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UREASE FROM LEAVES OF *GLYCINE MAX* AND *ZEA MAYS*

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Key Word Index—*Glycine max*; Fabaceae; soybean; *Zea mays*; Gramineae; maize; urease; properties; inhibitors; ureides.

Abstract—Extracts of *Glycine max* and *Zea mays* leaves catalysed the release of $^{14}CO_2$ from [^{14}C] urea with multiple pH maxima (5.5 and 9.0 for *G. max*; 5.5, 7.5 and 8.8 for *Z. mays*). Evidence was obtained that the principal activities, at pH 5.5 and 8.8–9.0, catalysed the same reaction stoichiometry as did urease purified from jackbean seeds (EC 3.5.1.5). The urease activities with these pH optima were not resolved by ammonium sulphate fractionation, DEAE-cellulose chromatography, or gel filtration chromatography. Many structural analogues of urea inhibited leaf urease, the most effective being amino acid hydroxamates, hydroxyurea and selenourea. Allantoic acid and ureidoglycolate are probably not alternative substrates because they showed at most only weak competitive inhibition with respect to radioactive urea.

INTRODUCTION

Seed and cell culture ureases have received much more attention than ureolytic activities present in roots and leaves (e.g. [1–3]). Leaf ureolytic reactions are of considerable interest in relation to the use of foliar-applied urea fertilizers and in legumes, the use of endogenous ureide nitrogen [4].

Because different types of urea catabolism have been described (reviewed in ref. [4]), careful characterization is required when urease (EC 3.5.1.5) activity is claimed in impure preparations, solely on the basis of ammonia or of CO_2 release from urea. Leaf 'urease' was not well characterized in early reports. Matsumoto *et al.* [5] claimed to have found an inducible urease in jackbean leaves and rice seedlings, but induction was transient despite the continued presence of external urea. Ammonia was considered to repress urease levels but it is difficult to be confident about the specificity of the effect described. Durzan [6] described the histochemical localization of a ureolytic activity in white spruce seedlings, but the suggestion of light-specific urea stimulation of 'urease' activity does not appear to have been substantiated. Omura *et al.* [7] went considerably further in claiming a new enzyme, 'urea dehydrogenase' which awaits full characterization.

In more detailed investigations, Hogan *et al.* [8] developed *in vivo* and *in vitro* assays for leaf ureolytic activity, and compared such activities from many species. Again the assay was non-specific, however, and the actual metabolic events were not established. Kerr *et al.* [4], examining ureolytic activity from the leaves of nodulated soybean plants, tested for activities other than urease itself. A particularly interesting feature was the unusual dual pH maximum of this activity. In the present paper we demonstrate 'classical' urease activity in the leaves of non-nodulated soybeans and maize, and show multiple pH maxima for these ureases.

RESULTS AND DISCUSSION

The data in Table 1 show typical yields of maize and soybean leaf ureolytic activities, determined using the $^{14}CO_2$ evolution assay. In general, higher activities were obtained, on a fresh weight or protein basis, from soybean

Table 1. Extraction of leaf ureolytic activity from soybean and maize ($^{14}CO_2$ evolution assay)

Preparation	% Saturation $(NH_4)_2SO_4$	Total activity (nkat)	Activity/g fr. wt tissue	Total protein (mg)	Sp. act. (nkat/mg protein)	Yield (%)	Purification (fold)
Soybean	Crude	130.0	4.32	1000	0.13	(100)	—
	40	26.2	0.872	120	0.22	12	1.7
	40–55	31.7	1.06	150	0.058	15	0.4
Maize	Crude	8.13	0.272	230	0.035	(100)	—
	40	1.87	0.062	52.5	0.035	23	1.0
	40–55	3.42	0.113	27.0	0.13	42	3.6

Data shown apply to the use of extraction Buffer 2 and resuspension/dialysis Buffer 4.

leaves than from maize leaves. Storage of leaves at $-70°$ did not affect yields. On a specific activity basis, a 40% saturation ammonium sulphate fractionation was a useful concentrating step for the soybean activity. Fractionation at 40–55% saturation was used with the maize preparation. Subsequent 55–80% saturation fractions yielded no activity from soybean or maize extracts. Considerable activity loss accompanying ammonium sulphate fractionation was shown not to result from the overnight dialysis step. Extraction and dialysis buffers of two different pH values were compared after a report indicated that soybean leaf 'urease' had a pH optimum (pH 8.8) significantly different from that of the seed enzyme (pH 7.0) [9]. The pH 7.0 buffer system would probably also enable the detection of some of the activity which peaked at pH 5.2 [9], the latter low pH being unsuitable for extraction buffers. We found that the use of phosphate (pH 7.0) extraction and dialysis buffers (Buffers 1 and 3, respectively, using either Tris pH 8.8 or phosphate pH 7.0 for assay) gave slightly lower activities than the pH 8.8 buffers (Buffers 2 and 4) in some experiments, and comparable activities in others.

With maize preparations, several variations of extraction Buffer 2 were explored. These included addition of 2 mM phenylmethylsulphonyl fluoride, 10 mM iodoacetamide in the absence of 2-mercaptoethanol, 20% (v/v) glycerol [with 10% (v/v) glycerol in the resuspension/dialysis Buffer 4], 1% (w/v) soluble polyvinylpyrrolidone, or 5 mM sodium diethyldithiocarbamate. These concentrations were selected on the basis of their minimal interference with activity in the $^{14}CO_2$ evolution assay. None improved yields and the presence of 2-mercaptoethanol was found to be essential.

Coloured components in the 40–55% ammonium sulphate fraction of maize preparations were removed by batchwise DEAE-cellulose fractionation, 75% of the ureolytic activity being recovered between 0.25 and 0.3 M potassium chloride washes. The resulting preparation could be concentrated 54-fold by pressure ultrafiltration, with 100% recovery. Only 43% of the activity remained after a further ten-fold concentration by the same method. Soybean dialysed ammonium sulphate fractions could be stored at $-20°$ with frequent thawing, with little activity loss. Maize fractions, whether concentrated by ultrafiltration or not, showed typically a 10–20% activity loss with each thawing. Ammonium sulphate fractions, concentrated by ultrafiltration, were used for all experiments except where indicated.

pH profiles

Figure 1 shows the effects of a range of assay buffer systems on soybean and maize leaf ureolytic activities in the $^{14}CO_2$ evolution assay. All buffers were included at 50 mM to overcome contributions from the small amount of buffer introduced with the preparation. The soybean double maxima (at pH 5.5 and 8.8) confirm results reported for nodulated soybeans [4, 9] (indicating also that this intriguing feature of the activity is not related to the nodulated condition). The maize results indicate that multiple pH maxima are not a unique aspect of soybean leaf activity; in fact, three maxima were obtained for maize, at 5.0–5.5, 7.5 and 8.8. The minimum at pH 6.5 corresponds exactly with the central minimum in the soybean profile. The maize experiments were repeated using a different series of

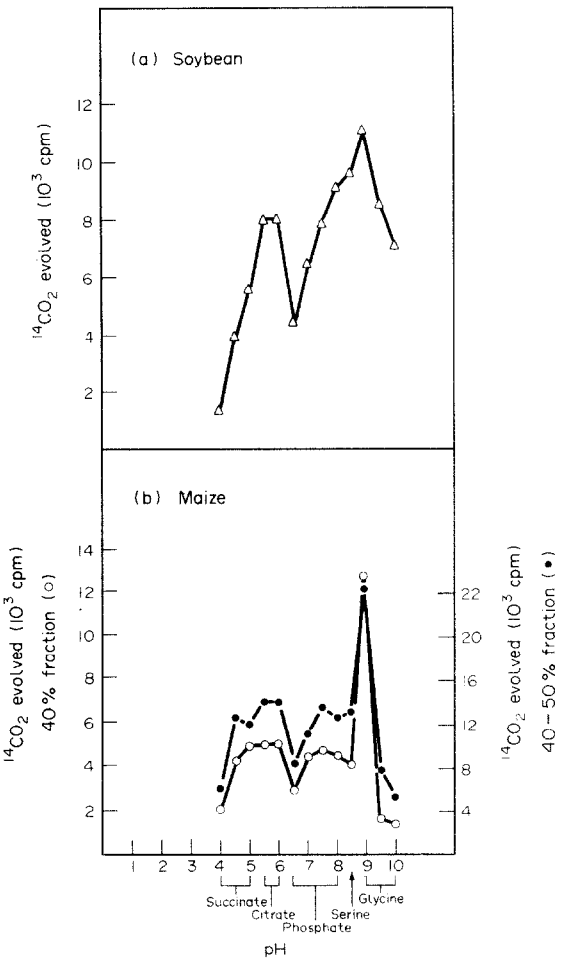

Fig. 1. Effects of different assay buffers on (a) soybean and (b) maize leaf ureolytic activity. (○) Maize activity obtained in 40% saturation ammonium sulphate fraction; (●) maize activity obtained in 40–55% fraction.

buffers (fumarate–HCl, histidine–HCl, maleate–HCl, Na_2HPO_4, pyrophosphate–HCl, serine–KOH, boric acid–KOH). Although relative peak sizes were different (as noticed also with different enzyme preparations), the positions of the maxima were essentially the same. We therefore interpret the results in terms of pH rather than in terms of buffer or ionic strength variations.

Multiple pH maxima could be explained by the existence of more than one enzyme species, by more than one type of active site on one molecular species, or by a catalytic mechanism in which both acidic and basic residues were involved. Although we were not interested in extensive purification, we did examine some partial purification procedures to see whether they had the potential to resolve the maize activities with pH maxima of 5 and 8.8. Ammonium sulphate fractions of 40% and 40–55% saturation gave nearly identical pH profiles (Fig. 1b), suggesting that ammonium sulphate precipitation provided no such resolution. Maize leaf ureolytic activity from DEAE-cellulose (batchwise and column procedures) was active at both pH maxima. Several gel filtration column procedures were also

explored covering fractionation ranges in which the maize activity was partially excluded, and substantially included (e.g. Bio-Gel A50M, Sepharose 6B-100, Sephadex G200-120; 1.5 × 30 cm columns equilibrated with Buffer 4). Assays of effluent fractions were performed at pH 8.8 using the $^{14}CO_2$ evolution assay described, and at pH 5.0 by replacing the Tris–HCl assay buffer by 25 mM sodium succinate–HCl, pH 5.0. Elution profiles from all these columns were broad, but had distinct peaks which were in the same fraction whether assayed at pH 5.0 or 8.8. Thus far, therefore, we have found no evidence that the maize activities with these pH maxima can be physically separated.

Comparison of CO_2 and NH_3 release

To examine the reaction stoichiometry at the two principal pH maxima, we compared activities in the $^{14}CO_2$ evolution assay with those in two types of assay for ammonia production. Incubation conditions were duplicated as closely as possible in both assays (at each pH) and it was found, consistently, that the rate of product formation by jackbean seed urease was three-fold higher in the ammonia evolution assay than in the CO_2 release assay (Table 2). On stoichiometric considerations alone one would expect a two-fold difference, so the enzyme activity must have been different also. The only known difference in conditions was the absorption of CO_2 in the CO_2 release assay, and it is possible that this lowers enzyme activity. This would mean that CO_2 trapping assays tend to underestimate urease. (Other possible differences can be ruled out, e.g. the pH 5.5 assays were both done in the same water bath; at both pHs ammonia was dealt with in the same way in the ammonia and CO_2 assays. Perhaps the first reaction product, carbamate [10], accumulates under conditions in which CO_2 is not absorbed, creating a disproportionate release of ammonia in the ammonia assay, although the enzyme is believed to act rapidly on carbamate.) Maize and soybean leaf preparations gave the same activity ratio as the commercial jackbean seed urease, at both pH values (Table 2). Since stoichiometry is a determinant of this ratio, we take this as evidence that these impure leaf ureolytic activities catalysed the same reaction as jackbean seed urease and were therefore ureases.

Inhibition

Apparent K_m values for urea were 0.50 mM (pH 5.5), 0.90 mM (pH 8.8) for soybean, and 0.45 mM (pH 5.5), 0.50 mM (pH 8.8) for maize. A series of structural analogues of urea were screened for inhibitory activity at pH maximum 8.8 (Table 3) in the $^{14}CO_2$ evolution assay. Pre-incubation, where shown, was for 60 min at 30°. The urea concentration was the standard assay value of 5 mM, which is just at the saturation level. Amino acid hydroxamates, well known as inhibitors of seed and plant cell culture ureases [3, 10], were more inhibitory than the other urea analogues (Table 3) but became less so with increasing size of the amino acid substituent. Pre-incubation markedly increased inhibition, suggesting slow and/or irreversible interaction. (Affinity chromatography was attempted with immobilized amino acid hydroxamates, but useful purification was not possible owing to an excess of non-specific interactions.) The inhibition of other ureases by hydroxamates has been extensively studied, but leaf ureases may interact with these compounds differently; Kobashi *et al.* [11] reported that lowering the pH from 7–10 to 5–6 completely removed the inhibition of jackbean seed urease, but we were unable to find such relief of inhibition with our maize preparation. Dixon *et al.* [12] postulated that hydroxamate inhibition results from chelation of enzyme-bound nickel ions. We were unable to find free nickel ion competition with hydroxamate inhibition of our maize leaf urease. Kerr *et al.* [4] cited reports of inhibition of seed ureases by hydroxyurea and reported that 4 mM hydroxyurea had no effect on their soybean leaf activity. In our hands, hydroxyurea was significantly inhibitory and much more so after pre-incubation with the extract (Table 3).

We were not able to find any inhibition of soybean leaf urease by the reaction products (up to 10 mM), even with the urea concentration set at the apparent K_m. This contrasts with reports (e.g. [5]) of product inhibition by ammonium.

Finally, we attempted to inhibit leaf urease activity in the $^{14}CO_2$ evolution assay with allantoin, allantoic acid and ureidoglycolate. These compounds in the pathway proposed for ureide catabolism [4] are urea analogues, and it was therefore of interest to examine whether the activity of leaf urease was restricted to the final step. With

Table 2. Products of leaf ureolytic activity

Buffer system	Extract	NH_3 evolution rate (nmol/min)	CO_2 evolution rate (nmol/min)	Rate ratio NH_3:CO_2
Glycine–KOH, pH 9.0	Urease standard*	2.0	0.62	3.2
	Soybean	1.0	0.32	3.1
	Maize	1.1	0.35	3.1
Citric acid–KOH, pH 5.5	Urease standard*	0.52	0.17	3.1
	Soybean	0.43	0.13	3.3
	Maize	0.34	0.10	3.4

*Urease standard was commercially available urease purified from jackbean (*Canavalia ensiformis*) seeds.

In the NH_3 evolution assays, background (minus urea) rates of change of absorbance were at least 3-(pH 9) or 4- (pH 5.5) fold lower than the 'plus urea' rates.

Table 3. Effects of urea analogues on soybean and maize leaf urease

	% Inhibition					
	Soybean			Maize		
Analogue	1	10	50 (mM)	1	10	50 (mM)
Hydroxyurea	19	71	91	13	67	87
Hydroxyurea (P)*	85	93	97	77	80	86
Thiourea	19	21	26	2	5	0
Selenourea	17	43	80	—	—	—
Semicarbazide	9	14	48	0	4	31
Hydroxylamine	12	8	23	0	0	31
L-Glutamine	0	0	0	0	0	14
Hydroxamates:						
Acetohydroxamate	54	88	96	56	92	97
Acetohydroxamate (P)	95	98	97	96	98	98
L-Glycine hydroxamate	97	99	99	94	92	97
L-Aspartyl-4-hydroxamate	0	0	19	2	2	43
L-Aspartyl-4-hydroxamate (P)	88	98	98	85	98	98
L-Glutamyl-5-hydroxamate	0	0	0	3	7	34

*P = Pre-incubation.

both soybean and maize preparations, allantoic acid and ureidoglycolate were inhibitory, but only at high concentrations, e.g. 60–80% at 50 mM and pH 5. Considerably less inhibition occurred at pH 8.8. Allantoin was not inhibitory. Allantoic acid and ureidoglycolate therefore resemble poor inhibitors, rather than good alternative substrates which would 'compete' with radioactive urea. We conclude that the leaf urease activity does not contribute significantly to the catalysis of ureide catabolism earlier than at the urea hydrolysis step.

EXPERIMENTAL

Plant material. Soybeans (*Glycine max* cv Forrest; not nodulated) were grown in a growth chamber (26°; 75% relative humidity, 12 hr day at 710 μE/m^{2}hr) and corn plants (*Zea mays* cv Northrup King PX74k Singlecross) in a greenhouse. Both were planted in peat–vermiculite–sand (3:2:1) and supplied with 30% (v/v) Murashige and Skoog salts (soybean) or Plantex 15/15/18 fertilizer (Plantex Inc., Bramalea, Ontario, Canada) soln daily. All leaves were harvested for enzyme extraction at *ca* 3 weeks after planting.

Enzyme extraction. A typical procedure for urease extraction from soybean or corn leaves involved the following steps (variations are discussed in Results and Discussion). The leaves were frozen in liquid N and then ground with acid-washed sand and 3 ml/g fr. wt extraction buffer at 0°. The buffer comprised either 50 mM KH_2PO_4–K_2HPO_4, 5 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.0 (Buffer 1), or 50 mM Tris–HCl, 5 mM EDTA, 10 mM 2-mercaptoethanol, pH 8.8 (Buffer 2). The mixture was blended for 1 min at top speed in a domestic blender, strained through 4 layers of cheesecloth, and centrifuged at 10000 *g* for 20 min (these, and all subsequent steps, at 0–4°). There followed fractionation with 40% saturation, and 40–55% saturation $(NH_4)_2SO_4$, resuspension in Buffer 3 or 4 (see below) and overnight dialysis against the same buffer (with one change at 3 hr). Resuspension/dialysis buffers were 10 mM KH_2PO_4–K_2HPO_4, 1 mM EDTA, 8 mM 2-mercaptoethanol, pH 7.0 (Buffer 3; used following Buffer 1) or 10 mM Tris–HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 8.8 (Buffer 4; used following Buffer 2). After dialysis, the preparations were centrifuged at 16000 *g* for 10 min, and in some experiments processed further (see Results and Discussion). Ultrafiltration was performed using an Amicon Diaflo pressure cell and PM10 membrane (Amicon Corp., Danvers, MA, U.S.A.).

Assays of ureolytic activity. A convenient, disposable test-tube assembly was used for $^{14}CO_2$ evolution assays developed from ref. [13]. A 21G11/2 disposable hypodermic needle was inserted all the way through the snap-on cap of a 12 × 75 mm disposable polypropylene test tube. Four filter discs (Schleicher & Schuell Analytical Paper 740-E, 0.25 in diameter) were placed together onto the needle and loaded with 50 μl α-phenylethylamine. The assay mixture was placed in the test tube and the cap fitted, placing the discs on the needle into the air space above the soln.

The standard assay contained 5 mM urea, 4630 Bq [^{14}C]urea, 10 mM Tris–HCl (pH 8.8), 2 mM EDTA and leaf preparation in a total vol. of 210 μl. Incubation was for 40 min at 30° and then 0.5 ml 0.4 M HCl was injected down the syringe needle to terminate the reaction and evolve $^{14}CO_2$. After 45 min of further incubation at 30°, the discs were transferred to 0.5 ml scintillation fluid ('Aquasol II', New England Nuclear Corp.) for scintillation counting. The use of phenylethylamine to trap CO_2 permitted immediate counting without chemiluminescence [14]. In a calibration experiment using excess jackbean seed urease, 2% of the supplied radioactivity remained in the soln and 88% was recovered reproducibly on the discs after correction for 68% counting efficiency (the remaining 10% probably resided in the atmosphere within the tube).

Two types of assay were used to measure NH_3 formation, according to the pH required. For activity at pH 8.8–9.0, NH_3 release was followed with time by coupling to glutamate dehydrogenase (GDH) [15]. Reactions were run at 30° and the mixture comprised 20 mM glycine–KOH (pH 9.0), 0.7 mM ADP, 0.1 mM NADH, 1 mM 2-ketoglutarate, 5 mM urea, 16.5 units GDH (Sigma type II) and leaf extract (sufficient activity for 2000 cpm in the $^{14}CO_2$ evolution assay) in a total vol. of 0.5 ml. The initial rate of decrease of *A* at 340 nm was measured and corrected for background rates in the absence of urea. The

standard curve was linear up to 40 nmol NH_3 (80 μM) with a slope of 0.0124/nmol.

GDH is not functional at pH 5.5 and so an alternative coupled assay was devised for use at this pH. Advantage was taken of the ability of acetohydroxamate to inhibit the ureolytic activity (see Results and Discussion), and accumulated NH_3 was subsequently estimated by addition of GDH in a conc. buffer which raised the pH. The initial incubation mixture contained 9.5 mM citric acid–KOH (pH 5.5), 10.5 mM urea and various amounts of NH_3 or leaf extract (typically sufficient for 500 cpm in the $^{14}CO_2$ evolution assay) in a total vol. of 228 μl. After 40 min incubation at 30°, 80 μl 0.375 M acetohydroxamate was added to stop the reaction. Coupling reaction mixture was then added; 0.3 ml 70 mM Tris–HCl (pH 7.5) containing ADP, NADH, 2-ketoglutarate and GDH for the above final concns, and the *A* at 340 nM was read after 30 min at 30°. In both types of NH_3 assay, corrections were made for control determinations in which urea had been omitted. For the stoichiometry investigations, the $^{14}CO_2$ evolution assay was undertaken in the presence of the NH_3 release assay constituents (excluding coupling reaction mixture in the pH 5.5 assay), and with the sp. radioactivity of the [^{14}C]urea increased five-fold. Jackbean seed urease was obtained from Sigma (type VII) and included in NH_3 and $^{14}CO_2$ assays for calibration purposes at 10 ng/ml. Solns of inhibitors for inclusion in the $^{14}CO_2$ evolution assay were first set to pH 8.8 with solid Tris.

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