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Purification and Characterization of Two Forms of Glutamine Synthetase from the Pedicel Region of Maize (*Zea mays* L.) Kernels

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ABSTRACT

Maize (*Zea mays* L.) kernel pedicels, including vascular tissues, pedicel parenchyma, placento-chalazal tissue, and the surrounding pericarp, contained two forms of glutamine synthetase (EC 6.3.1.2), separable by anion exchange chromatography under mildly acidic conditions. The earlier-eluting activity (GS_{p1}), but not the later-eluting activity (GS_{p2}), was chromatographically distinct from the maize leaf and root glutamine synthetases. The level of GS_{p1} activity changed in a developmentally dependent manner while GS_{p2} activity was constitutive. GS_{p1} and GS_{p2} exhibited distinct ratios of transferase to hydroxylamine-dependent synthetase activities (5 and 23, respectively), which did not change with kernel age. Purified pedicel glutamine synthetases had native relative molecular masses of 340,000, while the subunit relative molecular masses differed slightly at 38,900 and 40,500 for GS_{p1} and GS_{p2}, respectively. Both GS forms required free Mg^{2+} with apparent K_ms = 2.0 and 0.19 millimolar for GS_{p1} and GS_{p2}, respectively. GS_{p1} had an apparent K_m for glutamate of 35 millimolar and exhibited substrate inhibition at glutamate concentrations greater than 90 millimolar. In contrast, GS_{p2} exhibited simple Michaelis-Menten kinetics for glutamate with a K_m value of 3.4 millimolar. Both isozymes exhibited positive cooperativity for ammonia, with $S_{0.5}$ values of 100 and 45 micromolar, respectively. GS_{p1} appears to be a unique, kernel-specific form of plant glutamine synthetase. Possible functions for the pedicel GS isozymes in kernel nitrogen metabolism are discussed.

During seed development, nitrogen and carbon compounds are translocated from the vegetative portions of the plant to the seed where they are converted into water-insoluble storage polymers. In maize (*Zea mays* L.), the vascular system terminates in the pedicel tissue of the kernel, and assimilates are unloaded and pass symplastically through this basal maternal tissue region before entering the endosperm via the basal endosperm transfer cells (11). The form in which nitrogen is transported to the developing seed is species specific (28) and the results of amino acid pool analysis (3, 15, 16) and radiolabeled precursor studies (26) suggest that in maize nitrogen is transported predominantly as aspartate, serine, glutamate, glutamine, and alanine.

In both legumes (22) and cereals (7, 15, 16), considerable metabolism of the incoming nitrogenous transport compounds appears to take place in the seed-associated maternal tissues prior to their uptake into the developing endosperm or cotyledons. In the pedicel region of the maize kernel, the free amino acid pool is altered such that the relative glutamine and glutamate levels are increased and aspartate, glycine, and serine levels are decreased when compared to the cob vascular sap (15). Incubation of whole kernels in [^{14}C]aspartic acid-containing media showed that the carbon from aspartate is rapidly metabolized into organic acids within the pedicel (16). The most striking change in the transport free amino acid pool, however, is in the relative glutamine levels, which constitutes approximately 25% of the cob vascular sap free amino acids, but up to 50% of the pedicel free amino acids (16). Also, Pernollet *et al.* (26) have shown a dramatic transient increase of radioactivity in glutamine from $^{14}CO_2$ fed to the subtending leaf as assimilates move into maize kernels. Consistent with these metabolic conversions taking place in the pedicel, extracts from kernel pedicel tissues contain a number of enzymes of nitrogen metabolism, including GS,[1] glutamate synthase, glutamate dehydrogenase, and aspartate and alanine transaminases (16). Recently, I reported (21) that pedicel GS activity increases in the kernel pedicel region in a developmentally related manner such that GS activity reaches a maximum coincidentally with the period of maximum nitrogen assimilation by the kernel. In this paper, two forms of pedicel GS, which are differentially expressed during maize kernel development, are described. The two isozymes have been purified and some of their properties have been examined.

MATERIALS AND METHODS

Plant Materials

Hybrid maize (*Zea mays* L., W64AxA619) was grown in the field in the summers of 1986 and 1987. Ears were hand pollinated. Kernels were harvested from the cobs with a scalpel to ensure that the pedicel region was included. The kernels were then quickly frozen in liquid nitrogen and stored at −70°C until use. Maize seedlings were grown for 10 d in flats of vermiculite in a glasshouse at the Northern Regional Research Center, Peoria, Il.

[1] Abbreviations: GS, glutamine synthetase (EC 6.3.1.2); DAP, days after pollination; FPLC, fast protein liquid chromatography, a trademark of Pharmacia, Inc; MSO, DL-methionine sulfoximine.

Chemicals and Chromatographic Materials

High resolution hydroxyapatite and imidazole were purchased from Calbiochem-Behring Diagnostics,[2] while Matrex Blue A gel was obtained from Amicon Corporation. Protein standards for SDS-PAGE were from Diversified Biotech, Newton Centre, MA. Protein reagent was obtained from Bio-Rad. Certified-grade ethylene glycol was obtained from Fisher Scientific. Q-Sepharose, PD-10 Sephadex G-25 columns, Superose 6 gel filtration, Mono Q HR 5/5 anion exchange columns and other FPLC system components were from Pharmacia Inc. Phenyl Sepharose, native mol wt standards and all other chemicals were purchased from Sigma Chemical Co.

Enzyme Isolation and Anion Exchange Chromatography

Previously harvested kernels were held on dry ice for hand dissection with a scalpel. Pedicel regions (basal maternal kernel tissues, including vascular tissues, spongy and pedicel parenchyma, placento-chalazal tissue, and the surrounding pericarp) were collected on ice. Glutamine synthetase was extracted from 25 to 50 pedicel sections or from freshly harvested leaf and root samples as described previously (21). Centrifuged tissue homogenates were desalted on PD-10 Sephadex G-25 minicolumns equilibrated in anion exchange starting buffer (50 mM imidazole/HCl [pH 6.5], 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 15% [v/v] ethylene glycol). Portions of the desalted samples were loaded onto a Mono Q anion column which was then washed with 5 mL of starting buffer and eluted with a linear gradient of 0 to 225 mM NaCl in starting buffer. The total gradient volume was 40 mL, and 0.5-mL fractions were collected. The flow rate was maintained at 0.5 mL/min. The anion exchange elution profiles of pedicel extracts from kernels harvested 15, 30, and 45 DAP (Fig. 2) were from the same year's harvest as used in the previous study (21). Similar results were obtained from kernels harvested in the following year.

Purification of Maize Kernel Pedicel Glutamine Synthetase

The purification procedure for maize pedicel GS was adapted from Ericson (9). Pedicel regions were isolated as described above from maize kernels that had been harvested 25 to 30 DAP. The pedicels were immediately refrozen in liquid nitrogen and stored at −70°C. Approximately 500 to 600 pedicels were homogenized as described previously. The homogenate was centrifuged at 27,000*g* for 30 min and the supernatant was applied directly to a 3.5 × 5.5 cm column of Q-Sepharose which had been equilibrated with 50 mM Tris/HCl (pH 7.6), containing 10 mM $MgCl_2$, 1 mM EDTA, 15% (v/v) ethylene glycol, 1 mM DTT, and 28 mM 2-mercaptoethanol. The column was washed with 70 mL of the same buffer then eluted overnight at 1 mL/min with a 500 mL linear 0 to 400 mM NaCl gradient, all in starting buffer. Five-mL fractions were collected. The fractions were assayed for GS activity using the transferase assay (described below), and the GS-containing fractions were pooled and loaded directly onto a 12 × 2.6 cm hydroxyapatite column equilibrated in 50 mM imidazole/HCl buffer (pH 7.6), containing 5 mM $MgCl_2$, 1 mM EDTA, 15% ethylene glycol (v/v), 200 mM NaCl, 1 mM DTT, and 28 mM 2-mercaptoethanol. After washing the column with 50 mL of the equilibration buffer, GS was eluted with a 500 mL, linear 0 to 250 mM sodium arsenate gradient in equilibration buffer overnight at a flow rate of 0.5 mL/min. The gradient was followed with an additional 200 mL of 250 mM sodium arsenate in equilibration buffer to finish eluting GS from the column. Five-mL fractions were collected and assayed for transferase activity. All GS-containing fractions were pooled and loaded directly onto a 20 × 1.6 cm phenyl Sepharose column equilibrated in 50 mM imidazole/HCl buffer (pH 7.0), containing 250 mM sodium arsenate, 10% ethylene glycol, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 28 mM 2-mercaptoethanol. The column was washed with 50 mL of the equilibration buffer, and GS was eluted with 50 mM imidazole/HCl buffer (pH 7.0), containing 35% (v/v) ethylene glycol, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and 28 mM 2-mercaptoethanol. Fractions were assayed and pooled as above, then dialyzed overnight in dialysis buffer A (20 mM imidazole/HCl buffer [pH 7.6], containing 6.67 mM $MgCl_2$, 0.33 mM EDTA, 15% [v/v] ethylene glycol, 0.33 mM DTT, and 28 mM 2-mercaptoethanol). After dialysis, the enzyme-containing solution was passed through a 15 × 1 cm Blue A Matrex gel column previously equilibrated in dialysis buffer A. Fractions containing GS activity were pooled and dialyzed overnight in dialysis buffer B (50 mM imidazole/HCl [pH 6.5], containing 10 mM $MgCl_2$, 15% [v/v] ethylene glycol, and 1 mM each EDTA and DTT). After dialysis, the two forms of pedicel GS were separated by chromatography on a 12 × 1.6 cm column of Q-Sepharose as follows: the enzyme was applied to the column, which had been previously equilibrated with dialysis buffer B, washed with 25 mL of the same buffer and eluted with a 250 linear mL 0 to 225 mM NaCl gradient (all in dialysis buffer B), collecting 2.75-mL fractions. Pedicel GS isozymes were pooled separately, dialyzed overnight in dialysis buffer A, then aliquoted and stored at −70°C.

Relative Molecular Mass Determinations

Native M_r was estimated with a Superose 6 gel filtration column calibrated with the following standards: yeast alcohol dehydrogenase (M_r = 150,000); sweet potato β-amylase (200,000); Jack bean urease trimer (272,000); *Escherichia coli* α-galactosidase (465,000); and Jack bean urease hexamer (545,000). Two-hundred-μL samples of pedicel GS or standards were applied to the column via a sample loop. The column was equilibrated and run in 50 mM Tris/HCl (pH 7.6), containing 1 mM $MgCl_2$, 10% ethylene glycol (v/v), and 1 mM DTT, at a flow rate of 0.2 mL/min. One-half-mL fractions were collected. Standards were detected by measuring the absorbance at 280 nm of the fractions while GS was detected by the transferase assay.

Subunit M_r was estimated by SDS-PAGE. Protein samples were precipitated by the addition of equal volumes of 20% TCA, followed by incubation overnight at 4°C. Protein was

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that might also be suitable.

collected by centrifugation, and pellets were resuspended with 1 volume of dissociation solution (8 M urea, 4% SDS, 4% 2-mercaptoethanol) and 1 volume of separation gel buffer (1.5 M Tris, 0.4% SDS) plus 2 μL of 0.1% bromphenol blue tracking dye. Samples were boiled for 5 min, clarified by centrifugation, applied to a 12.5% polyacrylamide gel, and electrophoresed at 10 mA for 30 min and 20 mA until the bromphenol blue marker reached the bottom of the gel (about 4 h). Gels were stained for protein with a 40% methanol solution containing 0.08% (w/v) Coomassie brilliant blue G-250, 0.08% (w/v) Coomassie brilliant blue R-250, and 0.07% $HClO_4$ and were then destained with 10% acetic acid.

Enzyme Assays, Kinetic Studies, and Protein Determination

The semibiosynthetic (hydroxylamine-dependent biosynthetic) GS assay method has been described previously (21). Transferase activity was measured as described by O'Neal and Joy (24). The concentrations of the assay components were: imidazole/HCl (pH 7.0), 50 mM; $MnSO_4$, 4 mM; ADP, 5 mM; glutamine, 50 mM; sodium arsenate, 40 mM; and hydroxylamine (pH 7.0), 25 mM. An enzyme-coupled, continuous biosynthetic GS assay that measures ADP production (10) was used for kinetic studies on the purified pedicel GS isozymes. Substrate and cofactor concentrations for the coupled assay are given in the figure legends. An assay which quantitates phosphate liberation (10) was used for studying the effects of added ADP on GS activity. Substrate concentrations used for establishing known free Mg^{2+} concentrations were calculated as described by Morrison (20), using the stability constants of O'Sullivan and Smithers (25).

Initial rate data for the determination of K_m values were collected by maintaining one of the three substrates at high, but not saturating, levels while the other two substrates were varied (2). K_m values were determined from replots of intercepts from the analysis of initial rate data. Estimates of kinetic parameters were obtained using a computer program for nonlinear regression analysis of the Michaelis-Menten equation (8).

Protein was quantitated by the method of Bradford (4) using fraction V BSA as the standard.

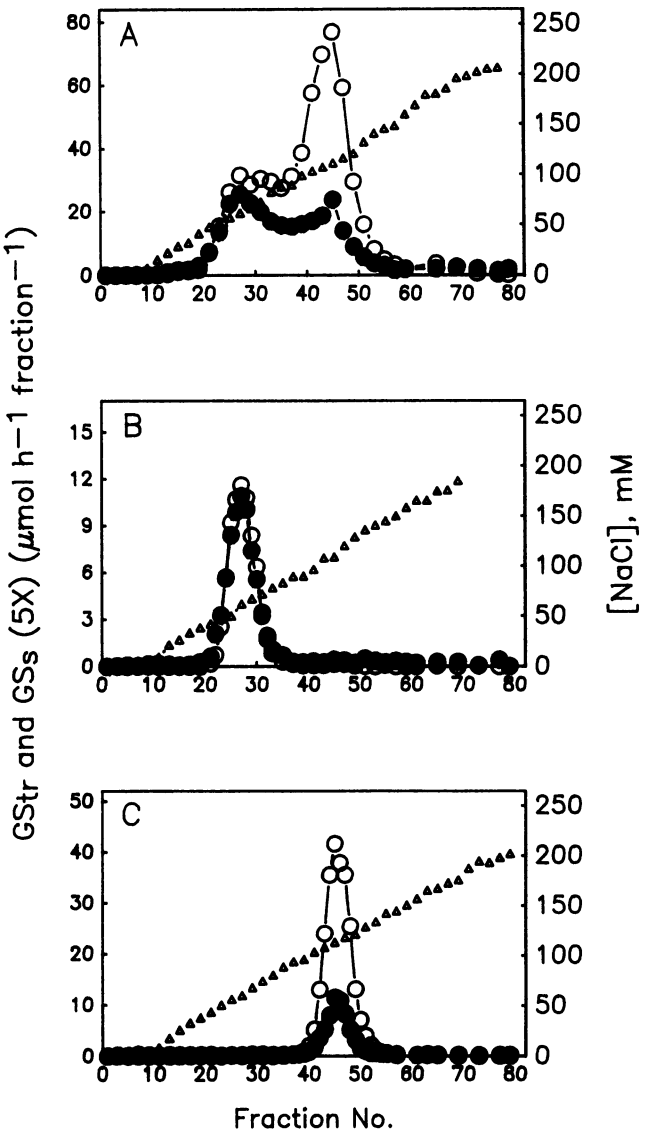

Figure 1. Anion exchange chromatography of a maize pedicel extract (A). Rechromatography of GS_{p1} (B) and GS_{p2} (C). (○), GS_{tr} (transferase) activity; (●), GS_s (semibiosynthetic) activity; (△), [NaCl].

RESULTS

Anion Exchange Chromatography

When extracts of the pedicel region of maize were subjected to anion exchange chromatography at 6.5, GS activity eluted as two peaks, designated GS_{p1} and GS_{p2} for the earlier and later eluting activities, respectively (Fig. 1). These two GS isozymes had different ratios of transferase to semibiosynthetic activities, 5 for GS_{p1} and 23 for GS_{p2}, which did not vary with kernel age. Upon rechromatography, each form eluted as a single peak at the same position at which it eluted when the two activities were initially separated (Fig. 1). Moreover, the presence of two pedicel GS activity peaks was not due to the hybrid nature of the maize variety used since anion exchange profiles of extracts of the inbred W64A contained the same two peaks of GS activity (data not presented). Each enzyme form exhibited its own characteristic developmental pattern, *i.e.* GS_{p1} activity was relatively low in very young kernels (15 DAP), high in kernels harvested in the middle stage of development (30 DAP) and once again low in mature (45 DAP) kernels (Fig. 2). In contrast, GS_{p2} activity was similar in kernels harvested at 15 and 30 DAP, but greatly diminished in kernels harvested 45 DAP (Fig. 2).

Separation of the two forms of pedicel GS by anion exchange chromatography required 10-fold greater hydrogen ion concentrations and a less steep salt elution gradient than is typically reported in the literature for separating the cytoplasmic and chloroplastic leaf GS isozymes (1, 18). A comparison of anion exchange elution profiles of GS activity from maize kernel pedicel, root, and leaf extracts chromatographed under the conditions required to separate the two pedicel GS activities is shown in Figure 3. Kernel GS_{p1} eluted much

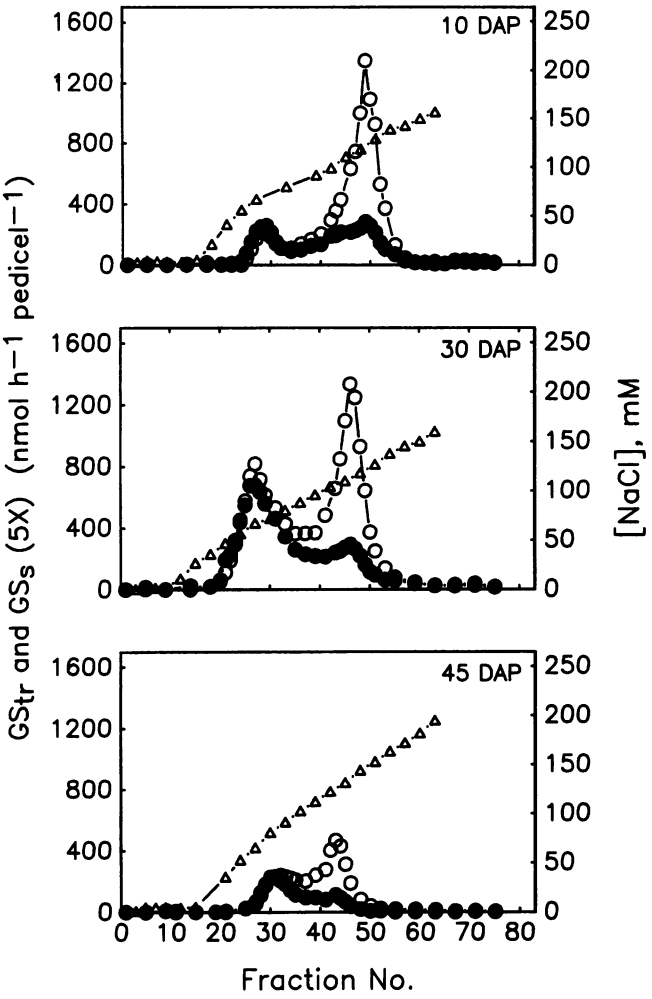

Figure 2. Glutamine synthetase activity elution profiles of 10, 30, and 45 DAP maize kernel pedicel extracts during anion exchange chromatography at pH 6.5 on a Mono Q column. (○), GS_{tr} (transferase) activity; (●), GS_s (semibiosynthetic) activity; (△), [NaCl].

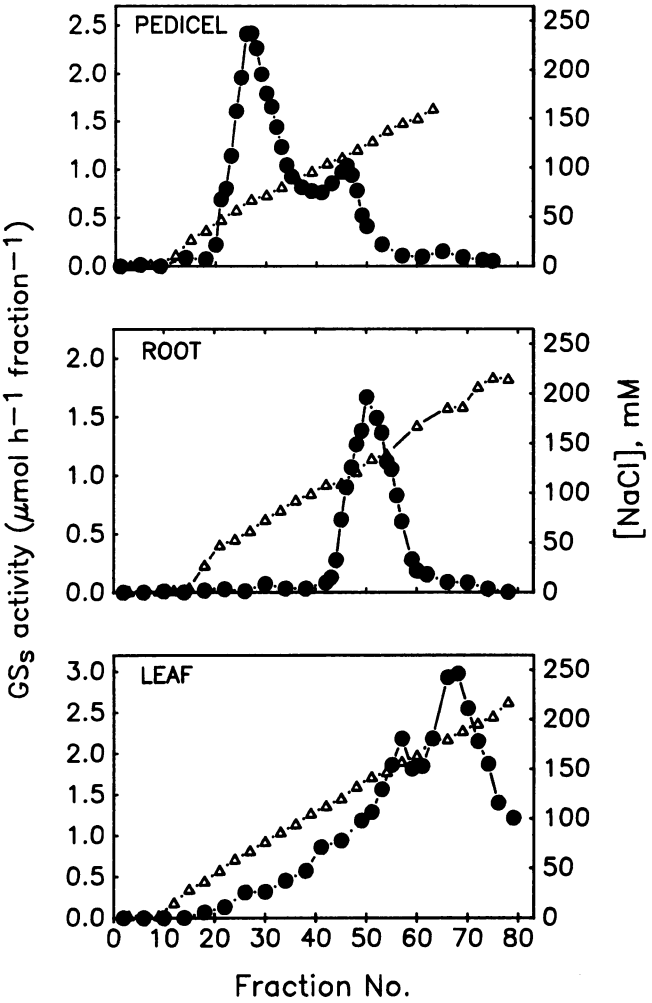

Figure 3. Comparison of elution profiles for maize kernel pedicel, root and leaf glutamine synthetase activities during anion exchange chromatography at pH 6.5 on a Mono Q column. (●), GS_s (semibiosynthetic) activity; (△), [NaCl].

earlier than either the root or the leaf GS isozymes. In contrast, GS_{p2} eluted in a NaCl concentration range similar to that of the root or the first-eluting leaf GS isozyme. The modified chromatographic conditions required for separation of GS_{p1} and GS_{p2} resulted in poor resolution of the two leaf GS isozymes.

Purification and Characterization of Maize Kernel Pedicel GS

Glutamine synthetase from maize kernel pedicels was purified by a combination of anion exchange, hydroxyapatite, hydrophobic interaction, and dye-ligand chromatographies, yielding GS_{p1} and GS_{p2} preparations with specific activities of 73 and 38 μmol min^{-1} mg $protein^{-1}$, respectively (Table I). When subjected to SDS-PAGE and stained with Coomassie blue, purified GS_{p1} and GS_{p2} preparations exhibited single major protein bands of M_r 38,900 and 40,500, respectively (Fig. 4). Despite their relatively high specific activities, both isozyme preparations contained several minor additional bands and therefore have not been purified to homogeneity.

The two pedicel isozymes were indistinguishable upon gel filtration on a Superose 6 column under nondenaturing conditions (not shown). Native M_r for both GS_{p1} and GS_{p2} was estimated to be 340,000 ± 14,000 (n = 4). When purified, both forms of pedicel GS had broad pH optima centered around 7.0 and exhibited the same transferase to semibiosynthetic activity ratios as they did when simply separated from each other by anion exchange chromatography of total homogenates (data not presented).

The kinetic properties of the two purified pedicel GS forms were analyzed. GS_{p2} followed simple Michaelis-Menten kinetics for both glutamate and ATP, but exhibited positive cooperativity for ammonia (n_H = 1.8). Double reciprocal plots of GS_{p2} activity *versus* glutamate at various fixed ATP concentrations (Fig. 5A) or *versus* ATP at various fixed glutamate concentrations (Fig. 5B) resulted in a family of linear curves which intersected in the second quadrant. Intercept replots of Figure 5, A and B (not shown) were linear, with correlation coefficients >0.99, and K_m values of 0.68 mM (SE = 0.06 mM) and 3.4 mM (SE = 0.06 mM) were obtained for ATP and

Table I. *A Typical Purification of Glutamine Synthetase from Pedicel Portions of Maize Kernels Harvested 25 to 30 DAP*

Step	Total Activity[a]	Protein	Specific Activity	Purification Factor	Recovery
	μmol min^{-1}	mg	μmol min^{-1} mg^{-1}	X	%
Clarified homogenate	83.2	119	0.7	1	
Q-Sepharose	88.2	50	1.8	2.6	106
Hydroxyapatite	64.7	4.2	15.4	22	78
Phenyl-Sepharose	72.7	1.5	48.5	69	87
Blue A + Q-Sepharose					
GS_{p1}	13.2	0.18	73.3	105	16
GS_{p2}	5.1	0.14	37.5	54	6

[a] Semibiosynthetic assay.

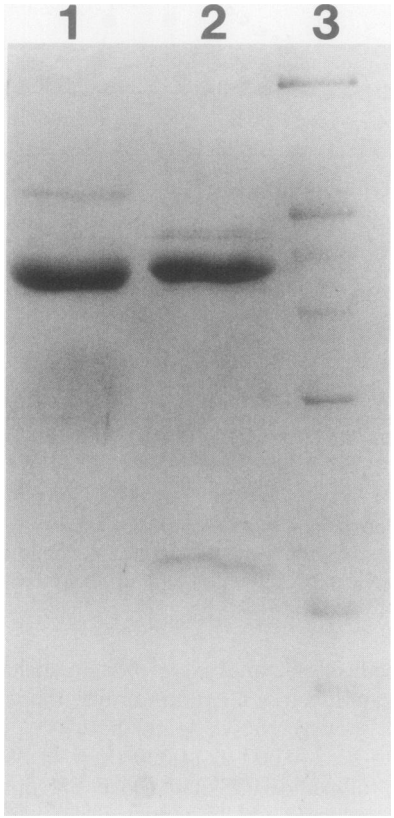

Figure 4. SDS-PAGE of purified GS_{p1} (lane 1) and GS_{p2} (lane 2). M_r standards (lane 3) are: Cyt *c* (12,400), lactoglobulin (18,400), carbonic anhydrase (29,000), lactate dehydrogenase (36,000), ovalbumin (43,000), glutamate dehydrogenase (55,000), and phosphorylase B (95,000). Fifteen μg of protein were loaded onto lanes 1 and 2.

glutamate, respectively. When assayed at saturating concentrations of glutamate and ATP, the $S_{0.5}$ value for ammonia was estimated at 45 μM.

The kinetic characteristics of GS_{p1} differed greatly from those of GS_{p2} and appeared to be quite complex. Glutamate exhibited typical hyperbolic saturation kinetics up to 90 mM (apparent K_m = 35 mM), but was inhibitory at concentrations greater than 90 mM (Fig. 6A). In contrast, glutamate concentrations up to 360 mM did not inhibit GS_{p2} activity (data not presented). GS_{p1} exhibited nonhyperbolic saturation curves for ATP and ammonia (Fig. 6, B and C), denoting positive cooperativity in both cases (n_H = 1.8 and 1.5, respectively). When assayed at 90 mM glutamate and saturating ATP concentration, the $S_{0.5}$ values for ammonia was 100 μM.

The apparent positive cooperativity of ATP binding to GS_{p1} could be the result of changing free Mg^{2+} concentrations when ATP and $MgCl_2$ are supplied at a fixed ratio. To test this possibility, GS_{p1} (as well as GS_{p2}) activity was determined as a function of free Mg^{2+} concentrations (Fig. 7). The results show that both pedicel GS isozymes require free Mg^{2+} for activity, with apparent K_m values of 2.0 (SE = 0.05) and 0.19 (SE = 0.02) mM for GS_{p1} and GS_{p2}, respectively. When GS_{p1} activity was measured as a function of MgATP concentration at saturating, constant free Mg^{2+} (20 mM), the enzyme exhibited typical Michaelis-Menten kinetics, with an apparent K_m of 0.76 mM (data not presented).

The effects of a number of compounds previously shown to inhibit the activities of various plant GSs were tested on purified GS_{p1} and GS_{p2}. Neither pedicel GS activity was greatly affected by most of these potential inhibitors (Table II). GS_{p1} activity was less sensitive to inhibition by the irreversible inhibitor MSO than was GS_{p2} activity. Inhibition by MSO was competitive with respect to glutamate for both isozymes, with K_i values of 1.3 and 0.17 mM for GS_{p1} and GS_{p2}, respectively. Five-mM ADP inhibited approximately 25% of both pedicel GS activities. Double-reciprocal plots of GS_{p1} activity *versus* ATP concentration at various fixed ADP concentrations resulted in a family of lines which converged on the Y-axis, indicative of competitive inhibition (not shown). A K_i value of 0.7 mM was obtained from a replot of slope values *versus* ADP concentration. ADP was also a competitive inhibitor of GS_{p2} (data not presented).

DISCUSSION

Total pedicel GS activity increases between 10 and 30 DAP in developing maize kernels, concomitant with the period of rapid kernel N accumulation (21). Beyond 30 DAP, pedicel GS activity decreases steadily as the kernels reach maturity (21). Comparison of the anion exchange profiles for pedicel extracts from various age kernels suggests that the increase in

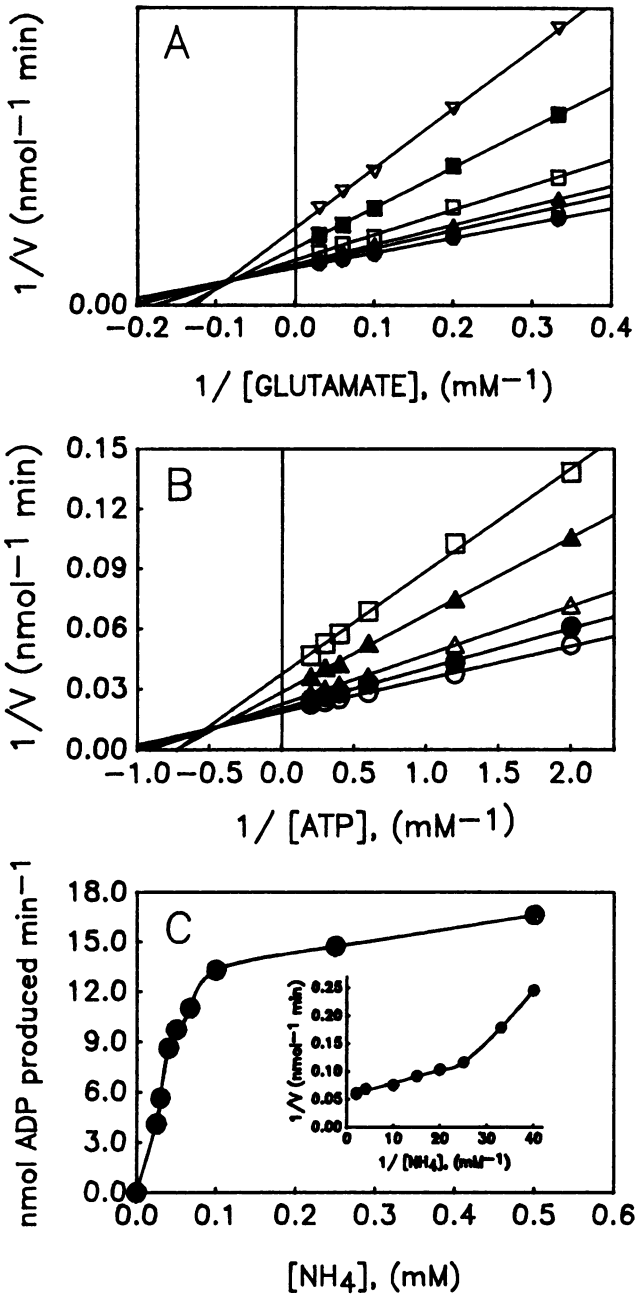

Figure 5. Kinetic analysis of maize kernel GS_{p2}. A, Double reciprocal plot of enzyme rate *versus* [glutamate] at various fixed [ATP]. [ATP] = (▽), 0.5 mM; (■), 0.83 mM; (□), 1.66 mM; (▲), 2.5 mM; (△), 3.33 mM; (●), 5 mM. [NH_4Cl] = 1 mM. B: Double reciprocal plot of enzyme rate *versus* [ATP] at various fixed [L-glutamate]. [L-glutamate] = (□), 3 mM; (▲), 5 mM; (△), 6.7 mM; (●), 10 mM; (○), 33.3 mM. [NH_4Cl] = 1 mM. C: Enzyme rate *versus* [NH_4Cl]. [L-glutamate] = 35 mM, [ATP] = 4 mM. Inset: Double reciprocal plot of enzyme rate *versus* [NH_4Cl]. Mg:ATP = 2 in all three panels.

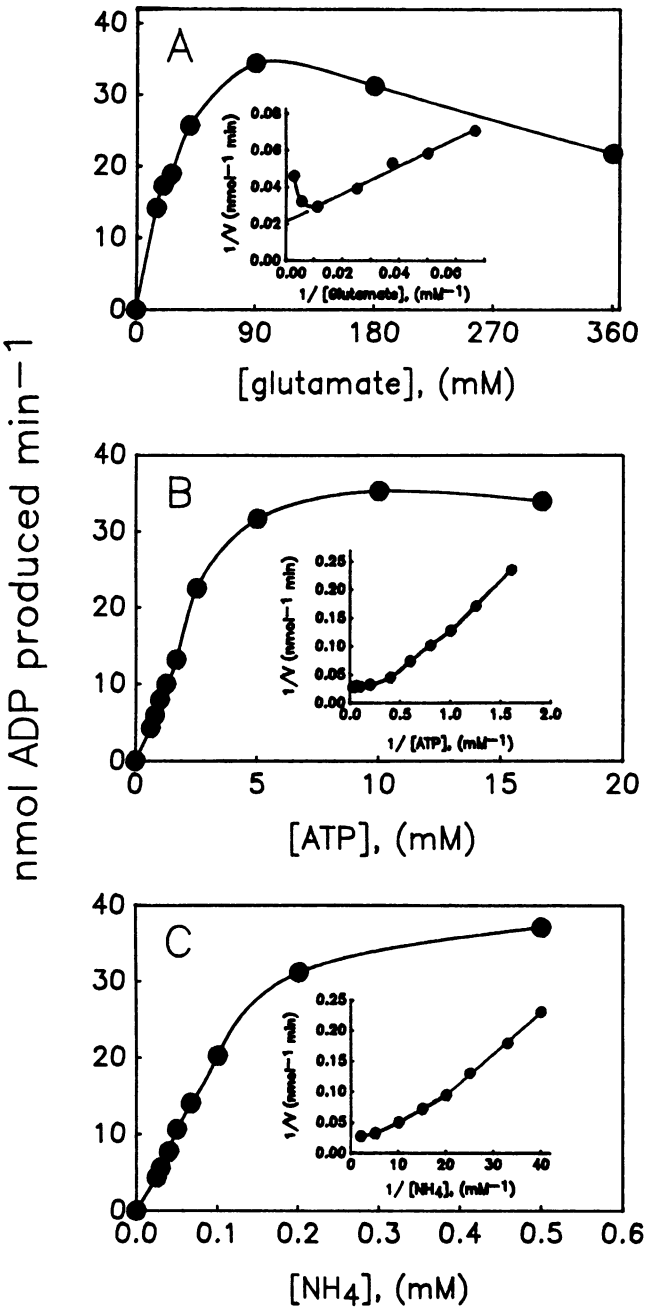

Figure 6. Kinetic analysis of maize kernel GS_{p1}. A, Enzyme rate *versus* [L-glutamate]. [NH_4Cl] = 1 mM, [ATP] = 15 mM. Inset, Double reciprocal plot of enzyme rate *versus* [L-glutamate]. B, enzyme rate *versus* [ATP]. [L-Glutamate] = 90 mM, [NH_4Cl] = 1 mM. Inset, Double reciprocal plot of enzyme rate *versus* [ATP]. C, Enzyme rate *versus* [NH_4Cl]. [L-Glutamate] = 90 mM, [ATP] = 15 mM. Inset, Double reciprocal plot of enzyme rate *versus* [NH_4Cl].

total GS activity between d 10 and 30 DAP is due solely to increased GS_{p1} activity. GS_{p1} activity was clearly resolved from GS_{p2} activity as well as from the GS activities of maize root and leaves upon anion exchange chromatography (Fig. 3). Its distinct elution profile, along with its unusual kinetic properties with respect to glutamate suggest that GS_{p1} is a unique, kernel-specific form of maize GS. Interestingly, a somewhat analogous situation has been described for *Phaseolous vulgaris* root nodules, where there are two GS forms with differing transferase to synthetase activity ratios, only one of which is developmentally regulated (6, 13).

The native (340,000) and subunit (38,900 and 40,500 for

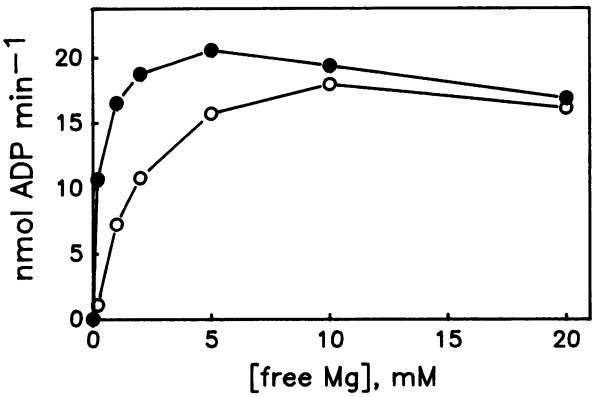

Figure 7. Effects of free Mg^{2+} on pedicel GS activities. Assay components included imidazole/HCl (50 mM, pH 7.0), L-glutamate (90 mM), NH_4Cl (1 mM), MgATP (5 mM), free Mg^{2+} as indicated. (○), GS_{p1}; (●), GS_{p2}.

Table II. *Effects of Various Compounds on Purified GS_{p1} and GS_{p2}*

Semibiosynthetic assays were conducted in microtiter plates, using 0.72 μg of GS_{p1} or 0.80 μg of GS_{p2} in a final volume of 0.2 mL. Assays incubated at 35°C for 15 min and stopped with 0.15 mL of ferric chloride solution (24). Two hundred μL portions of the assay solutions were transferred to a new microtiter plate and the absorbances at 540 nm were measured. Control activities were 0.221 and 0.146 μmol of γ-glutamylhydroxymate produced h^{-1}. $n = 2$.

Compound	Control	
	GS_{p1}	GS_{p2}
mM	%	
ADP (5)	78	76
AMP (5)	89	93
CTP (5)	98	104
NAD (5)	106	101
NADH (5)	110	109
Asp (10)	97	95
Asn (10)	100	99
Gln (10)	100	116
Gly (10)	98	86
Ala (10)	102	90
Ser (10)	99	97
His (10)	106	89
Trp (10)	105	89
Phospho-SER (10)	94	103
α-Ketoglutarate (20)	84	93
Citrate (20)	73	104
Malate (20)	106	103
Carbamoyl-phosphate (5)	105	89
GlcN-6-phosphate (5)	107	101
MSO (5)	81	52

GS_{p1} and GS_{p2}, respectively) M_r values of the purified pedicel GS isozymes are in the range typically reported for plant GS (27) and are consistent with both pedicel isozymes being homooctomers. Purified GS_{p2} exhibited typical Michaelis-Menten kinetics for glutamate and ATP (Fig. 5) and the families of intersecting lines suggest a sequential enzyme mechanism for Mg:ATP and glutamate binding, as has been found for lupin GS (17) as well as for bacterial (19) and mammalian (2) glutamine synthetases. K_m values for glutamate (3.4 mM) and for ATP (0.68 mM) are similar to those obtained by McCormack *et al.* for lupin nodule GS (17), and well within the range of apparent K_m values listed for various plant species in (27). Positive cooperativity of NH_4 binding, as exhibited by both pedicel GS isozymes, has previously been observed with the two GS forms from *Phaseolus* nodules (6), but was not observed for lupin nodule GS (17). Substrate inhibition of GS_{p1} activity by glutamate has not been reported previously for a plant GS.

Both pedicel isozymes of GS required free Mg^{2+}, with the K_m value for GS_{p1} being approximately 10-fold higher than that for GS_{p2}. In the reaction mechanism of *Escherichia coli* GS, free Mg^{2+} is thought to play a role in coordinating the γ-carboxyl group of glutamate and the γ-phosphate group of ATP within the active site (5). Although a free Mg^{2+} requirement for plant GS has been suggested previously, based on the stimulation of enzyme activity when Mg^{2+} is added in excess of ATP (27), the present study represents the first time that the effects of free Mg^{2+} on the activity of a plant GS have been specifically considered. The free Mg^{2+} requirement for plant GS enzymes explains the sigmoidal saturation kinetics that have been described for some plant GS activities with respect to total Mg^{2+} (*e.g.* 23) in that enzyme activity would be low at Mg^{2+} concentrations below the fixed ATP concentration as most of the added Mg^{2+} would be chelated by ATP. As Mg^{2+} is added in excess of ATP, however, there would be increasing amounts of free Mg^{2+}, which would stimulate activity in a hyperbolic fashion as free Mg^{2+} becomes saturating. When the data in Figure 7 were plotted as a function of total rather than free Mg^{2+}, both curves appeared sigmoidal (not shown).

The competitive nature of the ADP inhibition of both pedicel GS isozymes is in contrast to the rather complex effects of ADP on pea seed GS activity (12). Knight and Langston-Unkefer (12) presented evidence suggesting that the pea seed GS is activated by ADP via interaction at allosteric sites on the enzyme. The simple competitive inhibiton of GS_{p1} by ADP suggests that maize pedicel GS may not contain such ADP-binding sites, or if it does, these sites are already filled, since ADP acts as a simple competitive inhibitor of pea seed GS activity when its ADP-binding allosteric sites were filled (12). Experiments to test this possibility are in progress. Regardless of possible allosteric effects, the K_i of 0.70 mM for ADP inhibition of GS_{p1} is in the same range as the apparent K_m value for ATP (0.76 mM) and therefore could affect GS_{p1} activity *in vivo*.

Comparison of the free amino acid pools of the cob vascular sap, the pedicel region, and the endosperm (15), metabolism of radiolabeled aspartate and glutamate in the pedicels of isolated kernels (16), and pulse-chase studies with intact plants (26) all suggest that considerable metabolism of the transport forms of nitrogen takes place prior to their incorporation into storage proteins. Nitrogen is not assimilated only once in higher plants, but may be released and reassimilated several times before it enters the seed protein, including during the catabolism of transport compounds in the developing fruit

(14). As GS_{p1} increases during the period of kernel development wherein most of the nitrogen assimilation takes place, it seems likely that the function of this kernel-specific form of GS is to recapture ammonia released during this process. As glutamate is one of the major forms in which nitrogen is delivered to the pedicel region from the phloem (15, 26), GS_{p1} could presumably still efficiently catalyze glutamine synthesis despite a relatively high apparent K_m for this substrate. It seems unlikely, however, that substrate inhibition of GS_{p1} *in vivo* by high glutamate concentrations (>90 mM) would be significant. The level of GS_{p2} activity apparently remains constant from early to midkernel development, when nitrogen assimilation is maximal. Whether GS_{p2} also participates in the recapture of ammonia released during the metabolism of nitrogenous transport compounds is not known. Perhaps GS_{p2} is not involved in kernel nitrogen assimilation, but rather is a constitutive enzyme required for cell maintenance. Determination of the specific tissue localization of the individual pedicel isozymes would be useful in helping to understand the roles that these GS forms play in developing maize kernels.

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