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KINETICS OF FENUGREEK PEROXIDASE ISOENZYMES

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Key Word Index—*Trigonella foenumgraecum*; Leguminosae; fenugreek; peroxidase isoenzymes; purification; *o*-dianisidine oxidation; catechol oxidation.

Abstract—Peroxidase from fenugreek seedlings was separated into 6 isoenzymes; 4 on CM-cellulose and 2 on DEAE-cellulose. The kinetics of these peroxidase isoenzymes with regard to *o*-dianisidine and catechol are described.

INTRODUCTION

Despite the widespread distribution and large amount of work done on peroxidase, the mechanism of their action is not clearly understood. Another perplexing feature is that there are conflicting reports describing peroxidase catalysed reactions, due mainly to the existence of several isoenzymes. While all peroxidase isoenzymes may catalyse the same reaction, the individual isoenzymes differ markedly in biochemical properties. In studies on the role of peroxidase during growth and development in fenugreek, individual isoenzymes showed differing specificity towards peroxidatic and oxidatic types of reactions [1]. In order to understand the function of peroxidase isoenzymes the oxidation of *o*-dianisidine and catechol was studied.

RESULTS AND DISCUSSION

Separation of peroxidase isoenzymes

The crude enzyme was first passed through a CM-cellulose column to obtain 4 active peaks, B (tubes 8–13), C (tubes 14–17), D (tubes 20–31) and E (tubes 40–45). The protein which was not adsorbed on CM-cellulose was subsequently passed through a DEAE-cellulose column to obtain two peaks, viz. A-1 (tubes 14–19) and A-2 (tubes 21–31). The summation of peroxidase activity of these 6 fractions accounted for *ca* 28% of the activity in the crude enzyme extract. Attempts to purify peroxidase isoenzymes have been made by earlier workers [2–5]. Shannon *et al.* [4] isolated 7 homogenous isoenzymes from horseradish roots. The elution profile of the enzyme in the present study showed little contamination by closely related isoenzymes. The reproducibility of elution profiles and the failure to detect interconversions suggested that the various peroxidase components obtained were authentic isoenzymes rather than artifacts. The isoenzymes were stable when stored frozen at −20° for several months. However, at low pH values (< 3.5) the isoenzymes lost activity in storage rapidly.

o-Dianisidine oxidation

The individual peroxidase isoenzymes catalysed the oxidation of *o*-dianisidine and showed an absolute dependence on added H_2O_2. The reaction did not require a metal or aromatic cofactor.

In the peroxidatic reaction, most of the isoenzymes had pH optima at 5.5, but for isoenzyme E the optimum pH was 6 (Table 1). However, the peroxidases were active over a fairly wide pH range between 4.5 and 9. At alkaline pH values activity decline rapidly and was completely inhibited beyond 9. Chance [6] reported that no change occurred in the heme linkage of horseradish or lactoperoxidase between pH 3.6 and 8.8 and

Abbreviations—α-iodoacetamide, IA-NH_2; iodacetate, IAA; *p*-hydroxy mercuri benzoate, *p*HMB; *N*-ethylmaleimide, *N*-EM; 5,5′-dithiobis-(2-nitrobenzoic acid, DTNB; horseradish peroxidase, HRP.

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Table 1. Properties of fenugreek peroxidase isoenzymes in *o*-dianisidine oxidation

Isoenzyme	pH optima	K_{app} for *o*-dianisidine	Optimum H_2O_2 concn (mM)	H_2O_2 concn required for 50% inhibition (mM)
A-1	5.5	1.25 ± 0.06*	0.30	2.75
A-2	5.5	0.52 ± 0.04	0.15	3.35
B	5.5	1.43 ± 0.03	0.30	1.00
C	5.5	1.43 ± 0.02	0.30	2.00
D	5.5	0.55 ± 0.05	0.30	1.15
E	6.0	0.52 ± 0.03	0.15	2.60

* Mean of 3 replicates ± s.d. Significance between means of isoenzymes was tested using the '*t*-test' and found that A-1 is significantly more than A-2, D and E and less than B and C. Also B and C are significantly more than A-2, D and E, whereas among A-2, D and E there was no significant difference.

Table 2. Effect of SH inhibitors on *o*-dianisidine oxidation expressed as % of control (activity remaining)

SH reagent*	A-1	A-2	B	C	D	E
DTNB	0.0†	40.0	15.0	52.0	11.4	ND‡
N-EM	60 0	90.0	106.0	115.0	108.0	ND
IA-NH_2	54.0	21.0	106.0	124.0	108.0	ND
*P*HMB	0.0	22.0	20.0	24.0	31 0	ND
IAA	54.0	65.0	128.0	103.0	108.0	ND

* All SH reagents were used at a final concn of 2 mM in reaction mixture.

† Values for controls: A_{460}/min/ml: A-1, 0.070: A-2, 0.410, B, 0.230: C, 0.365: D 0.175.

‡ Not determined.

there was nearly constant enzymatic activity in this region. It was reported for HRP [5] that in the peroxidatic reaction, pH optima were higher for isoenzymes A-1, A-2 and A-3 than isoenzymes B, C, D and E. It was earlier reported that isoenzymes A-1, A-2 and A-3 from HRP had lower affinity for azide and H_2O_2 [5]. However, isoenzymes B and D from fenugreek had a higher affinity for H_2O_2.

The peroxidase activity was inhibited by SH inhibitors, viz. DTNB, *P*HMB, IA-NH_2, N-EM and IAA. Isoenzymes B, C and D were however, insensitive to *N*-EM, IA-NH_2 and IAA (Table 2). $HgCl_2$ (1 mM) completely inhibited the activity of all isoenzymes (Table 3). It was noteworthy that a precipitate was formed immediately after *o*-dianisidine addition in the reaction mixture containing $HgCl_2$. The cations Fe^{3+}, Zn^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} generally stimulated oxidation.

Fenugreek peroxidase could catalyse the oxidation of catechol. However, when H_2O_2 was incorporated into the reaction mixture, activity was enhanced *ca* 3-fold (Table 4). This indicated that catechol oxidation might be mediated by a peroxidase-oxidase system rather than as polyphenol oxidase. Crystalline peroxidase and *Sorghum* preparations have been shown to catalyse mercaptoethanol dependent oxidation of *o*-dihydroxy cinnamic acid and this was probably due to a peroxidase–oxidase activity [7, 8].

Table 3. Effect of cations on *o*-dianisidine oxidation expressed as % of control (activity remaining)

	A-1	A-2	B	C	D	E
$FeCl_3$*	0†	105	105	109	114	ND‡
$ZnSO_4$	85.7	161	167	170	120	ND
$CaCl_2$	64 3	274	111	119	177	ND
$MgCl_2$	78.6	128	105	152	85	ND
$MnCl_2$	78.6	124	106	112	100	ND
$HgCl_2$	0	34	0	26	0	ND

* Used at a final concn of 1 mM.

† Values for controls as in Table 2.

‡ Not determined.

Table 4. Effect of catechol concentrations on rate of catechol oxidation with and without H_2O_2 by isoenzymes A-2, B and C

Catechol concn(mM)	A_{420}/min/ml A-2	B	C
8 (+ H_2O_2)	0.105	0.215	0 170
16 (+ H_2O_2)	0.140	0.230	0.210
32 (+ H_2O_2)	0.210	0.250	0.205
16 (− H_2O_2)	0.040	0.070	0.070
32 (− H_2O_2)	0.105	0.100	0.100

EXPERIMENTAL

Preparation of crude extract. The extraction and purification procedures were carried out at 5–7°. The seedling axis (15 g) from 3-day-old fenugreek seedlings (*Trigonella foenumgraecum* L.) were ground in 30 ml 5 mM NaOAc buffer pH 4.5 and squeezed through 2 layers of muslin cloth. The crude homogenate was centrifuged for 10 min at 5000 rpm at 0°. The supernatant was decanted and centrifuged at 12000 rpm (6000 *g*) for 20 min at 0–4°.

CM-cellulose chromatography. The supernatant (15 ml) was loaded on to a CM-cellulose column (2 × 20 cm) equilibrated with 5 mM NaOAC buffer pH 4.5. The unadsorbed protein fraction was collected and stored in a deep freeze until used. A linear gradient of 5 mM NaOAc pH 4.5 and 0.2 M acetate pH 4.5 containing 0.2 M NaCl (250 ml + 250 ml) was applied to elute the adsorbed proteins. Fractions (10 ml) at a flow rate of 1.3 ml/min were collected and the fractions monitored at 280 nm for protein. The fractions containing peroxidase activity were pooled and designated as B, C, D, and E. The pooled fractions were dialysed against 1 l of 50 mM NaOAc buffer pH 4.5 for 24 hr with 2 changes.

DEAE-cellulose chromatography. The unadsorbed protein (40 ml) was dialysed against 3 changes of 5 mM Tris–HCl buffer 7.8 for 22 hr. The sample was then loaded on to a DEAE-cellulose column (2.5 × 12 cm) previously equilibrated with 5 mM Tris-HCl pH 7.8. A linear gradient of 5 mM Tris–HCl pH 7.8 and 5 mM Tris–HCl pH 7.8 containing 0.2 M NaCl (250 ml + 250 ml) was applied and 10 ml fractions were collected at a flow rate of 2 ml/min. The fractions containing peroxidase activity were pooled and stored at −15°. The pooled fractions were designated as A-1 and A-2. The isoenzymes A-1 and A-2 were dialyzed against 5 mM Tris–HCl pH 7.8 for 24 hr.

Enzyme assays. Peroxidase activity was assayed with o-dianisidine as hydrogen donor at 460 nm at 25° [9]. To a 1 cm cuvette was added 2.7 ml 0.2 M Pi buffer pH 6, 0.1 ml H_2O_2 (0.3%) and 0.1 ml enzyme. A was calibrated at zero time and 0.1 ml of 0.1% dianisidine (in MeOH) was added. The change in A at 0, 30 and 60 sec was recorded. When catechol was used as substrate the A was recorded at 420 nm. Lineweaver-Burk reciprocal plots were drawn to determine the K_m values. The cations were used at a final concn of 1 mM and SH reagents at 2 mM in the reaction mixture.

Protein was estimated with Folin-phenol reagent [10].

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