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PARTIAL PURIFICATION AND PROPERTIES OF CATECHOL OXIDASES IN GRAPES

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Abstract—Grapes contain a high level of catechol oxidase activity. The enzyme is located in a particulate fraction of the cell, apparently in the plastids. It resembles catechol oxidases from other fruits in many aspects but differs in its relatively high activity towards caffeic and *p*-hydroxycinnamic acids and in its high affinity towards the latter. The enzyme is relatively insensitive to most inhibitors of catechol oxidase. The enzyme was solubilized with 1% Triton X-100 and purified 115 fold by ammonium sulphate fractionation and column chromatography. The enzyme from grapes, like other catechol oxidases, can be resolved into several fractions by gel electrophoresis.

WHILE studying catechol oxidases in fruits, we noticed a relatively high level of catechol oxidase activity in grapes. Review of the literature revealed only few papers dealing with phenol oxidizing activity in grapes. All are concerned with technological aspects of the grape and wine industry.[1-7] A more detailed study of this enzyme seemed therefore to be justified. We shall report partial purification and characterization of the catechol oxidase from grapes. A subsequent paper will deal with studies on the multiple forms of the enzyme.

RESULTS

Most of the activity towards 4-methylcatechol in grapes was found in a particulate fraction of the cell precipitating at 1000 g for 10 min. The supernatant fraction after centrifugation at 20,000 *g* for 30 min, was devoid of catechol oxidase activity.

The change of enzyme activity with pH is shown in Fig. 1. There is a peak in activity at pH 5·0 and a small shoulder around pH 7·3. Enzyme activity was comparatively high, and varied with the variety of grapes and the degree of ripening. The activities measured were 700–1500 μl O_2/g fr. wt./min, determined by an oxygen electrode at pH 5·0 with 5 mM 4-methylcatechol as substrate. This activity is several fold higher than that recorded for apple chloroplasts[8] and similar to that found in apricots.[9] The activity of the particulate fraction towards *p*-cresol was 24% of the activity towards 4-methylcatechol, but decreased considerably during storage of the fruit or with ripening.

The enzyme was solubilized by extracting the particulate fraction with 1% Triton X-100 in 0·005 M phosphate buffer pH 7·3. No activation resulted from the detergent treatment and 60–70% of the activity was recovered in the extract. Recovery could be

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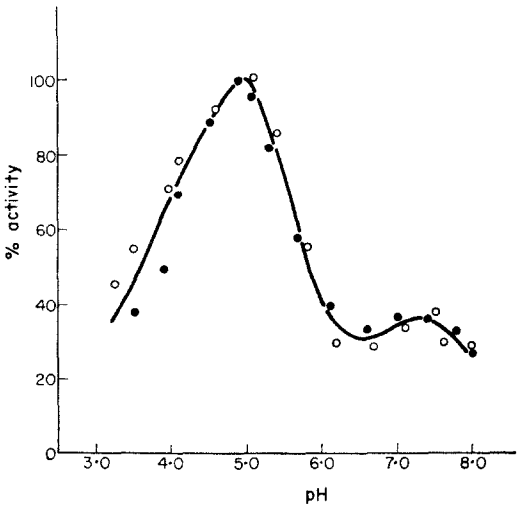

FIG. 1. THE CHANGE OF GRAPE CATECHOL OXIDASE ACTIVITY WITH pH.
Enzyme preparation 40–95% ammonium sulphate fraction prepared as described in Table 1. Activity determined in 0·1 M Na phosphate–citrate buffer, using an oxygen electrode, with 10 mM 4-methylcatechol as substrate. The shaded and open circles represent results from two different experiments.

increased by re-extracting the precipitate with 1% Triton X-100. Solubilization resulted in a drastic loss of the activity towards *p*-cresol. The enzyme was partially purified by ammonium sulphate fractionation and chromatography on DEAE-cellulose. Table 1 summarizes the steps in the purification procedure. A 155 fold purification was achieved

TABLE 1. PARTIAL PURIFICATION OF CATECHOL OXIDASE FROM THE PARTICULATE FRACTION OF GRAPES

	Step	Spec. act.	Purification	yield (%)
I	Particulate fraction	578	—	—
II	Triton extract	983	1·7	65
III	40–95% $(NH_4)_2SO_4$	2890	5·0	52
IV	1st elution on DEAE-cellulose	46067	79·7	46
V	2nd elution on DEAE-cellulose	66586	115·2	40

Spec. act. as μl O_2/mg protein/min at pH 5·0, 5 mM 4-methylcatechol as substrates, at 26°.

The particulate fraction (I) was prepared as described in the Experimental. It was extracted with 1% Triton X-100 in 0·005 M phosphate buffer pH 7·3, and the extract centrifuged at 20,000 *g* for 30 min, the supernatant (II) fractionated with $(NH_4)_2SO_4$ and the fraction precipitating between 40 and 95% saturation dissolved in 0·005 M Na phosphate buffer pH 8·0 and dialysed against the same buffer overnight at 2° (III). The preparation was applied to a column of DEAE-cellulose-GS (Serva) equilibrated with 0·005 M sodium phosphate buffer pH 8·0. The enzyme was eluted with 0·05 M of the same buffer (IV), precipitated with $(NH_4)_2SO_4$, dialysed and subjected to chromatography on a DEAE-cellulose-SS column equilibrated with 0·005 M sodium phosphate buffer pH 8·0. On elution with phosphate buffer pH 8·0, the bulk of catechol oxidase activity emerged between 0·02 and 0·05 M phosphate buffer pH 8·0 (V).

TABLE 2. SUBSTRATE SPECIFICITY OF CATECHOL OXIDASE FROM THE PARTICULATE FRACTION OF GRAPES

Substrate	Rel. act. (%)	
	Particulate fraction	Partially purified enzyme
Diphenols		
4-Methylcatechol	100	100
Chlorogenic acid	128	134
3,4-Dihydroxyphenylalanine	14	10
3,4-Dihydroxyphenylethylamine	10	6
3,4-Dihydroxybenzoic acid	8	10
(—)-Epicatechin	69	56
Caffeic acid	87	54
3-Methylcatechol	0	0
Quinol	0	0
p-Phenylenediamine	0	0
Monophenols		
p-Cresol	100	
p-Hydroxycinnamic acid	180	
o-Hydroxycinnamic acid	0	
m-Hydroxycinnamic acid	0	
Tyrosine	0	

Enzyme activity determined in 0·05 M phosphate–citrate buffer pH 5·0. Substrates concentration 5 mM, except for 2·5 mM in the case of caffeic acid, the hydroxycinnamic acids and tyrosine. Activity towards diphenols as per cent of the activity towards 4-methylcatechol. Activity towards monophenols as per cent of the activity towards *p*-cresol.

when the specific activity is compared to that of the particulate fraction. Further purification was obtained by gel filtration on Sephadex G-100.

The properties of the partially purified enzyme were compared to those of the particulate fraction. Table 2 summarizes the relative activities of the two preparations towards several mono- and diphenols. The enzyme resembles catechol oxidases from other fruits.[8–10] Noticeable, however, are the relatively low activity towards Dopa and Dopamine and the high activity towards caffeic acid and *p*-hydroxycinnamic acid.

The K_m values of the enzyme for three phenolic substrates and for oxygen in the presence of a mono- and a diphenol are given in Table 3. The K_m values for 4-methylcatechol and for oxygen in the presence of 4-methylcatechol for similar to the values obtained for apple chloroplasts[8] and for other catechol oxidases from fruits. A higher affinity to oxygen in the presence of monophenols as compared to *o*-diphenols was also noted for apple catechol oxidase.[8] The enzyme has a relatively high affinity towards *p*-hydroxycinnamic acid. The enzyme from grapes is relatively insensitive to most known inhibitors of catechol oxidases, with the exception of phenylthiourea (Table 4). Inhibition by *N*-vinyl-2-pyrrolidone of the purified enzyme is markedly higher than that of the particulate fraction. This could be due to permeability effects or to changes in the configuration of the enzyme caused by solubilization.

The behaviour of the enzyme in column chromatography and gel filtration at various steps of its purification was inconsistent. In some cases the enzyme was eluted from the columns as one peak, while in others it was separated into two or more peaks. The relative

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TABLE 3. K_m VALUES OF GRAPE CATECHOL OXIDASE FOR OXYGEN AND SOME PHENOLIC SUBSTRATES

K_m for	Particulate fraction	Partially purified enzyme
4-Methylcatechol	$6{\cdot}5 \times 10^{-3}$ M	$7{\cdot}7 \times 10^{-3}$ M
p-Cresol	$1{\cdot}4 \times 10^{-3}$ M	—
p-Hydroxycinnamic acid	$0{\cdot}6 \times 10^{-3}$ M	—
O_2 (4-methylcatechol)	19·6%	17·1%
O_2 (*p*-Hydroxycinnamic acid)	5·3%	—

Activity determined in 0·05 M phosphate–citrate buffer, pH 5·0. K_m values for oxygen were determined with no gas phase in the reaction bottles, with 12·5 mM 4-methylcatechol or 2·5 mM *p*-hydroxycinnamic acid.

TABLE 4. THE EFFECT OF VARIOUS INHIBITORS ON CATECHOL OXIDASE FROM THE PARTICULATE FRACTION OF GRAPES

Inhibitor	Concentration	% Inhibition	
		Particulate fraction	Partially purified enzyme
Diethyl dithiocarbamate	$2{\cdot}5 \times 10^{-4}$ M	14	15
Salicylaldoxime	$2{\cdot}5 \times 10^{-3}$ M	8	35
Phenylthiourea	$2{\cdot}5 \times 10^{-4}$ M	78	89
Potassium ethylxanthate	$2{\cdot}5 \times 10^{-3}$ M	28	32
2,3-Naphthalenediol	$5{\cdot}0 \times 10^{-3}$ M	19	27
N-Vinyl-2-pyrrolidone	2·5%	5	57

Activity determined with 5 mM 4-methylcatechol a substrate, at pH 5·0.

activity of the various peaks also differed from one experiment to another. It appeared that the situation might be similar to the multiplicity and interconversions between fractions in catechol oxidases from other sources.[10–14] The enzyme was resolved into at least eight bands on electrophoresis in acrylamide gel. While the electrophoretic mobilities of the various bands were extremely reproducible, their relative activity differed, depending on the treatment of the preparations before electrophoresis. Thus, the electrophoretic pattern of a Triton X-100 extract of the particulate fraction was markedly different from that of the 40–95% ammonium sulphate fraction or the enzyme eluted from a DEAE-cellulose column. Figure 2 shows the electrophoretic pattern of two such preparations, as well as a semi-quantitative determination of catechol oxidase activity along the gel. There were no qualitative differences in activity towards various mono-and *o*-diphenols. Although all fractions were active towards *p*-cresol, *p*-hydroxycinnamic acid, 4-methylcatechol,

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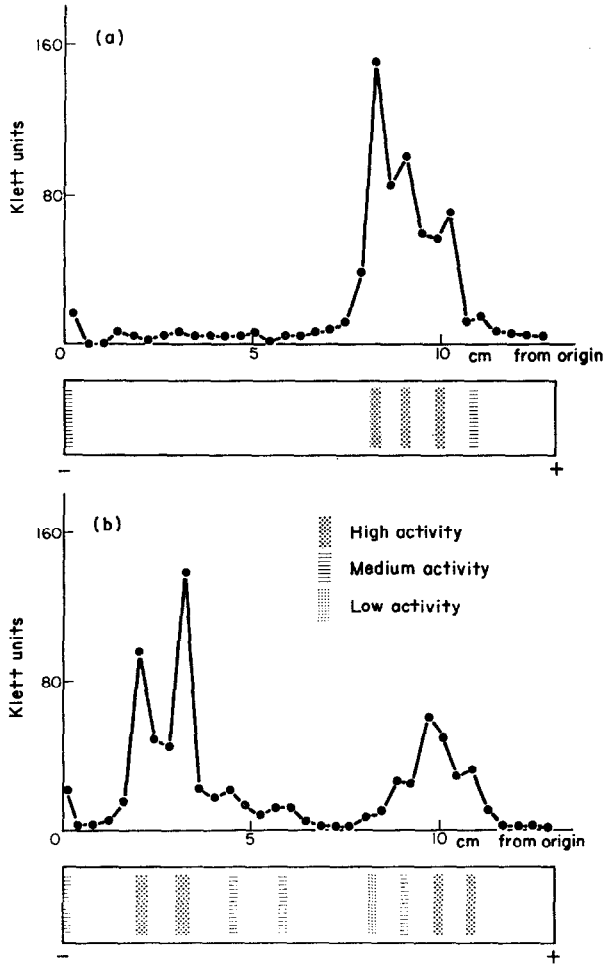

FIG. 2. ELECTROPHORESIS OF GRAPE CATECHOL OXIDASE IN ACRYLAMIDE GEL
Gel patterns and semi-quantitative determination of enzyme activity. (a) 40–95% Saturation with ammonium sulphate (Step III in Table 1). (b) 1% Triton X-100 extract of the particulate fraction (Step II in Table 1). 6% acrylamide gel in 0·02 M tricine buffer (N-Tris-(hydroxymethyl)-methylglycine) pH 7·9. Dimensions of gel slab: 19 × 13 × 0·3 cm. Electrophoresis run for 175 min at 350 V, 95 mA. For other details, see Experimental.

chlorogenic acid and 3,4-dihydroxyphenylalanine, considerable differences were apparent in their relative activity. From experiments to be described in a subsequent paper it appears that the multiplicity in grape catechol oxidase results from interconversions between various degrees of aggregation of one or more subunits of the same enzyme.

DISCUSSION

The catechol oxidase from grapes is located in subcellular particles of the cell. Insoluble polyvinyl pyrrolidone (Polyclar AT)[15] does not affect the localization of the enzyme. It is

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therefore unlikely that the enzyme originates in the soluble fraction but precipitates during extraction as a result of interaction with the endogenous phenols.[16] The particulate nature of the enzyme is also apparent from the work of Poux[3] who reported that the greater part of the activity of catechol oxidase in grapes precipitates on centrifugation at 1500 *g* for 6 min. It is probable that the enzyme is located in plastids as Ivanov[7] reported a much higher activity in the skin as compared to the pulp.

The enzyme from grapes is a typical catechol oxidase. It can hydroxylate monophenols and oxidize *o*-diphenols. *p*-Diphenols as well as monophenols and *o*-diphenols substituted in position 3 are not oxidized. The properties of the enzyme resembles those of the catechol oxidases from apple chloroplasts[8,10] the particulate fraction of peaches[9,17] and apricots.[9] It differs from other fruit catechol oxidases in its relatively high activity towards *p*-hydroxycinnamic, 3,4-dihydroxycinnamic and chlorogenic acids, and in its low sensitivity to inhibitors. It is interesting to note that the enzyme has a high activity towards both *p*-hydroxycinnamic acid and the product of its hydroxylation—caffeic acid.

The enzyme is resolved into eight bands on electrophoresis in acrylamide gel. Under the same conditions, the catechol oxidase from apple chloroplasts yields only three bands. While the multiplicity of apple catechol oxidase appears to be relatively simple (monomer-dimertetramer interconversions),[13] the situation in grapes is apparently far more complex and requires further studies.

EXPERIMENTAL

Grapes (white varieties: Hymonatica, Tamar) were purchased at a local supermarket and stored at 2°. Fruit samples were homogenized in a Waring blender in 0·4 sucrose–0·1 M phosphate buffer, pH 7·3, containing 0·01 M sodium ascorbate. The homogenate was filtered through gauze and the filtrate centrifuged at 1000 *g* for 15 min. The precipitate was suspended in 0·4 M sucrose–0·1 M phosphate–citrate buffer pH 5·0. For solubilization of the enzyme the particulate fraction was extracted with 1% Triton X-100 in 0·005 M phosphate buffer pH 7·3.[10]

Catechol oxidase activity was determined by the use of a polarographic oxygen electrode, as previously described.[8,18]

DEAE-cellulose was prepared for use according to Peterson and Sober.[19] The columns were coated with dimethyl dichlorosilane before packing. Protein was determined according to Lowry *et al.*[20] Acrylamide gel electrophoresis was carried out as previously described.[21] At the end of the run, the gels were dipped in 0·1 M phosphate–citrate buffer pH 5·0 for 10–15 min and then developed for catechol oxidase activity by spraying with a 10 mM solution of the desired phenolic substrate containing 0·05 per cent *p*-phenylenediamine in 0·1 M phosphate–citrate buffer pH 5·0.

For semi-quantitative determination of catechol oxidase activity in the gel, 2 cm wide gel strips were cut into 4 mm sections. The sections were dipped into 1·6 ml cold 0·05 phosphate–citrate buffer pH 4·8 in small tubes (7 mm I.D., 10 cm long). The gel sections were homogenized with a glass rod and the suspensions were allowed to stand for 20 min at 2°. 1 ml was then withdrawn from each tube with a syringe and added to a reaction mixture containing 20 mM 4-methylcatechol and 5 mM *p*-phenylenediamine in 5 ml 0·05 M phosphate–citrate buffer pH 5·0. The tubes were shaken for 10 min at 26° and the intensity of the colour read in a Klett colorimeter, filter No. 540.

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