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AN IMMUNOLOGICAL AND ENZYMOLOGICAL SURVEY OF ASPARAGINASE IN SEEDS OF *LUPINUS*

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Key Word Index—*Lupinus*; Leguminosae; lupins; asparaginase; immunology; distribution in varieties.

Abstract—Rabbit antiserum was raised against potassium-independent asparaginase purified from *Lupinus polyphyllus*. A survey of 54 lines of *Lupinus* showed that only 11 contained the enzyme in maturing cotyledons with activities > 0.5 μmol/hr per g fr. wt. Potassium-dependent asparaginase activity was detected in a number of the remaining varieties.

INTRODUCTION

Asparagine is a major transport compound in higher plants, although the mechanism by which the stored nitrogen is released for protein synthesis in the various plant tissues is still not clear [1].

Asparaginase (EC 3.5.1.1) catalyses the hydrolysis of the amide group of asparagine to yield aspartate and ammonia. The enzyme has been extensively studied in mammals and micro-organisms because of its potential antineoplastic activity [2, 3]. The presence of the enzyme was suggested in plant tissues by early workers, but never confirmed [4, 5]; later the enzyme was detected in crude extracts of roots and root nodules [6–8] and in young pea leaves where the activity declined to zero after 6 days growth [9].

Asparagine is the major nitrogen transport compound in both the xylem and the phloem of *Lupinus albus*, and labelling data and enzymological studies clearly show the presence of an asparaginase in the maturing seeds [10]. However, initial attempts by Lea *et al.* [11] to demonstrate the enzyme in the maturing seeds of a wide range of legumes were unsuccessful, a result confirmed in pea [12] and soybean [13]. An active enzyme could only be detected in the seeds of a garden variety of *Lupinus polyphyllus* [11]; this enzyme has been purified and antibodies raised to the enzyme.

Later studies indicated the presence of another form of asparaginase activity in a number of legumes that was totally dependent upon potassium ions [14]. The extreme potassium-dependence of such activity accounts for the earlier inability of workers to assay the enzyme when extractions were carried out in Tris buffers [11, 13], at the same time allowing the enzyme to be readily detected when potassium phosphate buffers were employed [10, 15]. Lower levels of activity of the enzyme were also detected in the developing seeds of maize and barley [14], a result later confirmed in maize [16].

A more recent study by Chang and Farnden [17] examined potassium-independent asparaginase in *L. arboreus* and *L. angustifolius* (cv Uniwhite); the properties of the two enzymes were very similar to that of *L. polyphyllus* [11]. The authors were also able to detect asparaginase activity in root tips, young leaves and flower buds that required the presence of both DTT and potassium ions.

The early work therefore suggested that within one genus the existence of either a potassium-dependent or -independent asparaginase activity or both was possible [11, 14, 17]. To confirm this proposal, studies were undertaken in conjunction with the University of Reading, Wolfson Oilseed Group who were carrying out a series of field trials on a wide range of varieties of *Lupinus* [18]. The aim of this study was to assay for asparaginase activity in as wide a range of *Lupinus* varieties as possible; at the same time assaying for the potassium-independent asparaginase protein by standard immunological techniques.

RESULTS

Specificity of rabbit antisera to L. polyphyllus *asparaginase*

Antisera to *L. polyphyllus* asparaginase was tested by Ouchterlony double immunodiffusion analysis (Figs. 1a and 1b). Serial dilutions of the antisera formed a single precipitin line with both purified asparaginase protein and crude extracts of maturing cotyledons of *L. polyphyllus*. Further studies showed that the asparaginase protein could only be detected during the maturation period when asparaginase activity exceeded 5 μmol/hr per g fr. wt. No cross-reaction was detected with pure asparaginase isolated from the bacteria *Erwinia carotovora*. There was no evidence of the presence of cross-reacting asparaginase protein in mature roots, stems or leaves of *L. polyphyllus*.

Asparaginase activity could be precipitated from crude extracts of the maturing seeds of *L. polyphyllus* by increasing concentrations of antisera raised to the enzyme (Fig. 2). No precipitation was detected with non-immune serum or with antiserum raised to *E. carotovora*. Potassium-dependent asparaginase activity from *L. albus* (Kievskij mutant) and *Pisum sativum* was not precipitated by either antiserum.

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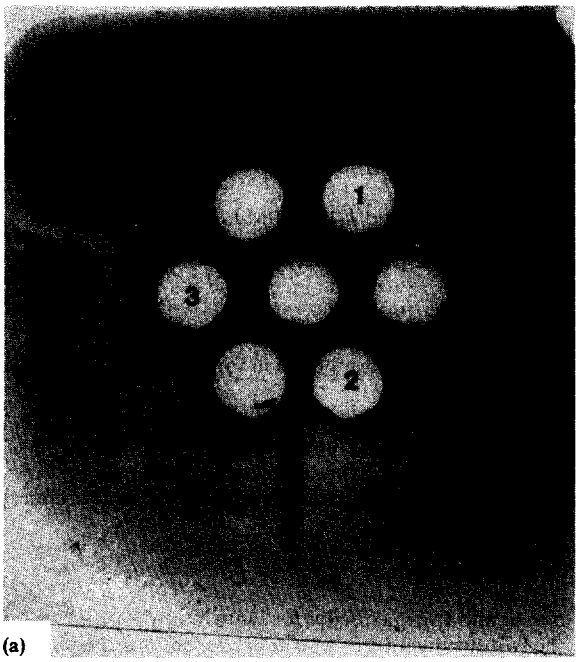
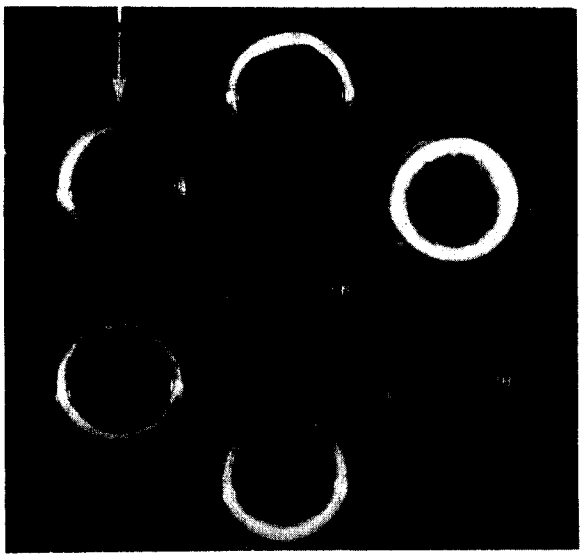


(a)



(b)

Fig. 1. Ouchterlony double diffusion plates using antiserum raised against *L. polyphyllus* asparaginase. (a) 1, Undiluted antiserum; 2, 1/4 dilution; 3, 1/16 dilution; the other wells contain a *L. polyphyllus* extract. (b) Antiserum in centre well. The outer wells contain the following lupin extracts clockwise from the arrowed well: *L. polyphyllus*, *L. mutabilis* LM113, *L. albus* cv Lupini Bean, *L. angustifolius* cv Kievskij mutant, *L. angustifolius* cv Kubesa, *L. angustifolius* cv Unicrop.

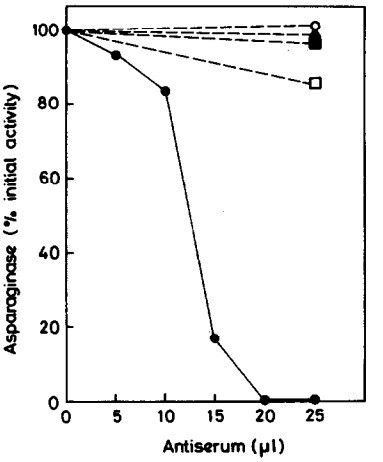


Fig. 2. Immunotitration of asparaginase activity with antisera (method as described in text). ○, *L. polyphyllus* enzyme and control serum; ●, *L. polyphyllus* enzyme and antiserum raised against the enzyme; ▲, *L. polyphyllus* enzyme and antiserum raised against *E. carotovora* asparaginase; □, *L. albus* cv Buttercup enzyme and antiserum raised against *L. polyphyllus* asparaginase; ■, *L. albus* cv Buttercup enzyme and antiserum raised against *E. carotovora* asparaginase.

Detection of potassium-independent asparaginase activity in a range of Lupinus *varieties*

Extracts of a wide range of varieties of maturing seeds of *Lupinus* were tested for potassium-independent asparaginase activity (Table 1). Only 11 varieties out of a total of 54 yielded extracts with an activity greater than 0.5 μmol/hr per g fr. wt. A positive antibody reaction was detected with all extracts having an asparaginase activity of > 5 μmol/hr per g fr. wt. Extracts from a complete maturation series of both *L. albus* cv Kievskij mutant and cv Buttercup showed no evidence of the presence of potassium-independent asparaginase activity.

Sufficient material was not available to test all varieties for the presence of potassium-dependent asparaginase activity. However, extracts of maturing seeds of the following varieties: *L. albus* (Buttercup and Kievskij mutant) and *L. mutabilis* (Tabor) exhibited potassium-stimulated activities of > 1 μmol/hr per g fr. wt. There was no evidence that potassium-independent asparaginase activity was stimulated by the addition of potassium ions.

DISCUSSION

The antisera raised to purified potassium-independent asparaginase isolated from maturing cotyledons of *L. polyphyllus* [11] appear to be specific for the enzyme as seen from Figs. 1 and 2. High levels of the enzyme protein and activity are apparently only present in maturing cotyledons at times of rapid protein synthesis, and are not present in the other organs of the plant.

A survey of 54 lines of *Lupinus* suggests that only a maximum of 11 varieties contain potassium-independent asparaginase activity (Table 1). However, it must be made clear that only one cotyledon sample of the majority of the varieties was tested. It is possible that potassium-independent asparaginase activity was present at levels below the limits of both the enzyme and immunodiffusion assay. However, a survey of a complete maturation series of the *L. albus* varieties Kievskij mutant failed to suggest that either potassium-independent asparaginase activity or the inactive protein was present at any time. As potassium-dependent asparaginase activity could be detected in a number of varieties and has been demonstrated

Table 1. Activity of asparaginase isolated from maturing cotyledons of a range of *Lupinus* lines determined in the absence of potassium

Variety	Asparaginase activity (μmol/hr per g fr. wt)
L. albus	
Lupini Bean*	54
L. angustifolius	
Unicrop*	6.7
Uniharvest	4.1
*L. tennuis**	
ex Sudan	12
L. mutabilis	
LM53*	27
LM99	2.9
LM111*	6.2
LM113*	29
LM115*	5.3
LM120*	27.5
LM127*	8.0

Extracts from lines marked * showed one precipitin line when tested by Ouchterlony double immunodiffusion analysis with antiserum raised against *L. polyphyllus* asparaginase.

Cotyledons from the following varieties of *Lupinus* contained potassium-independent activities of less than 0.5 μmol/hr per g fr. wt.

L. albus: Buttercup; Rimpans SSW; Neuland; Jena; Blanca; Kievskij mutant; Jorizont; SSK-79; Nahrquell; ex Shinfield; Kalina; Pflugs; Pflugs Gela; Kievskij Skoropely; Gynlatanyai; Kali; Kraftquell; Sweet White; Terre; 117 Greenhouse; von tapiozzele; Hausa; ex Tifton; Hope; VLS; Prizebedawski; Stredniocesny No 384; Arkansas.

L. angustifolius: Svalof Borre; Maresa; Nemnchinou-schoff; Kubesa.

L. luteus: Refusa; Sulfa.

L. mutabilis: ex Argentina; 24 ex France; LM96; LM100; LM105; LM114; LM126.

in the maturing seeds of a wide range of plants [14], it must be assumed that, within the *Lupinus* varieties, two forms of asparaginase are present which are immunologically different and therefore presumably coded for by different genes. There was no evidence that both enzymes were present in extracts of the same variety.

It is difficult from Table 1 to establish any pattern as to those varieties that are in the minority and contain the potassium-independent asparaginase activity. Although only cv Lupini Bean of the 29 varieties of *L. albus* tested contained the potassium-independent activity, approximately equal numbers of *L. mutabilis* contained the enzyme. Clearly a more detailed study of asparaginases in relation to the evolution of the species and varieties of *Lupinus* is required.

EXPERIMENTAL

Plant material. Samples of the different varieties of *Lupinus* were collected from the Wolfson Oilseed Group (Department of Agricultural Botany, University of Reading, Whiteknights, Reading RG6 2AS, U.K.) field trials at Shinfield in the summer of 1976/1977. Varieties were personally identified by Mr. Charles W. Looker, and stored as pods at −20° before use.

Enzyme isolation. Approximately 1 g of cotyledons (testas removed) were ground with 15 ml of extraction buffer (10% glycerol, 1 mM phenylmethyl-sulphonyl fluoride, 12.5 mM 2-mercaptoethanol, 50 mM Tris–HCl, pH 8) in a pestle and mortar at 0°. The homogenate was squeezed through muslin and the residue re-extracted in 10 ml of extraction buffer. The pooled extracts were centrifuged at 15 000 *g* for 20 min and 12.5 g of $(NH_4)_2SO_4$ was added to the supernatant. The precipitated protein was resuspended in 1 ml of buffer and dialysed overnight against extraction buffer at 4°; the final vol. of the extract was made up to 1.5 ml.

Asparaginase assay. Aliquots of the dialysed extracts (0.1 ml) were incubated with 100 mM asparagine in extraction buffer for 1 hr at 30° and the reaction was stopped by the addition of EtOH. Asparagine was separated from aspartate by PC in 75% (w/v) PhOH in NH_3 vapour and the aspartate formed determined by the methods of ref. [19]. Blank values for aspartate formed in the absence of asparagine were subtracted from all rates determined. A constant aliquot of 200 nmol of aspartate was run on each PC in order to standardize the assays. In a small number of expts, potassium-dependent and -independent asparaginase activities were assayed by the modified [^{14}C]asparagine method as described in ref. [14]. Essentially similar results were obtained with both assay methods.

Immunological studies. Potassium-independent asparaginase was purified from the maturing seeds of *L. polyphyllus* as described in ref. [11]. A sample (1.5 mg in Freund's adjuvant) with a sp. act. of 3420 nmol/min per mg protein (1000-fold purification) was utilized for antibody production in a rabbit. Ouchterlony immunodiffusion tests were carried out in 1% agar in PBS (phosphate-buffered saline; 0.01 M NaPi, pH 7, 0.85% NaCl). Purified asparaginase was reacted with a dilution series of antisera in PBS. The antibody activity (dilution end-point) of the first three antisera obtained at 2 weekly intervals corresponded to dilution factors of 1/4, 1/16 and 1/64, respectively. The fourth antiserum showed similar activity to the third sample and antisera produced in the sixth and eighth weeks were used for all expts quoted.

Immunotitration was performed by incubating various vols. of antisera with 100 μl of enzyme extracts for 1 hr at 37° and for 12 hr at 4°. The antigen–antibody complex was removed by centrifugation at 10 000 *g* for 10 min and the supernatant fraction used for the enzyme assays.

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