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Journal of Chromatography A, 667 (1994) 141–153

JOURNAL OF CHROMATOGRAPHY A

New approaches for separating and purifying apple polyphenol oxidase isoenzymes: hydrophobic, metal chelate and affinity chromatography

F. Richard-Forget*,[a], P. Goupy[a], J. Nicolas[b]

[a]*Institut National de la Recherche Agronomique, Station de Technologie des Produits Végétaux, Domaine Saint Paul BP 91, F-84143 Montfavet Cedex, France*

[b]*Conservatoire National des Arts et Metiers, Chaire de Biochimie Industrielle et Agroalimentaire, 292 Rue Saint-Martin, F-75141 Paris Cedex 03, France*

(First received September 28th, 1993; revised manuscript received December 16th, 1993)

Abstract

Apple polyphenol oxidase (PPO) was subjected to hydrophobic, metal chelate and affinity chromatography. Among numerous hydrophobic supports, Phenyl-Sepharose CL4B appeared to be the most appropriate matrix for the purification of highly hydrophobic proteins such as PPO. With immobilized copper affinity chromatography, four fractions were obtained. Using electrophoresis experiments, it was shown that these fractions differed from the isoenzymes separated by ion exchange on DEAE-Sepharose CL6B. Apple PPO was also adsorbed on synthetized affinity resins with competitive inhibitors (*p*-coumaric and *p*-hydroxybenzoic acid) coupled via an azo linkage to hexamethylenediamineagarose. These affinity matrices were also used to evaluate the inhibition constants of the ligands. Lastly, apple PPO was successively chromatographed on hydrophobic, metal chelate and affinity columns. This protocol led to a 280-fold purified fraction representing 25% of the crude extract activity.

1. Introduction

Browning reactions which occur during the handling, storage, processing and cooking of fruits or vegetables are mainly initiated by the enzyme polyphenol oxidase (*o*-diphenol: O_2 reductase, EC 1.10.3.1; PPO). Because of the deleterious effect of the enzymatic browning on food products, numerous studies have been devoted to PPO, and more precisely to its purification. Nevertheless, only a few PPOs have been purified to apparent homogeneity. These included PPOs from peach, grape, spinach, tomato and potatoes and several fungal PPOs [1].

The first step in the purification that has been commonly applied was the removal of inactive proteins by fractionation with ammonium sulphate. Further purification frequently involved conventional chromatographic methods of separation such as adsorption, gel filtration and ion-exchange chromatography. Several papers reported also the use of hydrophobic chromatography as a rapid, reproducible and effective

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E0060-8

purification step. This last method, introduced in 1978 by Jen and Flurkey [2] for peach PPO purification, was successfully applied to PPOs from different sources, *e.g.*, PPO from grape [3], pear [4] and broad bean [5]. Moreover, systematic studies on alkyl- and aminoalkylagarose efficiency have been carried out by Iborra *et al.* [6], Flurkey and Jen [7] and Ingebrigtsen and Flurkey [8].

Affinity chromatography was described as early as 1953 by Lerman [9] for mushroom PPO purification. However, subsequent studies on the affinity chromatography of PPO were mainly applied to fungal enzymes and only a small part to PPOs from fruits and vegetables. The ligands employed included substrates [10], competitive inhibitors [6,11,12] and antibodies [8,13]. Another affinity method, referred to as metal chelate or immobilized metal affinity chromatography (IMAC), introduced in 1975 by Porath *et al.* [14], has been successfully applied to the purification of PPO from Jerusalem artichoke [15] and carrot [16]. Nevertheless, although this technique has frequently been used to purify metalloenzymes such as superoxide dismutase [17,18] and carboxypeptidase [19], there has been no application to PPOs from other origins.

Concerning apple PPO, Harel *et al.* [20] and Walker and Hulme [21] partially purified the enzyme extracted from the peel using DEAE-cellulose chromatography, whereas Stelzig *et al.* [22] used calcium phosphate gel adsorption. Janovitz-Klapp *et al.* [23] reported the use of hydrophobic chromatography for purifying the cortex enzyme. Fractionation by ammonium sulphate precipitation followed by filtration on Phenyl-Sepharose CL4B gel resulted in 120-fold purified PPO from Red Delicious cortex, with a total yield close to 40%. However, no systematic study on the efficiency of different hydrophobic supports has been carried out by previous workers. It is not evident that Phenyl-Sepharose CL4B was the most appropriate gel for purifying apple PPO. Moreover, to our knowledge, affinity chromatographic methods have not been used to obtain highly purified apple PPO preparations. Hence, the purpose of this work was, on the one hand, to analyse the hydrophobic behaviour of apple PPO with different chromatographic supports as influenced by experimental parameters (temperature, pH and ionic strength of the elution buffer) in order to establish the optimum conditions for the enzyme purification. In addition, we applied for the purification of apple PPO the two affinity chromatographic methods, *i.e.*, IMAC and affinity chromatography using a competitive inhibitor as the ligand on agarose. Ion-exchange chromatography is also required in order to compare the heterogeneity of the apple PPO system in terms of copper affinity and isoelectric point value. This should also clarify the discrepancies that exist among the number of isoforms, which varied between one [24,25], two [21,26] and three [20,23] for apple PPO.

2. Experimental

2.1. Materials

The apples, variety Red Delicious, were picked at their commercial maturity and used as an enzyme source. DEAE-Sepharose CL6B, chelating Sepharose CL6B, Phenyl-Sepharose CL4B and Octyl-Sepharose CL4B were obtained from Pharmacia (Uppsala, Sweden). Phenylagarose, phenylaminoagarose and the two kits of alkyl- and aminoalkylagaroses, were purchased from Sigma (St. Louis, MO, USA). HMD-Ultrogel was supplied by IBF (Clichy, France). All other chemicals were of analytical-reagent grade from Sigma.

2.2. Initial purification step

The PPO was extracted with McIlvaine's buffer at pH 7.2, containing 0.5% Triton X-100 and 15 m*M* ascorbic acid, according to the method described by Janovitz-Klapp *et al.* [23]. Inactive proteins were partially removed by ammonium sulphate precipitation (30% saturation), the resulting supernatant being labelled "S_{30} extract".

2.3. PPO and protein assays

PPO activity was assayed polarographically according to the method of Janovitz-Klapp *et al.* [23]. Activity was expressed as nanomoles of oxygen consumed per second (nanokatals) under the assay conditions.

The protein content was determined according to Bradford [27] with bovine serum albumin as a standard.

2.4. Hydrophobic chromatography

On micro-columns

Chromatography was carried out at 4 and 25°C. Each hydrophobic support, *ca.* 1 ml, was packed into a small column (0.75 cm^2 section) and equilibrated with 50 m*M* sodium phosphate buffer (pH 6.5) containing 2 *M* ammonium sulphate. A 1-ml volume of "S_{30} extract" was dialysed overnight against the equilibration buffer and then applied to each column. Unbound proteins were thoroughly washed from the columns with equilibration buffer (5 × 1 ml). The adsorbed enzyme was eluted stepwise with 50 m*M* phosphate buffers (pH 6.5) containing ammonium sulphate with gradually decreasing concentration from 1.8 to 0.2 *M* in 0.1 *M* steps (5 × 1 ml). The columns were washed with water (5 × 1 ml) and 50% ethylene glycol (5 × 1 ml) to remove tightly bound enzyme. The PPO activity was determined in each 1-ml fraction.

On an analytical column

A 90-ml volume of the "S30 extract" dialysed overnight against 50 m*M* phosphate buffer (pH 6.5) containing KCl and $(NH_4)_2SO_4$ (both 0.5 *M*) was loaded on to a Phenyl-Sepharose CL4B column (8 × 2.5 cm I.D., 40-ml bed) equilibrated with the former buffer at a flow-rate of 100 ml h^{-1}. After elution of unbound proteins with the equilibration buffer, the PPO was eluted using the same buffer containing KCl and $(NH_4)_2SO_4$ (both 0.1 *M*). Proteins still bound to the gel were removed by washing with water and 50% ethylene glycol. The absorbance at 280 nm and the PPO activity were determined in each 7-ml fraction. The active fractions were combined and labelled as "first purified PPO extract".

2.5. Affinity chromatography

Preparation of the p-coumaric and p-hydroxybenzoic acid HMD-Ultrogel (p-COU-HMD and p-HBZ-HMD)

p-Coumaric and *p*-hydroxybenzoic acid were coupled to hexamethylenediamineagarose (HMD-Ultrogel) via an azo linkage, with a protocol adapted from Cuatrecasas [28], Cuatrecasas and Anfinsen [29] and Cohen [30]. This protocol included three steps, as follows.

Preparation of p-aminobenzamidohexylagarose. A 20-ml volume of hexamethylenediamineagarose was suspended in two volumes of 0.2 *M* sodium borate (pH 9.3) and cooled to 4°C. A solution of *p*-nitrobenzoyl azide (0.1 *M* in 50% dimethylformamide) was added gradually to the gel suspension with continuous stirring. The reaction mixture was then stirred at 4°C for 1 h and at 25°C for an additional 3–4 h. Completion of the acylation reaction can be checked by the loss of colour reaction with trinitrobenzenesulphonic acid [28]. The gel was then washed thoroughly with five volume of 50% dimethylformamide and ten volumes of distilled water.

The resulting *p*-nitrobenzamidohexylagarose was suspended in a solution of 0.2 *M* dithionite in 0.5 *M* sodium hydrogencarbonate (pH 8.5) and the mixture was kept at 40°C for 1 h with continuous stirring. Decoloration has to be total. The product obtained was washed with ten volumes of distilled water and in this form was stored at low temperature for later use.

Diazotation of the p-aminobenzamidohexylagarose support. The previous derivative was suspended in an equal volume of ice-cold 0.5 *M* hydrochloric acid. A solution of sodium nitrite (0.1 *M*) was added and the mixture was stirred for 7 min in an ice-bath. The gel was then

washed thoroughly with ten volumes of cold distilled water.

Coupling of phenolic ligand. The diazotized support was resuspended in an equal volume of cold 0.2 *M* acetate buffer (pH 5.5). A solution of *p*-coumaric or *p*-hydroxybenzoic acid (20 m*M*) dissolved in a similar volume of acetate buffer was added in one portion to the suspension of diazonium agarose and stirred in an ice-bath. Formation of the red azo gel began immediately and coupling was achieved in 30 min. The *p*-COU-HMD and *p*-HBZ-HMD gels were filtered, washed thoroughly with five volumes of 50% dimethylformamide followed by ten of distilled water and stored at 4°C as a suspension in distilled water supplemented with thymol (1%).

The concentrations of bound phenolic ligand were evaluated spectrophotometrically at 287 nm (*p*-coumaric acid) or 250 nm (*p*-hydroxybenzoic acid) by measuring the difference in absorbance between the initial solution at 20 m*M* and the filtered solution.

Column chromatography

A 5-ml volume of *p*-COU-HMD or *p*-HBZ-HMD was packed into a small column (1 cm^2 section) and equilibrated with 25 ml of McIlvaine's buffer (pH 4.5) at a flow-rate of 80 ml h^{-1}. A 6-ml volume of the "first purified PPO extract", dialysed overnight against the equilibration buffer, was loaded on to the column. After elution of unbound proteins by the equilibration buffer, the PPO was eluted with 0.1 *M* phosphate buffer (pH 7.5). The absorbance at 280 nm and PPO activity were determined in each 1-ml fraction.

N.B.: In order to determine non-specific or ion-exchange adsorption, the previous protocol was carried out with control columns, packed with activated but no ligand-coupled matrix.

Evaluation of inhibition constants

Protocol adapted from Graves and Wu [31]. The experiment was carried out at 4°C. A 1.6-ml volume of affinity support was equilibrated with McIlvaine's buffer (pH 4.5) in a total volume of 6 ml. A 200-μl volume (50 nkatal) of the "first purified extract" dialysed overnight against the former buffer were added after each 40 min. After each addition followed by a homogenisation step and when equilibrium was assumed to have been achieved, 2×100 μl were taken from the supernatant in order to assay in duplicate unbound PPO activity. The ratio between the two enzymatic fractions, free and trapped in the gel, was calculated and reported graphically for each enzyme addition number. Once the experiment was completed, 5 ml of McIlvaine's buffer (pH 7.5) were added to the filtered matrix in order to evaluate the total amount of bound enzyme.

Protocol adapted from Dunn and Chaiken [32]. Volumes of 4 ml of affinity supports were packed into a small column (0.75 cm^2 section) and equilibrated with 20 ml of McIlvaine's buffer (pH 4). A 1-ml volume of apple PPO "first purified extract" dialysed overnight against the former buffer was loaded on to the column. Enzyme elution was immediately achieved with McIlvaine's buffer (pH 7.5). The same experiment was repeated with nine different pH values of the elution buffer, *i.e.*, 7.5, 7.0, 6.6, 6.15, 5.9, 5.8, 5.7, 5.5 and 5.1. The pH value and PPO activity were determined in each 0.5-ml fraction. Decreasing pH values were employed to obtain different concentrations of effective bound inhibitor. As inhibition is mainly due to the protonated form of carboxylic acid [33], the concentration of effective inhibitor varied with the pH and followed the equation $[I_e] = [I_t]/(1 + 10^{pH-pK})$, where I_e and I_t represent the effective (protonated form) and total inhibitor (protonated and ionized form). For each chromatographic step, the elution volume of PPO activity was plotted against the concentration of effective inhibitor.

2.6. Ion-exchange chromatography

A 50-ml volume of the "first purified extract" was dialysed overnight against 10 m*M* sodium

phosphate buffer (pH 6.5) and applied to a DEAE-Sepharose CL6B column (10 × 2 cm I.D.) pre-equilibrated with the same buffer. The column was eluted with the equilibration buffer and the eluted protein was monitored by measuring the absorbance at 280 nm. After the absorbance had returned to the baseline, further elution was carried out with a linear salt gradient from 0 to 0.16 *M* $(NH_4)_2SO_4$ in 10 m*M* phosphate sodium buffer (pH 6.5). Proteins that were still bound to the gel were removed with 40 ml of the equilibration buffer successively supplemented with 0.16 and 0.66 *M* $(NH_4)_2SO_4$. The flow-rate was fixed at 80 ml h^{-1} and the absorbance at 280 nm and PPO activity were determined in each 5-ml fraction.

2.7. Immobilized copper affinity chromatography (IMAC)

IMAC was carried out using a method adapted from those described by Miyata-Asano *et al.* [34] and Zawistowsky *et al.* [15]. Chelating Sepharose CL6B was packed in a working column (20 × 1 cm I.D.) and in a guard column (6 × 1 cm I.D.). The working column was loaded with an aqueous solution of $CuSO_4 \cdot 5H_2O$ (6 mg ml^{-1}) until metal was observed in the eluate. The excess of copper was washed from the column with 50 m*M* phosphate buffer (pH 6.5) containing 0.1 *M* KCl at a flow-rate of 120 ml h^{-1}. Both columns coupled in sequence were equilibrated with the previous buffer. An 18-ml volume of the "first purified PPO extract" was applied to the working column. After removal of the unbound protein with 50 ml of the equilibration buffer, the PPO activity was eluted stepwise by using three eluent solutions (75 ml each) which corresponded to the equilibration buffer successively supplemented with 30 and 125 m*M* glycine and 10 m*M* histidine. The guard column was used to adsorb any copper ion that leaked from the working column during the enzyme elution. Columns were regenerated with 75 ml of the equilibration buffer containing 50 m*M* EDTA. The absorbance at 280 nm and PPO activity were determined in each 3.5-ml fraction.

2.8. Electrophoretic experiments

Isoelectrofocusing and electrophoresis in polyacrylamide gels were performed with a Phast system (Pharmacia) using Phastgels IEF 4–6.5 or Phastgels gradient 8–25. The migration conditions were those specified by Pharmacia. Gels stained for PPO activity were immersed in the substrate solution supplemented with *p*-phenylenediamine (0.05%). The specific detection of some isoforms required the addition of SDS (0.55%) to the staining solution.

3. Results and discussion

3.1. Purification of apple PPO by hydrophobic chromatography

Different factors involved in hydrophobic chromatography were analysed, *i.e.*, matrix hydrophobicity (type of spacer arm, length of alkyl or aminoalkyl head chain), temperature and pH. Applying the protocol described under Experimental to each type of gel, we measured, for each $(NH_4)_2SO_4$ concentration of the elution buffers, the ratio of cumulated eluted activity to the loaded activity. Typical graphs are shown in Fig. 1 for Sepharose CL4B, the Phenyl-Sepharose CL4B and Octyl-Sepharose CL4B at 4 and 25°C. To characterize the different packing materials, a hydrophobicity parameter termed "C50" was introduced; this represents the ammonium sulphate concentration required to elute 50% of the loaded activity, thus a low C50 value corresponded to a strong hydrophobic matrix.

Fig. 1 illustrates considerable adsorption of apple PPO on unsubstituted Sepharose CL4B. Moreover, the C50 value of 1.76 attributed to this matrix was not affected by the temperature. Because of the high salt concentrations of the equilibration buffer, this absorption, already reported by Ingebrigtsen and Flurkey [8], could not be explained by ionic interactions but was presumably due to hydrophobic interactions with methyl groups inside the matrix and the 3–6 methylene diether bridges present in every sec-

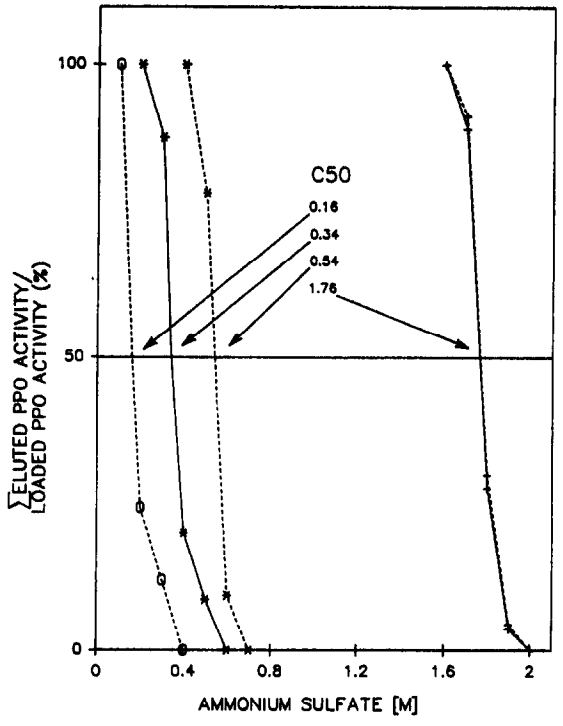

Fig. 1. C50 values determined for CL4B (+) Sepharose, (*) Phenyl-Sepharose and (○) Octyl-Sepharose at 4°C (dashed lines) and 25°C (full lines). For Octyl-Sepharose at 25°C, ethylene glycol was required to elute PPO activity.

ond galactose residue in polysaccharide chains [35,36].

Apple PPO was strongly retained on Octyl-Sepharose CL4B (Fig. 1) as ethylene glycol was required to partially elute enzyme activity at 25°C. At 4°C, full elution was achieved with very low ammonium sulphate concentrations. Under these conditions, a C50 value of 0.16 was associated with Octyl-Sepharose. Such results explain the choice of Octyl-Sepharose for the separation of proteins with low hydrophobicity. It has been successfully used for the purification of wheat gliadins [37]. Phenyl-Sepharose, for which higher C50 values (0.34 at 4°C and 0.54 at 25°C) were determined, is to be preferred for more hydrophobic proteins such as PPO [23,38,39], peroxidase [40,41] or lipoxygenase [42]. When the temperature was increased from 4 to 25°C, an increase in PPO adsorption was observed with Octyl- and Phenyl-Sepharose CL4B. Thus, the C50 value decreased by 0.2 *M* when the temperature was increased from 4 to 25°C.

Nature of the spacer arm

Three gels differing in the nature of their spacer arm were tested with apple PPO, namely an ether group (Phenyl-Sepharose purchased from Pharmacia), an amino group (phenyl-aminoagarose from Sigma) and a butyl propyl ether group (phenyl agarose ether from Sigma). The structures and C50 values associated with the three gels are given in Table 1. The highest hydrophobicity was observed for Phenyl-Sepharose. The introduction of an amino group led to considerably weaker hydrophobic interactions, in agreement with the works of Flurkey and Jen [7], who suggested the probable existence of additional electrostatic interactions in these matrices. However, under the high salt conditions employed in this investigation, the electrostatic effects should have been minimized. The lowest hydrophobicity was observed for phenyl agarose ether (C50 = 1.67 at 4°C and 1.48 at 25°C) and could be explained by intrinsic hydrophobic interactions occurring in these long chains [43].

Table 1
Structures and C50 values associated with Phenyl-Sepharose, phenylaminoagarose and phenyl agarose ether

Material	Structure[a]	C50	
		4°C	25°C
Phenyl-Sepharose (Pharmacia)	Aga–O–CH_2–CHOH–CH_2–O–C_6H_5	0.54	0.34
Phenyl-aminoagarose (Sigma)	Aga–O–CH_2–CHOH–NH–C_6H_5	1.12	1
Phenyl agarose ether (Sigma)	Aga–O–$(CH_2)_3$–O–$(CH_2)_4$–O–CH_2–CHOH–CH_2–O–C_6H_5	1.67	1.48

[a] Structures are those given by the manufacturers.

Length of the alkyl or amino alkyl head chains

The effect of chain length was investigated by the use of two hydrophobic series, alkyl- and aminoalkylagarose kits, purchased from Sigma. Because of the lack of a corresponding unsubstituted matrix, Sepharose 4B was selected as a reference. The C50 values associated with each chromatographic support are reported in Fig. 2. Within each series, an increasing chain length resulted in higher hydrophobicity. Concerning alkylagarose, a linear relationship was observed up to ten carbon atoms (the C50 value decreased from 1.88 to 1). With longer chains, a lower temperature or the use of ethylene glycol was required to elute the adsorbed PPO activity. Moreover, in this series, the influence of temperature appeared only after six carbon atoms. Similar results were reported by Shalthiel [44], Iborra *et al.* [6], Flurkey and Jen [7] and Ingebrigtsen and Flurkey [8]. Flurkey and Jen [7] reported that peach PPO was slightly retained on hexyl and fully retained on octyl columns. Iborra *et al.* [6] and Ingebrigtsen and Flurkey [8] observed that larger amounts of Dopa oxidase or mushroom tyrosinase were adsorbed as the alkyl chain length increased. Concerning the aminoalkyl kit at 25°C, a small increase in hydrophobicity was observed from no to six carbon atoms and then a larger increase for gels from six to twelve carbons, whereas at 4°C the increase in hydrophobicity was only apparent between six and twelve carbons (Fig. 2B). Concerning peach PPO, a less hydrophobic protein than apple PPO, a slight retention was observed by Flurkey and Jen [7] only after eight carbon atoms. The negative effect of introducing a charged group was revealed again by the previous studies. Despite the numerous reports in the literature on hydrophobic chromatography, no real explanation for this effect has been proposed.

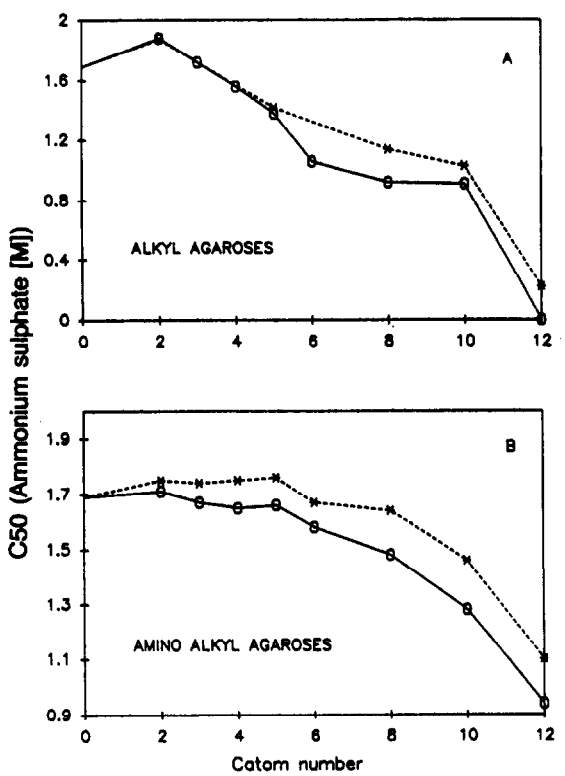

Fig. 2. Effect of head chain length on (A) alkyl- and (B) aminoalkylagarose hydrophobicity at 4°C (dashed lines) and 25°C (full lines).

pH of the elution buffer

The influence of elution pH was analysed between 4.5 and 8 for the alkyl- and aminoalkylagarose series. Concerning the alkylagarose kit, a typical result is illustrated in Fig. 3 with Phenyl-Sepharose CL4B. A negative effect of increasing pH on the gel hydrophobicity was observed. However, the modification of protein charge by changing the pH conditions has also to be taken in account. On the other hand, with aminoalkylagarose, no clear relationship was

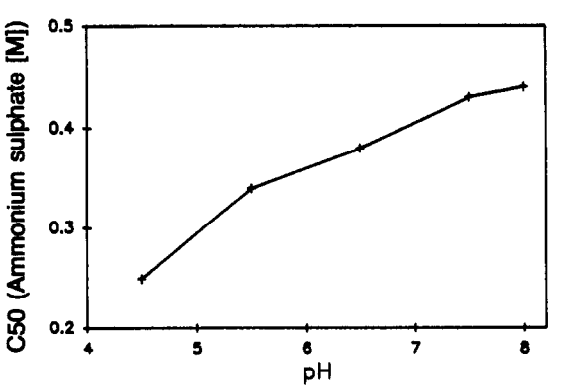

Fig. 3. Effect of pH on Phenyl-Sepharose hydrophobicity at 25°C.

obtained between pH and C50 values. Although Schmück *et al.* [45] observed maximum hydrophobic binding at a pH value equal to the isoelectric point of ovalbumin, the C50 value was not minimum for pH values close to 5, which corresponded to the isoelectric point region of apple PPO isoenzymes [23].

In conclusion to this study, for the correct application of hydrophobic chromatography to the purification of enzymatic proteins such as PPO, two main factors should be considered: first, the nature and hydrophobicity of both the adsorbent and the protein, and second, the elution conditions, including pH and temperature.

Moreover, it appears that for the purification of strong hydrophobic proteins such as apple PPO at normal temperature (25°C), Phenyl-Sepharose was the best support. Nevertheless, Octyl-Sepharose, used at 4°C, could also represent a convenient adsorbent. These conclusions were in agreement with earlier reports dealing with the suitability of Phenyl-Sepharose for grape [3], apple [23] and peach [38] PPO purification.

3.2. *DEAE-Sepharose CL6B ion-exchange chromatography*

A typical elution pattern of the "first purified extract" from a DEAE-Sepharose CL6B column is illustrated in Fig. 4. The eluted activity representing 90% of the loaded activity was recovered in three fractions, two major peaks labelled B and C (35 and 60%) and one minor peak labelled A (5%). These results suggested the presence of three isoenzymes characterized by different isoelectric points, lower than 6.5. The elution of inactive proteins, before and after the gradient step, reflected the further purification resulting from this procedure. The purification factor was about 3- and 7.5-fold for B and C, respectively (Table 2). These results, attesting to the purification efficiency of this chromatographic step, have been exploited in different PPO purification protocols [46–50]. Electrophoretic patterns of the "first purified extract" and of fractions A, B and C are shown in Fig. 5A

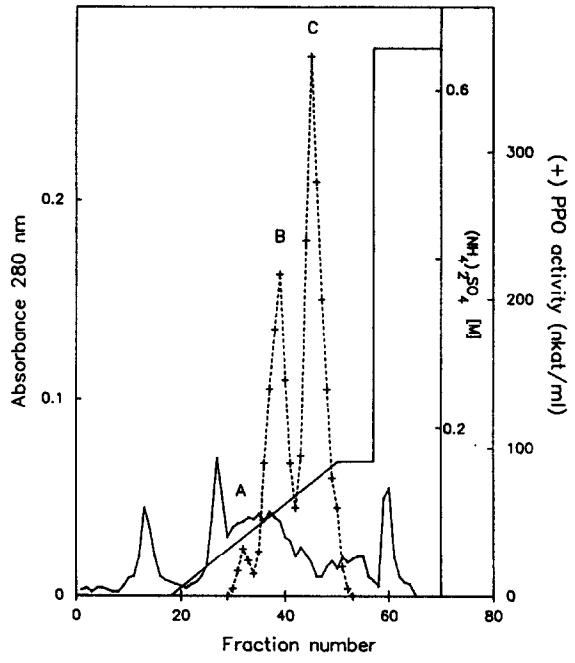

Fig. 4. Ion-exchange chromatography on DEAE-Sepharose CL6B of apple PPO.

(isoelectrofocusing pH 4–6.5) and B (normal electrophoresis). By isoelectrofocusing, the initial extract revealed two major bands in the pH 4.5–5 region and one minor, faint band whose isoelectric point was near neutral, in agreement with the results of Janovitz-Klapp *et al.* [23]. Fraction B contained one isoenzyme characterized by an isoelectric point of 5 and fraction C contained the other isoenzyme corresponding to an isoelectric point of 4.8. Concerning fraction A, the addition of SDS (0.55%) was required in order to reveal the presence of the band with an isoelectric point close to 6.5. SDS led to an increase in PPO activity, as has already been pointed out [51].

3.3. *Immobilized copper affinity chromatography (IMAC)*

A typical elution profile of an apple PPO "first purified extract" obtained with IMAC is shown in Fig. 6. When the column was eluted with the equilibration buffer, a small part (less than 5%) of unbound PPO activity was obtained. This

Table 2
Purification of apple PPO using DEAE-Sepharose CL6B ion-exchange chromatography, IMAC and affinity chromatography with competitive inhibitors as ligands

Method	Extract or fraction	Proteins (mg ml^{-1})	Activity (nkat ml^{-1})	Specific activity (μkat mg^{-1})	Purification factor (-fold)
	Crude extract	0.830	250	0.30	1
	First purified extract	0.058	285	4.91	16.4
DEAE-Sepharose CL6B ion-exchange chromatography	*Most actives fractions*:				
	A	0.006	30	5.33	17.8
	B	0.017	210	14.47	48
	C	0.009	360	36.4	120
IMAC	*Most actives fractions*				
	F1, 30 m*M* glycine	0.010	80	7.78	25.9
	F2, 125 m*M* glycine	0.020	425	20.1	67
	F3, 10 m*M* histidine	0.015	30	1.85	6.2
Affinity chromatography	*Most actives fractions*				
	p-COU-HMD	0.020	320	16	79
	p-HBZ-HMD	0.022	210	9.4	49

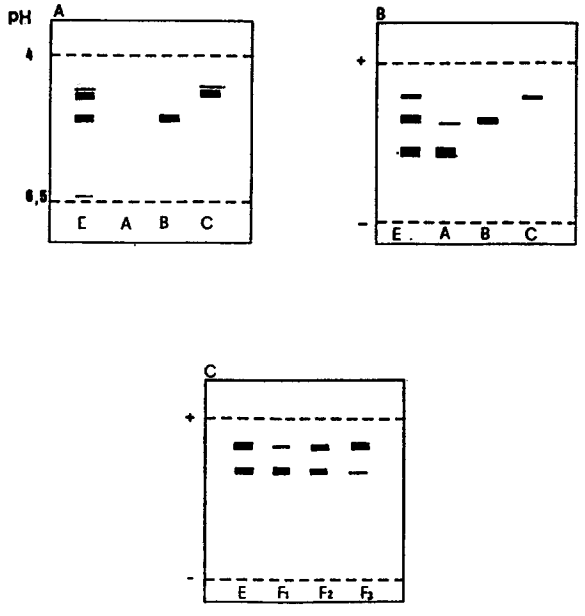

Fig. 5. Electrophoresis profiles of the "first purified extract" (E), fractions A, B and C separated by ion exchange and fractions F_1, F_2 and F_3 separated by IMAC. All gels were revealed by specific staining (PPO activity). (a) IEF (pH 4–6.5) applied to E, A, B and C; (b) electrophoresis (Phastgel 8–25, 0.55% SDS in the staining solution) applied to E, A, B and C; (c) electrophoresis (Phastgel 8–25) applied to E, F_1, F_2 and F_3.

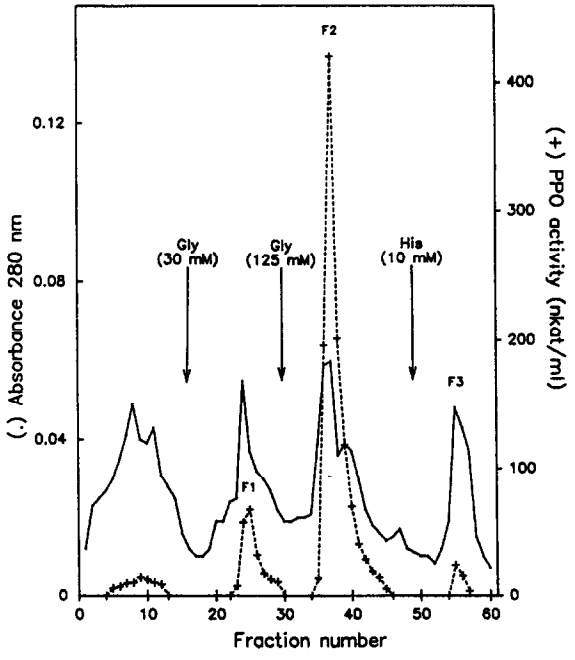

Fig. 6. IMAC of apple PPO on chelating Sepharose CL6B.

activity was not the result of overloading as the unbound percentage remained constant when the loaded activity was varied. Stepwise elution with glycine (30 and 125 m*M*) and then with 10 m*M*

histidine resulted in three additional fractions (F_1, F_2 and F_3). The bulk of the activity was recovered in fraction F_2, which contained 75% of the recovered activity, while fractions F_1 and F_3 represented 15 and 7%, respectively. Moreover, a high yield close to 100% compared with the loaded activity was obtained. Concerning the purification efficiency of this procedure (Table 2), the most active fraction (F_2) showed a purification rate of 67, F_1 was 1.6-fold purified compared with the initial extract while the purification factor of F_3 was decreased.

The difference in copper affinity among the three eluted fractions did not reflect differences in isoelectric points. Each electrophoretic pattern (Fig. 5C) revealed two bands (corresponding to the two isoenzymes characterized by isoelectric points of 4.8 and 5), the proportion of which varied with the fraction considered. According to this result, it appeared that the DEAE-Sepharose CL6B ion exchanger resolved the three isoenzymes more selectively than IMAC. The isoenzyme with an isoelectric point of 5 was predominant in F_1. An opposite result was observed for F_3. Thus, the former isoenzyme is also characterized by a weak copper affinity compared with that associated with an isoelectric point of 4.8. This difference in copper affinity presumably resulted from different numbers of copper chelating amino acid groups exposed on the surface of the enzyme molecule, in accordance with the work of Sulkowsky [52].

3.4. *Affinity chromatography on p-COUM- and p-HBZ-HMD*

p-Coumaric and *p*-hydroxybenzoic acid are known to be competitive inhibitors of PPO [33,53,55]. These acids were coupled to HMD-Ultrogel using the protocol described previously. The *p*-coumaric and *p*-hydroxybenzoic acid content of the synthetized affinity resins were determined spectrophotometrically to be close to 9 μmol ml^{-1} of packed gel. Considering the concentration of free NH_2 residues in the HMD-Ultrogel (between 7 and 10 μmol ml^{-1}) as stated by the manufacturer, these results corresponded to good binding yields.

Preliminary studies on activated but non-ligand-coupled matrices have shown that no adsorption due to non-specific or ion-exchange interactions occurred with apple PPO.

Column chromatography

Adsorption PPO on the derivatives was achieved at pH 4.5. At this pH, close to the p*K* value of the two carboxylic acids, the inhibition constants of *p*-coumaric and *p*-hydroxybenzoic acid were determined by Janovitz-Klapp *et al.* [33] to be 0.04 and 0.57 m*M*, respectively. According to these workers, the competitive inhibition of PPO was mainly due to the protonated form of carboxylic aromatic acids. This led us to elute enzyme activity at pH 7.5, at which *p*-coumaric and *p*-hydroxybenzoic acid were in the carboxylate form. Under these conditions, typical elution patterns obtained for apple PPO "first purified extract" on the two affinity supports are illustrated in Fig. 7. When the columns were eluted with equilibration buffer, a major

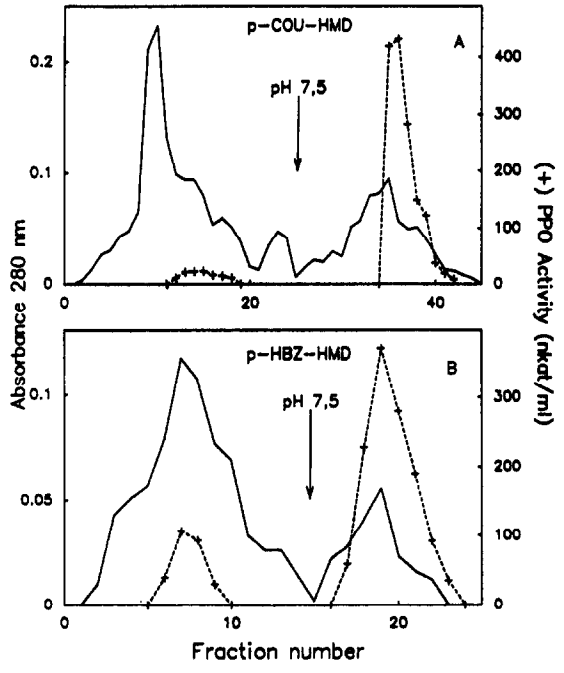

Fig. 7. Affinity chromatography of apple PPO on Ultrogel coupled with (a) *p*-coumaric acid or (b) *p*-hydroxybenzoic acid.

protein peak was eluted concomitantly with a small part of unbound PPO activity. This small percentage remained constant whatever the loaded activity was, equal to 5% and 25% with *p*-COU-HMD and *p*-HBZ-HMD, respectively. Such a difference was explained as a direct result of the value of the inhibition constants. Nevertheless, the bulk of the activity was eluted in a sharp peak when the elution buffer pH was increased to 7.5; excellent yields of recovered activity, close to 100% were obtained with the two types of resin. A fivefold purification was achieved with the chromatographic step on *p*-COU-HMD, while the purification factor was only *ca.* 3 with *p*-hydroxybenzoic acid as ligand (Table 2). This probably resulted from the higher percentage of unbound PPO activity with this affinity resin.

During the course of this study, we encountered two problems similar to those reported by O'Neill *et al.* [54] and Ingebrigtsen and Flurkey [8] concerning affinity chromatography, *i.e.*, the purified fraction was unstable after its elution and the synthetized affinity supports were found to deteriorate. The gel changed from brilliant red to brown after 2 months at 4°C. We demonstrated (data not shown) that the instability of the purified fraction can be partially ascribed to the pH variation (4.5–7.5) required for enzyme elution. Consequently in the following investigation, freshly synthesized supports were used and PPO activity was assayed immediately after its elution.

Evaluation of inhibition constants

We first applied the equilibrium model of adsorption described by Graves and Wu [31] to the two types of affinity matrices. With this model, these workers answered the simple question of how an enzyme is distributed between a solution phase (volume V) and a solid ligand-containing phase (volume v) for a fixed value of the equilibrium constant (K_i). They developed an equation that related the ratio of free to trapped enzyme with the inhibition constant of the ligand:

$$\alpha = E_0V/([L_0]v) + (K_i/L_0)[(V + v)/v] \quad (1)$$

where L_0 = concentration of bound ligand, E_0 = enzyme concentration in the solution phase and α = ratio between the two enzymatic fractions, free and trapped.

Following the protocol described under Experimental, the α values were plotted against each enzyme addition and a straight line was obtained (Fig. 8). Considering Eq. 1, K_i values of the bound carboxylic acids were estimated from the intercept with the ordinate. The values obtained (0.3 and 11 m*M* for *p*-coumaric and *p*-hydroxybenzoic acid, respectively) were higher than those obtained for the corresponding free acids by kinetic studies but close to those calculated for ferulic and vanillic acid (0.29 and 10 m*M*, respectively) [33]. This could be due to the *ortho* substitution (as regards the hydroxyl function of the aromatic ring) which occurred during the binding of the *p*-coumaric and *p*-hydroxybenzoic acid to the gel, leading to a similarity in the substitution patterns and therefore in the inhibition constants observed for the free ferulic and vanillic acid.

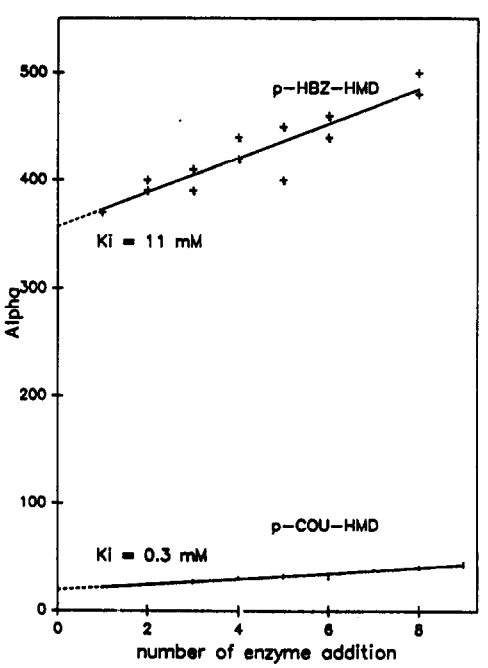

Fig. 8. Values of α *vs.* number of enzyme additions on *p*-COU-HMD and *p*-HBZ-HMD. Assay of activity was duplicated for each enzyme addition.

The second procedure aimed at determining the inhibition constant of bound *p*-coumaric acid was adapted from the work of Dunn and Chaiken [32], who considered, in their model, a variable bound ligand concentration. According to this model, the elution volume (v) of a given protein was directly linked to the bound ligand concentration ($[I_b]$):

$$v = V_0 + (V_0 - V_m)[I_b]/K_{i_b} \quad (2)$$

where V_0 = elution volume of an unretarded protein, V_m = dead volume and K_{i_b} = inhibition constant of the bound ligand.

In our experimental protocol (see Experimental), different bound ligand concentrations were obtained by varying the pH of elution buffer. Fig. 9 shows the elution profile obtained for each pH condition. An increase in PPO elution volume was observed with decreasing pH. Excellent yields higher than 80% were obtained for each chromatographic step when the pH was higher than 5.5. When elution volumes (maximum of the peak) were plotted against the corresponding bound ligand concentration, a straight line was observed. In accordance with Eq. 2, the K_i value was determined from the slope. The value obtained for *p*-coumaric acid was 0.6 m*M*, again close to that of vanillic acid. The difference observed between the two K_i values for *p*-coumaric acid obtained by the two previous protocols could result from an incorrect determination of the amount of protonated *p*-coumaric acid. For this determination, we assumed that the p*K* values of the free and bound *p*-coumaric acid were similar.

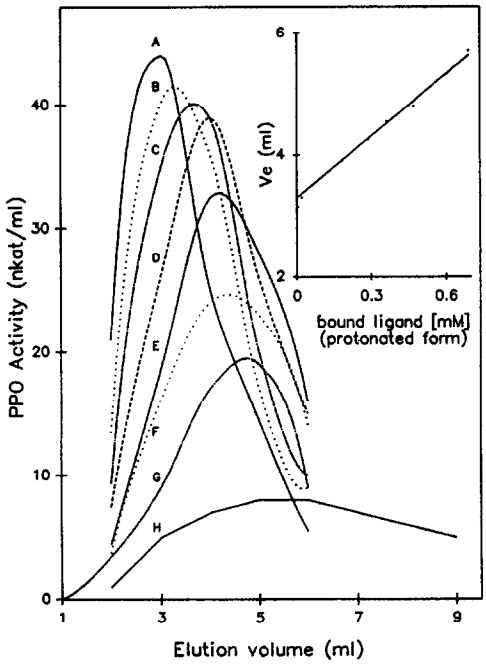

Fig. 9. Effect of buffer pH on elution volume of apple PPO on *p*-COU-HMD. Inset: elution volume *vs.* effective bound inhibitor concentration (protonated form). The pH values and inhibitor concentrations in the various experiments were as follows: experiment A, pH 7.67, 0.005 m*M*; B, pH 7.11, 0.02 m*M*; C, pH 6.61, 0.06 m*M*; D, pH 6.13, 0.18 m*M*; E, pH 5.93, 0.29 m*M*; F, pH 5.83, 0.36 m*M*; G, pH 5.71, 0.47 m*M*; and H, pH 5.53, 0.69 m*M*.

In conclusion, we have developed an easy method for the preparation of affinity gels. These gels can be used either for the purification of PPO (mainly *p*-COU-HMD) or for the determination of the inhibition constant of the ligand.

4. Conclusions

During the course of these studies, different chromatographic steps were applied to apple PPO purification and different isoforms were separated. To obtain the best result, we recommend combining the procedures in the order hydrophobic chromatography, IMAC and affinity chromatography. Applying this scheme, we obtained a fraction representing 25% of the crude extract and characterized by a 240-fold purification factor (283-fold for the most active fraction).

5. Acknowledgements

We greatly appreciate the skilful assistance of F. Gauillard and S. Gevaudan. Thanks are due to V. Mathieu (CTIFL) for supplying Red Delicious apples and to W. Oleszek for language assistance.

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