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A Novel Dipeptidyl Aminopeptidase from *Pseudomonas* sp. Strain WO24

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An activity similar to that of dipeptidyl aminopeptidase I (DAP I) which releases dipeptide from Gly-Arg-p-nitroanilide (Gly-Arg-pNA) was detected in a *Pseudomonas* sp. An enzyme was isolated and purified about 400-fold by a series of column chromatographies. The enzyme, named DAP BI (DAP from bacteria, type I), was revealed to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing. The molecular mass was estimated to be 82 kDa by SDS-PAGE and 65 kDa by gel filtration, suggesting that the enzyme may be a monomer. The enzyme had an isoelectric point of 4.7. It is optimally active at pH 9.0. The K_m and V_{max} of the enzyme for Gly-Arg-pNA were 0.25 mM and 195 μmol/min/mg, respectively. The purified enzyme did not hydrolyze Gly-Phe-pNA, which was also a substrate for DAP I, whereas it hydrolyzed Arg-Arg-4-methoxy-β-naphthylamide (Arg-Arg-MNA), a model substrate for DAP III. The K_m and V_{max} for Arg-Arg-MNA were 0.019 mM and 145 μmol/min/mg, respectively. This purified enzyme can also catalyze the removal of Asp-Arg from the N termini of angiotensins I and II. The enzyme activity was completely inhibited by Zn(II) (0.5 mM), tosyl-L-Lys-chloromethyl ketone (0.1 mM), and leupeptin (0.1 mM) and partially inhibited by Co(II) (0.5 mM) and chymostatin (0.1 mM), whereas the enzyme was not affected by general serine protease inhibitors (phenylmethylsulfonyl fluoride and diisopropylfluorophosphate) and thiol protease inhibitors. The substrate specificity, classification of catalytic site, and other enzymatic properties demonstrate that this enzyme is distinct from the previously described mammalian DAPs I and III and *Saccharomyces cerevisiae* DAP III. These results indicate that DAP BI may be a new type of the DAP family.

Dipeptidyl aminopeptidase (DAP) is capable of sequentially removing dipeptides from the amino termini of peptides and proteins. In mammalian cells, four distinct enzymes with DAP activity have been purified from the liver, brain, and pituitary on the basis of substrate specificity by using unique naphthylamide (NA) substrates, specific pH optima, and catalytic classification (13, 15). DAP I (cathepsin C) cleaves Gly-Arg-β-naphthylamide (Gly-Arg-βNA) and Gly-Phe-βNA at pH 6, and DAP II cleaves Lys-Ala-βNA with a pH optimum of 5. DAP III degrades Arg-Arg-βNA with a pH optimum of 8 to 9, while DAP IV hydrolyzes Gly-Pro-βNA at pH 8. DAP I is a lysosomal cysteine protease belonging to the papain superfamily. It possesses a broad specificity (20) for liberating dipeptides whose amino-terminal amino acid is not basic (Arg or Lys) and whose penultimate amino acid is not proline and that are not linked to proline through a peptide bond. DAP I appears to play an important role not only in intracellular protein degradation, but also in cell growth and processing and activation of several enzymes (18). On the other hand, DAP III shows relatively narrow substrate specificity on dipeptide NAs but a wide range of activity on peptide substrates. In particular, DAP III prefers angiotensins and enkephalins as substrates (13), suggesting that it plays a physiological role in their disposition.

In microorganisms, X-prolyl DAP (DAP IV) has been well studied with a number of organisms, such as bacteria (2, 21, 23, 28), yeasts (3), and fungi (25). The enzyme appears to have a substrate specificity and catalytic classification similar to those of enzymes of mammalian origin. DAP I and II have not been found in microorganisms, except for recent reports that *Bacteroides ruminicola* (19) and *Dictyostelium discoideum* (4) may possess DAP I-like activities capable of hydrolyzing Gly-Arg-4-methoxy-βNA (Gly-Arg-MNA) and Ala-Ala-Ala-Ala and hydrolyzing Gly-Arg-4-methylcoumarinamide (Gly-Arg-MCA), respectively. Concerning other types of DAPs, DAP III was found to be present only in *Saccharomyces cerevisiae* (27) and *D. discoideum* (7). Although these enzymes have been shown to resemble mammalian DAP III, some differences were noted and their molecular nature remains obscure.

DAPs may serve as tools in some fields, such as dietetics and the food industry (for production of oligopeptides, which are more absorbable than amino acids [6], from proteins) and analytical biochemistry (as a tool for peptide sequencing [10]). Furthermore, if various DAPs, including DAP IV, could be easily isolated in quantity from microorganisms, it would be feasible to investigate the relationship between the mechanism of dipeptide liberation and the molecular structure, compared with those for aminopeptidases (APs) and endopeptidases. Therefore, we have screened microbial DAP I-like enzymes, since they have significant advantages over mammalian enzymes for these studies.

In this study, we isolated a bacterial strain having an enzyme that exhibited N-terminal Gly-Arg-releasing activity on Gly-Arg-p-nitroanilide (Gly-Arg-pNA). The bacterium was tentatively identified as a *Pseudomonas* sp. strain. We also purified and characterized the DAP. The results obtained in this study indicate that this DAP is distinct from DAPs previously described. Therefore, we named the enzyme DAP BI (for DAP from bacteria, type I).

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TABLE 1. Purification of DAP from *Pseudomonas* sp. strain WO24[a]

Purification step	Protein (mg)	Total activity (U)	Yield (%)	Sp act (U/mg)	Purification (fold)
Cell extract	3,530	619	100	0.175	1.0
Ammonium sulfate (40–70%)	2,500	490	79	0.196	1.1
DEAE-Cellulofine	139	392	63	2.82	16
Butyl-Sepharose	11.1	172	28	15.5	89
Sephacryl S-300HR	3.54	110	18	31.1	178
Q-Sepharose FF	1.42	98.4	16	69.3	396

[a] The enzyme activities were assayed with Gly-Arg-pNA as the substrate and are expressed as units (micromoles of pNA released per min).

MATERIALS AND METHODS

Materials. Arg-pNA, Phe-pNA, Lys-pNA, Gly-Arg-pNA, Gly-Phe-pNA, Ala-Ala-pNA, Ala-Ala-Ala-pNA, Gly-Arg-MNA, Ser-Tyr-MNA, Arg-Arg-MNA, Phe-Arg-βNA, Ala-Ala-Ala, Ala-Ala-Ala-Ala, and angiotensins I and II were obtained from Sigma Chemical Co. Ala-pNA, Pro-pNA, Lys-Ala-MCA, Gly-Pro-pNA, Z-Arg-Arg-MCA, Boc-Gly-Arg-Arg-MCA, and 7-amino-4-methylcoumarin (AMC) were obtained from Peptide Institute Inc. (Osaka, Japan). Diisopropyl fluorophosphate (DFP), *N*-ethylmaleimide, and E-64 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), *N*-tosyl-L-lysyl chloromethyl ketone (TLCK), iodoacetate, chymostatin, leupeptin, and pepstatin A were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). DEAE-Cellulofine A-500 was acquired from Seikagaku Co. (Tokyo, Japan). Butyl-Sepharose 4B, Sephacryl S-300HR, and Q-Sepharose FF were products of Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of the highest purity available.

Screening of microorganisms. Microorganisms from soil, wastewater, and foods were screened. The samples were suspended in saline solution. A drop of suspended solution was diluted, plated on bouillon medium (0.7% meat extract, 1.0% peptone, 0.3% NaCl, 2.0% agar; pH 7.0), and incubated at 37°C. Microorganisms capable of hydrolyzing Gly-Arg-βNA were obtained by a plate-staining procedure the reverse of that used for the isolation of mutants lacking AP (1). We screened microorganisms that hydrolyzed Gly-Arg-βNA but not Arg-βNA. Candidate microorganisms that were positive in the assay were further cultivated in 300-ml shaking flasks containing the same medium at 37°C on a reciprocal shaker. After 1 day of cultivation, the cells were harvested by centrifugation at 9,500 × *g* for 20 min at 4°C. They were then suspended in 10 ml of cold saline solution and disrupted by ultrasonic treatment as described below. DAP and AP activities of the disrupted cell suspension were assayed by the hydrolysis of Gly-Arg-pNA and Arg-pNA over a wide range of reaction pHs, respectively.

Characterization of strain. The strain isolated after screening (strain WO24) was cultured at 37°C in a nutrient medium composed of 1.0% peptone, 0.7% meat extract, 0.5% yeast extract, and 0.3% NaCl (pH 7.2). The strain was characterized by a standard method (9). Utilization of various sugars as the sole source of carbon was tested by using the commercial API NE20 system of Biomerieux S.A. (Marcy l'Etoile, France).

Preparation of cell extracts. Preparation and purification of the enzyme were carried out at 4°C. Strain WO24 was aerobically grown in bouillon medium at 37°C. When the A_{600} reached 1.3, the cells were harvested by centrifugation at 9,500 × *g* for 20 min at 4°C. The harvested cells were washed twice with cold 50 mM Tris-HCl buffer (pH 8.0) and suspended in the same buffer containing 1 mM dithiothreitol so that the cell concentration was 1/10 the volume of the original culture. The cells were disrupted by ultrasonic treatment at 10 kHz for 10 min (model UD201; Tomy, Tokyo, Japan). Cell extract was obtained by centrifugation at 12,000 × *g*.

Purification of DAP. Table 1 shows the overall procedure for purification of the DAP from *Pseudomonas* sp. strain WO24. Cells (112 g [wet weight]) were obtained from 14 liters of culture. Proteins in the cell extract were fractionated by salting out with solid ammonium sulfate to 40 to 70% saturation. The precipitate formed was collected by centrifugation at 18,000 × *g* for 20 min, dissolved in a minimum volume of 50 mM Tris-HCl buffer (pH 9.0), and then dialyzed for 12 h against the same buffer (pH 9.0) at 4°C.

The dialyzed fraction was subjected to ion-exchange chromatography on a column (2.5 by 20 cm) of DEAE-Cellulofine A-500 which had been equilibrated with 50 mM Tris-HCl buffer (pH 9.0). After the application of the sample, the column was first washed with 5 bed volumes of the equilibration buffer and then eluted with a 1,000-ml linear gradient of 0 to 0.2 M NaCl in the same buffer at a flow rate of 60 ml/h. The eluate was collected at 10-ml intervals. Each fraction was tested for protein concentration by measuring the A_{280} and for DAP activity. Fractions with DAP activity were pooled.

The combined active fractions from DEAE-Cellulofine chromatography, which were brought to 30% ammonium sulfate saturation, were applied to a Butyl-Sepharose 4B column (2.5 by 12 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) 30% saturated with ammonium sulfate. After a washing with 5 bed volumes of equilibration buffer, fractions were eluted from the column with 1,000 ml of a linearly decreasing gradient of 30 to 0% saturation with ammonium sulfate in 10 mM Tris-HCl buffer (pH 8.0) at a flow rate of 60 ml/h. The eluate was collected in 10-ml fractions, and each fraction was assayed for DAP. Fractions containing DAP activity were pooled and concentrated to 5.0 ml by ultrafiltration with a Diaflo PM-10 membrane.

Gel filtration of the concentrated Butyl-Sepharose pool was performed on a Sephacryl S-300HR column (2.5 by 100 cm) with 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl at a flow rate of 17 ml/h. Fractions of 2.5 ml were collected. The fractions containing DAP activity were pooled, concentrated, and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) with a Diaflo PM-10 membrane.

The enzyme fractions obtained by Sephacryl S-300HR chromatography were applied to a Q-Sepharose FF column (1.0 by 25 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was washed with 5 volumes of the same buffer and further washed with 10 volumes of the 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. The fractions were then eluted with a 500-ml gradient of 0.1 to 0.3 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 50 ml/h and collected in 5-ml volumes. The fractions containing DAP activity were pooled and concentrated to 8.0 ml with a Diaflo PM-10 membrane. The subsequent characterization of DAP from strain WO24 reported in this paper was performed with this preparation.

Enzyme assay. DAP activity was routinely assayed by the hydrolysis of Gly-Arg-pNA (one of the substrates of DAP I). The incubation mixture consisted of 100 μl of 3 mM substrate, 500 μl of 0.1 M Tris-HCl buffer (pH 9.0), 390 μl of water, and 10 μl of an appropriately diluted enzyme solution, and the reaction was initiated by the addition of the enzyme solution. After incubation at 37°C for 10 to 60 min, over which time the reaction rate was linear, the reaction was stopped by the addition of 50 μl of 100% (wt/vol) trichloroacetic acid, and the extent of hydrolysis was measured at an A_{385}. AP activity was measured by the hydrolysis of Arg-pNA as a substrate. One unit of DAP and AP activity was defined as the amount of enzyme that liberates 1 μmol of pNA per min at 37°C.

Hydrolysis of pNA derivatives of amino acids and peptides was carried out by the standard enzyme assay procedure described above. Enzyme activity was also analyzed by a method using derivatives of βNA and MNA as substrates (29). The assay mixture contained 500 μl of 100 mM Tris-HCl buffer (pH 9.0), 300 μl of water, and 100 μl of 3 mM βNA or MNA derivative, and the reaction was initiated by the addition of 100 μl of an enzyme solution. After incubation of the assay mixture at 37°C for 20 min, the reaction was stopped by the addition of 500 μl of 0.1% Fast Garnet GBC prepared in 1 M CH_3COOH-CH_3COONa buffer (pH 4.0) containing 10% Triton X-100, and then the A_{550} was measured after 10 min. One unit of this DAP activity was defined as the amount of enzyme that liberates 1 μmol of βNA and MNA per min at 37°C. DAP activity was also determined by using peptide-MCAs as substrates according to the method described by Kato et al. (8). The assay mixture contained 100 μl of 100 mM Tris-HCl buffer (pH 9.0), 20 μl of 3 mM peptide-MCA, and 70 μl of water, and the reaction was initiated by the addition of 10 μl of an enzyme solution. After incubation for 20 min, the reaction was terminated by addition of 10 μl of 100% trichloroacetic acid. Subsequently, the reaction mixture was added to 1.9 ml of water, and the liberated AMC was determined fluorometrically with a model F4010 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) using excitation and emission wavelengths of 380 and 460 nm, respectively. The observed fluorescence was converted into micromoles of AMC by using a standard curve. One unit of this activity was defined as the amount of enzyme that liberates 1 μmol of AMC per min at 37°C. All assays were done in triplicate, and the mean variation between samples was approximately 5%.

The steady-state kinetic constants for Gly-Arg-pNA, Gly-Arg-MNA, and Arg-Arg-MNA were determined under the same conditions as described above except for substrate concentrations. These activity measurements were made for incubation mixtures ranging in substrate concentration from 0.01 to 3 mM. The K_m and V_{max} were obtained from Eadie-Hofstee plots and expressed as the means of three different experiments, respectively.

Temperature and pH dependence of DAP activity. The effect of temperature on DAP activity was measured in the range of 4 to 70°C. The enzyme mixture was equilibrated for 5 min at the temperature tested before the addition of Gly-Arg-pNA substrate. The optimum pH for hydrolyzing Gly-Arg-pNA was determined

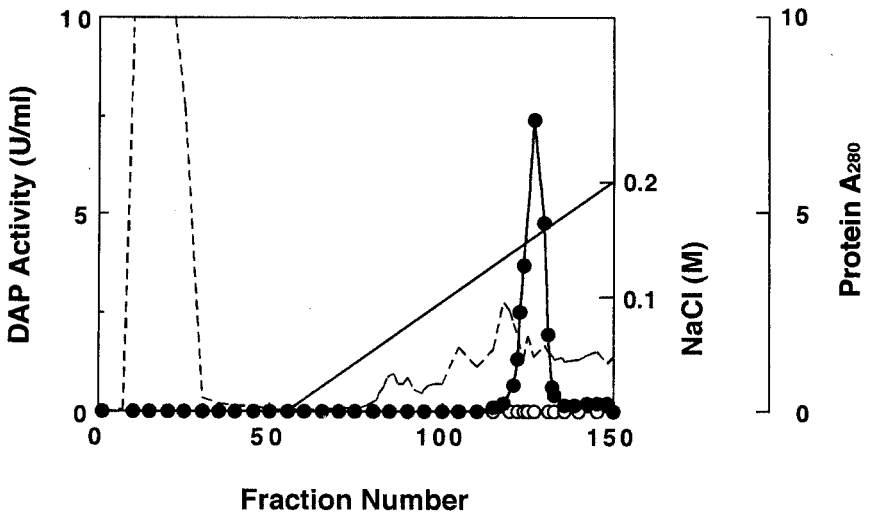

FIG. 1. DEAE-Cellulofine column chromatography of the DAP from *Pseudomonas* sp. strain WO24. Chromatography was conducted as described in Materials and Methods. Elution was performed with a linear gradient of 0 to 0.2 M NaCl (——) with 50 mM Tris-HCl buffer (pH 9.0). Enzyme activities were assayed with Gly-Arg-pNA (●) and Arg-pNA (○) as described in the text. The protein concentration (– – –) was monitored by A_{280}.

in the pH range of 5.5 to 11 by using buffer consisting of 50 mM (each) morpholineethanesulfonic acid (MES) (pH 5.5 to 7.0), morpholinepropanesulfonic acid (MOPS) (pH 7.0 to 8.0), Tricine (pH 8.0 to 9.0), cyclohexylaminoethanesulfonic acid (CHES) (pH 9.0 to 10.0), and cyclohexylaminopropanesulfonic acid (CAPS) (pH 10.0 to 11.0). The thermostability of the enzyme was demonstrated under the routine assay conditions after the enzyme solution was treated for 30 min at the temperature tested. For measurements of pH stability, the activity was routinely assayed except at a final concentration of 250 mM Tris-HCl buffer (pH 9.0) after the enzyme solution had been preincubated for 30 min at the concentration of the above buffers of 50 mM (final concentration, 5 mM in the assay mixture).

HPLC analysis. The enzymatic reaction was stopped after 30 or 60 min of incubation by the addition of 250 μl of 6% perchloric acid, and the mixture was neutralized with 75 μl of 1 N KOH. The breakdown products in the digested sample were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) with a Shodex octyldecyl silane C_{18}-5B column (0.46 by 25 cm). For Gly-Arg-pNA, the column was equilibrated with 12% acetonitrile, 0.017 M boric acid, and 0.1 M sodium perchlorate, and the sample was eluted with a linear gradient of acetonitrile from 12 to 36% for 20 min. For angiotensins I and II, the reaction mixture contained 100 μg of angiotensin and 0.09 μg of purified enzyme in 500 mM Tris-HCl buffer (pH 9.0). The reaction mixture was incubated at 37°C for 30 or 60 min, and then the reaction was stopped by boiling for 5 min. The column was equilibrated with 0.1% trifluoroacetic acid, 10 μl of the reaction mixture was injected, and then sample was eluted with a linear gradient of acetonitrile from 0 to 60% with 0.1% trifluoroacetic acid for 40 min. The HPLC was performed at 30°C at a flow rate of 1 ml/min. The A_{200} was monitored with a model SPD-6A UV detector (Shimadzu Co., Kyoto, Japan).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (13) with 7.5% polyacrylamide. The protein samples were mixed in a ratio of 4:1 with sample buffer (0.05 M Tris-HCl [pH 6.8], 10% SDS, 22% glycerol, 10% β-mercaptoethanol, and 0.18% bromophenol blue) and applied to the gel. The gel was stained with Coomassie brilliant blue.

Isoelectric focusing. The isoelectric point of DAP was determined by isoelectric focusing with 2% amphorite (pH 3.5 to 10) using a model 3230 apparatus according to the isoelectric focusing manual of Atto Co. (Tokyo, Japan).

Determination of molecular mass. Subunit molecular masses were determined by the SDS-PAGE procedure described above, using the following proteins as standards: glutamic dehydrogenase (53 kDa), transferrin (76 kDa), β-galactosidase (116 kDa), α-macroglobulin (170 kDa), and myosin (212 kDa). The molecular mass of the native enzyme was determined by its elution volume from a column of Sephacryl S-300HR (2.5 by 100 cm) relative to those of the standards thyroglobulin (670 kDa), catalase (232 kDa), gamma globulin (158 kDa), bovine serum albumin (68 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa).

Protein determination. The quantitative estimation of protein in various concentrated enzyme samples was carried out by the method of Lowry et al. (15), with bovine serum albumin as the standard.

N-terminal amino acid sequencing. The purified enzyme was subjected to SDS-PAGE (8% polyacrylamide gel) and electroblotted onto a polyvinylidene difluoride membrane. After being stained with amido black 10B, the purified enzyme band was identified, cut out, and directly analyzed with a 470A protein sequencer (Applied Biosystems).

RESULTS

Screening of DAP activity and identification of strain WO24. Seven strains exhibited enzyme activity capable of releasing dipeptide from Gly-Arg-pNA as a result of the screening. The crude enzyme from microbial strain WO24, isolated from industrial wastewater, had the highest hydrolytic activity on Gly-Arg-pNA but almost no activity on Arg-pNA. Strain WO24 is a motile, gram-negative, non-spore-forming rod-shaped bacterium and forms acid aerobically from glucose. Tests for oxidase, catalase, urease, nitrate reduction, and gelatin hydrolysis were positive, and tests for indole formation and starch hydrolysis were negative. D-Glucose, L-arabinose, maltose, and sucrose were utilized as the sole sources of carbon. According to the criteria of *Bergey's Manual of Systematic Bacteriology* (24), strain WO24 seems to belong to the genus *Pseudomonas*. Thus, this strain was tentatively identified as a *Pseudomonas* sp. and named *Pseudomonas* sp. strain WO24.

Purification of DAP. Figure 1 shows that the peak activity hydrolyzing Gly-Arg-pNA did not degrade Arg-pNA. Therefore, this result suggested the presence of DAP activity. The pooled fractions from DEAE-Cellulofine chromatography (Fig. 1) were found to cleave Gly-Arg-pNA but not Arg-pNA by HPLC analysis of the reaction products. As shown in Fig. 2, Gly-Arg and pNA peaks were detected and free amino acids were not observed after incubation for 30 min.

The active fraction was purified successively by a series of column chromatographies with Butyl-Sepharose 4B, Sephacryl S-300HR, and Q-Sepharose FF. The enzyme purification procedures are summarized in Table 1. The enzyme was purified about 400-fold, with a recovery of 16% of the activity from the cell extract. The final preparation apparently exhibited a single band in SDS-PAGE and isoelectric focusing (Fig. 3). A single band of DAP activity was also detected at the same position of the protein band in isoelectric focusing by the activity staining method with Gly-Arg-βNA as a substrate (data not shown).

Molecular mass and isoelectric point. The molecular mass of the enzyme was estimated to be 82,000 Da by SDS-PAGE on a 7.5% polyacrylamide gel (Fig. 3) and 65,000 Da by gel filtration on a Sephacryl S-300HR column using molecular mass markers (data not shown), suggesting that the native enzyme occurs as a monomer. The pI of the enzyme was

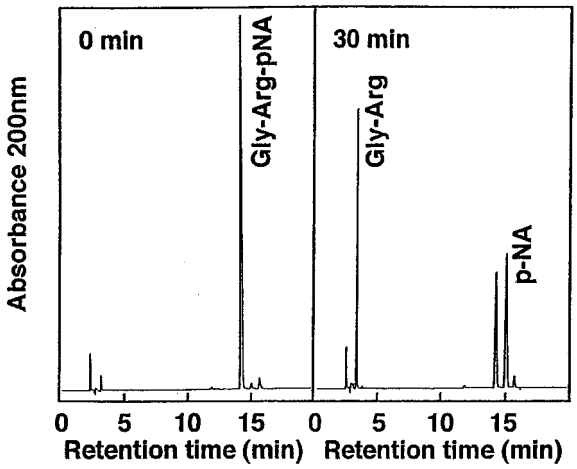

FIG. 2. Reverse-phase HPLC analysis of hydrolyzed products of Gly-Arg-pNA by the DAP from *Pseudomonas* sp. strain WO24. The reaction mixture contained 0.3 mM Gly-Arg-pNA and 1.0 μg of DAP in 1.0 ml of 50 mM Tris-HCl buffer (pH 9.0). The reaction mixture was incubated at 37°C for 30 min. HPLC analysis was carried out as described in Materials and Methods.

TABLE 2. Hydrolytic activities of the DAP on various substrates

Substrate	Sp act (U/mg)
Ala-pNA	0.0
Arg-pNA	0.0
Phe-pNA	0.0
Lys-pNA	0.0
Pro-pNA	0.0
Gly-Arg-pNA	69.3
Gly-Phe-pNA	0.0
Ala-Ala-pNA	0.0
Lys-Ala-MCA	0.0
Gly-Pro-pNA	0.0
Gly-Arg-MNA	85.3
Arg-Arg-MNA	98.1
Phe-Arg-βNA	76.8
Z-Arg-Arg-MCA	0.390
Boc-Gly-Arg-Arg-MCA	0.354
Suc-Ala-Ala-pNA	0.0
Ala-Ala-Ala-pNA	0.0
Ala-Ala-Ala	0.0
Ala-Ala-Ala-Ala	0.0

determined to be approximately 4.7 by isoelectric focusing (Fig. 3).

Enzymatic properties. The effects of temperature and pH on DAP activity were examined. The purified enzyme was stable for at least 30 min below 20°C, and its maximum activity was found to be exhibited between 35 and 40°C under the routine assay conditions (data not shown). The optimum pH for hydrolyzing Gly-Arg-pNA appeared to be 9.0 under the assay conditions, and no hydrolysis of Gly-Arg-pNA was detected below pH 5.5 or above pH 11.5. The enzyme was stable over a broad pH range of between 7.5 and 10.0 (data not shown).

The purified enzyme was incubated with pNA, βNA, MNA, and MCA derivatives of amino acids, dipeptides of free and N-terminally blocked amino acids, and oligopeptides or free-form oligopeptides. The K_m and V_{max} values of the enzyme for Gly-Arg-pNA and Gly-Arg-MNA at pH 9.0 and 37°C were 0.25 and 0.052 mM and 195 and 95 μmol/min/mg, respectively. Table 2 shows the substrate specificity of the purified DAP, which did not hydrolyze substrates of AP, DAP II (Lys-Ala-MCA), and DAP IV (Gly-Pro-pNA) or Ala-Ala-Ala or Ala-Ala-Ala-Ala. Although Gly-Arg-pNA (substrate of DAP I) was cleaved by the purified enzyme, Gly-Phe-pNA (also substrate of DAP I) was not hydrolyzed at all. Surprisingly, the enzyme cleaved Arg-Arg-MNA (K_m, 0.019 mM; V_{max}, 145 μmol/min/mg), a substrate of DAP III, but the optimum pH was similar to that for Gly-Arg-pNA. All good substrates of the enzyme caused substrate inhibition (or product inhibition) at high concentrations. Therefore, the specific activities for these substrates were lower than these V_{max} values. Furthermore, a slight enzyme activity on N-terminally blocked peptide derivatives which contain arginine residues at the P1 position was observed, showing that this enzyme possesses endopeptidase activity besides DAP activity. The substrate specificities for bioactive peptides such as angiotensins I and II and oxidized insulin B chain were also confirmed by HPLC analysis. The dipeptide fragment released by treatment of angiotensins I and II with the purified enzyme was the N-terminal one (Asp-Arg) alone. No degradation of oxidized insulin B chain, which has the hydrophobic dipeptide Phe-Val as an N terminus and an arginine residue in the internal sequence, Glu-21–Arg-22, was observed for up to 2 h of incubation.

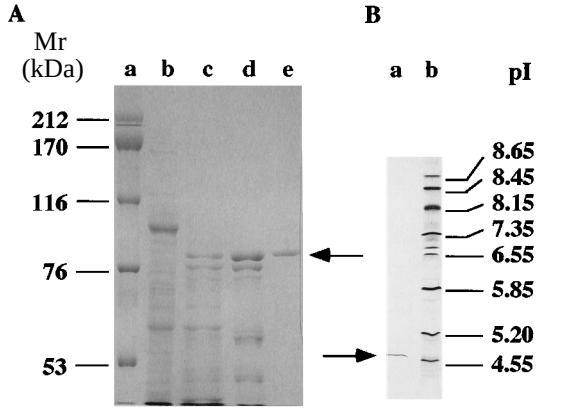

FIG. 3. SDS-PAGE of the DAP-active fractions obtained in the different purification steps and isoelectric focusing of the purified DAP. The DAP is indicated (arrows). (A) Electrophoresis was performed on an SDS–7.5% polyacrylamide gel with high-molecular-mass protein standards (see Materials and Methods). Lane a, marker; lane b, cell extract (50 μg of protein); lane c, DEAE-Cellulofine column chromatography (30 μg of protein); lane d, Butyl-Sepharose column chromatography (7.5 μg of protein); and lane e, Q-Sepharose FF column chromatography (2 μg of protein). (B) The isoelectric point of DAP was determined as described in Materials and Methods. Lane a, purified enzyme (2 μg of protein); lane b, marker. The markers are as follows: pI 8.65, lentil lectin (basic); 8.45, lentil lectin (middle); 8.15, lentil lectin (acidic); 7.35, myoglobin (basic); 6.55, human carbonic anhydrase B; 5.85, bovine carbonic anhydrase B; 5.20, β-lactoglobulin A; and 4.55, soybean trypsin inhibitor.

Effects of various chemical reagents. The influences of various agents on DAP activity on Gly-Arg-pNA as a substrate are summarized in Table 3. The enzyme activity was inhibited completely by treatment with 0.5 mM $ZnCl_2$ and inhibited considerably with 0.5 mM $CoCl_2$. $CaCl_2$ and EDTA had no effect on the enzyme activity. Little or no inhibition was seen in the presence of serine protease inhibitors (DFP, PMSF, and TPCK), whereas the enzyme activity was completely inhibited by 0.1 mM TLCK. Microbial protease inhibitors (chymostatin and leupeptin) also remarkably decreased the enzyme activity. Cysteine protease inhibitors, such as *N*-ethylmaleimide, iodoacetic acid, and E-64, had no effect on the enzyme activity. Similar results were obtained with Arg-Arg-MNA as a substrate (data not shown).

TABLE 3. Effects of inhibitors on hydrolysis of Gly-Arg-pNA by purified DAP[a]

Reagent	Concn (mM)	Relative activity (%)
None		100
$ZnCl_2$	0.5	1.3
$CoCl_2$	0.5	20.4
$CaCl_2$	5	92
EDTA	10	107
DFP	0.1	108
PMSF	1	94
TPCK	0.1	82
TLCK	0.1	1.0
Chymostatin	0.1	11
Leupeptin	0.1	1.0
N-Ethylmaleimide	10	85
Iodoacetate	1	103
E-64	0.01	104
Pepstatin A	0.01	109

[a] Purified DAP BI (0.18 μg) was preincubated with each reagent under routine assay conditions at 37°C for 15 min, and the remaining activities were assayed.

N-terminal amino acid sequencing. The N-terminal sequence of the purified enzyme was ASATPPDVAKKPHVV KAP. This sequence was used to search for homologous sequences in the PIR international protein database and showed no significant homology to any enzymes or proteins, including proteases.

DISCUSSION

The major finding of this study was that a DAP other than DAP IV was found in bacteria. Another significant finding was that the purified DAP was a novel one so far, in view of its substrate specificity and some of its enzymatic properties. Also, the results strongly suggest that the classification of DAP in microorganisms may be different from that of mammalian DAP.

In this study, we have identified, purified, and characterized an enzyme from *Pseudomonas* sp. strain WO24 which shows an activity of releasing Gly-Arg from Gly-Arg-pNA (DAP I-like activity). The purified DAP seems to have activity profiles different and distinctive from those of DAP I, on the basis of substrate specificity and inhibitors of the enzyme (15, 16). The enzyme did not hydrolyze Gly-Phe-pNA or Ala-Ala-Ala-Ala, whereas high activity was observed on Arg-Arg-MNA, the preferred substrate of DAP III. Previous reports on partially purified murine and bovine DAP I demonstrated that the enzyme cleaved Gly-Phe-βNA at 5% of the rate of Gly-Arg-βNA hydrolysis, while DAP I from the human spleen was reported to exhibit higher specific activity on Gly-Phe-MNA than on Gly-Arg-MNA (17). On the other hand, DAP III cannot hydrolyze Gly-Arg derivatives but can degrade Ala-Ala-Ala-Ala and Ala-Ala-βNA (15). With respect to these substrate specificities of DAPs, *Pseudomonas* DAP is distinct from DAPs I and III.

The DAP we have purified had an optimum activity at pH 9.0 and a molecular mass of about 82,000 or 65,000 Da as estimated by SDS-PAGE and gel filtration, respectively. The difference in the molecular masses may be due to the interaction between the enzyme molecule and the carrier for gel filtration, Sephacryl S-300HR, or the irregular form of the purified enzyme. These properties (optimum pH and molecular mass) of the DAP appeared to have some resemblance to those of mammalian and *S. cerevisiae* DAP III rather than to those of mammalian DAP I. However, the enzyme differs from DAP III with respect to inhibitor sensitivity (27). The enzyme is not inhibited by EDTA or thiol, carboxy, and general serine protease inhibitors (DFP and PMSF), but it is inhibited by TLCK, chymostatin, and leupeptin. Since the latter inhibitors affect a variety of serine and cysteine peptidases, further studies are needed to assign the DAP to one of four classes of active site structure. Furthermore, while Co(II) inhibited the DAP activity, it stimulated mammalian and yeast DAP III to hydrolyze enkephalin and Arg-Arg-βNA, respectively (27). These results show that the purified DAP from *Pseudomonas* sp. strain WO24 seems to be different from previously described DAPs I to IV in substrate specificity and some enzymatic properties. Therefore, we named this enzyme DAP BI.

Although the peptide and arylamide substrates tested in this study were quite limited, it is nonetheless reasonable to conclude that DAP BI prefers substrates composed of a basic amino acid, such as arginine, at the P1 position, because it could liberate Asp-Arg from angiotensins I and II and no further breakdown of the resulting product (data not shown) was found. Furthermore, the enzyme also has a little endopeptidase activity as well as DAP activity. Kuribayashi et al. (11) reported recently that cathepsin C (DAP I from the bovine spleen), which is primarily a DAP, can also behave as an endopeptidase. Furthermore, it has been shown that cathepsin B possesses an endopeptidase activity together with a dipeptidyl carboxypeptidase activity (26), and thermolysin, a microbial, thermostable, zinc-containing endopeptidase, shows dipeptidyl carboxypeptidase activity on certain N-blocked peptides with a free carboxyl terminal (5). These results suggest that some exopeptidases could have an endopeptidase activity or vice versa.

In unpublished work of our laboratory, two other types of DAPs which hydrolyze Gly-Phe-pNA but not Gly-Arg-pNA or Arg-Arg-MNA were purified from the same bacterial strain. These three DAPs do not fall under the classification of mammalian DAPs. Thus, another classification for bacterial DAPs is needed. At the present time, the physiological role of bacterial DAP is not clear, and further investigation is needed for its clarification. A preliminary work with a new DAP from a *Streptomyces* sp. was reported during the course of the preparation of this paper (22). From substrate specificities and inhibitor sensitivities, the authors suggested that the enzyme may be classified as mammalian DAP II. The purified DAP in this study is thus obviously different from the *Streptomyces* sp. DAP.

In order to investigate the physiological role and the molecular structure of DAP BI, we are continuing the cloning and sequencing of its gene, using oligonucleotide probes deduced from the N-terminal amino acid sequences described above.

REFERENCES

1. **Atlan, D., P. Laloi, and R. Portalier.** 1989. Isolation and characterization of aminopeptidase-deficient *Lactobacillus bulgaricus* mutants. Appl. Environ. Microbiol. **55:**1717–1723.
2. **Atlan, D., P. Laloi, and R. Portalier.** 1990. X-prolyl-dipeptidyl aminopeptidase of *Lactobacillus delbrueckii* subsp. *bulgaricus*: characterization of enzyme and isolation of deficient mutants. Appl. Environ. Microbiol. **56:**2174–2179.
3. **Bordallo, C., J. Schwencke, and M. S. Rendueles.** 1984. Localization of the thermosensitive X-prolyl dipeptidyl aminopeptidase in the vacuolar membrane of *Saccharomyces cerevisiae*. FEBS Lett. **173:**199–203.
4. **Chan, S. A. T., K. Toursarkissian, J. P. Sweeney, and T. H. D. Jones.** 1985. Dipeptidyl-aminopeptidases and aminopeptidases in *Dictyostelium discoideum*. Biochem. Biophys. Res. Commun. **127:**962–968.
5. **Fukuda, M., and S. Kunugi.** 1984. Mechanism of dipeptidyl carboxypeptidase activity of thermolysin. Bull. Chem. Soc. Jpn. **57:**2965–2970.
6. **Ganapathy, V., and F. H. Leibach.** 1983. Role of pH gradient and membrane potential in dipeptide transport in intestinal and renal brush-border membrane vesicles from the rabbit. J. Biol. Chem. **258:**14189–14192.
7. **Huang, J., J. Kim, P. Ramamurthy, and T. H. D. Jones.** 1992. The purifica-

tion, specificity, and role of dipeptidyl peptidase III in *Dictyostelium discoideum*. Exp. Mycol. **16:**102–109.
8. **Kato, T., T. Nagatsu, T. Kimura, and S. Sakakibara.** 1978. Fluorescence assay of X-prolyl dipeptidyl-aminopeptidase activity with a new fluorogenic substrate. Biochem. Med. **19:**351–359.
9. **Komagata, K.** 1985. Section 8 bacteria (I): aerobic bacteria, 99–161. *In* T. Hasegawa (ed.), Classification and identification of microorganisms (II). Gakkai Syuppan Center, Tokyo.
10. **Krutzsch, H. C., and J. J. Pisano.** 1977. Analysis of dipeptides by gas chromatography-mass spectrometry and application to sequencing with dipeptidyl aminopeptidases. Methods Enzymol. **47:**391–404.
11. **Kuribayashi, M., H. Yamada, T. Ohmori, M. Yanai, and T. Imoto.** 1993. Endopeptidase activity of cathepsin C, dipeptidyl aminopeptidase I, from bovine spleen. J. Biochem. **113:**441–449.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Lee, C. M., and S. H. Snyder.** 1982. Dipeptidyl-aminopeptidase III of rat brain. J. Biol. Chem. **257:**12043–12050.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
15. **McDonald, J. K., and C. Schwabe.** 1977. Intracellular exopeptidases, p. 311–391. *In* A. J. Barret (ed.), Proteases in mammalian cells and tissues. Elsevier Scientific Publishing Co., Amsterdam.
16. **McDonald, J. K., B. B. Zeitman, T. J. Reilly, and S. Ellis.** 1969. New observation on the substrate specificity of cathepsin C (dipeptidyl aminopeptidase I). J. Biol. Chem. **244:**2693–2709.
17. **McGuire, M. J., P. E. Lipsky, and D. L. Thiele.** 1992. Purification and characterization of dipeptidyl peptidase I from human spleen. Arch. Biochem. Biophys. **295:**280–288.
18. **McGuire, M. J., P. E. Lipsky, and D. L. Thiele.** 1993. Generation of active myeloid and lymphoid granule serine proteases requires processing by the granule thiol protease dipeptidyl peptidase I. J. Biol. Chem. **268:**2458–2467.
19. **McKain, N., R. J. Wallace, and N. D. Watt.** 1992. Selective isolation of bacteria with dipeptidyl aminopeptidase type I activity from the sheep rumen. FEMS Microbiol. Lett. **95:**169–174.
20. **Metrione, R. M., A. G. Neves, and J. S. Fruton.** 1966. Purification and properties of dipeptidyl transferase (cathepsin C). Biochemistry **9:**1597–1604.
21. **Mineyama, R., and K. Saito.** 1991. Purification and characterization of dipeptidyl peptidase IV from *Streptococcus salivarius* HHT. Microbios **68:** 37–52.
22. **Murao, S., T. Kitade, H. Oyama, and T. Shin.** 1994. Isolation and characterization of a dipeptidyl aminopeptidase from *Streptomyces* WM-23. Biosci. Biotech. Biochem. **58:**1545–1546.
23. **Nardi, M., M. C. Chopin, A. Chopin, M. M. Cals, and J. C. Gripon.** 1991. Cloning and DNA sequence analysis of an X-prolyl dipeptidyl aminopeptidase gene from *Lactococcus lactis* subsp. *lactis* NCDO 763. Appl. Environ. Microbiol. **57:**45–50.
24. **Palleroni, N. J.** 1984. Family I. *Pseudomonadaceae* Winslow, Broadhurst, Buchanan, Krumweide, Rogers and Smith 1917, 555^{AL}, p. 141–219. *I*n N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.
25. **Tachi, H., H. Ito, and E. Ichishima.** 1992. An X-prolyl dipeptidyl-aminopeptidase from *Aspergillus oryzae*. Phytochemistry **31:**3707–3709.
26. **Takahashi, T., A. H. Dehdarani, S. Yonezawa, and J. Tang.** 1986. Porcine spleen cathepsin B is an exopeptidase. J. Biol. Chem. **261:**9375–9381.
27. **Watanabe, Y., Y. Kumagai, and Y. Fujimoto.** 1990. Presence of a dipeptidyl aminopeptidase III in *Saccharomyces cerevisiae*. Chem. Pharm. Bull. **38:**246–248.
28. **Yoshimoto, T., and D. Tsuru.** 1982. Proline-specific dipeptidyl aminopeptidase from *Flavobacterium meningosepticum*. J. Biochem. **91:**1899–1906.
29. **Yoshimoto, T., R. Walter, and D. Tsuru.** 1980. Proline-specific endopeptidase from *Flavobacterium*. J. Biol. Chem. **255:**4786–4792.