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Purification and Properties of Glutamine Synthetase in Leaves and Roots of *Pinus banksiana* Lamb.[1]

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ABSTRACT

A method is described for the purification of glutamine synthetase (GS; EC. 6.3.1.2) from the leaves and roots of *Pinus banksiana* Lamb., a conifer which utilizes ammonium as its primary nitrogen source. The enzyme was purified to apparent homogeneity by a procedure involving salt fractionation as well as ion-exchange, size exclusion, and affinity chromatography. Since the final preparation produced two bands on SDS polyacryamide gels but only one band on a nondenaturating gel, it is concluded that the two subunits (44 and 40 kilodaltons, respectively) are part of a single enzymatic protein which shows GS activity. The pH optimum for leaf GS ranged between 6.2 and 6.5, one pH unit lower than the values reported for higher plants which utilize primarily nitrate nitrogen. Magnesium requirements for GS in *P. banksiana* were different for leaves and roots, showing $V_{max}/2$ values of 2.5 and 8 millimolar, respectively at 5 millimolar ATP. Furthermore, K_m values for ammonium were higher for the enzyme in leaves (33.1 micromolar) than in roots (19.2 micromolar). K_m values for ATP and for glutamate, on the other hand, were similar for the two tissues. A polyclonal antibody was produced against the purified leaf GS. Western blots of leaf homogenates produced two bands, the lighter one being more abundant. The same pattern was found when immunodetection was performed using an anti GS IgG produced against purified GS from *Phaseolus* nodules thus indicating common antigenic determinants. At least 30% of total GS was recovered in a plastid-fraction of dark-grown calli produced from the basal part of *P. banksiana* hypocotyls.

Conifers such as jack pine (*Pinus banksiana* Lamb.) grow on acidic soils in the boreal forest region where very little nitrification occurs. They are thus forced to rely on ammonium as their primary nitrogen source (4, 6). Upon its entry into the root, free ammonium has to be assimilated into organic molecules because small increases in its concentration can easily result in physiological and metabolic disorders (22). In higher plants as well as in many other eukaryotic and prokaryotic organisms, ammonium is assimilated mainly through the action of the GS[2]/GOGAT cycle (22). Although the nitrogen assimilatory pathways have been well characterized in ammonium growing unicellular algae and aquatic multicellular plants (27), very little information is available on the major assimilatory pathways for nitrogen in higher land plants which grow primarily on ammonium-nitrogen.

It has been recently demonstrated that GS is found in roots and leaves of *P. banksiana* (32) as well as other conifers (30). Since reliable techniques now exist to extract and measure the activity of GS in conifers, it is now possible to obtain information about the regulatory characteristics of this enzyme in conifer tissue. We believe that conifers represent an interesting model with which to study the regulation of the GS enzyme. This is because GS in conifer roots is located in a tissue where the assimilatory flow of its primary substrate, free ammonium ions, is far more important than that found in most other higher plants.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical Co. The DEAE-Sephacel, G-50, and Sepharose 4B matrices were obtained from Pharmacia. The hydroxyapatite matrix, the Bio-beads and all SDS-PAGE and immunoblotting material were purchased from Bio-Rad Laboratories. The ^{125}I-labeled anti-rabbit IgG was purchased from Amersham.

Purification of GS from Roots and Leaves

Plant Material

Two-year-old *Pinus banksiana* Lamb. seedlings were grown in an outdoor nursery according to the standard procedure for containerized seedling production, as described previously (19). Plants were harvested in early September, their leaves and roots were separated, and then frozen immediately at −80°C. For the intracellular localization of GS activity, *P. banksiana* Lamb. seeds were germinated in vermiculite and kept in an environment-controlled propagation chamber under a day/night temperature regime of 22°C/18°C, a 16 h photoperiod and a light intensity of about 350 $\mu mol \cdot s^{-1} \cdot m^{-2}$. They were allowed to grow until the cotyledons were fully expanded. The seedlings were watered regularly throughout this period but no fertilizer was added.

Extraction Procedure

Either 100 g of frozen leaf tissue or 500 g of frozen root tissue was pulverized in 3 volumes of a medium consisting of 100 mM Tris (pH 7.8), 10 mM $MgSO_4$, 5 mM Glu, 0.75% (w/

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[2] Abbreviation: GS, glutamine synthetase; GOGAT, glutamate synthase.

v) Nonidet P-40, 5% (v/v) glycerol, and 6 mM β-mercaptoethanol using a Polytron ultrasonic homogenizer. The homogenate was squeezed through four layers of cheese cloth and centrifuged at 30,000*g* for 20 min.

Protamine Sulfate and Ammonium Sulfate Fractionation

A freshly prepared 1% aqueous solution of protamine sulfate (7.5% of the volume) was added by drops to the clarified supernatant. The precipitate was removed by centrifugation at 30,000*g* for 15 min. The clarified extract was brought to 35% saturation $(NH_4)_2SO_4$ and the precipitate was removed by centrifugation at 30,000*g* for 15 min. Then, the supernatant was brought to 65%-saturation $(NH_4)_2SO_4$. The solution was centrifuged and the precipitate was dissolved in 20 mL of buffer containing 20 mM Tris (pH 7.8), 5 mM Glu, 10 mM $MgSO_4$, and 5 mM β-mercaptoethanol. This buffer was used in all subsequent steps unless otherwise stated. One g of Bio-beads SM-2 was then added per 10 mL of the resuspended ammonium sulphate precipitate and the mixture was placed on a rotary shaker at 4°C for 30 min. The Bio-beads with the absorbed detergent were removed by filtration on a 20 μM nylon membrane. The solution containing the dissolved proteins without the detergent was then desalted on a G-50 column pre-equilibrated with buffer.

DEAE-Sephacel Chromatography

The desalted protein extract was loaded on a DEAE-Sephacel column (2.5 × 20 cm) preequilibrated with buffer. The column was washed with 100 mL of buffer and GS was eluted by a 250 mL linear KCl gradient (0–0.5 M). Fractions of approximately 2.5 mL were collected at 28 $mL \cdot h^{-1}$, and those containing GS activity were pooled (approximately 20 mL). The solution containing GS activity was then brought to 80%-saturation $(NH_4)_2SO_4$, the precipitate was removed by centrifugation and the pellet resuspended in 5 mL of buffer.

Sepharose-4B Chromatography

The extract was next applied to a column (2.5 × 30 cm) of Sepharose 4B preequilibrated in 10 mM KH_2PO_4 (pH 7.0), 5 mM Glu, 1 mM $MgSO_4$ and 6 mM β-mercaptoethanol (phosphate buffer) and the protein eluted at 20 $mL \cdot h^{-1}$ in the same buffer. Fractions of approximately 5 mL were collected and the ones containing GS activity were pooled (20 mL).

Hydroxyapatite Chromatography

The extract was then loaded on a Bio-gel HTP column (0.5 × 7 cm) preequilibrated in phosphate buffer. Glutamine synthetase was then eluted by a 50 mL-linear KH_2PO_4 gradient (10–250 mM). Fractions of 1 mL were collected at 28 $mL \cdot h^{-1}$ and those containing GS activity (10 mL) were pooled. The purified protein was further concentrated by precipitating it at 80%-saturation $(NH_4)_2SO_4$ and resuspending the pellet in 10 mM KH_2PO_4 (pH 7.0), 5 mM Glu, 10 mM $MgSO_4$, and 6 mM β-mercaptoethanol.

Production of Pine-Leaf GS Antibodies

Four rabbits were immunized by four repeated subcutaneous injections each containing 250 μg of the purified leaf protein in 250 μL saline solution and 250 μL complete Freund's adjuvant. Booster injections were given every 2 weeks with incomplete Freund's adjuvant and the purified protein. Rabbits were bled 1 week after the last injection.

SDS-PAGE and Western Blots

Denaturating polyacrylamide electrophoresis was performed according to Laemmli (17) using a Mini-protean electrophoresis unit from Bio-Rad. Proteins were electrotransferred on nitrocellulose membranes using a Mini-Transblot unit from Bio-Rad. Immunodetection was performed as described previously (33) using the IgG raised against the protein purified from *P. banksiana* leaves or an IgG raised against *Phaseolus* nodule GS (10) to determine GS subunit composition in different fractions and the cross-reactivity of pine leaf GS.

Protein Determination

Protein level in extracts was determined according to the method of Lowry (18).

Native PAGE

Nondenaturating electrophoresis of the purified fractions was performed on slab gels as described by Davis (12) for cylindrical gels. Minigels with stacking gels were run for 80 min on the Mini-Protean system at 200 V. Glutamine synthetase activity was detected by the transferase assay as de-

Table I. *Purification of Glutamine Synthetase from P. banksiana Leaves*

	Total Protein	Synthetase Activity	Specific Activity	Purification	Recovery
	mg	*μmol·min⁻¹*	*μmol·min⁻¹·mg⁻¹ protein*	*-fold*	*%*
Crude extract	2,660	3.80	0.0014	1.0	100
Protamine sulphate supernatant	2,492	4.05	0.0016	1.13	106
35–65% saturated $(NH_4)_2SO_4$	36.2	1.73	0.048	33.5	45
DEAE-sephacel	13.7	0.84	0.061	42.9	22
Sepharose 4B	3.9	0.47	0.121	84.3	12
Hydroxyapatite	0.8	0.24	0.302	210	6

scribed by Barratt (3). Glutamine synthetase and other proteins were detected on acrylamide gels by staining with Coomassie blue R.

Intracellular Localization

Being highly lignified, even in its juvenile state, conifer root tissue is not a suitable source of intact organelles for the study of enzyme intracellular localization. To avoid the inherent problem of organelle extraction from this type of tissue, the cellular distribution of GS activity was determined by a coarse sedimentation procedure on homogenates obtained from dark-grown calli.

Callus Culture

At the end of the growing period (see "Plant Material"), a 1-cm section was cut off the basal portion of the hypocotyl. These sections were sterilized by immersion (30 s) in a 5% (v/v) sodium hypochlorite solution. They were rinsed and transferred on a nutritive agar prepared as described by Durzan *et al.* (13). They were kept in total darkness at 21°C. Calli were subsequently divided and subcultured at 3-week intervals.

Fractionation of Cellular Constituents

Two-and-a-half g of calli were gently homogenized in a prechilled mortar pestle with 5 volumes of an extraction buffer consisting of 100 mM Tricine-KOH at pH 7.9, 396 mM sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 0.1% BSA, and 6 mM β-mercaptoethanol. The brei was filtered through one layer of Miracloth and centrifuged for 1 min at 600*g* to remove cell debris. The resulting supernatant was then centrifuged at 5,000*g* for 5 min, the pellet removed, and the supernatant centrifuged again at 12,000*g* for 15 min. Both pellets (5,000 and 12,000*g*) were resuspended in 5 mL of a buffer (A) consisting of 100 mM Tricine-KOH at pH 7.9, 330 mM sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 0.1% BSA, and 6 mM β-mercaptoethanol, and the two suspensions were centrifuged for 5 min at 5,000*g* and for 15 min at 12,000*g*, respectively. The resulting washed pellets were resuspended in 3 mL of buffer A with the addition of 0.5% Nonidet P-40. Aliquots of the initial homogenate and the final supernatant were also brought to 0.5% Nonidet P-40. All fractions remained at +4°C for at least 20 min with the added nonionic detergent to allow the solubilization of organelle membranes prior to the enzyme measurements.

Enzyme Assays

In all the fractions, GS activity was determined by the semibiosynthetic assay as described by Wallsgrove *et al.* (34), nitrite reductase was assayed according to Dalling *et al.* (11), triosephosphate isomerase according to Burton and Waley (7) and Cyt *c* oxidase as described previously (33).

Enzyme Kinetics

Kinetic measurements of leaf and root GS were made on protein purified by the gel filtration step of the purification

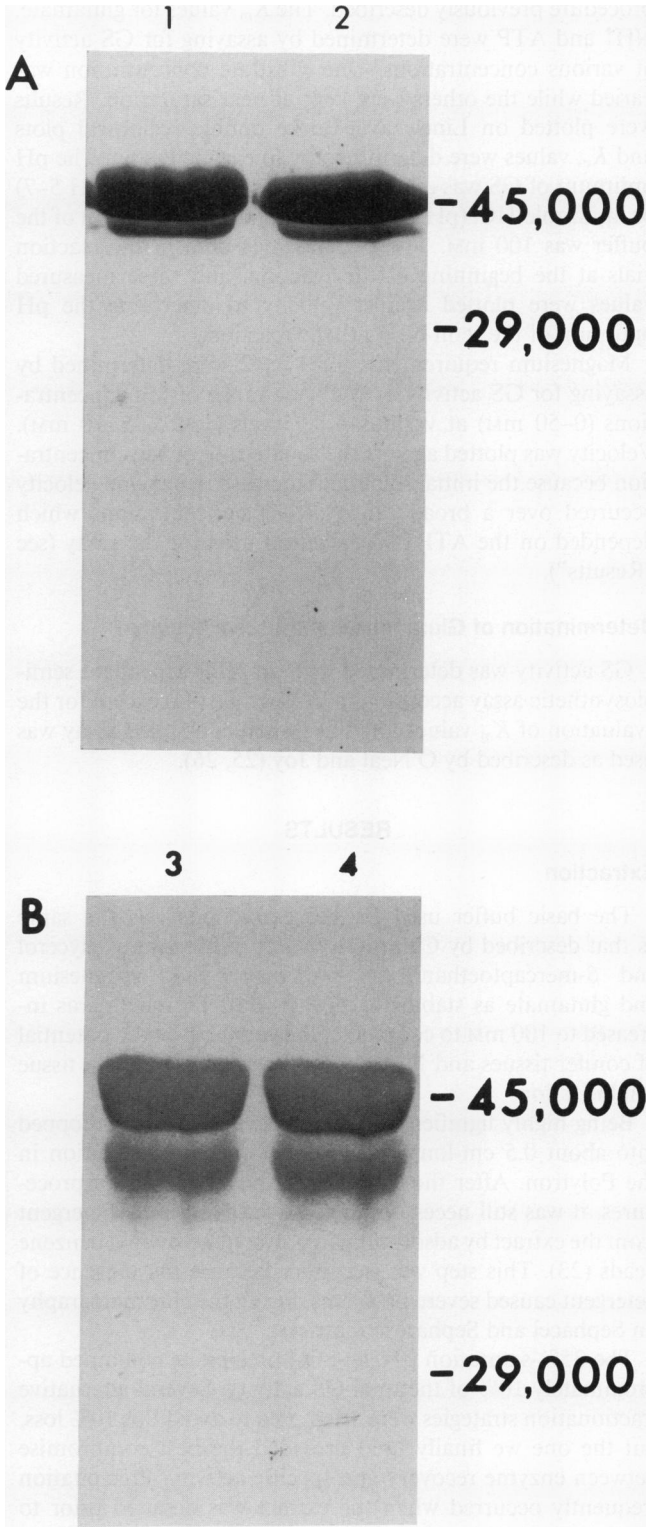

Figure 1. Denaturating PAGE of the purified GS enzymes from *P. banksiana*. Purification procedures were as described in "Materials and Methods." A, Purified GS (30 μg) from needles were loaded in each well (lanes 1 and 2); B, 20 μg of purified GS from roots were loaded in each well (lanes 3 and 4). Proteins were separated by electrophoresis and stained with Coomassie blue.

procedure previously described. The K_m values for glutamate, NH_4^+ and ATP were determined by assaying for GS activity at various concentrations. One substrate concentration was varied while the others were kept at near saturation. Results were plotted on Lineweaver-Burke double reciprocal plots and K_m values were determined by linear regression. The pH optimum of GS was established by assaying in Mes (pH 5–7) or imidazole-HCl (pH 6–8.5). The initial concentration of the buffer was 100 mM. The pH was measured in the reaction vials at the beginning of the reaction, and these measured values were plotted against velocity to determine the pH optimum of the semi-biosynthetic reaction.

Magnesium requirements for $V_{max}/2$ were determined by assaying for GS activity over a wide range of Mg concentrations (0–50 mM) at various ATP levels (1, 2.5, 5, 10 mM). Velocity was plotted against the square root of Mg concentration because the initial sigmoidal increase in enzyme velocity occurred over a broad range of Mg concentrations which depended on the ATP concentration used for the assay (see "Results").

Determination of Glutamine Synthetase Activity

GS activity was determined with an ATP-dependent semi-biosynthetic assay according to Wallsgrove (34) except for the evaluation of K_m values for ATP, where a coupled assay was used as described by O'Neal and Joy (25, 26).

RESULTS

Extraction

The basic buffer used for the extractions was the same as that described by Cullimore *et al.* (9). It contains glycerol and β-mercaptoethanol as protectants, and magnesium and glutamate as stabilizing agents. Buffer strength was increased to 100 mM to counteract the very high acidic potential of conifer tissues and Nonidet P-40 was added to help tissue solubilization.

Being highly lignified, roots and leaves had to be chopped into about 0.5 cm-long segments before homogenization in the Polytron. After the three successive precipitation procedures, it was still necessary to remove the residual detergent from the extract by adsorption on polystyrene-divinyl benzene beads (23). This step was necessary because the presence of detergent caused severe problems during the chromatography on Sephacel and Sepharose matrices.

The 35%-saturation [$(NH_4)_2SO_4$] precipitate contained approximately 10% of the total GS activity. Several alternative fractionation strategies were attempted to avoid this 10% loss, but the one we finally used provided the best compromise between enzyme recovery and specific activity. Precipitation frequently occurred when the extract was desalted prior to loading on the ion-exchange column, and thus in this case, an additional centrifugation step (20,000*g*, 10 min) had to be included in the procedure in order to clarify the extract. No GS activity was found in the precipitate so formed.

Purification

Glutamine synthetase was purified to apparent homogeneity from leaves and roots of *P. banksiana*, by a procedure involving ion-exchange, size exclusion, and affinity chromatography (Table I). Six hundred to 800 μg of the purified protein was usually obtained from 100 g of leaf tissue, and 300 to 450 μg from 500 g of root tissue. Since no effort was made to isolate any specific leaf- or root-cell compartments prior to extraction, it is believed that both cytosolic and plastid GS isoforms are present in the final preparations. In their final form, both the purified leaf GS and the purified root GS produced two bands on SDS polyacrylamide gels (Fig. 1, A and B) of similar mol wt. When the purified leaf GS was run on a native gel, only one band stained with Coomassie blue (Fig. 2) and this same band also showed glutamine synthetase activity. The specific activity in the final preparation was 0.302 and 4.31 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein for the leaf and the root enzyme respectively. It is interesting to note that the final specific activity of leaf GS is relatively low compared to the root preparation or to that obtained from pea leaves (≃60 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein) (25) or *Phaseolus* sp. nodules (3.6

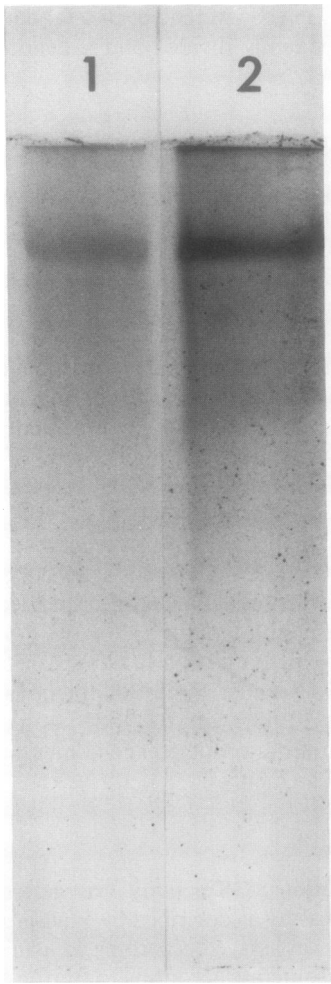

Figure 2. Nondenaturating PAGE of the purified GS enzyme from *P. banksiana* needles. Purification procedures were as described in "Materials and Methods"; 30 μg of protein was loaded in each well and separated by electrophoresis. Lane 1 contains the protein stained with Coomassie blue; lane 2 contains the band exhibiting *in situ* glutamine synthetase activity.

Table II. *Apparent K_m Values of the Root and Leaf Glutamine Synthetase of P. banksiana*

Post-Sepharose preparations of the enzyme were used in these determinations. Kinetic measurements were made by assaying for GS activity at various concentrations of ammonia (5–1000 μM), ATP (1–10 mM) and glutamate (250 μM–10 mM). (Data shown here represent the mean of three experiments). Figure in parentheses indicate the coefficient obtained for each linear regression of the Lineweaver-Burk plots.

K_m	Leaf Enzyme	Root Enzyme
Ammonia (μM)	33.1 (0.92)	19.2 (0.96)
ATP (mM)	2.9 (0.96)	2.3 (0.98)
Glutamate (mM)	1.14 (0.98)	1.85 (0.96)

and 11.3) (9). Although the yield of purified leaf GS is quite high in weight (about 700 μg from 100 g of tissue), it seems that a substantial amount of activity is lost during purification and, therefore, the actual specific activity is probably underestimated. In aqueous solution, the activity of the enzyme decreased steadily at −20°C at about 30% per month, even with addition of ATP (8 mM) or supplementary glutamate (20 mM), $MgSO_4$ (50 mM), β-mercaptoethanol (10 mM), and glycerol (50%).

The whole purification procedure took approximately 30 h but the enzyme could be frozen overnight between the column-chromatography steps without significant loss of activity. Calculated on the basis of activity, the yield was 6% for the leaf GS (Table I) and 14% for the root GS.

Enzyme Characteristics

Pine leaf GS has an apparent K_m of 33.1 μM for ammonia, 2.9 mM for ATP and 1.14 mM for glutamate (Table II). Pine root GS has an apparent K_m of 19.2 μM for ammonia, 2.3 mM for ATP and 1.85 mM for glutamate (Table II). The only major difference between the two enzymes is that the affinity for ammonium is lower for the leaf (K_m = 33.1 μM) than for the root enzyme (K_m = 19.2 μM). However, all the K_m values obtained were within the range of the values reported for GS in other plants (29).

Magnesium requirements, on the other hand, were noticeably different for root and leaf GS in *P. banksiana* (Fig. 3, A and B). GS activity was determined over a wide range of Mg concentrations at several different ATP concentrations, and in all cases the root enzyme needed significantly more Mg^{2+} to reach $V_{max}/2$ than the leaf enzyme (Fig. 3, A and B). With the root GS preparation, $V_{max}/2$ was attained at 0.64, 3.4, and 8.4 mM $MgCl_2$ at 1, 2.5, and 5 mM ATP, respectively. With the leaf GS preparation, $V_{max}/2$ was reached at 0.36, 1.2, and 2.13 mM $MgCl_2$ at 1, 2.5, and 5.0 mM ATP, respectively. However, with both leaf and root GS, the highest activity was obtained at 5.0 mM ATP and, at 10 mM ATP, V_{max} was still not attained even at 50 mM $MgCl_2$ (Fig. 3, A and B).

The pH optimum was around 6.5 (±0.2) and 6.2 (±0.2) when imidazole-HCl and Mes were used as buffering agents, respectively (Fig. 4). There was no difference between the optimum pH obtained when Mn^{2+} or Mg^{2+} were used as divalent cations (results not shown). The activity was, how-

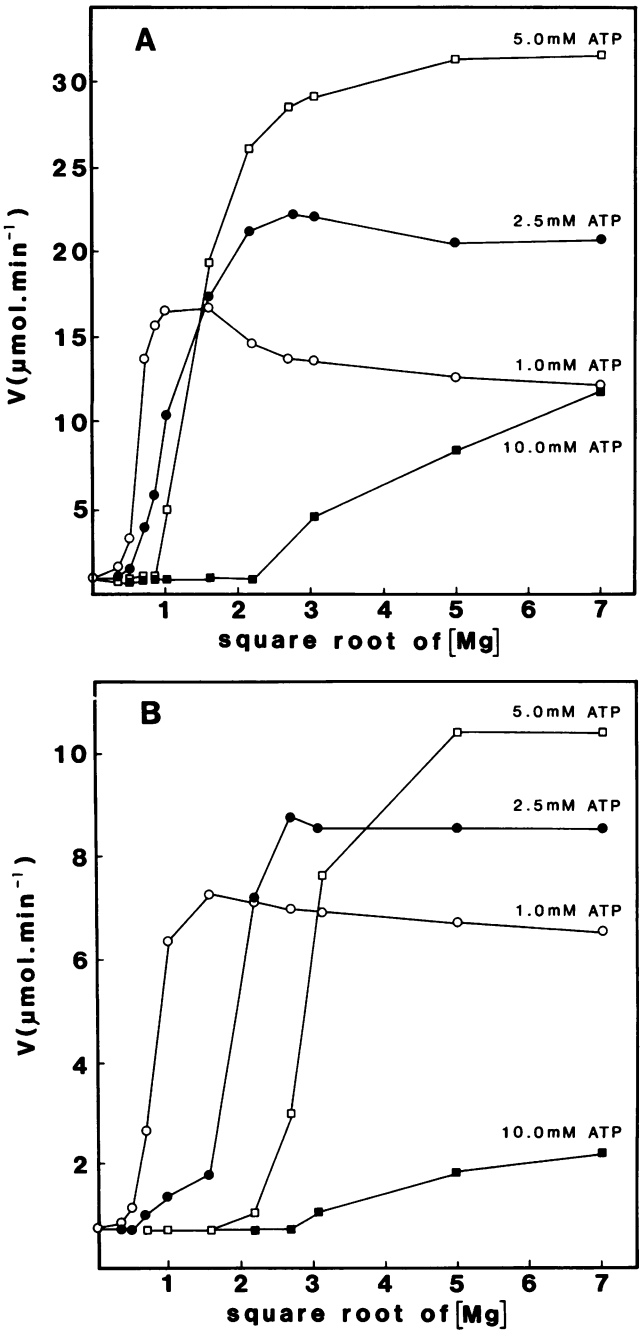

Figure 3. Effect of Mg^{2+} concentration on the biosynthetic activity of glutamine synthetase. The assay mixture contained glutamate (80 mM), hydroxylamine (6 mM) and Mes (100 mM) at a pH of 6.4. A, Leaf enzyme; B, root enzyme. ATP concentration was adjusted to (○), 1 mM; (●), 2.5 mM; (□), 5.0 mM; and (■), 10 mM. Velocity was plotted against the square root of Mg^{2+} concentration to illustrate the effects at both the micromolar and the millimolar level under different ATP concentrations.

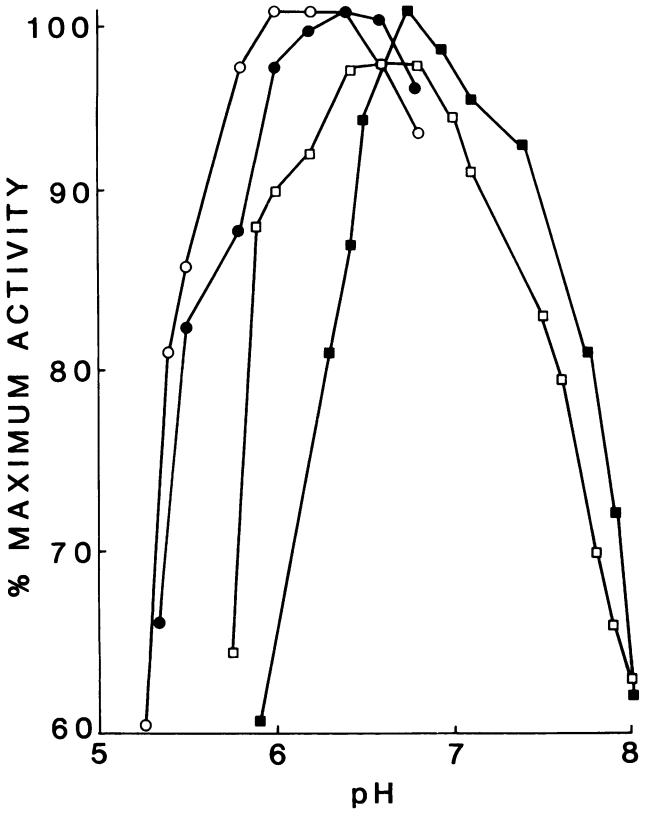

Figure 4. Effect of pH on the biosynthetic activity of glutamine synthetase. The assay mixture contained $MgCl_2$ (50 mM), glutamate (80 mM), ATP (8 mM), hydroxylamine (6 mM) and (○, ●), 100 mM Mes, or (□, ■) 100 mM imidazole-HCl. Open circles and squares, root GS; filled circles and squares, leaf GS.

ever, 30% lower at optimum pH when Mn^{2+} was used instead of Mg^{2+}.

Subcellular Distribution of Glutamine Synthetase

The results of differential centrifugation of an organelle suspension obtained from dark-grown calli of *P. banksiana* are shown in Table III. The 5,000*g* pellet contained almost all NiR activity, a marker enzyme for plastids (14), and the 12,000*g* pellet showed a large proportion of the Cyt *c* oxidase activity, a marker enzyme for mitochondria (14). The 5,000*g* pellet also contained 19% of the total triose-phosphate isomerase activity, a marker enzyme present in both the cytosol and the plastids (14).

The distribution of NiR activity indicates that an extremely high proportion of the plastids were intact after the homogenization of the calli. All the NiR activity was recovered in the two particulate fractions, with no detectable activity in the remaining supernatant (Table III). Therefore, our results indicate that approximately 30% of the extractable GS activity was located in plastids, 70% of which were pelleted by the first centrifugation (5,000*g*).

Subunit Composition

The immunodetection demonstrated that the polyclonal antibody produced against leaf GS from pine recognized the two GS subunits extracted from pine leaves (Fig. 5). A polyclonal antibody raised against *Phaseolus* nodules GS also recognized the same two GS subunits extracted from pine leaves (Fig. 5).

DISCUSSION

The GS enzymes isolated and purified from leaves and roots of *Pinus banksiana* have a similar subunit composition to that of leaf GS from angiosperms (Fig. 1, A and B), *i.e.* both have one predominant peptide of 44 kD and one lighter, less abundant peptide of 40 kD (15, 31). The migration of the enzyme on nondenaturating gels demonstrates that these two peptides (40 kD, 44 kD) represent the constitutive subunits of a single enzymatic protein which exhibits glutamine synthetase activity *in situ* (Fig. 2). The GS enzyme isolated from leaves of *P. banksiana* also have common antigenic determinants with the *Phaseolus* nodule GS.

The GS enzymes isolated from *P. banksiana* leaves and roots have several kinetic properties in common. They have similar K_m values for glutamate and ATP. They share these properties with most other GS from higher plants (15, 20, 21, 25, 26, 29). However, the pine leaf enzyme has a higher K_m for ammonia and its activity requires a lower Mg concentration than the enzyme isolated from pine roots.

It has been demonstrated that adenine nucleotides bind to pea seed GS both at the active site and at an allosteric site, and can therefore act as allosteric effectors (16). These authors (16) also demonstrated that ATP and ADP bind as Mg^{2+}-complexes to one allosteric site per enzyme subunit in peas (eight in all), and that this process occurs in two kinetic

Table III. *Distribution of GS Activity and Marker Enzyme in Fractions from P. banksiana Dark-Grown Calli*

	GS	NiR	Triose-P Isomerase	Cyt *c* Oxidase	Protein
					mg
Crude homogenate	0.083[a]	0.014	5.72	0.271	41.32
5,000 pellet	0.018 (22)[b]	0.012 (83)	1.08 (19)	0.019 (7)	13.81 (33)
12,000 pellet	0.007 (8.5)	0.003 (21)	0.67 (12)	0.165 (61)	4.99 (12)
Supernatant	0.054 (65.5)	ND[c]	3.73 (65)	0.057 (21)	20.90 (50)
Total recovery	96%	104%	96%	89%	96%

[a] All figures in $\mu mol \cdot min^{-1}$. [b] All figures in parentheses are percentages of total initial activity in crude homogenate. [c] Not detected.

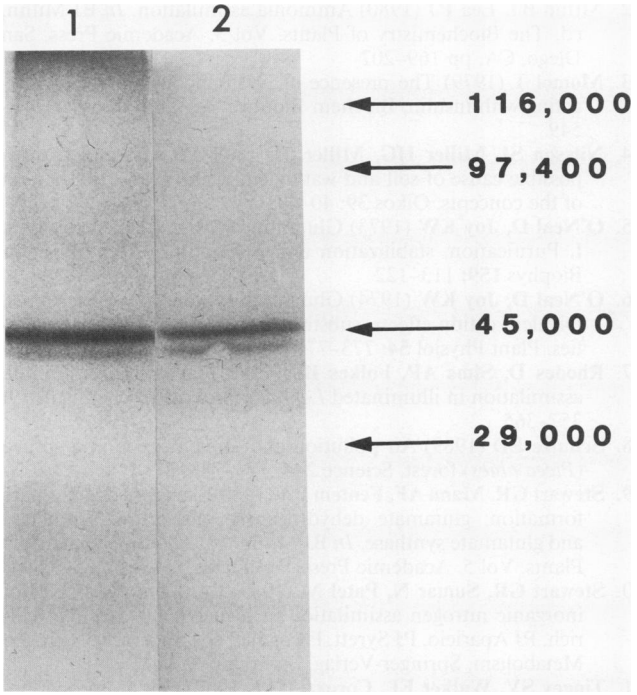

Figure 5. Subunit composition of *P. banksiana* glutamine synthetase. Proteins were obtained from whole homogenates of pine leaves, separated by SDS-PAGE and electrotransferred on a nitrocellulose membrane. Line 1, immunodetection was performed with an anti-GS IgG raised against *P. banksiana* leaf glutamine synthetase. Lane 2, immunodetection was performed with an anti-GS IgG raised against *Phaseolus* nodule GS.

phases. There was no attempt in our study to demonstrate the allosteric effect of the Mg^{2+}-ATP complexes on the enzymes purified from *P. banksiana*. However, ATP concentrations greater than 5 mM had an inhibitory effect on pine GS activity and all the saturation curves obtained with Mg^{2+} as divalent cation were sigmoidal. Since the length of the assay period was sufficiently long to allow ATP to bind to the allosteric site, our results demonstrate that ATP acts as an allosteric effector for pine GS as it does for pea GS.

For a given ATP concentration, Mg^{2+} requirements were always lower for the leaf enzyme than for the root enzyme. The observed difference between the two forms of GS could be due to small differences in binding of ATP^{-4} and Mg^{2+}-ATP species. GS from plants, unlike GS from microbes and mammals, seem to be the only known types of GS whose activity can be regulated by cell energy charge (ADP/ATP ratio). It is possible therefore, that root and leaf GS isoenzymes in pine have differentially adapted to variations in the energy charge as well as the Mg^{2+} availability of their respective tissues.

The most striking difference between the two GS enzymes purified from pine tissue, and the purified GS from other plants is their pH optimum for the true Mg^{2+}-dependent biosynthetic reaction (Fig. 4). The optimum pH values are at least one unit lower than the values for the Mg-dependent reaction for the pea leaf GS (pH 7.4 and 8.2), *Phaseolus* nodule GS (pH 7.7) or GS from leaves and roots of rice (pH 7.5) (9, 15, 25, 26, 29).

There have been very few measurements of the actual pH of the cytosol or of the plastids of plant cells and, to our knowledge, none for conifers. Where data are available, they tend to show that the proton concentration in the cytosol or the plastids remain fairly constant and are close to neutrality. Extracts of conifer tissue, however, are considerably more acid than that of most other plants and our results indicate that this may be indicative of the cytoplasm as well as the vacuoles. It is possible that this lower pH optimum for the GS enzyme may represent an ecological adaption of boreal conifers to growth in acid soils with low nitrification potentials (1, 2, 6, 8, 24).

Because of the highly lignified nature of pine roots, it was not possible to study the intracellular distribution of GS in the same root tissue from which the two GS subunits had been extracted. However, our results show that plastids isolated from dark-grown calli of *P. banksiana* contain a large proportion of the total GS activity (30% or more). Although callus tissue cannot be taken as a reliable indicator of any tissue *in planta*, our results from the calli do indicate that a large part of total glutamine synthesis can occur in pine plastids in the absence of light. It has yet to be demonstrated that the GS enzyme found in the plastids of dark-grown calli is also present in pine root plastids.

The cause of dieback symptoms in *Picea abies* L. Karst in Germany has recently been demonstrated to be a result of Mg deficiency (28). Our results suggest that both conifer roots and needles are more prone to metabolic disruption of GS activity from Mg deficiency. This could be a particularly important physiological disruption under conditions, such as those in central Europe, where there is substantial nitrogen input to the ecosystem from atmospheric deposition and conditions of ammonium toxiates would be more likely to occur.

ACKNOWLEDGMENTS

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