**Short Communication**

**Diurnal Variation of Asparaginase in Developing Pea Leaves**

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**ABSTRACT**

Levels of asparaginase activity from developing pea leaves (*Pisum sativum*) were found to change on a daily basis, increasing during the light period and decreasing in the dark. During extended periods of light, high levels of activity were maintained, while prolonged dark reduced activity to a low value. Half-expanded leaves exhibited the greatest change in activity over the photoperiod. Very young or mature leaves displayed little or no diurnal variation in asparaginase activity.

Asnase hydrolyzes the amide group from asparagine to produce aspartate and ammonia, and provides a route for the utilization of asparagine, a nitrogen transport compound in many plants. Asnase has been found in developing seeds of lupin (1, 3, 8, 9), peas (6, 16), and developing roots and leaves of lupin (3) and pea (6). In young pea leaves, Asnase is high but decreases during leaf expansion and in later stages asparagine aminotransferase appears to be the predominant route for asparagine utilization (6).

In yeast, the synthesis of an extracellular Asnase II is regulated by nitrogen catabolite repression (5, 15) and, in a euryhaline *Chlamydomonas* species, the derepression of an intracellular plasmid Asnase has been reported to be regulated via a light-dependent process (14). In higher plants, regulation of Asnase is not well documented. In a more detailed study of the changes in Asnase levels in developing pea leaves, it has been found that in the expanding leaf Asnase activity shows a marked diurnal variation.

**MATERIALS AND METHODS**

**Plant Material.** Effectively nodulated *Pisum sativum* L. (cv Little Marvel) plants were grown in soil in growth cabinets. A 16-h period of light (12,000 lux, 23°C) and an 8-h dark period (18°C) was provided. Nonnodulated *P. sativum* were grown hydroponically as described previously (2) except that a 16-h light period (9000 lux, 28°C) and an 8-h dark period (18°C) was employed. Plants were used when 3 weeks old, and had four fully expanded leaves. The young (fifth) leaves were selected as they began to emerge from the stipules of the fourth leaf (stage 3 [17]). Developing seeds were obtained from plants 4 to 7 weeks old.

**Enzyme Extractions.** All operations were carried out at 4°C.

Leaves of selected ages were ground in a mortar for 30 s in either 5 or 10 volumes of 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl and 2 mM DTT. The extracts were filtered through four layers of moistened cheesecloth and centrifuged for 10 min at 20,000g. Extracts used for Asnase determinations were prepared either by (NH₄)₂SO₄ precipitation (60% saturation) followed by a 10-min 20,000g centrifugation, with the pellet resuspended in the above buffer, or the supernatant from the initial clarification step was desalted (Sephadex G-25) and the protein fraction used directly for enzyme assay.

Tissue harvested for glutaminase synthetase assay was extracted in 50 mM Tris-HCl buffer (pH 8.0), 10 mM MgSO₄, 1 mM EDTA, and 1 mM DTT. After centrifugation, the desalted (G-25) protein fraction was used for assays.

**Enzyme Assays.** Asnase assays contained enzyme, 20 mM asparagine (purified from contaminating aspartate by passage through Dowex 1 formate, pH 6.8) at pH 8.0, 40 mM KCl, 50 mM Tris-HCl (pH 8.0), and 1 mM DTT in a total volume of 0.3 ml. Mixtures were incubated at 34°C for 30 min at 0 time and at 30 min. 0.2-ml samples were removed and subject to microdiffusion for ammonia analysis, or 25 mg 5-sulfosalicylic acid was added to precipitate proteins, and the aspartate produced was determined via amino acid analysis. GS was assayed by the biosynthetic (glutaminyl hydroxamate) method (13), with 1 mM EDTA in place of 1 mM diethylene triamine pentaacetate in the reaction medium.

**RESULTS AND DISCUSSION**

Asnase extracted from young developing pea leaves was less stable in storage at 4°C than the enzyme derived from developing seed tissue (activity declined to less than 50% of initial activity in 2 d, compared to 5 d for seed). This is in agreement with the report for lupin Asnase (3). The leaf enzyme displayed a requirement for K⁺ (3, 6, 16) although almost half the activity was still detectable when extracted in the absence of K⁺. The addition of K⁺ to extracts prepared without K⁺ only partially restored activity. Activity of preparations extracted without K⁺ was also partially restored by precipitation with (NH₄)₂SO₄. Both K⁺ and DTT were required to maintain activity at 4°C.

Previous reports have indicated, as found here (Fig. 3), that levels of Asnase activity are high in young leaves and decrease during leaf maturation (3, 6). Upon further analysis it became apparent that the extractable Asnase activity not only changes with leaf age, but also varies on a daily basis. When assayed over 24 h, Asnase activity from young leaves increased steadily through the photoperiod (Fig. 1). In the dark period, the levels decreased to those observed at the onset of the photoperiod. These experiments were performed upon nodulated plants; a similar trend was observed in nonnodulated plants. The diurnal change in activity was also observed when activity was expressed on a per mg protein or per leaf basis, and was therefore not a result of changes in fresh weight over the 24-h period.

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3 Abbreviations: Asnase, asparaginase; GS, glutamine synthetase.
The levels of asparagine within young leaf tissue was also monitored over a 24-h period (Fig. 1). An inverse pattern in the levels of asparagine, when compared with Asnase activity, is evident with the levels decreasing during the photoperiod and increasing in the dark. The leaves used for this analysis (Fig. 1) were still enclosed within the stipules of the next older leaf and transpiration rates would have been minimal (17). The decreased levels of asparagine during the photoperiod may indicate either reduced translocation into the developing leaf, or increased utilization in the light.

Both nodulated and nonnodulated plants were subjected to extended light or dark periods to determine whether the diurnal change in activity was an entrained circadian rhythm. A prolonged light period resulted in the maintenance of high levels of activity (Fig. 2). When the dark period was extended, the levels of extractable activity continued to decrease slowly to a low value of 20 to 40 nmol aspartate/min·g fresh weight (Fig. 2). This was observed both when the temperature remained unchanged (at 18°C) during the extended dark period, and also when there was a switch to 23°C (the temperature of the usual light period) in the extended dark period. Thus, the diurnal increase in activity of Asnase is a light-dependent process. A light-dependent increase in Asnase activity via derepression of Asnase synthesis has been reported in *Chlamydomonas* (14), and required a 40-h period to achieve a four-fold increase in activity. In the pea leaf system, a similar increase in activity was obtained in 16 h, and little further increase was obtained in prolonged light (Fig. 2).

Since the variation of Asnase could result in a diurnal variation in ammonia release, it was of interest to look for daily changes in activity of glutamine synthetase which would be responsible for ammonia reassimilation. Earlier reports have indicated that the chloroplastic isozyme GS\(_2\), increased in activity when etiolated leaf tissue was exposed to light (cf. 12). Other workers have also demonstrated both diurnal variations of GS activity in *Hordeum vulgare* leaf tissue (10) and ultradian rhythms in leaves of *Glycine max* (4) and roots of *Helianthus annuus* (7).

When GS was analyzed over a 24-h period, no consistent diurnal pattern was evident. Extractable activity did fluctuate, but from day to day there was no consistent time for a peak; this may indicate the possibility of an ultradian cycle (data not shown). The levels of ammonia remained low and essentially constant (at about 1 to 1.5 µmol/g fresh weight) throughout the day, indicating that the levels of GS in the tissue appear to be adequate for reassimilation of ammonia produced by Asnase activity and by photorespiration. The maximum potential rate for Asnase in these tissues was approximately 2.5 µmol/h·mg
Chl. This is comparable with rates of inorganic nitrogen assimilation in leaves and about one-tenth of the estimated rates of nitrogen cycling in photorespiration (20–40 µmol/h·mg Chl [11]).

Since Asnase activity decreases with leaf age (6), the light-dependent increase in activity over the photoperiod was investigated during leaf development. Leaf tissue was harvested at five different ages and examined for Asnase activity at the end of the dark and light periods (Fig. 3). During development, there was a steady decrease in Asnase activity when measured at the end of the dark period, and this is in agreement with that reported earlier (6). However, activity measured at the end of the light period showed a different relationship. Maximum activity first increased, then remained high during early stages of development, and finally fell rapidly (Fig. 3). The greatest stimulation of activity was observed in half-expanded leaves approximately 3 d after emergence from the stipule. Very young (younger than that used for earlier experiments; Figs. 1 and 2) or mature leaf tissue did not exhibit a diurnal change in extractable activity. This pattern was observed in nodulated and nonnodulated plants.

Changes in Asnase in pea leaf may be due to a daily variation in the rate of enzyme synthesis or turnover, or to activation/deactivation of an existing protein; this is currently under investigation, and preliminary results suggest that both processes may be involved.

LITERATURE CITED