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PURIFICATION AND PROPERTIES OF INDOLE 2,3-DIOXYGENASE FROM MAIZE LEAVES

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Abstract—An indole 2,3-dioxygenase was purified *ca* 38-fold from maize leaves The enzyme had an MW of about 98 000, an optimum pH of 5 0 and the energy of activation was 9 1 kcal/mol The K_m for indole was $1\,4 \times 10^{-4}$ M The enzyme was inhibited by diethyldithiocarbamate, salicylaldoxime and sodium dithionite The inhibition by diethyldithiocarbamate was specifically reversed by Cu^{2+} The dialysed enzyme was stimulated by Cu^{2+} Four atoms of oxygen were utilized in the disapperance of 1 mole of indole Inhibition of the enzyme by -SH compounds and -SH group inhibitors, and their partial removal by Cu^{2+} only, suggested the involvement of -SH groups in binding of Cu^{2+} at the catalytic site

INTRODUCTION

Metabolism of indole is important since it serves as the precursor of tryptophan in microorganisms [1–4] and higher plants [5–9] The biosynthesis of indole occurs from the shikimic acid pathway and tryptophanase reaction [10] At least two enzymatic reactions are known for indole utilization conversion into tryptophan and oxidation into anthranilic acid and anthranil The former reaction catalysed by tryptophan synthetase has received intensive study [11–15] The latter reaction catalysed by indole oxidase or indole 2,3-dioxygenase (EC 1 1 3 17) has been reported in certain *Pseudomonas* species adopted to indole [16], *Aspergillus niger* [17] and the leaves of *Tecoma stans*, a common Indian hedge plant [18] and is comparatively less investigated [19] Chauhan *et al* from this laboratory have reported the presence of an indole oxidizing system in maize leaves and suggested its possible role in controlling the tryptophan level in maize [20] So far, however, the indole 2,3-dioxygenase has not been purified and fully characterized The aim of the present study was to purify and characterize an indole 2,3-dioxygenase from maize leaves

RESULTS AND DISCUSSION

Table 1 shows the specific activity of indole 2,3-dioxygenase in 15 000 *g* supernatant of maize genotypes at various growth stages No activity was detected in the seeds of any genotype Low activity appeared with germination which continued to increase until day 20 The activity remained high up to day 40 and then declined Highest activity was observed in Vijay composite on day 20 and this was therefore chosen for purification

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Purification

Indole 2,3-dioxygenase has been purified *ca* 38-fold (Table 2) from 20-day-old leaves of maize Low activity of indole 2,3-dioxygenase in the 15 000 *g* supernatant has been attributed to the presence of some natural inhibition [21] A 50–80% ammonium sulphate fractionation resulted in 4-fold purification but with a great loss (*ca* 64%) of the activity When this fraction was subjected to Sephadex G-100 chromatography, it resulted in a major peak of activity with two shoulders on either side A total of 1300 enzyme units were recovered in this major peak against 818 units loaded on the Sephadex G-100 column It is possible that a natural inhibitor (specific or non-specific) is concentrated during the ammonium sulphate fractionation and is subsequently resolved from the enzyme during gel filtration Elution of the post Sephadex G-100 enzyme fractions from DE-52 cellulose gave a single peak of enzyme activity at 0 204 M sodium chloride The enzyme was stable at 4° for 2 weeks

Properties

The MW of the enzyme determined on a calibrated Sephadex G-100 column according to the method of Andrews [22] was *ca* 98 000

The enzyme showed a pH optimum at 5 0 in citrate–phosphate buffer, like the *Tecoma* enzyme [18] The enzyme was stable in the pH range from 3 to 6 The enzyme activity was maximal at 45°, after which it showed a rapid decline A continuous Arrhenius plot obtained from activity measurement between 30° to 45°, gave the activation energy of 9 1 kcal/mol

The rate of indole disappearance was linear up to 30 min, after which it was constant Therefore, in all enzyme assays, the incubation time was 30 min A hyperbolic relationship between indole concentration in the range of $1\,25 \times 10^{-5}$ M to 3×10^{-4} M and indole disappearance was obtained, like the *Tecoma* enzyme [18]

Table 1 Specific activity* of indole 2,3-dioxygenase in 15 000 g supernatant of maize genotypes at various growth stages

Growth stage	Plant parts analysed	Genotype				
		Vijay composite	Ganga-5	Ganga-2	D741	Opaque-2
Coleoptile stage (3-day old)	Epicotyls	0 30	0 26	0 35	0 27	0 17
5-day old	Shoot tissue	0 47	0 45	0 41	0 23	0 27
10-day old	Shoot tissue	0 62	0 96	0 68	0 26	0 32
20-day old	Middle leaves	1 20	1 16	0 51	0 40	0 38
40-day old	Middle leaves	0 68	0 55	0 47	0 34	0 30
Flowering stage (60-day old)	Middle leaves	0 53	0 37	0 30	0 50	0 21
Milking stage (80-day old)	Middle leaves	0 27	0 26	0 30	0 28	0 12

*Activity values represent the mean of three separate determinations

Standard assay conditions were used Specific activity denotes the number of enzyme unit per mg protein One unit of enzyme is defined as the amount of enzyme which utilized 1 nmol of indole per min

Table 2 Purification of indole 2,3-dioxygenase from maize leaves

Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)*	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
1 15 000 g supernatant	225	1930 0	2300	1 18	1 0	100
2 50–80% $(NH_4)_2SO_4$ precipitate	8	170	818	4 82	4 0	35 6
3 Sephadex G-100 eluate	24	44 8	739	16 5	14 0	32 6
4 DE-52 cellulose eluate	30	6 3	278	44 1	37 4	12 1

*One enzyme unit is defined as the amount of enzyme which utilized 1 nmol of indole per min

Standard assay conditions were used except for the enzyme preparation as indicated

Lineweaver–Burk plot of $1/v$ and $1/[S]$ was linear and the K_m obtained for indole was $1\ 40 \times 10^{-4}$ M A plot of enzyme concentration versus indole disappearance was linear over the range used for these assays

The enzyme was inhibited by diethyldithiocarbamate (DIECA) and salicylaldoxime (Table 3) indicating the requirement of Cu^{2+} for enzyme activity Inhibition caused by DIECA was partly reversed by Cu^{2+} only (Table 3), thus confirming the preliminary observations by Chauhan *et al* [20] The requirement of Cu^{2+} has also been suggested for the *Tecoma* enzyme [18]

Dialysed enzyme was used to study the effects of metals and coenzymes The enzyme was dialysed against 0 02 M sodium phosphate buffer (pH 7) containing EDTA (10^{-4} M) in the cold for 6 hr The enzyme did not lose much of its activity after dialysis and was stable at 4° for 1 week Of the several metal ions tested, only Cu^{2+} stimulated the enzyme activity Fe^{2+}, Fe^{3+} and Hg^{2+} caused slight inhibition at 5×10^{-4} M (Table 3) Other metals (Mn^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Sr^{2+} and Mo^{6+}) had no effect. Similar results were obtained for the *Tecoma* enzyme [18]

Amongst the coenzymes tested, only FAD gave slight stimulation in the activity of dialysed enzyme (Table 3) However, FMN, NAD^+ and $NADP^+$ had no effect at 7×10^{-4} M Addition of both Cu^{2+} and FAD together exhibited a cumulative increase in the dialysed enzyme activity, which was similar to the stimulation produced by Cu^{2+} alone (Table 3) These results do not indicate that maize indole 2,3-dioxygenase could be a flavoprotein, as suggested by Chauhan *et al* [20] Further, at least the purified enzyme did not show a spectrum of a typical flavoprotein Nair and Vaidyanathan [18] showed an absolute requirement of Cu^{2+} and FAD for the maximum activity of *Tecoma* leaf indole oxidase and classified it as a cuproflavoprotein

An almost complete inhibition by sodium dithionite (10^{-3} M) (Table 3) showed the requirement of O_2 for the reaction O_2 uptake study of the enzyme reaction carried out in Warburg flasks revealed that for disappearance of 1 mole of indole, 4 atoms of oxygen were utilized (Table 4) These findings suggest that the enzyme could be a dioxygenase

Identification of products of the reaction

Two products of the reaction were detected in the form of UV fluorescent spot A_1 (R_f value = 0 83) and spot A_2

Table 3 Effect of metal chelators, metals and FAD on maize leaf indole 2,3-dioxygenase

Enzyme preparation	Compound added	Final concentration	Effect (%)
DE-52 cellulose eluate	Diethyldithiocarbamate	5×10^{-4} M	−63
	Salicylaldoxime	5×10^{-4} M	−34
	Diethyldithiocarbamate $CuSO_4$	5×10^{-4} M 5×10^{-4} M	−30
	Diethyldithiocarbamate $MnSO_4$	5×10^{-4} M 5×10^{-4} M	−63
	Diethyldithiocarbamate $FeSO_4$	5×10^{-4} M 5×10^{-4} M	−67
	Sodium dithionite	1×10^{-3} M	−96
DE-52 cellulose eluate (dialysed)*	$CuSO_4$	5×10^{-4} M	+23
	$FeSO_4$	5×10^{-4} M	−16
	$FeCl_3$	5×10^{-4} M	−17
	$HgCl_2$	5×10^{-4} M	−6
	FAD	7×10^{-4} M	+3
	FAD $CuSO_4$	7×10^{-4} M 5×10^{-4} M	+27

*The enzyme was dialysed in 0 02 M sodium phosphate buffer (pH 7) in the cold for 6 hr
−, Inhibition, +, stimulation Standard assay conditions were used except for the addition of compound(s) as indicated

Table 4 O_2 uptake in the reaction of maize leaf indole 2,3-dioxygenase at different time

Time (min)	μmol O_2 utilized	μmol indole oxidized
15	0 422	0 201
30	0 668	0 334
45	0 810	0 386
60	0 870	0 396

The assay was carried out at 40° in Warburg flasks containing 90 μmol citrate phosphate buffer (pH 5) 2 μmol indole and concentrated enzyme in a total vol of 3 ml Both O_2 uptake and indole disappearance were measured at the times indicated

(R_f value = 0 42) Spots A_1 and A_2 were identified as anthranil and anthranilic acid respectively, on the basis of the following tests

(i) Spots A_1 and A_2 gave identical R_f values and UV fluorescence (violet) on paper chromatograms as those of synthetic anthranil and authentic anthranilic acid, respectively

(ii) The UV spectrum of spot A_1 in methanol showed maximum A at 266 nm and 310 nm, similar to that of synthetic anthranil in methanol Similarly, the UV spectrum of spot A_2 in methanol closely corresponded with that of authentic anthranilic acid in methanol as both showed maximum absorption at 248 nm and 335 nm

Earlier, using the crude preparation of indole oxidase from leaves, only anthranil in *Tecoma* [18] and anthranilic acid and anthranil in maize [20] could be detected as the major products of indole oxidation

These results of O_2 uptake study and identification of products of the reaction could be explained on the basis of the following scheme of indole oxidation in maize proposed by Chauhan *et al* [20] (Fig 1)

The cleavage of indole by maize leaf indole 2,3-dioxygenase is different from the cleavage of the indole ring of tryptophan by rat liver tryptophan 2,3-dioxygenase [23], as the former is not inhibited by catalase (0 5 mg/ml of reaction mixture) It indicates that there is no hydrogen peroxide formation in indole oxidation in maize Witkop *et al* [24, 25] reported the cleavage of the pyrrole ring of indole by oxidizing agents like perbenzoic acid, ozone and peracetic acid, which resulted in the formation of *N*-formylaminobenzaldehyde The electron density of the indole ring at carbons 2 and 3 favours this type of cleavage By analogy of the chemical oxidation of indole, the first product of biological oxidation of indole in maize could be *N*-formylaminobenzaldehyde which is formed by the cleavage of the indole ring at carbons 2 and 3 by consuming both atoms of oxygen *o*-Aminobenzaldehyde, possibly derived from *N*-formylaminobenzaldehyde, could be oxidized to anthranil and anthranilic acid by this enzyme system Sakamoto *et al* [16] reported the bacterial cleavage of indole via isatin, which gives rise to *N*-formylanthranilic acid It is clear from this scheme that two atoms of oxygen would be required for the initial cleavage of indole and one each for the oxidation of *o*-aminobenzaldehyde to anthranil and anthranilic acid, simultaneously The O_2 uptake of the enzyme reaction also supports this type of scheme, as four atoms of O_2 were utilized per mole indole disappeared (Table 4)

Role of –SH *groups*

Amongst the –SH reagents, glutathione, mercaptoethanol and cysteine were found to be strong inhibitors Cu^{2+} protected to some extent against this inhibition (Table 5). The addition of the –SH reagents may remove Cu^{2+} from the catalytic site and thereby inhibit the enzyme activity However, 2,3-dimercaptopropanol

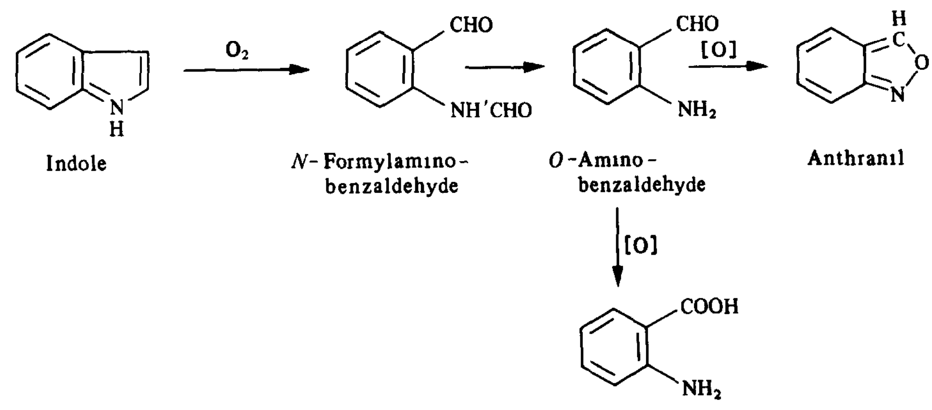

Fig 1 The sequence of reactions catalysed by maize leaf indole 2,3-dioxygenase

Table 5 Effect of -SH compounds and -SH group inhibitors with or without Cu^{2+} on purified maize leaf indole 2,3-dioxygenase

Compound added (5×10^{-4} M)	% Inhibition
Glutathione	81
Mercaptoethanol	80
Cysteine	78
Glutathione–$CuSO_4$	71
Mercaptoethanol–$CuSO_4$	73
N-Ethylmaleimide	34
N-Ethylmaleimide–$CuSO_4$	ND

ND, Not detected Standard assay conditions were used except for addition of compound(s) as indicated

(5×10^{-4} M) had no effect Of the -SH groups blocking agents, *N*-ethylmaleimide (5×10^{-4} M) caused 34% inhibition of the enzyme which was reversed by addition of Cu^{2+} (Table 5) Sodium arsenite (5×10^{-4} M), a powerful inhibitor of two vicinal -SH groups had no effect These results suggest that -SH groups are not directly involved in the enzyme activity Even so, -SH groups may facilitate the binding of Cu^{2+} at the catalytic site during the reaction Similar conclusions based upon a preliminary study of indole oxidation in *Tecoma* leaf were reached by Nair and Vaidyanathan [18]

Physiological role

A comparison of the specific activity of indole 2,3-dioxygenase in 15 000 *g* supernatant of 20-day old leaves of maize genotypes as shown in Table 1, revealed that the activity in normal genotype (viz Vijay composite, Ganga-5) known for their low tryptophan content [26, 27] was 3–4 times greater than mutant (Opaque-2) known for its high tryptophan content [27] A preliminary study of tryptophan synthetase in developing kernel of two maize genotypes in this laboratory showed that tryptophan synthetase activity was highest at the 18-day growth stage when the activity of indole 2,3-dioxygenase was lowest and with the advancing maturity of the kernel, the tryptophan synthetase activity declined, whereas the indole 2,3-dioxygenase activity was more pronounced [28] These correlative data emphasize that indole 2,3-dioxygenase, in conjunction with tryptophan synthetase, plays an important role in directing the flow of indole into tryptophan, or its oxidative breakdown

EXPERIMENTAL

Plant material Maize seeds (*Zea mays* L genotypes Vijay composite, Ganga-5, Ganga-2, D741 and Opaque-2) were soaked in H_2O for 24 hr and then surface sterilized with 0 01 % $HgCl_2$ and washed thoroughly with H_2O The seeds were germinated at $33 \pm 2°$ at Crop Research Centre of the University Epicotyls at the coleoptile stage, shoot tissue at 5 and 10-day old stages and middle leaves at 20-day, 40-day, 60-day (flowering stage) and 80-day (milking stage) after germination were collected and stored at $-20°$ until used

Isolation of indole 2,3-dioxygenase Indole 2,3-dioxygenase from various plant material was prepared by the method of ref [20] The material was homogenized with 2 vols of cold H_2O in a chilled pestle and mortar The homogenate was squeezed through a double layer of cheese cloth and centrifuged at 15 000 *g* for 30 min in the cold The supernatant was collected and used as the source of the enzyme It was stored at 4° until used

Assay of indole 2,3-dioxygenase Indole 2,3-dioxygenase activity was determined by estimating unused indole by the method of ref [29] Unless specified otherwise, the assay system consisted of 60 μmol citrate–Pi buffer (pH 5), 0 2 μmol indole and enzyme in a total vol of 2 ml After incubating at 40° for 30 min, the reaction was terminated by adding 3 ml toluene The tubes were shaken immediately and the indole in the toluene layer was estimated [29] One unit of enzyme activity was defined as the amount of enzyme which utilized 1 nmol indole per min, under the standard conditions of assay

Purification of indole 2,3-dioxygenase All purification steps were carried out at 4° A 50% satd $(NH_4)_2SO_4$ soln of 15 000 *g* supernatant prepared from 20-day old leaves of Vijay composite genotype, was centrifuged at 10 000 *g* for 30 min The $(NH_4)_2SO_4$ concn of the supernatant was increased to 80% satn and centrifugation was performed as before The ppt was taken up in 0 02 M NaPi buffer (pH 7) Gel filtration of this $(NH_4)_2SO_4$ fraction was affected on Sephadex G-100 column (2 5 cm × 25 cm) equilibrated and eluted with 0 02 M NaPi buffer (pH 7) at a flow rate of 0 5 ml per min Each fraction (3 ml) was monitored for indole 2,3-dioxygenase activity and protein The fractions containing maximum sp act were pooled and applied to DE-52 cellulose column (1 25 cm × 6 cm) equilibrated with 0 02 M NaPi buffer (pH 7) The enzyme was eluted with 200 ml of the same buffer with a linear gradient of NaCl between 0 1 M and 0 4 M concentration Each fraction (3 ml) was analysed for activity and protein The pooled fractions containing maximum activity constituted the purified enzyme, which was stored at 4° until use The protein content of enzyme fractions was determined by the method of ref [30]

MW of the enzyme was estimated by gel filtration on a Sephadex G-100 column (2 5 cm × 25 cm) equilibrated and eluted with 0 02 M NaPi buffer (pH 7) at a flow rate of 0 5 ml/min The following markers were used cytochrome *c* (12 500), hemoglobin (68 000), aldolase (150 000) and catalase (250 000)

O_2 *uptake* of the reaction was measured by a Warburg manometric technique Each flask contained 90 μmol citrate–Pi buffer (pH 5), 2 μmol indole, concd enzyme and H_2O in a total vol of 3 ml Both O_2 uptake and indole disappearance were measured at various time intervals.

The products of the enzyme reaction were identified by the method of ref [18] The reaction mixture (100 ml) consisted of buffer, indole, H_2O and enzyme in the same proportion as used in the routine assay After incubation at 40° for 30 min, the reaction mixture was extracted × 3 in 50 ml toluene to remove unutilized indole from the reaction mixture The aq phase was pooled and extracted with twice the vol of Et_2O The Et_2O layers were separated and concd *in vacuo* The residue was dissolved in 3 ml EtOAc, dried over Na_2SO_4 and concd again *in vacuo* up to 1 ml A light yellow coloured liquid was obtained An aliquot of this liquid (20 μl) was run on Whatman No 1 paper using MeOH, NH_3 and H_2O (18 1 1) as solvent system and authentic anthranilic acid and synthetic anthranil as standards Anthranil was prepared by the method of ref [31] Spots on the chromatogram were visualized under UV light

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