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Glutamate Synthetase in Developing Cotyledons of *Pisum sativum*[1]

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ABSTRACT

Glutamate synthetase (glutamine[amide]:α ketoglutarate amino transferase oxidoreductase) activity has been demonstrated in the developing cotyledons of *Pisum sativum* L. cv. Burpeeana. The enzyme appears to be soluble and is specific for glutamine as amide donor. The enzyme activity is greater with NADH than with NADPH as electron donor.

It is suggested that glutamate synthetase in the developing cotyledon provides a mechanism by which the amide nitrogen of glutamine, from the translocatory stream, is converted into the amino nitrogen of glutamate; transamination reactions involving this synthesized glutamate could provide the amino groups for the biosynthesis of the seed protein amino acids.

The cotyledons of *Pisum sativum* L. are the sites of deposition of large quantities of proteins. These proteins classically are characterized as the water-soluble albumins and the saline-soluble globulins. The globulins are considered to be reserve proteins whereas the albumins contain enzymic proteins (4). Seed development and protein deposition occur concurrently with leaf senescence and it might be expected that the amino acids produced during proteolysis in the senescing leaf could be translocated and serve as the precursors for reserve globulin synthesis in the developing cotyledon. However, the rerserve globulins have a different amino acid composition from that normally encountered in proteins from leaf tissue. Lewis and Pate (12) have indicated that the translocatory stream supplying the cotyledons does not contain the complete spectrum of amino acids required for protein synthesis. The translocatory stream is enriched in asparagine and glutamine (16). It has been suggested (12) that the majority of the amino acids required for protein synthesis during cotyledonary development are synthesized *(de novo)* by utilizing the amide nitrogen.

Lewis (11) has indicated that a pathway which readily makes the amide nitrogen available for amino acid biosynthesis could involve a transamidation reaction to α-ketoglutarate via glutamate synthetase (glutamine[amide]:α-ketoglutarate amino transferase oxidoreductase) to produce glutamate. Transaminase reactions involving the synthesized glutamate could provide the amino groups for the biosynthesis of the seed protein amino acids.

The operation of this scheme depends upon the demonstration of the occurrence of the key enzyme glutamate synthetase in cotyledonary tissue. The enzyme has previously been demonstrated in cell tissue cultures (5), root (7, 10, 17) and leaf tissue and has been implicated in conjunction with glutamine synthetase as providing a principal point of entry of inorganic nitrogen into organic combination (9).

In this communication we report on the occurrence of glutamate synthetase in developing cotyledons of *Pisum sativum*. The observation is consistent with the speculation of Lewis (11) and it is suggested that activities of the enzyme facilitate metabolism of the amide nitrogen of glutamine into the amino nitrogen of the amino acids utilized in reserve protein biosynthesis.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L. cv. Burpeeana) were planted in pots containing Vermiculite (Terra-Lite, W. R. Grace and Co., Cambridge, Mass.) and grown in a growth chamber at 3,000 ft-c during a 16-hr photoperiod at a day temperature of 23 C and a night temperature of 13 C. The developing plants were irrigated daily with either a modified Hoagland's solution or distilled H_2O. The plants were staked and labeled 14 days after germination and flowering dates were recorded daily as reported by Beevers and Poulson (3). Pods were collected 21 days after flowering; this is the time of most active protein deposition in the seeds. Seeds were collected from the pod and the testa and embryonic axis removed; the remaining cotyledons were used for enzyme extraction.

Extraction Procedure A. Cotyledons were homogenized for 2 min at high speed and 3 min at low speed in 50 mM tris-HCl, 1 mM of ethylene diamine tetraacetic acid disodium salt (EDTA), 5 mM 2-mercaptoethanol, pH 7.5, in a Virtis tissue homogenizer at a tissue to buffer ratio of 1:2.5 (w/v). The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 1,000*g* for 10 min. The resulting supernatant was centrifuged at 20,000*g* for 15 min and was used for the enzyme assay.

Extraction Procedure B. In a modified extraction procedure cotyledons were homogenized with a mortar and pestle in 1:2.5 (w/v) cold buffer solution consisting of 50 mM tris-HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 1% BSA, and 0.4 M sucrose, pH 7.5. The homogenate was passed through Miracloth and centrifuged at 1,000*g* for 10 min. The resulting supernatant was centrifuged at 20,000*g* for 15 min and the post 20,000*g* supernatant was centrifuged at 100,000*g* for 30 min. Aliquots of the 1,000*g*, 20,000*g*, and the 100,000*g* supernatants were saved for enzyme assay while the material pelleted during each centrifugation was suspended in the homogenization buffer.

For those enzyme assays in which paper chromatography was used, the 100,000*g* supernatant was brought to 70% saturation with solid ammonium sulfate and stirred at 4 C for 30 min. The ammonium sulfate-precipitable material was recovered by centrifugation at 20,000*g* for 15 min. The pellet was suspended in 50 mM tris-HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5, and then dialyzed against 100 volumes of the same buffer for 1 hr at 4 C.

Enzyme Assay. Glutamate synthetase activity was usually measured spectrophotometrically at room temperature using a

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Gilford 240 spectrophotometer and Gilford 6040 recorder. Except where noted, the decrease in absorbance at 340 nm was followed in 3-ml reaction mixtures in which 0.1 ml of enzyme extract (the 100,000*g* supernatant from extraction B) was added to 50 mM tris-HCl, 1 mM EDTA, pH 7.5, containing 15 μmol of α-ketoglutarate and 15 μmol of L-glutamine, L-asparagine or NH_4Cl. The reaction was normally initiated by the addition of 250 nmol of NAD(P)H. The resulting decrease in absorbance at 340 nm was taken to indicate oxidation of reduced pyridine nucleotides. Optimum conditions for the assay were determined and the enzyme activities are expressed as nmol of NAD(P)H oxidized/min taken from the slope of the initial linear portion of the reaction curve. Saturation curves were obtained by varying the concentration of the experimental substrate at fixed saturating concentrations of the other components. Estimates of apparent Michaelis constants (*Km*) were obtained from Lineweaver-Burk plots of saturation curve data. These plots were linear and regression analysis of the points was performed with the aid of a Hewlett-Packard 9810A Calculator and 9862A Calculator Plotter to determine the slope and intercepts of the least square curve by a poly regression program.

Paper Chromatography. In some assays, in addition to following NAD(P)H oxidation, the formation of glutamate, resulting from glutamate synthetase activity, was detected by paper chromatography. The enzyme reactions, performed as described above, were terminated by the addition of ethanol to an 80% (v/v) final concentration. The resulting precipitated material was removed by centrifugation at 20,000*g* for 15 min. One milliliter of the supernatant was spotted onto Whatman 3MM chromatography paper and subjected to descending chromatography in the solvent; 1-butanol, acetic acid, water (12:3:5). The chromatograms were dried and after being dipped in a cadmium ninhydrin solution (1) were allowed to develop in a darkened glass air tight container containing a beaker of concentrated H_2SO_4.

Protein Determination. Protein was precipitated from the enzyme extracts with 5% trichloroacetic acid. The protein pellet recovered by centrifugation at 20,000*g* was suspended in 0.1 N NaOH, and the suspension was used for the determination of protein by using the Folin phenol procedure of Lowry *et al.* (13).

Table I. *Effect of Extraction Procedure on Substrate Dependence of Glutamate Synthetase Activity from Pea Cotyledons*

Complete reaction mixture is 0.2 ml of enzyme extract added to 50 mM tris-HCl, 1 mM EDTA, pH 7.5, containing 15 μmol of α-ketoglutarate (αKG); 15 μmol of L-glutamine (Gln) were replaced by 15 μmol of L-asparagine or 15 μmol of NH_4Cl as indicated.

Reaction Mixture	Activity			
	Extraction procedure A		Extraction procedure B	
	NADH	NADPH	NADH	NADPH
	nmol oxidized/min			
Complete	62	42	22	11
−αKG	37	29	0	0
−Gln	37	30	0	0
−αKG, −Gln	37	30	0	0
−Gln, +Asn	37	29	0	0
−Gln, $+NH_4^+$	44	33	4	3
Glutamate synthetase[1]	25	12	22	11

[1] Estimated by subtracting −αKG, −Gln from complete.

RESULTS AND DISCUSSION

Enzyme extracts prepared according to procedure A oxidized NAD(P)H in the presence of α-ketoglutarate and L-glutamine (Table I). However, these extracts also oxidized the reduced pyridine nucleotides in the absence of α-ketoglutarate and glutamine. Thus the activity of glutamate synthetase was calculated from the differences in the rate of NAD(P)H oxidation in the presence and absence of glutamine and α-ketoglutarate (Table I).

The oxidation of NADH in the absence of glutamine and α-ketoglutarate has been ascribed to an NADH oxidase (5, 7, 8, 14) but also could be due to the activities of any NADH-dependent dehydrogenases present in the crude extract.

In attempts to eliminate the high substrate-independent oxidation of NADH, homogenates were prepared with a pestle and mortar in the presence of a buffered sucrose solution as described in procedure B. The 100,000*g* supernatant of such extracts showed strict dependence for the presence of α-ketoglutarate and glutamine for NAD(P)H oxidation and thus glutamate synthetase activity could be related directly to pyridine nucleotide oxidation. Analysis of the 1,000*g*, 20,000*g* and 100,000*g* pellets produced in procedure B indicated that glutamine and α-ketoglutarate independent NADH oxidation was associated with the 1,000*g* and 100,000*g* pellets (Table II). The activity in the 1,000*g* fraction probably represents the enzyme associated with intact cells. The NADH oxidation, independent of α-ketoglutarate and glutamine, in the 100,000*g* pellet is indicative of an endoplasmic reticulum-associated NADH oxi-

Table II. *Distribution of Glutamate Synthetase and NADH Oxidase Activity in Cell Fractions from Pea Cotyledons*

Fractionation by procedure B as described under "Materials and Methods." Glutamate synthetase reaction mixture of 0.1 ml enzyme extract added to 50 mM tris-HCl, 1 mM EDTA, pH 7.5, containing 15 μmol of L-glutamine, 15 μmol of α-ketoglutarate, and 250 nmol of NADH. NADH oxidase assay was performed in the same reaction mixture minus L-glutamine and α-ketoglutarate. Specific Activity = nmol NADH oxidized/min·mg protein; total Activity = nmol NADH oxidized/min.

ENZYME	ACTIVITY PER CELL FRACTION						
	Crude homogenate	1000 g pellet	1000 g supernatant	20,000 g pellet	20,000 g supernatant	100,000 g pellet	100,000 g supernatant
Glutamate Synthetase							
Specific Activity	43.5	9.8	48.6	2.6	50.6	12.6	54.0
Total Activity	5722.1	182.0	4280.4	92.0	3997.9	68.0	3827.2
Per Cent Yield	100.0	3.2	74.8	1.6	69.8	1.1	66.0
NADH Oxidase							
Specific Activity	78.3	48.4	48.3	0	37.9	68.3	0
Total Activity	10,296.5	900.0	5356.3	0	2878.4	368.9	0
Per Cent Yield	100.0	8.74	52.0	0	27.9	3.6	0

dase. No NADH oxidation in the presence of glutamine or α-ketoglutarate was demonstrated by the 20,000*g* pellet (Table II).

Under optimal conditions, glutamate synthetase activity was found to be directly proportional to increased amounts of enzyme extract added up to 0.6 mg of protein in the reaction mixture (Fig. 1). The pH optima for glutamate synthetase was found to be 7.5.

Both NADH and NADPH were oxidized by the plant extracts in the presence of α-ketoglutarate and glutamine (Table I, Figs. 1, 2, and 4). A similar lack of reduced pyridine nucleotide specificity has been observed by Fowler *et al.* (7) and Dougall (5). In contrast, Meers *et al.* (14) indicate that glutamate synthetase activity in several bacterial species is NADPH-specific, whereas that in *Rhizobium* bacteroids is NADH-dependent (17). Robertson *et al.* (17), in contrast to the observations of Fowler *et al.* (7), indicate that the glutamate synthetase activity in extracts from lupin roots is NADH-specific. At all concentrations tested the rate of oxidation of NADH was greater than that of NADPH (Fig. 2). Lineweaver-Burk plots of these values were linear over a range 0 to 200 nmol NAD(P)H and yielded apparent Michaelis constants (K*m*) of 13.3 μM for NADH and 27.7 μM for NADPH. It is possible that the functioning of both NADH and NADPH as electron donors, observed in the present study, represents a lack of pyridine nucleotide specificity of the glutamate synthetase as suggested by Fowler *et al.* (7) and Dougall (5). It is also possible that the activity of a pyridine nucleotide phosphatase could be converting NADPH to NADH (6, 18) which may be the preferred electron donor. The lower efficiency of NADPH in supporting the glutamate synthetase activity favors this latter alternative. Saturation curves for L-glutamine, α-ketoglutarate, and NH_4Cl are shown in Figure 3. Lineweaver-Burk plots of these values were linear and yielded estimates of apparent Michaelis constants of 24.4 mM for NH_4, 1.43 mM for L-glutamine, and 0.96 mM for α-ketoglutarate. No glutamate synthetase activity was found at any of the concentrations of L-asparagine tested in the presence of 15 μmol of α-ketoglutarate.

Asparagine and glutamine occur in the translocatory stream (2, 12, 16) and Millerd *et al.* (15) have indicated that asparagine is a more efficient nitrogen source than glutamine for the production of proteins and nucleic acids in the cultured cotyledons of *Pisum sativum* L. These observations imply that the amide group of asparagine is an effective nitrogen source for synthesis of nitrogenous components in developing cotyledons. Fowler *et al.* (7) originally indicated that asparagine was an effective amide donor for glutamate synthetase in extracts from pea roots. Such a reaction would provide a mechanism for the utilization of the amide group of asparagine in the synthesis of other nitrogenous components. Lea and Miflin (10) have indicated recently that the findings of Fowler *et al.* (7) should be questioned since commercially available asparagine is frequently contaminated with aspartic acid which can give erroneous results in glutamate synthetase assays. The glutamate synthetase from the developing pea cotyledon specifically utilized glutamine, and other mechanisms must be invoked to account for the metabolism of the amide group of asparagine. The current findings are consistent with the recent report of Atkins *et al.* (2) which failed to demonstrate glutamate synthetase activity, in extracts of white lupin (*Lupinus alba* L.), when asparagine was the amide donor. Further confirmation of the inactivity of asparagine in the glutamate synthetase reaction is demonstrated by the inability to detect glutamate formation in the reaction products when asparagine replaced glutamine as the amide donor (Fig. 5G).

The oxidation of NADPH in the presence of α-ketoglutarate and L-glutamine remained linear for at least 5 min (Fig. 4) when 0.1 ml of freshly prepared extract (extraction procedure B) was used. However, routinely, assays were conducted for only 3 min.

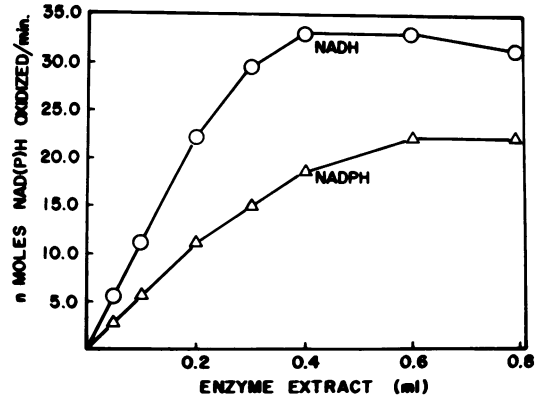

FIG. 1. Influence of enzyme level on the oxidation of NADH (○) or NADPH (△) by glutamate synthetase from pea cotyledons.

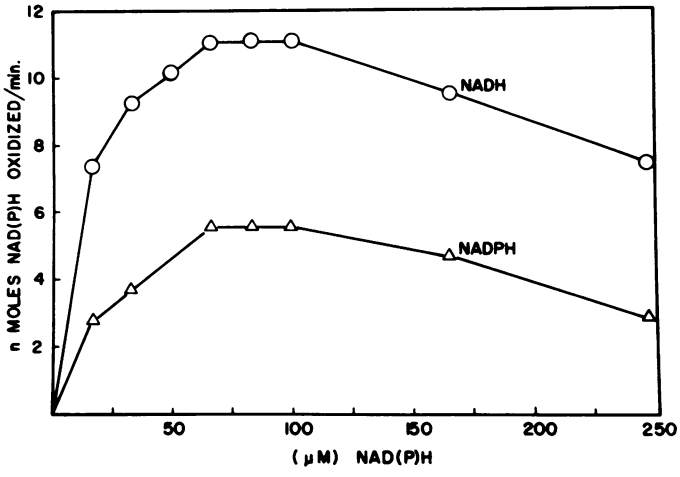

FIG. 2. Saturation curves of NADH and NADPH. Glutamate synthetase was assayed as described under "Materials and Methods" but at the indicated concentrations of the reduced pyridine nucleotides. NADH (○); NADPH (△).

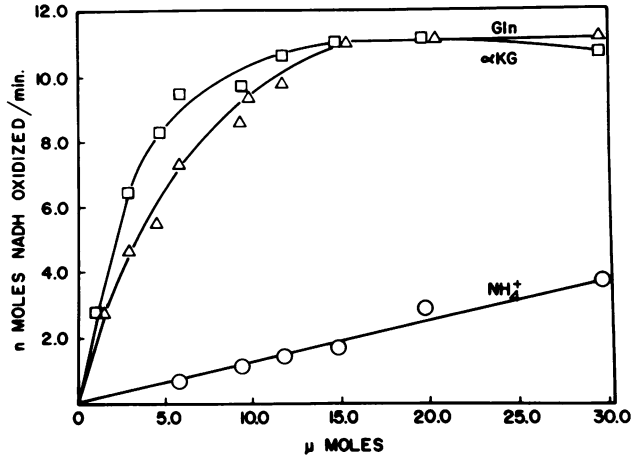

FIG. 3. Influence of alteration of the concentration of component substrates on the rate of oxidation of NADH by glutamate synthetase. α-ketoglutarate (□), L-glutamine (△), NH_4^+ (○). No NADH oxidation was observed when L-asparagine replaced L-glutamine.

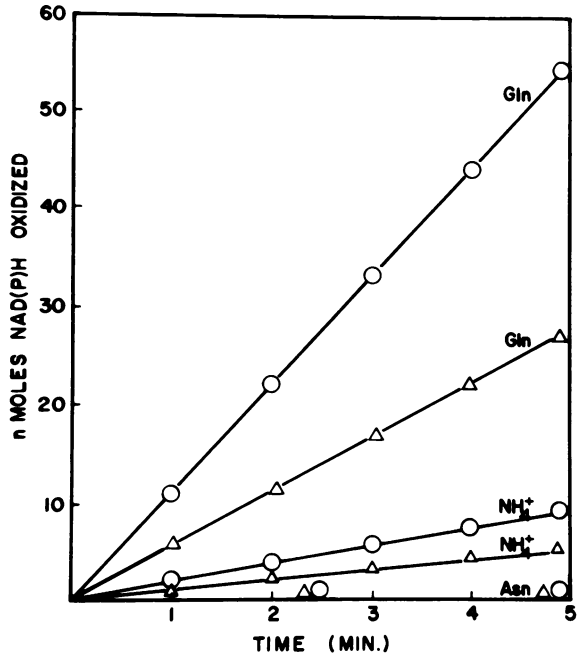

FIG. 4. Influence of various nitrogen donors. L-Glutamine, NH_4^+, and asparagine on the time course oxidation of NADH (○) or NADPH (△) by glutamate synthetase in extracts from pea cotyledons.

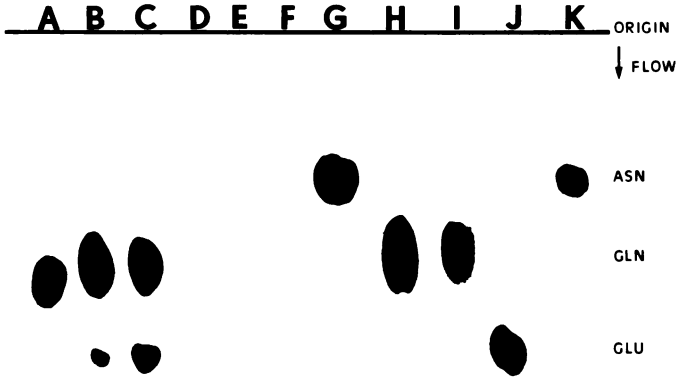

FIG. 5. Paper chromatography of the amino acids in the reaction products of assays for glutamate synthetase activity. Assays were made by using a partially purified enzyme preparation as described under "Materials and Methods." A: Complete system, 0 time; B: complete system, 10-min incubation; C: complete system, 20-min incubation; D: complete system − L-glutamine, α-ketoglutarate; E: complete system − L-glutamine, + NH_4Cl; F: Enzyme extract only; G: complete system − L-glutamine, + L-asparagine; H: complete system − α-ketoglutarate; I: glutamine standard; J: glutamate standard; K: asparagine standard.

The short incubation times were dictated by the fact that, during prolonged storage (1–2 hr) of the enzyme extracts in ice, protein slowly and progressively precipitated from solution. It is believed that this cryoprecipitating protein is a reserve protein component present in the pea cotyledon and its precipitation interfered with the spectrophotometric detection of glutamate synthetase. The precipitate could be removed by centrifugation at 20,000*g* for 15 min with no detectable loss of glutamate synthetase activity. However, after a subsequent period of storage on ice, precipitation resumed, necessitating further centrifugation. Oxidation of NADH in the presence of α-ketoglutarate by the plant extract was stimulated slightly by high concentrations of ammonium ions (Fig. 3). Thus the extracts apparently contained some glutamic acid dehydrogenase activity. It is possible that a glutaminase was converting the supplied glutamine to ammonia and glutamic acid; the released ammonia could then bring about the oxidation of NADH in a glutamic acid dehydrogenase catalyzed reaction. However, that the NADH oxidation measured in the presence of glutamine was attributable to glutamate synthetase and not to the coupled activities of glutaminase and glutamic dehydrogenase is indicated by the observation that glutamine was much more efficient than ammonium in stimulating the oxidation of NADH. Moreover, no glutamate was produced when the enzyme extracts were incubated with glutamine in the absence of α-ketoglutarate (Fig. 5H), thus demonstrating the absence of glutaminase activity.

Although the glutamine-stimulated oxidation of NADH in the presence of α-ketoglutarate and plant extracts is indicative of glutamate synthetase activity, confirmation of the enzymic activity requires the demonstration of the production of glutamate during the reaction. This evidence was provided by paper chromatography of the reaction products from assays which utilized partially purified enzyme extracts. The partial purification of the enzyme removed endogenous amino acids (Fig. 5F) which would have interfered with the detection of the products of the glutamate synthetase reaction. By using the dialyzed suspension of the 70% ammonium sulfate-precipitable material from the 100,000*g* supernatant it was possible to demonstrate glutamine and α-ketoglutarate dependent oxidation of NADH. Paper chromatography demonstrated the progressive production of glutamate during the course of the reaction (Fig. 5, A, B, and C). No detectable glutamate was produced when α-ketoglutarate and ammonium chloride (Fig. 5E) or α-ketoglutarate and L-asparagine (Fig. 5G) or glutamine (5H) were incubated under similar reaction conditions.

CONCLUSIONS

The developing pea cotyledons contain a glutamate synthetase which shows specificity for glutamine as the amide donor. Both NADPH and NADH serve as reductant in the reaction. It is possible that NADPH is converted by a pyridine nucleotide phosphatase to NADH and thus the glutamate synthetase from the pea cotyledon may show a pyridine nucleotide specificity similar to that reported in lupin roots (17).

The presence of glutamate synthetase in the developing cotyledons provides a mechanism whereby the amide group of the glutamine translocated into the cotyledon can be metabolized into the amino nitrogen of glutamate. This glutamate, via transamination reactions, could furnish the amino nitrogen which is utilized in the biosynthesis of the amino acids required for the production of the reserve proteins in the developing cotyledons.

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