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A LACCASE-LIKE ENZYME IN PEACHES

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Abstract—A soluble, laccase-like enzyme from peaches is described. A purification procedure involving removal of pectic substances resulted in a 160-fold purification. Its substrate specificity, pH optimum, K_m and reaction with inhibitors, are described. The molecular weight as isolated is about 70,000–90,000.

INTRODUCTION

LACCASE (*p*-diphenol oxygen-oxido reductase E.C. 1.10.3.2) from a number of plant tissues has been described (see for example the review by Levine[1]) although most detailed investigations deal with fungal laccases.[1–6] The most detailed recent studies of laccase in higher plants are those of Omura[7, 8] and Nakamura and Ogura,[9] who studied these enzymes in various *Rhus* species. We have previously demonstrated that peaches contain at least two enzymes which are capable of oxidizing phenolic compounds. One of these was present in the particulate fraction of the cell and was of the catechol oxidase type (E.C. 1.10.3.2[10]). This was very similar to the catechol oxidase present in apples, which has been described previously.[11, 12] The second enzyme was laccase-like and its reported presence appears to be the only one about the occurrence of laccase in fruit.[10] Moreover, the simultaneous presence of a laccase-like enzyme and a catechol oxidase is unusual. An attempt was therefore made to purify and characterize more fully the soluble laccase-like enzyme from peaches.

RESULT AND DISCUSSION

Purification of the laccase-like enzyme from peaches is complicated by two factors. One is the presence of large amounts of pectins in the fruit which clog up columns and tend to co-precipitate with proteins. The second difficulty is due to the presence of a catechol oxidase in the particulate fraction, which tends to leach out of the particle, during extraction and purification. We sought to overcome these difficulties and purify it the laccase, using as criterion for purification the ratio of activity towards 4-methylcatechol and quinol. CTAB

[1] W. G. LEVINE in *The Biochemistry of Copper* (edited by J. PEISACH, P. AISEN and W. E. BLUMBERG), p. 371. Academic Press, New York (1966).
[2] K. ESSER, S. DICK and W. GIELEN, *Arch. Mikrobiol.* **48**, 306–318 (1964).
[3] G. FAHRAEUS, *Physiol. Plantarum* **7**, 704 (1954).
[4] G. FAHRAEUS and H. LJUNGGREN, *Biochim. Biophys. Acta* **46**, 22 (1961).
[5] B. MALMSTROM, *Z. Naturwissenschaft.-med. Grundl.* **2**, 259 (1965).
[6] H. MASON, *Ann. Rev. Biochem.* **34**, 264 (1961).
[7] T. OMURA, *J. Biochem.* **50**, 264 (1961).
[8] T. OMURA, *J. Biochem.* **50**, 305 (1961).
[9] R. NAKAMURA and Y. OGURA, in *The Biochemistry of Copper* (edited by J. PEISACH, P. AISEN and W. E. BLUMBERG), p. 389. Academic Press, New York (1966).
[10] A. M. MAYER, E. HAREL and Y. SHAIN, *Phytochem.* **3**, 447 (1964).
[11] E. HAREL, A. M. MAYER and Y. SHAIN, *Phytochem.* **4**, 783 (1965).
[12] E. HAREL, A. M. MAYER and Y. SHAIN, *Physiol. Plantarum* **17**, 921 (1964).

(cetyl trimethyl ammonium bromide) was used to precipitate the pectic compounds[13] after a short alkaline hydrolysis. This procedure is effective provided the amount of CTAB applied to the preparation is carefully determined prior to treating the bulk of the extract. Excess CTAB tends to precipitate proteins as well as pectic compounds. The final procedure for purification of the enzyme was as follows. One kg of peaches var. Orley was destoned and then disintegrated in 800 ml 0·4 M sucrose 0·1 M phosphate buffer, pH 7·3, containing 0·05 M sodium ascorbate. The homogenate was filtered through gauze and centrifuged at pH 6·0. The pectic substances were hydrolysed at pH 10·5 at room temperature for 30 min. Five g CTAB were now added to the hydrolysate and the pH of the solution was reduced to pH 5·0 with citric acid. After 10 min at room temperature, the precipitate was removed by centrifugation at 20,000 *g* for 30 min. The precipitate was extracted with 100 ml 0·05 M phosphate–citrate buffer, pH 5·0, the suspension was centrifuged again at 20,000 *g* and the two supernatants combined. Excess CTAB was removed by adding potassium iodide to the supernatant till turbidity reached a constant level. The complex formed by the pectins with CTAB was removed by centrifugation at 20,000 *g* for 30 min. The supernatant was fractioned with $(NH_4)_2SO_4$, and the protein fraction precipitating between 60 and 95 per cent saturation collected. The precipitate was dissolved in 30 ml 0·005 M phosphate buffer, pH 7·2, and dialysed against the same buffer for 24 hr. The resulting solution was passed through a DEAE-cellulose column equilibrated with 0·005 M phosphate buffer, pH 7·2. The enzyme was not absorbed by the column. The resulting eluate was subjected to a second fractionation with ammonium sulphate and the fraction precipitating between 65 and 95 per cent saturation again redissolved and then dialysed. The preparation was subjected to gel filtration on a Sephadex G-100 column (1·8 cm in dia., 60 cm height) in 0·02 M phosphate buffer, pH 6·0, and the fractions having a relatively high activity towards quinol were collected (41–52 ml elution volume).

The steps in the purification procedure are summarized in Table 1.

TABLE 1. PARTIAL PURIFICATION OF THE LACCASE-LIKE ENZYME FROM THE SOLUBLE FRACTION OF PEACHES VARIETY: "ORLEY"

Step	Specific* activity	Purification	Yield (%)	Activity towards quinol (%)†
Supernatant 20,000 *g* × 20 min	8·1	—	—	50
After acetone and alkaline hydrolysis	7·5	—	71	48
After CTAB and KI	15·4	1·9	65	41
First fractionation with $(NH_4)_2SO_4$	127·2	15·7	52	53
After DEAE-cellulose	407·0	50·2	43	71
Second fractionation with $(NH_4)_2SO_4$	610·5	75·4	39	75
After filtration on a Sephadex G-100 column	1343·0	165·8	34	76

* Activity determined by Warburg technique, at 26°, in 0·05 M phosphate buffer, pH 6·0. Specific activity: μl_2/mg protein/min in the presence of 5×10^{-3} M 4-methylcatechol.

† Activity towards quinol (5×10^{-3} M) as percent of the activity towards 4-methylcatechol.

[13] J. C. SCOTT, in *Methods of Biochemical Analysis* (edited by D. GLICK), Vol. 8, p. 145. Interscience, New York (1960).

The partially purified fraction obtained by this means, came of the Sephadex column in a position corresponding to a molecular weight of between 70,000–90,000. This value could also be confirmed using thin-layer gel filtration on Sephadex G-200.

Electrophoresis of the purified preparation on 1 per cent agar gel at pH 7·2, in phosphate buffer, 0·04 M showed it to move as a single band, towards the cathode.

The activity of the partially purified enzyme had a clear optimum at pH 6·0 (Fig. 1), both towards 4-methylcatechol and quinol. The enzyme oxidized a number of dihydric phenols, such as 3-methylcatechol, 4-phenylcatechol, 4-chlorocatechol and chlorogenic acid readily. It has about 50 per cent of the activity towards pyrogallol as to 4-methylcatechol. The partially purified enzyme had virtually no activity towards *p*-cresol both in the absence and pres-

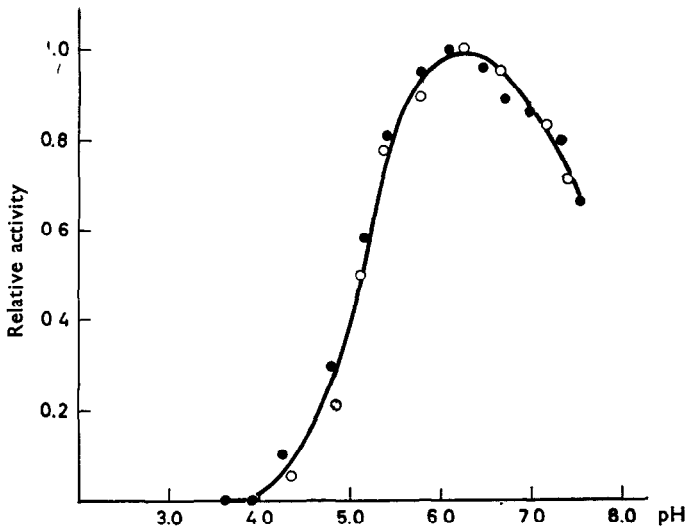

FIG. 1. DEPENDANCE ON pH OF OXIDATION OF 4-METHYLCATECHOL AND QUINOL, BY PARTIALLY PURIFIED SOLUBLE ENZYME FROM PEACHES.

Substrate concentration 5×10^{-3} M.
Activity determined by Warburg technique in phosphate–citrate buffer.

● 4-Methyl catechol
○ Quinol.

ence of ascorbic acid (2 and 3 per cent respectively, compared to 4-methylcatechol) and very little activity towards *p*-phenylene diamine.

The preparation had the following K_m values at pH 6·0: 4-methylcatechol 2×10^{-3} M, quinol $1{\cdot}2 \times 10^{-2}$ M. K_m values for O_2 in the presence of these substrates were 22 and 11 per cent respectively in the presence of 5×10^{-3} M phenolic substrate.

The response of the partially purified enzyme towards a number of inhibitors of catechol oxidase was studied (Table 2).

The results justify our earlier conclusion[10] that the enzyme present in the soluble fraction of peaches is laccase-like in nature. It is entirely different from the catechol oxidases which are present in apples.[11, 12] The enzyme has a relatively low affinity for quinol and unlike fungal laccase described by Fahraeus and Ljunggren[4] is unable to oxidize *p*-cresol to an appreciable extent. Laccases from *Rhus* species, and of other fungi also lack the ability to oxidize *p*-cresol.[2, 7, 8] The enzyme is unusual in that it is not absorbed on DEAE cellulose

columns under the conditions used and does not migrate on electrophoresis in starch gel. However, it did move as a single band in agar gel. The molecular weight of the enzyme in our partially purified preparations correspond to about 80,000, which falls into the range of molecular weights recorded for other laccases.[5–8]

TABLE 2. THE EFFECT OF INHIBITORS ON THE LACCASE FROM PEACHES, VAR. ALBERTA

Inhibitor	Concentration (M)	% Inhibition
2,3-Naphthalenediol	5×10^{-3}	0
Salicylaldoxine	5×10^{-3}	0
Phenylthiourea	5×10^{-4}	12
Dieca	5×10^{-5}	0
Dieca	2×10^{-4}	33
$NH_2OH.HCl$	5×10^{-3}	0
N-Vinyl-2-Pyrrolidone	3%	0
KCN	1×10^{-5}	14

Activity determined using oxygen electrode at 26°, in 0·05 M phosphate buffer pH 6·0.
Substrate: 4-methylcatechol 5×10^{-3} M.

The physiological role of the laccase in peaches (*Prunus persica*) is entirely obscure. In the closely related species apricots (*P. armeniaca*) we have been able to detect only very small traces of a soluble laccase although the apricots do contain a soluble catechol oxidase (Harel and Mayer, unpublished). The simultaneous occurrence of a laccase-like enzyme and a catechol oxidase, in the same tissue, although in different subcellular fractions, is of considerable interest and requires further study.

The method of purification for this enzyme is still not satisfactory and the yield is rather poor. The main obstacle is the presence of the large amounts of pectic substances present in the fruit. Because of the difficulty of purifying the enzyme its behaviour in the ultracentrifuge and certain other features have not yet been studied.

EXPERIMENTAL

Peaches of several varieties were used. The content of the laccase-like enzyme in various varieties is very different, but since the availability of peach varieties is very brief, it was impossible to carry out all the experiments with a single variety. Most of the experiments were made with peaches variety Alberta or Orley. The method of purification of the enzyme is described under Results and Discussion.

Activity of the enzyme towards the various substrates was determined using a polarographic oxygen electrode.[14] All other experimental techniques were as previously described.[10–12]

[14] A. M. MAYER, E. HAREL and R. BEN SHAUL, *Phytochem.* 7, 783 (1966).