Measuring Asparagine Synthetase Activity in Crude Plant Extracts

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Asparagine synthetase B (AS) is the primary enzyme responsible for asparagine synthesis in plants. Routine biochemical studies of this enzyme's activity have been hindered by several problems including enzyme instability and rapid physiological turnover, endogenous inhibitors, competing pathways, and asparaginase activity. We describe an extraction procedure and assay conditions that provide a reliable, direct assay for the determination of AS activity in crude plant extracts. This assay performed well with several leguminous species and the enzyme preparation retained activity for up to 3 weeks when stored at −80 °C. Radio-HPLC detection enabled quantitative measurement of de novo aspargine synthesis in the extracts. Optimal activity was obtained with 1 mM glutamine and 10 mM ATP in the reaction assay. Aminooxyacetic acid (AOA, 1 mM) which prevents the assimilation of aspartate into the TCA cycle, was necessary to measure AS activity in peas, but not in lupine or soybean.

Keywords: *Asparagine synthetase; aspartate; enzyme activity; regulation; lupine; soybean; pea*

INTRODUCTION

Asparagine synthetase (AS; EC 6.3.5.4) is the primary enzyme involved in the production of asparagine (asn). There are two principal classes of AS; a prokaryotic form that employs ammonia as the sole source of nitrogen and eukaryotic asparagine synthetases that utilize either glutamine or ammonia as their nitrogen source (Richards and Schuster, 1998). In plants, AS-A is found mainly in roots and uses either glutamine (gln) or NH_3 as a substrate (Streeter, 1973; Oaks and Ross, 1984). AS-B, the primary asparagine-producing enzyme in plants (Ireland and Lea, 1999), is distributed throughout the plant and it catalyzes an ATP-dependent reaction where the amine group of gln is transferred directly to aspartate (asp) (Sieciechowicz et al., 1988; Tsai and Coruzzi, 1990).

Other than being used as a building block for protein synthesis, the high N:C ratio of asn makes it a key molecule involved in nitrogen transport in plants in darkness (Ireland and Lea, 1999). Expression of AS is repressed in plants exposed to light and is stimulated during period of darkness (Rognes, 1975; Lam et al., 1994). The increased level of activity occurring in darkness is not due to circadian rhythms but is instead associated with phytochrome regulation (Rognes, 1975; Ireland and Lea, 1999). Additional roles for asn include acting as the anchor for N-glycosidic linkages (Kaplan et al., 1987) and providing certain TCA cycle intermediates, such as OAA (Richards and Schuster, 1998). Catabolic intermediates are important in gluconeogenesis as well as transamination reactions.

In the past, measurement of AS activity has proven extremely difficult (Richards and Schuster, 1998; Ireland and Lea, 1999). Problems associated with assaying AS in plants include the relatively short half-life of the enzyme and its highly regulated activity. The rapid turnover and deactivation associated with plant AS make extraction of this enzyme in an active form difficult. Protectants, such as glycerol and thiol-containing compounds (Joy et al., 1983; Snapp and Vance, 1986), are required to maintain this highly labile enzyme in its active dimerized conformation during the extraction process. Other difficulties associated with biochemical studies of AS include the presence of endogenous natural inhibitors (Rognes, 1980) and relatively high cytoplasmic asparaginase (Hughes et al., 1997) and glutamine synthetase activities (Sieciechowicz et al., 1988) competing for similar substrates. Several studies have measured AS activity by providing radiolabeled [^{14}C]asp to whole etiolated cotyledon sections (e.g., Streeter, 1973), but in general, in vivo conversion of exogenous asp to asn has been difficult to quantify (Joy et al., 1983). Other assays determining AS activity by monitoring PP_i released from ATP during the activation of asp prior to the transamination step do not account for the numerous other enzymatic processes using ATP or PPi concurrently in the crude plant extracts (Dembinski et al., 1996; Hughes et al., 1997). In general, there are no reliable, reproducible AS assays for crude extracts with which we are familiar.

Previous studies have determined that rapid metabolism of asp to TCA intermediates may contribute to the low observable conversion rates of radiolabeled asp to asn (Joy et al., 1983; Snapp and Vance, 1986). Aspartate enters the TCA cycle as oxaloacetic acid via aspartate aminotransferase (AAT), therefore treatments which inhibit AAT activity may enhance the conversion of asp to asn. The addition of aminooxyacetate (AOA), an aminotransferase inhibitor, has been documented to decrease the flow of [^{14}C]asp to organic acids in pea leaves (Joy et al., 1983).

In this paper we present an improved extraction protocol yielding highly active AS as well as a reproducible enzymatic assay using radiolabeled [^{14}C]asp to monitor de novo asn formation. This method uses crude plant extracts and circumvents the time and inconve-

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nience of purifying AS. We optimized enzyme assay conditions and documented significant product inhibition by asn. Finally, we compared and contrasted differences between pea and lupine AS and the effect of the aminotransferase inhibitor AOA.

MATERIALS AND METHODS

Plant Material. Soybean (*Glycine max* L. Merr. v. DP3588), lupine (*Lupinus albus* v. Victoria), and peas (*Pisum sativum* v. Little Marvel) were grown in moist commercial potting soil (Miracle Grow) for 6 days at 27 °C in the dark. Lupine and soybean were obtained as gifts from various sources. Peas were obtained from W. Atlee Burpee & Co. (Warminster, PA).

Preparation/Extraction Methods. Extraction methods are modified from Dembinski et al. (1996). Etiolated cotyledons of either soybean, lupine, or the first leaves of peas were harvested in the dark and homogenized in a Waring blender (3 × 30 s pulse on high) at 4 °C in 2.5 v/w extraction buffer (100 mM Tris HCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM ATP, 2 mM asp, 20% glycerol, 0.5 mM DTT, and 67 mM β-mercaptoethanol; pH was adjusted to 7.8 at 4 °C). The homogenate was filtered through two layers each of cheesecloth and Miracloth (CalBiochem, La Jolla, CA), and the resultant filtrate was centrifuged at 16000*g* for 15 min a 4 °C. The supernatant was collected, avoiding the lipid layer floating on top when soybean was used. Proteins were precipitated from the cell-free crude extract with 65% $(NH_4)_2SO_4$ for 30 min on ice. The pH of the extraction buffer was carefully monitored and maintained at pH 7.8 with 10 M NaOH during the salting-out process. The precipitate was collected via centrifugation for 15 min at 20000*g* at 4 °C. For soybean and lupine, the protein layer containing AS activity was floating on top of the supernatant. For peas, AS activity was found in the pellet at the bottom of the tube. The pellet was resuspended in extraction buffer (vol. 30% fresh weight) and desalted on a PD-10 Sephadex G-25M column (Supelco, Bellefonte, PA) that had been preequilibrated with extraction buffer. The protein fractions were combined and concentrated with Centricon 30 concentrators (Amicon, Inc., Beverly, MA). Protein concentration in the extracts was determined according to Bradford (1976) and adjusted to 15−20 mg mL^{-1}. The concentrated extracts were frozen at −25 °C overnight and assayed for AS activity the following day, unless otherwise indicated.

Standard Enzyme Assay. AS activity was assayed by mixing 250 μL of concentrated protein extract (normalized to 15 mg protein mL^{-1}) with 200 μL of assay buffer [100 mM Tris-HCl (pH 7.5), 1 mM gln, 1.6 mM asp, 10 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, and 0.25 μM [^{14}C(U)]asp {Moravek Biochemicals, Inc., Brea CA} (final activity of 0.5 μCi)] in 10-mL glass test tubes. Samples were incubated at 25 °C and the conversion of asp to asn was monitored over time. The reaction was terminated with the addition of 500 μL of 100% EtOH to the reaction mixture and boiling for 5 min. Samples were transferred to screw cap 1.5-mL Eppendorf microcentrifuge tubes, and the denatured protein was pelleted from the sample by centrifugation (13000*g*) for 15 min. The supernatant was kept for quantitative analysis of asn synthesized de novo.

Optimization of Enzyme Assay Conditions. ATP was tested between 0.1 and 33 mM, gln between 0.03 and 10 mM, and asn between 0.01 and 100 μM. The AS activity found in the lupine extract was assessed at fixed intervals for a maximum of 240 min. Also, the effect of 1.0 mM AOA on the observed rate of asn synthesis was tested in lupine and pea. The storage stability of AS in the semicrude extracts was determined over time after storing the samples at −25 and −80 °C for varying amounts of time.

HPLC Methods. The HPLC system consisted of Waters Associates components: Model 717 autosampler, Model 600 controller, and Model 996 photodiode array (PDA) detector. An IN/US Systems inline β-RAM radioisotope detector equipped with a 400 μL lithium glass packed dry cell (LiGl) was connected in tandem with the PDA detector. A guard column (BioRad ODS 5S) was mounted onto a 250 × 4.6 mm Spherisorb S5 SCX cation exchange column (Waters Associates). The chromatographic conditions were optimized to obtain complete separation of asp and asn within each 8-min run. The solvent system was isocratic 100% 10 mM KH_2PO_4 (pH 3.0). Each injection consists of 100 μL of supernatant obtained as previously described. Between each sample, 100 μL of methanol was injected and run for 8 min by using the same isocratic solvent system to remove unlabeled contaminants remaining in the column. This step was necessary to obtain reproducible retention times for asp and asn. The amount of radiolabeled asn synthesized by AS was calculated by using a standard curve established with technical grade ^{14}C[U]asn (Moravek Biochemicals, Inc., La Brea, CA). Minimum asn detection for this system above background was 5 pmol asn (1.0 nCi). Chromatographic separation obtained with this system appears to be specific for ^{14}C-asn, since no other putative radiolabeled metabolite with R_f values similar to asn were detected when AS activity was completely inhibited (data not shown).

Statistical Analysis. Statistical analysis for significance between treatments (ANOVA) was performed in SAS (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

The difficulties involved in extracting active AS from plants and reliably assaying its activity have been reported numerous times in the literature (Streeter, 1973; Rognes, 1975; Kaplan et al., 1987). This extraction and assay protocol attempts to address factors limiting AS activity in order to simplify and optimize the measurement of AS activity in crude plant extracts.

The buffer conditions required to stabilize AS during the extraction process, such as high concentrations of $MgCl_2$, glycerol, and thiol-containing compounds, have been reported previously (Joy et al., 1983; Snapp and Vance, 1986). However, there were many discrepancies about actual amounts of stabilizing compounds, particularly glycerol and β-mercaptoethanol, required for optimum activity. We determined the required amount of glycerol to be 20−25% (v/v) and β-mercaptoethanol to be 65−70 mM. It should be noted that, in addition to stabilizing AS during storage at low temperature, glycerol was also required for optimum activity in the assay prior to freezing the samples.

The rapid desalting of the enzyme achieved with the PD-10 Sephadex column also improved AS activity by removing many endogenous inhibitors of AS known to be present in the tissue homogenate (Rognes, 1980). Moreover, this step diluted the protein fraction by only 40%, whereas traditional column chromatographic methods can dilute the protein sample by up to 400%. Furthermore, concentrating the enzyme preparation by centrifugation increased the relative activity of AS in the assay and allowed for high reproducibility.

Unlike previous extraction methods requiring that the enzyme preparations be utilized as quickly as possible, preferably within 24−48 h of the time of extraction (Rognes, 1975) because no AS activity could be measured with longer periods of storage, the protocol described above significantly improved the stability of AS in the crude extracts. AS retains 80% of its activity within the first 3 days. Activity was measurable for up to 12 days, although it decreased slowly throughout that period of time to a level of ca. 35% of original activity (Figure 1A). Storage half-life of the samples was 9−10 days when stored at −25 °C, and the enzyme retained ca. 70% of its activity for over 3 weeks when stored at −80 °C (Figure 1B).

Optimal [^{14}C]asn de novo synthesis was attained after 2 h incubation at 25 °C (Figure 2), although detectable

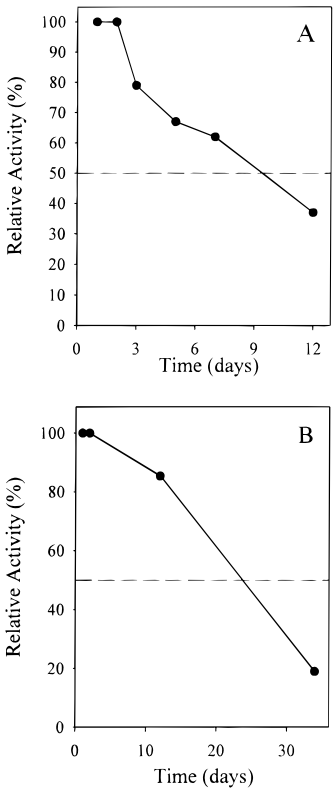

Figure 1. Storage half-life of AS in lupine crude extract at −25 °C (A) and −80 °C (B). The experiment was performed by using standard assay conditions as described under Materials and Methods. Data are the means of three measurements ± SD. Note the different time scale on the abscissa.

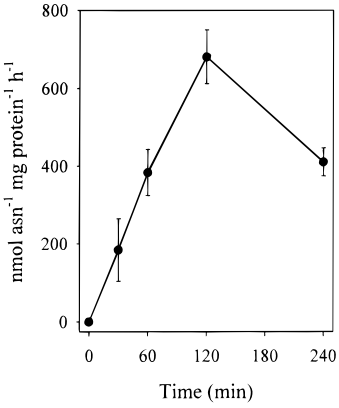

Figure 2. Asparagine synthetase activity (nmol of asn mg^{-1} of protein h^{-1}) in lupine observed at four different incubation times. The experiment was performed by using standard assay conditions as described under Materials and Methods. Data are the means of nine measurements ± SD.

levels of asn were observed within the first hour. Asparagine levels began to decrease with longer period of incubation. The amount of gln present in the various reaction assay buffers reported in the literature varied greatly, with recommended gln concentration ranging between 10 and 100 mM (Streeter, 1973; Rognes, 1975; Hughes et al., 1997; Richards and Schuster, 1998). Under the conditions of this assay, maximum activity was achieved with 1 mM gln (Figure 3A). AS activity in lupine decreased with either lower or higher gln concentrations.

AS activity was also dependent on ATP concentration. A semilog dose−response study showed a narrow range

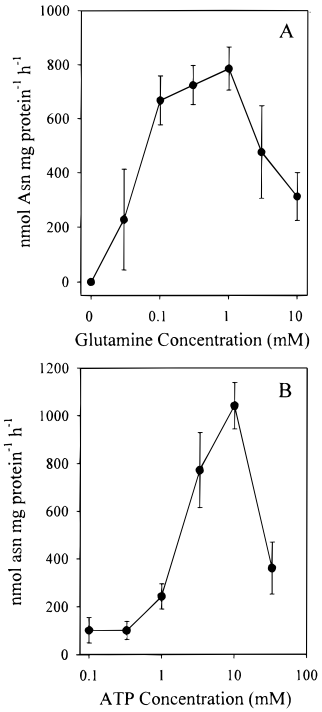

Figure 3. Asparagine synthetase activity from lupine assayed under standard conditions (see Materials and Methods), except that the effects of (A) 0.03−10 mM gln and (B) 0.1−33 mM ATP were tested. Data are the means of six measurements ± SD for both graphs.

of 3−10 mM ATP for optimum activity (Figure 3B). AS activity decreased substantially at concentrations lower than 3 mM and greater than 10 mM.

The negative substrate effect observed with higher gln and ATP concentrations suggests that AS is sensitive to substrate inhibition. The reduction in overall ^{14}C-asn synthesized with high gln concentration may be due to possible competition for the binding site of aspartate on AS. The negative effect observed with high ATP concentration may be associated with the fact that the energetics of reactions catalyzed by enzymes converting ATP to AMP and PPi (i.e., asparagine synthetase) is not favorable, in particular if there is not enough pyrophosphatase activity in the extract to pull the reaction forward by converting PPi into 2 Pi.

We treated subsamples of lupine and pea extracts with 1mM aminooxyacetic acid (AOA) in order to inhibit various aminotransferases present in the crude extracts. This compound was used to prevent AAT from diverting [^{14}C]asp toward other biosynthetic pathways such as the TCA cycle (Richards and Schuster, 1998; Ireland and Lea, 1999). AOA did not have any effect on AS activity in crude extracts of lupine (Figure 4A). In contrast, the presence of AOA resulted in a significant increase in net production of asn in the pea extract. Enhanced asn levels were detectable within 60 min of incubation (Figure 4B). The effect of AOA was even more dramatic after 2 h incubation. It should be noted that detectable [^{14}C]asn levels in the nontreated control peas diminished over the time of the experiment, illustrating the underlying competing pathways using asn as a substrate in this extract (Joy et al., 1983; Snapp and Vance, 1986).

The difference in AAT activity between the lupine and pea extracts was unexpected. However, the extraction of AS from pea was strikingly different from the other

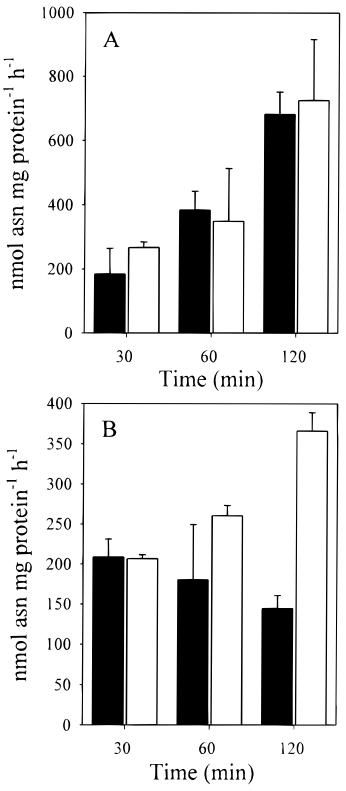

Figure 4. Effects of aminooxyacetate (AOA) on observed rate of ^{14}C-asn synthesis in crude extracts of (A) lupine (*Lupinus albus*) (data are the means of six measurements ± SD) and (B) pea (*Pisum sativum*) (data are the means of five measurements ± SD). Black = standard assay and white = standard assay with 1 mM AOA.

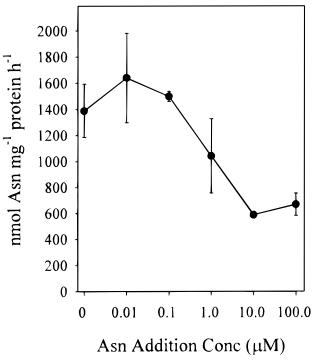

Figure 5. Asparagine synthetase activity from lupine assayed under standard conditions (see Materials and Methods), except that unlabeled asn was added to the reaction mixture. Data are the means of six measurements ± SD.

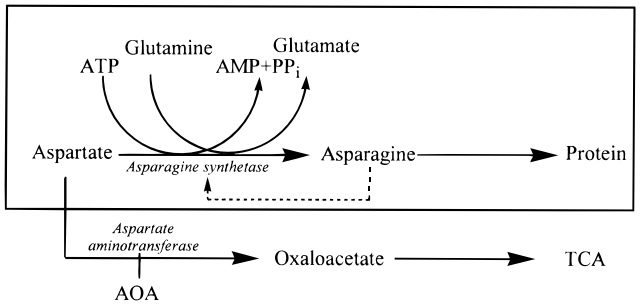

Figure 6. Model noting the reaction catalyzed by AS, the diversion of asp by aspartate amino transferase toward the TCA cycle, and the putative product inhibition of AS by asn.

species. In pea, AS activity was found in the pellet after the 65% ammonium sulfate precipitation, whereas the activity was in the supernatant in the other two species. Therefore, it is likely that with pea, AAT was copurified with AS in the pellet, whereas AAT activity was precipitated out with the other two species, leaving AS activity free of AAT in the supernatant. Further research needs to be done in order to determine what ammonium sulfate concentration would cause AAT to precipitate while retaining AS in the supernatant.

This decrease was also visible in lupine, however, it occurred over a longer time interval [120 min (Figure 1)]. As a result, one must consider that, even when de novo biosynthesis of asn is observed, as with lupine, soybean, and pea (with AOA), other enzymes, such as asparaginase, in the crude protein extract utilize a portion of the asn synthesized, resulting in an apparent level of activity that is lower than the true AS activity in the extracts. We attempted to minimize this effect by providing nonradiolabeled asn to the reaction assay solution, with the intention of supplying a source of asn undetectable to our assay system to these degradative enzymes. However, the dose−response curve indicated that rather than minimizing the effect of asparaginase and subsequently increasing the levels of [^{14}C]asn detected, adding unlabeled asn to the assay reduced overall ^{14}C-asn synthesis when tested at concentrations above 1 μM (Figure 5). This suggests that AS activity is sensitive to product inhibition by asn (dotted line in Figure 6).

Conclusions. The study of highly regulated and unstable enzymes such as AS is problematic. In particular, any length of time spent purifying the enzyme is counterproductive because of the concurrent loss of activity. Several of the limitations involved in measuring plant AS activity have been addressed by the method described. The extraction is rapid and yields a relatively stable enzyme preparation free of endogenous inhibitors. Moreover, the concentration of the reactants has been optimized and permits a sensitive and reproducible detection of de novo asn synthesis.

Detection of AS activity by radio-HPLC is more quantitative than the TLC method (Richards and Schuster, 1998). It also provides an easy, definitive means of identifying the substrate and the product formed during the assay and enables direct measurements of de novo asn synthesis rather than indirect measurements attained using either secondary asparaginase assays (Dembinski et al., 1996) or measuring PP_i release (e.g., Hughes et al., 1997).

ABBREVIATIONS USED

AAT, aspartate aminotransferase; AOA, aminooxyacetic acid; AS, asparagine synthetase; asp, aspartate; asn, asparagine; gln, glutamine.

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