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PYRROLOOXYGENASES FROM PEPPER AND POINSETTIA LEAVES

ROSALIA B. FRYDMAN, MARIA L. TOMARO and BENJAMIN FRYDMAN
Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires, Junin 956, Buenos Aires, Argentina

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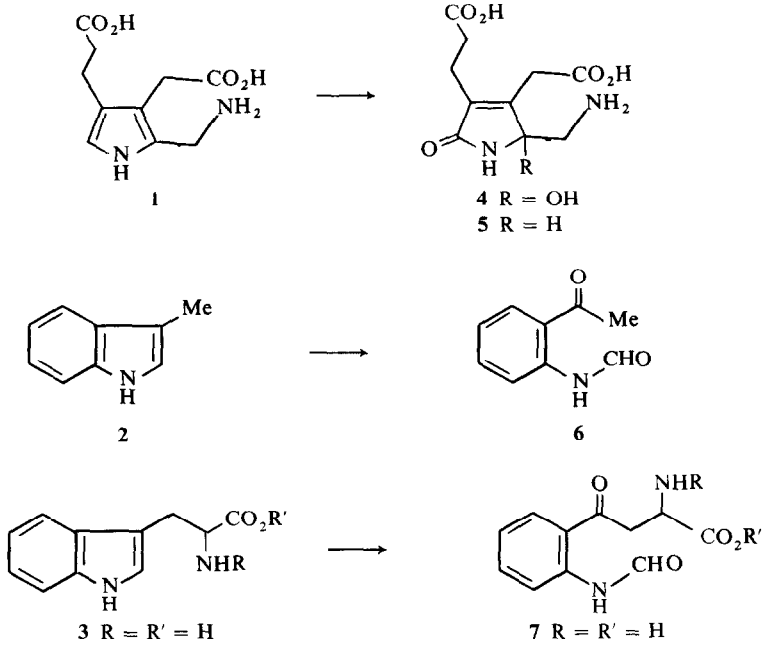
Key Word Index—*Capsicum annuum*; Solanaceae; *Euphorbia pulcherrima*; Euphorbiaceae; porphobilinogen oxygenase; skatole pyrrolooxygenase; tryptophan pyrrolooxygenase; leaves; fruiting plants.

Abstract—Leaf extracts of pepper (*Capsicum annuum*) and poinsettia (*Euphorbia pulcherrima*) contained pyrrolooxygenases which varied in activity according to the age of the leaves and the origin and physiological condition of the plants. An inhibition of the pyrrolooxygenases was present in the crude extracts of senescent leaves. Fruiting enhanced pyrrolooxygenase activity and added a new ionic form of greater negative charge to the usual cationic form of the enzymes. Pyrrolooxygenases of *C. annuum* leaves from greenhouse-grown plants showed three forms of different ionic charge which exhibited multiple MW forms for porphobilinogen oxygenase and skatole pyrrolooxygenase. The cationic form of porphobilinogen oxygenase had sigmoidal kinetics, while the anionic forms had Michaelis kinetics. Skatole and tryptophan pyrrolooxygenase showed Michaelis kinetics. Pyrrolooxygenase activities in *E. pulcherrima* were lower than those in *C. annuum* and the former were also found to be more unstable.

INTRODUCTION

Pyrrolooxygenases are mixed function oxidases which have been isolated from wheat germ and from rat liver microsomes and were also detected in Swiss chard and spinach chloroplasts [1]. Three enzymes of this type were identified within the group; porphobilinogen oxygenase which oxidizes porphobilinogen **1** [2, 3] (Scheme 1), skatole pyrrolooxygenase which oxidizes skatole **2** [4] and tryptophan pyrrolooxygenase which oxidizes free and bound forms of tryptophan **3** [4, 5]. The oxidation of porphobilinogen (PBG) **1** by the oxygenase forms 2-hydroxy-5-oxoporphobilinogen **4**, and 5-oxoporphobilinogen **5**, the oxidation of skatole **2** gives 2-formamidoacetophenone **6** and the oxidation of tryptophan **3** gives *N*-formylkynurenine, or substituted *N*-formylkynurenines **7** if substituted tryptophanes are used (Scheme 1). Porphobilinogen oxygenase from wheat germ was the most extensively studied of the 3 pyrrolooxygenases and it was found to be a cationic iron sulfur protein containing 8 mol of non-heme iron and 8–10 mol of labile sulfide per mol of enzyme (MW 100 000) [6]. The



Scheme 1

Table 1. Pyrrolooxygenase activities in young and senescent leaf extracts of *C. annuum* and *E. pulcherrima*

Species	Leaf	Pyrrolooxygenase activity*		
		Porphobilinogen	Skatole	Tryptophan
C. annuum (field-grown)	young	3.2	6.8	19.4
	senescent	1.6	3.6	15.6
E. pulcherrima (field-grown)	young	3.2	8.0	14.5
	senescent	2.2	3.2	10.0
C. annuum† (greenhouse grown)	young	1.6	1.2	—
	young (fruiting plants)	3.6	3.4	—
	senescent	0.8	1.0	—
	senescent (fruiting plants)	2.4	2.0	—

* nmol of substrate consumed/50 μl of enzyme/30 min.
† Tryptophan pyrrolooxygenase activity was not measured.

oxygenase exists in multiple MW forms [2, 3, 6], a feature also of skatole pyrrolooxygenase [1]. Porphobilinogen oxygenase, either from wheat germ or from rat liver, was found to have allosteric kinetics. In rats it is induced by a number of steroids [3] and its activity is submitted to a regulatory control under different physiological conditions [7, 8].

Pyrrolooxygenases are usually inhibited in the crude extracts due to presence of a proteic inhibitor [2, 4]. The inhibitor is inactivated by storage and can be separated from the enzymes by a DEAE-cellulose purification step.

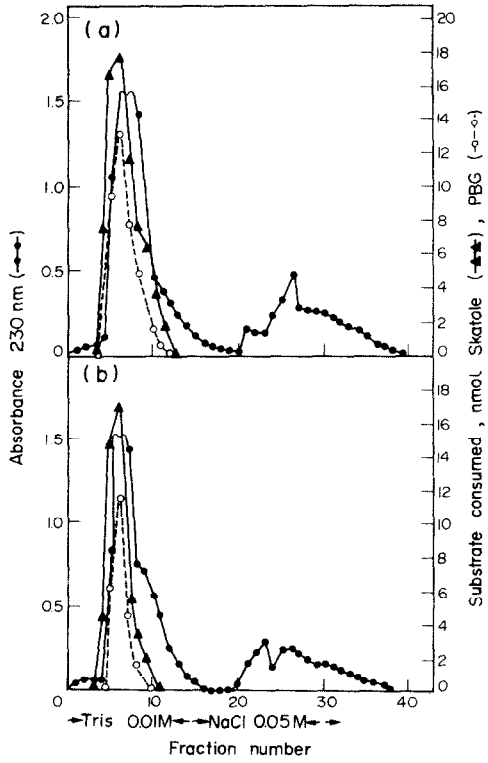


Fig. 1. DEAE-cellulose elution profiles of porphobilinogen oxygenase and skatole pyrrolooxygenase of (a) senescent and (b) young leaves of field-grown *C. annuum* plants. The crude extracts (7 ml) prepared as described in Experimental were applied on DEAE-cellulose column (2 × 18 cm) and eluted stepwise. Fractions of 7 ml were collected. Activities were assayed as described using 100 μl of enzyme.

To examine the properties of these enzymes in plant material, as well as the possible variations of their activity under the different physiological conditions, they were isolated from leaves of pepper (*Capsicum annuum*) and poinsettia (*Euphorbia pulcherrima*). The leaves were collected from plants grown under different conditions, as well as from fruiting and non-fruiting plants, and comprised young and senescent leaves.

RESULTS

Leaf extracts of *C. annuum* and *E. pulcherrima* contained pyrrolooxygenases. Their activity varied according to the age of the leaves, and the origin and physiological condition of the plants. The activity in the crude extracts of young leaves of field-grown plants (*E. pulcherrima* extracts were prepared by addition of nicotine, 0.1%), was higher than in the senescent leaves (Table 1). The

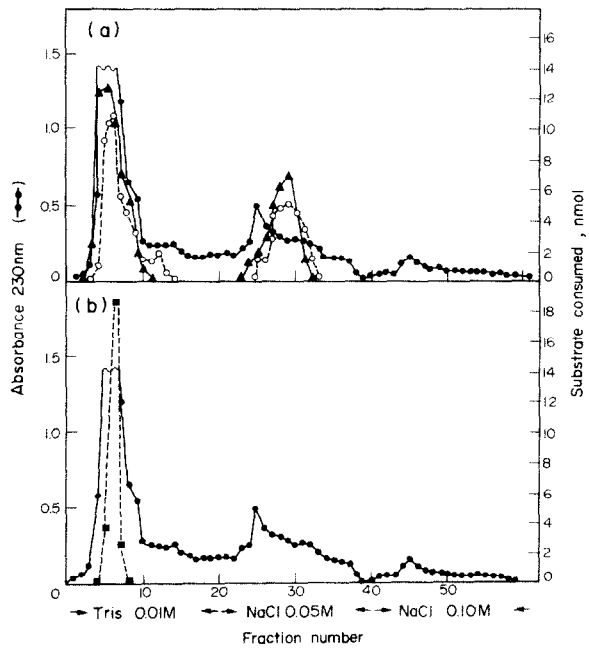


Fig. 2. DEAE-cellulose elution profiles of (a) porphobilinogen oxygenase (○ - - - ○), skatole pyrrolooxygenase (▲——▲) and (b) tryptophan pyrroloxygenase (■ - - - ■) in senescent leaves of field-grown fruiting *C. annuum* plants.

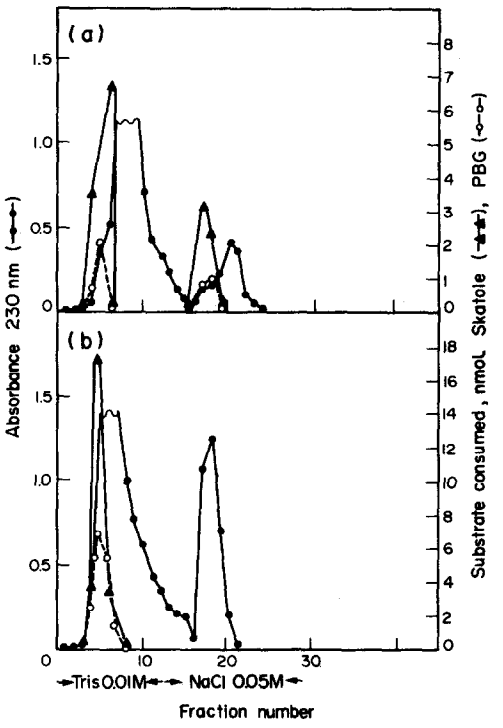


Fig. 3. DEAE-cellulose elution profiles of porphobilinogen oxygenase and skatole pyrrolooxygenase from leaves of *E. pulcherrima*. (a) After flowering; (b) before flowering. *E. pulcherrima* extracts were prepared with addition of nicotine (0.1 % v/v).

same was observed with leaves of greenhouse-grown *C. annuum*, and in these plants fruiting enhanced pyrrolooxygenase activity in the extracts (Table 1). This effect was absent in the field-grown plants. The oxygenases were usually localized in leaf chloroplasts isolated at 5000 *g* from leaf homogenates in 0.3 M sucrose 0.05 M phosphate buffer (pH 7.4). Activity was also found in the supernatant. The crude extracts were purified by DEAE-cellulose. It was known [2, 4] that porphobilinogen oxygenase and skatole pyrrolooxygenase (of either wheat germ or rat liver microsomes) are eluted in one cationic form with multiple MW forms. When the leaf extracts of the field-grown *C. annuum* were purified by DEAE-cellulose chromatography, using a stepwise elution from 0 to 0.2 M NaCl in 0.01 M Tris–HCl buffer (pH 7.4), the active peak was eluted with the buffer (Fig. 1). The elution pattern of the proteins (as measured at 230 nm), and the pattern of pyrrolooxygenase activity of the young and senescent leaves was very similar, although a higher pyrrolooxygenase activity was found in the senescent leaves. When equivalent leaves of fruiting plants were analysed, a new activity peak of porphobilinogen oxygenase and skatole pyrrolooxygenase was eluted with 0.05 M NaCl from the extracts of the senescent leaves (Fig. 2a). It was absent in the extracts of the young leaves of the fruiting plants, which displayed the same elution pattern as those of non-fruiting plants (Fig. 1). Tryptophan pyrrolooxygenase activity of the senescent leaves of the fruiting plants did not show this new cationic form (Fig. 2b). When the extracts of *E. pulcherrima* leaves (young or senescent), were purified by DEAE-cellulose as described above, it was found that the pyrrolooxygenase elution pattern was similar to that found in field-grown pepper when the leaves were collected before flowering (Fig. 3b). When the leaves were collected after flowering, porphobilinogen oxygenase and skatole pyrrolooxygenase showed an additional ionic form (Fig. 3a), while tryptophan pyrrolooxygenase showed only the original cationic form. These results strongly resemble those obtained with senescent fruiting pepper leaves (see above).

The pyrrolooxygenase from poinsettia leaves were less stable than those from pepper. Although their activity can be readily measured in the crude extracts, it was lost after storage (4 or −15°) for a few days. More active preparations were obtained in the presence of nicotine (0.1 % v/v), or PVP (5 % w/v). The enzyme purified on DEAE-cellulose lost its activity after a few days, while the *C. annuum* enzyme was stable (4°) for several weeks.

When the DEAE-cellulose purification was performed on the extracts of the greenhouse-grown *C. annuum* leaves, an entirely different elution pattern of the pyrrolooxygenases was obtained (Fig. 4). This was valid for the young as well as for the senescent leaves of the fruiting

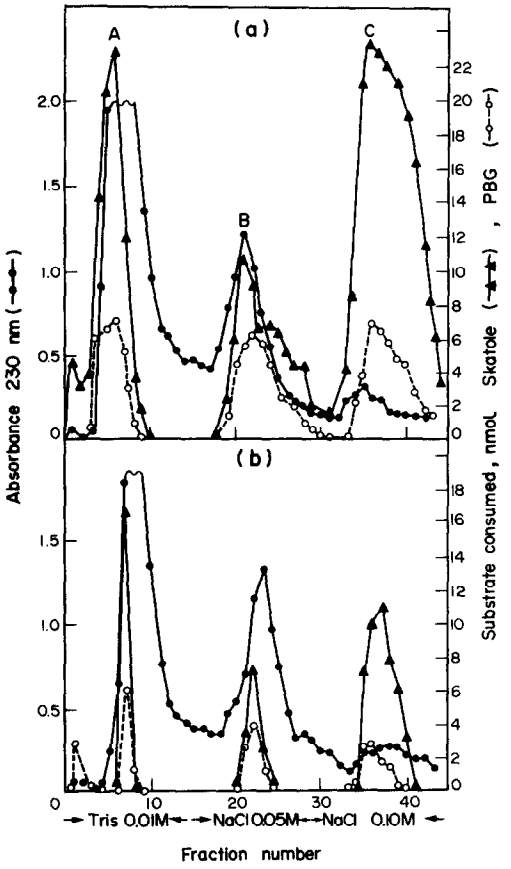


Fig. 4. DEAE-cellulose elution profiles of porphobilinogen oxygenase and skatole pyrrolooxygenase of senescent leaves from greenhouse-grown *C. annuum*. (a) Fruiting; (b) non fruiting plants. The DEAE-cellulose column and elution conditions were as described for Fig. 1. Three different peaks of enzymatic activities were eluted. Peak A was eluted with 10 mM Tris–HCl buffer, peak B with 0.05 M NaCl in the same buffer and peak C with 0.1 M NaCl.

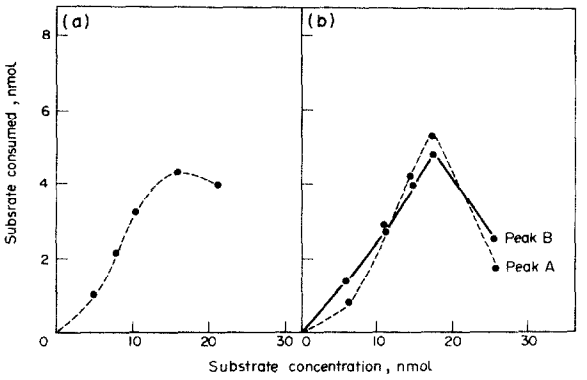


Fig. 5. Effect of substrate concentration on porphobilinogen oxygenase activity. Activity was assayed as described in Experimental by incubating 50 μl of enzyme at 37° for 15 min. (a) DEAE-cellulose purified porphobilinogen oxygenase (Fig. 1a) was used, (b) the most active fractions of peak A and peak B (Fig. 4) were used.

and non-fruiting plants. Pyrrolooxygenase activity was eluted in three peaks; the usual cationic peak (eluted with 10 mM Tris–HCl buffer), a less cationic form (eluted with 0.05 M NaCl) and an anionic form (eluted with 0.1 M NaCl). The intermediate form (Fig. 4, peak B) was similar to the ionic form which appeared in senescent leaves of fruiting plants grown in the field (Fig. 2a). The anionic form (Fig. 4, peak C) was the most abundant for skatole pyrrolooxygenase. The 3 differently charged forms were not interconvertible. When each was rechromatographed on DEAE-cellulose using a 10 mM Tris–HCl buffer–0.5 M NaCl gradient, they were recovered unchanged.

The highest total pyrrolooxygenase activity was found in the extracts of the senescent leaves of fruiting plants (Fig. 4a). The activities recovered after the DEAE-cellulose chromatography were higher than the original activities applied (Table 1), which suggest the removal of an inhibitor. The relative activities of porphobilinogen oxygenase vs skatole pyrrolooxygenase greatly varied in the different active fractions, a further indication that both enzymes are different [1, 9]. The cationic form of the pyrrolooxygenase (Fig. 4, peak A), was concentrated by ultrafiltration and then filtered through Sephadex G-100. Porphobilinogen oxygenase activity was mainly eluted in a fraction with a MW of 100 000 ± 5000; peaks with a MW of 75 000 ± 5000 and with a MW of 50 000 ± 5000 with much lower activities were also eluted. The intermediate form (Fig. 4, peak B) gave 4 fractions of different MW when filtered through Sephadex G-100. Porphobilinogen oxygenase and skatole pyrrolooxygenase activities were evenly distributed among the peaks with a MW of 100 000 ± 5000 and with a MW of 75 000 ± 5000 while the form with a MW of 25 000 ± 5000 had the highest activity. A form with a MW of 13 000 of low activity was also found for porphobilinogen oxygenase, but was absent in skatole pyrrolooxygenase. It was known [1, 6] that both enzymes exist in multiple MW forms, although the relationship of these forms and the differently charged forms is still obscure. In porphobilinogen oxygenase, the multiplicity in the MW forms is closely related to its allosteric kinetics [6, 2], although this is not the case with skatole pyrrolooxygenase. The cationic form of porphobilinogen oxygenase from leaves of *C. annuum* (from either origin), showed allosteric kinetics and was inhibited by high substrate concentrations (Fig. 5a).

When the two charged forms of the same enzymatic extract (Fig. 4, peaks A and B) were assayed with increasing substrate concentrations, it was found that peak A had a sigmoidal kinetics while peak B showed Michaelis kinetics (Fig. 5b). Both were inhibited by high substrate concentrations. These results agree with the data obtained with the wheat germ enzyme [6], where the allosteric kinetics were only obtained with the high MW form and not with the stabilized (by succinylation) form with a MW of 25 000.

The pyrrolooxygenases from *E. pulcherrima* were too unstable for detailed kinetic studies. The activity of porphobilinogen oxygenase from *C. annuum* increased with time and enzyme concentration when a DEAE-cellulose purified enzyme was used (Fig. 6). The enzyme was inhibited by α,α′-dipyridyl (5 mM inhibited 80% of the activity), and EDTA (0.5 mM) exerted the same

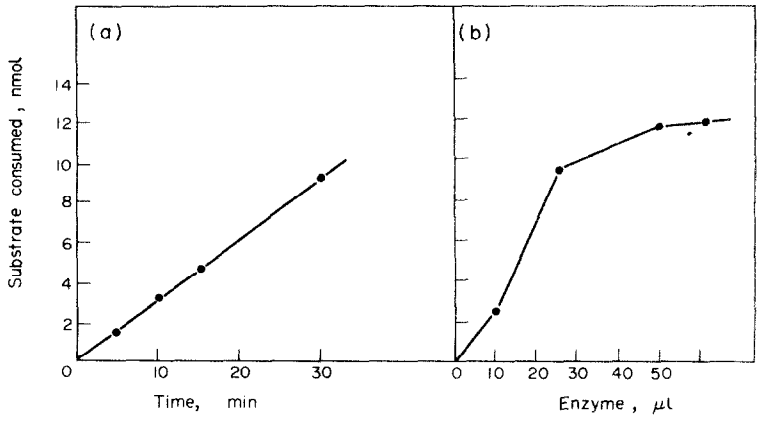


Fig. 6. Effect of (a) time and (b) enzyme concentration on porphobilinogen oxygenase activity. DEAE-cellulose purified enzyme (eluted with 10 mM Tris–HCl buffer) was used. Incubations were performed as described in Experimental. The time curve was obtained with 25 μl of the enzyme. The effect of enzyme concentration was measured by incubating during 30 min.

inhibition. Cd^{2+}, Fe^{3+} and Zn^{2+} (1 mM), inhibited 50% of the enzymatic activity. In these properties the enzyme resembles the wheat germ oxygenase [2].

Tryptophan pyrrolooxygenase activity of *C. annuum* leaves increased linearly with up to 180 nmol tryptophan in the standard assay. Using chymotrypsin as substrate [5], it was found that the enzyme oxidized 20–30% of the substrate, as measured by the decrease in *A* at 280 nm for tryptophan.

DISCUSSION

Pyrrolooxygenases are more active in the crude extracts of young leaves of pepper and poinsettia than in senescent leaves and this is due to the presence in the latter of an inhibitor which can be removed by DEAE-cellulose exchange. This is not unlike the case found with wheat germ extracts and with rat liver microsomes [1]. Fruiting increased the activity of pyrrolooxygenases and added a new ionic form of greater negative charge to the usual cationic form of porphobilinogen oxygenase and skatole pyrrolooxygenase in senescent leaves (Figs. 2a and 3a). Pyrrolooxygenases of *C. annuum* leaves from greenhouse-grown plants showed three forms of different ionic charge and fruiting again increased the peak of lesser cationic character (Fig. 4a).

The usual cationic form of porphobilinogen oxygenase in wheat germ and in *C. annuum* leaves which is eluted with 10 mM Tris–HCl buffer, has a predominant high MW form (100 000) and exhibits allosteric kinetics. In the wheat germ enzyme, the forms with lower MW (25 000) readily associate to give the higher MW polymer and can only be stabilized by succinylation [6]. The succinylated enzyme shows Michaelis kinetics. The weaker cationic form found in the leaves of *C. annuum* (Fig. 4, peak B), has however a predominant MW of 25 000 and exhibits Michaelis kinetics. Hence, it is very likely that this form does not associate in solution to give the higher MW polymer.

EXPERIMENTAL

Materials. Porphobilinogen was obtained by synthesis [10] and skatole was recrystallized from EtOH. L-Tryptophan, sodium dithionite and all other chemical reagents used were commercial products of analytical grade. α-Chymotrypsin was obtained from Sigma, DEAE-cellulose from Eastman Kodak and used after a treatment according to the method of ref. [11].

Plant material. Leaves from *C. annuum* growth either in a greenhouse or field were collected. Leaves from *Euphorbia pulcherrima* Willd. grown in a garden before and after flowering, were harvested in the morning and used within 30 min. When *C. annuum* leaves were used, the first and second leaves starting from the apex were called young leaves and the first 3 leaves from the bottom upward were called senescent leaves. Equivalent leaves from several plants were used. None of the leaves were yellowing. Equivalent leaves from fruiting plants were also used. When *E. pulcherrima* leaves were used, the first and the second leaves from the top downward (small size and light green), were collected and called young leaves. The 4th and 5th leaves from the top downward (large size and dark green) were collected and called mature leaves. Equivalent leaves from several branches of the same tree were used.

Enzyme preparation. The deribbed leaves were cut into small pieces and thoroughly ground with a pestle in an ice-cold mortar until no fibrous residue was left. The grinding medium (3 ml/g fr. wt) consisted of a 10 mM Tris–HCl buffer soln (pH 7.4), and sand in the case of *C. annuum*. When *E. pulcherrima* leaves were used the enzyme was prepared with the above-mentioned grinding medium supplemented with either nicotine (0.1% v/v) or PVP (5% w/v). The homogenate was filtered through several layers of nylon cloth and centrifuged at 20 000 *g* for 15 min. All operations were performed at 4°. The supernatant was used as enzyme source either for direct activity measurements (crude extracts) or for further enzyme purifications. Protein was estimated by the method of ref. [12].

Enzyme assays. Assay of skatole pyrrolooxygenase and porphobilinogen oxygenase. The standard reaction mixture contained, in a final vol. of 100 μl, 10 μmol of Pi buffer (pH 7.4), 0.1 μmol of Na dithionite, porphobilinogen (13 nmol) or skatole (28–32 nmol), and 50 μl of enzyme (150–250 μg of protein in the crude extracts, 2–20 μg in the purified prepns). Incubations were usually run for 30 min at 37°. Two blanks, omitting either dithionite or enzyme, were run simultaneously. Enzymatic activity was assayed by measuring substrate consumption with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in HOAc–60% $HClO_4$ (21:4)), at 552 nm in the case of porphobilinogen and at 540 nm in the case of skatole, after previous addition of Hg^{2+}.

Assay of tryptophan pyrrolooxygenase. The incubation mixture contained, in a final vol. of 100 μl, 10 nmol of Pi buffer (pH 7.4), 0.1 μmol of Na dithionite and L-tryptophan (100 nmol) or α-chymotrypsin (50–100 μg). Incubations were carried out at 37° for 30 min. Two blanks were run simultaneously, omitting dithionite or enzyme (which is added after incubation). Incubations were stopped by diluting the reaction mixture with 1 ml of H_2O. Tryptophan consumption was estimated by *A* at 280 nm using 5500 as the molar extinction coefficient.

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