performed with 5.9 units of the purified enzyme. The relative activity was expressed as a percentage of the activity without an inhibitor. The reagents and the final concentrations employed were 1 mM PMSF, 1 mM *N*-ethylmaleimide (NEM), 1 mM transepoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), 1 mM pepstatin, 1 mM $HgCl_2$, 5 mM EDTA, 10 mM penicillin G, and 10 mM ampicillin. The action of the enzyme on penicillin G and ampicillin was also checked with a Shimadzu LC-9A HPLC system by monitoring the generation of 6-aminopenicillanic acid with a SPD-6A uv detector set at 220 nm.

Substrate Specificity—The substrate specificity of the enzyme was investigated with amino acid methyl esters, fatty acid methyl esters, amino acid amides, and dipeptides as the substrates. The activity toward amino acid esters was measured with 200 mM amino acid methyl ester dissolved in 50 mM phosphate, pH 7.0. Methanol released from the esters incubated with 13.5 units of the enzyme for 5 min at 25°C was quantified as described under "Enzyme Assay." The action on 200 mM fatty acid methyl ester was examined in 3 ml of 50 mM phosphate, pH 7.0, with constant stirring at 500 rpm for 30 min at 25°C. After the enzyme reaction, 20 ml of ethanol was added, and then the fatty acids released during the incubation were titrated with 50 mM KOH. One unit of the activity toward fatty acid methyl ester was defined as the activity releasing 1 μmol of fatty acid per min under the above conditions. Amidase activity was measured with 200 mM amino acid amide under the same conditions as those for the esterolytic assay described above. Ammonia released from amide was determined by the method of Weatherburn (*32*). One unit of amidase activity was defined as the activity releasing 1 μmol of ammonia

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per min under the above conditions. Peptidase activity was assayed with 10 mM dipeptide in the same manner as above, and the hydrolysis was monitored with an ALC-1000 amino acid analyzer (Shimadzu, Kyoto) according to the method of Ishida *et al.* (*33*). When necessary, D-/L-amino acid analysis was performed by HPLC on the Cadenza CD-C18 column with a mobile phase of 5–45% of methanol/acetonitrile (6:4, v/v) in 50 mM phosphate, pH 5.7. Samples were hydrolyzed with twice-distilled HCl for 16 h at 105°C, and then derivatized with *o*-phthalaldehyde and *N*-isobutyryl-L-cysteine (*34*). Proteolytic activity was measured according to the method of Wilcox with some modification with casein as the substrate (*35*). One milliliter of the enzyme solution was added to 5 ml of 0.6% (w/v) casein in 50 mM Tris-HCl, pH 7.0. After incubation at 25°C for an appropriate time, the reaction was stopped by the addition of 5 ml of a mixture composed of 36 ml of 50% TCA, 220 ml of M CH_3COONa, and 330 ml of M CH_3COOH. The reaction mixture was allowed to stand for a further 20 min. The resulting precipitate was removed by filtration with a filter paper No. 131 (Advantec Toyo, Tokyo), and then the absorbance of the filtrate at 275 nm was measured with a UV-1600 spectrophotometer (Shimadzu) against a control prepared by mixing the enzyme solution, TCA, and the substrate in that order. One unit of proteolytic activity was defined as the activity yielding soluble peptides equivalent to 1 μmol of L-tyrosine per min under the above conditions.

Enzymatic Peptide Synthesis—Homooligopeptide synthesis was carried out with 200 mM amino acids or their methyl esters as the substrate. Each substrate was incubated with 20 units of the enzyme in 50 mM phosphate, pH 7.0, for 10 min at 25°C. The ability of the enzyme to form diastereomers of Ac-Phe-Phe-NH_2 and Ac-Phe-Leu-NH_2 was examined using a mixture of 150 mM Ac-D-/L-Phe-OCH_3 and 300 mM D-/L-Phe-NH_2 or 300 mM D-/L-Leu-NH_2, respectively. Each mixture was incubated with 20 units of the enzyme for 10 min at 25°C in 50 mM phosphate, pH 7.0, containing 20% dimethylformamide. The reaction products were analyzed by HPLC as described under "Substrate Specificity."

Fast Atom Bombardment (FAB) Mass Spectrometry—Enzymatically synthesized peptides were purified by HPLC as above, and their masses were measured with a JMS-AX505HA mass spectrometer (JEOL, Tokyo) in the FAB mode using a mixture of thioglycerol and HCl as the matrix.

RESULTS

Purification of α-Amino-Acid Esterase—Table I summarizes the purification of the α-amino-acid esterase. The enzyme was purified approximately 3,900-fold with a yield of 3.1% as to the initial activity. The low activity yield can be ascribed in part to the poor fractionation on DEAE ion-exchange chromatography caused by irreversible adsorption of the enzyme to the chromatographic support. The subsequent electrofocusing resulted in precipitation of much of the contaminating proteins, and hence greatly increased the specific activity of the enzyme preparation (Fig. 1). The final enzyme preparation was homogeneous, as judged on SDS-PAGE, there being a single protein band corresponding to a molecular mass of 39 kDa (Fig. 2). Gel filtration on the Superdex 200 column, performed in the presence of 0.5% Tween 80 to minimize the enzyme adsorption to the support, gave a value of 160 kDa as the molecular weight of the enzyme, suggesting that the enzyme is a tetrameric protein.

Properties of the Enzyme—Electrofocusing of the enzyme after ion-exchange chromatography afforded a single active peak corresponding to pI of 7.0 (Fig. 1). The optimum pH of the enzyme estimated with D-Phe-OCH_3 as the substrate was around 7.6 (Fig. 3). The optimum temperature was around 47°C (Fig. 4). After hydrolysis of D-Phe-OCH_3 at pH

TABLE I. **Purification of α-amino-acid esterase.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture supernatant	138,000	76,800	0.56	100
10–75% $(NH_4)_2SO_4$	17,800	36,100	2.03	47
DEAE-Toyopearl	3,070	14,600	4.76	19
Electrofocusing	76.5	10,400	136	14
Disc electrophoresis	1.1	2,370	2,160	3.1

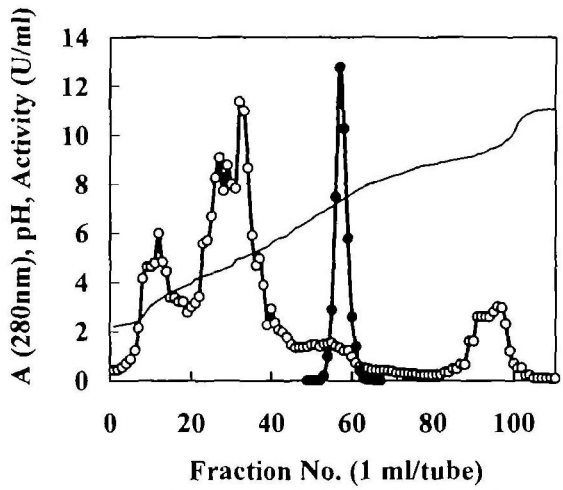

Fig. 1. **Sucrose density-gradient electrofocusing of α-amino-acid esterase.** The enzyme preparation after ion-exchange chromatography was subjected to electrofocusing in a 110 ml apparatus with Pharmalyte 3-10 as the carrier ampholytes. The electrofocusing was performed at 450 V for 40 h at 2°C. ○, absorbance at 280 nm; ●, hydrolytic activity toward D-Phe-OCH_3; —, pH.

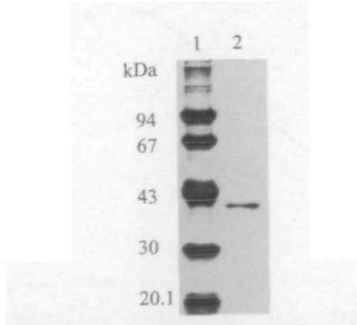

Fig. 2. **SDS-PAGE of the purified α-amino-acid esterase.** SDS-PAGE was performed on a 12% gel. Lane 1, the purified enzyme; lane 2, molecular weight marker proteins consisting of phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa). The gel was stained with Quick CBB.

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7.0 and 25°C, Lineweaver-Burk plotting based on an enzyme molecular weight of 160 kDa gave 6,160 s^{-1} and 5.6 mM as the molecular activity and apparent Michaelis constant of the enzyme, respectively. The substrate specificity of the enzyme is shown in Table II. The enzyme exhibited broad specificity towards D- and L-amino acid esters. In contrast, fatty acid methyl esters were very poor substrates. On the other hand, the enzyme exhibited no detectable hydrolytic activity toward the amino acid amides tested, *viz.*, amides of D-/L-Ala, D-/L-Arg, D-/L-Asp, D-/L-Glu, D-/L-Gln, D-/L-His, D-/L-Ile, D-/L-Leu, D-/L-Lys, D-/L-Met, D-/L-Phe, D-/L-Pro, D-/L-Ser, D-/L-Thr, D-/L-Trp, D-/L-Tyr, D-/L-Val, and Gly. None of the diastereomers including DD-, DL-, LD-, LL-diphenylalanine, and DD-, LL-dialanine were hydrolyzed by the enzyme. Similarly, casein did not undergo enzyme-catalyzed hydrolysis even after 8 h incubation. The effects of some typical inhibitors and the two β-lactams were also investigated (Table III). Out of these chemicals, only PMSF at 1 mM had a drastic inhibitory effect, suggesting that the enzyme is a serine hydrolase. Incubation of the enzyme with 10 mM penicillin G or ampicillin did not cause any inhibition. Moreover, the two inhibitors were not hydrolyzed by the enzyme.

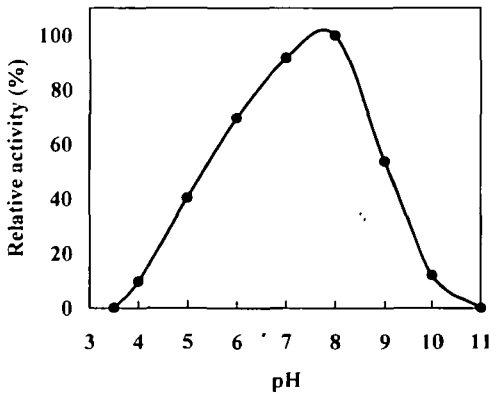

Fig. 3. **Effect of pH on the activity of α-amino-acid esterase.** The hydrolytic activity toward 200 mM D-Phe-OCH_3 was assayed in Britton-Robinson buffers of different pHs at 25°C. Activity was expressed by taking that at pH 7.8 as 100.

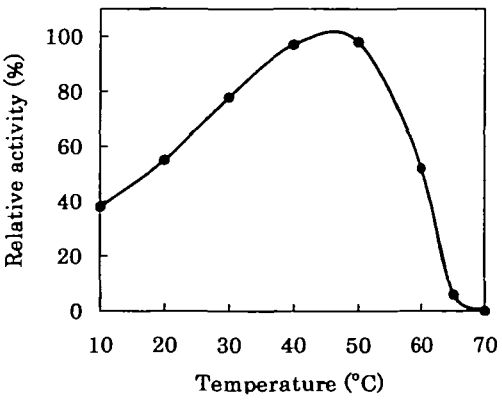

Fig. 4. **Effect of temperature on the activity of α-amino-acid esterase.** The hydrolytic activity toward 200 mM D-Phe-OCH_3 was assayed in 50 mM phosphate, pH 7.0, at different temperatures. Activity was expressed by taking that at 47°C as 100.

Enzymatic Peptide Synthesis—Of the amino acid esters, amino acid amides and free amino acids tested, four methyl esters, *i.e.* those of D-Phe, D-Try, D-Tyr, and D-Asp(OCH_3), gave some products on incubation with the enzyme (Fig. 5). The structures of the products were confirmed by FAB mass spectrometry (Table IV). As can be seen, the enzyme synthesized methyl esters of oligopeptides under the reaction conditions used. The products derived from D-aspartic acid dimethyl ester were separated into at least 25 peaks on HPLC, indicating the formation of many different species of oligopeptides.

When a mixture of Ac-D-Phe-OCH_3 and D-/L-Phe-NH_2 was incubated with the enzyme, a single new peak appeared on each HPLC. Figure 6 shows HPLC and FAB mass spectra of the reaction product obtained with Ac-D-Phe-OCH_3 and L-Phe-NH_2. It is clear that the enzyme catalyzed the synthesis of Ac-D-Phe-L-Phe-NH_2. In the same way, Ac-D-Phe-L-Leu-NH_2 was obtained from Ac-D-Phe-OCH_3 and L-Leu-NH_2 through the enzyme action (Fig. 7). Table V summarizes the retention times, yields, masses, and proposed structures of the reaction products. The proposed structures were derived from the results of mass and amino acid analyses. With Ac-L-Phe-OCH_3 as the C-component, even trace amounts of Ac-L-Phe-D-/L-Phe-NH_2 and Ac-L-Phe-D-/L-Leu-NH_2 were not detected.

TABLE II. **Substrate specificity of α-amino-acid esterase.**

Substrate	Hydrolytic activity (%)	Substrate	Hydrolytic activity (%)
D-Phe-OCH_3	100	Methyl caproate	2.0
L-Phe-OCH_3	99	Methyl caprylate	1.1
Ac-D-Phe-OCH_3	119[a]	Methyl laurate	0.3
Ac-L-Phe-OCH_3	111[a]	Methyl oleate	0.5
D-Trp-OCH_3	96	D-Phe-NH_2	3.6
L-Trp-OCH_3	96	D-Phe-D-Phe-OH[b]	ND
D-Tyr-OCH_3	93	D-Phe-L-Phe-OH[b]	ND
L-Tyr-OCH_3	95	L-Phe-D-Phe-OH[b]	ND
D-Asp(OCH_3)-OCH_3	107	L-Phe-L-Phe-OH[b]	ND
L-Asp(OCH_3)-OCH_3	106	L-Ala-L-Ala-OH	ND
D-Met-OCH_3	102	D-Ala-D-Ala-OH	ND
L-Met-OCH_3	98	D-Phe-L-Ala-OH	ND
Methyl acetate	1.1	L-Phe-L-Ala-OH	ND
Methyl propionate	2.7	L-Ala-Gly-OH	ND
Methyl butyrate	3.6	Casein	ND

The following amides were inert as substrates: amides of D-/L-Ala, D-/L-Arg, D-/L-Asp, D-/L-Asn, D-/L-Gln, D-/L-Glu, D-/L-His, D-/L-Ile, D-/L-Leu, D-/L-Lys, D-/L-Met, L-Phe, D-/L-Pro, D-/L-Ser, D-/L-Thr, D-/L-Trp, D-/L-Tyr, D-/L-Val, and Gly. The relative activity was expressed as a percentage of the activity obtained with D-Phe-OCH_3 as the substrate. ND: not detected. [a]In the presence of 20% DMF. [b]In the presence of 10% DMF.

TABLE III. **Effects of inhibitors on α-amino-acid esterase.**

Inhibitor	Remaining activity (%)[a]
None	100
1 mM PMSF	1
1 mM NEM	98
1 mM E-64	99
1 mM pepstatin	92
1 mM $HgCl_2$	88
5 mM EDTA	102
10 mM penicillin G	101
10 mM ampicillin	99

[a]After incubation for 1 h at 25°C and pH 7.0. Relative activity was expressed as a percentage of the activity without an inhibitor.

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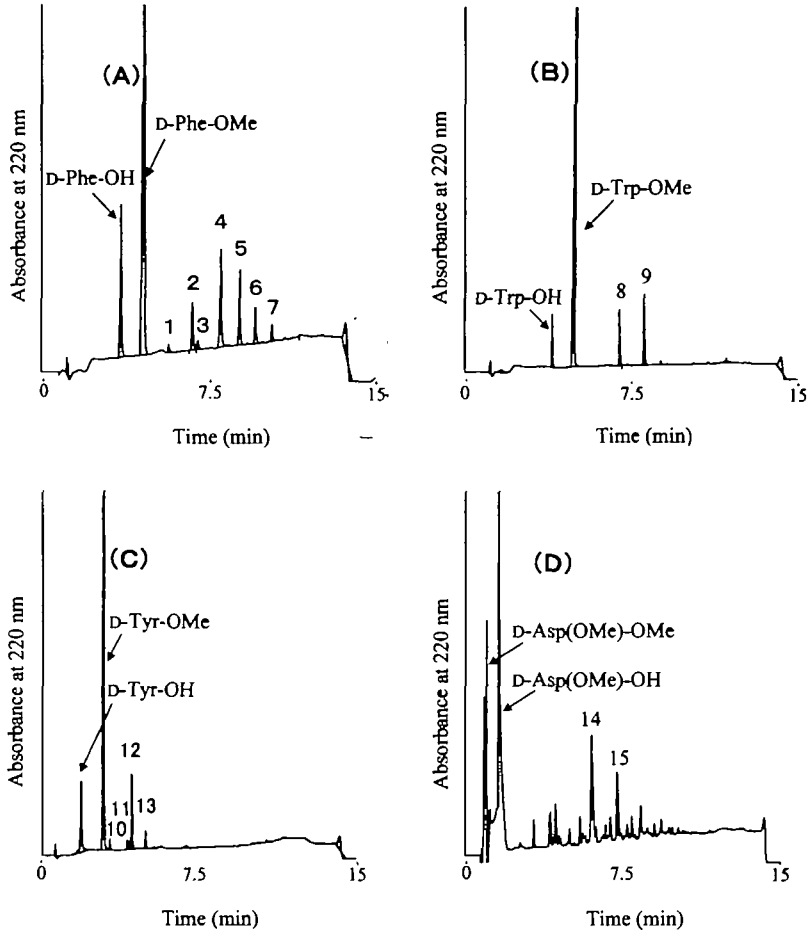

Fig. 5. **HPLC of the reaction products derived from D-amino acid esters.** The reaction products were derived from (A) D-Phe-OCH_3, (B) D-Trp-OCH_3, (C) D-Tyr-OCH_3, and (D) D-Asp(OCH_3)-OCH_3. The reaction was conducted by incubating 200 mM D-amino acid methyl ester with 20 units of the α-amino-acid esterase for 10 min in 50 mM phosphate, pH 7.0, at 25°C. The numbered peaks indicate those subjected to amino acid analysis and FAB mass spectrometry.

TABLE IV. **Retention times, molecular masses and proposed structures of the reaction products derived from D-Phe-OCH_3, D-Trp-OCH_3, D-Tyr-OCH_3, and D-Asp(OCH_3)-OCH_3.**

Peak No.[a]	Retention time (min)	Molecular mass[b] (Da)	Structure[c]
1	5.7	312	(D-Phe)$_2$-OH
2	6.7	326	(D-Phe)$_2$-OCH_3
3	7.0	460	(D-Phe)$_3$-OH
4	8.0	473	(D-Phe)$_3$-OCH_3
5	8.9	620	(D-Phe)$_4$-OCH_3
6	9.6	768	(D-Phe)$_5$-OCH_3
7	10.4	915	(D-Phe)$_6$-OCH_3
8	7.0	404	(D-Trp)$_2$-OCH_3
9	8.1	591	(D-Trp)$_3$-OCH_3
10	3.6	344	(D-Tyr)$_2$-OH
11	4.4	358	(D-Tyr)$_2$-OCH_3
12	4.6	522	(D-Tyr)$_3$-OCH_3
13	5.2	683	(D-Tyr)$_4$-OCH_3
14	6.1	932	(D-Asp(OCH_3))$_7$-OCH_3
15	7.3	1,446	(D-Asp(OCH_3))$_{11}$-OCH_3

[a]Designated in Fig. 3. [b]Determined by FAB mass spectrometry. [c]Determined by amino acid analysis and FAB mass spectrometry.

DISCUSSION

We have isolated *B. mycoides* from soil that extracellularly produces a novel hydrolase acting on a variety of D- and L-amino acid esters. Since the culture broth showed hydrolytic activity toward both D- and L-amino acid esters, complete enzyme purification was necessary to determine if the activity was due to a single enzyme. The purification work demonstrated that the esterolytic activity in the culture broth was due to the newly isolated single enzyme. The hydrolysis of α-amino acid esters is effected by several hydrolases such as beef liver carboxylesterase (*36*), leucine aminopeptidase (*37*), trypsin (*38*), chymotrypsin (*39*), and *Ochrobactrum anthropi* aminopeptidase (*40*). The enzyme from *B. mycoides* should not be referred to as a carboxyl esterase, amidase, peptidase, or protease since it exhibited little hydrolytic activity toward fatty acid methyl esters, amino acid amides, peptides including diastereomers of dipeptides, and casein. From the results of inhibition experiments involving peptidyl antibiotics, the enzyme was considered not to be a penicillin-binding protein, as are many of the D-amino acid–specific peptidases so far isolated.

Takahashi *et al.* have reported a partially purified enzyme from *Acetobactor turbidans* that catalyzes not only aminoacyl transfer from D-/L-α-amino acid esters to 7-amino-3-deacetoxy-cephalosporanic acid, but also the hydrolysis of such α-amino acid esters (*41*). The enzyme was given the name α-amino-acid esterase (α-amino-acid-ester aminoacylhydrolase, EC. 3.1.1.43). Later, Kato *et al.* isolated and characterized a different α-amino-acid esterase from *Xanthomonas citri*, which was shown to catalyze similar reactions, but to have no caseinolytic activity (*42–44*). The enzyme was not inhibited by EDTA, DFP, NEM, *p*-chloromercuribenzoate, or any protease inhibitors including pepsinostreptin and plasminostreptin. These two enzymes have been the only α-amino-acid esterases subjected to

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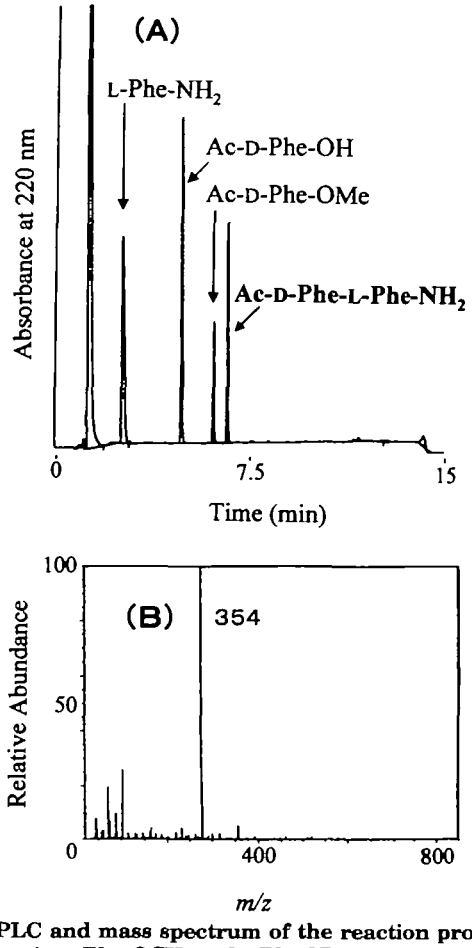

Fig. 6. **HPLC and mass spectrum of the reaction product derived from Ac-D-Phe-OCH_3 and L-Phe-NH_2.** A mixture of 150 mM Ac-D-Phe-OCH_3 and 300 mM L-Phe-NH_2 was incubated with 20 units of the α-amino-acid esterase for 10 min at 25°C and pH 7.0, in the presence of 20% DMF. After the reaction, HPLC was performed on a Cadenza CD-C18 column, and the reaction product was subjected to FAB mass spectrometry.

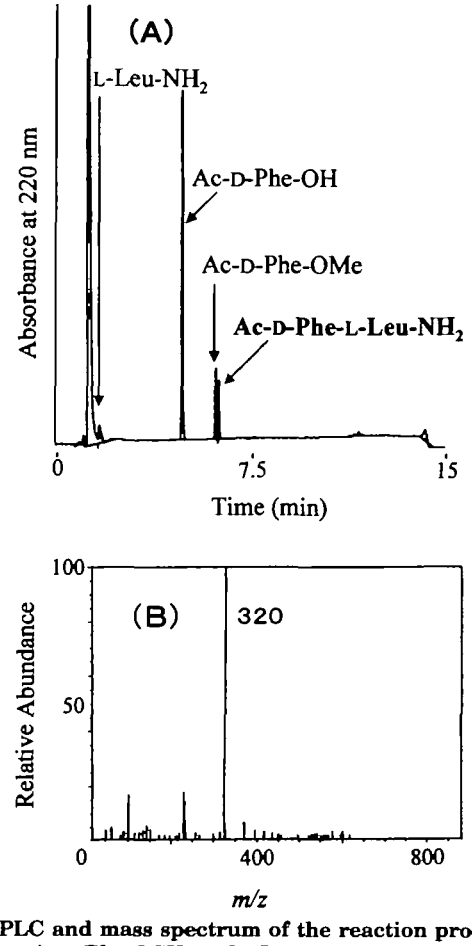

Fig. 7. **HPLC and mass spectrum of the reaction product derived from Ac-D-Phe-OCH_3 and L-Leu-NH_2.** A mixture of 150 mM Ac-D-Phe-OCH_3 and 300 mM L-Leu-NH_2 was incubated with 20 units of the α-amino-acid esterase for 10 min at 25°C and pH 7.0, in the presence of 20% DMF. After the reaction, HPLC was performed on a Cadenza CD-C18 column, and the reaction product was subjected to FAB mass spectrometry.

purification. Comparison of the substrate specificity and catalytic functions of the enzyme from *B. mycoides* with those of the above two enzymes led to the conclusion that the enzyme from *B. mycoides* is a member of the α-amino-acid esterase group. One millimolar PMSF completely inhibited the enzyme after 1 h incubation, suggesting a serine residue plays an important role in the catalysis.

As is well-known, serine and cysteine enzymes have the ability to synthesize peptides from amino acid esters or amides through a kinetic approach. Hence we examined the ability of the enzyme from *B. mycoides* to synthesize peptides. As expected, the enzyme formed oligopeptides from some of the D-amino acid esters, although it did not act on free D- or L-amino acids. Out of the ten D-amino acid esters tested, the methyl esters of D-Phe, D-Trp, and D-Tyr, and D-Asp(OCH_3) were accepted for homooligopeptide formation. With regard to D-Asp esters, only the α-,β-diester was involved in oligopeptide formation, *i.e.* not the α- or β-ester. The enzyme may not accommodate negatively charged amino acid esters in the active site. On the other hand, none of the L-amino acid esters tested were recognized as a substrate for oligomer synthesis.

TABLE V. **Retention times on HPLC, masses, and yields of the products derived from Ac-D-Phe-OCH_3 and D-/L-Phe-NH_2, or D-/L-Leu-NH_2.**[a]

N-Component	Product[b]	Retention time[c] (min)	Mass[d] (Da)	Yield (%)
D-Phe-NH_2	Ac-D-Phe-D-Phe-NH_2	6.3	353	4.7
L-Phe-NH_2	Ac-D-Phe-L-Phe-NH_2	6.6	353	21.3
D-Leu-NH_2	Ac-D-Phe-D-Leu-NH_2	5.8	319	2.1
L-Leu-NH_2	Ac-D-Phe-L-Leu-NH_2	6.3	319	4.8

[a][Ac-D-Phe-OCH_3] = 150 mM, [N-component] = 300 mM, with 20 units of enzyme at pH 7.0 and 25°C for 10 min in the presence of 20% DMF. [b]Deduced from the results of amino acid analysis and FAB mass spectrometry. [c]Obtained by HPLC. [d]Determined by FAB mass spectrometry.

It has been well documented that many biologically important peptides such as dermorphin, bombinin, and fulicin are composed of a single D-amino acid and several L-amino acids. In protease-catalyzed peptide synthesis, the enzymes are very specific for L-amino acids as the P1 residue (C-component). The P1′ residue (N-component) is more flexible, and both L- and D-amino acids are acceptable, although

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D-amino acids usually only react about one-tenth as fast as the L-counterparts (*12–14*). Thus, the synthesis of peptides of LD-configuration can be accomplished without much difficulty. On the contrary, peptides of DL-configuration are much more difficult to synthesize. Though the enantioselectivity of the enzyme catalysis can be altered by solvent engineering, as typically shown by the synthesis of Ac-D-Phe-L-Phe-NH_2 using subtilisin in anhydrous *tert*-amyl alcohol (*15*), this approach is not always applicable to all proteases owing to their general instability or ineffectiveness in such an environment. As mentioned earlier, an increasing number of enzymes specifically acting on D-amino acid-containing peptides has been isolated, and some of these enzymes were shown to hydrolyze peptides of DL- as well as DD-configuration. Though the synthesis of peptides of DD-configuration has been reported, there have been no examples of the synthesis of peptides of DL-configuration by use of these enzymes. Hence we examined if the newly isolated α-amino-acid esterase from *B. mycoides* could easily form peptides of DL-configuration. In this study we used Ac-D-Phe-OCH_3 as the C-component, and L-Phe-NH_2 or L-Leu-NH_2 as the N-component. Though L-Phe-OCH_3 and L-Leu-OCH_3 were not accepted for homooligopeptide formation, we expected they might be used as the P1′ residue since the configuration of this residue is flexible with conventional proteolytic enzymes. It is noteworthy that the enzyme efficiently synthesized Ac-D-Phe-L-Leu-NH_2 as well as Ac-D-Phe-L-Phe-NH_2. It should be added that the enzyme catalyzed the synthesis of the corresponding peptides of DD-configuration when D-Phe-NH_2 or D-Leu-NH_2 was used as the N-component. These observations, together with the fact that homooligopeptides were formed only from D-amino acid methyl esters, suggest that in peptide synthesis the enzyme demands a D-amino acid as the P1 residue, and that both D- and L-amino acids are acceptable as the P1′ residue.

Despite the fact that α-amino-acid esterase was first discovered by Takahashi *et al.* in 1974 (*41*), the enzymes in this group have never been used for peptide synthesis. This is because, in addition to only a few reports on the enzymes, no information has been available on the activity as to the synthesis and hydrolysis of peptides. In this study we have demonstrated for the first time that an α-amino-acid esterase from *B. mycoides* is capable of forming peptides of DD- and DL-configurations, but it lacks peptidase activity leading to secondary hydrolysis. All these findings indicate the significant but unrealized utility of the enzyme for the synthesis of D-amino acid–containing peptides of biologically importance. However, it is necessary to check if α-amino-acid esterases from different sources share these desirable properties. Detailed studies on the peptide synthesis stereospecificity and on the structure-function relationship of the enzyme are now in progress for a better understanding of the reaction mechanism.

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