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MULTIPLE FORMS OF *VITIS VINIFERA* CATECHOL OXIDASE

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Key Word Index—*Vitis vinifera*; Vitaceae; grape; catechol oxidase; multiple forms; MW determination; gel electrophoresis.

Abstract—Grape catechol oxidase shows multiple forms upon ion exchange chromatography, acrylamide gel electrophoresis and gel filtration. Conversion of some bands into others, which occurs during isolation and storage, is enhanced by dilution and by treatment with urea or acid pH. Estimation of MWs suggested that the conversions might be due to dissociation of the enzyme into subunits, but attempts to induce reassociation were unsuccessful. The effects of urea and acid pH could be imitated by partial enzymic proteolysis of the enzyme. Analysis of the various bands observed in gel electrophoresis suggested that some of the enzyme forms have the same MWs but differ in charge distribution.

INTRODUCTION

IN A previous paper,[1] we reported the partial purification and some properties of catechol oxidase from grapes. The relatively high activity found in the fruit was located exclusively in a particulate fraction, probably chloroplastic. The enzyme could be solubilized by extracting the particulate fraction with 1% Triton X100 and purified more than 100-fold by ammonium sulphate fractionation and chromatography on DEAE-cellulose. Like catechol oxidases from other sources,[2-10] the enzyme from grapes was resolved into several forms differing in their behaviour during ion exchange chromatography and in their gel electrophoretic mobility. These multiple forms were apparent in both Triton extracts of the particulate fraction and in the various stages of purification of the enzyme. Furthermore, the complement and relative activity of the various fractions varied, depending apparently on conditions existing during the extraction and purification of the enzyme.[11] We now report on attempts at understanding the multiplicity of grape catechol oxidase.

RESULTS

Acrylamide gel electrophoresis of Triton X100 extracts of the particulate fraction showed up to 8 bands having catechol oxidase activity (Fig. 1a). The four fast moving bands (F_1–F_4)

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predominated after ammonium sulphate fractionation of the Triton extract. An increase in the strength of the slow moving bands (S_1–S_4) was noted after storing the enzyme at 2° for several weeks, especially in dilute solutions (Fig. 1b). The pH during ammonium sulphate fractionation had a marked effect on the electrophoretic pattern of the enzyme. If the pH was maintained between pH 7·0 and 7·5, the resulting preparation contained only the fast moving bands (Fig. 1c). When a preparation, containing only the fast bands, was kept at pH 5·0 for several days, the slow moving bands appeared. Exposure to acid pH at 2° for several weeks resulted in a complete conversion of the fast to the slow moving bands (Fig. 1d). All attempts to induce formation of the fast moving bands from the slow ones failed.

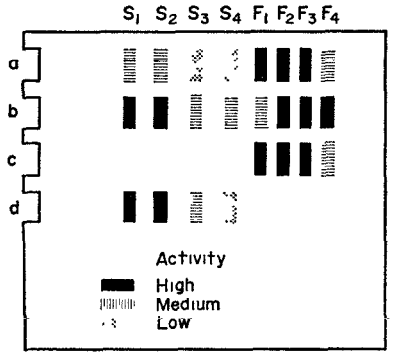

FIG. 1. ACRYLAMIDE GEL ELECTROPHORESIS OF GRAPE CATECHOL OXIDASE SHOWING BAND MULTIPLICITY AFTER VARIOUS TREATMENTS.
(a) Triton X100 extract of lyophilysed 1000 g × 10 min ppt. (b) Enzyme as in (a) but diluted 4-fold and stored at 2° for 2 weeks. (c) Fraction precipitated from (a) at 40–95% saturation with $(NH_4)_2SO_4$. (d) Enzyme as in (c) but stored for 3 weeks in 0·1 M citrate buffer pH 5·0. Electrophoresis performed for 4 hr at 300 V (17 V/cm) in 7·5% acrylamide in 0·02 M tricine buffer pH 8·5.

All 8 fractions observed in the gels were active towards 4-methylcatechol, chlorogenic acid, 3,4-dihydroxyphenylalanine, caffeic acid, *p*-cresol and *p*-hydroxycinnamic acid. Differences were however observed in their relative activity towards various substrates and in the activity of various bands at different pH values.[11]

Conversion of the fast moving bands (F) to the slow ones (S) was observed also during ion exchange chromatography. When a preparation containing only the F bands was chromatographed on a column of DEAE-cellulose, the enzyme was resolved into several peaks (Fig. 2a) which have well defined mobilities in gel electrophoresis (Fig. 2b). However, activity from each peak was resolved into several bands, with one or two of the bands predominating. Moreover, the slow bands, which were absent from the preparation applied to the column, were clearly present after elution.

Conversion of F to S bands, similar to those caused by dilution or acid pH, was also obtained by treating the enzyme with 5 M urea. Gel filtration of the enzyme, before and after the treatment with urea showed that the conversion of F to S bands was accompanied by conversion of a high MW peak into two additional peaks having lower MWs (Fig. 3). Estimation of the MW of the peaks obtained by gel filtration in Sephadex G75 and G100 gave values of 55 000–58 400; 31 400–33 000 and 20 000–21 300; corresponding, respectively, to the four F bands, the S_2 and the S_1 band.

From the results presented in Figs. 1–3 it was clear that the apparent dissociation of *F* into *S* bands must have caused a considerable change in the distribution of charges on the enzyme surface. The mobilities in acrylamide gel are considerably reduced in spite of the smaller size of the dissociated species. Unfolding and possibly refolding into a new conformation, resulting from exposure to urea or acid pH, might cause the reduced mobilities.

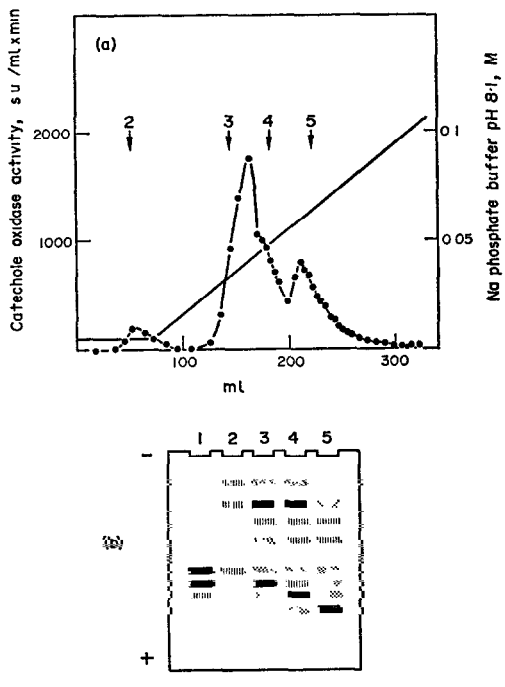

FIG. 2. CHROMATOGRAPHY ON A DEAE-CELLULOSE COLUMN AND ACRYLAMIDE GEL ELECTROPHORESIS OF GRAPE CATECHOL OXIDASE.

(a) A 35-ml sample (prepared as in Fig.1c) containing 12 200 scale units/ml min was applied to the column (2·5 cm dia., 14 cm ht) in 5 mM Na phosphate buffer pH 8·1. Flow rate: 1·3 ml/min. Arrows indicate fractions taken for electrophoresis. (b) Acrylamide gel electrophoresis of fractions from DEAE-cellulose column (a). Electrophoresis performed in 6% acrylamide gel in 0.02 M tricine buffer pH 7.9 at 20 V/cm, 6·3 mA/cm width for 2·5 hr. Samples were brought to identical concentrations of phosphate buffer before run. (1) Enzyme preparation before column chromatography. (2–5) Fractions obtained from column at points indicated by arrows in Fig. 2a.

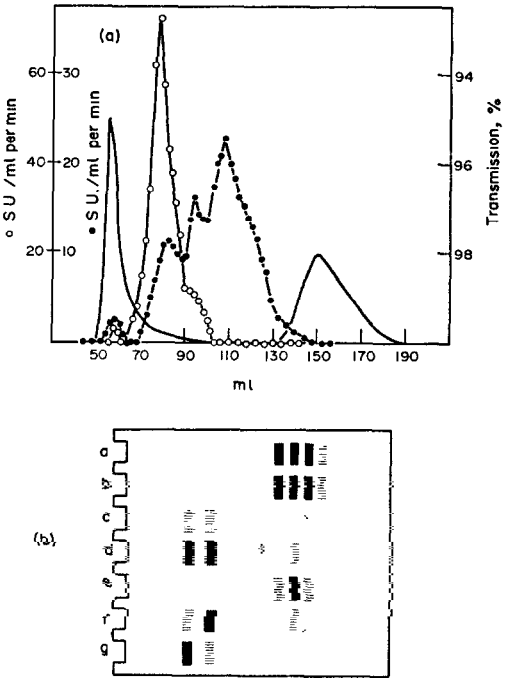

FIG. 3. THE EFFECT OF UREA ON THE MULTIPLICITY OF GRAPE CATECHOL OXIDASE–GEL FILTRATION AND ACRYLAMIDE GEL ELECTROPHORESIS.

(a) Gel filtration in a column (2·5 cm dia., 44 cm ht) of Sephadex G100 in 0·01 M K phosphate buffer pH 7·3. Samples containing 7·2 mg protein in 0·01 M phosphate buffer pH 7·3 were applied to the column. ○ 40–95% $(NH_4)_2SO_4$ fraction from Triton extract; ● 40–95% $(NH_4)_2SO_4$ fraction after standing in 5 M urea at 2° for 48 hr;—— Protein. (b) Acrylamide gel electrophoresis of fractions obtained by gel filtration (Fig. 3a). Condition of electrophoresis as described in Fig. 1. (a–c) Untreated preparations; (a) before gel filtration (b) 74 ml elution vol.; (c) 96 ml elution vol. (d–g) After treating with 5 M urea: (d) before gel filtration; (e) 78 ml; (f) 94 ml; (g) 112 ml elution volume.

Orthacryl two dimensional gel electrophoresis[12,13] has been used to resolve problems of enzyme multiplicity. Following orthacryl runs, the mobilities of the various bands fell on a straight line which passed through the origin, a pattern which according to Raymond[12]

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[13] RAYMOND, S. and NAKAMICHI, M. (1964) *Anal. Biochem.* **7**, 225.

suggested a group of molecular species having identical MW but differing in their distribution of charges. It was also evident from the orthacryl runs that conversions among fractions occurred during electrophoresis. The patterns obtained indicated interconversion among F_1, S_1 and S_2 as well as among F_4, S_3 and S_4.

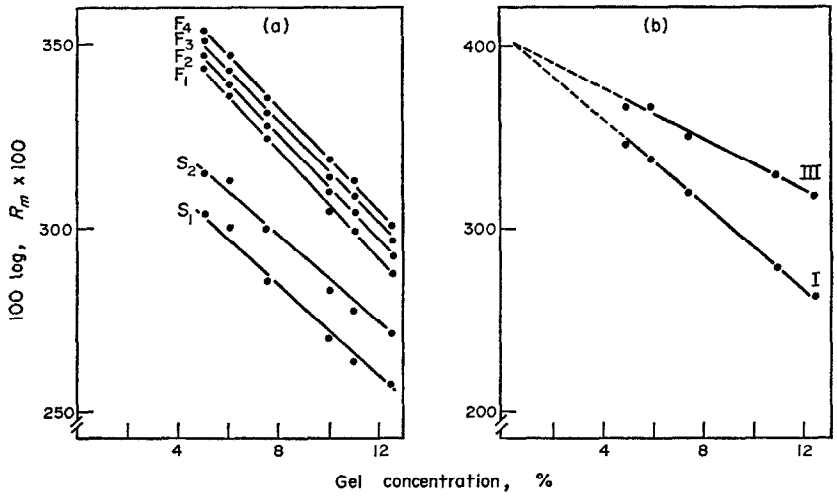

FIG. 4. CHANGE IN ELECTROPHORETIC MOBILITIES OF THE VARIOUS BANDS OF GRAPE AND APPLE CATECHOL OXIDASES WITH ACRYLAMIDE GEL CONCENTRATION.
Electrophoresis performed as in FIG. 1. (a)—grape catechol oxidase; (b)—partially purified catechol oxidase from apple chloroplasts.

Analysis of the behaviour of the different bands upon running in different concentrations of acrylamide gels[14,15] suggested that the F bands have indeed identical MW, but differ in charge distribution (Fig. 4a). However, no simple aggregation–deaggregation relationships seemed to exist between the various slow and fast moving bands. The migration behaviour of partially purified catechol oxidase from apples, run simultaneously with grape catechol oxidase in the various concentrations of acrylamide gel (Fig. 4b) corresponded to that expected according to Smithies[14] and Hedrick and Smith[15] for polymeric forms of a given protein. This is in agreement with our earlier findings on apple catechol oxidase obtained by interconversions and starch gel electrophoresis and by estimation of MW using gel filtration.[9]

Multiplicity in an enzyme preparation could result from the action of hydrolytic enzymes, which partly degrade the enzyme protein or carbohydrates attached to it in the case of glycoproteins. Laccase, which is related to catechol oxidase, is a glycoprotein and microheterogeneity in *Podospora* laccase is due to differences in the carbohydrate part of the molecule.[16] Favorable pH conditions or exposure of some sites after treatment with urea or acid pH could accelerate such degradations.

We examined the possibility that the multiplicity of grape catechol oxidase is a result of a hydrolytic activity present in the enzyme preparation. Exposure of a preparation containing only the F bands to purified α- and β-glucosidase had no effect on the pattern of bands

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upon subsequent electrophoresis. Exposure of the same preparation to a number of purified proteolytic enzymes resulted in conversion of *F* to *S* bands (Fig. 5). Purified trypsin was particularly effective while α-chymotrypsin, papain and carboxypeptidase *A* had little or no effect. The course of disappearance of the *F* bands and appearance of the *S* bands with time resembled the changes in band pattern caused by urea, acid pH, etc. It seemed possible that the conversions of *F* to *S* bands observed during the preparation of the enzyme might have resulted from an endogenous proteolytic activity. The similar results obtained by urea or acid pH treatment could be brought about by exposure of certain sites in the enzyme which are relatively sensitive to proteolytic attack. However, attempts to prevent the conversions caused by urea and acid pH by adding various inhibitors of proteolytic enzymes (phenylmethylsulfonyl fluoride, $HgCl_2$, iodoacetate, all at 10^{-3} M and 10^{-2} M) were only partly successful.

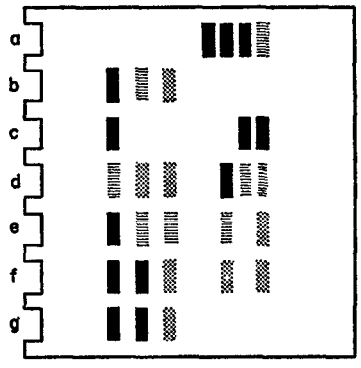

FIG. 5. THE EFFECT OF PROTEOLYTIC ENZYMES ON THE BAND MULTIPLICITY OF GRAPE CATECHOL OXIDASE.

Electrophoresis as in Fig. 1 except that the gels were run for 3 hr. Enzyme fraction was obtained by precipitation at 40–95% saturation with $(NH_4)_2SO_4$. Samples containing 400 μg protein in 0·05 M phosphate–citrate buffer pH 5·0 were used for each treatment and incubated at room temp. (a) Control, enzyme incubated in buffer for 2 hr. (b) Enzyme incubated with 5 M urea for 2 hr. (c) Enzyme incubated with 2·5 μg pepsin for 2 hr. (d–g) Enzyme incubated with 2·5 μg trypsin for: 5 min (d); 20 min (e); 40 min (f); 2 hr (g).

DISCUSSION

The multiplicity of grape catechol oxidase differs from that observed in other fruits. Multiplicity in apples[6,9] and apparently also in apricots (Harel and Mayer, unpublished) is caused by various degrees of aggregation of subunits. Similar situations were reported for catechol oxidases from mushrooms,[10] *Neurospora*[4] and potatoes.[7]

Our results indicate that in grapes multiplicity is due to the presence of true isoenzymes. The various *F* bands differ in charge distribution with no appreciable differences in size. It is difficult to determine the physiological significance of the multiple forms, as we have not been able to isolate them in a pure form. There seem to be no qualitative differences with regard to substrate specificity; differences seem to exist, however, in the pH optima of some of the fractions.[11]

It was previously reported[11] that short exposure to acid pH or urea induces a rapid reversible, 4- to 10-fold, activation of grape catechol oxidase. Such treatments also induce

marked changes in the electrophoretic behaviour of the enzyme. Therefore changes in electrophoretic mobility might be directly related to the activation phenomenon. However, the changes in electrophoretic behaviour are irreversible. It seems therefore that mobility is changed due to the irreversible changes occurring in enzyme structure, which have also been described.[11]

The *S* bands observed in gel electrophoresis after dilution of the enzyme or exposure to urea or acid pH result apparently from partial degradation of the *F* bands. Multiplicity of forms differing in size, resulting from proteolytic degradation, has been reported by Wilgus *et al.*[17] and by Pringle[18] for yeast hexokinase, by Saeed *et al.*[19] for plasma cholinesterase and by Gardner *et al.*[20] for rye and oat phytochrome. A similar situation might also exist under our experimental conditions, although the evidence is not conclusive. It is evident that great care should be taken in interpreting multiplicity and conversions in partly purified enzyme preparations. Though it might be tempting to assume a dissociation–association relationship, the cause for multiplicity might be much more commonplace.

EXPERIMENTAL

Isolation of catechol oxidase from grapes, including Triton X100 extraction of the 1000 *g* ppt, $(NH_4)_2SO_4$ fractionation and column chromatography on DEAE-cellulose was performed as previously described.[1,11] Catechol oxidase from apple chloroplast was partially purified as described by Harel *et al.*[6] and its activity determined by the use of a polarographic oxygen electrode [21,22] Acrylamide gel electrophoresis was carried out and gels developed for catechol oxidase activity as described by Harel and Mayer.[1,23] Orthacryl gel electrophoresis was performed according to Raymond.[12] Gel filtration was performed as described by Harel and Mayer.[9] Estimation of MWs of catechol oxidase fractions from gel filtration was done by calibrating the Sephadex columns with proteins of known MWs[9] and by calculations according to Determann.[24] Both methods gave almost identical results, using either Sephadex G75 or G100.

Trypsin (type I from bovine pancreas), α-chymotrypsin (type III from bovine pancreas), pepsin (2× crystallized, from hog stomach mucosa), papain (2× crystallized, from Papaya latex), α-glucosidase (type I from yeast) and β-glucosidase (from almonds) were purchased from Sigma. Carboxypeptidase A (2× crystallized, from bovine pancreas) was obtained from Worthington Biochemical Corporation.

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