Plant Physiol. (1977) 60, 235-239

Asparaginase and Asparagine Transaminase in Soybean Leaves and Root Nodules[1]

Received for publication February 8, 1977 and in revised form April 21, 1977

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ABSTRACT

Asparaginase activity (≤1 μmol/mg protein·hr) was detected in extracts of soybean (*Glycine max* [L.] Merr.) leaf blades, but, even after efforts to optimize extraction and assay of the enzyme, specific activity was not sufficient to metabolize the estimated amount of asparagine translocated to leaves. Asparagine transaminase activity with glyoxylate or pyruvate was at least 52 and 62 nmol/mg protein·hr, respectively. This estimate of transaminase activity is based on the analysis of the reaction product α-ketosuccinamate. Formation of glycine and alanine was confirmed by amino acid analysis. α-Ketosuccinamate deamidase had a specific activity of 85 nmol/mg protein·hr in leaf blade extracts.

A large amount of asparaginase (300-500 nmol/mg protein·hr) was found in root nodules. The enzyme is stable in 75% ethanol at room temperature, has a *Km* of 5 μM for asparagine, and was six times more active (protein basis) in bacteroids than cytosol. The relatively high activity, stability, and *Km* of the enzyme complicate efforts to study asparagine synthesis in the nodule, an organ known to export large amounts of this amino acid.

Asparagine was the principal component of stem exudate collected from field-grown soybean plants (21) and is also the major component in the bleeding sap of excised soybean root nodules (27). It is clear that ASN[2] plays a central role in nitrogen translocation and in the nitrogen nutrition of the soybean plant. The extensive studies of *Pate* and co-workers (see [1] and references therein) suggest that this generality also applies to other legumes.

Evidence for the soybean plant suggests the presence of an active asparagine-synthesizing system in root nodules. Numerous attempts to demonstrate the formation of labeled ASN in nodule tissue by feeding a variety of radioactive precursors under a wide range of experimental conditions have failed (Streeter, unpublished). These failures are probably related to the presence of a very active asparaginase in nodules, as described in this report.

Since ASN does not accumulate in soybean leaves or stems, except in seedlings (22), shoots apparently possess an active system for metabolism of ASN. While asparaginase could be detected in extracts of soybean leaves, ASN breakdown in some experiments could not be accounted for by aspartate formed. Pursuit of this problem led to the realization that leaf extracts contained much more ASN transaminase than asparaginase activity. A preliminary account of this portion of the work was reported previously (24).

[1] Approved for publication as Journal Article 7-77 of the Ohio Agricultural Research and Development Center.

[2] Abbreviations: ASN: asparagine; AKS: α-ketosuccinamate.

MATERIALS AND METHODS

Plant Material. Soybean plants (*Glycine max* [L.] Merr., cv. Beeson), inoculated with a commercial source of *Rhizobium japonicum*, were grown in a greenhouse in silica sand using a nitrogen-free nutrient solution. Tissue from 30- to 70-day-old plants was used in all experiments.

Extraction and Assay of Asparaginase in Leaf Blades. To optimize extraction of asparaginase, Na-phosphate (pH 7.3), tris-HCl (pH 8), and Tricine (pH 8) buffers were compared and Tricine was found superior. Addition of 1 mM EDTA, 0.5% (v/v) Triton X-100, 1 mg/ml BSA, or insoluble (Polyclar AT, thoroughly washed, approximately 0.5 g dry material/g fresh wt) to 10 mM Tricine buffer (pH 8) did not significantly improve the extraction of asparaginase activity. However, use of Polyclar AT in the extraction buffer reduced the loss of enzyme activity in storage, so the practice was continued.

Leaf blade tissue was weighed, chilled, and ground with a mortar and pestle in 50 mM Tricine (pH 8.2); Polyclar was mixed in and the slurry was allowed to stand 5 to 10 min before filtration through four layers of cheesecloth. Crude extract was centrifuged at 40,000*g* for 15 min and 10-ml portions of supernatant were passed through columns of Sephadex G-25 (23) using 20 mM Tricine (pH 8.2). All operations prior to the assay of enzymes were carried out at 2 C.

Asparaginase activity was determined by measuring the conversion of uniformly labeled [^{14}C]asparagine to [^{14}C]aspartate. [^{14}C]ASN was purchased from several sources and all lots contained small amounts of contaminants which interfered with assays and which were removed by passage of the material through small columns of Dowex 1 (200-400 mesh) ion exchange resin in the formate form.

Tris, HEPES, Tricine, and phosphate buffers (0.1 M, pH 8.2) were compared and reaction mixtures containing Tricine resulted in twice as much enzyme activity as any other buffer. Addition of 5 μmol dithiothreitol to the reaction mixture did not increase enzyme activity. Reaction mixtures contained 0.3 ml gel-filtered enzyme preparation (less than 1 mg protein, in 20 mM Tricine, pH 8.2) and about 1 μCi [^{14}C]ASN (10-20 nmol). The control consisted of boiled enzyme preparation. Reaction mixtures were incubated for 60 min at 30 C, after which the reaction was stopped by placing tubes in a boiling water bath for 10 min.

Initially, the assay of Martin (12) was adapted to determine [^{14}C]aspartate formation. The method involves binding of aspartate to DEAE-cellulose paper discs (DE81 discs, Reeve Angel & Co.) which do not bind ASN at neutral pH. The assay is usable but will give slightly different results depending on type of buffer, buffer concentration, and pH used in reaction mixtures. Most of the work reported here was done using the assay described by Prusiner and Milner (15), involving small columns of ion exchange resin. The accuracy of both assays was regularly checked with two-dimensional TLC or paper chromatography (23).

Asparagine Transaminase Assay. Keto acids were purchased from Sigma Chemical Co. and were used as the sodium salts. α-Ketosuccinamate (AKS) was synthesized (13, 20). Reaction mixtures contained 0.90 ml gel-filtered enzyme preparation (in 50 mM Tricine buffer, pH 8.2), about 0.5 μCi [^{14}C]ASN (1 μmol ASN), and 1 μmol of keto acid in a total volume of 1 ml. Controls lacking keto acid or protein, or containing boiled extract, were employed. Mixtures were incubated for 1 hr at 30 C.

Attempts were made to analyze radioactive AKS directly by TLC or paper chromatography or by passage of mixtures through columns of Dowex 50-H^+. Chromatography of reaction mixtures yielded more than one radioactive spot (in addition to ASN), presumably due to the formation of AKS dimer (20), and ASN was not well separated from AKS in chromatography systems tried.

A satisfactory assay for AKS formation involves the formation of AKS dinitrophenylhydrazone. After incubation, 20 to 40 μg of unlabeled AKS and 0.2 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 3 N HCl were added to each tube and mixtures were incubated for another hr at 30 C. Hydrazones were purified by extraction with four 1-ml portions of ethyl acetate, extraction of combined ethyl acetate fractions with four 1-ml portions of 10% (w/v) $NaHCO_3$, acidification of the combined bicarbonate fractions with cold concentrated HCl, and extraction with three 1-ml portions of ethyl acetate. Radioactivity in the final ethyl acetate fraction is an accurate representation of radioactivity in AKS as determined by paper chromatography of dinitrophenylhydrazones (19). Amino acids formed in transamination reactions were analyzed qualitatively by descending paper chromatography using 1-butanol-acetic acid-water (12:3:5, v/v/v) as a solvent and ninhydrin for location of spots.

α-Ketosuccinamate Deamidase Assay. Addition of oxaloacetate and NADH to gel-filtered enzyme preparations resulted in rapid decline in A_{340}, indicating the presence of malate dehydrogenase. Endogenous malate dehydrogenase was used to measure the amount of oxaloacetate formed by AKS deamidase in a reaction mixture containing 65 μmol of Tricine buffer (pH 8), 5 to 10 μmol of AKS, 0.5 mol of NADH, and 1.5 ml of enzyme preparation (2-3 mg protein) in a total volume of 3 ml. Controls lacked AKS. ΔA_{340} was monitored for 5 to 20 min, during which ΔA was linear.

Extraction and Assay of Enzymes in Nodules. Extraction of nodules was the same as described for leaf blades, except that 10 mM Tricine (pH 8.2) was used for gel filtration.

Asparaginase was assayed in a reaction mixture containing 5 μmol Tricine buffer (pH 8.2), about 0.5 μCi [^{14}C]ASN (80-100 nmol ASN), and enzyme preparation (0.1-0.4 mg protein) in a total volume of 0.30 ml. [^{14}C]Aspartate formed was determined using resin columns (15) or TLC (23).

Asparagine transaminase was assayed as described above.

Glutamine synthetase was measured by the γ-glutamyltransferase assay described by Kurz *et al.* (8). The amount of extract used in the assay must be carefully adjusted to avoid complete consumption of ADP; generally 10 μl extract and a 15 min incubation were used.

Glutamate synthase assay was similar to that described by Dunn and Klucas (3). Reaction mixtures contained 200 μmol of HEPES (pH 7.5), 10 μmol of α-ketoglutarate, 3 μmol of EDTA, 0.5 μmol of NADH, 20 μmol of glutamine, and enzyme preparation (0.5-4 mg protein) in a total volume of 2.7 ml. Controls lacked glutamine. ΔA_{340} was determined with two or more protein concentrations.

Glutamate dehydrogenase was assayed in a reaction mixture containing 200 μmol of HEPES (pH 7.5), 20 μmol of α-ketoglutarate 100 μmol of NH_4Cl, 0.5 μmol of NADH and enzyme preparation (0.5-4 mg protein) in a total volume of 2.4 ml. Controls lacked NH_4Cl and A_{340} was determined at two or more protein concentrations.

Glutaminase was assayed in a reaction mixture containing 20 μmol of HEPES (pH 7.5), about 0.5 μCi (2.6 μmol) of uniformly labeled [^{14}C]glutamine and enzyme preparation (0.1-0.6 mg protein) in a total volume of 0.25 ml. Boiled enzyme was used as a control. After incubation for 1 hr at 30 C, mixtures were boiled and radioactivity in glutamate was determined using TLC (23).

Invertase was assayed in reaction mixtures containing 20 μmol of HEPES (pH 7.5), 10 μmol of sucrose, and enzyme preparation (0.2-1.5 mg protein) in a total volume of 0.40 ml. Boiled enzyme was used in controls. After incubation for 30 min at 30 C, mixtures were boiled and portions were analyzed by gas chromatography (25). Since our "pure" sucrose contained traces of glucose, the quantity of fructose formed was used to estimate enzyme activity.

In all experiments reported, protein concentration of extracts was determined by method of Lowry *et al.* (11).

RESULTS

Asparagine Metabolism in Leaves. The response of asparaginase to pH and substrate concentration was checked in early experiments. The pH response was determined in 50 mM Tricine buffers having a range of pH values from 7.3 to 9.1. Maximum activity was observed at pH 8.2 but the peak was not pronounced; activity at pH 7.3 and 9.1 was about 75% of the activity at pH 8.2. Response to substrate (ASN) concentration was determined in reaction mixtures having a range of substrate concentration from 8 to 340 μM. Reciprocal substrate concentration ($1/[S]$) and velocity ($1/V$) values were calculated. The regression of $1/V$ on $1/[S]$ was calculated and the value of $1/[S]$ when $1/V = 0$ was used to estimate a $Km = 16$ μM. The correlation coefficient relating $1/[S]$ and $1/V$ values was 0.986.

When portions of crude extract (before gel filtration) were added to reaction mixtures, breakdown of [^{14}C]ASN was markedly increased but radioactivity was not recovered in aspartate after chromatography of reaction mixtures. Further analysis of this result led to tests of enzyme activity in the presence of keto acids and to direct analysis of the radioactive product, AKS (Table I). Although transaminase activity could be detected with several keto acids, substantial enzyme activity was found only with pyruvate and glyoxylate. Paper chromatography of reaction mixtures showed that alanine and glycine were formed in mix-

Table I. Asparagine transaminase activity, extracted from soybean leaves, with various keto acids

Radioactivity in AKS-dinitrophenylhydrazone was measured after paper chromatography and essentially no radioactivity was detected in oxaloacetate dinitrophenylhydrazone.

Keto acid added	Radioactivity in AKS-dinitrophenylhydrazone
	cpm x 10^{-3}
None	0.4
α-Ketobutyric	2.4
α-Ketoisocaproic	1.2
α-Ketovaleric	1.8
α-Ketoisovaleric	1.1
β-Phenylpyruvic	3.5
α-Ketoglutaric	4.5
Oxaloacetic	10.0
Pyruvic	30.2
Glyoxylic	25.2
Glyoxylic (no enzyme preparation)	0.4

tures containing pyruvate and glyoxylate, respectively. Oxaloacetate spontaneously decarboxylates to pyruvate in solution. For this reason, plus the fact that the formation of aspartate was not confirmed in mixtures containing oxaloacetate, I suspect that formation of radioactive AKS with oxaloacetate may be due to the presence of pyruvate.

In spite of efforts to optimize the extraction and assay of asparaginase, activity was generally about 0.7 nmol/mg protein·hr. In contrast, activity of asparagine transaminase was easily measured. Since the extraction and assay of the transaminases may not have been optimum, transaminase activity in soybean leaves would appear to be at least 50 times greater than the asparaginase activity (Table II). α-Ketosuccinamate deamidase (AKS → oxaloacetate + NH_3) was found with a level of activity similar to the level of transaminase.

An attempt was made to confirm the activity of ASN transaminase and AKS deamidase *in vivo* by feeding radioactive ASN to soybean leaves. After incubation for 0, 30, 60 or 120 min, leaves were ground in 75% (v/v) ethanol, and a portion of the extract was immediately reacted with saturated solution of 2,4-dinitrophenylhydrazine in 3 N HCl. After purification and chromatography of hydrazone (19), no accumulation of label was found in AKS or in oxaloacetate. Analysis of amino acids and organic acids (23) revealed slightly more radioactivity in malate than aspartate but it was not possible to tell which metabolite was labeled first.

Asparagine Metabolism in Nodules. Radioactive metabolites such as pyruvate, succinate, acetate, aspartate, and glycerate have been supplied to soybean nodules by injection of whole nodules using a μl syringe, vacuum infiltration of whole nodules, or incubation of nodule slices on blotter paper saturated with a solution of radioactive metabolite. Tissue was routinely extracted with 75% (v/v) ethanol in these experiments. Results (unpublished) of these studies indicated rapid synthesis of organic acids and amino acids but essentially no synthesis of labeled asparagine. The importance of asparaginase in soybean nodules became apparent when [^{14}C]ASN was supplied to nodule tissue and the result was a rapid hydrolysis of ASN to aspartate. Nearly quantitative conversion of ASN to aspartate occurred even when nodule tissue was ground in 75% ethanol prior to mixing the extract with [^{14}C]ASN. After trying several extraction media, it was found that ethanol containing 10% (v/v) acetic acid or formic acid or 15 μmol HCl/ml will rapidly destroy asparaginase activity. There was no significant acid-catalyzed hydrolysis in 75% ethanol containing as much as 65 μmol HCl/ml.

Experiments with [^{14}C]ASN indicated the presence in soybean nodules of asparaginase which retains activity in 75% (v/v) ethanol at room temperature. This finding led to extraction and assay of the enzyme as described under "Materials and Methods." The stability of asparaginase in ethanol was confirmed by adding increasing amounts of absolute ethanol to gel-filtered, buffer extracts of nodules. Assay of the enzyme after exposure to ethanol concentrations as high as 50% (v/v) at room temperature for 30 min resulted in only small decreases in specific activity. Heating a preparation containing 67% ethanol (v/v) to 40 C for a few min resulted in complete loss of activity. Thus, extraction of nodules with warm ethanol in *in vivo* studies of asparagine synthesis may inactivate asparaginase as efficiently as dilute HCl.

The amount of asparaginase activity in nodules can be judged by comparing it to the activity of other enzymes commonly assayed in nodule extracts (Table III). There was essentially no asparagine transaminase activity in nodules. Nodule extracts contained more asparaginase than glutamate synthase or glutaminase and there was several hundred times as much asparaginase in nodules as in soybean leaves (Table II). The concentration of asparaginase in bacteroids was approximately six times as great as the concentration in cytosol (Table III). Recovery of bacteroids by the methods used is variable and incomplete, but it is possible to estimate that total asparaginase activity in cytosol was about double the activity in bacteroids.

Response of nodule asparaginase to substrate concentration was determined in reaction mixtures having a range of substrate concentrations from 1.4 to 20 μM. It was necessary to use a very low protein concentration (40 μg protein/assay) and short incubations (20 min) in order to determine accurate initial velocities. The regression of $1/V$ on $1/[S]$ was calculated and the value of $1/[S]$ when $1/V = 0$ was used to estimate a $Km = 4.9$ μM. The correlation coefficient relating $1/[S]$ and $1/V$ values was 0.987. The very small Km for nodule asparaginase is similar to the Km

Table II. Activity of four enzymes extracted from soybean leaves

Activity of each enzyme is the highest observed in several different experiments; i.e. all four enzymes were not assayed in the same extract.

Enzyme	Enzyme activity nmol mg protein^{-1} hr^{-1}
Asparaginase	1.0
Asparagine-pyruvate transaminase	52.0
Asparagine-glyoxylate transaminase	62.0
α-Ketosuccinamate deamidase	85.0

Table III. Activity of enzymes extracted from whole soybean nodules or present in bacteroids and cytosol

In Expt. II, bacteroids were prepared by the method of Evans, et al. (4). After washing, bacteroids were disrupted by 10 to 12 30-sec. periods of sonication over 20 minutes. In Expt. III, nodules were ground in 0.25 M Tricine buffer, pH 7.5 containing 10 mM mercaptoethanol. Otherwise, preparation of bacteroids was the same as in Expt. II. After sonication, Triton X-100 was mixed with the bacteroid preparation (10 μl Triton/ml prep.) and allowed to stand 15 min. In all experiments all preparations were gel-filtered (23) using 10 mM Tricine pH 8.2; mercaptoethanol (1 mM) was added to the filtration buffer in Expt. III.

Enzyme Activity	Experiment I	Experiment II		Experiment III		Ratio of activities
	Whole nodules	Bacteroid	Cytosol	Bacteroid	Cytosol	Bacteroid ÷ cytosol (Avg. 2 expts.)
	μmol mg protein^{-1} hr^{-1}					
Glutamine synthetase (transferase)	47.1	5.6	48.4	15.8	70.9	0.17
Invertase[1]	3.44	0	1.72	0	3.01	0
Glutamate dehydrogenase	0.36	2.4	0.24	4.9	0.41	11.
Asparaginase	0.28	0.81	0.14	1.35	0.24	5.7
Glutamate synthase	0.23	0.26	0.26	0.53	0.45	1.1
Glutaminase	0.11	0.27	0.06	0.57	0.17	3.9
Asparagine:pyruvate transaminase[2]	0.0068	NA[3]	NA	NA	NA	-
Asparagine:glyoxylate transaminase	0.0027					

[1] μmol sucrose hydrolyzed

[2] α-ketoglutarate was also tested but activity was less than 1.0 nmol mg protein^{-1} hr^{-1}

[3] Not assayed

values reported for the enzyme from several species of bacteria (7, 28).

DISCUSSION

Asparagine Metabolism in Leaves. There are few, if any, reports which demonstrate conclusively any mechanism for asparagine metabolism in green plant tissues. In a recent study of two legumes, Lees and Blakeney (10) have demonstrated the presence of asparaginase in roots and nodules and their data indicate a trace of enzyme activity in shoots. However, specific activities were not reported. Atkins *et al.* (1) have recently reported the presence of an active asparaginase in embryos of developing *Lupinus alba* seeds. The reported *Km* of this enzyme was quite high (10 mM) but was similar to the recently reported *Km* for asparaginase from *Lupinus polyphyllus* seeds (9).

The effect of keto acids on the breakdown of asparagine *in vitro* was first noted by Greenstein and Price (6) in studies with rat liver extracts. Later work by Meister *et al.* (13, 14) explained the effect as not keto acid stimulation of asparaginase, but as resulting from transamination and subsequent deamidation of the transamination product α-ketosuccinamate.

Several studies conducted since Meister's reports have suggested the presence of asparagine transaminase activity in plant tissues (5, 26, 29). These workers used an assay for formation of an amino acid corresponding to the keto acid added to reaction mixtures. It is possible that their results can be explained by the presence of asparaginase plus the transamination of the aspartate formed, or by the presence of a trace of some amino acid other than asparagine in reaction mixtures. This latter possibility seems especially likely because significant proteolysis could have occurred during the long incubations (2–3 hr) which were employed. With these potential complications, it seems obvious that an assay for AKS is required where asparagine transamination is suspected.

Based on the data in Table II, it is suggested that although asparaginase may be present in green plant tissues, transamination is a more likely route for asparagine metabolism. AKS deamidase was also demonstrated in soybean leaves. The presence of this enzyme in plant tissues has previously been reported by Meister (13). My initial attempt to demonstrate the operation of the transaminase *in vivo* was unsuccessful. This work needs to be repeated with more effort directed toward trapping radioactivity from ASN in AKS; transamination and deamidation reactions may be closely coupled (14) so that it may be possible only to show the labeling of oxaloacetate prior to the labeling of aspartate.

Based on the seasonal average asparagine concentration in stem exudate and the average exudate flow rate (21), it was possible to estimate the influx of asparagine to soybean leaves as 25 nmol/g fresh wt·hr. The average protein concentration of 10 enzyme preparations and the weight of the leaf tissue extracted were used to calculate the buffer-extractable protein in soybean leaves (23 mg/g fresh wt). Although there are many obvious limitations to this approach, these calculations led to the conclusion that there is insufficient asparaginase to metabolize asparagine entering soybean leaves, whereas the transaminase activity is more than adequate to metabolize incoming asparagine.

Asparagine Metabolism in Nodules. Lees and Blakeney (10) have previously reported the presence of asparaginase in legume root nodules. This report confirms and extends their observations. Whereas the activity of asparagine transaminase was almost nil, the activity of asparaginase in soybean nodules is approximately the same order of magnitude as other nodule enzymes which have recently received attention (Table III and refs. 2, 3, 8, 16, 17). The specific activities and the distribution of activity in bacteroid *versus* cytosol are comparable to results reported by others, with the exception of glutaminase and glutamate dehydrogenase. The activity and distribution of glutaminase in legume nodules have not previously been reported. I found much higher glutamate dehydrogenase activity in soybean nodules than Dunn and Klucas (3) and also in contrast to their results, found the highest concentration of the enzyme in bacteroids. Apparently, the amount and distribution of glutamate dehydrogenase activity in nodules are highly variable among legume species (2).

Soybean nodule asparaginase was concentrated in bacteroids suggesting that the enzyme is of bacterial origin. Activity in the cytosol may have been the result of loss from the bacteroids during the isolation procedure. The fact that large amounts of asparagine are synthesized in and exported from nodules (21, 27) also makes it attractive to suggest that asparaginase is localized in bacteroids allowing asparagine synthesis to occur in uninfected tissue, perhaps in the nodule cortex near the vascular bundles.

The remarkable stability and the extremely small *Km* (5 μM) of the nodule asparaginase seriously complicate efforts to demonstrate asparagine synthesis *in vitro* or *in vivo* because of the potential for rapid destruction of the product, ASN. My experience indicates that ASN hydrolysis occurs whenever the nodule is cut or punctured in order to supply radioactive materials. However, the knowledge that this enzyme is present will make it possible to adopt measures to circumvent it in future studies of asparagine synthesis.

An enzyme from lupin nodules which catalyzes glutamine-dependent asparagine synthesis has recently been reported (18). Lupin nodules also contain asparaginase and it is interesting to note that asparagine synthetase was not detected until a stage of nodule development where asparaginase could no longer be detected. Although I have not systematically studied asparaginase activity as a function of nodule age, asparaginase was found in many different samples of soybean nodules ranging in age from 20 to 60 days after initiation of nodule growth.

Ackowledgments – I thank A. Meister for advice on the synthesis of α-ketosuccinamate, M. Bosler for general technical assistance, and D. Murphy for suggesting the feeding of [^{14}C]asparagine to nodules.

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