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Fractionation of phenolase from green table olives *(Ascolana tenera* var.) by immobilized copper affinity chromatography.

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RESUMEN

Fraccionamiento de fenolasa de aceitunas verdes de mesa (variedad *Ascolana tenera)* mediante cromatografía de afinidad sobre cobre inmovilizado.

Fenolasa de aceitunas verdes de mesa de la variedad *Ascolana tenera* ha sido fraccionada mediante cromatografía de afinidad sobre cobre inmovilizado. Se han obtenido cuatro fracciones cromatográficas con elevada actividad específica. La fracción obtenida con tampón L-histidina fue la que presentó mayor actividad. Las cuatro fracciones activas se caracterizaron por sus respuestas a la especificidad y concentración del sustrato.

PALABRAS-CLAVE: *Aceituna verde de mesa — Ascolana tenera var. — Cromatografía de afinidad sobre cobre inmovilizado — Fenolasa (fraccionamiento).*

SUMMARY

Fractionation of phenolase from green table olives *(Ascolana tenera* var.) by immobilized copper affinity chromatography.

Phenolase from green table olives *(Ascolana tenera* var.) was fractionated by immobilized copper affinity chromatography. Four chromatographic fractions with high specific activities were obtained. The highest activity was found for the fraction eluted with L-histidine buffer. The four active fractions were characterized by their response to both specificity and concentration of the substrate.

KEY-WORDS: *Ascolana tenera var. — Green table olive — Immobilized copper affinity chromatography — Phenolase (fractionation).*

1. INTRODUCTION

Immobilized metal affinity chromatography, introduced a short time ago (Porath et al., 1975), is becoming a standard tool for the isolation of proteins. This modern chromatographic technique is based on the ability of proteins to form coordination bonds with metal due to the presence of surface chelating amino acids (metal combining sites). Heavy metal ions like Cu^{2+} and Ni^{2+} have been involved in the isolation and purification of oligopeptides and nucleotides and compounds to them related as enzymes (Hubert et al., 1980; Hemdam et al., 1985; Krishnan et al., 1985).

As regards the isolation and purification of the phenolase (E.C. 1.14.18.1.), also known as polyphenolase, polyphenoloxidase, tyrosinase, catechol oxidase, cresolase and catecholase, and widely distributed in many vegetable products, several methods have been used with varying degrees of success. Purification procedures including ammonium sulfate fractionation, ion exchange chromatography, gel filtration, acrylamide gel electrophoresis or high performance liquid chromatography (HPLC) have been reported for studies of phenolase in wheat (Interesse et al., 1983), apples (Richard-Forget et al., 1994), bananos (Montgomery et al., 1975), grapes (Yokotsuka et al., 1988; Lamikanra et al., 1992), potatoes (Matheis et al., 1977), artichokes (Zawistowski et al., 1987) and carrots (Söderhäll et al., 1989).

Studies of phenolase in green olives concerning its localization (Ben-Shalom et al., 1977) and its changes (Ben-Shalom et al., 1978) during fruit development have also been reported for enzyme preparations from differential centrifugation. With other separation procedures, other aspects of green olives phenolase may be studied. To-date, to our knowledge, separation by immobilized metal affinity chromatography of green olives phenolase has never attempted.

Therefore, the present study was undertaken to investigate the green olives phenolase by immobilized copper affinity chromatography.

2. MATERIALS AND METHODS

2.1. Plant material

In September 1993, green olives (Olea Europaea L.) of the variety "Ascolana tenera", utilized for Sevillan style olive preparation (Sciancalepore et al., 1984 a), were hand-picked from trees, 15 years old, grown at a modern private field of Campobasso (Italy) and used immediately throughout this investigation. All procedures, unless otherwise indicated, were carried out at 4°C.

2.2. Purification procedures

An acetone powder was prepared from fresh olive tissues as reported in a previous paper (Sciancalepore et al., 1984 b) and used to extract the enzyme. The extraction was performed using 0.1 M phosphate buffer (1:30, w/v), pH 6.5, containing 20 mM L-ascorbic acid. The slurry was stirred for 2 hr and then centrifuged at 10,000 x g for 20 min. The supernatant was treated with $(NH_4)_2S0_4$. The protein fraction which precipitated between 20 and 80% of salt concentration was resuspended in 10 mL of 0.05 M

phosphate buffer, pH 6.5, dialyzed against three changes of 2 L of the same buffer and then loaded on a Sephadex G-100 column (2.4 x 75 cm) previously equilibrated with 0.1 M phosphate buffer, pH 6.5. Fractions having phenolase activity were pooled and concentrated to 5 mL against polyethylene glycol (MW 40,000; Sigma Chemical Co., St. Louis, MO), and used for the following step.

2.3. Copper-chelate affinity chromatography

The biscarboxymethylamino-Sepharose 6B, prepared as described by Vijayalakshmi et al. (1979), was packed into a column (10 x 1.2 cm I.D.) with total volume of 10 mL. The column was washed with water prior to loading with an aqueous solution of $CuSO_4 \cdot 5H_2O$ (1 mg/mL).

After saturation had been reached, excess of copper was removed by washing with 2 bed volumes of water. At this stage, the amount of Cu^{2+} bound was 756 μmoles per gram of dry gel. The copper chelate adsorbent was equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. The enzyme preparation was then applied on the column which was eluted stepwise, first with 50 mL of equilibration buffer and successively with 50 mL of the same buffer containing 20 mM glycine, 20 mM histamine and 15 mM L-histidine. The flow-rate was maintained at 15 mL/h. Fractions of 5 mL were collected and assayed spectrophotometrically for the protein content and the phenolase activity. All chemicals used in the copper-chelate affinity chromatography experiments were of analytical grade.

2.4. Protein quantitation

Protein was estimated by absorbance at 280 nm or by the method described by Lowry et al. (1951), with bovine serum albumin (Sigma) as a standard.

2.5. Phenolase activity determination

All measurements of phenolase activity were carried out essentially as reported in a previous paper (Sciancalepore, 1985). The incubation mixture contained 0.5 mL enzyme preparation, 1.5 mL 0.1 M phosphate buffer (pH 6) and 1 mL 0.02 M 4-methylcatechol (substrate) in the same buffer. The assay mixture without the enzyme solution served as the control. The reaction mixture was oxygenated for 5 min prior to the addition of the enzyme solution. One unit of enzymatic activity was defined as the amount of the enzyme giving, under the abovementioned conditions, a change in absorbance of 0.05 min^{-1}.

Absorbance at 410 nm was recorded with a Varian DMS 100S spectrophotometer. Activity was linear with time for the first 60 sec of the reaction.

3. RESULTS AND DISCUSSION

3.1. Initial purification

Table I summarizes briefly the initial purification of the phenolase. After extraction, the ammonium sulphate treatments and the dialysis allowed a four-fold purification with a yield of 76%. Despite the heterogeneity of the green olives phenolase system (Ben-Shalom et al., 1978; Sciancalepore et al., 1983; Chen et al., 1991), it always eluted in a single peak during gel filtration using a Sephadex G-100 column. The yield of gel filtration was 57% with an overall purification factor of 6.6 for the pooled active fractions.

Table I
Initial purification of the phenolase

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification factor (fold)
Crude extract	265	1124	4,2	100	1
20-80% $(NH_4)_2S0_4$	46	854	18,6	76	4,4
Sephadex G-100	23	645	28,0	57	6,6

3.2. Fractionation by copper-chelate affinity chromatography

Figure 1 shows the elution profile obtained when the partially purified enzyme (23 mg protein, 645 activity units) from gel filtration step was applied to the column (10 x 1.2 cm I.D.) of copper-biscarboxymethylamino-Sepharose 6B, whereas Table II summarizes some properties of active fractions obtained from this column.

Table II
Properties of active fractions A, B, C and D of green olives phenolase recovered by copper-chelate affinity chromatography on column

Fractions	Total volume (mL)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification factor (fold)
A (tubes 3-8)	30	0.88	56	63.6	15.1
B (tubes 12-17)	30	0.49	92	186.2	44.3
C (tubes 22-26)	25	0.20	67	336.0	80.0
D (tubes 32-37)	30	0.30	109	361.2	86.0

After affinity chromatography, approximately 50% of the initial activity applied to the column was recovered (Table II). In particular, when the column was eluted with

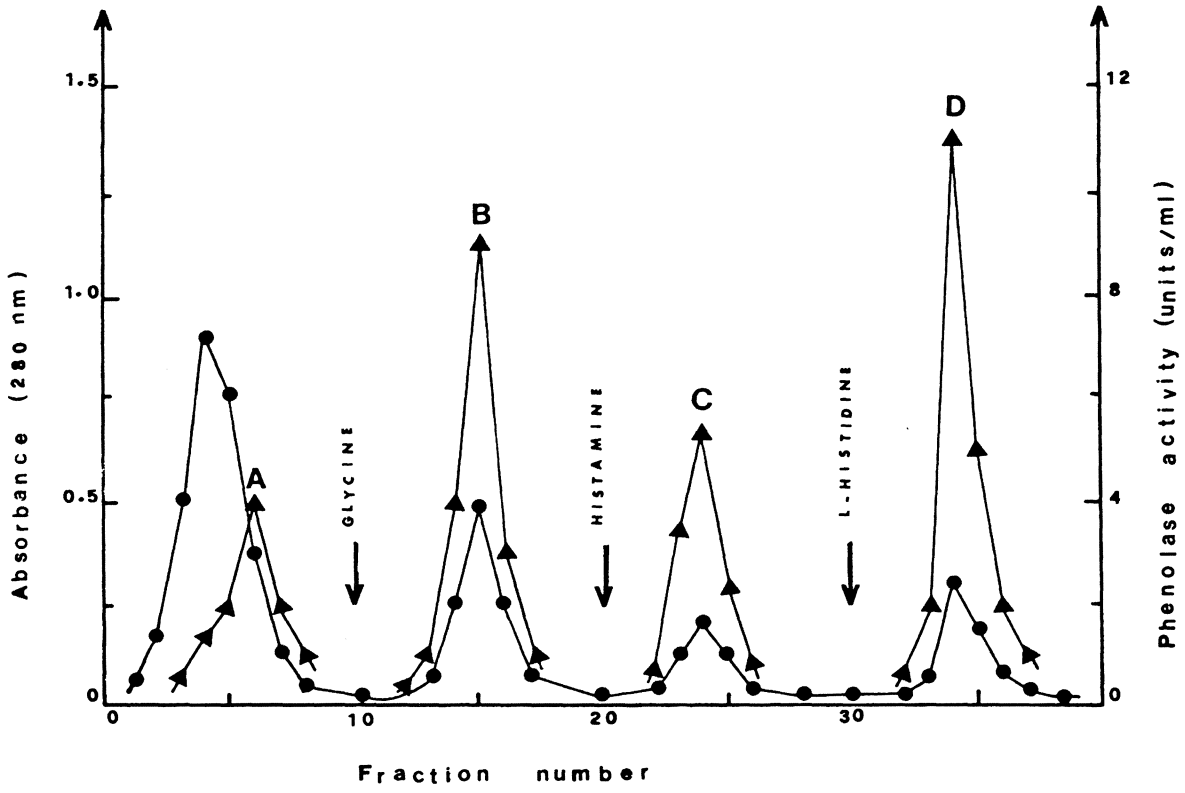

Fig. 1
Copper-chelate affinity chromatography of phenolase. A 5 mL sample (23 mg protein, 645 activity units) from Sephadex G100 chromatography was applied to the column after equilibration and then eluted stepwise as described in "Materials and Methods" section. -●-●-, absorbance; -▲-▲-, phenolase activity.

equilibration buffer, only one flow-through fraction (A) of activity containing 56 units with a purification factor of 15.1 was obtained (Fig. 1). It is likely that the enzyme components present in this fraction do not have effective copper ion binding sites on their molecular surfaces. To recover the adsorbed enzymatic proteins, the column was eluted stepwise using equilibration buffer containing various specific ligands. Because the order of stability constant to copper ion is glycine<histamine<histidine (Kikuchi et al., 1981), the elution buffers which contained these respective ligands were used in this order. The amino acid residues of the adsorbed proteins were gradually replaced by the ligands with greater stability constants, and the adsorbed proteins were eluted from the column. Three additional fractions (B, C and D) of activity were obtained. The majority of the activity was present in the B and D fractions which contained 92 units with a purification factor of 44.3, and 109 units corresponding to a purification factor of 86, respectively. The intermediate fraction C, eluted with 20 mM histamine in equilibration buffer, resulted in a 80-fold purification with 67 units of activity. These results suggest that the enzyme components of B, C and D fractions bind to the metal chelate gel via structural sites possibly composed of glycine, histamine and histidine residues, respectively.

To characterize partially the four active fractions (isoenzymes), recovered by this chromatographic technique, substrate specificity as well as K_m and V_{max} values of each fraction were determined. The specificity of all these fractions were tested using some phenolic compounds purchased from Sigma Chemical Co. (St. Louis MO) except oleuropein which was isolated in our laboratory from olive leaves with the method of Panizzi et al. (1960). Assay procedures were the same as described in the enzyme assay except that readings were taken at different wavelengths, given in Table III, for the various substrates used. The results of these assays, expressed as a percentage of activity towards 4-methylcatechol (Table III), show that the active forms of green olives phenolase had high catecholase activities. This phenomenon has been observed in phenolase of many plants (Benjamin et al., 1973; Rivas et al., 1973; Hasegawa et al., 1980). Fractions C and D were very active towards oleuropein and caffeic acid, and moderatly active towards protocatechuic acid, whereas the other two fractions A and B had low activities towards the same diphenolic compounds. Very low activity was found towards polyphenolic compounds tested for all active fractions.

Activity of all four active fractions as a function of substrate concentration was determined as described in enzyme assay for 4-methylcatechol concentrations ranging from 1 to 20 mM (Table IV). By using Lineweaver-Burk reciprocal plots, the K_m values obtained for C and D fractions were, respectively, 7.13 and 6.46 mM, which are in general agreement with the K_m values of phenolase from other plants for the same phenolic substrate (Lerner et

al.,1976; Kidron et al.,1978; Interesse et al., 1983). A lower affinity to 4-methylcatechol was noted for A and B active fractions.

Table III
Substrate specificity of active fractions A, B, C and D of green olives phenolase recovered by copper-chelate affinity chromatography on column

Substrate*	Relative activity (%) compared to 4-methylcatechol	A	B	C	D
diphenols:					
4-methylcatechol	(410 nm)**	100	100	100	100
Oleuropein	(392 nm)**	65	56	95	98
Caffeic acid	(395 nm)**	67	55	88	94
Protocatechuic acid	(430 nm)**	19	10	27	38
polyphenols:					
(-) Epicatechin	(395 nm)**	17	9	12	14
Chlorogenic acid	(444 nm)**	7	4	6	9

*Substrate concentration was 10 mM.
**Wavelength used for determining phenolase activity.

Table IV
K_m and V_{max} values* of active fractions A, B, C and D of green olives phenolase recovered by copper-chelate affinity chromatography on column

Fractions	Km (mM)	Vmax ($\Delta_{O.D.}$/min)
A (tubes 3-8)	16.24	0.085
B (tubes 12-17)	13.42	0.092
C (tubes 22-26)	7.13	0.105
D (tubes 32-37)	6.46	0.124

*All determinations were performed using 4-methylcatechol as substrate

4. CONCLUSIONS

The results reported in this study indicate that the copper-chelate affinity chromatography technique, applied for the first time to the study of green olives phenolase, shows itself to be very useful both for bringing the multiple active forms into evidence, and for the recovery of significant amounts of enzymatically active proteins using an easy and simple procedure of isolation and purification of the enzyme.

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