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THE PURIFICATION AND PROPERTIES OF ASPARAGINASE FROM *LUPINUS* SPECIES

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Key Word Index—*Lupinus* species, Leguminosae; maturing seeds; asparagine; asparaginase; ammonia assimilation; protein nitrogen.

Abstract—Asparaginase, which catalyses the conversion of asparagine to ammonia and aspartate, has been purified from maturing *Lupinus* seeds. The enzyme has a high K_m for asparagine (12.2 mM) and is able to utilise a number of asparagine analogues as substrates although glutamine and its analogues are not reactive. The possible route for the reassimilation of the liberated ammonia is discussed.

INTRODUCTION

Asparagine is a major constituent of the free non-protein nitrogen pool of legumes [1]. It is synthesized from aspartate and glutamine by asparagine synthetase which has been purified from germinating legume cotyledons [2–4]. Asparagine is also transported up the stem in the xylem [5] and phloem [6] after synthesis in the root. The nitrogen required for asparagine synthesis may be obtained from (1) nitrate reduction; (2) nitrogen fixation or (3) protein breakdown. Earlier results suggested that asparagine was not utilised as a nitrogen source for protein synthesis [1]. However, the elegant feeding experiments of Pate *et al.* [5–7] with ^{14}C and ^{15}N (amide) labelled asparagine have confirmed that asparagine is slowly metabolized to other constitutents of the soluble pool of maturing lupin seeds. A range of amino acids in the seed protein also derived both carbon and nitrogen from the applied asparagine [6]. Labelling data suggested that the amide nitrogen was metabolised in a different manner than the four carbon skeleton.

Three possible routes of asparagine breakdown have been suggested by Lea and Fowden [1]. Asparagine transaminases with different oxoacid acceptors have been detected in crude extracts of a number of plants [8–10], but the enzyme has not yet been purified. Asparagine (amide), 2-oxoacid amino transferases which transfer the amide group of asparagine to 2-oxoacids to form 2-amino acids in a reductant dependent step similar to glutamate synthase (GOGAT) have been suggested [11, 12], but further work has indicated that this may be due to the use of asparagine contaminated with aspartate [13]. The direct transfer of the amide of asparagine to glutamine by a mechanism similar to a reversal of asparagine synthetase has been suggested by the labelling data of Joy *et al.* [14], but no enzyme capable of carrying out this reaction has yet been isolated.

The enzyme known to degrade asparagine; (L-asparagine amido hydrolase EC 3.5.1.1) catalyses the following reaction: asparagine + H_2O ⟶ aspartate + NH_3. The enzyme has been extensively studied in mammals and microorganisms because of its potential antineoplastic activity [15]. The conversion of asparagine to aspartate has been demonstrated in crude extracts of seedlings [16, 17], although in neither case was the presence of the enzyme asparaginase unequivocably demonstrated. During the course of this investigation Atkins *et al.* [6] detected activity of an asparaginase from a similar tissue (the maturing seeds of *L. albus*). These studies report the properties of a highly purified plant asparaginase and discuss the possible routes of reassimilation of the ammonia liberated by the enzyme. A preliminary report of this investigation has previously been published [9].

RESULTS

Evidence for the enzymatic formation of aspartate from asparagine

The formation of aspartate in the the presence of aspartate-free asparagine [13] and a *L. polyphyllus* seed extract was demonstrated by PC. The product chromatographed with standard aspartate using 3 different solvents. A stoichiometric quantity of ammonia was also liberated, as determined by direct Nesslerization [18]. No other products were detected with the purified enzyme, but in crude extracts there was evidence of the formation of alanine derived from aspartate. No cofactors were required for the reaction, although certain salts had a slight stimulatory effect (see later section). Evidence that the reaction was carried out by an enzyme was that the non-dialysable factor was precipitated (1) by heating at 80° for 5 min; (2) 10% TCA; (3) 50% ethanol. Antiserum prepared against the purified enzyme was shown to totally inhibit the breakdown of asparagine (Lea, P. J. Festenstein, G. Hughes, J. S. and Miflin, B. J. unpublished results).

Enzyme purification and stability

The enzyme purification was carried out as described in the experimental section and set out in Table 1. As large quantities of maturing seeds were taken during the period of maximum protein synthesis, there was some

Table 1. Purification of asparaginase from *Lupinus polyphyllus*

Preparation	Total volume (ml)	Total protein (mg)	Total asparaginase (nmol/min)	Specific activity (nmol/min /mg protein)
Crude	380	2250	7870	3.51
Centrifuged crude	380	1680	7870	4.69
Ammonium sulphate precipitate	20	620	6920	11.20
Ultrogel 22 eluate	50	74	6180	83.40
DEAE-cellulose eluate	22	18	5520	307
Ultrogel 34 eluate	35	11.2	5170	461
Polyacrylamide gel eluate	6.5	2.82	4650	1650

variation in the total protein content of different batches used for enzyme purification, giving rise to some variation in the sp. act. of the enzyme in crude homogenates. In the buffer used the enzyme was stable at 4° for up to 4 days, less than 10% of the activity was lost after storage at −20° for 3 months.

Optimal assay conditions

The reaction rate was linear up to 40 min at 30°, and linear up to 0.2 mg of protein added in a crude homogenate. Tris–HCl (50 mM) was found to be the best buffer tested with a pH optimum of 8–8.2. There was little activity below pH 6, but the rate only dropped by 25% at pH 9.6. The reaction rate was very dependent upon the asparagine concentration, and the enzyme was still not saturated at the maximum concentration obtainable (100 mM), the apparent K_m for asparagine was calculated as 12.2 mM. The products ammonia (75 mM) and aspartate (1 mM the maximum possible by the assay method) had no inhibitory action on the enzyme.

Inhibitors and activators

Stimulation of up to 50% of the asparaginase activity could be obtained with certain salts (Table 2). Difficulties were encountered in the chromatographic separation as high salt concentrations tended to cause asparagine to run with aspartate and give spuriously high activities. Salts of the transition elements were strong inhibitors of the enzyme, but lead and mercury salts including *p*-chloromercuribenzoate had little effect. The enzyme was unaffected by a number of compounds, e.g. glucose, sucrose, fructose, the other 18 protein amino acids and nucleotides ATP, ADP, AMP, NAD, NADH, NADP and NADPH. The enzyme was unable to catalyse transamination between asparagine and any of the 4

Table 2. Action of cations and anions on the activity of asparaginase purified form *Lupinus polyphyllus*

Cation	Concentration (mM)	Relative activity
Sodium	70	122
Calcium	35	116
Mercuric	1	112
Potassium	70	107
Magnesium	70	63
Plumbic	16.6	42
Cupric	8.3	13
Ferric	8.3	0
Cobaltic	8.3	0
Stannic	8.3	0
Nickel	8.3	0
Cadmium	8.3	0
Anion		
Sodium tartarate	35	161
Ferricyanide	70	138
Hydrogen phosphate	70	136
Pyrophosphate	70	125
p-Chloromercuribenzoate	1	111
Chloride	70	107
Acetate	70	102
Nitrate	70	101
Iodide	70	90
Sulphate	70	71
Bromide	70	26
Dichromate	70	20

All cations were supplied as the chloride, except cadmium, lead and nickel as the acetate. All anions were supplied as the potassium salt. The reaction rate assayed as described in the text was taken as a 100 in the presence of distilled water.

Table 3. Substrate specificity of asparaginase isolated from *Lupinus polyphyllus*

Analogue	Concentration	V_{max}
L-Asparagine	50	100
N^4-Methoxy-L-asparagine	50	154
DL-Aspartate-3-hydroxamate	50	52
threo-3-Hydroxy-L-asparagine	10	36*
N^4-Methyl-L-asparagine	50	12
N^4-Ethyl-L-asparagine	50	8
L-Glutamine	100	0

*Product *threo*-3-hydroxy-L-aspartate.

The enzyme was assayed as described in the text, only asparagine was replaced by an analogue as shown. The following compounds were not utilized as a substrate by the enzyme: 4-methylene-L-glutamine, *S*-carbamoyl-L-cysteine, L-albizziine, N^5-2-hydroxyethyl-L-glutamine, L-glutamate-4-methyl ester, DL-methionine sulphoximine, L-glutamate-4-hydrazide, DL-azaserine, *erythro*-3-hydroxy-L-asparagine, 3-methyl-L-asparagine, N^2-methyl-L-asparagine, L-2-amino-2-carboxyethane sulphonamide, 3-cyano-L-alanine, *meso*-diamino succinic acid monoamide, 5-diazo-4-oxo-L-norvaline, 5-bromo-4-oxo-L-norvaline, N^4-isopropyl-L-asparagine, N^4-benzyl-L-asparagine, L-alanyl-asparagine and L-glycyl-L-asparagine.

major 2-oxoacids, 2-oxoglutarate, oxaloacetate, pyruvate or glyoxylate (although non-enzymic transamination was detectable with the latter oxoacid). Aminooxyacetate, an inhibitor of pyridoxal phosphate-dependent enzymes had no effect on asparaginase activity.

Substrate specificity of asparaginase

A large number of asparagine and glutamine analogues were incubated with asparaginase, and the reaction products separated and identified by chromatography in phenol–NH_3 (Table 3). N^4-Methoxy-L-asparagine was rapidly converted to aspartate, and the K_m for the analogue (6 mM) was lower than that of asparagine. *Threo*-3-hydroxy-L-asparagine was extremely insoluble and the maximum concentration obtained was 10 mM, thus it is possible that the V_{max} is much higher than recorded. No evidence was obtained for the formation of glutamate from glutamine. The ability of an analogue to act as an inhibitor was tested by incubating the analogue with 50 mM asparagine and enzyme. Both derivatives of 4-oxo-L-norvaline were potent inhibitors of the enzyme, as was 3-cyanoalanine (Table 4).

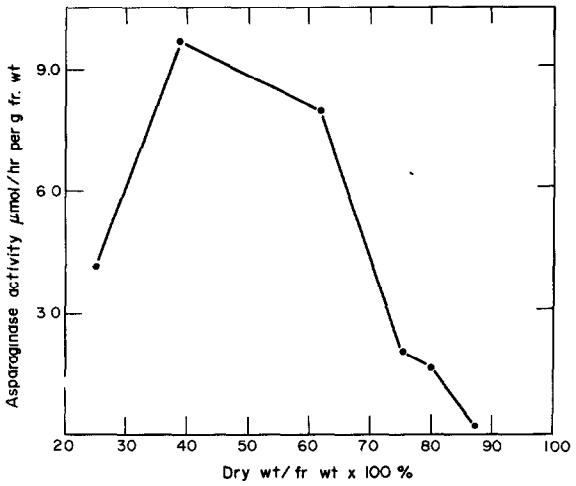

Fig. 1. The distribution of asparaginase activity during the maturation of *L. angustifolium* seed.

Distribution and localisation of enzyme activity within the plant.

The presence of asparaginase with a high K_m could only be detected in the maturing cotyledons of certain lupin species. The precise levels of activity during the maturation process of *L. polyphyllus* can be seen in Fig. 1. The ratio of dry wt/fr. wt has been used as a measurement of the maturation stage of the seeds. No activity could be detected in the leaves, stems, roots or pods of all plants tested.

A study of the distribution of asparaginase activity within *Lupinus* species (Lea, P. J., Festenstein, G., Hughes, J. S. and Miflin, B. J., unpublished results) indicates that activity resides in a limited number of varieties. Attempts to demonstrate an organelle localization of the enzyme using the sucrose density gradient developed by Miflin and Beevers [19] were unsuccessful, and it appears likely that the enzyme is located in the cytoplasm or vacuole of the plant cell.

Table 4. Ability of amino acid analogues act as inhibitors of asparaginase isolated from *Lupinus polyphyllus*

Analogue	Concentration (mM)	% Inhibition
5-Diazo-4-oxo-L-norvaline	30	100
5-Bromo-4-oxo-L-norvaline	30	100
3-Cyano-L-alanine	30	100
2-Amino-5-chloro-4-oxo pentanoic acid	30	50.8
DL-Aspartate-3-hydroxamate	50	45.2
N^4-Hydroxyethyl-L-asparagine	50	24.8
4-Methylene-L-glutamine	50	22.1

Asparagine was supplied as a substrate at 50 mM and the enzyme assayed as described in the text. The following compounds did not act as inhibitors:
L-glutamine, *S*-carbamoyl-L-cysteine, L-albizziine, N^5-hydroxyethyl-L-glumatine, L-glutamate-4-methyl ester, L-glutamate-4-ethyl ester, DL-methionine sulphoximine, L-glutamate-4-hydrazide, DL-azaserine, *threo*-3-hydroxyl-L-asparagine, N^2-methyl-L-asparagine L-2-amino- 2-carboxyethane sulphonamide, N^4-ethyl-L-asparagine, N^4-isopropyl-L-asparagine, *meso*-diaminosuccinic acid monoamide, L-alanyl-L-asparagine and L-glycyl-L-asparagine.

Table 5. Activity of various enzymes involved in ammonia assimilation at the time of maximum asparaginase activity (average seed dry wt 12 mg) in maturing cotyledons of *Lupinus polyphyllus*

Enzyme	Activity (μmol/hr/g fr. wt
Glutamine synthetase	
(a) transferase	39.1
(b) synthetase	8.4
Glutamate dehydrogenase	
(a) NADPH	2.5
(b) NADH	16.3
Glutamate synthase	
(a) NADPH	0.35
(b) NADH	0.51
(c) ferredoxin	0.46

The enzymes were extracted and assayed as described in Experimental.

The levels of various enzymes involved in ammonia assimilation in maturing seeds

Maturing seeds with a dry wt/fr. wt ratio of 40–50% were selected corresponding to the stage of maximum asparagine activity. As asparaginase has been shown to liberate ammonia, the levels of various enzymes involved in ammonia assimilation were measured (Table 5). Two recent reviews by Miflin and Lea [20, 21] have discussed the significance of glutamine synthetase (GS), glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) in detail. For the enzymes isolated from *L. angustifolium* the apparent K_m for ammonia measured for glutamine synthetase was 2×10^{-4} M and for glutamate dehydrogenase (NADH) 6.5×10^{-3} M.

MW determination

The sucrose density gradient method of Martin and Ames [22] was employed using bovine catalase (MW 250 000) as a marker. A value of *ca* 72 000 was determined for the MW of *L. Polyphyllus* asparaginase.

Polyacrylamide gel electrophoresis

Asparaginase activity immediately behind the bromophenol blue marker band could be detected after the electrophoresis of crude extracts on 6% polyacrylamide gels, pH 8.2, by incubating 1 mm slices in 0.2 ml 0.1 M asparagine. With pure preparations of asparaginase, activity could be detected in the intact gel by the presence of a white precipitate caused by the reaction of the ammonia liberated and sodium tetraphenylboron. The precipitate was only formed immediately behind the bromophenol blue marker band and coincided with the single protein band obtained by staining the gel with Coomassie blue. The purified enzyme was denatured by heating to 80° in 1% SDS and 0.5% mercaptoethanol and electrophoresed on 12.5% acrylamide 0.1% SDS gels. Two equally staining bands running close together with MW of *ca* 38 000 were detected.

DISCUSSION

An enzyme converting asparagine to aspartate has been clearly demonstrated in extracts of maturing lupin seeds. The enzyme closely resembles that reported by Atkins *et al.* [6] during the course of this investigation. In their detailed studies on the nitrogen balance of *L. albus*, Atkins *et al.* [6] calculated that the concentration of asparagine in the phloem was 30 mM, which would permit an asparaginase with a high K_m to work efficiently. They also calculated that during the period of maximum protein synthesis over 80% of the asparagine entering the pod was metabolized. As the maximum asparaginase activity is present at this time, it may be assumed (although not proved) that the enzyme is involved in asparagine breakdown *in vivo*.

Feeding of asparagine -[^{14}C(U), ^{15}N-amide] to *L. albus* has shown that the amide nitrogen is metabolised in a different way to the carbon skeleton [6]. Such a result is consistent with asparaginase action. If asparaginase is functioning then liberated ammonia must be reassimilated. Two possible routes of ammonia assimilation are shown in Fig. 2; the merits of the two pathway have been extensively reviewed [20, 21]. The studies of Atkins *et al.* [6] with asparagine -[^{15}N-amide] show that after 8 hr only ammonia and glutamine are labelled but that after a longer period (24–60 hr) glutamine acquires further label, and glutamate, alanine, valine and serine are also labelled but to a lesser extent. Such labelling data would suggest that the GS/GOGAT pathway (route 3,4) was operating in the maturing lupin seed. However, as the minimum metabolic period investigated was 8 hr, it could be argued that glutamine was synthesised as a secondary reaction after the formation of glutamate from 2-oxoglutarate by GDH.

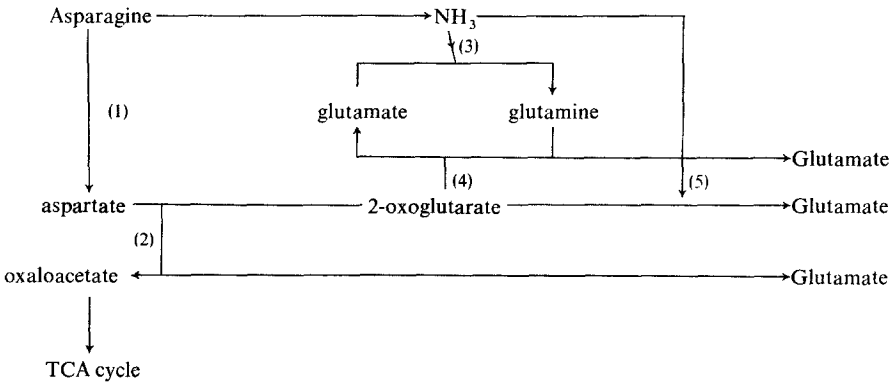

Figure 2. Scheme for the conversion of the nitrogen containing groups of asparagine to the 2-amino group of glutamate in the developing seed of *Lupinus polyphyllus*. (1) Asparaginase (2) Aspartate: 2-oxoglutarate aminotransferase (3) Glutamine synthetase (GS) (4) Glutamate synthase (GOGAT) (5) Glutamate dehydrogenase (GDH).

Table 3 shows that both GS and GDH are present at high levels in the maturing seed. GS has a 30-fold higher affinity for ammonia than GDH, which suggests that GS would be a more important enzyme at ammonia levels below 5 mM. Values of 20–70 mM have been reported for the ammonia concentration the endosperm fluid, but this is extracellular and its pH of 4.4 would not allow either enzyme to function.

The second enzyme in the GS/GOGAT pathway is present at very low level (Table 3); both NAD(P)H and ferredoxin-dependent GOGAT are apparently present, although it is not certain whether they are separate enzymes. Beevers and Storey [23] have isolated an NAD(P)H-dependent GOGAT from maturing pea cotyledons where the GDH levels reported were smaller than GOGAT. It is possible that the authors underestimated the activity of GDH by having EDTA present in their assay mixture.

Thus it is not possible to decide by the present enzymological and labelling data which of the two pathways are operating. More detailed labelling studies with short time courses are required preferably with maturing cotyledons grown in culture [24, 25]. The inhibitors azaserine and methionine sulphoximine could be used in a similar manner to that used with blue–green algae [26] and *Lemna* [27]. Further studies are also required to see if the low levels of GOGAT detected are due to the presence of inhibitors which have been suggested in maize and *V. faba* [28].

The major difference between asparaginases isolated from animals and microorganisms and that of *Lupinus* is the K_m for asparagine. The previous highest K_m reported was 4.7 mM from *Bacillus coagulans*, whilst the majority varied from 0.0048–0.03 mM [15]. It would appear from the kinetic data in Fig. 1 that at levels below 1 mM, asparagine would be little metabolized. However, at concentrations above this value the rate of breakdown of asparagine would be directly proportional to its concentration in the seed. As the concentration in the phloem transporting asparagine to the seed is 30 mM, such levels would permit efficient functioning of the enzyme. The MW of 72000 is low, *ca* half that of the majority of asparaginases from other sources. Preliminary investigations on 'Ultrogel 34' suggested that the enzyme may undergo aggregation. The SDS gel data would suggest that the enzyme contains two subunits of *ca* equal MW. It is not possible to ascertain whether the second protein band is an impurity or due to slight differences in the MW of the subunits. The inability of mercury salts and *p*-chloromercuribenzoate to inhibit the enzyme suggested that a cysteine residue is not present at or near the active site of the enzyme. Asparaginase was, however, very susceptible to the action of transition elements. The crucial result of the analogue studies on the *L. polyphyllus* enzyme is that only compounds having C_4-chains were bound to the enzyme. Glutamine was not a substrate thus preventing a futile cycle being set up in which ammonia is liberated and reassimilated via GS with the expenditure of ATP. As neither azaserine nor methionine sulphoximine had any effect on the enzyme, these compounds may be used to test *in vivo* assimilation of the ammonia liberated by asparaginase. 4-Methylene- glutamine was an inhibitor. It is possible that the compound is able to position itself so that the 4-methylene group is able to bind in place of the amide group of asparagine.

Derivatives of 4-oxonorvaline were potent inhibitors of the enzyme as for other asparaginases: N^4-methoxy-L-asparagine was a better substrate for the enzyme than asparagine itself. An enzyme capable of hydrolysing 3-cyanoalanine to asparagine has been isolated from blue lupins [29]. The asparaginase is presumably different, as 3-cyanoalanine is not a substrate but a potent inhibitor.

Although the presence of the enzyme asparaginase has been demonstrated in a limited number of species of *Lupinus*, the enzyme does not exist in all maturing seeds. No other enzyme capable of metabolizing asparagine at the rates required has yet been isolated, and the pathway of asparagine breakdown in the majority of legumes has still not been elucidated.

EXPERIMENTAL

Plant material. Maturing seeds (dry wt 5–15 mg) of the garden lupin *Lupinus polyphyllus* were selected for enzyme purification. Maturing seeds of *L. angustifolium* cv Unicrop grown in field plots at Rothamsted were utilized for some expts. Pods were stored at −20° before use.

Chemicals All amino acids were tested for purity by PC Commercial samples of L-isomers of the protein amino acids were of high purity except asparagine from which the contaminating aspartic acid was removed by passing through a Dowex-1 resin column [13]. The majority of amino acid analogues were obtained as described in an earlier paper [4]. N^4-Benzyl-L-asparagine, N^4-isopropyl-L-asparagine, L-2-amino-4-oxo-5-chlorovaleric acid and N^4-methoxy-L-asparagine were a generous gift of Professor F. Schneider, Institut der Universitat, Marburg, Lahn, W. Germany 30. 'Ultrogel' resins used in the enzyme purification were products of L.K.B.

Enzyme isolation. One standard buffer was used throughout the purification procedure (10% w/v glycerol, 1 mM phenylmethyl-sulphonyl fluoride, 12.5 mM 2-mercaptoethanol, 50 mM Tris–HCl pH 8). All operations were carried out at 0–4°. Maturing seeds were ground in buffer in a 'Kenwood' homogenizer (1 g tissue, 7 ml buffer) and the homogenate squeezed through cheesecloth. The crude extract was centrifuged at 30000*g* for 20 min. The $(NH_4)_2SO_4$ fraction precipitating between 24–44 g/100 ml was collected and applied to a column of DEAE-cellulose (34 × 2.5 cm) and eluted with a gradient of 0–0.4 M KCl (400 ml); the enzyme showed a peak of activity at 280 ml. The asparaginase-containing fractions were dialysed and concd against polyethylene glycol and applied to an Ultrogel 34 column (80 × 3 cm), when the enzyme eluted after 168 ml. Further purification was carried out using a Birchover Macro-PAGE apparatus (Birchover Instruments Letchworth) on a 6 cm gel. The enzyme eluted from the gel just behind the bromophenol blue tracker dye. Relatively crude extracts were used for assaying total enzyme levels. Seeds were ground in a pestle and mortar with buffer, squeezed through muslin, centrifuged at 10000*g* for 15 min and $(NH_4)_2SO_4$ was added at 50 g/100 ml. The resuspended pellet was passed through a column of Sephadex G-75, equilibrated with extraction buffer.

Enzyme assays. Asparaginase was detected by incubating the enzyme in 100 mM asparagine for 15 min at 30°, the reaction was stopped by the addition of EtOH. Asparagine was separated from aspartate by PC in 75% (w/v) PhOH in the presence of NH_3 vapour, and the aspartate formed was determined by the method of ref. [31]. Glutamine synthetase (transferase and synthetase activity) was determined by a modified method of ref. [32]. Glutamate synthase (ferredoxin dependent) was assayed by the method of ref. [33] and the NAD(P)H-dependent enzyme by the method of ref. [13]. Glutamate dehydrogenase (NAD(P)H-dependent) was assayed as described in ref. [34]. Protein was determined by the method of ref. [35] using BSA as a standard.

MW determinations. The method described by ref. [22] was employed using a linear sucrose density gradient of 5–20% (w/v). The gradients were centrifuged on a Beckman L2-65B

with a SW 27 rotor at 25000 rpm for either 24 or 40 hr. Beef liver catalase was used as a standard marker.

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REFERENCES

1. Lea, P. J. and Fowden, L. (1975) *Biochem. Physiol Pflanzen.* **168**, 3.
2. Streeter, J. G (1973) *Arch. Biochem. Biophys* **157**, 613.
3. Rognes, S. E. (1975) *Phytochemistry* **14**, 1975.
4. Lea, P. J. and Fowden, L (1975) *Proc. Roy. Soc. B.* **192**, 13.
5. Pate, J. S. (1971) in *Nitrogen-15 in Soil Plant Studies* p. 165. International Atomic Energy Authority, Vienna.
6 Atkins, C. A., Pate, J S and Sharkey, P J (1975) *Plant Physiol.* **56**, 807.
7. Pate, J. S., Walker, J. and Wallace, W. (1965) *Ann. Bot* **29** 475.
8 Streeter, J. G. (1974) *Plant Physiol.* **54**, 65S
9. Lea, P. J., Fowden, L. and Miflin, B. J (1976) *Plant Physiol.* **57**, 213S.
10. Joy, K. W. Personal communication.
11. Dougall, D. K. (1974) *Biochem. Biophys. Res. Commun.* **58**, 639.
12. Fowler, M. W., Jessup, W. and Sarkissian, G S. (1974) *FEBS Letters* **46**, 340.
13. Miflin, B. J. and Lea, P. J. (1975) *Biochem J* **149**, 403.
14. Bauer, A., Joy, K. W. and Urquart, A A. (1977) *Plant Physiol* **59**, 920.
15. Wriston, J. C. Jr. and Yellin, T. O. (1973) in *Advances in Enzymology* (Meister, A. ed.) Vol. **39**, p 185. Interscience, New York.
16. Kretovitch, W. L. (1958) in *Advances in Enzymology* (Nord, F. F. ed.) Vol. **20**, p. 319. Interscience, New York.
17. Lees, E. M. and Blakeney, A. B (1970) *Biochim. Biophys Acta* **215**, 145.
18 Wriston, J. C. Jr. (1970) *Methods Enzymol.* **17**A, 732
19. Miflin, B. J. and Beevers, H. (1974) *Plant Physiol.* **53**, 870
20. Miflin, B. J. and Lea, P. J. (1976) *Phytochemistry* **15**, 873.
21. Miflin, B J. and Lea, P. J (1977) *Ann. Rev. Pl Physiol.* **28** 299
22. Martin, R. G. and Ames, B. N (1961) *J. Biol. Chem* **236**, 1372
23. Beevers, L. and Storey, R. (1976) *Plant. Physiol.* **57**, 862.
24. Millerd, A., Spencer, D., Dudman, W. F. and Stiller, M. (1975) *Australian J. Plant Physiol* **2**, 51.
25. Thompson, J. F., Madison, J T and Meunster, A. E (1977) *Ann. Bot.* **41**, 29.
26. Stewart, W. D. P. and Rowell, P (1975) *Biochem. Biophys. Res. Commun.* **65**, 846.
27. Stewart, G. R. and Rhodes, D. (1976) *FEBS Letters* **64**, 296.
28. Wallsgrove, R. M., Harel, E., Lea, P. J. and Miflin, B J. (1977) *J. Exp. Bot.* in press.
29. Castric, P A., Farnden, K J. F and Conn, E. E. (1972) *Arch. Biochem. Biophys.* **152**, 62.
30. Herman, V., Rohm, K. H. and Schneider, F. (1972) *FEBS Letters* **39**, 214.
31. Atfield, G. N. and Morris, C. J. O. R. (1961) *Biochem. J.* **81**, 604.
32. Rhodes, D., Rhendon, G. A. and Stewart, G. R. (1976) *Planta* **129**, 203.
33. Lea, P. J. and Miflin, B. J. (1974) *Nature* **251**, 614.
34. Miflin, B. J. (1974) *Plant Physiol.* **54**, 550
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R J. (1951) *J. Biol. Chem.* **193**, 265.