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Note

Purification of grape polyphenoloxidase with hydrophobic chromatography

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The oxidation reaction associated with the darkening of damaged tissue in fresh fruits and vegetables is catalyzed by the enzyme polyphenoloxidase (PPO) (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase; E.C. 1.14.18.1). To better control the action of PPO in different fruit and vegetable products, it is necessary to characterize the enzyme isolated from those different sources. However, the isolation of PPO is difficult[1,2], and several methods for the purification of PPO have been reported with varying degrees of success[3–10].

In this report, hydrophobic chromatography is used to isolate and purify grape PPO. The use of hydrophobic chromatography for the separation of proteins[11–15] is relatively new; Flurkey and Jen[16] appear to be the first to use such columns to purify PPO. The use of hydrophobic chromatography as a rapid, reproducible and successful purification technique for grape PPO is presented in this paper.

MATERIALS AND METHOD

Enzyme extraction

The two varieties of grapes, Ravat 51 and Niagara, used in this study were grown in the vineyards at the New York State Agricultural Experiment Station, Geneva, N.Y.

The PPO was initially extracted from the grapes with acetone[16,17]. Acetone powders were prepared by homogenizing 200 g of fresh grapes with 300 ml of cold (−20°) acetone and 1% of polyethylene glycol (average mol.wt. 3000–3700). The seeds were discarded, and the remainder of the homogenate was filtered under suction, and washed with 150 ml of cold acetone. The marc was further extracted with 300 ml of cold acetone three more times.

The dried acetone powders were suspended in 100 volumes (w/v) of 0.05 *M* K_2HPO_4–1.0 *M* KCl buffer (pH 6.5) and stirred for 30 min at ambient temperature. The suspensions were then centrifuged in a Sorvall RC-5 refrigerated centrifuge at 12,000 *g* at 0° for 15 min and then filtered through glass wool. The filtrate was made

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1 *M* with respect to ammonium sulfate by adding solid crystals of the salt, adjusted to pH 6.5 and filtered through Whatman No. 4 filter paper (labeled "before-column extract").

Chromatography on phenyl Sepharose CL-4B

PPO was isolated and purified on a hydrophobic column in a manner similar to that of Flurkey and Jen[16] and Flurkey *et al.*[18]. Pre-swollen phenyl Sepharose CL-4B (Pharmacia, Uppsala, Sweden), a hydrophobic resin, was de-gassed and equilibrated in four volumes of de-aerated buffer, consisting of 1 *M* ammonium sulfate, 1 *M* KCl and 0.05 *M* K_2HPO_4 (pH 6.5). A 20-ml bed-volume column (20 × 1 cm I.D.) was packed, rinsed with buffer, and then 300 ml of the "before-column extract" were applied on to the column. The proteins bound to the hydrophobic resin were eluted by using eluents decreasing in buffer concentration in a batchwise manner: (A) 60 ml of buffer; (B) 32 ml of buffer + 8 ml of distilled and deionized water; (C) 24 ml of buffer + 16 ml of water; (D) 16 ml of buffer + 24 ml of water; (E) 8 ml of buffer + 32 ml of water; (F) 40 ml of a 50% solution of ethylene glycol; and (G) 40 ml of water. Each fraction (160 drops) collected was assayed for PPO activity, and its protein content was estimated by measuring the absorbance at 280 nm in a Varian Cary UV/visible spectrophotometer (Model 219) against a reference of the buffer solution. The most active fractions were combined, dialyzed against three changes of 0.01 *M* Na_2HPO_4 buffer of pH 6.5, freeze-dried, and then suspended in 10 ml of Na_2HPO_4 buffer (labeled "purified PPO extract").

Specific activity

The PPO activity was determined on 0.2 ml of the enzyme extract in 2.4 ml of 0.01 *M* Na_2HPO_4 buffer of pH 6.5 and 0.4 ml of 0.5 *M* catechol also prepared in the buffer. The increase in absorbance at 420 nm, at 25°, was measured against a reference of the same solution without the enzyme[19]. One unit of PPO activity is defined as the amount of enzyme that caused a 0.001 unit change in absorbance per min at 420 nm. The protein content (mg/ml) of the extract was determined by the method of Sutherland *et al.*[20] using bovine serum albumin as standard. The specific activity was expressed as Units of activity per mg of protein.

RESULTS AND DISCUSSION

The elution profile from the hydrophobic column is shown in Fig. 1 for Ravat PPO. The largest portion of PPO activity contained in one peak (fractions 68–75) was clearly separated from the bulk of the other material. The reproducibility of this purification step was excellent for both the Ravat and Niagara enzymes.

The purification procedure for Ravat PPO is summarized in Table I. Although the crude extract was not the primary step in the isolation process, it is included in Table I to emphasize the merits of the procedure. The preparation of the acetone powder achieved a 23-fold purification, and after the entire isolation an increase in the PPO specific activity of over 250-fold was obtained. The recovery (better than 100%) of PPO indicates the removal of an inhibitory substance or an activation of the enzyme during the isolation procedure. If the "before-column extract" is set at 100%, a 53% yield of PPO activity for Ravat was recovered in the most active

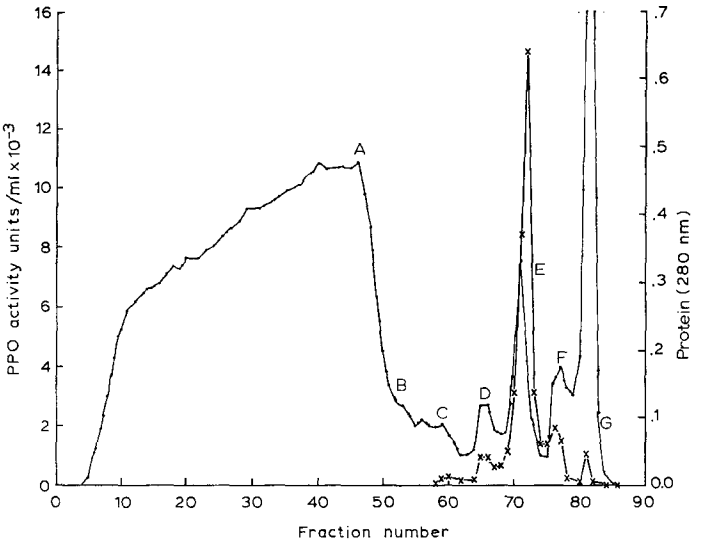

Fig. 1. Elution profile from a phenyl Sepharose CL-4B hydrophobic column for Ravat PPO activity (×—×, Units per ml); ●—●, protein content. The letters A–G indicate addition of a different eluent (see text).

TABLE I

PURIFICATION TABLE OF RAVAT 51 POLYPHENOLOXIDASE

Sample	*Volume (ml)*	*PPO activity (Units/ml)*	*Total Units*	*Protein (mg/ml)*	*Specific activity (Units/mg)*	*Yield (%)*	*Purification (α-fold)*
Crude extract*	200	908	181,600	6.50	140	—	1.0
Acetone powder buffer extract	350	1083	379,050	0.34	3185	—	23
"Before-column extract"	337	3250	1,096,875**	0.31	10,484	100**	75
After-column fractions 68–75	56	9267	583,821	0.28	33,096	53	236
"Purified PPO extract"	10	46,900	570,956	1.33	35,263	52	252

* The crude extract was prepared by homogenizing 100 g of grapes with 100 ml of Na_2HPO_4 buffer.

** There was an increase in the total Units, therefore the percentage yield was based on the "Before-column extract".

fractions. Further studies on the biochemical properties of the Ravat and Niagara PPO enzymes, using disc gel electrophoresis revealed that the purified PPO extract was almost nearly homogeneous.

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