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5-Oxoprolinase (L-Pyroglutamate Hydrolase) in Higher Plants

PARTIAL PURIFICATION AND CHARACTERIZATION OF THE WHEAT GERM ENZYME

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MENDEL MAZELIS AND RICHARD K. CREVELING
Department of Food Science and Technology, University of California, Davis, California 95616

ABSTRACT

5-Oxoprolinase has been found to be widely distributed in higher plants. This enzyme catalyzes the ATP-dependent hydrolysis of 5-oxo-L-proline (L-pyrollidone carboxylate, L-pyroglutamate) to glutamate. The enzyme has been purified almost 60 fold from wheat germ (*Triticum aestivum* L). This enzyme requires a divalent cation, either Mn^{2+} or Mg^{2+}, and a combination of both appears to be the most effective. There is also an absolute requirement for a monovalent cation best fulfilled by either NH_4^+ or K^+. The K_m for ATP is 0.4 mM and for 5-oxo-L-proline is 14 μM. A small amount of activity is observed when other purine nucleotides such as ITP and GTP replace ATP. The substitution of the pyrimidine nucleotides CTP and UTP for ATP yield almost completely inactive preparations. The enzyme appears to have an active sulfhydryl group since there is an increase in activity in the presence of dithioerythritol. Preincubation with reagents such as N-ethylmaleimide or iodoacetamide lead to complete inactivation. The presence of this enzyme leads to the speculation of the possible presence of a γ-glutamyl cycle in higher plants.

5-Oxoproline (pyrollidone carboxylate, pyroglutamate) has often been identified as a constituent of extracts from biological materials (9). Because of the ease with which it can be formed from glutamic acid or glutamine during the extraction, it was customarily considered to arise as an artifact of the isolation procedure. In recent years, however, it has been shown to be a normal metabolite in animal tissues (6, 10, 15). A number of enzymes have been found which will form 5-oxo-L-proline from glutamic acid, glutamine, or their derivatives (1, 4, 7, 9, 10). The discovery of the enzyme L-5-oxoprolinase (L-pyroglutamate hydrolase) in various mammalian tissues (14) led to the formulation of the γ-glutamyl cycle by Meister (5). 5-Oxoprolinase catalyzes the ATP-dependent hydrolysis of 5-oxo-L-proline to glutamate as follows:

$$\text{5-oxo-L-proline} + \text{ATP} + 2H_2O \xrightarrow[K^+ \text{ or } NH_4^+]{Mg^{2+}} \text{glutamate} + \text{ADP} + \text{Pi}$$

The occurrence of this enzyme in a prokaryote has also been reported (12).

The *in vivo* catabolism of 5-oxoproline by higher plants has been examined recently. After infiltration of L-5-[U-^{14}C]oxoproline into various tissues of a number of different higher plant species, labeled glutamate and glutamine were produced readily (3). Early attempts to obtain cell-free systems from higher plants which would convert 5-oxoproline to other products were unsuccessful. This report now demonstrates the presence of 5-oxoprolinase in preparations from a number of higher plants and describes the partial purification and properties of the enzyme from wheat germ.

MATERIALS AND METHODS

Chemicals. L-5-[U-^{14}C]Oxoproline was purchased from New England Nuclear Corp. ATP was a product of P-L Biochemicals. Cellex-D was obtained from Bio-Rad. Dowex 50 resin, Sepharose 6B, and DTE[1] were purchased from Sigma Chemical Co. Tricine was obtained from Calbiochem. Ammonium sulfate was the special enzyme grade available from Mann Co. 2-Imidazolidone-4-carboxylic acid was purchased from Aldrich Chemical Co. All other chemicals were the highest available grade from commercial sources. The wheat germ (*Triticum aestivum* L.) was a gift from General Mills Co., Vallejo, Calif.

Acetone Powder Preparation. The wheat germ was blended in the cold using 5 ml of −20 C acetone per g of germ. Blending was done at high speed for 1 to 2 min and the slurry then filtered by means of a vacuum filter flask. The precipitate was washed several times with cold acetone and kept on the filter funnel under suction until dry. The precipitate was spread on paper towels and left at room temperature for several hr, then ground to a fine powder with mortar and pestle and stored at −20 C. The powder retains the enzyme activity for several months under these conditions.

Enzyme Distribution Studies. Crude extracts of a number of plant species and tissues were tested for 5-oxoprolinase activity. The preparation procedure was to blend the plant material in the cold in 0.02 M HEPES (pH 7.4) which also contained 0.005 M DTE and then centrifuge the homogenate for 15 min at 20,000*g*. An aliquot of the supernatant solution was tested for the 5-oxoprolinase activity by the standard assay described below.

Standard Enzyme Assay. The complete reaction mixture consisted of the following components in a final volume of 1.0 ml: Na glycinate, 100 mM (pH 9.5); ATP, 5 mM; $MnCl_2$, 2.5 mM; $MgCl_2$, 2.5 mM; $(NH_4)_2SO_4$, 20 mM; DTE, 5 mM; L-5-[U-^{14}C]oxoproline, 0.2 mM (1,000 cpm/nmol); enzyme. The mixture was incubated in a 30 C water bath with constant shaking for the selected time period, usually 15 min. The reaction was stopped by the addition of 0.1 ml of 1 M acetic acid and heating for 5 min at 100 C. The heated reaction mixture was centrifuged and an aliquot of the supernatant solution assayed for [^{14}C]glutamate by the procedure of Van der Werf *et al.* (14). A unit of enzyme activity is that which will produce 1 nmol of glutamate/min. The reaction rate was linear with respect to both time and amount of enzyme under the conditions used.

Protein Assay. Protein was determined by the Lowry procedure (2).

RESULTS

Purification of 5-Oxoprolinase. All of the following steps were carried out in the cold.

a. Twenty g of wheat germ acetone powder was extracted with 200 ml of 0.02 M HEPES (pH 7.4) for 25 min with constant

[1] Abbreviations: DTE: dithioerythritol; NEM: N-ethylmaleimide.

stirring. The slurry was passed through several layers of cheesecloth and centrifuged for 30 min at 20,000*g*.

b. The supernatant solution was decanted and 15 ml of a 1% solution of protamine sulfate added for each 100 ml of supernatant with constant stirring. The resulting mixture was centrifuged for 20 min at 20,000*g* and the precipitate discarded.

c. The solution was made to 35% saturation with solid $(NH_4)_2SO_4$. After stirring for 20 min the suspension was centrifuged and the precipitate discarded. More ammonium sulfate was added until the solution was at 55% saturation. The precipitate was isolated by centrifugation.

d. The precipitate was dissolved in a minimal volume of 0.02 M HEPES (pH 7.4) which contained 20% ethylene glycol. The solution was heated in a 50 C water bath for 1 min then cooled and the precipitate removed by centrifugation and discarded.

e. The enzyme solution was then placed on a Sepharose 6B column (2.5 × 60 cm) and eluted with the HEPES-ethylene glycol buffer, collecting 6-ml fractions.

f. The active fractions were combined and put on a Cellex-D column (2.5 × 20 cm) which had been packed in a 0.05 M Tris (pH 8.0) which contained 20% ethylene glycol. The enzyme was eluted by a linear gradient between the Tris-ethylene glycol buffer described above and the same buffer containing 1 M NaCl. The mixing reservoir contained a volume of 200 ml. Approximately 4-ml fractions were collected and those with the highest activity were combined and used as the enzyme source. The enzyme was stable for several weeks at −15 C.

A typical purification is summarized in Table I. In this particular instance a 59-fold purification with an over-all yield of 10% was obtained. The purest fraction was still very heterogeneous when analyzed by disc gel electrophoresis.

Requirement for Individual Reaction Components. In addition to enzyme, substrates, and buffer, there was a need for a number of other constituents to obtain a maximal reaction rate. The effect of each component of the reaction mixture on the 5-oxoproline hydrolysis is summarized in Table II. As would be expected there was a complete dependence on the presence of ATP. The absence of the divalent cations, Mg^{2+} and Mn^{2+}, also resulted in no hydrolysis. In addition to the divalent cation requirement there was an absolute requirement for either NH_4^+ or K^+ as a monovalent cation. The reaction was also stimulated in the presence of DTE.

Identification of Reaction Product. The identification of the product as a glutamate was made by use of paper chromatography. After eluting the product from the ion exchange column in the assay devised by Van der Werf *et al.* (14), aliquots were spotted on filter paper strips and developed in an ascending direction. Three different solvents were compared, phenol-water (61:39), butanol-acetic acid-water (52:13:35), and methanol-water-pyridine (20:5:1). Known markers of glutamate, glutamine, and 5-[^{14}C]-oxoproline were spotted alongside. At the end of the development the paper was dried and the strip corresponding to the reaction mixture was cut into a series of 1-cm pieces along its length. Each piece was placed in a scintillation vial with scintillation fluid and counted. The glutamate and glutamine markers were visualized by ninhydrin and the radioactive 5-oxoproline located in the same way as the unknowns above. The only product in the reaction mixture was found at an R_F identical to that of the glutamic acid marker.

pH Optimum. The pH optimum was at 9.5 with either glycine or β-alanine buffers. A comparison of glycine, Tricine, and Tris buffers was made at pH 8.5 which is in the buffering region of each of these buffers. The highest activity was found in the presence of glycine buffer in every case.

Cation Requirements. The first experiments clearly showed that the divalent cation Mg^{2+} was necessary for 5-oxoprolinase activity. The only cation which could replace it was Mn^{2+}, and in fact the latter was much more effective. Saturation of the enzyme occurred at a lower concentration of Mn^{2+} than Mg^{2+}. However, at saturating concentrations of Mn^{2+} there was still an increase in activity with the addition of an increment of Mg^{2+}. Table III shows that the combination together of 2.5 μmol of each cation is more effective than 5 μmol of either individually or together.

Previous work by Van der Werf *et al.* (11) had reported a requirement for K^+ or NH_4^+ for the rat kidney enzyme. Since our buffers were normally present as the K salt, we switched to Na buffers and found that the wheat germ enzyme activity was completely dependent on the presence of either NH_4^+ or K^+. NH_4^+ was more effective since it saturated the reaction at a much lower concentration and consistently resulted in higher activity. The maximum rate was obtained between 20 and 40 mM NH_4^+. If K^+ was used a maximum activity was reached at 150 to 200 mM. Other monovalent cations were tested, but only Rb^+ gave a positive response, being almost as effective as K^+. The results are summarized in Table IV.

Nucleotide Effects. The reaction was dependent on the presence of ATP (Table II). Other nucleotide triphosphates were tested with the results shown in Table V. Purine triphosphates such as ITP and GTP could replace ATP to a slight extent, however the

Table I. Summary of the purification of the enzyme from a wheat germ acetone powder.

Procedure	Fraction No.	Total units	Specific activity (units/mg protein)	Yield %	Purification
Original extract	1	1061	0.34	100	1.0
Protamine SO_4 treatment	2	1126	0.39	106	1.1
35-55% $(NH_4)_2$ SO_4 fraction	3	1283	1.4	121	4.1
Heat-treated $(NH_4)_2SO_4$ fraction	4	1236	1.6	115	4.7
Gel filtration	5	540	15.7	51	46
DEAE-cellulose eluate	6	106	20.1	10	59

Table II. Reaction mixture requirements for 5-oxoprolinase activity.

Fraction 6 protein (0.73 units) was used as the enzyme. The reaction was performed and assayed as described in Materials and Methods. Reaction time was 15 min.

Reaction mixture	Glutamate produced nmoles
Complete	9.2
Complete minus Mn^{++}, Mg^{++}	0.3
Complete minus ATP	0.3
Complete minus NH_4^+ (or K^+)	0.2
Complete minus Dithioerythritol	5.4

Table III. Interaction of Mn^{++} and Mg^{++} in obtaining maximal reaction rates.

The standard reaction assay was utilized except for variation of the levels of Mn^{++} and Mg^{++} as indicated. Fraction 6 enzyme (2.1 units) was used and the incubation time was 15 min.

Experiment	Divalent cation present	Concn mM	Glutamate produced nmoles
1	Mn^{++}	2.5	17.9
2	Mn^{++}	5.0	20.2
3	Mg^{++}	2.5	5.3
4	Mg^{++}	5.0	9.1
5	Mn^{++}	2.5	27.8
	Mg^{++}	2.5	
6	Mn^{++}	2.5	26.3
	Mg^{++}	5.0	
7	Mn^{++}	5.0	20.6
	Mg^{++}	2.5	
8	Mn^{++}	5.0	23.0
	Mg^{++}	5.0	

Table IV. Monovalent cation requirement for 5-oxoprolinase activity.

The standard reaction mixture was used except for the monovalent cation added. The reaction mixture always contained 50 mM Na^+ due to the buffer. The activity with NH_4^+ added was used as the reference.

Cation	Final concentration (mM)	Relative activity
NH_4^+	40	100
K^+	150	61
Rb^+	150	41
Ce^+	150	4
Li^+	150	6

Table V. Comparison of ATP with other nucleoside triphosphates in 5-oxoprolinase reaction.

The standard assay system was utilized except for the substitution of ATP by other nucleotides.

Nucleotide	Relative activity
ATP	100
ITP	40
GTP	28
UTP	4
CTP	2

pyrimidine triphosphates, UTP and CTP, gave only negligible activities. In the presence of ATP the other nucleotide triphosphates neither stimulated nor inhibited the reaction. ADP did inhibit the reaction with ATP and at equimolar concentrations produced an inhibition of greater than 40%. This was probably due to product inhibition since AMP, NAD, and NADP had no effect on the reaction.

Michaelis Constants. The K_m for each substrate, ATP and 5-oxoproline, was determined in the presence of a saturating concentration of the other. The constant was determined by use of a linear regression analysis of a straight line form of the Michaelis-Menten equation. The K_m for L-5-oxoproline was 14 μM and for ATP was 0.4 mM. Apparent K_m values of 4.2 mM for Mg^{2+} and 0.5 mM for Mn^{2+} were obtained by the same procedure.

Inhibition Studies. The stimulation of activity in the presence of DTE suggested that a sulfhydryl group was required in the enzyme for maximum activity. Preincubation of the enzyme at 30 C for 15 min with 10 or 50 mM iodoacetamide was carried out before the addition of the other components of the reaction mixture. There was 84% inhibition of the reaction in the presence of 10 mM iodoacetamide and complete inhibition with 50 mM. A more specific sulfhyryl reagent is NEM. Preincubation of the enzyme with 1 mM NEM completely inactivated the enzyme. A final concentration of 0.1 mM NEM inhibited the reaction more than 86%.

The possible protection of the reactive sulfhydryl group by one of the reaction components was examined. The enzyme was preincubated with each individual component except DTE for 5 min. NEM was then added at a final concentration of 1 mM and preincubation continued for 10 min more, at which time all of the remaining reaction constituents were added and the assay carried out as usual. None of the constituents protected the enzyme from inhibition by NEM. There was complete inhibition of the reaction after treatment with 1 mM NEM. Preincubation with other reaction components single or in combination prior to the addition of the NEM did not prevent inactivation.

L-2-Imidazolidone-4-carboxylate has been shown to be an effective inhibitor of the rat kidney enzyme (15). At 1 mM, this compound inhibited the wheat germ enzyme by 80%.

Distribution of 5-Oxoprolinase Activity. A brief survey was made of the distribution of this enzyme in higher plants. Using various plant tissues obtained from local markets, the enzyme was prepared as described under "Materials and Methods." The enzyme was found to be quite common, although a number of plants tested showed little or no activity (Table VI).

DISCUSSION

The demonstration of 5-oxoprolinase activity in higher plant extracts provides further evidence for the widespread occurrence of this enzyme in biological material. It had been found in mammalian tissue (13) and prokaryotes (13) previously. Based on the properties described above the wheat germ enzyme is much more like the rat kidney enzyme than the bacterial enzyme. The K_m for ATP of the plant enzyme was 0.4 mM compared with 0.17 mM for the mammalian enzyme (11) and 1 mM for that from the bacteria (12). The K_m for 5-oxo-L-proline was 14 μM for the wheat germ enzyme compared to 50 μM for that from rat kidney (11) and 0.14 mM for the bacterial enzyme (12). The levels of K^+ or NH_4^+ for maximum activation were again closer to the values found for the enzyme from rat kidney rather than the bacterial system.

The mol wt of the rat kidney enzyme has been estimated at 325,000 (11) or 460,000 (16) by use of calibrated Sephadex G-200 columns. Zonal sedimentation in a sucrose gradient gave a mol wt of 230,000 (16). The plant enzyme would appear to have the same order of mol wt since it is excluded from Sephadex G-150. Unfortunately, the Sepharose 6B column could not be adapted to give a calibrated gel filtration column for mol wt.

The plant enzyme was inhibited by L-2-imidazolidine-4-carboxylate as was the kidney enzyme (11). The bacterial enzyme was inhibited to a much lesser extent by this compound (12). The wheat germ enzyme appeared to be even more sensitive to this inhibitor than the kidney enzyme. An inhibition of 80% was obtained at 1 mM concentration of inhibitor with the plant enzyme whereas only a 20% inhibition at this same inhibitor concentration was seen with the kidney enzyme (11). The wheat germ 5-oxoprolinase was more akin to the kidney enzyme in regard to its pH optimum and also had a similar sensitivity to sulfhydryl reagents.

What could be the possible function of this enzyme in plant tissue? In recent years a role has been proposed for this enzyme in kidney tissue as a member of the γ-glutamyl cycle proposed by Meister (5). The suggested function of this cycle is to transport amino acids across the kidney cell membrane by means of the breakdown and resynthesis of glutathione. The cycle and enzymes required are outlined in Figure 1. All of the enzymes in this cycle

Table VI. Distribution of 5-oxoprolinase activity in crude extracts of higher plants.

Family	Species	Organ	Specific activity (units/mg)
Graminae	Wheat (*Triticum aestivum*)	Germ	0.32
Cruciferae	Turnip (*Brassica rapa*)	Root	0.82
	"	Leaf	0.17
	Cauliflower (*Brassica oleracea*)	Bud	0.72
	Radish (*Raphanus sativus*)	Root	0.82
Chenopodiaceae	Spinach (*Spinacia oleracea*)	Leaf	0.10
Cucurbitaceae	Squash (*Cucurbita pepo*)	Fruit	0.36
Leguminosae	Pea (*Pisum sativum*)	Seed	0.23
Solanaceae	Potato (*Solanum tuberosum*)	Tuber	0.02
Compositae	Lettuce (*Lactuca sativa*)	Leaf	0
Umbelliferae	Carrot (*Daucus carota*)	Root	0.37

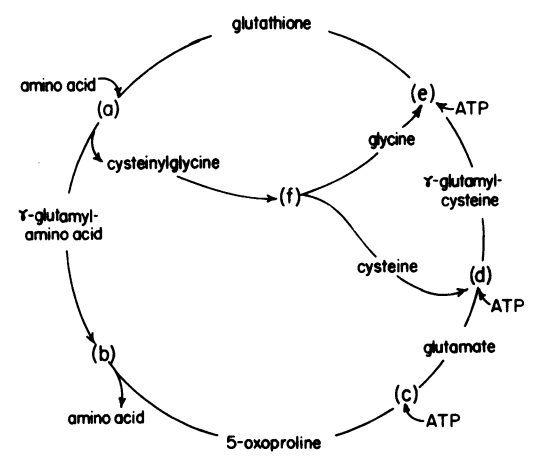

FIG. 1. Schematic representation of the γ-glutamyl cycle proposed by Meister (5). Enzymes catalyzing the various steps are: (a): γ-glutamyl transpeptidase (EC 2.3.2.2); (b): γ-glutamylcyclotransferase (EC 2.3.2.4); (c): 5-oxoprolinase; (d): γ-glutamyl cysteine synthetase (EC 6.3.2.2); (e): gutathione synthetase (EC 6.3.2.3); (f): cysteinyl glycine peptidase (EC 3.4.13.6).

have been reported in plants save γ-glutamyl cyclotransferase and a specific peptidase hydrolyzing cysteinyl glycine. Amino acids are readily taken up from the external medium into whole plant tissue (8) but the mechanism has never been elucidated. It would be of interest to seek evidence for the cycle enzymes not yet discovered in plant tissues and to see whether the cycle enzymes already reported are widely distributed in plants in order to consider whether a mechanism analogous to that proposed to kidney tissue might be concerned with amino acid transport into higher plant cells.

LITERATURE CITED

1. AKITA S, K TANAKA, S KINOSHITA 1959 Enzymic dehydration of L-glutamic acid. Biochem Biophys Res Commun 1: 179–181
2. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
3. MAZELIS M, HM PRATT 1976 *In vivo* conversion of 5-oxoproline to glutamate by higher plants. Plant Physiol 57: 85–87
4. MEISTER A 1968 The specificity of glutamine synthetase and its relationship to substrate conformation at the active site. Adv Enzymol 31: 183–218
5. MEISTER A 1973 On the enzymology of amino acid transport. Science 180: 33–39
6. MEISTER A, SS TATE 1976 Glutathione and related γ-glutamyl compounds: biosynthesis and utilization. Annu Rev Biochem 45: 559–604
7. MESSER M, M OTTESEN 1965 Isolation and properties of glutamine cyclotransferase of dried papaya latex. C R Trav Lab Carlsberg 35: 1–24
8. OAKS A, RGS BIDWELL 1970 Compartmentation of intermediary metabolites. Annu Rev Plant Physiol 21: 43–66
9. ORLOWSKI M, A MEISTER 1971 Enzymology of pyrrolidone carboxylic acid. *In* PD Boyer, ed, The Enzymes, Ed 3 Vol 4. Academic Press, New York, pp 123–151
10. ORLOWSKI, O, PG RICHMAN, A MEISTER 1969 Isolation and properties of γ-L-glutamylcyclotransferase from human brain. Biochemistry 8: 1048–1055
11. VAN DER WERF P, OW GRIFFITH, A MEISTER 1975 5-Oxo-L-prolinase (L-pyroglutamate hydrolase). Purification and catalytic properties. J Biol Chem 250: 6686–6692
12. VAN DER WERF P, A MEISTER 1974 Isolation of 5-oxoprolinase from a prokaryote. Biochem Biophys Res Commun 56: 90–96
13. VAN DER WERF P, A MEISTER 1975 The metabolic formation and utilization of 5-oxo-L-proline (L-pyroglutamate, L-pyrrolidone carboxylate). Adv Enzymol 43: 519–556
14. VAN DER WERF P, M ORLOWSKI, A MEISTER 1971 Enzymatic conversion of 5-oxo-L-proline (L-pyrrolidone carboxylate) to L-glutamate coupled with cleavage of adenosine triphosphate to adenosine diphosphate, a reaction in the γ-glutamyl cycle. Proc Nat Acad Sci USA 68: 2982–2985
15. VAN DER WERF P, RA STEPHANI, M ORLOWSKI, A MEISTER 1973 Inhibition of 5-oxoprolinase by 2-imidazolidone-4-carboxylic acid. Proc Nat Acad Sci USA 70: 759–761
16. WENDEL A, U FLÜGGE, L FLOHE 1976 5-Oxoprolinase from rat kidney. *In* IM Arias, WB Jakoby, eds, Glutathione: Metabolism and Function. Raven Press, New York, pp 71–75