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PYRROLOOXYGENASE ISOENZYMES FROM WHEAT GERM

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; porphobilinogen oxygenase; skatole pyrrolooxygenase; isoenzymes; wheat germ.

Abstract—Both porphobilinogen oxygenase and skatole pyrrolooxygenase of wheat germ have isoenzyme forms of different charge. The more cationic isoenzymes were eluted from DEAE-cellulose with 10 mM Tris–HCl buffer (pH 7.6) and the less cationic were eluted with 50 mM NaCl in the same buffer. The former had almost twice as many free amino groups (per mg of protein) as the latter. The more cationic isoenzyme was more sensitive to chelating agents and to acid treatment. They were differently inhibited by sodium dodecyl treatment and by temperature inactivation. Porphobilinogen oxygenase isoenzymes showed different activities with different buffers and also differed in their kinetics.

INTRODUCTION

Porphobilinogen oxygenase and skatole pyrrolooxygenase have been isolated from wheat germ [1,2], from pepper and *Poinsettia* leaves [3] and from wheat grain endosperm [4]. The former oxidizes porphobilinogen (PBG) to oxoporphobilinogens, while the latter oxidizes skatole and 3-indoleacetic acid to 2-aminoacetophenone. Both enzymes are inhibited in the crude extracts by proteic inhibitors which are inactivated by storage, or can be separated by DEAE-cellulose filtration [1, 2]. The oxygenases were eluted from the DEAE-cellulose as cationic proteins, which were purified and shown to be iron–sulfur proteins having multiple MW forms [5]. A systematic analysis carried out with wheat flours of different Argentine wheat varieties showed that the oxygenases of the endosperm have isoenzyme forms of different charge [4]. These results, as well as the finding that in green leaves they also have isoenzymes [3], led us to look for possible isoenzyme forms of both oxygenases also in wheat germ.

RESULTS AND DISCUSSION

Porphobilinogen oxygenase and skatole pyrrolooxygenase isoenzymes in wheat germ

The wheat germ used in these studies was obtained from wheat mixtures of the traditional and the new Mexican varieties. They were all harvested after 1973. The enzymes were obtained as described in Experimental and the fraction 30–50% $(NH_4)_2SO_4$ was used for the DEAE-cellulose purification step. The elution patterns of the oxygenases from the DEAE-cellulose column (Fig. 1) showed the presence of two activity peaks of different ionic charge in both enzymes. The relative proportion of the peaks varied with the year of the harvest and probably reflects the continuous shift among the wheat varieties used. The same elution patterns were obtained when the stepwise elution from the DEAE-cellulose was performed in the presence of 4 M urea.

To exclude the possibility that peaks A and B were artifacts of the stepwise elution, they were rechromatographed separately and as a mixture on DEAE-cellulose columns using a gradient of NaCl 0–0.1 M in 10 mM Tris–HCl buffer (pH 7.6). Both were again eluted as two different peaks (Fig. 2). To ascertain that both peaks are isoenzymes of different charge, they were analysed by polyacrylamide gel electrophoresis at pH 7. While peak A of porphobilinogen oxygenase had a M_D of 0.05 (indi-

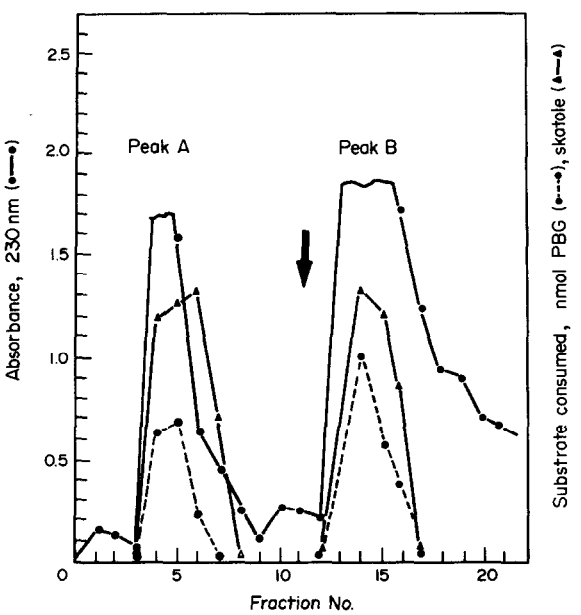

Fig. 1. DEAE-cellulose elution profiles of porphobilinogen oxygenase and skatole pyrrolooxygenase isoenzymes from wheat germ. The 30–50% $(NH_4)_2SO_4$ fraction was used (see Experimental) and eluted stepwise with 10 mM Tris–HCl buffer (pH 7.6) (peak A), and 50 mM NaCl in the same buffer (peak B). Fractions of 40 ml were collected and activity was assayed using 25 μl of each fraction.

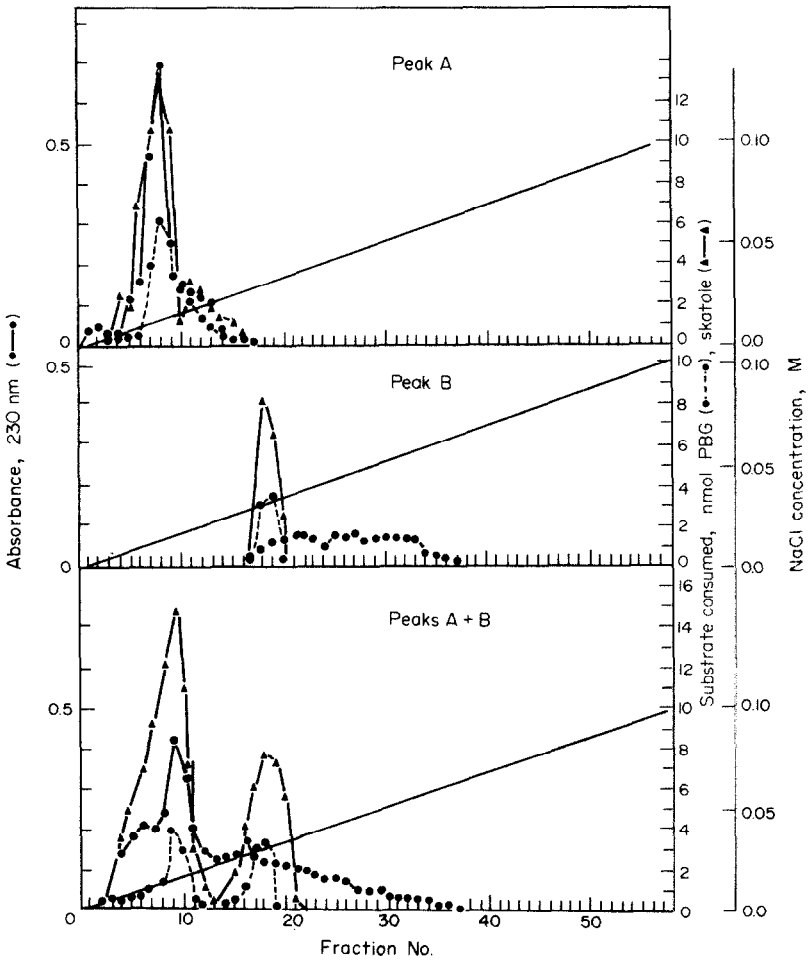

Fig. 2. Elution pattern of peaks A, B and A + B on a second DEAE-cellulose column (2 × 18 cm). 5 ml of each isoenzyme separated on the first column (Fig. 1) were applied on the column and eluted with a gradient of NaCl (0–0.1 M) in 10 mM Tris–HCl buffer (pH 7.6). Fractions of 10 ml were collected and the activity assayed with 50 μl of enzyme.

cating a cationic character), peak B had a M_D of 0.42 which showed its anionic character. When the free amino groups of peak A were measured, it was found to have almost twice as many free amino groups (per mg of protein) as peak B (Table 1). The content of free amino groups agrees with the observed electrophoretic mobility of both peaks. When both isoenzymes were succinylated with succinic anhydride at pH 8, isoenzyme B was entirely succinylated while the succinylation of isoenzyme A was incomplete (Table 1). The succinylated isoenzymes were eluted from DEAE-cellulose as anionic proteins with 0.45 M NaCl in a gradient 0–0.5 M NaCl in Tris–HCl buffer (pH 7.6).

Table 1. Activity and free amino groups of the native and succinylated porphobilinogen oxygenase isoenzymes

Isoenzyme	Free amino groups (μmol/mg protein)	Activity (nmol/30 min)
A Native	1.2	4.7
Succinylated	0.22	4.8
B Native	0.63	4.0
Succinylated	None	3.8

The DEAE-cellulose enzymes were used. The activity was assayed using 25 μl of enzyme (20 μg of protein).

Properties of the isoenzymes of porphobilinogen oxygenase and skatole pyrrolooxygenase

A number of properties indicate that the two isoenzymes are different proteins. They are differentially inhibited by EDTA (Fig. 3) and α,α′-dipyridyl; two chelating agents which are known to inhibit pyrrolooxygenases [2, 3]. The inhibitory effect of α,α′-dipyridyl is similar to that obtained with EDTA, but lower concentrations of the former are needed. Isoenzyme A was more sensitive to the chelating agents, probably due to a greater exposure of the iron–sulfur chromophore. Preincubation of isoenzymes A and B of both oxygenases at pH 4, followed by incubation at pH 7.4, showed that the isoenzyme A was more labile to acid treatment. Porphobilinogen oxygenase isoenzyme A was 50% inhibited, while isoenzyme B was inhibited only 30%. The corresponding values for skatole pyrrolooxygenase were 70 and 40%. These data again lend support to the suggestion that the iron–sulfur chromophore is more exposed in the isoenzyme A.

Isoenzymes A and B of both oxygenases are differently inhibited by sodium dodecyl sulfate (Fig. 4). The differences were larger for the porphobilinogen oxygenase isoenzymes. The isoenzymes were different in their time course of temperature inactivation at 60°. The relative

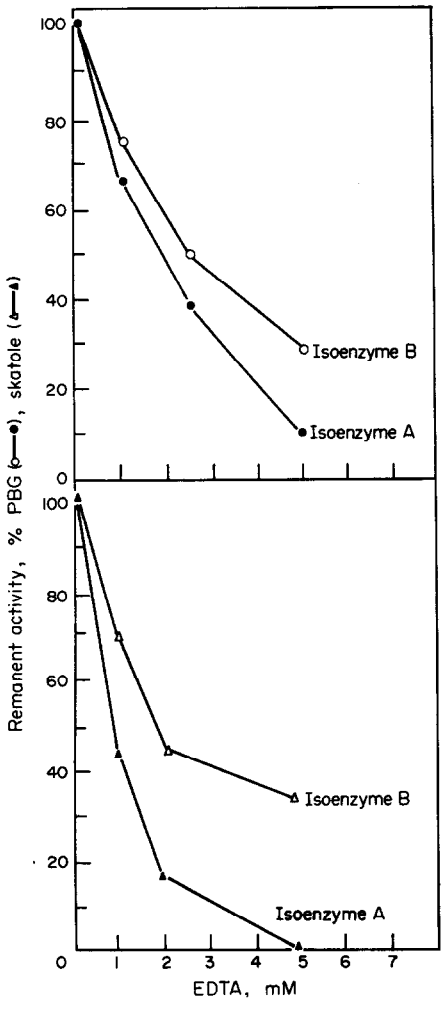

Fig. 3. Effect of EDTA on the activities of porphobilinogen oxygenase and skatole pyrrolooxygenase isoenzymes. DEAE-cellulose purified enzymes were used (25 µl, 20 µg, of protein).

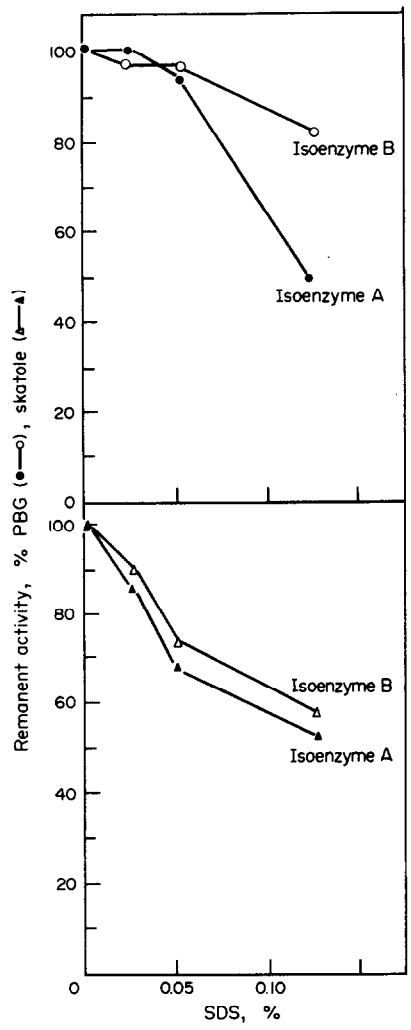

Fig. 4. Effect of sodium dodecyl sulfate on the activities of porphobilinogen oxygenase and skatole pyrrolooxygenase isoenzymes.

stability of both isoenzymes was different for the two enzymes (Fig. 5).

The isoenzymes of porphobilinogen oxygenase, although they had the same optimum pH, showed differences in their activities with the different buffers used (Fig. 6). The largest differences were found in barbital and Tris–maleate buffers. They also differed in their kinetics. Isoenzyme A showed the typical sigmoidal kinetics already described for porphobilinogen oxygenases of various sources [1, 3, 5], that was lost after succinylation. Isoenzyme B had Michaelis kinetics with a K_m = 0.5 mM and a V_{max} = 2.5 nmol/min. Both isoenzymes of skatole pyrrolooxygenase showed Michaelis kinetics; isoenzyme A had a K_m = 0.5 mM and a V_{max} = 2.5 nmol/min, and isoenzyme B had a K_m = 0.8 mM and a V_{max} = 5 nmol/min. The kinetic data were always determined with the isoenzymes eluted from the same column.

It can be concluded that wheat germ porphobilinogen oxygenase and skatole pyrrolooxygenase exist as two isoenzymes. The isoenzymes are similar to those found in the wheat grain endosperm [4].

EXPERIMENTAL

Materials. Porphobilinogen was obtained by synthesis [6]. Skatole was recrystallized from EtOH. All other chemical reagents used were of analytical grade. DEAE-cellulose was obtained from Eastman Kodak and used after treatment according to the method of ref. [7]. Wheat germ was a gift of Molinos Rio de La Plata.

Enzyme preparation. Wheat germ (100 g) was extracted with 400 ml of H_2O, filtered through nylon cloth and centrifuged for 15 min at 20000 *g*. The supernatant was adjusted to pH 5 with N HOAc and kept for 1 hr at 0–4°. The ppt. was removed by centrifugation at 20000 *g* and the supernatant was fractionated by addition of $(NH_4)_2SO_4$. The fraction precipitating between 30–50% was dissolved in *ca* 20 ml of 10 mM Tris–HCl buffer (pH 7.6) and dialysed overnight against 4 l. of the same buffer. The dialysed enzyme was centrifuged to remove any ppt. which appeared during the dialysis. Two of these prepns were pooled and applied to a DEAE-cellulose column (3 × 20 cm), previously equilibrated with 10 mM Tris–HCl buffer (pH 7.6). Elutions were performed as indicated in Results. Polyacrylamide gel electrophoresis was performed as described [5].

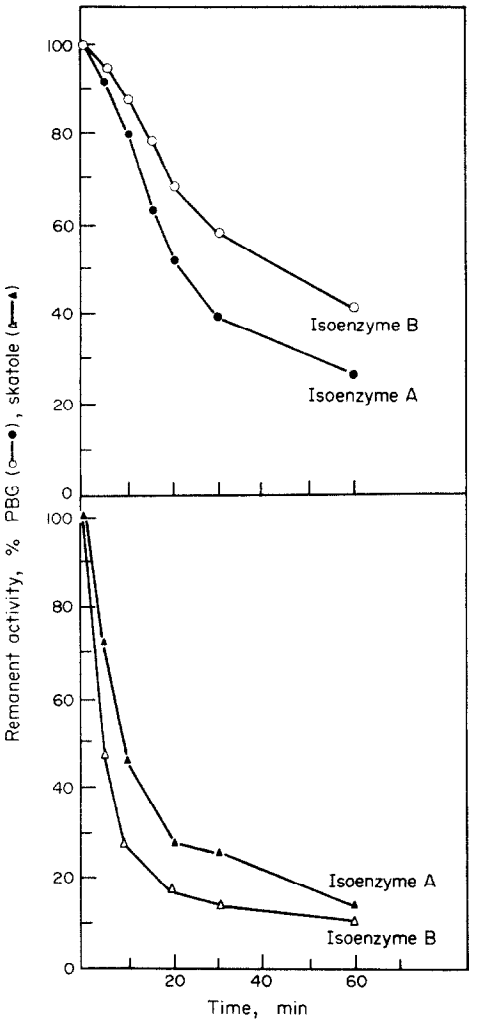

Fig. 5. Heat stability of porphobilinogen oxygenase and skatole pyrrolooxygenase isoenzymes. The enzyme (25 μl) was preincubated in the presence of 10 μmol Tris–maleate buffer (pH 7.8) in a final volume of 90 μl at 61° for the indicated times. Substrate (either porphobilinogen or skatole) and dithionite were added, and the incubations were carried out at 37° for 30 min.

Assay of skatole pyrrolooxygenase and porphobilinogen oxygenase. The standard reaction mixture contained in a final vol. of 100 μl; 10 μmol Na Pi buffer (pH 7.4), 0.1 μmol Na dithionite, porphobilinogen (15 nmol) or skatole (28–32 nmol), and 25 μl of enzyme (2–20 μg of protein 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 [1, 2].

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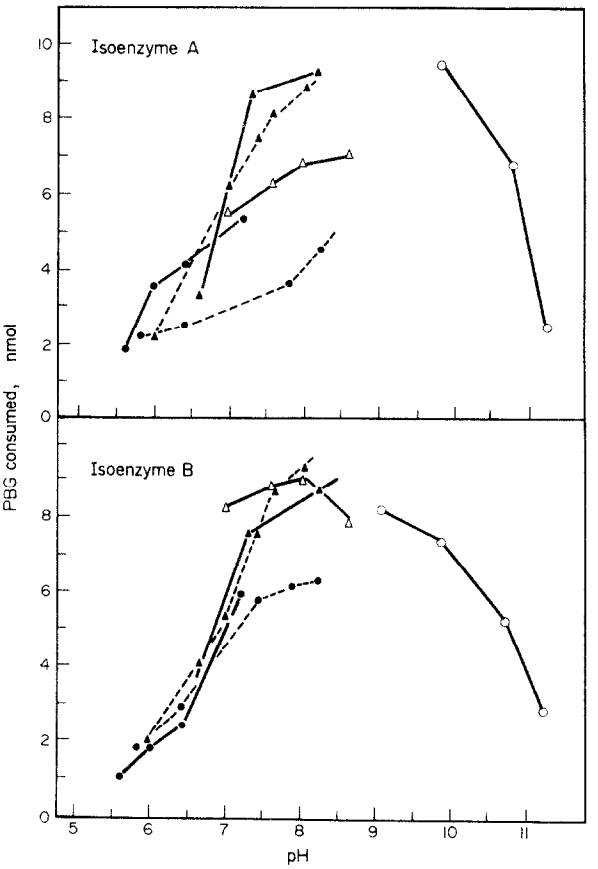

Fig. 6. Effect of pH and different buffers on the activities of porphobilinogen oxygenase isoenzymes. Citrate (●—●); Tris–maleate (●- - -●); barbital (—△—△—); Tris–HCl (—▲— ▲—); glycine (○—○); phosphate (▲- - -▲).

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