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Production and Characterization of *N*-Acyl-D-Glutamate Amidohydrolase from *Pseudomonas* sp. Strain 5f-1

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***N*-Acyl-D-glutamate amidohydrolase from *Pseudomonas* sp. strain 5f-1 was inducibly produced by D isomers of *N*-acetylglutamate, glutamate, aspartate, and asparagine. The enzyme has been purified to homogeneity by DEAE-cellulose, $(NH_4)_2SO_4$ fractionation, and chromatofocusing followed by gel filtration on a Sephadex G-100 column. The enzyme was a monomer with molecular weight of 55,000. The enzyme activity was optimal at pH 6.5 to 7.5 and 45°C. The isoelectric point and the pH stability were 8.8 and 9.0, respectively. *N*-Formyl, *N*-acetyl, *N*-butyryl, *N*-propionyl, *N*-chloroacetyl derivatives of D-glutamate and glycyl-D-glutamate were substrates for the enzyme. At pH 6.5 in 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer at 30°C, a K_m of 6.67 mM and a V_{max} of 662 μmol/min/mg of protein for *N*-acetyl-D-glutamate were obtained. None of the metal ions stimulated the enzyme activity. Na^+, K^+, Mg^{2+}, and Ba^{2+} acted as stabilizers. Hg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, and EDTA were strongly inhibitory.**

D-Aminoacylase (*N*-acyl-D-amino acid amidohydrolase; EC 3.5.1) catalyzes the hydrolysis of *N*-acyl derivatives of various neutral D-amino acids. The enzyme has been characterized from *Pseudomonas* spp. (11, 12, 14), *Streptomyces* spp. (26, 27), and *Alcaligenes* spp. (19, 24, 29). The physiological role of the enzyme is still unknown, but the enzyme is of importance in producing neutral D-amino acids through the optical resolution of neutral DL-amino acids.

D-Amino acids are known to occur in bacterial cell walls, peptide antibiotics, and higher plants (4). A protein containing D-aspartic acid (Asp) has been shown to accumulate with age in human tooth enamel, eye lens, and brain (8, 9, 18). There has been increasing interest in the physiological functions of D-amino acids, many of which are produced as industrial materials of semisynthetic antibiotics and other medical compounds.

Recently, we have discovered novel bacterial enzymes which act on *N*-acyl derivatives of acidic D-amino acids (19, 25). This is the first report describing the production and characterization of *N*-acyl-D-glutamate (Glu) amidohydrolase from *Pseudomonas* sp. strain 5f-1.

MATERIALS AND METHODS

Cultivation. *Pseudomonas* sp. strain 5f-1 was grown on a medium containing 1.0% carbon source, 1.0% nitrogen source, 0 to 1.5% inducer, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.01% yeast extract (pH 7.0). The culture was grown at 30°C with shaking in a 500-ml Sakaguchi flask containing 100 ml of medium or in a 30-liter jar fermentor containing 20 liters of medium.

Assay of *N*-acyl-D-Glu amidohydrolase. The enzyme activity was determined at 30°C by using a standard reaction mixture containing 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 6.5), 10 mM *N*-acyl-D-Glu, 0.4 mM $MgCl_2$, and enzyme. D-Glu liberated was analyzed by high-performance liquid chromatography or by its reaction with 2,4,6-trinitrobenzenesulfonic acid (5). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of D-Glu per min. Specific activity is expressed as units per milligram of protein. Protein was estimated by the method of Lowry et al. (17), with crystalline egg albumin as the standard.

Purification of the enzyme. All steps were done at 0 to 5°C.

(i) Preparation of cell extract. The cells (26.3 g [wet weight]) were suspended in 131 ml of 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM $MgCl_2$ (KP_i buffer) and disrupted by ultrasonic oscillation (19 kHz) by pulsing for 3 min and cooling for 5 min. The total sonication time was 30 min. The supernatant obtained by centrifugation was dialyzed against 10 mM KP_i buffer.

(ii) DEAE-cellulose column chromatography. The dialysate was applied to a column of DEAE-cellulose (4 by 20 cm) equilibrated with 10 mM KP_i buffer. The active enzyme was eluted with 10 mM KP_i buffer.

(iii) Ammonium sulfate fractionation. The eluate was fractionated with ammonium sulfate (20 to 80% saturation) in the presence of 10% glycerol and 0.1% 2-mercaptoethanol. The precipitate was dissolved in a small volume of 25 mM ethanolamine-HCl buffer (pH 10.0) and dialyzed against the same buffer.

(iv) Chromatofocusing. The dialysate was applied to a chromatofocusing (PBE 94; Pharmacia LKB Biotechnology, Uppsala, Sweden) column (0.8 by 41 cm) which was equilibrated with 25 mM ethanolamine-HCl buffer (pH 10.0). The active enzyme was eluted with the equilibration buffer.

(v) First Sephadex G-100 column chromatography. A Sephadex G-100 (Pharmacia LKB Biotechnology) column (2.2 by 130 cm) was equilibrated with 10 mM KP_i buffer. The enzyme (3.2 ml) was applied to the column, which was then eluted with 10 mM KP_i buffer. The active fractions were pooled and concentrated in an Amicon filtration unit with a YM-10 membrane (Amicon Corp., Lexington, Mass.).

(vi) Second Sephadex G-100 column chromatography. To remove the inpurity proteins, the concentrate (1.0 ml) was applied to a second Sephadex G-100 column (1.0 by 128 cm) which had been equilibrated; the column was eluted as described above. The major peak of activity was concentrated on a Diaflow apparatus with a YM 5 membrane.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (15) with gels containing 12.5% acrylamide. Phos-

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TABLE 1. Effect of the nitrogen and carbon sources on the production of N-Ac-D-Glu amidohydrolase from *Pseudomonas* sp. strain 5f-1[a]

Carbon source (1%)	Nitrogen source (1%)	Cell yield (mg [wet wt])	Sp act (U/mg of protein)	Total activity (U)
None	$(NH_4)_2SO_4$	0.263	4.0	64
None	Peptone	0.732	2.2	113
None	L-Glutamate	0.630	0.14	6.0
None	Casamino Acids	0.717	1.2	68
None	Yeast extract	0.799	0.67	40
Glucose	$(NH_4)_2SO_4$	0.320	3.0	61
Glucose	Peptone	0.633	1.8	81
Glucose	L-Glutamate	0.625	0.21	8.7
Glucose	Casamino Acids	0.589	0.57	24
Glucose	Yeast extract	0.750	0.50	30
Glycerol	$(NH_4)_2SO_4$	0.465	2.6	57
Glycerol	Peptone	0.837	2.2	94
Fructose	$(NH_4)_2SO_4$	0.309	1.9	32
Fructose	Peptone	0.755	1.6	76
Citric acid	$(NH_4)_2SO_4$	0.543	1.4	26
Citric acid	Peptone	0.652	0.9	36

[a] Cells were grown in 100 ml of medium at 30°C for 22 h. DL-Asp (0.3%) was used as an inducer.

phorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lysozyme (14,400) (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) were used as molecular weight markers.

Determination of molecular weight. The method of Andrews (1) was used to estimate the molecular weight by the gel filtration method on a calibrated Sephadex G-150 column (1.6 by 119.5 cm) with bovine serum albumin (68,000), egg albumin (45,000), chymotrypsinogen A (25,000), and cytochrome *c* (12,500) (Boehringer GmbH, Mannheim, Federal Republic of Germany).

Chemicals. Various *N*-acyl derivatives of D-Glu were synthesized as described in the literature (2, 6, 20, 21, 28). All chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Characteristics of *Pseudomonas* sp. strain 5f-1. Strain 5f-1, which was isolated from soil, closely resembles *Pseudomonas pickettii* biovar I, except that generally reduces nitrate and grows at 41°C. Therefore, this isolate was identified as *Pseudomonas* sp. strain 5f-1.

Production of the enzyme. The effect of various inducers on production of the enzyme was examined. The enzyme was inducibly produced by D-isomers of *N*-acetyl (Ac)-Glu, Glu, Asp, and asparagine (Asn), but not by *N*-Ac-D- (or L-) Asp, *N*-Ac-L-Glu, L-Asp, or L-Asn. The enzyme was also produced in the absence of inducer. The two active peaks (pI 8.8 and 8.2) of the enzyme were eluted with a chromatofocusing column of the cell extract. Table 1 shows the effect of the carbon and nitrogen sources on the production of the enzyme. The highest specific activity was obtained when DL-Asp was used as the carbon source and inducer and with $(NH_4)_2SO_4$ as the nitrogen source. Enzyme induction by various concentrations (0.1 to 1.5%) of DL-Asp is shown in Fig. 1. Increasing the concentrations of DL-Asp resulted in inhibition of growth and a decrease in the total units of activity, although the specific activity was constant.

Purification of the enzyme. Purification of the enzyme with

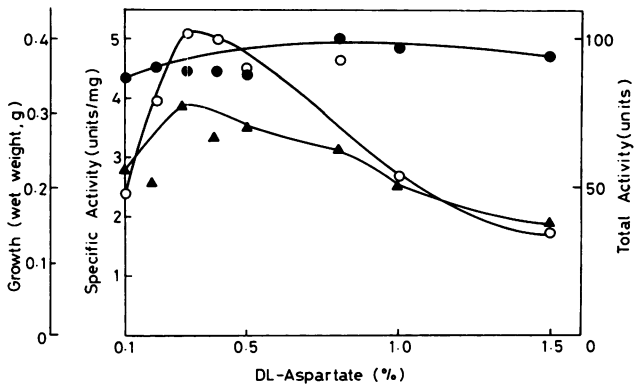

FIG. 1. Effect of DL-aspartate concentration on the production of the enzyme. The cells were grown in 100 ml of medium containing DL-Asp (carbon and inducer sources) and $(NH_4)_2SO_4$ (nitrogen source) for 22 h. Symbols: ●, specific activity; ○, total activity; ▲, growth.

pI 8.8 from *Pseudomonas* sp. strain 5f-1 is summarized in Table 2. The enzyme was purified about 214-fold in a yield of 16.2% and was found to be homogeneous as judged by one band on SDS-PAGE (Fig. 2).

Molecular weight determinations. The apparent molecular weight of the native enzyme was 44,000 as determined by gel filtration. On SDS-PAGE, an estimated molecular weight of 55,000 was obtained.

Substrate specificity and kinetic parameters. *N*-Ac-D-Glu, *N*-butyryl-D-Glu, *N*-propionyl-D-Glu, *N*-chloroacetyl-D-Glu, and glycyl-D-Glu, each at 10 mM, were about 58, 21, 16, 7.5, and 0.79% as active, respectively, as *N*-formyl-D-Glu. *N*-Ac-L-Glu and *N*-Ac-D- or *N*-Ac-L- derivatives of Asp, alanine, phenylalanine, valine, tryptophan, and methionine were inert as substrates. The enzyme had an apparent K_m of 6.67 mM for *N*-Ac-D-Glu, with a V_{max} of 662 μmol/min/mg of protein when assayed at pH 6.5 in 100 mM HEPES buffer at 30°C.

Other properties. The enzyme was extremely labile at around neutral pH, but it was stable on storage as a suspension in 10 mM KP_i buffer containing 80% saturated ammonium sulfate, 10% glycerol, and 0.1% 2-mercaptoethanol. The rapid loss of activity after treatment at pH 7 and 30°C for 120 min was completely prevented by the addition of NaCl, KCl, $MgCl_2$, or glycerol. The presence of 10 mM $MgCl_2$ protected the enzyme against inactivation during extraction from the cells by sonication at 0 to 4°C. The thermostability of the enzyme was also affected by $MgCl_2$ (Fig. 3). Thus, half of the enzyme activity was lost within 10 min at 48°C in the presence of $MgCl_2$ and at 41°C in its absence, respectively. The stability of the enzyme as a function of pH was determined by incubating the enzyme for 10 min at 45°C in 66 mM buffer at pHs ranging from 4.5 to 11.0 (pH 4.5 to 6.0 for acetate buffer, pH 6.0 to 8.5 for HEPES buffer, pH 8.0 to 9.0 for barbital buffer, pH 9.0 to 11.0 for carbonate-bicarbonate buffer). The enzyme was quite stable at pH 9.0 to 9.5, regardless of the presence or absence of $MgCl_2$. The enzyme activity was optimal between pH 6.5 and 7.5 in HEPES buffer and at 45°C. Stimulation of the enzyme activity by metal ions (10 mM) was not observed. The enzyme activity was completely inhibited by Hg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, and EDTA, each at 10 mM. D-Glu (2 mM), D-Asp (2 mM), *N*-Ac-L-Glu (10 mM), *N*-Ac-D- or *N*-Ac-L-Asp (10 mM), and *N*-Ac derivatives of neutral D- or

TABLE 2. Purification of *N*-Ac-D-Glu amidohydrolase from *Pseudomonas* sp. strain 5f-1[a]

Step	Amt of protein (mg)	Sp act (U/mg of protein)	Total activity (U)	Yield (%)	Purification (fold)
Cell extract	4,194	2.75	11,525	100	1
DEAE-cellulose	1,140	9.97	11,376	98	3.6
20–80% $(NH_4)_2SO_4$ fractionation	530	7.87	4,171	36	2.8
Chromatofocusing	24.3	132	3,208	27	48
1st Sephadex G-100	4.57	574	2,628	22	209
2nd Sephadex G-100	3.17	589	1,867	16	214

[a] From 26.3 g (wet weight) of cells.

L-amino acids (10 mM) did not affect the enzyme activity. It was, however, 34% inhibited by 100 mM acetate.

DISCUSSION

In this report we present data on the biochemical characterization of a novel *N*-acyl-D-Glu amidohydrolase from *Pseudomonas* sp. strain 5f-1. The enzyme did not hydrolyze *N*-acyl derivatives of neutral D-amino acids or D-Asp and was specific for *N*-acyl-D-Glu. The enzyme was designated as *N*-acyl-D-Glu amidohydrolase (EC 3.5.1).

Until now, the enzymes hydrolyzing *N*-acyl-L-Glu have been found in *Pseudomonas* spp. (3, 7, 10, 16) and *Streptomyces coelicolor* (13). One of them, *N*-formyl-L-Glu amidohydrolase, catalyzes the terminal reaction in the five-step pathway of histidine metabolism (3, 10, 13, 16) and is induced by *N*-formyl-L-Glu and urocanate, the first product in the histidine pathway (10). The other, *N*-Ac-L-Glu deacetylase, is believed to be involved in arginine biosynthesis (7) and is induced by *N*-Ac-L-Glu and L-Glu, but not by L-ornithine, an intermediate in arginine biosynthesis (7). However, the role of *N*-acyl-D-Glu amidohydrolase remains unknown. This enzyme was induced by *N*-Ac-D-Glu, D-Glu, D-Asp, and D-Asn and was different in inducer specificities from those of *P. putida* and *P. aeruginosa*.

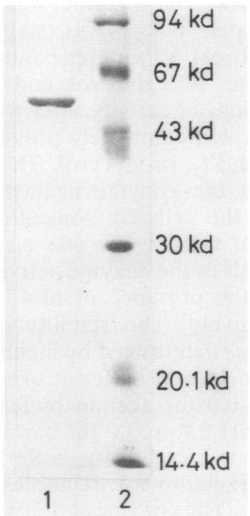

FIG. 2. SDS-PAGE analysis of purified enzyme. Lane 1 contains purified enzyme (10 μg of protein). Lane 2 contains marker proteins: phosphorylase *b* (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100), and lysozyme (M_r 14,400). Protein was stained with Coomassie brilliant blue. kd, Kilodaltons.

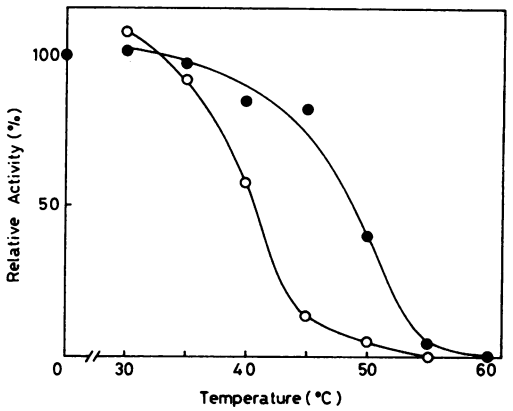

FIG. 3. Effect of $MgCl_2$ on the thermostability of the enzyme. The enzyme was incubated in 10 mM potassium phosphate buffer (pH 7.0) in the presence (●) or absence (○) of 10 mM $MgCl_2$ at various temperatures for 10 min. The residual activity was measured.

The *Pseudomonas* sp. strain 5f-1 enzyme was D-specific and active with various *N*-acyl-D-Glu derivatives and the dipeptide glycyl-D-Glu. The enzyme of *P. putida* was specific for *N*-formyl-L-Glu (10). The *P. aeruginosa* enzyme hydrolyzed various *N*-acyl-L-Glu and *N*-acetylated intermediates of arginine biosynthesis, but neither *N*-acyl-D-amino acids nor dipeptides were hydrolyzed (7). Both enzyme activities of *P. aeruginosa* and *P. putida* depended on Co^{2+} (7, 10). In contrast, the *Pseudomonas* sp. strain 5f-1 enzyme was not stimulated by metal ions, which positively affected its stability. We have no explanation for the role of metal ions in enhancing the stability of this enzyme.

In conclusion, the data presented here suggest that *N*-acyl-D-Glu amidohydrolase is clearly different from *N*-formyl-L-Glu amidohydrolase and *N*-acetyl-L-Glu amidohydrolase.

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