Purification and Characterization of 5-Oxo-L-prolinase (L-Pyroglutamate Hydrolase) from *Alcaligenes* sp. F-137

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5-Oxo-L-prolinase (L-pyroglutamate hydrolase, EC 3.5.2.9) from *Alcaligenes* sp. F-137 has been purified to homogeneity as judged by acrylamide gel electrophoresis and ultracentrifugation. The molecular weight of the enzyme was about 106,000 and 123,000 by gel filtration and sedimentation equilibrium, respectively. Upon disc electrophoresis in 0.1% sodium dodecyl sulfate the purified preparation migrates as a single band of molecular weight 126,000. The sedimentation coefficient ($s_{20,w}^0$) of the enzyme was 6.82S by ultracentrifugation and its isoelectric point was pH 5.1 by isoelectric focusing. The enzyme catalyzed the hydrolysis of 5-oxo-L-proline to glutamate coupled with the hydrolysis of ATP to ADP and inorganic phosphate, stoichiometrically. K^+ and Mg^{2+} were required for the enzyme reaction. Michaelis constants of the enzyme were 0.07 mM for 5-oxo-L-proline and 0.32 mM for ATP. The enzyme was maximally active at pH 7.8 and 50°C. The enzyme was inhibited by *p*-chloromercuribenzoate of *N*-ethylmaleimide.

5-Oxo-L-prolinase (L-pyroglutamate hydrolase, EC 3.5.2.9) catalyzes the hydrolysis of 5-oxo-L-proline (L-pyroglutamate; L-2-pyrrolidone-5-carboxylate) to glutamate coupled with the hydrolysis of ATP to ADP and inorganic phosphate (Pi):

The enzyme has been found in animal tissues,^{1~4)} plants,⁵⁾ and microorganisms.^{6~8)} 5-Oxo-L-prolinase from rat kidney was purified about 1700-fold by Williamson and Meister.⁹⁾ The enzyme has a molecular weight of 325,000 and is composed of two apparently identical subunits. The enzyme catalyzes the glutamate synthesis coupled with ATP hydrolysis to ADP and Pi. The microbial 5-oxo-L-prolinase from *Pseudomonas putida* is composed of two protein components (A and B).⁸⁾ Neither component alone can catalyze the 5-oxo-L-prolinase reaction, but the reaction is effectively catalyzed when both components are

mixed. Component A and Component B have molecular weights of about 750,000 and 650,000, respectively. Thus, the reaction catalyzed by 5-oxo-L-prolinase is of particular interest in that the cleavage of the internal peptide bond of 5-oxo-L-proline is coupled with the cleavage of ATP.

Recently, the author found an 5-oxo-L-prolinase from a strain of soil bacteria, *Alcaligenes* sp. F-137. This bacterial enzyme had different properties from those of rat kidney and *Pseudomonas* 5-oxo-L-prolinases. The purification and characterization of the enzyme from *Alcaligenes* sp. F-137 are described here.

MATERIALS AND METHODS

Materials. The following were obtained from Boehringer Mannheim-Yamanouchi, Tokyo: ATP, ADP, β -NAD, β -NADH, glutamate dehydrogenase, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate tri-cyclohexylammonium salt, and molecular weight standards. Dithiothreitol and 5-oxo-L-proline (L-pyroglutamate; L-2-pyrrolidone-5-carboxylate) were purchased from the Sigma Chemical Co., St. Louis. Sephadex

G-200, Sepharose 6B, DEAE-Sepharose CL-6B and AH-Sepharose 4B were obtained from Pharmacia, Uppsala. 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride was purchased from Nakarai Chemicals, Ltd., Kyoto. Ampholine pH 3.5~10.0 was obtained from LKB Produkter, Bromma, Sweden. All other chemicals were of spectral or analytical grade.

Microorganism and cultivation. The bacterium, *Alcaligenes* sp. F-137, was isolated from soil by an enrichment-culture technique with a medium containing 5-oxo-L-proline, and was taxonomically characterized as a strain of genus *Alcaligenes* according to "Bergey's Manual."¹⁰ Cells were grown aerobically for 20 hr at 30°C in a medium (pH 5.6) containing 1% 5-oxo-L-proline, 0.2% yeast extract, 0.1% KH_2PO_4 , 0.1% K_2HPO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Cells were harvested by centrifugation, washed twice with 10 mM Tris-HCl buffer, pH 7.2, containing 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and stored at -15°C .

Enzyme assay. The standard reaction mixture for the assay of 5-oxo-L-prolinase activity contained 10 μmol of ATP, 5 μmol of magnesium chloride, 80 μmol of potassium chloride, 100 μmol of Tris-HCl buffer, pH 7.8, and the enzyme in a total volume of 1.0 ml. The reaction was started by the addition of 20 μmol of 5-oxo-L-proline. The reaction mixture was incubated at 37°C for 10~60 min and stopped by boiling in a water bath for two min. After removal of precipitated protein by centrifugation, the supernatant was then used for a measurement of the product. Glutamate formation was mostly measured by the method of Kikuchi *et al.*¹¹ using glutamate dehydrogenase and β -NAD. The ninhydrin method of Rosen¹² was also used for glutamate measurement. Inorganic phosphate was measured by the method of Fiske and Subbarow.¹³ Nucleoside triphosphatase activity was measured by following nucleoside diphosphate formation continuously using a pyruvate kinase-lactate dehydrogenase coupled assay.⁹ One unit of the enzyme was defined as the amount of enzyme required to form 1 μmol of L-glutamate from 5-oxo-L-proline per min.

Protein measurement. Protein was measured by the method of Lowry *et al.*¹⁴ with bovine serum albumin as a standard. Elution patterns of protein upon column chromatography were monitored by the measurement of absorption at 280 nm.

Preparation of 5-oxo-L-proline-AH-Sepharose 4B. AH-Sepharose 4B was coupled with 5-oxo-L-proline in the presence of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide by the conventional method of Cuatrecasas *et al.*¹⁵

Polyacrylamide gel electrophoresis. Analytical electrophoresis on polyacrylamide gel was done at 4°C by the

method of Davis.¹⁶ Gels were stained for protein with Coomassie brilliant blue and destained in 12.5% trichloroacetic acid.

Ultracentrifugal analysis. All sedimentation experiments were done in a Hitachi UCA-1A analytical ultracentrifuge (Hitachi, Ltd., Tokyo). Sedimentation coefficients were corrected to standard conditions and extrapolated to infinite dilution as described by Schachman.¹⁷ Diffusion coefficient measurement was done at 10490 rpm with a capillary-type synthetic boundary cell as described by Kawahara.¹⁸ The molecular weight of the native enzyme was calculated by the meniscus depletion sedimentation equilibrium method of Yphantis.¹⁹ The partial specific volume of 0.75 ml/g was used in this experiment.¹⁷

Molecular weight measurements. The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-200 column (2.3 $\times$ 90 cm) at 4°C by the method of Andrews.²⁰ The column was standardized with catalase, aldolase, bovine serum albumin, and ovalbumin. The molecular weight of the subunit of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Weber and Osborn.²¹ Electrophoresis was done using a 5% polyacrylamide gel column containing 0.1% sodium dodecyl sulfate. After electrophoresis for 4 hr at 8 mA per gel at room temperature, the gel was stained and then destained as described in "Polyacrylamide gel electrophoresis."

Other analytical methods. Absorption spectra were measured with a Hitachi 323 recording spectrophotometer (Hitachi, Ltd., Tokyo) at 25°C. Isoelectric focusing of the enzyme in polyacrylamide gel was done by the method of Righetti and Drysdale²² with carrier ampholytes from pH 3.5 to 10.0 at 4°C.

RESULTS

Purification of 5-oxo-L-prolinase

All the procedures for isolation and purification of the enzyme were done at 0~8°C. Washed cells (320 g as wet weight) were suspended in 10 l of 10 mM Tris-HCl buffer, pH 7.8, containing 1 mM dithiothreitol (buffer T) and disrupted by a Dyno-mill KDL (Willy A. Bachofen Maschinefabrik, Basel). The suspension of disrupted cells was centrifuged at 8,000 rpm for 60 min and solid ammonium sulfate was added slowly to the cell-free supernatant solution to give a final concentration of 50% saturation. After 60 min, the precipitate was collected by centrifugation, resuspended in a minimal volume of buffer T, and then

dialyzed against the same buffer overnight. The dialysate was put on a DEAE-cellulose column (15×40 cm) previously equilibrated with buffer T. The column was washed with buffer T containing 0.05 M NaCl and then eluted with a linear gradient of NaCl from 0.05 M to 0.4 M in buffer T. Active fractions were concentrated by the addition of ammonium sulfate (50% saturation). The concentrated preparation was put on a column (2.6×100 cm) of Sepharose 6B previously equilibrated with buffer T containing 0.05 M NaCl (buffer S). Gel filtration was done with the same buffer at a flow rate of 35 ml/hr. The active fractions were put on a DEAE-Sepharose CL-6B column (3.6×20 cm) and eluted with a linear gradient of NaCl from 0.1 M to 0.4 M in buffer T. Active fractions were concentrated, dialyzed against to buffer T, and put on a column (2.6×10 cm) of 5-oxo-L-proline-AH-Sepharose 4B equilibrated with buffer T. The column was eluted with a linear gradient of NaCl from 0.05 M to 0.3 M in buffer T. The concentrated active fraction was put on a column (2.6×100 cm) of Sephadex G-200 previously equilibrated with buffer S. The second gel filtration was done at a flow rate of 20 ml/hr. The overall purification achieved was approximately 181-fold with a yield of 2.2%. The purified preparation catalyzed the formation of 3.8 μ mol of glutamate/min/mg of protein under the standard assay conditions. The results of the enzyme purification are summarized in Table I. Ultracentrifugation of the purified enzyme showed only a single symmetrical peak (Fig. 1A). Disc electrophoresis in acrylamide gel also showed a single band of protein (Fig. 1B).

Molecular weight and other physicochemical properties

The molecular weight of *Alcaligenes* 5-oxo-L-prolinase was about 106,000 by Sephadex G-200 gel filtration (Fig. 2). The subunit structure was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which showed a single band of stained protein. The molecular weight of the protein was about

TABLE I. PURIFICATION OF 5-Oxo-L-PROLINASE

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	38,000	800	0.021	100
Ammonium sulfate	17,300	656	0.038	82
DEAE-cellulose	1,782	392	0.22	49
Sepharose 6B	891	312	0.35	39
DEAE-Sepharose CL-6B	182	144	0.79	18
5-Oxo-L-proline-AH-Sepharose 4B	18.8	46.4	2.47	5.8
Sephadex G-200	4.6	17.6	3.80	2.2

FIG. 1. Ultracentrifugal Schlieren Diagram and Polyacrylamide Gel Electrophoresis of Purified *Alcaligenes* 5-Oxo-L-prolinase.

A: Centrifugal analysis was done 55,430 rpm at 20°C. The photograph was taken 40 min after reaching full speed. B: About 12 μ g of the purified enzyme was put on the column. The direction of migration is from the cathode (top) to the anode (bottom). An arrow represents the position of the tracking dye.

126,000. These results suggested that the enzyme was a single polypeptide chain protein. Upon ultracentrifugal analysis, the sedimentation coefficient ($s_{20,w}^0$) at 20°C was calculated to be 6.82 s by extrapolating the protein concentration to zero. The Stokes' radius and the diffusion coefficient ($d_{20,w}^0$) were 41 Å and 4.78×10^{-7} cm²/sec, respectively. The molecular weight of the enzyme was also calculated to be $123,000 \pm 2,300$ by sedimentation equilibrium studies. The isoelectric point of the 5-oxo-L-prolinase was pH 5.1 by isoelectric focusing.

FIG. 2. Estimation of Molecular Weight by Gel Filtration.

Standard proteins; a, catalase; b, aldolase; c, bovine serum albumin; d, ovalbumin. Sample; ●, 5-oxo-L-prolinase.

Enzyme reaction and kinetics

Table II shows the formations of glutamate, ADP, and inorganic phosphate (Pi) from 5-oxo-L-proline and ATP. One mole each of 5-oxo-L-proline and ATP were converted to 1 mol each of glutamate, ADP, and Pi. These results showed that the enzyme catalyzed the hydrolysis of 5-oxo-L-proline to glutamate coupled with the hydrolysis of ATP to ADP and Pi. However, in the absence of 5-oxo-L-proline under the same conditions, ADP and Pi formations from ATP were not observed and, in the absence of ATP, no activity was also found with 5-oxo-L-proline either. The reaction rates of glutamate formation were measured at various substrate concentrations under the standard conditions. The plots of the reaction velocity against 5-oxo-L-proline or ATP concentration gave a normal Michaelis-Menten curve. Michaelis constants of the enzyme were 0.07 mM for 5-oxo-L-proline and 0.32 mM for ATP.

Effects of monovalent and divalent cations

The effects of various monovalent and divalent cations on the *Alcaligenes* 5-oxo-L-prolinase reaction were examined under the standard assay conditions in the absence of K^+ (80 mM) or Mg^{2+} (5 mM) (Tables III and IV). K^+ (80 mM) was essential for maximal

TABLE II. REACTION OF *Alcaligenes* 5-OXO-L-PROLINASE

The reaction mixture (1.0 ml) contained 5 μ mol $MgCl_2$, 80 μ mol KCl, 100 μ mol Tris-HCl buffer (pH 7.8), 5 μ mol ATP, 0.2 μ mol and 0.4 μ mol 5-oxo-L-proline and the enzyme. The reaction mixture was incubated at 37°C for 20 min and then used for a measurement of products as described in MATERIALS AND METHODS. In Experiments 3 and 4, 5-oxo-L-proline and ATP were omitted respectively.

Exp.	Reaction conditions	Product formed		
		Glutamate	ADP	Pi
			μ mol	
1	5-Oxo-L-proline (0.2 μ mol) + ATP	0.214	0.198	0.211
2	5-Oxo-L-proline (0.4 μ mol) + ATP	0.418	0.421	0.406
3	ATP	—	0	0.005
4	5-Oxo-L-proline (0.2 μ mol)	0	—	—

TABLE III. EFFECTS OF MONOVALENT CATIONS

The reaction mixture (1.0 ml) contained: 100 μ mol Tris-HCl buffer (pH 7.8), 5 μ mol $MgCl_2$, 10 μ mol ATP, 20 μ mol 5-oxo-L-proline, the enzyme, and the monovalent cation listed. After incubation for 10 min at 37°C, portions of each reaction mixture were analyzed for glutamate. The activities are expressed as percentages of the activity obtained under the standard assay conditions.

Monovalent cation	Concentration (mM)	Relative activity (%)
KCl	80	100
	40	72
	5	32
NH_4Cl	40	41
	5	72
RbCl	40	56
	5	49
CsCl	40	16
	5	16
LiCl	40	0
	5	0
NaCl	40	0
	5	0
None	—	0

activity of the enzyme. NH_4^+ , Rb^+ , and Cs^+ also promoted the activity. Na^+ and Li^+ , however, could not replace K^+ . Among divalent cations, Mg^{2+} , Mn^{2+} , and Co^{2+} could

TABLE IV. EFFECTS OF DIVALENT CATIONS

The reaction mixture (1.0 ml) contained: 100 μ mol Tris-HCl buffer (pH 7.8), 80 μ mol KCl, 10 μ mol ATP, 20 μ mol 5-oxo-L-proline, the divalent cation listed, and the enzyme dialyzed against metal free 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM dithiothreitol. Glutamate formation was measured by the glutamate dehydrogenase method and the ninhydrin method.

Divalent cation (1 mM)	Relative activity (%)
MgSO ₄	100
CoSO ₄	98
MnSO ₄	75
Ca(CH ₃ COO) ₂	61
FeSO ₄	26
NiSO ₄	24
CuSO ₄	0
ZnSO ₄	0
None	0

FIG. 3. Effects of pH on 5-Oxo-L-prolinase Activity.

Enzyme activities were assayed under the standard conditions with 100 mM buffer as follows: Δ , acetate; $\bullet$, Tris-maleate; $\circ$, Tris-HCl.

satisfy the requirement of the enzyme. Mg^{2+} was most effective. No other common divalent cations tested could be substitutes.

Effects of pH and temperature

The pH dependence for 5-oxo-L-prolinase was observed in various buffers (Fig. 3). The pH optimum of the enzyme was around pH 7.8. 5-Oxo-L-prolinase activity was assayed at

FIG. 4. Effects of Temperature on 5-Oxo-L-prolinase Activity.

Enzyme activities were measured at various temperatures under the standard assay conditions.

TABLE V. EFFECTS OF INHIBITORS ON 5-Oxo-L-PROLINASE ACTIVITY

The enzyme activity was measured under the standard assay conditions after an incubation period of 10 min at 37°C with a reagent. Glutamate formation was measured by the glutamate dehydrogenase method and the ninhydrin method.

Reagent	Concentration (mM)	Relative activity (%)
None	—	100
EDTA	10	13
<i>o</i> -Phenanthroline	10	0
<i>N</i> -Bromosuccinimide	1	0
K ₂ S ₂ O ₈	1	70
SDS	1	3
Iodoacetamide	1	87
<i>N</i> -Ethylmaleimide	1	0
<i>p</i> -Chloromercuribenzoate	0.1	0

various temperatures (Fig. 4). The optimum temperature was about 50°C under the standard assay conditions.

Effects of inhibitors

Various reagents were investigated for possible inhibitory effects on the enzyme activity as shown in Table V. *N*-Bromosuccinimide strongly inhibited the enzyme activity. Metal

binding reagents and sodium dodecyl sulfate significantly inactivated the enzyme. When the enzyme was treated with *p*-chloromercuribenzoate and *N*-ethylmaleimide, 100% loss of the activity was observed. The loss of activity caused by the treatment with *p*-chloromercuribenzoate (0.1 mM) was almost restored by subsequent treatment with excess dithiothreitol (1 mM).

DISCUSSION

A 5-oxo-L-prolinase has been isolated from *Alcaligenes* sp. F-137 and purified to apparent homogeneity. The molecular weight of the enzyme was 106,000, 123,000 and 126,000 by gel filtration, sedimentation equilibrium centrifugation, and SDS-polyacrylamide gel electrophoresis, respectively. Subunit structure was not observed and various physicochemical properties were examined. Wendel and Flugge^{23,24} reported the molecular weight of 5-oxo-L-prolinase from rat kidney to be 460,000 and concluded that the enzyme consists of four subunits ($M_r = 115,000$). Williamson and Meister⁹ showed that homogeneous rat kidney enzyme has the molecular weight of 325,000 and that it is composed of two identical subunits. Bacterial 5-oxo-L-prolinase from *Pseudomonas putida* was found to be composed of two functionally different proteins (Components A and B), neither of which alone can catalyze the conversion of 5-oxo-L-proline to glutamate.⁸ Component A, which catalyzes the 5-oxo-L-proline dependent ATPase reaction, is composed of two dissimilar polypeptide chains (molecular weights of 51,000 and 64,000) implying a minimum molecular weight 115,000. The molecular weight of Component A under native conditions is estimated to be 750,000, suggesting that Component A is composed of six heterodimers. Component B, when added to Component A, couples hydrolysis of ATP with that of the internal peptide of 5-oxo-L-proline to yield glutamate. It has a molecular weight of 650,000 and consists of eight identical subunits with a molecular weight of 82,000.⁸ Thus, the native

molecular weight and subunit structure of *Alcaligenes* 5-oxo-L-prolinase was apparently different from those of the enzymes from rat kidney and *Pseudomonas*. Furthermore, *Alcaligenes* enzyme did not show the presence of two functionally different proteins, which were observed in *Pseudomonas* 5-oxo-L-prolinase.

Our studies showed that *Alcaligenes* 5-oxo-L-prolinase catalyzed the cleavage of 5-oxo-L-proline to glutamate coupled with the cleavage of ATP to ADP and Pi. This preparation did not have ATPase activity in the absence of 5-oxo-L-proline. Van Der Welf *et al.*²⁵ and Griffith and Meister²⁶ reported that purified rat kidney 5-oxo-L-prolinase has ATPase activity in the absence of 5-oxo-L-proline. However, the rat kidney enzyme highly purified by Williamson and Meister⁹ has much less ATPase. Component A of *Pseudomonas* 5-oxo-L-prolinase has no ATPase activity alone but has full ATPase activity when 5-oxo-L-proline is added.⁸ The Michaelis constants of *Alcaligenes* 5-oxo-L-prolinase were 0.07 mM for 5-oxo-L-proline and 0.32 mM for ATP, and the optimum pH of the enzyme was 7.8, but the rat kidney enzyme has a *K_m* value for 5-oxo-L-proline of 8 μ M and that for ATP of 0.17 mM, and is maximally active at pH 8.2.²⁵

Rat kidney 5-oxo-L-prolinase has a number of sulfhydryl groups, at least one of which is required for ATP cleavage.⁹ Component A of *Pseudomonas* enzyme required dithiothreitol for stability.⁸ Our inhibitory studies on the *Alcaligenes* enzyme with sulfhydryl reagents suggests that this enzyme also contains sulfhydryl groups essential for its activity. Further studies on *Alcaligenes* 5-oxo-L-prolinase will be required to understand the complex reaction mechanism of 5-oxo-L-prolinase containing two cleavages.

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