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PEROXIDASES OF *SOLANUM MELONGENA* LEAVES

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Abstract—Three major peroxidases, designated as *A*, B_1 and B_2 from *Solanum melongena* leaves have been reported. Peroxidases-*A*, -B_1 and -B_2 were considered to be true peroxidases on the basis of $k_1:k_4$ ratio. The pH optima for the three enzymes were found to be 7·0, 6·0 and 6·0 respectively. These peroxidases differ in their $k_1:k_4$ ratio, in the effect of pH on this ratio and in the uric acid/guaiacol and *o*-dianisidine/guaiacol activity ratio.

INTRODUCTION

PLANT peroxidases have been known for a long time and have been purified from many different sources.[1–11] Peroxidase from horse-radish,[1,2] turnip[6,12] and Japanese radish[7] have been extensively purified and the purified preparations are usually found to contain more than one active component. From commercial horse-radish peroxidase preparations seven active peroxidases were identified,[13,14] and at least three components were isolated from turnip peroxidase preparations.[15] The significance of the multiplicity is not clear, since the possibility that the components may arise as artifacts during isolation has not been eliminated.

This work describes a preparative procedure for separation and purification of three major peroxidases from *Solanum melongena* (egg plant) leaves and some of their properties.

RESULTS

In a typical elution profile for the chromatography of peroxidatic activity in the ammonium sulfate fraction on an agar column two peaks of activity were found. The pooled fractions from peak *A* (after 180 ml) were designated as peroxidase-*A* and the pooled fractions from peak *B* (after 250 ml) were designated as peroxidase-*B*. A summary of the initial purification steps are shown in Table 1.

Although peroxidase-*A* was not retained by the TEAE-cellulose column, much of the inert fraction was adsorbed and a significant purification was achieved. Furthermore, two

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minor peaks with very low specific activity were detected. The data in Table 1 indicate at least a 4-fold purification over the agar step and 30-fold over the crude extract.

Peroxidase-*B* was separated from the major protein contamination, and two types of cationic peroxidases were detected. The first peak was designated as peroxidase-B_1 and eluted from the cellulose phosphate column with 0·1 M NaCl; the second peroxidase (B_2) was eluted with 0·3 M NaCl. Data in Table 1 show the achievement of at least 5-fold purification over the agar step, 99-fold over the crude extract for peroxidase-B_1, at least 3-fold over the agar step and 73-fold over the crude extract for peroxidase-B_2.

TABLE 1. PURIFICATION AND SEPARATOIN STEPS OF PEROXIDASES-*A*, -B_1 AND -B_2 FROM *Solanum melongena* LEAVES

Step			Total activity (units)*	Total protein (mg)	Recovery (%)	Specific activity (Units/mg protein)
Crude extract			418 000	44 000	100	9·4
1st Ammonium sulfate			364 000	36 400	87·7	10
Acetone			130 000	3210	31·0	40
2nd Ammonium sulfate			85 500	1170	20·4	75
Peroxidase-*A*	Agar column		14 800	210	3·5	70·5
	Acetone		10 000	148	2·4	70·0
	TEAE-cellulose		6600	23·4	1·58	282
Peroxidase-*B*	Agar column		48 400	252	11·1	191
	Dialysis		42 100	215	10·1	196
	Cellulose phosphate	B_1	14 500	15·6	3 47	930
		B_2	3250	4·75	0 77	684

* Absorption change/min.

pH Optima

In phosphate buffer peroxidase *A* has a pH optimum of 7·0, while both peroxidases-B_1 and -B_2 have maxima in phosphate buffer at pH 6·0.

Relative Activities toward o-*Dianisidine and Uric Acid*

The relative activities toward the hydrogen donors *o*-dianisidine, uric acid and guaiacol indicated marked differences for the three peroxidases (Table 2). The *o*-dianisidine/guaiacol activity ratios are 2·32, 7·57 and 0·08 for peroxidases-*A*, -B_1 and -B_2 respectively; also, the uric acid/guaiacol relative activities of these three enzymes are different for each enzyme.

Effect of pH on k_1/k_4 *ratios*

The rate constants k_1 and k_4 for peroxidases-*A*, -B_1 and -B_2 were determined at different hydrogen ion concentrations and plotted as $k_1:k_4$ ratio versus pH. The data indicate that the $k_1:k_4$ ratio for the three peroxidases responded similarly to the change of pH, which is expected, since for peroxidases -*A*, -B_1 and -B_2, hydrogen peroxide is the natural substrate

and the binding site involves a haem molecule. However, the ratio for peroxidase-*A* started to increase above pH 6·0, and started to increase above pH 5·0 for peroxidases-B_1 and -B_2. It is of interest to note that the optimum pH for peroxidase-*A* is 7·0 while it is 6·0 for the other two enzymes.

TABLE 2. ENZYMATIC PROPERTIES OF PEROXIDASES-*A*, -B_1 AND -B_2 FROM *Solanum melongena* LEAVES

Peroxidase	pH optimum	k_1/k_4 (guaiacol) pH 5·6	pH 6·0	pH 7·7	*o*-Dianisidine/ guaiacol	Uric acid/ guaiacol
A	7·0	38·0	40 0	94·0	2·32	0·12
B_1	6·0	5·5	5·4	31·6	7·57	0·038
B_2	6·0	5·2	7·45	18·9	0·08	0·014

DISCUSSION

Most of the new peroxidase isoenzymes have been detected in many other plant tissues[16–19] only on the basis of their electrophoretic behaviour and have not been significantly purified. Previous work with Alaska pea leaves[16] indicated the presence of two major peroxidases and two minor fractions. In *Solanum melongena* (egg plant) leaves three major peroxidases were detected, namely peroxidases-*A*, -B_1 and -B_2. In addition, two minor fractions with very low specific activity associated with peroxidase-*A* were separated on a TEAE-cellulose column. On application of this purification scheme for the separation of peroxidases from Artichoke leaves, only two major fractions were detected, in addition to two minor fractions.[11] Since peroxidatic activity is a property of all haem-proteins[20] these two minor fractions could be an artifact and not true peroxidases.

A useful tool for the differentiation of true peroxidases from other haem-proteins is the $k_1:k_4$ ratio for a given hydrogen donor. For true peroxidases this ratio appears to be several orders of magnitude higher than for other haem-proteins. Thus, Kurozumi,[21] using leucomalachite green as hydrogen donor found the $k_1:k_4$ ratio for horse-radish peroxidase, metheomyoglobin and cytochrome-*c* to be 40, $2{\cdot}3 \times 10^{-2}$ and $8{\cdot}4 \times 10^{-4}$ respectively. With guaiacol as hydrogen donor, the $k_1:k_4$ ratio for all four Alaska pea peroxidases ranged from values as low as 7·0 up to 24·0.[16] However, the $k_1:k_4$ ratios determined for peroxidases-*A*, -B_1 and -B_2 from egg plant leaves with guaiacol as hydrogen donor at pH values 5·6, 6·0 and 7·7 ranged from 5 to 94 (Table 2) which indicates that these three peroxidases are true peroxidases. Chance[22] presented the peroxidatic oxidation of hydrogen donor (AH_2) in the following reaction sequences:

$$\text{Peroxidase } (Fe^{3+}) + H_2O_2 \xrightarrow[I]{k_1} \text{Compound I} \xrightarrow[k_7]{AH_2} \text{Compound II} \xrightarrow[k_4]{AH_2} \text{Peroxidase } (Fe^{3+})$$

He demonstrated that usually steps I and II are rate limiting, depending on the relative concentrations of H_2O_2 and the donor AH_2. At very low H_2O_2 concentration step I will

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be the rate limiting step and k_1 can be determined; also, in the presence of low concentrations of AH_2, II will be the rate-limiting step and k_4 can be determined. On this basis, Chance and Maehly[23] described a method that approximates measurement of k_1 and k_4 using guaiacal as a hydrogen donor. Using this information k_1 and k_4 values were determined at different hydrogen concentrations for peroxidases-A, -B_1 and -B_2. The results indicate that for peroxidases-A, -B_1 and -B_2 the $k_1:k_4$ ratio as a function of pH reaches its highest values above pH 6·5. The ratio increased sharply above pH 6·0 for peroxidase-A, and above pH 5·0 for both peroxidases-B_1 and -B_2. Based on this observation we suggest that the difference in the degree of ionization of the different binding sites for the hydrogen donor in the isolated peroxidases as reflected in the behaviour of the velocity constant k_4, play an important role in the control and determination of the pH optima.

The broad hydrogen donor specificity of peroxidases complicates the application of the isoenzyme concept to this group of enzymes. The data reported by Hosoya[15] on the three crystalline peroxidases from turnip indicate that they have completely different kinetic constants. Also, Shannon and Kay[14] were able to classify the seven peroxidases from horse-radish roots into two groups on the basis of their chromatographic behaviour, electrophoretic migration, spectrophotometric properties and amino acid and carbohydrate composition. On the other hand, Klapper and Hackett[13] demonstrated that the four major components of horse-radish peroxidases have the same specific activities, sedimentation constants, amino acid composition and absorption spectrum. Macnicol,[16] was reluctant to accept the application of the isoenzyme concept to plant peroxidases because of the above mentioned contradiction and the fact that peroxidatic activity is a property of all haem-proteins.[20] He suggested that for two different peroxidases the identical ratio of specific activities toward two different hydrogen donors is enough for them to be characterized as isoenzymes, even in partially purified preparations. Thus the three major peroxidases-A, -B_1 and -B_2 isolated from egg plant leaves are not isoenzymes by Macnicol's definition, since they differ in: (a) pH optima, (b) $k_1:k_4$ ratio values as well as in the effect of pH on this ratio, and (c) in the uric acid/guaiacol activity and o-dianisidine/guaiacol activity ratios (Table 2).

EXPERIMENTAL

Solanum melongena (Egg plant) leaves, freshly harvested at Dokki, were used. Oxoid agar No. 3 (Oxoid Division of Oxo-Ltd, London), was used for column chromatography. Cellulose phosphate, was prepared according to the method of Leggett Bailey.[24] All other chemicals used were obtained from commercial sources.

Peroxidase activity. Peroxidase activity was assayed (unless stated otherwise) by measuring the change in absorbance (A) at 470 nm due to guaiacol oxidation in the presence of H_2O_2 and the enzyme at 30 sec intervals. The reaction mixture contained in a vol. of 3 ml 8 μmol H_2O_2, 60 μmol guaiacol, 50 μmol NaOAc buffer pH 5·6, and peroxidase at concentrations which gave a linear response over a period of 3 min The reaction was initiated by introducing the enzyme and mixing. All the assays were carried out at 25°. A unit of peroxidase activity was defined as that amount of enzyme which cause a change of 1 A unit per min at 25°.

Protein concentration was determined using the modified Folin method.[25]

Determination of the rate constants $k_1:k_4$. The rate constant for the reaction of peroxidase with H_2O_2 (k_1), and the rate constant for the reaction of peroxidase complex II with hydrogen donor (k_4) were determined according to the method of Chance and Maehly.[23]

Uric acid activity. The reaction mixture contained in a vol. of 5 ml: 5 μmol H_2O_2, 2·5 μmol, uric acid and 225 μmol NaOAc buffer pH 5·6. The reaction was started by introducing 10 units of peroxidase, mixing and incubating at 25°. After 1 min, samples (0·2 ml) were withdrawn and the reaction stopped by the addition of 0·1 ml of 2 N NaOH, the vol. was adjusted to 3 ml with dist. H_2O and the absorbance eas read at 290 nm.

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The uric acid concentration was determined from a standard curve for uric acid. *o*-Dianisidine activity was measured according to the method described in the Worthington Enzyme Manual.[26]

Buffers used in this work were prepared according to Gomori.[27]

Extraction and purification of enzyme. All purification steps were performed at 4–7°. Unless otherwise stated 0·05 M NaOAC buffer was used throughout the purification scheme. Well washed, freshly harvested leaves (2 kg) were grated into small pieces, suspended in 1200 ml buffer and homogenized for 10 min. The filtrate obtained by straining through cheese cloth (the crude extract) was treated with solid $(NH_4)_2SO_4$ to 75% saturation and the ppt. formed was suspended in the buffer. Pre-cooled acetone was added to this protein solution equivalent to 60% of the original vol. and the ppt. formed was removed by centrifugation and discarded. An additional amount of pre-chilled acetone was added to the supernatant, to make the total vol. of the added acetone equivalent to 240% of the original vol. The protein ppt. was dissolved in the acetate buffer. The peroxidase activity in the acetone fraction was concentrated by adding $(NH_4)_2SO_4$ to 80% of saturation. The protein ppt. was dissolved in the least vol. of buffer.

Chromatography on agar column. Oxoid agar No. 3 was used after the removal of the fine particles by decantation several times, and soaking for 48 hr in dist. H_2O. The packed column (132 × 1·8 cm) was equilibrated with NaOAc buffer and the protein fraction obtained from the second $(NH_4)_2SO_4$ step was placed on the column and developed with the same buffer. The eluate was collected in 10 ml fractions and after measuring the activity in the different fractions, those which contained most of the activity in each peak were collected separately.

Further purification of peroxidase-A. Active peroxidase-*A* obtained by chromatography on the agar column was subjected to the following purification steps. Pre-chilled acetone equivalent to 240% of the original vol. was added with mixing. The ppt. was collected by centrifugation and dissolved in the least vol. of 0·05 M buffer. When this fraction was chromatographed on a cellulose phosphate column after dialysis against 0·02 M NaOAc buffer (pH 5·6) overnight, the enzyme neither exchanged with the column material nor was any purification achieved. The active fractions from the agar column, were dialyzed against 0·02 M sodium phosphate buffer (pH 7·5), and chromatographed on TEAE-cellulose column (50 × 0·8 cm), pre-treated as recommended by Peterson and Sober[28] and equilibrated with the same buffer. The enzyme was eluted with a NaCl stepwise gradient ranging from 0 to 1·0 M in 0·02 M sodium phosphate buffer (pH 7·5).

Further purification of peroxidase-B. Pooled active peroxidase-*B* fractions were subjected to further purification steps after dialysis against 0·02 M NaOAc buffer pH 5·6 overnight. Cellulose phosphate was pre-treated as recommended by Peterson and Sober[28] and equilibrated with 0·02 M buffer. The dialyzed protein solution was placed on the column (50 × 0·8 cm) and eluted with NaCl in a stepwise gradient ranging from 0 to 0·3 M in the same buffer.

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