A GLUTAMINE-DEPENDENT ASPARAGINE SYNTHETASE FROM YELLOW LUPINE SEEDLINGS

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1. Introduction

Studies on asparagine formation in seedlings are classical in plant biochemistry, dating back more than one century [1,2]. Despite the vast amount of physiological work done on this subject, the true pathway of asparagine biosynthesis in higher plants has remained obscure. Purified bacterial asparagine synthetase (ASase) (L-aspartate:ammonia ligase (AMP), EC 6.3.1.1) converts aspartate to asparagine in the presence of NH_3, ATP and Mg^{2+}, with concomitant production of AMP and inorganic pyrophosphate [3–5]. The amide nitrogen atom of glutamine rather than ammonia is primarily utilized by ASases of mammalian origin [6–9].

No comparable, well-characterized enzyme has been purified from higher plant sources [10,11]. Some authors have claimed that asparagine synthesis in plant tissues [12–14] and yeast [15,16] takes place by a mechanism analogous to the glutamine synthetase (EC 6.3.1.2) reaction, but even recent attempts in other laboratories to carry out such a reaction, or demonstrate conversion of aspartate to asparagine *in vitro*, have failed [17–19].

Since a thorough reexamination of this problem at the enzyme level appeared highly desirable, the present study was undertaken. As a first step, this paper describes some general properties of a partially purified enzyme extracted from lupine seedlings, catalyzing the formation of asparagine from aspartate in the presence of ATP and Mg^{2+}, glutamine being far superior to ammonia as donor of amide group nitrogen.

2. Materials and methods

2.1. *Source of seed and chemicals*

Yellow lupine seed (*Lupinus luteus* L.), of a low-alkaloid variety grown in Denmark, was purchased from L.O.G./H. Nielsen A/S, (Oslo). Uniformly labeled L-^{14}C-aspartic acid and 4-^{14}C-aspartic acid were obtained from ICN (Irvine, California), Calbiochem (Los Angeles), New England Nuclear (Boston), and the Radiochemical Centre (Amersham). Prior to use, the radioactive materials were diluted with cold L-aspartic acid (neutralized with KOH). Tris base, disodium ATP, L-aspartic acid, L-asparagine monohydrate (homogenous by electrophoresis), L-glutamine and other L-amino acids were from Sigma (St. Louis), as well as *E. coli* L-asparaginase (EC 3.5.1.1) (40 I.U./mg). Sephadex was from Pharmacia (Uppsala).

2.2. *Germination of seeds and enzyme preparation*

The lupine seeds were rinsed, soaked overnight, treated with 0.1% $HgCl_2$, and washed repeatedly before sowing in trays on moist vermiculite. Germination took place in darkness at 25° for 6–8 days. The hypocotyls were cut, and the following steps were performed at 0–5°. 60 g of etiolated shoots were homogenized with 120 ml of 100 mM tris-Cl, pH 7.5 (containing 10% glycerol and 7 mM 2-mercaptoethanol) in a Waring Blendor operated at full speed for 2.30 sec. The homogenate was strained through cheesecloth, followed by centrifugations, first for 10 min at 2000 *g* and then for 30 min at 45,000 *g*. The precipitates were discarded. Cold,

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saturated $(NH_4)_2SO_4$ solution (brought to pH 7.5 with NH_3, containing 7 mM 2-mercaptoethanol and 0.5 mM EDTA) was added to the 45,000 *g* supernatant to 60% saturation. The precipitate was collected by centrifugation and washed with cold 60% saturated $(NH_4)_2SO_4$, with additions as above. The well-drained protein pellets could be stored in the (eight) centrifuge tubes for at least two weeks at – 25° with little decrease in ASase activity, but freezing and thawing of enzyme solutions led to serious losses. Before assays, the protein in two tubes was dissolved in 4 ml cold 50 mM tris-Cl, pH 7.5, with 10% glycerol and 7 mM 2-mercaptoethanol, and passed through a 2.11 cm Sephadex G-25 column previously equilibrated against the same buffer. Protein fractions free of NH_4^+ were pooled and used as the source of the enzyme.

Protein was estimated by the Lowry method [20].

2.3. *Enzyme assay*

The standard assay mixtures were incubated at 37° for 60 min and contained 8 mM L-4-^{14}C-aspartate (3.7×10^5 cpm/μmole), 10 mM ATP, 15 mM $MgCl_2$, 20 mM glutamine, 5 mM 2-mercaptoethanol, 100 mM tris-Cl, lupine protein (Sephadex G-25 eluate) and water in a final volume of 200 μl. Final pH at 37° was 8.0. The reaction was stopped by addition of 50 μl of 10% trichloroacetic acid, followed by 2 μmoles of cold carrier asparagine. After centrifugation, 25 μl aliquots of the supernatant were spotted on Whatman no. 3 paper, and asparagine was separated from aspartate by electrophoresis (0.05 M Na-acetate, pH 5.0, 48 V/cm, 40 min). A 2.3 cm strip bearing the asparagine spot was cut out and counted in a Philips Liquid Scintillation Analyzer, using 4 g PPO and 0.2 g POPOP per litre toluene. Figures obtained were corrected for the value of control tubes without enzyme (≈ 150 cpm), representing a neutral impurity in the ^{14}C-aspartate, not destroyable by asparaginase. Activity is expressed as total cpm in asparagine per incubation tube.

3. Results and discussion

When seedling homogenates were incubated with ATP, Mg^{2+}, glutamine and 4-^{14}C-aspartate at pH 8, and the reaction products were separated by ion exchange [21] or electrophoresis, a small, but distinct enzymatic formation of neutral, radioactive material was detected (2-4 nmoles/mg protein/hr). After centrifugation, all of this activity could be accounted for in the 45,000 *g* supernatant. Fractionation (section 2.2) resulted in 15-20 fold increase in specific activity, and a 2-3 fold apparent increase in yield, thus permitting characterization of the radioactive product. Its formation was more than 98% inhibited when *E. coli* L-asparaginase (0.25 I.U.) was included in standard assay mixtures. When asparaginase was added to the complete assay after 60 min, and the tubes were incubated for another 15 min at 37°, 97% of the labeled product was destroyed. After elution from electrophoresis strips, the radioactive material migrated as a discrete spot, exactly overlapping authentic asparagine during paper chromatography and electrophoresis in three systems. This evidence showed that the product measured was L-asparagine.

Shown in table 1 are the requirements of the partially purified ASase. No activity was observed in the absence of ATP or Mg^{2+}, or with boiled enzyme. Activity was 93% dependent upon added glutamine, even when no rigid precautions were taken to eliminate ammonia from the reaction components. Ammonium ions substituted rather poorly, being only one sixth as effective as glutamine at this pH.

Mn^{2+} could not successfully replace Mg^{2+}. 3, 10 and 15 mM $MnCl_2$ gave 9, 6 and 0%, respectively, of the activity obtained with 15 mM $MgCl_2$ under standard conditions.

The synthesis of ^{14}C-asparagine was linear with time for at least 60 min, and with the amount of protein added to the system up to 1 mg. The system was saturated at 8 mM aspartate, half-maximal rates being reached at 2.5 mM (fig. 1A). 10 mM ATP was optimal for asparagine synthesis with this relatively crude preparation (fig. 1B), which still catalyzed independent adenine nucleotide interconversions.

A high affinity of the enzyme for glutamine was evident. This nitrogen donor was essentially saturating at 2 mM, with an apparent K_m around 0.5 mM. Higher concentrations of NH_4^+ (5–10 mM) were required for saturation (fig. 2A). However, a striking difference was noted in the maximal rates obtained, glutamine giving 4–10 times more reaction at pH 7.5–8.2, with an optimal pH close to 8.0 (fig. 2B). Relative effectiveness of NH_4^+ was highest at the highest pH-values, decreasing to zero at pH 7.0. At

Table 1
Requirements for synthesis of ^{14}C-asparagine.

Deviations from	^{14}C-asparagine formed	
standard assay	cpm	nmoles
None	13,280	36.0
– $MgCl_2$	0	0
– ATP	0	0
– Glutamine	980	2.6
– Glutamine + 20 mM NH_4Cl	2,345	6.3
– Enzyme, or boiled enzyme	0	0

Standard assay conditions (see section 2.3), pH 7.8, 0.72 mg lupine protein per tube.

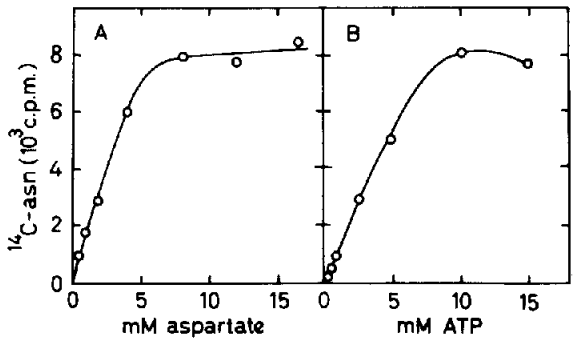

Fig. 1. Lupine ASase activity as function of the concentrations of aspartate and ATP. A) aspartate concentration variable, otherwise standard conditions, 0.51 mg protein per tube. B) ATP concentration variable, otherwise standard conditions, 0.51 mg protein per tube.

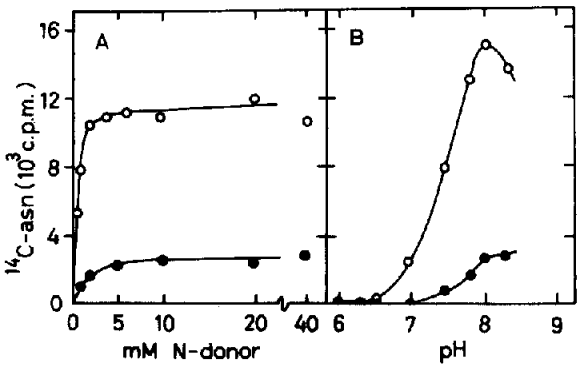

Fig. 2. Comparison between glutamine and NH_4^+ as donors of amide group nitrogen in the lupine ASase system. A) glutamine concentration variable (○), glutamine omitted and replaced by varying amounts of NH_4Cl (●), otherwise standard conditions, 0.60 mg protein in all tubes. B) Activity as function of pH, using 20 mM glutamine (○) or 20 mM NH_4Cl (●) as nitrogen donor. Tubes containing 0.72 mg protein were incubated under standard conditions, except that tris-acetate buffers were used, giving the indicated final pH-values at 37°.

Table 2
Effect of amino acids on enzyme activity.

Amino acid added	Concn. (mM)	ASase activity (percent of control)
None	–	100
L-Asparagine	5	64
	50	41
L-Threonine	50	86
L-Lysine	50	90
L-Methionine	50	77
L-Isoleucine	50	80
L-Glutamate	50	69

Standard assay conditions, with amino acids added at the given concentrations, 0.70 mg protein per tube. Control tubes gave 14,110 cpm in asparagine = 100%.

this pH, glutamine still permitted significant reaction. The effectiveness of ammonia in this system seems to be much lower than observed for mammalian ASases [6,8,24]. Preliminary results indicate that NH_4^+ inhibits the glutamine-mediated reaction at low glutamine concentrations, suggesting competition between ammonia and glutamine for the same binding site(s). This behaviour of the lupine ASase toward the two substrates resembles that of the carbamoyl phosphate synthetase (EC 2.7.2.5) of pea seedlings [22].

The increase in yield resulting from the simple fractionation method employed suggested inhibition of the reaction by materials present in the crude extract. Since the lupine seedlings were loaded with asparagine at harvest [1], the effect of the amide on its own synthesis was tested (table 2). At 5 and 50 mM, asparagine inhibited 36 and 59%. The effect appeared to be specific, other amino acids tested giving only 10–23% inhibition at 50 mM. Glutamate, a likely reaction product, inhibited 31%. The apparent paradox, that lupine ASase is inhibited by asparagine, which is known to accumulate to the extent of 20%

of seedling dry weight [1,23], is probably explained by compartmentation phenomena and transport of newly formed asparagine to storage pools *in vivo*.

Glucose, at 5 or 25 mM, had no effect on the reaction. 8 mM NaF caused only 6% inhibition. Assayed in the absence of thiol, the enzyme was completely inactivated by 5 mM *p*-hydroxymercuribenzoate.

The enzymatic activity described here has several properties in common with ASases of animal cells [6–9,24], but differs appreciably from reported activities from plant tissues [12,14,15,21]. However, certain aspects of the work carried out prior to these studies should be mentioned. Initial experiments were performed with yeasts (Fleischmann baker's yeast and *Candida utilis* ATCC 8205). Several months were spent trying to confirm the earlier reports [15,16], but all attempts to demonstrate ASase in yeast extracts were totally negative, probably due to the very active asparaginase present. This enzyme interfered with the detection of ASase in *E. coli* [5] and guinea-pig tissues [24], inclusion of 5-diazo-4-oxo-norvaline or high salt concentrations being necessary in order to show activity. Enzymatic production of $^{14}CO_2$ and ^{14}C-alanine (interfering with the detection of ^{14}C-asparagine) from U-^{14}C-aspartate was shown, but fractionation of yeast extracts according to the purification procedures described [15], only led to enrichment of a mixture of asparaginase and a threonine-sensitive aspartate kinase (EC 2.7.2.4).

Uniformly labeled aspartate was also used in later studies with a number of etiolated seedling tissues. Virtually all the crude extracts examined, under ASase assay conditions, showed the presence of enzymatic activity forming $^{14}CO_2$ and neutral radioactive material. However, this labeled material, by chromatography indistinguishable from alanine*, was not destroyed by *E. coli* asparaginase, nor was its formation ATP-dependent.

Similar labeling of materials different from asparagine during ASase assays with U-^{14}C-aspartate has been reported by others [8,24]. Lees et al. [18] also identified alanine as a major product of U-^{14}C-aspartate metabolism in crude extracts of wheat shoots. The magnitude of such interference prevented the detection of ASase in crude systems by relatively simple assay methods. By abandoning U-^{14}C-aspartate and instead using 4-^{14}C-aspartate, these difficulties were largely eliminated.

Oaks [21], using U-^{14}C-aspartate and crude extracts of corn scutella, advanced a hypothesis that sugars, like glucose, regulate asparagine synthesis by direct inhibition of ASase. The method used for assaying ^{14}C-asparagine was non-specific, and no data for dependencies or product characterization were given. The present findings make the validity of her conclusions highly questionable.

4. Conclusion

This communication reports for the first time the existence of a glutamine-dependent enzyme system in a higher plant capable of converting aspartate to asparagine *in vitro*. No too far-reaching conclusions should be made at this stage, but in the present author's opinion, evidence definitely favours the hypothesis that asparagine synthesis in higher plants normally takes place by a mechanism similar to the one found in animal cells, as described by the equation:

$$\text{aspartate} + \text{glutamine} + \text{ATP} \xrightarrow{Mg^{2+}} \text{asparagine} + \text{glutamate} + \text{AMP} + PP_i .$$

Further studies with more purified enzyme are in progress.

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* Separate studies have given evidence for the identification of this activity as aspartate-4-decarboxylase (EC 4.1.1.12) (C. Rognes, unpublished data).

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