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ISOLATION AND PROPERTIES OF AN ASPARAGINASE FROM LEAVES OF *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; asparaginase; diurnal variation; developing leaves.

Abstract—Asparaginase (EC 3.5.1.1), which displays a diurnal variation in activity in developing leaves of *Pisum sativum*, has been isolated from half-expanded leaves obtained at the end of the dark (dark-harvested) or during the light (light-harvested) periods and its properties compared to determine if variation in activity is a result of changes to the protein. Both dark- and light-harvested asparaginase preparations required K^+ and Ca^{2+} for maximum activity and had similar M_r (58 000), pH optima (7.8–9.8), and K_m for asparagine (3.2–3.7 mM). Asparaginase activity from light- or dark-harvested leaves co-eluted during size-exclusion, ion-exchange and hydroxylapatite chromatography. Addition of MgATP to crude preparations inhibited activity non-competitively (K_{ies} 0.2 mM, K_{ie} 0.8 mM), but no inhibition of activity was noted by ATP in purified preparations. The activity of purified asparaginase was not affected by a range of monovalent or divalent ions (except for Zn^{2+}), or by any of the protein amino acids. These results indicate that changes in asparaginase activity during the photoperiod are not a result of post-translational modification of existing protein, and support our previous suggestion that increased activity is a result of *de novo* synthesis.

INTRODUCTION

The degradation of asparagine in developing roots, leaves, and cotyledons is important in supplying carbon and nitrogen for the growth of these structures [1]. Asparagine is catabolized through two routes in plants, one involves transamination (asparagine: pyruvate transaminase, EC 2.6.1.14) [2–4], and the other starts with the deamidation of asparagine producing aspartate and ammonia (asparaginase EC 3.5.1.1) [5, 6]. Transamination of asparagine is observed predominantly in leaves, with low levels of activity detected in pod tissues [2], whereas asparaginase activity is present not only in leaves but also in roots [2, 7], and cotyledons [8] and decreases during maturation of these organs [7, 8]. The activity of asparaginase in developing cotyledons is much higher than that of roots or leaves [2, 7] and has therefore commonly been the organ of choice for the purification and characterization of the enzyme [6, 7]. These latter studies [6, 7] examined properties of asparaginases that did not require K^+ for full activity.

In young leaves of *Pisum sativum* [2] and two *Lupinus* species [7] K^+ is required for maximum *in vitro* asparaginase activity. In leaves of *P. sativum* asparaginase activity decreases with leaf age and varies diurnally, with increased activity during the light period [9]. The increase in activity in half-expanded leaves is dependent upon photosynthetic electron transport [10], yet products of the light reactions of photosynthesis (e.g. NADPH, ATP, reduced ferredoxin) do not directly affect asparaginase activity *in vitro* [11]. We have therefore characterized asparaginase activity extracted from half-expanded leaves at the end of the dark (dark-harvested) or during the light (light-harvested) periods to determine if changes in the properties of the enzyme contribute to the observed diurnal variation in activity. This is the first report on the characterization of a K^+-dependent asparaginase isolated from leaf tissue.

RESULTS AND DISCUSSION

Crude preparations of asparaginase obtained from dark- or light-harvested half-expanded pea leaves were stable for only short periods of time, with losses in activity of 50% during 24 hr at 4°, or 80% over 10 min at 34° [11]. However, after partial purification following size exclusion or ion exchange chromatography, asparaginase activity was more stable with only a 10% loss of activity noted over four months storage at −20°, or 2 hr at 34°. During storage, a M_r 9 500 peak (detected by size exclusion HPLC), not present in freshly purified preparations following ion exchange chromatography, increased proportionally with storage time.

Purification typically yielded 10–15% of initial activity, with 70–90-fold purification (Table 1). The presence of one major band, (M_r 60 000) along with several minor contaminants after non-denaturing gel electrophoresis were observed, but attempts to localize asparaginase activity within the gel either by slicing the gel followed by elution and assay of asparaginase, or by activity staining in the presence of tetraphenylboron were not successful (see Experimental).

Properties of dark- and light-harvested asparaginase

In half-expanded pea leaves asparaginase displays a diurnal variation in activity [9], and the increase in

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Table 1. Purification of asparaginase from light-harvested leaves

Protein fraction	Total protein (mg)	Total activity (nmol asp/min)	Sp. act. nmol asp/min/mg protein	Purification fold	yield	fold (per step)
Crude	199	302	1.52	1	100	—
25–65%	139	296	2.13	1.4	98	1.4
Sephacryl S300	18.3	393	21.5	14.1	130	10.1
DEAE cellulose	2.7	181	66.5	43.8	60	3.1
Hydroxylapatite	0.3	36	113	74.2	12	2.6

activity during the light period is dependent on photosynthetic electron transport [10]. As asparaginase in other organisms is subject to modulation of activity, including autophosphorylation [12], protein synthesis/degradation [13], and sigmoidal kinetics in response to asparagine [14], it was important to determine whether changes in asparaginase activity in developing pea leaves resulted from alterations of existing protein, as well as from the synthesis of new asparaginase protein as recently proposed [11].

Asparaginase activity, obtained from dark- or light-harvested half-expanded pea leaves, co-eluted during open column size exclusion chromatography (S300), size exclusion HPLC, ion exchange, or hydroxylapatite chromatography. The M_r of leaf asparaginase (58 000) is less than that of asparaginases isolated from *Lupinus* (72 000 [6], 75 000 [7] or *Pisum* (68 000 [8]) cotyledons. After S300 chromatography, total asparaginase activity increased 30–40% from light-harvested leaves (Table 1) while no increase in total activity was observed after S300 chromatography of dark-harvested leaves. The pH optima of dark- or light-harvested preparations were broad, with 95% of the activity observed between pH 7.8 and 9.8, and this was observed in crude or purified extracts.

Michaelis–Menten kinetics were observed for both dark- and light-harvested preparations extracted in the presence of Tris, Tricine, or potassium phosphate buffers at pH 8 containing 10% glycerol, with a K_m of 2.5–4.9 mM for asparagine (Table 2). In the absence of glycerol the K_m for asparagine increased and varied between 7.5 and 14.8 mM and these values approximate previously published K_m's of asparagine for asparaginase activity in cotyledons [2, 8] and leaves [2, 7]. Hyperbolic kinetics were observed in the presence or absence of K^+, Ca^{2+}, or ATP (see below); there was no evidence of asparaginase responding allosterically to asparagine as reported in yeast [14]. The addition of glycerol to crude or purified enzyme preparations was essential for kinetic estimations, since in the absence of glycerol and at low asparagine concentrations (less than 7–8 mM) asparaginase is inactivated [11]. Aspartate also stabilizes asparaginase in the absence of glycerol and asparagine [11], but a 25% loss of activity in crude preparations was noted in the presence of 10 mM aspartate and no glycerol (data not presented). Ammonia (up to 3 M) had no effect on activity (also see [15]).

Asparaginase activity in crude preparations obtained from dark- or light-harvested leaves in Tris buffer (buffer A, see Experimental) was inhibited by MgATP noncompetitively (K_{ies} 0.2 mM, K_{ie} 0.8 mM), supporting the suggestion that asparaginase activity is not modulated by phosphorylation/dephosphorylation [11] as it is in *Leptosphaeria michotii* [12]. No inhibition of asparaginase activity by up to 1 mM MgATP was observed in purified preparations.

Table 2. The K_m for asparagine of asparaginase preparations extracted in different buffers

Extraction buffer	0 hr K_m (±s.d.)*		6 hr K_m (±s.d.)*	
Tris–HCl + KCl + $CaCl_2$				
crude extract	7.0	(0.6)	8.3	(0.9)
S300 extract	14.5	(1.9)	14.8	(1.8)
+ 10% glycerol				
crude extract	2.5	(0.2)	4.2	(0.4)
S300 extract	3.4	(0.8)	2.8	(0.6)
Potassium phosphate + 10% glycerol				
crude extract	3.7	(0.4)	4.9	(1.1)
S300 extract	—		2.0	(0.3)
Tricine + KCl + $CaCl_2$ + 10% glycerol				
Crude extract	3.6	(0.7)	3.2	(0.8)

*K_m asparagine mM.

Half-expanded leaves were harvested at the end of the dark period (0 hr) or during the light period (6 hr) in the indicated buffers, and assayed immediately, or assayed following partial (S300 chromatography) purification ($n = 3$, ± s.d.).

Effects of ions and metabolites on asparaginase activity

The response of asparaginase to a range of ions was complex. Potassium is required for maximum activity of asparaginase isolated from several plant tissues and species including *Pisum sativum* [2, 7, 8]. Dark- or light-harvested pea leaves (half-expanded) exhibited an 8–10-fold increase in asparaginase activity when assayed in 20–50 mM potassium chloride (Fig. 1). No increase in asparaginase activity was observed in the presence of sodium chloride. The effect of K^+ on asparaginase activity was reversible: K^+ could be removed and added back to extracts with varying degrees of recovery of the original activity depending on the time of extract preparation during the photoperiod. In the absence of K^+, a diurnal variation of activity was still observed (Table 3) suggesting that diurnal changes in activity are not dependent upon changes in subcellular K^+ ion concentrations during the photoperiod.

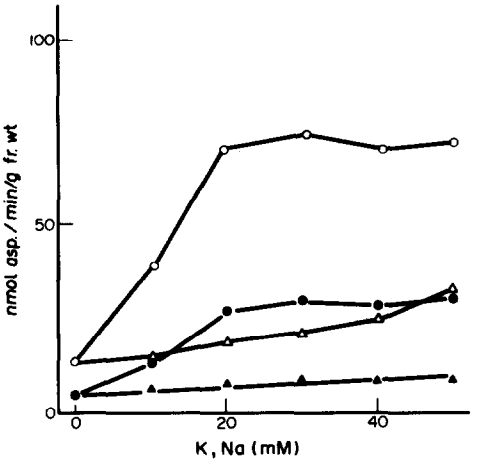

Fig. 1. Effect of K^+ and Na^+ on asparaginase activity. Half-expanded leaves were harvested at the end of the dark period (solid symbols), or after 6 hr in the light (open symbols) in the absence of K^+ or Na^+, and assayed in the presence of increasing KCl (●, ○) or NaCl (▲, △) concentrations along with 20 mM asparagine for 30 min. The experiment was repeated with similar results.

The addition of calcium chloride to crude dark- or light-asparaginase preparations harvested in the absence of Ca^{2+} had little effect on activity (Fig. 2a). A similar result was also found for asparaginases isolated from cotyledons [6, 8]. However, extracting dark- or light-harvested leaves in the presence of 0.5 mM Ca^{2+} resulted in increased asparaginase activity (Fig. 2b, c). Calcium ions had no effect on the K_m for asparagine (3.6 mM), but doubled the V_{max}. It has previously been reported that calmodulin is not involved in mediating asparaginase activity [11].

When dark- or light-harvested leaves were extracted in the absence of K^+ and crude preparations incubated with several divalent ions, a complete loss of activity was observed in the presence of 10 mM Mn^{2+}, Mg^{2+} (as the Cl^-), $NaNO_2$ and Zn_2SO_4. Only Zn^{2+} produced a complete loss of activity when leaves were extracted in the presence of K^+. However, the activity of purified asparaginase (either dark- or light-harvested) was not affected by the addition of any ion added to the reaction mixture at a 10 mM concentration [$MeCOO^-$, I^-, Cl^-, NO^{2-}, NO^{3-}, SO_4^{2-}, CO_3^{2-} supplied as ammonium, potassium, or sodium salts; Li^+, NH_4^+, Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cd^{2+} supplied as Cl^-, SO_4^{2-}, or $(MeCOO^-)_2$ salts].

The addition of protein amino acids and several non-protein amino acids and other compounds (homoserine, 2- and 4-aminobutyric acid, hydroxyproline, citrulline, allantoate, norvaline, methionine sulphoximine, aminooxyacetate, albizziine, fructose-2,6-bisphosphate; all at 20 mM; asparagine 5 mM) also had no effect on purified asparaginase activity.

Characterization of asparaginase harvested at the end of the dark, or during the light periods indicates that differences of activity are not a result of differences in the properties of the enzyme. The dark- or light-harvested preparations had similar M_r, pH optima, K_m for asparagine, as well as similar responses to added ions and metabolites in crude or purified preparations. These observations are consistent with the proposal that variation in asparaginase activity is not a result of modulation of existing protein; rather, it is due to the synthesis and degradation of asparaginase protein [11].

Once purified, asparaginase activity was more stable than in crude preparations, but degradation of asparaginase still took place since a peak of M_r 9 500, not detected in freshly purified preparations, increased during storage. Several observations suggest that decreased asparaginase activity may be a result of proteolytic degradation of existing protein. The first is that during purification of light-harvested preparations, an increase in the total activity of the asparaginase preparation took place following S300 chromatography. No increase in total activity was observed after chromatography of dark-harvested preparations suggesting that inhibitory components are removed following chromatography of light-harvested preparations. The second is that asparaginase is inhibited in crude preparations by ATP but this is not observed after enzyme purification, again suggesting that a factor removed during chromatography, possibly an ATP-dependent protease, may be involved in the degradation of asparaginase.

Table 3. Diurnal variation of asparaginase activity harvested in the presence or absence of K^+

Time of sampling in hr	with K^+	no K^+	K^+ added back*
	(asparaginase activity: nmol asp/min/g fr.wt)		
L 2	63.2	32.1	49.9
15	114 -	75.6	117
D 4	46.1	14.2	28.3
6	54.3	13.7	18.7

*Extracts were obtained in the absence of K^+, and K^+ was added to the reaction mixture (final concentration 50 mM).

Half-expanded leaves were obtained during the light (L) or dark (D) periods, desalted through G25 in the presence or absence of 50 mM K^+ in Tris buffer and assayed for asparaginase activity. The start of the light period is 0 hr. The experiment was repeated with similar results.

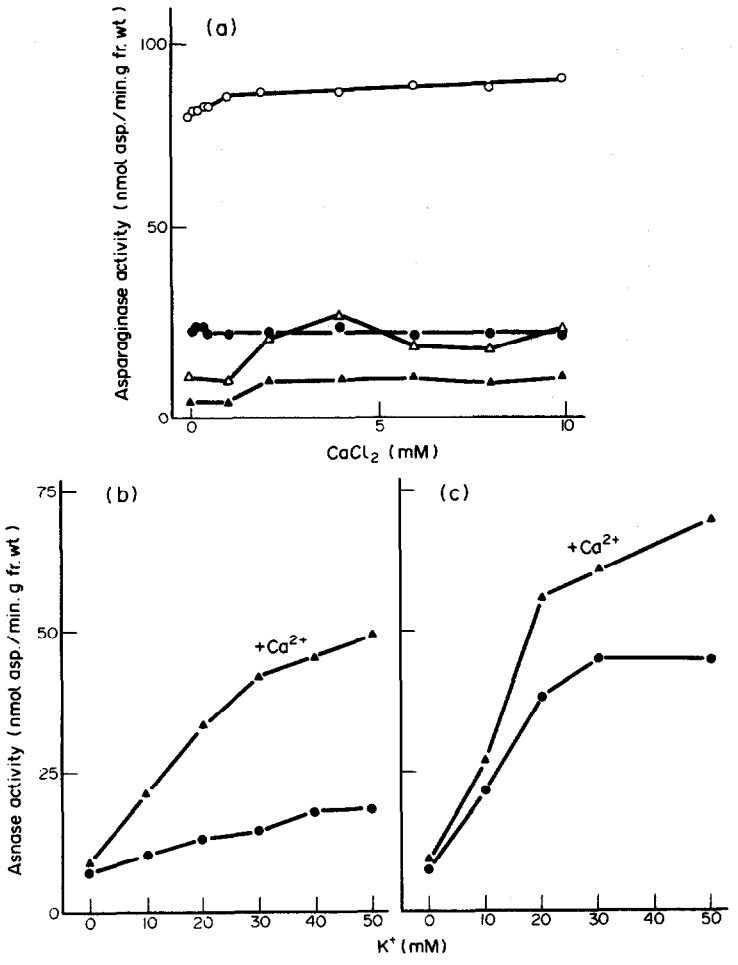

Fig. 2. Effect of Ca^{2+} on asparaginase activity. (A) Half-expanded leaves were harvested at the end of the dark period (solid symbols), or after 6 hr in the light (open symbols) in the presence (●,○) or absence (▲,△) of K^+, and in the absence of Ca^{2+}. Increasing concentrations of $CaCl_2$ were then added to assay mixtures in the presence of 20 mM asparagine for 30 min. (B) Half-expanded leaves were harvested at the end of the dark period in the presence (▲) or absence (●) of 0.5 mM $CaCl_2$, and in the absence of K^+. Increasing concentrations of KCl were then added during assay of asparaginase activity. (C) Half-expanded leaves were harvested after 6 hr in the light period in the presence (▲) or absence (●) of $CaCl_2$, and in the absence of K^+. KCl was then added back during the assay of asparaginase. All experiments were repeated with similar results.

EXPERIMENTAL

All biochemicals were obtained from Sigma, except for mono- and divalent ions (reagent grade) which were obtained from either Fisher or BDH. Sephacryl S300 was obtained from Pharmacia, DEAE cellulose and hydroxylapatite from BioRad. HPLC columns were obtained from Waters (Protein Pak 300SW) or Whatman (C18 Partisphere). All gel electrophoresis reagents were purchased from BioRad.

Plant material. Non-nodulated *Pisum sativum* L. were grown as described earlier [10]. Developing fifth leaves were used for all enzyme extractions and were obtained at the end of the dark period (dark harvested, with low levels of activity), or after 6 to 8 hr of light (light harvested, with 3- to 5-fold higher levels of activity).

Asparaginase isolation. All operations were carried out at 4°. Half-expanded leaves (10 to 15g) were ground in 10 vol. of 50 mM Tris–HCl (pH 8), 50 mM KCl, 1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), and 10% glycerol (buffer A). Extracts were filtered through 4 layers of cheese cloth and clarified at 20 000 *g* for 10 min. The $(NH_4)_2SO_4$ fraction precipitating between 25 and 65% was resuspended in *ca* 1 vol. of buffer A and applied onto Sephacryl S300 (2.5 × 80 cm) equilibrated in buffer A. The asparaginase-containing fractions were pooled and applied onto DEAE Sephacel (1 × 26 cm) equilibrated in buffer A, washed with 100 ml of buffer A, and developed with 300 ml linear gradient of 0.05 to 0.5 M KCl in buffer A; asparaginase eluted at 0.2 M KCl. Pooled fractions were diluted 5-fold in 5 mM Na-Pi buffer (pH 7.8) containing 50 mM KCl, and 20% glycerol (buffer B) and loaded onto hydroxylapatite (1 × 26 cm, equilibrated with buffer B). After a 100 ml wash with buffer B, asparaginase was eluted with a 300 ml gradient of Na-Pi (0.005–0.2 M); asparaginase eluted at 0.05 M Na-Pi. Pooled fractions were concd in dialysis tubing against polyethylene glycol 8000.

Size exclusion HPLC was carried out with a Protein Pak 300SW column (equilibrated in 75 mM K-Pi buffer, pH 7.5) and run at a flow rate of 0.6 ml per min at 22°. Up to 50 μg protein were injected in a total of vol. of 10 μl, and 0.3 ml fractions of eluant were collected in 1.5 ml microfuge tubes containing

asparagine (final concn 20 mM) and K-Pi buffer, pH 8 containing 20% glycerol, in a total vol. of 0.2 ml and processed as described below. Protein elution was followed at 218 nm.

Asparaginase assay. During open CC, asparaginase activity was assayed by measuring NH_3 production [16] after a 30 min incubation period (34°) in the presence of 20 mM asparagine in final vol. 0.25 ml. For all characterization work asparaginase was assayed in the presence of 20 mM asparagine (unless otherwise noted) along with components of interest, and aspartate production was determined by HPLC. This involved terminating reactions (0.25 ml final vol.) by adding 25 mg of 5-sulphosalicylic acid, followed by a 3 min spin in an Eppendorf microfuge. Aliquots (0.2 ml) of the supernatant were adjusted to pH 6 with 1 M NaOH, diluted with 10% HPLC grade MeOH, and 10 μl samples derivatized with *o*-phthaldialdehyde for chromatography by HPLC on a C18 Partisphere column.

Estimation of M_r. Open column S300, and HPLC 300SW columns were calibrated using blue dextran, alcohol dehydrogenase (M_r 150 000), bovine serum albumin (M_r 66 000), carbonic anhydrase (M_r 29 000), and cytochrome *c* (M_r 12 400) and run as above.

Electrophoresis was carried out using the method of ref. [17]. Attempts were made to maintain asparaginase activity during electrophoresis and these included substituting borate for glycine in the tank buffer [7], and/or adding 30 mM KCl to the gel and tank buffers. Two methods were used to assay asparaginase activity following electrophoresis. The first involved slicing gels into 2 mm sections and incubating these sections in buffer A for 2, 6, or 24 hr at 4° prior to assaying for asparaginase in the presence of asparagine (the production of aspartate was assayed using HPLC). The second method involved covering the gel with an agar overlay containing tetraphenylboron and asparagine in buffer (tetraphenylboron forms a white ppt. in the presence of NH_4^+ against a dark background) [18]. It was noted that tetraphenylboron formed a ppt. in the presence of K^+ which was included in gels or assay buffer since it is required for asparaginase activity. With either of these methods no asparaginase activity could be detected following electrophoresis.

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