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PYRROLOOXYGENASES FROM ARGENTINE WHEAT VARIETIES

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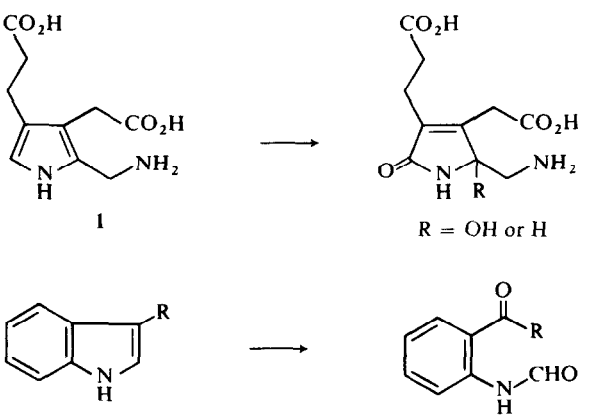
Key Word Index—*Triticum aestivum*; Gramineae; wheat; porphobilinogen oxygenase; skatole pyrrolooxygenase; tryptophan pyrrolooxygenase; wheat embryo; wheat endosperm; Argentine varieties.

Abstract—Pyrrolooxygenase activities were examined in different varieties of Argentine wheat (*Triticum aestivum*) which included the traditional Klein varieties and the new mixed Mexican and traditional varieties (DeKalb and Cargill). The enzymatic activities were variety-dependent and were more inhibited in some varieties than in others, while some (Cargill) were devoid of the proteic inhibitor. The enzymes were isolated from the flours as two isoenzymes of different charge whose relative proportions were dependent on the variety of wheat used. The more cationic isoenzymes were eluted with 10 mM Tris–HCl buffer (pH 7.6) from DEAE-cellulose and the less cationic were eluted with 50 mM NaCl in the same buffer. The protein inhibitor, when present, was associated with the more cationic isoenzymes. Porphobilinogen oxygenase and skatole pyrrolooxygenase activities were higher in the endosperm, while tryptophan pyrrolooxygenase activity was higher in the embryo. The proteic inhibitors were mainly concentrated in the embryo.

INTRODUCTION

Pyrrolooxygenases are mixed-function oxidases which oxidize pyrroles as well as indoles [1–4]. Three enzymes have been identified within the group; porphobilinogen oxygenase which oxidized porphobilinogen (PBG) **1**, skatole pyrrolooxygenase which oxidized skatole **2**, and tryptophan pyrrolooxygenase which oxidizes free and bound forms of tryptophan **3** (Scheme 1). They have been



Scheme 1.

isolated from wheat germ [2, 3], rat liver [5], *Poinsettia* and pepper leaves [6] and from human and avian erythrocytes [7]. The pyrrolooxygenases isolated from wheat germ are cationic proteins which are eluted from DEAE-cellulose columns with low ionic strength. When porphobilinogen oxygenase from wheat germ was purified, 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 [8]. The crude and partially purified wheat germ extracts also contain a proteic inhibitor of the oxidases [1–3]. The activity of tryptophan pyrrolooxygenase is particularly high in the extracts of wheat germ obtained from wheat harvested prior to 1973. In extracts prepared in the following years this activity decreased, a feature which could be attributed to the introduction in Argentina of new varieties of wheat. Therefore, pyrrolooxygenase activities were examined in different varieties of Argentine wheat, which included the traditional Klein varieties and the new mixed Mexican and traditional varieties.

RESULTS

Pyrrolooxygenase activities in different varieties of Argentine wheat (Triticum aestivum)

Wheat samples were obtained from three different breeders: DeKalb, Cargill and Klein. DeKalb and Cargill wheats are mixed Mexican and traditional varieties, while Klein wheats are traditional varieties with high gluten content. The wheat kernels (1976 harvest) were milled, the whole wheat flours were extracted with water (1 hr), the extracts were filtered, the filtrates were centrifuged (20000 *g* for 15 min) and the supernatant was fractionated by the addition of solid $(NH_4)_2SO_4$. Precipitations were performed between 0–30, 30–60, and 60–80% saturation. Most of the pyrrolooxygenase activities were found in the 30–60% fraction. The 30–60% fraction was therefore used, and porphobilinogen oxygenase, as well as skatole and tryptophan pyrrolooxygenase activities, were measured in the different wheat varieties (Table 1). It was known [1–3] that a protein inhibitor of the enzymes was present in the extracts of wheat germ; this could be readily detected by its inhibitory effect when larger amounts of enzyme were used during the incubations. Hence, all the incubations

Table 1. Pyrrolooxygenase activities in the whole flour of different varieties of *Triticum aestivum*

Breeder	Variety	Enzyme (μl)	Pyrrolooxygenase activity* (nmol/30 min) Porphobilinogen	Skatole	Tryptophan
DeKalb	Tala	25	8.3	15.5	11.0
		50	7.3	13.2	14.7
	E-2305	25	8.5	11.7	14.5
		50	5.7	10.0	12.0
Cargill	Trigal 700	25	9.6	16.8	7.3
		50	11.0	18.0	12.0
	Trigal 705	25	6.6	11.0	4.0
		50	7.8	13.0	8.6
Klein	Toledo	25	6.5	15.4	14.0
		50	5.2	12.0	17.0
	Atlas	25	9.1	17.7	15.0
		50	6.5	15.8	13.0
	Impacto	25	8.7	15.5	10.4
		50	7.2	15.5	13.0
	Sendero	25	7.9	16.5	10.0
		50	6.4	17.5	18.0
	Fortin	25	8.5	17.3	12.0
		50	6.8	15.0	12.4
	Granador	25	8.7	16.5	12.0
		50	7.7	13.4	12.4
	Rendidor	25	9.6	18.5	16.6
		50	7.8	15.7	14.0

* Each value is the mean of six different preparations obtained from the same wheat sample, each milled before the enzyme extraction. The incubation and reaction conditions were as described in Experimental.

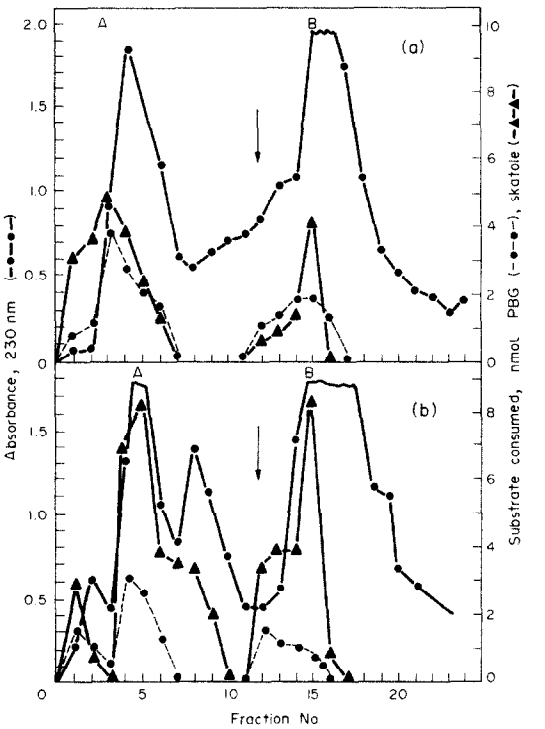


Fig. 1. DEAE-cellulose elution profiles of porphobilinogen oxygenase and skatole pyrrolooxygenase of DeKalb wheat; (a) Tala variety, (b) E-2305 variety. Two 30–60% $(NH_4)_2SO_4$ preparations obtained as described in Experimental were applied on a DEAE-cellulose column (3 × 25 cm) and eluted stepwise with 10 mM Tris-HCl buffer (pH 7.6) (peak A), and 50 mM NaCl in the same buffer (peak B). Changes in the ionic strength were made where indicated by an arrow. Fractions of 40 ml were collected. Activities were assayed as described using 50 μl of enzyme.

were carried out with two different enzyme concentrations. In the preparations that contained the inhibitor, less activity was found when the enzyme concentration was doubled (Table 1).

Pyrrolooxygenase activities were variety-dependent (Table 1). The inhibitor of porphobilinogen oxygenase activity was present in the extracts of 9 out of the 11 varieties examined, that of skatole pyrrolooxygenase was

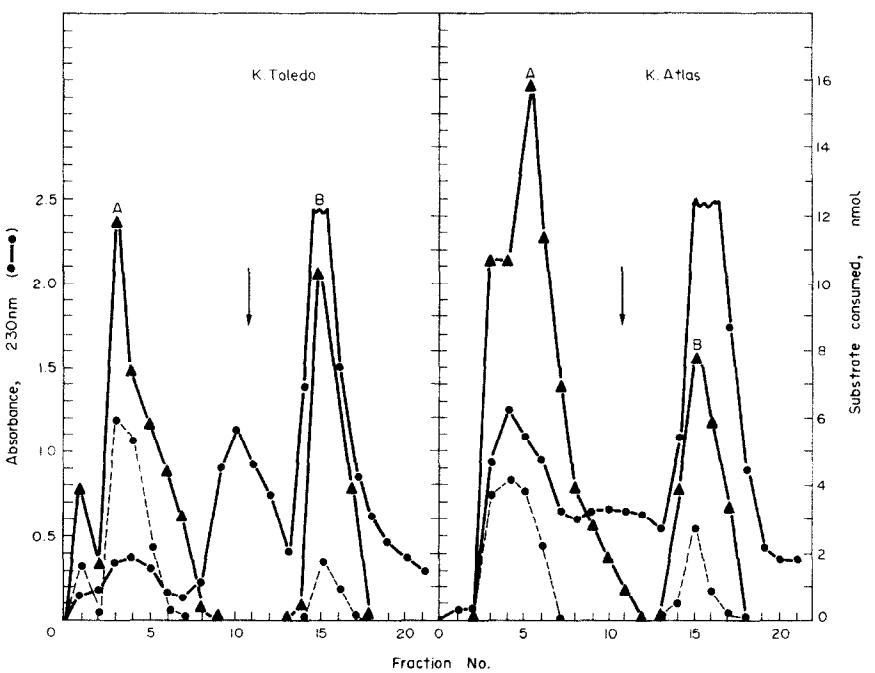


Fig. 2. DEAE-cellulose elution profile of porphobilinogen oxygenase and skatole pyrrolooxygenase of Klein Toledo and Klein Atlas wheats. Two 30–60% $(NH_4)_2SO_4$ fractions were pooled and applied to the column. Conditions were as described for Fig. 1. PBG (—●—●—), skatole (—▲—▲—).

found in 7 out of the 11 varieties and that of tryptophan pyrrolooxygenase was found in only 3 of them. Differences in the inhibitory effect on the 3 enzymes were found within the same varieties (Klein Impacto and Klein Sendero, Table 1), and the percentage of inhibition was different within each variety (e.g. DeKalb E-2305, Table 1). The Cargill varieties were devoid of the inhibitor and their tryptophan pyrrolooxygenase activity was the lowest. The inhibitor, when present, was detected only in the 30–60% $(NH_4)_2SO_4$ fractions.

Isoenzymes of porphobilinogen oxygenase and skatole pyrrolooxygenase

Pyrrolooxygenases have been isolated from wheat germ as proteins of strong cationic character [2, 3, 8]. In green leaves, however, they were found in two or three ionic forms; the usual strong cationic form, a less cationic form and an anionic form [6]. The pyrrolooxygenases isolated from the different varieties of wheat flour described above exhibited two ionic forms. Great variations were found in the relative proportions of both isoenzyme forms of porphobilinogen oxygenase and skatole pyrrolooxygenase. The proportions were mainly dependent on the variety of the wheat used. These results were obtained by performing DEAE-cellulose purifications of 30–60% $(NH_4)_2SO_4$ fractions. The more cationic isoenzyme (peak A) was eluted with 10 mM Tris buffer (pH 7.6), while the less cationic isoenzyme (peak B) was eluted with 50 mM NaCl in the same buffer (Figs. 1–4). In porphobilinogen oxygenase isolated from the DeKalb Tala variety (Fig. 1a), Klein Impacto, and Klein Sendero varieties, both peaks were found to have similar activities. In the DeKalb E-2305 variety (Fig. 1b), the Klein Toledo, and the Klein Atlas varieties (Fig. 2), as well as in the Cargill Trigal varieties, peak A was more active than peak B. Peak B was more active than peak A in the enzymes isolated from the Klein Granador, Klein Rendidor and Klein Fortin varieties (Figs. 3 and 4).

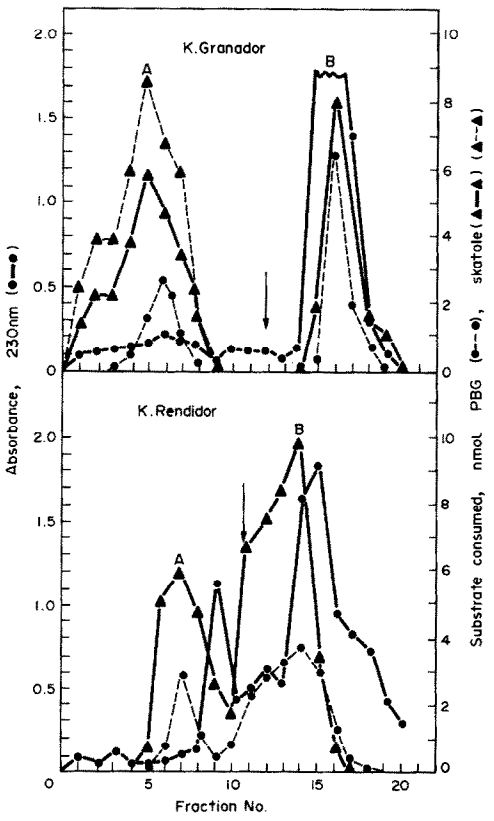


Fig. 3. DEAE-cellulose elution profile of porphobilinogen oxygenase and skatole pyrrolooxygenase of Klein Rendidor and Klein Granador wheats. (▲——▲) Skatole pyrrolooxygenase activities assayed after 48 hr of storage. No change in the porphobilinogen oxygenase was observed under the same conditions.

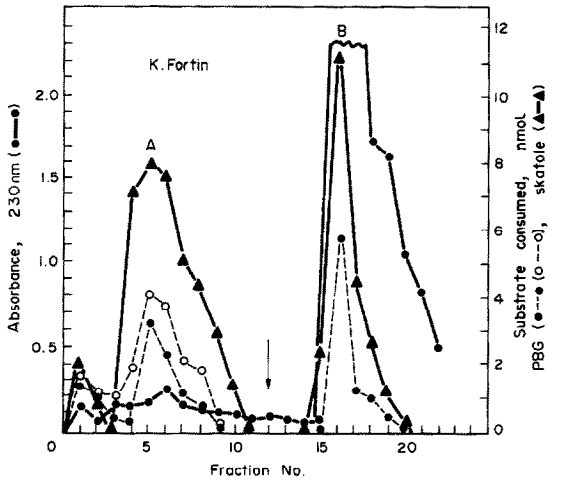


Fig. 4. DEAE-cellulose elution profile of porphobilinogen oxygenase and skatole pyrrolooxygenase of Klein Fortin wheat. (○——○) Porphobilinogen activities assayed after 48 hr of storage. No change in the skatole pyrrolooxygenase was observed under the same conditions.

Skatole pyrrolooxygenase also showed differences in the relative activities of both isoenzyme peaks in the different wheat varieties. The isoenzyme pattern of skatole pyrrolooxygenase was however different from that of porphobilinogen oxygenase within the same variety (Figs. 1–4).

The protein inhibitor, when present, is always associated with the more cationic isoenzyme (peak A). In Fig. 4, an increase in the activity of porphobilinogen oxygenase in peak A fractions is shown after 48 hr storage at 4° (inactivation of the inhibitor). A similar effect for skatole pyrrolooxygenase is depicted for the Klein Granador variety (Fig. 3). In the Cargill varieties the inhibitor was always absent.

Localization of enzymes

The high pyrrolooxygenase activities found in the wheat flours indicated that they were also localized in the endosperm and not only in the embryo. To separate the embryos from the endosperm, the wheat kernels (Klein Toledo) were soaked with water under aeration for 16 hr, when they began to germinate. The embryos were mechanically separated from the rest of the kernel and the enzymes were prepared from both as described in Experimental. Pyrrolooxygenase activities were found both in the endosperm and in the embryo (Table 2). Porphobilinogen oxygenase and skatole pyrrolooxygenase activities were higher in the former, while tryptophan pyrrolooxygenase was higher in the latter at low

Table 2. Localization of pyrrolooxygenases in wheat grain

Enzyme source	Enzyme (μl)	Substrate consumed (nmol/30 min) Porphobilinogen	Skatole	Tryptophan
Endosperm	10	3.2	15.1	6.9
	20	7.2	19.7	8.2
Embryo	10	2.6	8.5	15.0
	20	1.4	4.1	—

The incubation and reaction conditions were as described in Experimental.

Table 3. Inhibition of endosperm pyrrolooxygenases by embryo extracts

Inhibitor added (μl)	Substrate consumed (nmol/30 min) Porphobilinogen	Inhibition (%)	Skatole	Inhibition (%)
—	6.5	—	12.5	—
10	3.2	50	7.6	40
20	0.65	90	4	68

Incubations were carried out as described in Experimental. Fraction 30–80% $(NH_4)_2SO_4$ (10 μl) from endosperm was used as enzyme source. Embryo extract was used as inhibitor.

enzyme concentrations. The inhibitor was mainly localized in the embryo and only at high enzyme concentrations was it detected in the endosperm. When the embryo extracts were added to the enzyme of the endosperm a strong inhibitory effect was obtained (Table 3). Hence most of the enzymatic activities measured in the flours (Table 1) are of endosperm origin.

Properties of pyrrolooxygenase of wheat flour

The effect of enzyme concentration on the activities of the three pyrrolooxygenases can be seen in Fig. 5. The 30–60% $(NH_4)_2SO_4$ fraction of the Klein Toledo variety was used. While porphobilinogen oxygenase and skatole pyrrolooxygenase were inhibited at higher enzyme concentrations due to the presence of the inhibitor, tryptophan pyrrolooxygenase activity was not inhibited.

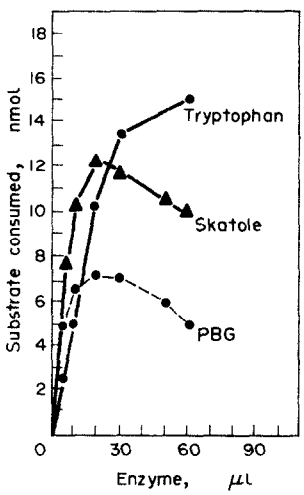


Fig. 5. Effect of enzyme concentration on the activities of pyrrolooxygenases.

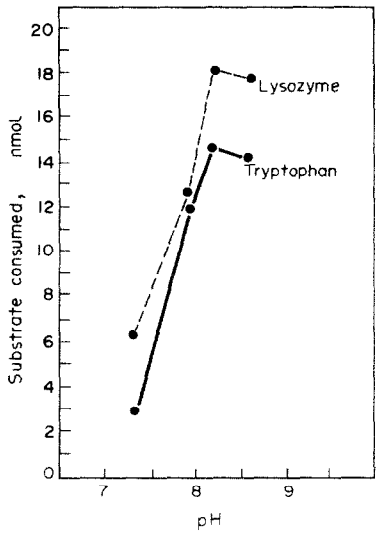


Fig. 6. Effect of pH on tryptophan pyrrolooxygenase activity. The 30–60% $(NH_4)_2SO_4$ fraction of DeKalb Tala wheat was used (50 μl; 200 μg protein). Tris–HCl buffer was used. Incubations were run as described in Experimental.

The activity of the enzymes increased linearly with time only up to 30 min. Tryptophan and skatole pyrrolooxygenases of all the wheat flours tested showed hyperbolic kinetics, while porphobilinogen oxygenase showed sigmoidal kinetics. These results were obtained by using the 30–60% $(NH_4)_2SO_4$ fractions and are similar to those obtained with the wheat germ enzymes [2, 3].

The effect of pH on the activity of tryptophan pyrrolooxygenase isolated from DeKalb Tala wheat using tryptophan and lysozyme as substrates [4] is shown in Fig. 6.

DISCUSSION

Pyrrolooxygenases were isolated from flours of different wheat varieties. Different varieties from the same breeder were used to ensure similar conditions of soil and climate. Since differences were found in the activities of the enzymes, the amount of inhibitor present and the isoenzyme composition of the pyrrolooxygenases among the varieties from the same breeder, the differences may be attributed to genetic factors.

The endosperm contained large amounts of the enzyme with relatively small amounts of inhibitors. The latter were mainly contained in the embryo (Table 2), which was already known to contain pyrrolooxygenases [2, 3]. The enzymes of the embryo are very possibly involved in porphyrin and amino acid metabolism during germination, and the presence of the inhibitors might keep them under a more strict regulatory control.

EXPERIMENTAL

Materials. Porphobilinogen was obtained by synthesis [9]. Skatole was recrystallized from EtOH. L-Tryptophan, Na dithionite and all other chemical reagents used were of analytical grade. DEAE-cellulose was obtained from Eastman Kodak and used after a treatment according to the method of ref. [10].

Enzyme preparation. Wheat grains (*T. aestivum*) of the 1976

harvest were used. The flour (100 g) was extracted with 400 ml H_2O for 1 hr with continuous mechanical stirring. The mixture was filtered through several layers of nylon cloth and centrifuged at 20000 *g* for 15 min. The supernatant was precipitated with solid $(NH_4)_2SO_4$ and the fractions between 0–30, 30–60 and 60–80% were obtained. The 30–60% fraction was used as enzyme source either for direct activity measurements, or for further enzyme purifications. The fraction was dissolved in 20 ml Tris–HCl buffer (pH 7.6) and dialysed overnight against the same buffer. The protein content [11] of this fraction was 5–6 mg/ml. In order to separate the embryo from the endosperm, the grains (10 g) were inbibed in H_2O for 16 hr and an air current was passed through the H_2O. The embryos were then separated and ground in a chilled mortar with 2 ml Tris–HCl buffer (10 mM, pH 7.6), the extract was centrifuged (20000 *g* for 20 min) and the supernatant was used as enzyme source. Protein content was 32 mg/ml. The endosperm was ground in a chilled mortar with 40 ml of the same buffer, then centrifuged as described and the supernatant was fractionated by addition of solid $(NH_4)_2SO_4$ between 0–30 and 30–80%. The latter fraction was dissolved in 4 ml Tris–HCl buffer (pH 7.6), dialysed overnight and used as enzyme source. Protein content was 35 mg/ml.

Assay of porphobilinogen oxygenase and skatole pyrrolooxygenase. The reaction mixture contained in a final vol. of 100 µl: 10 µmol of NaPi buffer (pH 7.4), 0.1 µmol of Na dithionite, porphobilinogen (15 nmol) or skatole (28–32 nmol) and enzyme (25 or 50 µl, 150–300 µg of protein in the $(NH_4)_2SO_4$ fraction and 20 µg of protein in the purified prepns). Incubations were run for 30 min at 37°. Blanks, omitting either enzyme or dithionite, were run simultaneously. Enzymatic activity was assayed by measuring substrate consumption with Ehrlich's reagent [2, 3].

Assay of tryptophan pyrrolooxygenase. The incubation mixture contained in a final vol. of 100 µl: NaPi buffer (pH 7.4), Na dithionite and enzyme (at the concns indicated above). L-Tryptophan (70 nmol), or lysozyme (50–100 µg), was used as substrate. Incubations were carried out at 37°/30 min. Two blanks were run simultaneously omitting either dithionite or enzyme (which was added after the incubation). The reaction was stopped by diluting the reaction mixture with 1 ml H_2O. Tryptophan consumption was estimated spectrophotometrically at 280 nm by using an $\varepsilon = 5500$.

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