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ISOELECTRIC POINT CHANGES IN *VITIS VINIFERA* CATECHOL OXIDASE

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Key Word Index—*Vitis vinifera*; Vitaceae; grape; catechol oxidase; tyrosinase; electrophoresis; isoelectric focussing.

Abstract—A comparison between electrophoretic and isoelectric focussing patterns of grape catechol oxidase is reported. A relationship exists between changes in electrophoretic mobility and isoelectric point of the enzyme; these changes are induced by acid shock, 5 M urea or by storing the enzyme for several weeks.

INTRODUCTION

THE OCCURRENCE of irreversible structural changes in grape catechol oxidase (or tyrosinase) was first shown by Harel *et al.*[1,2] using acrylamide gel electrophoresis. Changes were observed by treating the enzyme with an acid or 5 M urea or after storing the enzyme at 2° for several weeks, especially in dilute solution. The electrophoresis pattern showed two groups of bands, slow moving (S) and fast moving (F) bands. By treating a freshly prepared enzyme extract containing only F bands, S bands were irreversibly obtained; their different migration rates suggested that S and F bands differ only in charge distribution on the enzyme surface. Also Lerner[3] showed that the acid shock increased the Stokes' radius of grape catechol oxidase and this could explain the slower migration of S bands.

Using acrylamide gel disc electrophoresis and isoelectric focussing we have found evidence that structural changes of grape catechol oxidase are accompanied by changes in the isoelectric point of the enzyme protein.

RESULTS AND DISCUSSION

The electrophoretic pattern of grape catechol oxidase, freshly extracted from grape chloroplasts, is shown in Fig. 1a. Only F bands are present. After the enzyme has been stored for 3 months at 2°, S bands appear (Fig. 1b). S bands can also be obtained by treating the enzyme extract with 5 M urea for 16 hr or by using a low pH (4·75) for 2 days. The electrophoretic pattern is the same for all grape varieties tested, before or after treatments. Obviously, the phenomenon observed is the same as that described by Harel *et al.*[1,2] The isoelectric focussing pattern of catechol oxidase freshly extracted from grape chloroplasts is shown in Fig. 2. Three peaks can be distinguished, corresponding to isoelectric points of pH: A—4·7; B—4·9; C—5·1. In the isoelectric focussing pattern of a 3-month-old extract, peaks A and B are always present and, in some cases, peak C can be seen. Two

[1] LERNER, H. R., MAYER, A. M. and HAREL, E. (1972) *Phytochemistry* **11**, 2415.
[2] HAREL, E., MAYER, A. M. and LEHMAN, E. (1973) *Phytochemistry* **12**, 2649.
[3] LERNER, H. R. personal communication.

more peaks appeared (Fig. 3), corresponding to isoelectric points of pH D—5·5 and E—about 6. The same result is obtained after 5 M urea treatment and in this case all peaks are present.

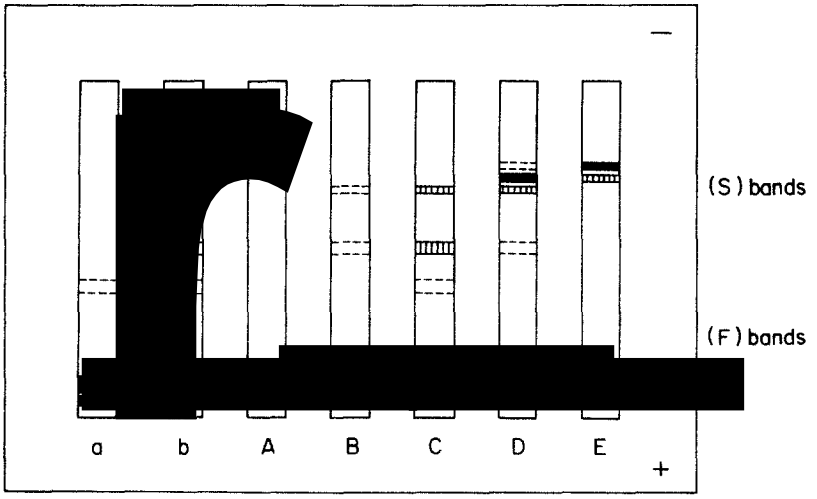

FIG. 1. ACRYLAMIDE GEL DISC ELECTROPHORESIS OF *Vitis vinifera* CATECHOL OXIDASE.
Enzyme extracted from grape chloroplasts with Triton X100. Current was 2·5 mA per tube. Front, marked with bromophenol blue was allowed to migrate about 5 cm in 2 hr. 0·05 ml enzyme preparation was separated in each tube. (a) Enzyme freshly extracted. (b) Enzyme stored for 3 months at 2°. (A–E) Fractions corresponding to peaks A–E obtained by isoelectric focussing.

The results obtained suggest that different electrophoretic migration rates (Fig. 1a and b) are correlated with changes in isoelectric point. In order to demonstrate a relationship between electrophoretic bands and peaks A, B, C, D, E obtained by isoelectric focussing, fractions corresponding to each peak were collected and placed on electrophoresis gels.

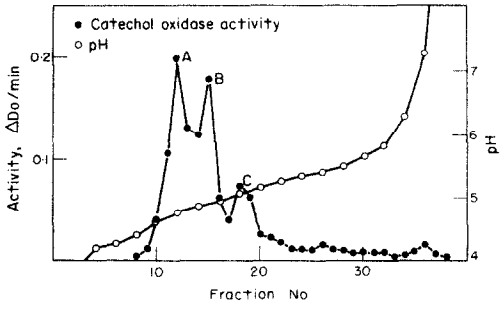

FIG. 2. ISOELECTRIC FOCUSSING OF FRESHLY EXTRACTED *Vitis vinifera* CATECHOL OXIDASE.
Enzyme extracted from grape chloroplasts with Triton X100 was precipitated by $(NH_4)_2SO_4$ (25–90% saturation). The ppt. was dissolved in 0·02 M K phosphate buffer pH 7 and the preparation was applied to a column of Sephadex G25 fine for desalting. The active fraction was dialysed for 5 hr against dist. H_2O before being applied to an isoelectric focussing column (pH range 4–6, focussing time 64 hr, voltage 500 V, current 2 mA).

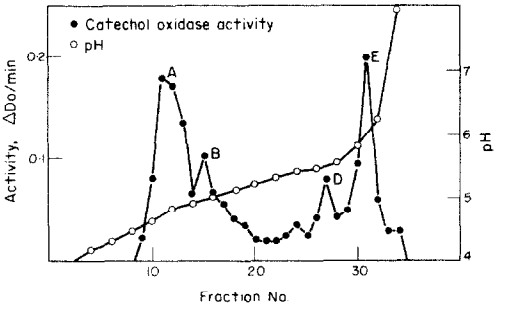

FIG. 3. ISOELECTRIC FOCUSSING OF A THREE-MONTH-OLD EXTRACT.
The extract was treated in the same way as Fig. 2.

The electrophoretic pattern of the different peaks is shown in Fig. 1A–1E. S Bands are present mainly in peaks D and E corresponding with higher isoelectric points, and F bands in peaks A, B and C corresponding to lower isoelectric points.

These findings indicate that irreversible structural changes of grape catechol oxidase are accompanied by considerable transformations in the enzyme structure. Changes in Stokes' radius, described by Lerner,[3] change the charge distribution of the enzyme protein and consequently its isoelectric point. However, in spite of these important changes in the enzyme molecule, the active site appears to be unaffected. For example, when structural changes are induced by storing the enzyme for several weeks at 2°, the activity of extracts towards 4-methylcatechol shows the same value all through the treatment. Our results support the suggestion of Harel and Mayer[2] that S bands result from partial degradation of the F bands.

EXPERIMENTAL

Mature grape of *Vitis vinifera* (var. Malbec, Cabernet-Sauvignon, Merlot and Semillon) were collected in the experimental vineyard of the Institut d'oenologie de Bordeaux. Crude enzyme extracts were obtained from 200 g samples of berries by the method of Harel and Mayer.[4]

Determination of catechol oxidase activity. To 2 ml 4-methylcatechol (10 mM) in phosphate–citrate buffer (0·1 M, pH 4·75), 0–1 ml enzyme extract was added and the vol. made up to 3 ml with buffer. O_2 concentration was stabilized by saturating the reaction mixture with air at 25°. The increase of absorption at 400 nm (λ_{max} of the corresponding quinone) was monitored and results expressed in ΔD_0/min/0·1 ml of enzyme extract.

Electrophoresis. Acrylamide gel electrophoresis was carried out according to Davis.[5] At the end of the run, gels were developed for catechol oxidase activity by immersion in 10 mM soln of 4-methylcatechol in 0·1 M phosphate–citrate buffer. Stained gels were then sprayed with 10 mM p-phenylenediamine to intensify the coloration of bands. Isoelectric focussing was performed in the LKB column 8101, 110 ml (LKB Stockholm, Sweden) using 1% Ampholine pH 4–6 according to LKB's suggestions and sucrose for density gradient.

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[4] HAREL, E. and MAYER, A. M. (1971) *Phytochemistry* **10**, 17.
[5] DAVIS, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404.