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Glutamate Synthase: A Possible Role in Nitrogen Metabolism of the Developing Maize Endosperm[1]

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ABSTRACT

Glutamate synthase activity was demonstrated in the developing endosperm of maize (*Zea mays* L.). The enzyme shows specificity for glutamine and α-ketoglutarate as amino donor and acceptor, respectively. Both NADH and NADPH function as electron donors although lower activities were often, but not always, obtained with NADPH. The apparent K_m values for glutamine, α-ketoglutarate, and NADH were 1.35 mM, 0.57 mM, and 7 μM, respectively.

The pattern of activity during endosperm development revealed a well defined peak coinciding with the period of most active N accumulation. Activity in general was related to the rates of accumulation throughout development. Maximum glutamate synthase activity was the order of 56 nmoles of glutamate formed per minute per endosperm compared with a rate of N accumulation of 9.5 nmoles per minute.

It is suggested that glutamate synthase plays a key role in the N nutrition of the maize endosperm providing a means whereby N transported in the form of glutamine is made available for the synthesis of other seed protein amino acids via transaminase reactions. Transaminase activity involving glutamate, the product of the glutamate synthase reaction, was also demonstrated.

The origin of amino acids utilized for the synthesis of reserve proteins in seeds has become a subject of increasing interest in recent years. Essentially, two mechanisms are possible. Amino acids could arrive ready-made via the translocation stream or they could be synthesized in the developing seeds themselves. Evidence for the first alternative has been provided principally by Pate's group (2, 19) who have demonstrated that virtually all protein amino acids are present in the translocation stream supplying the developing fruits. Moreover, several amino acids have been shown to be translocated and incorporated largely intact into the seed storage protein (2, 6, 11, 23).

The balance of the various amino acids in the translocation stream is quite different from the amino acid composition of the reserve proteins, asparagine and glutamine being the major N compounds entering the seeds (2, 13, 19). Studies with ^{15}N (2, 13, 14) have shown that the amide N of these amino acids is readily transferred to the amino group of other amino acids. This suggests an important role for the biosynthesis of amino acids in developing seeds. Indeed, ^{14}C tracer work has established that the biosynthetic pathways of protein amino acids are operative in developing seeds (4, 20, 22).

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The relative importance of the two mechanisms is difficult to evaluate, but the contribution of amino acid biosynthesis may be considerable in view of the high levels of amides and general imbalance of protein amino acids in the translocation stream. The question arises as to how the amide N is made available for amino acid biosynthesis, in the case of glutamine, Lewis (13) has suggested that the glutamate synthase reaction may be the key to this scheme. This enzyme catalyses the transfer of the amide group to α-ketoglutarate forming glutamate. The glutamate so formed may then provide the amino groups for the biosynthesis of other amino acids via transaminase reactions. Glutamate synthase is a new enzyme to biochemistry (24), but has already been demonstrated in several plant tissues including leaves (12), roots (8, 15, 21), and tissue culture cells (5). Beevers and Storey (3) recently reported the presence of this enzyme in developing pea cotyledons, thus bearing out Lewis' suggestion.

Our objectives were to demonstrate the presence of glutamate synthase in maize endosperm and to evaluate its importance in the N metabolism of this organ.

MATERIALS AND METHODS

A single cross hybrid of maize (*Zea mays* L.) designated HS 1406, synthesized from inbred lines of a Tuxpeño variety, was used in this study. The plants were grown in the field in the 1975 to 1976 growing season, and self-pollinated on the same day.

Ten days after pollination, and thereafter at 7-day intervals, four ears were collected and taken to the laboratory for immediate processing. An equal number of kernels were removed from the midregion of each ear for enzyme extraction and the remainder stored frozen for posterior lyophilization. Endosperms of 24-day-old maize were used in all experiments designed to study the properties of the enzyme.

Standard Extraction Procedure. Forty kernels were degermed and ground in a chilled mortar with 20 ml of 50 mM tris-HCl (pH 7.5) containing 1 mM EDTA and 10 mM 2-mercaptoethanol. The homogenate was filtered through muslin and centrifuged at 10,000*g* for 15 min. The resulting supernatant was decanted through muslin to remove the floating fat layer. An aliquot of 5 ml was then passed through a column of Sephadex G-25 (1.5 × 16 cm) equilibrated with extraction buffer. The protein fraction was collected in a volume of 8 ml, and aliquots containing about 300 μg of protein taken for enzyme assay.

Partial Purification Procedure. Forty kernels were degermed and homogenized with 10 ml of extraction buffer as in the standard extraction procedure. After centrifugation at 10,000*g* for 15 min the supernatant was brought to 40% saturation (value at 0 C) by the stepwise addition of solid ammonium sulfate. After standing for 20 min, the precipitate was removed by centrifugation at 10,000*g* for 10 min. The supernatant was

then brought to 55% saturation by the further addition of ammonium sulfate and the precipitated protein collected by centrifugation and taken up in 5 ml of extraction buffer. After filtration on Sephadex G-25, a partially purified preparation of glutamate synthase was obtained with a specific activity about three times that of the crude extract.

Enzyme Assays. Glutamate synthase was assayed spectrophotometrically by following the oxidation of NADH at 340 nm. Routinely, the 3-ml reaction mixture contained 50 mM tris-HCl (pH 7.5), 5 mM L-glutamine, 5 mM α-ketoglutaric acid, 0.1 mM NADH, and an aliquot of the enzyme preparation. The mixture was incubated in a waterbath at 30 C and briefly removed at 0, 15, and 30 min for reading in the spectrophotometer. Corrections were made for substrate-independent NADH oxidation.

For the determination of the stoichiometry of the reaction, a similar reaction mixture was used except that glutamine was present at 2 mM and NADH at 0.6 mM. NADH was determined spectrophotometrically and amino acids with an amino acid analyzer (16) using nor-leucine as internal standard.

Transaminase assays were performed in pH 7.5 buffer with various L-amino-acids and keto acids at 2 mM and 5 mM, respectively. TLC was used to detect the formation and disappearance of amino acids.

Apparent K_m values were determined from double reciprocal plots of the reaction velocity at different substrate concentrations, with other substrates maintained at saturating concentrations.

Chromatography. Amino acids of assay mixtures were analyzed by TLC after stopping the reaction with an equal volume of ethanol. Glass plates (20 × 20 cm) were coated with cellulose-silica mixtures (10:4, w/w) (25) at a thickness of 250 μm. Aliquots (50 μl) of the ethanol-treated reaction mixtures were applied to the plates and chromatographed in the solvent phenol-water (80:20, w/v). After drying, the plates were treated with1% ninhydrin in acetone and developed for 10 min in a hot oven to localize the amino acids.

Protein and Nitrogen. Protein was precipitated from the extracts with 5% trichloroacetic acid and determined by the method of Itzhaki and Gill (9) using 300 nm for readings and BSA as standard. N was determined on lyophilized samples by the Kjeldahl method (1).

RESULTS AND DISCUSSION

Crude extracts of maize endosperm (and embryo) were found to oxidize NADH in the presence of glutamine and α-ketoglutaric acid (Table I). Removal of either glutamine or α-ketoglutarate or both considerably reduced but did not eliminate the oxidation of NADH. This substrate-independent NADH oxidation varied noticeably between extracts, and was generally much lower when frozen material was used as the source of enzyme. Partial purification of the endosperm glutamate synthase by ammonium sulfate fractionation removed all of the nonspecific NADH oxidase, with a recovery of 90% of the original activity (Table I). This suggests that the calculation of glutamate synthase activity by subtraction of nonspecific NADH oxidation from the total activity (Table I) is justified when working with crude extracts.

The specificity of the enzyme for glutamine as amino donor is indicated by the fact that neither asparagine nor ammonium ions would substitute for glutamine (Table I). Some reports (5, 8) that asparagine effectively substitutes glutamine as amino donor may be in error (15). The failure of ammonium ions to substitute for glutamine suggests that the activity measured is not due to coupled glutaminase and glutamate dehydrogenase activities. Lack of glutaminase activity under the conditions used is also evident from the data shown in Figure 1, where no detectable glutamate was formed when NADH was removed from the reaction mixture.

Table I - Substrate specificity of glutamate synthase
The complete system was as described in "Materials and Methods". Other compounds were present at 5mM. The embryo extract was prepared in an identical manner to that of the endosperm.

reaction mixture	Activity	
	crude enzyme	partially purified enzyme
	nmol NADH oxidized/min/grain	
Endosperm:		
Complete system	44	30
- Gln	11	0
- αKG	11	0
- Gln, - αKG	11	0
- Gln, + Asn	11	0
- Gln, + NH_4^+	12	0
- αKG, + pyruvate	...	0
- αKG, + oxaloacetate	...	∞[2]
Gln, - αKG, + oxaloacetate	...	∞
Glutamate synthase [1]	33	30
Embryo		
Complete system	9	
- Gln, - αKG	2	
Glutamate synthase[1]	7	

[1]Calculated by substracting -Gln, -αKG from complete
[2]Oxidation instantaneous.

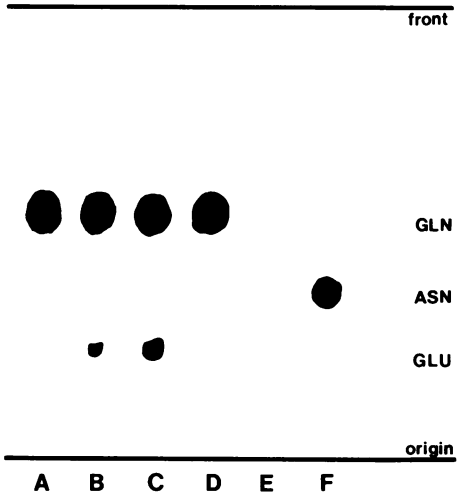

FIG. 1. TLC of amino acids in the reaction mixture of glutamate synthase assay using a crude enzyme preparation. The assay was as described under "Materials and Methods" except that NADH was 0.4 mM. A: Complete system, zero time; B: complete system 30-min incubation; C: complete system, 60-min incubation; D: complete system, NADH; E: complete system, L-glutamine; F: complete system, L-glutamine + L-asparagine.

Specificity for the amino acceptor is indicated by the inability of pyruvate to substitute for α-ketoglutarate (Table I). Oxaloacetic acid was also tested but its activity in the glutamate synthase system could not be evaluated due to the presence of a very active malate dehydrogenase, which caused an almost instantaneous oxidation of the NADH present.

In plants, glutamate synthase appears to be nonspecific with regard to pyridine nucleotide (5, 8) although reports are conflicting (21). Our own findings are not shown here because of the great variation in activity obtained with NADPH with different enzyme preparations. Some gave as little as 20% of the NADH activity while others produced 100% (both crude and partially purified preparations gave results in this range). Miflin and Lea (15) also noted variations in NADPH activity with different preparations. Beevers and Storey (3) reported lower activities with NADPH as electron donor than NADH, and speculated that a phosphatase could be converting NADPH to NADH as Wells and Hageman (26) had shown when dealing with a similar problem of pyridine nucleotide specificity with nitrate reductase. Wells and Hageman used fluoride to inhibit

phosphatase activity and thereby demonstrated specificity of their enzyme for NADH. The addition of fluoride to our preparation showing equal activity with NADH and NADPH was unsuccessful. Evidently further work is needed to clarify the question of pyridine nucleotide specificity of glutamate synthase.

As a further check that the substrate-dependent NADH oxidase activity was truly due to glutamate synthase, the products of the reaction were analyzed by TLC (Fig. 1). These data show that glutamate was the product accumulating in the reaction mixture. The formation of glutamate was dependent on the presence of glutamine, α-ketoglutarate, and NADH. Substitution of glutamine with asparagine in the enzyme assay did not lead to glutamate formation, confirming the result of a similar test made in the spectrophotometric assay. A quantitative analysis of the changes in substrates and products produced by a partially purified preparation of glutamate synthase revealed that for each mol of NADH oxidized, 1.05 mol of glutamine were lost and 2.34 mol of glutamate formed, which is reasonably close to the stoichiometry of the glutamate synthase reaction.

In order to determine optimal conditions for the assay of glutamate synthase, certain kinetic parameters were determined with the partially purified enzyme, from which it was possible to conclude the following. The pH optimum was 7.5 and the apparent K_m for the various substrates was 1.35 mM, 0.57 mM, and 7 μM, respectively, for glutamine, α-ketoglutarate, and NADH. These values compare favorably with those obtained for the enzyme from other plant sources (3, 15). Using crude extracts under optimal assay conditions, activity was linear with time and proportional to the amount of enzyme added, in the ranges used. The assay was therefore considered suitable for the quantitative measurement of glutamate synthase during development of the maize endosperm.

The pattern of glutamate synthase activity during endosperm development for a normal variety of maize is shown in Figure 2. The experiment was not repeated for the same single cross but almost identical patterns were obtained for an opaque-2 variety and a normal flint single cross. After the onset of endosperm development, there was a rapid increase in glutamate synthase activity reaching a peak at about 30 days after pollination. The peak coincides with the period of most active N accumulation. Enzyme activity subsequently falls and reaches zero at the time of formation of the abscission zone (black layer). Although the endosperm becomes harder and more

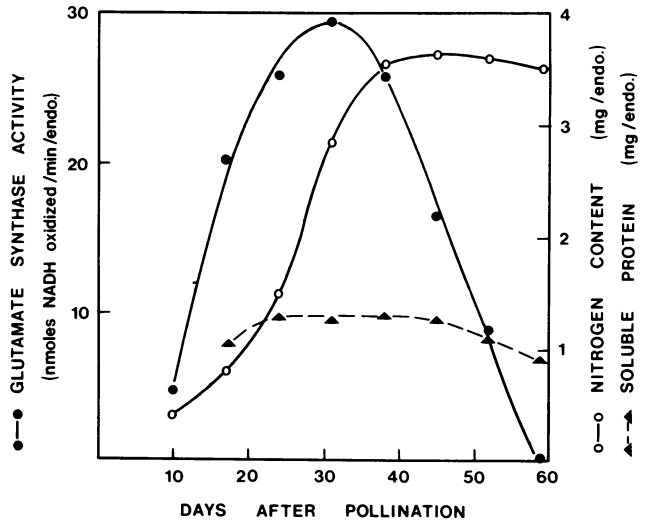

FIG. 2. Glutamate synthase, and soluble protein levels in maize endosperm during development.

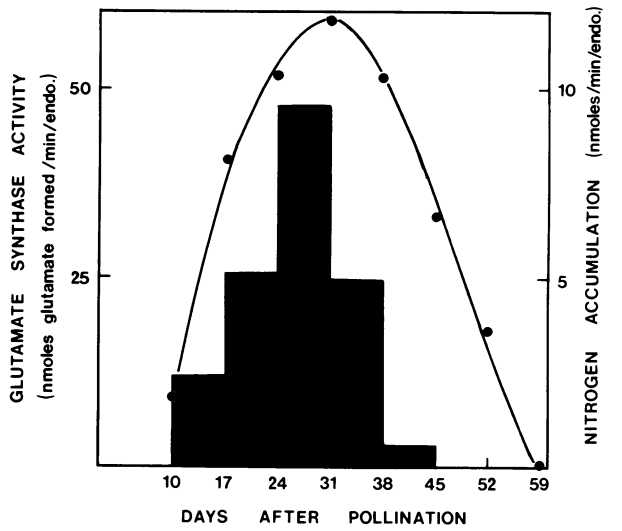

FIG. 3. Glutamate synthase activity (●——●) and rates of accumulation (histogram) in maize endosperm during development. Data calculated from Figure 2.

difficult to grind at more advanced stages of development, fairly constant levels of protein were extracted with the enzyme at all stages. Soluble protein would consist of the albumin and globulin classes which are known to remain at a fairly constant level during endosperm development (18). Evidently the fall in activity in the latter part of the curve cannot be attributed to a deficiency in the extraction procedure.

The fact that glutamate synthase activity does not simply accompany soluble protein levels suggests that the changes observed have some physiological significance. Lewis (13) has suggested that glutamate synthase may function in the utilization of translocated glutamine as a source of amino groups for the synthesis of seed protein amino acids. In this respect it is noteworthy that glutamate synthase activity is related to the rates of N accumulation in the endopserm during development (Fig. 3). Since N accumulation in the maize endosperm is closely associated with the synthesis and deposition of storage protein (17, 18), it is reasonable to infer from these data that the flow of N through the glutamate synthase system could be important for sustaining the biosynthesis of amino acids to meet the needs of protein synthesis. The activities of glutamate synthase are high enough for the enzyme to fulfill such a role, activities being many times higher than the rates of N accumulation. For example, when the rate of N accumulation is maximal, glutamate synthase activity per endosperm is in the order of 56 nmol of glutamate formed per min compared with an accumulation rate for N of 9.5 nmol/min. Evidently the activity of glutamate synthase is more than sufficient to process all of the incoming glutamine even if all of the N were transported to the developing kernels in this form. Although little information exists in the literature concerning the transport of amino acids in maize, there is some evidence that glutamine is the major N component transported to the kernels (10).

The utilization of the glutamate formed in the glutamate synthase reaction to provide amino groups for the synthesis of other amino acids depends on the presence of transaminases. Using crude extracts of 24-day-old endosperm, it was possible to demonstrate the presence of transaminase activity by a qualitative assay (data not shown). The glutamate-pyruvate and alanine-α-ketoglutarate transaminase system was very active when considered in relation to glutamate formation in the glutamate synthase system. Transaminase systems involving α-ketoglutarate and valine, isoleucine, leucine, phenylalanine, and tyrosine were also looked for but no significant activity was

detected by the chromatographic method used. Nevertheless, these activities might be expected to be much lower than the glutamate-pyruvate system (7) and may require a more sensitive assay for detection.

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LITERATURE CITED

1. Association of Official Agricultural Chemists 1965 Official Methods of Analysis Ed 10. Association of Official Agricultural Chemists, Washington, D.C. pp 744–745
2. Atkins CA, JS Pate, PJ Sharkey 1975 Asparagine metabolism—key to the nitrogen nutrition of developing legume seeds. Plant Physiol 56: 807–812
3. Beevers L, R Storey 1976 Glutamate synthetase in developing cotyledons of *Pisum sativum*. Plant Physiol 57: 862–866
4. Bilinski E, WB McConnell 1957 The utilization of acetate for amino acid biosynthesis. Can J Biochem Physiol 35: 357–364
5. Dougal DK 1974 Evidence for the presence of glutamate synthase in extracts of carrot cell cultures. Biochem Biophys Res Commun 58: 639–646
6. Finlayson AJ, WB McConnell 1969 The utilization of ^{15}N and phenylalanine-2-^{14}C by wheat plants. Can J Biochem 47: 415–418
7. Forest JC, F Wightman 1972 Amino acid metabolism in plants. II. Transamination reaction of free protein amino acids in cell-free extracts of cotyledons and growing tissues of bushbean seedlings (*Phaseolus vulgaris* L.). Can J Biochem 50: 538–542
8. Fowler MW, W Jessup, GS Sarkissian 1974 Glutamate synthase type activity in higher plants. FEBS Lett 46: 340–342
9. Itzhaki RF, DM Gill 1964 A micro-biuret method for estimating proteins. Anal Biochem 9: 401–410
10. Jelenic C, M Pencic, B Kerecki, L Zaric 1966 Changes in the content of amino acids and nitrogen in the sap of domestic corn varieties at different stages. Zemljiste Biljka 15: 29–41, cited in Chem Abstr 68: 57410 (1968).
11. Lawrence JM, DR Grant 1964 Incorporation of lysine-^{14}C into the developing grain of wheat. Arch Biochem Biophys 104: 73–78
12. Lea PJ, BJ Miflin 1974 Alternative route for nitrogen assimilation in higher plants. Nature 246: 614–616
13. Lewis OAM 1975 An ^{15}N-^{14}C study of the role of the leaf in the nitrogen nutrition of the seed of *Datura stramonium* L. J Exp Bot 26: 361–366
14. Lewis OAM, JS Pate 1973 The significance of transpirationally derived nitrogen in protein synthesis in fruiting plants of pea (*Pisum sativum* L.). J Exp Bot 24: 596–606
15. Miflin BJ, PJ Lea 1975 Glutamine and asparagine as nitrogen donors for reductant-dependent glutamate synthesis in pea roots. Biochem J 149: 403–409
16. Moore S, DH Spackman, WH Stein 1958 Chromatography of amino acids on sulfonated polystyrene resins: an improved system. Anal Chem 30: 1185–1189
17. Moureaux T, J Landry 1972 La maturation du grain du mais. Evolution qualitative of quantitative des différentes formes azotées. Physiol Vég 10: 1–18
18. Murphy JJ, A Dalby 1971 Changes in the protein fractions of developing normal and opaque-2 maize endosperm. Cereal Chem 48: 336–349
19. Pate JS, PJ Sharkey, OAM Lewis 1974 Phloem bleeding from legume fruits—a technique for study of fruit nutrition. Planta 120: 229–243
20. Pate JS, PJ Sharkey, OAM Lewis 1975 Xylem to phloem transfer of solutes in fruiting shoots of legumes, studies by a phloem bleeding technique. Planta 122: 11–26
21. Robertson JG, MP Warburton, KJ Farnden 1975 Induction of glutamate synthase during nodule development in lupin. FEBS Lett 55: 33–37
22. Sodek L 1976 Biosynthesis of lysine and other amino acids in developing maize endosperm Phytochemistry 15: 1903–1906
23. Sodek L, CM Wilson 1970 Incorporation of leucine-^{14}C and lysine-^{14}C into protein in the developing endosperm of normal and opaque-2 corn. Arch Biochem Biophys 140: 29–38
24. Tempest DW, JL Meers, CM Brown 1970 Synthesis of glutamate in *Aerobacter aerogenes* by a hitherto unknown route. Biochem J 117: 405–407
25. Turner NA, RJ Redgwell 1966 A mixed layer for separation of amino acids by thin-layer chromatography. J Chromatogr 21: 129–132
26. Wells GN, RH Hageman 1974 Specificity for nicotinamide adenine dinucleotide by nitrate reductase from leaves. Plant Physiol 54: 136–141