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Properties of a Novel D-Stereospecific Aminopeptidase from *Ochrobactrum anthropi**

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A novel aminopeptidase active toward D-amino acid-containing peptides, D-amino acid amides, and D-amino acid esters has been purified 2,800-fold to homogeneity from a bacterium *Ochrobactrum anthropi* SCRC C1-38, which had been isolated from soil. The enzyme has a molecular weight of about 122,000 and is composed of two identical subunits (M_r = 59,000). The optimal pH for activity was 8.0. It showed strict D-stereospecificity toward substrates including low molecular weight D-amino acid amides such as D-alanine amide, D-α-aminobutyric acid amide, and D-serine amide; D-alanine *N*-alkylamides such as D-alanine-*p*-nitroanilide, D-alanine benzylamide, and D-alanine *n*-butylamide; and peptides with a D-alanine at the NH_2 terminus such as D-alanylglycine, D-alanylglycylglycine, D-alanyl-L-alanyl-L-alanine, and D-alanine oligomers. Generally, the enzyme did not act on substrates composed of L-amino acid at the NH_2 terminus, although it showed low stereospecificity only toward substrates such as the methyl esters of L-alanine, L-serine, and L-alanine-*p*-nitroanilide. Comparing the K_m and V_{max} values for the major substrates, it is clear that the enzyme prefers peptides to amino acid arylamides or amino acid amides. The enzyme was tentatively named as "D-aminopeptidase." EDTA and divalent cations have no effect on the enzyme activity. The enzyme appears to be a thiol peptidase.

Aminopeptidase is an exopeptidase which catalyzes the stepwise cleavage of a single amino acid from the amino terminus of a peptide (1). A vast number of reports has been published on the properties of this group of enzymes, mostly dealing with peptides and amides consisting of NH_2-terminal L-amino acid as substrates (2).

The existence of an enzyme which catalyzes the stereospecific hydrolysis of peptides containing D-amino acid could hardly have been imagined. Aminoacylase (3, 4) and carboxypeptidase-like peptide hydrolase (5) of microbial origin are the only known D-specific examples. D-Amino acid-containing peptides are found in the bacterial peptidoglycan (6), D-alanine-containing dipeptides in rice plant (7), the peptide antibiotics gramicidin S, tyrosidine, and bacitracin (8), and dermorphin in the frog (9).

During the course of studies on the enzyme-catalyzed organic synthesis of D-amino acid derivatives, we needed a D-stereospecific aminopeptidase. We speculated that microorganisms could be a likely source for such an enzyme, as they play an important role in the environment as synthesizers and degraders of a wide variety of substances.

We report here the purification and characterization of an unusual aminopeptidase named "D-aminopeptidase" from *Ochrobactrum anthropi* SCRC C1-38, which had been screened and isolated for this purpose. The enzyme shows high stereospecificity toward D-amino acid containing peptides, amides, and esters.

EXPERIMENTAL PROCEDURES

Materials—DEAE-Toyopearl 650 M, Butyl-Toyopearl 650 M, and HPLC[1] columns G-3000 SW and DEAE-5PW were purchased from Tosoh Corp. (Japan); Sephacryl S-300 was from Pharmacia (Sweden); marker proteins for molecular weight determination were from Oriental Yeast (Japan). Coomassie Brilliant Blue R-250 was purchased from Fluka (Switzerland). Ampholytes used for isoelectric focusing were the products of LKB-Produkter AB (Sweden). The membrane filter (Diaflo Ultrafilter, PM-30) was obtained from Amicon. D-Alanylglycine, D-alanyl-D-alanine, DL-alanyl-DL-leucine, DL-alanyl-DL-methionine, D-tryptophan methyl ester HCl, N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, D-alanyl-D-alanyl-D-alanine, D-alanyl-L-alanyl-L-alanine, D-alanylglycylglycine, glycine amide HCl, L-alanylglycine, β-alanylglycine, β-alanyl-L-alanine, L-alanine-*p*-nitroanilide HCl, L-threonine amide HCl, L-serine amide HCl, L-valine amide HCl, L-leucine amide HCl, L-methionine amide HCl, L-phenylalanine amide HCl, L-tyrosine amide HCl, L-tryptophan amide HCl, L-lysine amide 2HCl, L-arginine amide 2HCl, L-aspartic acid amide (L-isoasparagine), L-glutamic acid amide (L-isoglutamine), L-threonine methyl ester HCl, D-amino acid oxidase (EC 1.4.3.3, hog kidney), L-amino-acid oxidase (EC 1.4.3.2, *Bothrops atrox*), and alanine dehydrogenase (EC 1.4.1.1, *Bacillus subtilis*) were purchased from Sigma. D-Alanyl-L-alanine, D-alanine-*p*-nitroanilide HCl, D-alanyl-D-alanyl-D-alanyl-D-alanine, D-alanine-β-naphthylamide HCl, D-threonine benzyl ester hemioxalate, D-aspartic acid-β-benzyl ester, D-glutamic acid-γ-benzyl ester, DL-alanyl-DL-serine, L-alanyl-D-alanine, L-alanyl-L-alanine, and L-alanylglycine amide HCl were from Bachem (Switzerland). D-Alanine methyl ester HCl, D-alanine amide HCl, D-arginine amide HCl, glycine methyl ester HCl, D-proline methyl ester HCl, L-alanine methyl ester HCl, L-alanine amide HCl, L-isoleucine amide HCl, L-aspartic acid amide (L-isoasparagine) HCl, L-histidine amide, L-proline amide HCl, $(Boc)_2O$ (di-*tert*-butyldicarbonate), Boc-ON (2-*tert*-butoxycarbonyloxyimino-2-phenylacetonitrile), and 4 N hydrogen chloride in ethylacetate were purchased from Kokusan Chemicals (Japan). D-Phenylglycine methyl ester HCl, D-α-amino-ϵ-caprolactam, L-α-amino-ϵ-caprolactam, DL-α-amino-γ-butyrolactone HBr, and hydroxyapatite were purchased from Wako Pure Chemicals (Japan), and DL-alanyl-DL-valine, DL-alanyl-DL-phenylalanine, DL-alanyl-DL-isoleucine, DL-alanyl-DL-asparagine, and L-methionine methyl ester HCl were from Tokyo Kasei (Japan). Peroxidase (EC 1.11.1.7, horseradish) was from Toyobo (Japan). *N*-Nitrosomethylurea was from ICN K&K Laboratories, Inc. (United Kingdom). Palladium-carbon (10%) was purchased from Nippon Engelhard Inc. (Japan).

Synthesis of Methyl Esters of Amino Acid Derivatives—The follow-

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl.

ing methyl esters of amino acids were synthesized by slowly dropping 1.2–5.0 eq of thionylchloride into amino acids suspended in methanol at −20 °C (10). The amino acid methyl esters synthesized included D-alanine methyl ester HCl, D-α-aminobutyric acid methyl ester HCl, D-leucine methyl ester HCl, D-norvaline methyl ester HCl, D-norleucine methyl ester HCl, D-methionine methyl ester HCl, D-phenylalanine methyl ester HCl, D-tyrosine methyl ester HCl, D-histidine methyl ester HCl, L-serine methyl ester HCl, L-α-aminobutyric acid methyl ester HCl, L-*allo*-threonine methyl ester HCl, L-norvaline methyl ester HCl, L-norleucine methyl ester HCl, and Z-D-alanine methyl ester (11). Boc-Amino acids were synthesized from free amino acids by the use of $(Boc)_2O$ (12) or Boc-ON (13). Methyl esters of the Boc-D-amino acids were synthesized by methylation of the Boc-D-amino acids by ethereal diazomethane, which was evolved from *N*-nitrosomethylurea. Boc-D-glutamic acid dimethyl ester, Boc-D-aspartic acid dimethyl ester, Boc-D-aspartic acid-β-benzyl ester-α-methyl ester, Boc-D-glutamic acid-γ-benzyl ester-α-methyl ester, Boc-D-serine methyl ester, Boc-D-threonine methyl ester, Boc-D-*allo*-threonine methyl ester, Boc-D-valine methyl ester, Boc-D-isoleucine methyl ester, Boc-DL-aminobutyric acid methyl ester, Boc-L-threonine methyl ester, and N^{α},N^{ϵ}-diBoc-D-lysine methyl ester were synthesized by this method.

Synthesis of Amino Acid Amides—Amino acid amides were synthesized by ammonolysis (10) of amino acid methyl esters HCl, or Boc-amino acid methyl esters in absolute methanol saturated with dry ammonia gas at room temperature. Deprotection of Boc and Z moieties were carried out in 4 N hydrogen chloride in ethylacetate and by catalytic hydrogenation in the presence of 10% palladium-carbon, respectively. The amino acid amides thus synthesized included Boc-D-alanine amide, D-alanine amide HCl, D-α-aminobutyric acid amide HCl, DL-β-aminobutyric acid amide HCl, D-serine amide HCl, D-threonine amide HCl, D-*allo*-threonine amide HCl, D-methionine amide HCl, D-norvaline amide HCl, D-norleucine amide HCl, D-phenylglycine amide HCl, D-proline amide HCl, D-glutamine amide HCl, D-lysine amide HCl, D-asparagine amide HCl, D-aspartic acid amide HCl, D-glutamic acid amide, D-valine amide HCl, D-isoleucine amide HCl, D-leucine amide HCl, D-histidine amide HCl, D-phenylalanine amide HCl, D-tyrosine amide HCl, D-tryptophan amide HCl, D-phenylglycine amide HCl, L-α-aminobutyric acid amide HCl, L-threonine amide HCl, and L-methionine amide HCl. The syntheses of D-alanine benzylamide HCl, D-alanine-3-aminopentane amide HCl, D-alanine *n*-butylamide HCl, D-alanine *n*-laurylamide HCl, and D-alanine anilide HCl are described elsewhere. All other chemicals were from commercial sources and used without further purification.

Screening of D-Aminopeptidase Producers—An enrichment culture technique (14) in a medium containing D-alanine amide as the sole source of nitrogen was applied for the selection of a desirable microorganism. A number of microorganisms utilizing D-alanine amide have been isolated from soil samples in Japan. The bacterial strains were aerobically cultured at 30 °C for 24 h in 200 ml of the following medium: the screening medium was composed of 5 g of glycerol, 2 g of D-alanine amide HCl, 2 g of K_2HPO_4, 1 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of yeast extract (Oriental Yeast, Japan), 0.02 μg of biotin, 4 μg of calcium pantothenate, 20 μg of inositol, 4 μg of nicotinic acid, 4 μg of thiamine HCl, 2 μg of pyridoxine HCl, 2 μg of *p*-aminobenzoic acid, 2 μg of riboflavin, and 0.1 μg of folic acid in 1 liter of tap water, pH 7.0. D-Alanine amide HCl was filter-sterilized and added after autoclaving. The cells were collected by centrifugation and disrupted by a Kubota-Syoji 9 kHz ultrasonic oscillator. The disrupted cells were removed by centrifugation at 14,000 × *g* for 20 min. The supernatant was dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol.

Enzyme Assay and Definition of Units—Activity of D-aminopeptidase was routinely assayed at 30 °C by measuring the production of D-alanine from D-alanine amide. The reaction mixture contained 50 μmol of Tris-HCl, pH 8.0, 5 μmol of D-alanine amide HCl, and the enzyme in 0.5 ml. The reaction was terminated after 10 min by boiling for 3 min. The hydrogen peroxide formed from D-alanine by the action of D-amino acid oxidase were determined by the oxidative coupling with 4-amino antipyrine and phenol in the presence of peroxidase (15). The standard reaction mixture (1.6 ml) containing 50 μmol of potassium phosphate buffer, pH 7.0, 0.72 unit of D-amino acid oxidase, 11 μmol of phenol, 0.8 μmol of 4-amino antipyrine, 68 units of peroxidase, and the sample was incubated with shaking at 30 °C for 1 h, unless otherwise specified. The formation of quinoneimine dye was measured at 500 nm and quantified by the standard curve obtained from authentic D-alanine. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of D-alanine in the hydrolysis reaction. The substrate specificity was examined qualitatively by thin layer chromatography first, and then quantitatively assayed by any one of the following methods. (*a*) The enzyme activity toward D-alanine amide, D-alanine peptides, and D-alanine *N*-alkylamides was measured as follows. A reaction mixture (0.5 ml) containing 50 μmol of substrate, 50 μmol of Tris-HCl, pH 8.0, and 0.2 unit of the purified enzyme was incubated at 30 °C for 10 min, and the formation of D-alanine was determined by the method described above. (*b*) The enzyme activity toward D-amino acid amides and L-amino acid amides (except for D-alanine amide) was measured with a reaction mixture containing 50 μmol of amino acid amide HCl, 50 μmol of Tris-HCl, pH 8.0, and 4.0 units of the enzyme and incubated for 2 h at the same temperature. Ammonia formed from the amides was determined by a decrease in absorbance at 340 nm of NADH by an assay kit for ammonia which utilizes NAD^+-dependent glutamate dehydrogenase (Kyowa Medex, Tokyo, Japan) (16). (*c*) The enzyme activities toward amino acid esters were measured with a reaction mixture containing 50 μmol of amino acid ester HCl, 50 μmol of Tris-HCl, pH 8.0, and 1.8 units of the enzyme and incubated for 10 min at the same temperature. The amount of the amino acid formed was determined by a color formation with ninhydrin, after paper chromatography on Advantec filter paper (number 51A, Tokyo, Japan) with a solvent system (1-butanol:acetic acid:water 4:1:1, by volume). (*d*) For substrates containing L-alanine at the NH_2 terminus, L-alanine released in the reaction mixture was measured by alanine dehydrogenase (17). The formation of a yellow color from D-alanine-*p*-nitroanilide (10 mM) was also followed at 405 nm (18) to detect enzyme activity.

Analytical Methods—Protein was assayed by the method of Lowry *et al.* (19) with bovine serum albumin as a standard or by measuring absorbance at 280 nm.

Polyacrylamide disc gel electrophoresis was performed in 6.0–10.0% polyacrylamide gel with Tris-HCl buffer, pH 8.9, at 5 mA/gel (20). The M_r of the subunits was determined by SDS-disc gel electrophoresis using 10% polyacrylamide with sodium phosphate buffer, pH 7.2, using oligomers of cytochrome *c*: hexamer (M_r = 74,400), tetramer (M_r = 49,600), trimer (M_r = 37,200), dimer (M_r = 24,800), and monomer (M_r = 12,400) (21). Electrophoresis was performed at 8 mA/gel. Protein bands were visualized with Coomassie Brilliant Blue R-250. To estimate the M_r of the enzyme, the enzyme samples were subjected to HPLC (Tosoh, Japan) at room temperature using a TSK G-3000 SW column (0.5 × 60 cm, Tosoh Corp., Japan) at a flow rate of 0.5 ml/min with an elution buffer consisting of a 0.1 M potassium phosphate buffer, pH 7.0, containing 0.2 M NaCl. A calibration curve was made with the following proteins: glutamate dehydrogenase (M_r = 290,000), lactate dehydrogenase (M_r = 142,000), enolase (M_r = 67,000), adenylate kinase (M_r = 32,000), and cytochrome *c* (M_r = 12,400).

To determine the NH_2-terminal amino acid sequence, the enzyme sample was passed through a TSK Phenyl-5PW column (0.75 × 7.5 cm, Tosoh Corp., Japan), and fractionated with a linear gradient of 20–80% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid. The enzyme sample (about 300 μg) was analyzed with an automatic protein sequencer 470A (Applied Biosystem) (22). Phenylthiohydantoin derivatives were determined with an HPLC system SP 8100 XR (Spectra Physics) on a reversed phase column, Senshu Pack Aquasil SEQ-4 (0.46 × 30 cm, Senshu Kagaku, Japan).

Isoelectric focusing was performed according to the method of Vesterberg (23), at 5 °C for 70 h using Ampholite in the pH range of 3.5–10.

The mode of action of the enzyme toward tripeptide D-alanylglycylglycine was determined with a reaction mixture (1.0 ml) containing 50 μmol of potassium phosphate, pH 7.5, 2 μmol of D-alanylglycylglycine, and 0.12 unit of the enzyme. The reaction mixture was incubated at 30 °C, and 100-μl aliquots were withdrawn and put into tubes containing 20 μl of 30% trichloroacetic acid. The deproteinized mixture was subjected to an amino acid analyzer (Kyowa Seimitsu, K-101, Japan).

Purification of D-Aminopeptidase from O. anthropi SCRC C1–38—*O. anthropi* SCRC C1-38 was cultivated aerobically at 30 °C for 18 h in a medium called TGY medium (5 g of Tryptone, 5 g of yeast extract, 1 g of glucose, and 1 g of K_2HPO_4 in 1 liter of tap water, pH 7.0 (24)). All the enzyme purification procedures were performed at temperatures lower than 5 °C. Potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol was used throughout the purification.

Cells (about 186 g wet weight) from 20 liters of culture were suspended in 0.1 M buffer. The cells were disrupted for 50 min by a

Kubota-Syoji 9 KHz sonic oscillator. The disrupted cells were removed by centrifugation at 14,000 × *g* for 20 min. To the cell-free extract was added 5% protamine sulfate, at a concentration of 0.1 g of protamine sulfate to 1 g of protein. After stirring for 30 min, the precipitate formed was removed by centrifugation at 14,000 × *g* for 20 min. To the supernatant was added ammonium sulfate to 30% saturation to remove inactive residues and then brought to 90% saturation. The active precipitate collected by centrifugation was dissolved in 0.01 M buffer and dialyzed against the same buffer. The dialyzed enzyme solution was applied to a DEAE-Toyopearl 650 M column and eluted with 0.1 M buffer containing 0.1 M NaCl. To the active fractions were added ammonium to 30% saturation. The enzyme solution was first placed on a column of Butyl-Toyopearl 650 M, equilibrated with 0.01 M buffer containing ammonium sulfate to 30% saturation. The active fractions were eluted with a linear gradient of ammonium sulfate (30–0% saturation) in 0.01 M buffer. The active fractions were combined, dialyzed against 0.01 M buffer and then ammonium sulfate was added to 30% saturation. The enzyme solution was placed on a second column of Butyl-Toyopearl 650 M. The active fractions were eluted with a linear gradient of ammonium sulfate (30–0% saturation) in 0.01 M buffer. The active fractions were dialyzed, concentrated by ultrafiltration, and applied to a column of Sephacryl S-300 (2.4 × 116 cm) equilibrated with the buffer (0.05 M) containing 0.1 M NaCl. The active fractions were combined, dialyzed, and applied to a hydroxyapatite column and eluted with a linear gradient of 0.01–0.15 M buffer. The active fractions were collected and concentrated by ultrafiltration DEAE-5PW column chromatography was carried out as described previously (64).

RESULTS

Purification of D-Aminopeptidase—A bacterial strain SCRC C1-38, isolated from a soil sample was chosen as a likely source of the enzyme. The strain is a Gram-negative, motile, obligatory aerobic, nonfermenting rod, and identified as *O. anthropi* (63).[2] The enzyme was formed intracellularly: when the strain was cultivated in TGY medium for 13 h, cell mass was about three times more than in the screening medium, and the specific activity in the cell-free extract was about 65% of the latter. Therefore, the enzyme appeared to be formed constitutively. TGY medium was chosen for a larger scale preparation of the enzyme. A summary of the purification procedures for the enzyme from *O. anthropi* SCRC C1-38 is shown in Table I. The enzyme was purified about 2800-fold with a 17% yield from the cell-free extracts.

Purity of the Enzyme—The enzyme was judged to be homogeneous by the criteria of native and SDS-polyacrylamide gel electrophoreses, HPLC with a TSK G-3000 SW column, and isoelectric focusing, as all of the results gave a single band or a single peak. The isoelectric point (pI) was 4.2. Fig. 1 shows the results of disc gel electrophoresis in the absence (*A*) and presence (*B*) of SDS.

Identification of Reaction Product and Mode of Action—A typical time course for the stereoselective hydrolysis of D-alanine amide is shown in Fig. 2, with racemic alanine amide as a substrate. D-Alanine amide was completely hydrolyzed by the action of the enzyme, while the L-enantiomer remained unhydrolyzed in the reaction mixture.

The identity of D-alanine formed by the D-stereospecific hydrolysis of DL-alanine amide was confirmed by its isolation. The reaction mixture contained 934 mg (7.5 mmol) of DL-alanine amide, 15 mmol of potassium phosphate, pH 7.0, 210 units of purified enzyme in a total volume of 75 ml. After the mixture was incubated at 37 °C for 1 h, alanine formed in the mixture was isolated by a procedure involving deproteinization by trichloroacetic acid and column chromatography on Amberlite IRA-400 (Cl^-) and Dowex 50W-X8 (H^+) columns.

[2] *O. anthropi* SCRC C1-38 is available from American Type Culture Collection (Rockville, MD) with the accession number ATCC 49237. The strain was originally classified in the CDC group Vd and also named as *Achromobacter* sp., according to the old criteria (25).

TABLE I

Summary of purification of D-aminopeptidase from O. anthropi SCRC C1-38

The concentration of the purified enzyme after the DEAE-Toyopearl step was determined from absorbance at 280 nm assuming an extinction coefficient ($A^{1\%}_{1cm}$ = 10.0).

Step	Total protein	Total activity	Specific activity	Yield
	mg	*units*	*units/mg*	*%*
Cell-free extract	7480	1600	0.214	100
Protamine sulfate and ammonium sulfate	2980	1170	0.392	73
DEAE-Toyopearl	233	1050	4.52	66
First Butyl-Toyopearl	67.0	1060	15.8	66
Second Butyl-Toyopearl	9.63	1040	108	65
Sephacryl S-300	4.30	664	155	42
Hydroxyapatite	1.06	538	507	34
DEAE-5PW	0.445	265	596	17

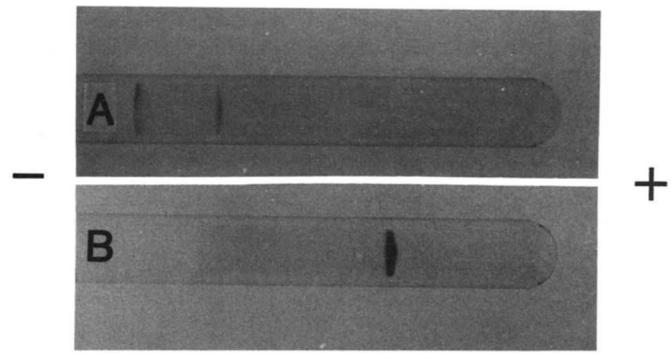

FIG. 1. **Polyacrylamide disc gel electrophoresis of the purified enzyme from *O. anthropi* SCRC C1-38.** *A*, purified enzyme was incubated in the presence of 1% SDS and 3% 2-mercaptoethanol at 95 °C for 3 min. The enzyme (8 μg) was electrophoresed in the presence of 0.1% SDS at a current of 8 mA. *B*, purified enzyme (15 μg) was electrophoresed in the absence of SDS at a current of 2 mA. The gels were stained with Coomassie Brilliant Blue R-250.

The isolated alanine (313 mg, 3.5 mmol) was recrystallized from water-methanol-isopropyl alcohol-ether. The results of the elementary analysis of the isolated alanine were:

Calculated for $C_3H_7NO_2$: C 40.44, H 7.92, N 15.72
Found: C 40.24, H 8.12, N 15.55

$[\alpha]_D^{20} = -14.15°$ (c 6.6, 1 N HCl).

The mode of action of the enzyme toward a peptide substrate was studied with D-alanylglycylglycine. When the reaction was followed with time, alanine and glycylglycine were released first until the substrate tripeptide was nearly completely consumed, then glycine was released, finally yielding alanine and glycine.

Molecular Weight, Isoelectric Point, and NH_2-terminal Amino Acid Sequence—The molecular weight of the native enzyme was calculated to be approximately 122,000 by gel filtration on HPLC. The molecular weight of the subunits were calculated to be 59,000 by comparing the mobility on SDS-polyacrylamide disc gel electrophoresis to that of standard proteins. The enzyme had an isoelectric point of pH 4.2. The NH_2-terminal amino acid sequence of the enzyme was determined to be Ser[1]-Lys-Phe-Asp-Thr[5]-Ser-Ala-Leu-Glu-Ala[10]-Phe-Val-*X*-*X*-Ile[15]-Pro-Gln-Asn-Tyr-Lys[20]-Gly-Pro-Gly-*X*-Val[25] (*X* denotes an undetermined amino acid residue). Thus, the native enzyme has a dimeric structure consisting of two identical subunits. The NH_2-terminal amino acid sequence should facilitate the synthesis of an appropriate oli-

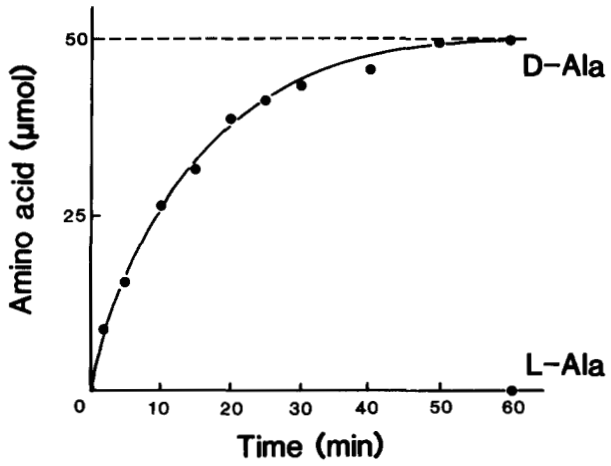

FIG. 2. **Kinetic resolution of DL-alanine amide by *O. anthropi* D-aminopeptidase.** A reaction mixture containing 0.5 mmol of Tris hydrochloride, pH 8.0, 0.1 mmol of DL-alanine amide hydrochloride, and 5 units of the purified enzyme in a total volume of 5.0 ml was incubated at 30 °C. The content of L-alanine was measured by L-alanine dehydrogenase.

gonucleotide for use as a probe in cloning DNA for the enzyme.

Absorption Spectrum—The absorption spectrum of the purified enzyme in 0.01 M potassium phosphate buffer, pH 7.0, showed the maximum absorbance at 281 nm.

Effect of Temperature and pH on the Enzyme Activity—The enzyme exhibited the maximal activity at 45 °C and pH 8.5. About 80% activity remained after incubation at 45 °C in 0.1 M potassium phosphate, pH 8.0, for 10 min. No loss of activity was found between pH 7.0 and 10.0 after incubation at 30 °C for 1 h in 0.05 M buffers of various pH values.

Substrate Specificity and Kinetic Properties—The enzyme showed strict chemo- and stereospecificities toward D-amino acid amides, peptides, and esters, as shown in Table II. Kinetic studies were also carried out to determine the Michaelis constants (K_m) and maximum reaction velocity (V_{max}) for some of the major substrates, from double-reciprocal plots. The enzyme acted on D-alanine amide (relative velocity, 100%; K_m value, 0.65 mM), glycine amide (44%, 22.3 mM), D-α-aminobutyric acid amide (30%, 18.3 mM), D-serine amide (29%, 27.0 mM), D-alanine 3-aminopentane amide (32%, 2.27 mM), D-alanine anilide (73%), D-alanine benzylamide (72%, 0.51 mM), D-alanine-*p*-nitroanilide (96%, 0.51 mM), D-alanine *n*-butylamide (66%, 0.73 mM), D-alanine methyl ester (75%), glycine methyl ester (229%), D-alanylglycine (95%, 0.98 mM), D-alanylglycylglycine (45%, 0.37 mM), D-alanyl-D-alanine (21%, 10.2 mM), D-alanyl-D-alanyl-D-alanine (92%, 0.57 mM), D-alanyl-D-alanyl-D-alanyl-D-alanine (89%, 0.32 mM), D-alanyl-L-alanine (46%, 1.03 mM), and D-alanyl-L-alanyl-L-alanine (100%, 0.65 mM), etc. However, as shown in Table III, some of the L-amino acid derivatives were active as substrates, albeit slowly: L-alanylglycine amide (releasing ammonia at 3.0% of the velocity for D-alanine amide), L-serine amide (1.1%), L-threonine amide (0.5%), L-alanine methyl ester (53%), L-serine methyl ester (26%), L-α-aminobutyric acid methyl ester (2.0%), L-threonine methyl ester (1.6%), and L-alanine-*p*-nitroanilide (42%). Enzymatic analysis with alanine dehydrogenase and D-amino acid oxidase of the alanine produced from L-alanine methyl ester and L-alanine-*p*-nitroanilide revealed that D-alanine was not contained in the product, ruling out the possibilities of enzymatic racemization prior to hydrolysis and contamination by a D-alanine derivative in the substrate. The compounds listed in Table IV were judged to be inactive as substrates with less than 0.05% the velocity for D-alanine amide. They include most of the D-amino acid amides with more than five carbon atoms, most of the L-amino acid amides, L-amino acid methyl esters, D- and L-α-amino-ε-caprolactams, low molecular weight aliphatic amides, β-alanyl dipeptides, and *N*-protected D-alanine derivatives. The rate of hydrolysis of L-alanine amide was less than 0.01% that of D-alanine amide.

TABLE II
Substrate specificity of D-aminopeptidase from O. anthropi SCRC C1-38

Substrate	Relative activity	K_m	V_{max}
	%	mM	units/mg
D-Alanine amide	100	0.65	600
Glycine amide	44	22.3	365
D-α-Aminobutyric acid amide	30	18.3	576
D-Serine amide	29	27.0	22.0
D-Threonine amide	9	100	60.3
D-Methionine amide	2		
D-Norvaline amide	1.8		
D-Norleucine amide	0.8		
D-Phenylglycine amide	0.7		
D-Alanylglycine	95	0.98	1000
D-Alanylglycylglycine	45	0.37	799
D-Alanyl-D-alanine	21	10.2	326
D-Alanyl-D-alanyl-D-alanine	92	0.57	866
D-Alanyl-D-alanyl-D-alanyl-D-alanine	89	0.32	702
D-Alanyl-L-alanine	46	1.03	312
D-Alanyl-L-alanyl-L-alanine	100	0.65	730
DL-Alanyl-DL-serine	27		
DL-Alanyl-DL-methionine	20		
DL-Alanyl-DL-phenylalanine	9		
DL-Alanyl-DL-asparagine	7		
DL-Alanyl-DL-leucine	1		
DL-Alanyl-DL-valine	0.5		
Glycine methyl ester	229		
D-Alanine methyl ester	75		
D-Alanine-β-naphthylamide	32		
D-Alanine benzylamide	72	0.51	768
D-Alanine anilide	73		
D-Alanine-*p*-nitroanilide	96	0.51	696
D-Alanine *n*-butylamide	66	0.73	670
D-Alanine-3-aminopentane amide	32	2.27	288
D-alanine *n*-laurylamide	19		
D-Threonine benzyl ester	3.2		

TABLE III
L-Amino acid derivatives active as substrates

Substrate	Relative activity
	%
L-Serine amide	1.1
L-Threonine amide	0.5
L-Alanylglycine amide	3.0[a]
L-Alanine methyl ester	53
L-Serine methyl ester	26
L-α-Aminobutyric acid methyl ester	2.0
L-Threonine methyl ester	1.6
L-Alanine-*p*-nitroanilide	42

[a] Determined by the formation of ammonia. No formation of L-alanine was detected.

Effect of Metal Ions and Inhibitors—The enzyme activity was measured after the enzyme was preincubated at 30 °C for 30 min with various compounds (at 1 mM unless otherwise noted). The enzyme activity was inhibited to 20–50% by Ca^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, 5,5′-dithiobis(2-nitrobenzoic acid), hydroxylamine (at 10 mM), and *N*-ethylmaleimide; and 25–100% by Ag^{+}, Hg^{2+} (at 0.02 mM), and *p*-chloromercuriben-

TABLE IV
Amino acid derivatives inactive as substrates

D-Amino acid derivative	L-Amino acid derivative
	L-Alanine amide
	L-Methionine amide
D-Aspartic acid amide (D-isoasparagine)	L-Aspartic acid amide (L-isoasparagine)
D-Glutamic acid amide (D-isoglutamine)	L-Glutamic acid amide (L-isoglutamine)
D-Asparagine	L-Asparagine
D-Glutamine	L-Glutamine
D-Asparagine amide	
D-Glutamine amide	
D-Leucine amide	L-Leucine amide
D-Isoleucine amide	L-Isoleucine amide
D-Valine amide	L-Valine amide
D-Phenylalanine amide	L-Phenylalanine amide
D-Tyrosine amide	L-Tyrosine amide
D-Tryptophan amide	L-Tryptophan amide
D-Histidine amide	L-Histidine amide
D-Lysine amide	L-Lysine amide
D-Arginine amide	L-Arginine amide
D-Proline amide	L-Proline amide
	L-Alanyl-D-alanine
	L-Alanyl-L-alanine
	L-Alanylglycine
	L-Methionine methyl ester
	L-Norvaline methyl ester
	L-Norleucine methyl ester
	L-*allo*-Threonine methyl ester
DL-Alanyl-DL-isoleucine	
D-α-Amino-ε-caprolactam	L-α-Amino-ε-caprolactam
DL-α-Amino-γ-butyrolactone	

Other amides

Acetamide
Propionamide
β-Alanyl-L-alanine
β-Alanylglycine
N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine
Boc-D-alanine amide
Z-D-Alanine methyl ester

zoate (at 0.074 mM). The activity of the enzyme was not lost upon incubating with the following agents: Li^{+}, Na^{+}, Ba^{2+}, Fe^{2+}, Mg^{2+}, Sn^{2+}, Al^{3+}, Fe^{3+}, Pb^{3+}, EDTA, 8-oxyquinoline, α,α′-dipyridyl, *o*-phenanthroline, sodium azide, KCN (at 0.2 mM), monoiodoacetate, phenylmethylsulfonyl fluoride, leupeptin (20 μM), and pepstatin A (20 μM). The effect of various compounds and metal ions on the enzyme activity indicated that the enzyme is a thiol peptidase.

DISCUSSION

We have described the purification and characterization of a novel aminopeptidase from *O. anthropi* SCRC C1-38, which had been isolated from soil. The microorganism was identified taxonomically and chosen as the enzyme producer. The enzyme was formed constitutively and intracellulary. Without the enzyme purification, it cannot be determined whether enzyme activity detected in the crude extract is the result of two enzymes, one active toward L-amino acid containing substrates and the other active toward those with D-amino acid, or the result of a single enzyme which is not highly stereospecific. To our surprise, almost no L-alanine amide-hydrolyzing activity was detected in the cell-free extracts of the bacterium (data not shown). The purification work has proved that the enzyme activity shown in the crude extract was due to a D-stereospecific peptidase.

We have characterized the aminopeptidase from *O. anthropi* SCRC C1-38 and found it differs from those described in the literature. The enzyme has a molecular weight of approximately 122,000 with two identical subunits having molecular weights of 59,000. The enzyme showed neither endopeptidase, nor carboxypeptidase activity, but exhibited a wide range of activities: (i) aminopeptidase activity which requires D-configuration at the NH_2 terminus (except for glycine amides), (ii) D-aminoacylamidase and aryl-D-aminoacylamidase activities, and (iii) amino acid esterase activity. With amino acid esters as substrates of the hydrolytic reaction, the enzyme showed low stereoselectivity, suggesting that they are not the true substrates of the enzyme. With the peptide substrates, the enzyme recognized the configuration of the NH_2-terminal D-amino acid and hydrolyzed not only the peptide bond between D-alanyl-D-alanine, but also that of D-alanyl-L-alanine.

A number of aminopeptidases have been purified and characterized, and their activities have been detected in several organisms, such as bacteria (see "Discussion"), fungi (26), muscles (27), brain (28), lens (29), etc. of vertebrates. However, there has been no report on the occurrence of an aminopeptidase preferentially active toward D-amino acid containing peptides, amides, and esters with a high D-stereospecificity. D-Aminoacylase (3, 4) and *Streptomyces* D-specific carboxypeptidase-like peptide hydrolase (5) are examples of moderately D-specific peptide hydrolases, apparently belonging to other categories. Among a number of reports on aminopeptidases, only a few described the substrate specificity toward D-amino acid amides and peptides, in addition to natural substrates with L-configuration. Robinson *et al.* (30) studied the substrate specificity of an aminopeptidase from kidney cellular particulates and concluded that the enzyme shows rather loose stereospecificity toward the carboxyl-terminal amino acid of oligopeptides, while it required an L-amino acid at the amino terminus. *Mycobacterium phlei* aminopeptidase, although not purified to homogeneity, catalyzes the hydrolysis of D-leucylglycine at less than 1% of the velocity toward L-leucylglycine (31). An intracellular aminopeptidase from *B. subtilis* (32), aminopeptidase from *Streptomyces griseus* Pronase (33), and aminopeptidase B from rat liver (34) hydrolyze D-amino acid-containing peptides at the NH_2 terminus nonselectively at much slower rates than L-amino acid-containing peptides, although the data are not quantitative. Measuring some of the K_m and V_{max} values for the major substrates including a number of synthetic substrates, it was clear that the enzyme in the present study prefers peptides more than amino acid arylamides or amino acid amides. Thus, the enzyme exhibits a mode of action typical of aminopeptidases which liberate an NH_2-terminal D-amino acid residue with a free amino group, placing it in the category of an aminoacyl-peptide hydrolase (EC 3.4.11) (1); we tentatively named the enzyme as "D-aminopeptidase." Considering the high stereospecificity and rather narrow substrate specificity toward derivatives of low molecular weight D-amino acid, the physiological role of the enzyme may be to hydrolyze the dipeptide D-alanyl-D-alanine which is a product of D-alanyl-D-alanine ligase (35), a fragment of bacterial peptidoglycan composed of D-alanine and glycine (6), or to degrade D-alanylglycine and D-alanyl-D-alanine synthesized in the rice plants (7).

The enzyme was inhibited by sulfhydryl reagents such as Ag^{+}, Hg^{2+}, and *p*-chloromercuribenzoate, but not by chelating agents such as *o*-phenanthroline or EDTA. The enzyme was not activated with divalent cations. These observations indicate the involvement of a thiol group at the active center of the enzyme. The fact that the enzyme catalyzed the aminolysis of D-amino acid ester or amide with 3-aminopentane in water-saturated organic solvents (data not shown) also sup-

ports the possible presence of a thiol group at the enzyme active site. Aminopeptidases which appear to have an active thiol group are relatively small in number as compared with metal-dependent ones (2). Sulfhydryl group-dependent aminopeptidases include, for example, proline iminopeptidase (EC 3.4.11.5) from *Bacillus megaterium* (36), arginine aminopeptidase (EC 3.4.11.6) from rat liver (34), and aminopeptidases from human placenta (37), *Plasmodium falciparum* (38), buckwheat seed (39), monkey brain (28), and skeletal muscle of the rat (40).

Aminopeptidase activity toward L-alanine-*p*-nitroanilide has been proposed to be one of the criteria for the classification of bacteria: nearly 80% of randomly chosen Gram-negative bacteria are aminopeptidase-positive (41). Arylamidases activities toward L-amino acid *p*-nitroanilides and L-amino acid 2-naphthylamides are also widely distributed among bacteria (42), although they are not always aminopeptidases. Aminopeptidases have been isolated from several bacterial sources. They include extracellular enzymes from *Aeromonas proteolytica* (EC 3.4.11.10) (43), *Vibrio* sp. (44), *B. subtilis* (45), *Bacillus licheniformis* (46), *S. griseus* (33), *Streptomyces rimosus* (47), *Streptomyces peptidofaciens* (48), *Clostridium histolyticum* (EC 3.4.11.13) (49), and *Empedobacter collagenolyticum* (50); intracellular membrane-bound enzymes from *Pseudomonas aeruginosa* (51), *Streptococcus cremoris* (52), and *Mycoplasma salivarium* (19); and intracellular soluble enzymes from *Escherichia coli* (53), *Salmonella typhimurium* (54), *Flavobacterium* sp. (55), *Neisseria meningitidis* (56), *Moraxella urethralis* (56), *B. megaterium* (EC 3.4.11.5) (36), *Bacillus stearothermophilus* (57), *Lactobacillus acidophilus* (58), *Bifidobacterium breve* (59), *Streptococcus durans* (60), *Mycobacterium tuberculosis* (61), and *Treponema phagedenis* (62). Among them, the enzymes from *Bacillus* proline iminopeptidase (36) and *S. durans* (60) are the only thiol aminopeptidases which are soluble and intracellularly formed. The former enzyme is a monomer enzyme with a molecular weight of 58,000. The latter has a molecular weight of 300,000 and is composed of six identical subunits having molecular weights of 49,400. Thus, the aminopeptidase characterized in this study is quite unusual for an NH_2-terminal exopeptidase, both in its stereospecificity, possible active center, and its occurrence in the bacterial world.

There is limited knowledge concerning the *in vitro* enzymatic synthesis of D-amino acid-containing substances. First, we showed that D-aminopeptidase can be used as a catalyst in the stereospecific synthesis of D-amino acid *N*-alkylamides in organic solvents, from an amine and a D-amino acid ester or a D-amino acid amide as substrates. In contrast to the hydrolytic reaction, L-alanine methyl ester was not utilized at all as a substrate in the D-specific aminolysis reaction, with a complete kinetic resolution of DL-alanine methyl ester. The details of the enzymatic synthesis of D-amino acid *N*-alkylamides and the behavior of the enzyme in organic solvents will be described elsewhere. Studies on the molecular cloning of the gene for the enzyme and the primary structure elucidation are in progress.

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