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AMINO OXIDASE FROM *TRIGONELLA FOENUM-GRAECUM* SEEDLINGS

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Key Word Index—*Trigonella foenum-graecum*; Fabaceae; fenugreek; amine oxidase; protein purification; substrate specificity and inhibitors.

Abstract—Amine oxidase was isolated from eight-day-old *Trigonella foenum-graecum* seedlings grown in the dark, using rivanol lactate and ammonium sulphate fractionations, heat denaturation and hydroxylapatite chromatography. The yellow enzyme is a monomer M_r of 80 000 with pI 6.8, A_{max} at 280 nm and a shoulder at 415 nm. Substrate specificity and inhibitor interactions were similar to those of pea amine oxidase. The *N*-terminal of the enzyme was blocked and no reaction was observed with antibody against amine oxidase either from *Escherichia coli* or *Micrococcus luteus*.

INTRODUCTION

Amine oxidase (EC 1.4.3.6) [amine: O_2 oxidoreductase (deaminating, copper containing)], has been found in microorganisms, and various plants and animals [1]. The enzyme is widespread in the Fabaceae. Amine oxidase catalyses the oxidative deamination of amines, producing the corresponding aldehyde, H_2O_2 and ammonia. Copper and a carbonyl cofactor, recently identified as the quinone 6-hydroxydopa (topa) [2, 3], mediate substrate reaction following a ping-pong mechanism [4]. In plants, amine oxidase acts in various physiological processes in connection with polyamine degradation [5]. We have now isolated and characterized amine oxidase from *Trigonella foenum-graecum* (fenugreek).

RESULTS AND DISCUSSION

Amine oxidase (T-AO) activity was followed in extracts from fenugreek grown in continuous light or in the dark during the first 16 days. Enzyme activity appeared soon after seed swelling and rose sharply until six–eight days of cultivation, mainly in the cotyledon. Higher activity was found in etiolated plants than in those cultivated in light. For amine oxidase purification, eight-day-old etiolated germinating seeds were found to be optimum.

The purification procedure is based on the method applied for pea amine oxidase (P-AO) [6]; rivanol lactate and ammonium sulphate fractionation, heat denaturation of inert proteins and hydroxylapatite chromatography. The summary of the purification procedure is presented in Table 1. SDS–PAGE showed a single band corresponding to M_r 80 000 and native PAGE showed an activity band corresponding to a single protein band for purified T-AO. Since gel HPLC on TSK gel showed a single peak corresponding also to M_r of 80 000, the monomeric structure of T-AO was confirmed. SDS–PAGE purified enzyme was subjected to Edman degradation after blotting to PVDF membrane, but the *N*-terminal sequencing of the enzyme was not possible probably due to blocking. The purified enzyme was yellow having A_{max} at 280 nm, and a shoulder at 415 nm. Copper content was *ca* 1 atom mole^{-1}.

The enzyme was active toward 1,4-diaminobutane (putrescine) with K_m 0.33 mM and 1,5-diaminopentane (cadaverine) with K_m 0.11 mM. The activity of the enzyme was measured over a pH range of 4–8; the optimum was *ca* 7.

T-AO is inhibited, as in other Fabaceae amine oxidases, by substrate analogues (1,5-diamino-3-pentanone, *E*- and *Z*-1,4-diamino-2-butene), Cu chelating agents (*o*-phenanthroline, 2,2′-bipyridyl), carbonyl reagents (aminoguanidine, phenylhydrazine) and some alkaloids (quinidine and L-lobeline). Inhibition by *E*- and *Z*-1,4-diamino-2-butene was determined as inhibition by an excess of the substrate. Surprisingly, spectral changes of amine oxidases giving a new A_{max} at 420–440 nm due to the formation of a Schiff base with the carbonyl cofactor were not observed in the reaction of T-AO with phenylhydrazine or *p*-nitrophenylhydrazine, although they showed total inhibition. Also, inhibition was relatively slow compared with P-AO, especially when *p*-nitrophenylhydrazine was used. Since phenylhydrazine can also inhibit flavine containing bovine kidney mitochondrial amine oxidase (EC 1.4.3.4) [7] bleaching A_{max} at 455, pargyline as a typical irreversible inhibitor of flavine containing amine oxidase was tested. Pargyline causes a new A_{max} at 410 nm with flavine inhibiting bovine kidney mitochon-

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Table 1. Purification of fenugreek amine oxidase

Purification step	Volume (ml)	Total protein (mg)	Total activity (μkat)	Specific activity (nkat mg^{-1})	Purification (fold)	Yield (%)
Crude extract	1500	3990	12.7	3.2	1	100
Rivanol lactate fractionation	1560	1260	11.4	9	2.8	89.3
Ammonium sulphate fractionation	96	345	5.5	16	5	43
Heat denaturation	90	139	5.4	39	12	42.3
Hydroxylapatite chromatography	88	27	2.9	106	33	22.5

drial amine oxidase [8]. Such a change was not observed with T-AO and inhibition by pargyline was very weak, so T-AO probably does not contain flavine. We performed Western blotting with antibodies against *Escherichia coli* (copper-containing) and *Micrococcus luteus* (flavine-containing) amine oxidases, but T-AO did not show a positive reaction with either of them. Considering the differences in reaction with hydrazines compared with P-AO, the structure of the T-AO active site seems to be slightly different, although both enzymes show similar substrate specificity. The difference may be related to the monomeric structure of T-AO, compared with the dimer of P-AO.

EXPERIMENTAL

Cultivation of the plants. Seeds of *Trigonella foenum-graecum* were sterilized by 5% chloramine-B for 20 min and then soaked in H_2O for 24 hr. The seeds were transferred on to Perlite and grown for 8 days at 23°, in the dark (germination 75–95%).

Enzyme prepn. All procedures were performed at 0–5°. Eight-day-old seedlings (760 g) were homogenized in a Waring blender with 1.5 l of 0.1 M K- Pi buffer (pH 7), for 5 min and the homogenate was filtered through a nylon cloth, centrifuged at 15 000 *g* for 30 min and ppt discarded. The supernatant was fractionated with 4% rivanol lactate (1 ml 80 mg^{-1} of protein). The mixt. was centrifuged 1 hr at 3000 *g* and the sediment was discarded. The supernatant was then decolorized with charcoal (*ca* 6 g) and re-centrifuged. Solid $(NH_4)_2SO_4$ was added to 70% satn and the soln stirred for 30 min in the cold. The ppt. was collected by centrifugation (1 hr, 3000 *g*), resuspended in 40 ml of 0.1 M K-Pi buffer, pH 6.4 and dialysed against the same buffer overnight. The dialysed soln was rapidly heated to 55–58° and kept at 60° for 5 min. Then the soln was rapidly cooled to 0°, and a ppt. with the remaining charcoal was removed by 30 min centrifugation at 5000 *g*. The supernatant was concd osmotically in dialysis tubing against polyethylene glycol (PEG 20 000), to a vol. of *ca* 15 ml. The concd soln was dialysed for 48 hr against 20 mM K-Pi buffer, pH 7, containing 1 μM $CuSO_4$. The dialysed enzyme was applied to a hydroxylapatite column (3.8 cm i.d. × 6.5 cm) equilibrated with the same buffer. After washing with 10 and 100 mM K-Pi buffers of pH 7 to remove inert proteins, the enzymes was eluted with 1M K-Pi buffer pH 7.

Activity and protein assay. Amine oxidase activity was measured by coupled reaction with peroxidase and guaiacol [9] with putrescine as substrate. Proteins were determined by Coomassie Brilliant Blue [10], BSA as a standard.

Electrophoresis. SDS–PAGE [11] was performed on slab gel (12.5%). Samples were treated with 6% SDS at 60° for 1 hr before application. Proteins with the indicated M_rs were used as references: lysozyme (14 400), carbonic anhydrase (31 000), ovalbumin (45 000), BSA (66 000) and phosphorylase b (97 400). Gel was stained with Coomassie Brilliant Blue G-250. Native enzyme electrophoresis was performed on slab polyacrylamide gel (7%). Gels were stained either with Coomassie Brilliant Blue G-250 for proteins or with putrescine, peroxidase and guaiacol for the activity.

Isoelectric focusing was performed on polyacrylamide disc gels [12] with Ampholine 3-11 (Pharmacia) and acetylated cytochrome c as pI marker, pI 4.1, 4.9, 6.4, 8.3, 9.7 and 10.6 (Oriental Yeast Japan). Sample gels were stained with putrescine, peroxidase and guiacol for the activity.

Gel HPLC. Performed with TSK 3000SW gel column (7.5 mm I. D. × 60 cm) and SPD M6A photodiode array detector, pH 7 (0.1 M) K-Pi buffer containing 0.2 M NaCl was used as a mobile phase. Proteins were eluted with a flow rate of 0.7 ml min^{-1} and detected at 214 and 280 nm.

Copper content. Determined according to ref. [13], by standard addition method with atomic absorption spectrophotometry.

N-terminal sequencing of T-AO by Edman degradation was performed on Shimadzu PSQ-2 protein sequencer after SDS–PAGE and PVDF membrane blotting of the enzyme.

Reaction with antibodies. Western blotting of T-AO with antibodies against amine oxidases from *Escherichia coli* (EC 1.4.3.6) and *Micrococcus luteus* (EC 1.4.3.4) donated by Prof. H. Kumagai from Department of Food Science and Technology, Kyoto University, Japan, showed no reactivity.

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