Plant Physiol. (1985) 78, 256–262
0032-0889/85/78/0256/07/$01.00/0

Purification of d'Anjou Pear (*Pyrus communis* L.) Polyphenol Oxidase[1]

Received for publication August 27, 1984 and in revised form February 1, 1985

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ABSTRACT

Polyphenol oxidase (PPO) was extensively purified to homogeneity from d'Anjou pear (*Pyrus communis* L.) by extraction in the presence of the phenolic binder AG 2-X8 andTriton X-100. Chlorophyll pigment was removed by chromatography resulting in a clear, colorless enzyme extract. Purification of pear PPO was achieved after chromatography on Phenyl Sepharose CL-4B, DEAE-cellulose, and hydroxylapatite columns. Only after the columns were run at room temperature rather than at 4°C were sharp peaks and good resolution obtained. Reproducibility of the entire scheme was excellent with chromatography on the hydrophobic resin as a key to successful purification. Three separate fractions of pear PPO were homogeneous when stained for protein with the silver stain after polyacrylamide slab gel electrophoresis and corresponded to relative mobilities of 0.41, 0.43, and 0.73. The effect of dimethylsulfoxide on enzyme activity was investigated and found to increase significantly the activity of purified pear PPO over the control.

Oxidative browning catalyzed by PPO[4] (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase; EC 1.14.18.1) that occurs when fresh fruits and vegetables are damaged is of economic concern to most fruit and vegetable processors. Information on the molecular structure and catalytic site would help to define the mode of action of PPO and methods to control its undesirable effects. Isolation and purification of PPO from contaminating proteins and phenolics is essential for these studies. Unfortunately, the purification of PPO has typically been a difficult task, and the number of PPO preparations that have been purified to homogeneity from higher plants and particularly from fruits is very limited (9, 16, 24). The main problem lies in the interference of endogeneous phenolic compounds during the extraction. Reaction of phenolics or quinones with the enzyme can inactivate or modify the protein molecule through covalent, hydrogen, hydrophobic, and ionic bonding (17).

Most effective methods to prevent the reaction of PPO with phenols include the use of phenol-complexing agents such as polycaprolactam powder, PEG, insoluble PVP, Amberlite XAD resins, and anion exchange resins. However, since all plant materials contain different types and quantities of phenolic compounds, there is no single compound or combination of compounds that is universally applicable (23). Therefore, each plant system must be examined individually to optimize the enzyme extraction and minimize protein-phenolic interactions. Smith and Montgomery (25) have developed an extraction procedure for d'Anjou pear based on a knowledge of the phenolics present in the fruit and various trials with different levels and combinations of insoluble PVP, Amberlite XAD, and anion exchange resins.

Once the phenolic materials have been removed and the enzyme is solubilized separation of the PPO from the other proteins which are present is necessary. Recently, the use of hydrophobic interaction chromatography of Phenyl Sepharose CL-4B has been shown to be very useful in the purification of PPO (8, 9, 28). This procedure allows for the separation of proteins based on the different size and shape of accessible hydrophobic regions on their surface (20). These hydrophobic pockets interact with immobilized hydrophobic groups supported on insoluble, hydrophilic matrices, such as beaded agarose (30). The optimum resolution of individual proteins is then achieved by gradually changing the nature of the eluting solvent, *i.e.* ionic strength, salt concentration, pH, polarity, or temperature. This report demonstrates use of phenol-complexing agents, hydrophobic chromatography, and other conventional purification techniques to obtain pure, colorless preparations of d'Anjou pear PPO.

MATERIALS AND METHODS

Plant Material. d'Anjou pears (*Pyrus communis* L.) used in this study were grown in the orchards of the Mid-Columbia Experiment Station, Hood River, OR during the 1981 season, picked in September, and placed in −1°C storage for 7 months. Upon removal from storage the pears were green, hard, and in good condition. After transporting to the laboratory, the pears were quartered, cored, frozen in liquid N_2, sealed under vacuum in Cryovac bags, and stored at −40°C until used.

Protein Determination and Enzyme Assay. Protein content was determined by the dye binding method of Bradford (3) using BSA as a standard. A microassay method for 1 to 20 μg protein was used with the purified extracts. To avoid the interference of Triton X-100 in the protein assay, samples containing 1% (v/v) or more of Triton X-100 were treated with Bio-Beads SM-2 (Bio-Rad Laboratories) according to the procedure of Holloway (15). A spectrophotometric scan from 400 to 200 nm, before and after the Bio-Bead treatment, revealed that the 275 nm absorbance peak for Triton X-100 had been removed. No loss in enzyme activity was observed.

PPO activity was determined by measuring O_2 uptake with a Clark-type O_2 electrode monitor (Yellow Springs Instruments) at 25°C. The instrument was calibrated daily with air-saturated water at 25°C. To determine PPO activity, 2.3 ml of 0.1 M citrate-0.2 M sodium phosphate buffer, pH 5.0, and 0.2 ml of enzyme extract were added to the reaction chamber and allowed to

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[4] Abbreviations: PPO, polyphenol oxidase; DMSO, dimethylsulfoxide; HA, hydroxylapatite.

equilibrate for 4 min. To initiate the reaction, 0.5 ml of 0.1 M 4-methyl catechol in 10 mM citrate-20 mM sodium phosphate buffer, pH 3.6, was added. The linear portion of the curve was used to calculate the initial rate of O_2 consumption. Enzyme activity was expressed as nmoles of O_2/min · ml of enzyme extract under the conditions described above.

Enzyme Extraction Procedure. Pear PPO was initially extracted by a modification of the procedure developed by Smith and Montgomery (25). Frozen pear quarters were chilled in liquid N_2 until brittle, broken into smaller pieces and ground under liquid N_2 in a stainless steel Waring Blendor until a fine white powder resulted. The white powder was poured into a Dewar flask and stored in liquid N_2 until used. Extraction buffer consisted of 0.1 M citrate-0.2 M sodium phosphate, pH 5.6, containing 2% (v/v) Triton X-100 and 5 mM phenylmethylsulfonyl fluoride. Either phenylmethylsulfonyl fluoride or trasylol was added throughout the entire isolation and purification procedure as recommended by Flurkey and Jen (10). Pear nitrogen power (12.5 g) was combined with 33.8 g AG 2-X8 (200 to 400 mesh, Bio-Rad Laboratories) equilibrated in 100 ml of the extraction buffer. The solution was stirred in the cold room (4°C) for 60 min, filtered through glass wool, and centrifuged at 7,700*g* for 15 min in a Sorvall refrigerated centrifuge. The supernatant was collected and 0.33 ml of trasylol (Aprotinin, Sigma) per 100 ml of supernatant was added. The crude enzyme extract, after centrifugation, had a slight green tint, presumably from the Chl in the skin of the green pears.

Purification Procedure. d'Anjou pear PPO was extracted and purified according to the procedure outlined in Figure 1. The purification scheme was designed after the work by Flurkey and Jen (8) and Wissemann and Lee (28). Glass-distilled water was used throughout the following purification procedure.

Step 1: Bio-Gel P-6 Chromatography. A Bio-gel P-6 (Bio-Rad Laboratories) desalting column with a 20-ml bed volume was prepared and equilibrated with 1 mM citrate-2 mM sodium phosphate, pH 5.0, buffer. The combined supernatants of two 100-ml crude enzyme extracts were passed through the column and collected in the void volume.

Step 2: Hydrophobic Chromatography. To the eluant collected from the Bio-gel P-6 column, 0.33 ml of Aprotinin was added per 100 ml of extract. This solution was made to 1 M $(NH_4)_2SO_4$ by adding solid crystals of the salt, adjusted to pH 6.5 with 10 N NaOH, and filtered through Whatman No. 1 filter paper. The filtrate was applied onto a Phenyl Sepharose CL-4B (Pharmacia Fine Chemicals) column equilibrated with equilibration buffer (1 M $(NH_4)_2SO_4$, 1 M KCl, and 50 mM sodium phosphate, at pH 6.5). The column was developed at room temperature and the proteins bound to the resin were eluted by decreasing the equilibration buffer concentration in a stepwise manner (Fig. 2). The most active fractions were combined and dialyzed overnight against two changes of 5 mM sodium phosphate, pH 7.0, buffer at 4°C. After dialysis, the samples in the dialysis bags were concentrated to 20 ml with Aquacide II A (Calbiochem).

Step 3: Ion-Exchange Chromatography. Aprotinin (0.33 ml/100 ml of extract) was added to the dialyzed and concentrated sample from the hydrophobic column. This solution was applied onto a DEAE-cellulose 32 (Whatman Ltd.) column and washed with 2 volumes of 5 mM sodium phosphate, pH 7.0, buffer. Proteins were eluted by two linear phosphate gradients (Fig. 3). Three peaks of PPO activity were eluted from the ion-exchange column and labeled DEAE 1, 2, and 3.

Step 4: Hydroxylapatite Chromatography. PPO samples DEAE 1 and DEAE 2 were each applied separately on a HA (Bio-Rad Laboratories) column equilibrated with 1 mM sodium phosphate, pH 7.0, buffer (Fig. 4). Fractions containing enzyme activity were combined and stored at 4°C until further use.

Electrophoresis. PAGE was performed under the discontinuous nondenaturing conditions described by Davis (6), in a Bio-Rad model Protean 16-cm slab gel unit. The slab gels were 0.15 cm thick and 14 cm wide and consisted of 12 cm of a 7.0% polyacrylamide separation gel and 2 cm of a 4.5% large pore stacking gel. All solutions used were filtered through a 1.2 μm Millipore filter. To 1 ml of enzyme extract was added 0.25 ml of a 40% sucrose solution containing a very small amount of bromophenol blue. One hundred μl of this solution were applied per sample well slot in the stacking gel. A current of 15 mamp/slab was employed at 4°C until the tracking dye migrated to within 1 to 2 cm from the bottom (about 5–6 h).

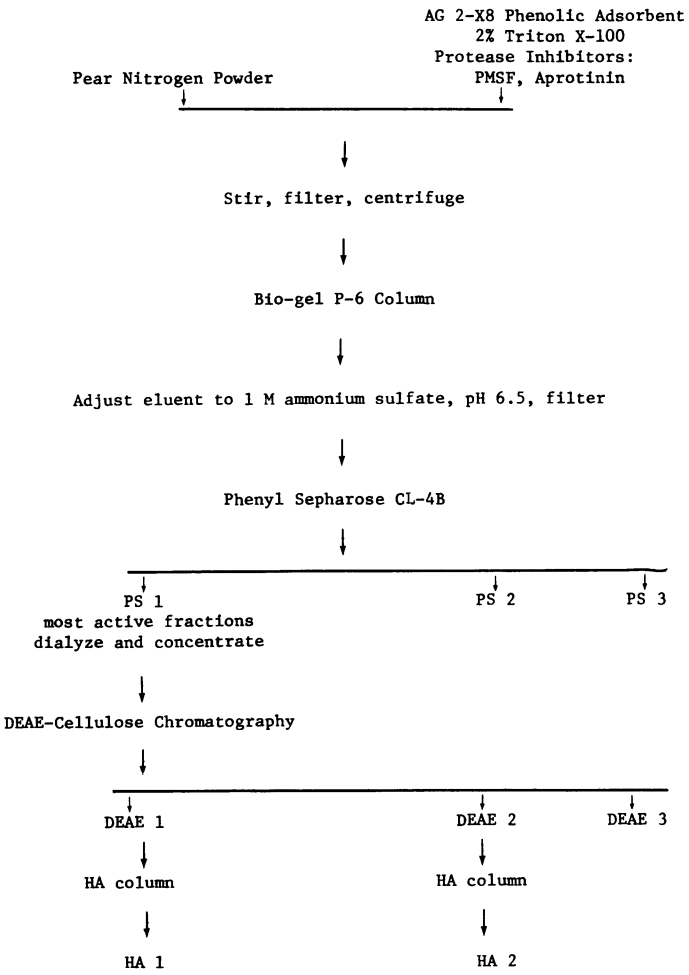

FIG. 1. Outline of the extraction and purification procedure for d'Anjou pear PPO. The first two steps were performed at 4°C; all of the subsequent chromatography was run at room temperature. PS 1, 2, and 3 represent peaks of enzyme activity from the Phenyl Sepharose column, and DEAE 1, 2, 3 and HA 1, 2 were separated on the DEAE-cellulose and hydroxylapatite columns, respectively.

Detection of PPO Isozymes. The procedure for detection of PPO activity in the electrophoresis gel slabs was similar to that described by Smith and Montgomery (25) and Benjamin and Montgomery (2). Slabs were immersed in 15 mM catechol in 0.1 M citrate-0.2 M sodium phosphate buffer, pH 5.0, containing 0.05% *p*-phenylenediamine for about 1 h. To stabilize the color and decrease the nonspecific background color, the slabs were rinsed in 1 mM ascorbic acid for 5 min, soaked in water overnight, and stored in 30% ethyl alcohol.

Protein Stain. Slab gels were stained for protein by a modification of the silver stain procedure (19, 29) developed by Einerson (7). Distilled, deionized H_2O was used to prepare all of the solutions. The slab gels were immersed in 10% TCA for 30 min,

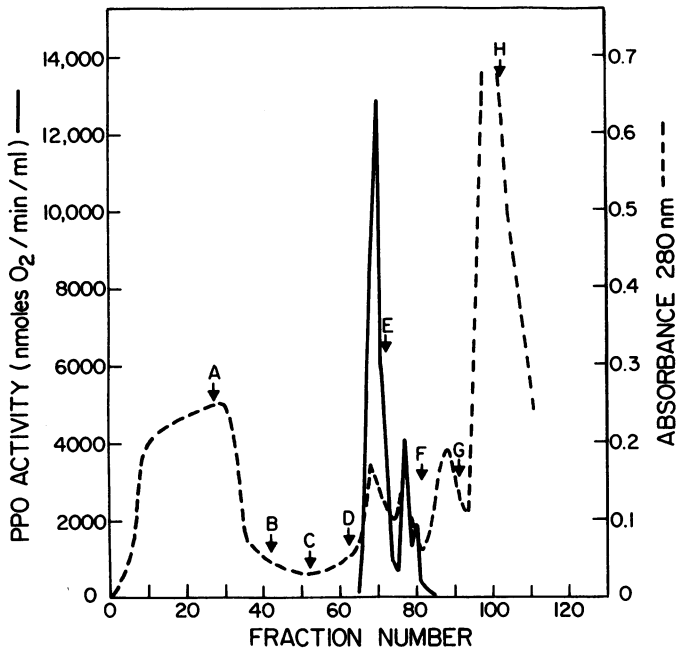

FIG. 2. Phenyl Sepharose CL-4B column chromatography of pear PPO. A 30 × 1 cm SR 10/50 column (Pharmacia) was packed at room temperature and rinsed with several volumes of equilibration buffer (1 M $(NH_4)_2SO_4$, 1 M KCl, and 50 mM sodium phosphate, pH 6.5). After sample application, the column was developed with: (A), 100 ml EB; (B), 60 ml 0.8 EB; (C), 60 ml 0.6 EB; (D), 60 ml 0.4 EB; (E), 60 ml 0.2 EB; (F), 60 ml 0.05 EB; (G), 60 ml 50% ethylene glycol; (H), water. Flow rate was regulated to 60 ml/h and 6-ml fractions were collected. PPO peaks: PS 1 fractions 66 to 72; PS 2 fractions 76 to 79; and PS 3 fractions 80 to 82.

rinsed in water for 20 min, and placed in 50% methanol, 0.05% formaldehyde overnight (about 12 h). The gels were then allowed to swell in water for 30 min, with one change of the water after 15 min, and transferred to a solution containing 50% methanol and 0.05% formaldehyde for at least 2 h after which the gels were placed in water for 10 min before the staining solution was added. Silver staining solution was prepared by dissolving 4 g silver nitrate in 20 ml water and adding this to 105 ml 0.35% NaOH and 7 ml concentrated NaOH dropwise with rapid mixing. The total was made up to 500 ml with water and used within 5 min of preparation. The gel was stained for 20 min with gentle agitation on a mechanical shaker, rinsed with water, and agitated in water for 5 min. After the water was removed, the gel was developed by the addition of freshly prepared developer solution that consisted of 2.5 ml 1% citric acid and 0.25 ml formaldehyde in a total volume of 500 ml. Development was usually completed in 15 to 30 min and the gel was returned to 50% methanol. To remove unwanted background, the gels were destained by immersion in Kodak Rapid Fix (film strength) containing 5% methanol. When the desired amount of background had been removed, the Rapid Fix was removed with a 5-min water wash, a 20-min wash in Kodak Hypo Cleaning Agent (film strength), another 5-min water wash, and finally the gel was returned to 50% methanol.

Effect of DMSO on Enzyme Activity. The effect of DMSO on enzyme activity was investigated using the most active peak from the Phenyl Sepharose column (PS 1). Five-ml solutions were prepared containing 1 ml of the enzyme fraction and from 1 to 80% (v/v) DMSO made to volume with 0.1 M citrate-0.2 M sodium phosphate buffer, pH 5.0. A 0.2-ml aliquot of this solution was assayed for enzyme activity in the usual manner. The control contained 1 ml of enzyme extract plus 4 ml of the citrate-phosphate buffer.

RESULTS

Purification of d'Anjou Pear PPO. The majority of this work was concerned with the purification of d'Anjou pear PPO. An outline of the procedure is illustrated in Figure 1. The anion exchange resin AG 2-X8 (1.0 g dry weight/g pear tissue) was used in the extraction procedure to remove endogenous pear phenolics. Initial work on the purification procedure was conducted without the use of 2% (v/v) Triton X-100 during extraction. Without Triton X-100, the supernatant of the crude extract was clear and colorless; however, when the detergent was added at least 30% more enzyme activity could be extracted and the elution patterns from the hydrophobic column were more consistent. Triton X-100 also extracted the green pigment from the unripe fruit; thus, the supernatant was light green. The crude enzyme extract was passed through a Bio-gel P-6 column and the enzyme was collected in the void volume. Column yields of 85 to 100% were obtained with a slight increase in the purification (Table I).

The remainder of the column chromatography steps were initially done in a cold room at 4°C and were unsuccessful. Several runs were attempted with the Phenyl Sepharose column

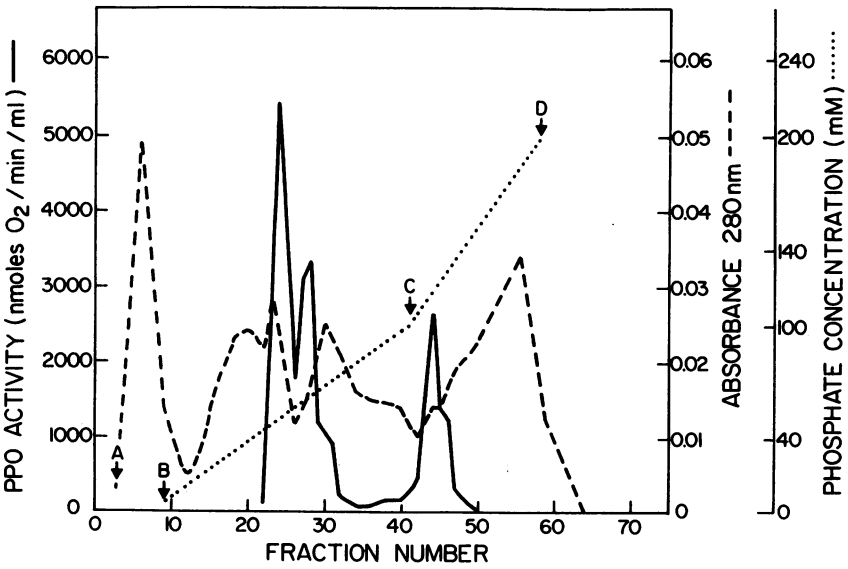

FIG. 3. DEAE-cellulose column chromatography of pear PPO. A DEAE-cellulose column (35 × 1 cm, SR 10/50) was packed at room temperature and rinsed with several washings of 5 mM sodium phosphate, pH 7.0 buffer. Sample PS 1 was applied with the following elutants: (A), 5 mM sodium phosphate pH 7.0; (B), 200 ml linear gradient from 5 to 100 mM sodium phosphate, pH 7.0; (C), 100 ml linear gradient from 100 to 200 mM sodium phosphate, pH 7.0; (D), 50 ml 400 mM sodium phosphate, pH 7.0. Flow rate was 60 ml/h and 6-ml fractions were collected. PPO peaks: DEAE 1 fractions 23 to 26; DEAE 2 fractions 27 to 29; and DEAE 3 fractions 43 to 46.

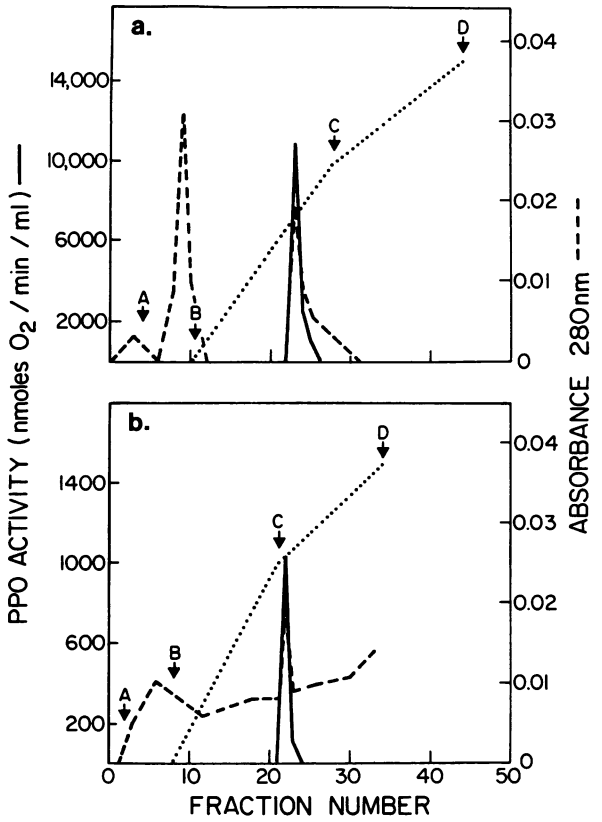

FIG. 4. Hydroxylapatite column chromatography of pear PPO. An HA column (30 × 1 cm, SR 10/50) was developed at room temperature at a flow rate of 50 ml/h, and 6-ml fractions were collected. The elutants were: (A), 1 mM sodium phosphate, pH 7.0; (B), linear gradient from 1 mM sodium phosphate to 50 mM sodium phosphate, 1 M KCl, pH 7.0; (C), linear gradient from 50 mM sodium phosphate, 1 M KCl to 50 mM sodium phosphate, 1 M KCl, 1 M $(NH_4)_2SO_4$, pH 7.0; (D), 400 mM sodium phosphate, pH 7.0; (·····), Linear gradients described above. a, Enzyme sample was DEAE 1; PPO peak is HA 1 fractions 23 to 25. b, Enzyme sample was DEAE 2; PPO peak is HA 2 fraction 22.

at 4°C using different pH levels, gradients, and elution buffers, but the enzyme was not released from the hydrophobic media and was not eluted. Chromatograms from the HA and DEAE columns showed that the enzyme was also not well separated on these columns at 4°C. Only when the columns were developed at room temperature were sharp and well-resolved peaks obtained.

The elution profile from the hydrophobic column, run at room temperature and with Triton X-100 (Fig. 2), shows that the majority of the PPO activity corresponded to one protein peak. This peak was labeled PS 1. The two smaller peaks of activity, following the major peak, were labeled PS 2 and PS 3, respectively. PPO in PS 1 was released from the column following the addition of elutant D, which was 0.4 concentration of the equilibration buffer. The remainder of the 280 nm adsorbing material was eluted off the column in large amounts before and after the PPO. Triton X-100 was eluted after the enzyme peak, when the ethylene glycol was used as an elutant. There was a 24-fold increase in the specific activity in PS 1 over the crude extract, and the total column yield was over 100% of the activity units applied to the column (Table I).

Chromatography of PS 1 on DEAE-cellulose columns at pH 7.0 resulted in the separation of three major chromatographic PPO forms (Fig. 3). These isozymes were labeled DEAE 1, 2, and 3 and eluted at 50, 62, and 118 mM phosphate concentration, respectively. Total column yield was 95% and the three forms were well resolved, with DEAE 3 appearing homogeneous by slab gel electrophoresis, as will be discussed later. The other two PPO forms (DEAE 1 and 2) were each applied onto a HA column, and the elution profiles are illustrated in Figure 4. PPO from DEAE 1 was resolved from the other 280 nm absorbing material and eluted in one protein peak at 36 mM phosphate concentration (pH 7.0) and 0.72 M KCl (Fig. 4a). The recovery of enzyme activity from this column was approximately 90% with 5-fold purification over the DEAE column. When PPO activity of DEAE 2 was subjected to HA chromatography (Fig. 4b) elution was at a higher phosphate and salt concentration (50 mM phosphate, 1 M KCl, 0.08 M $(NH_4)_2SO_4$ than DEAE 1, indicating that PPO of DEAE 2 had a greater affinity for the Ca^{2+} groups in the HA resin.

Table I is a summary of the purification of d'Anjou pear PPO. An overall purification of 148-fold was achieved for HA 1 and 103-fold for HA 2. DEAE 3 was only purified 7-fold which was due to the high (13.2 μg/ml) protein level and low enzyme activity for that fraction. The overall yield for HA 1 was 28% and 7% for HA 2, with respect to the crude extract. Highest increase in specific activity was due to the Phenyl Sepharose

Table I. *Purification of d'Anjou Pear Polyphenol Oxidase*

Sample	Vol	PPO Activity	Total Activity	Protein	Specific Activity	Yield	Purification
	ml	*nmol O_2/ min·ml*	*units*	*μg/ml*	*units/ μg*	*%*	*-fold*
Crude supernatant	182	1,410	257,000	135	10	100	1
Bio-gel P-6 eluent	180	1,220	224,000	69	18	87	2
$(NH_4)_2SO_4$ filtrate	176	1,180	212,000	75	16	83	2
Phenyl Sepharose							
Most active fractions[a]	24	6,140	151,000	26	236	59	24
After dialysis + concentration	20	8,650	181,000	30	288	69	29
DEAE—Cellulose							
Peak 1	24	3,090	92,700	10	297	36	30
Peak 2	18	900	20,200	8	118	8	12
Peak 3	24	880	26,400	13	68	10	7
Hydroxylapatite							
Peak 1	18	3,120	73,100	2	1,480	28	148
Peak 2	6	1,030	18,500	1	1,030	7	103

[a] Represents only PPO activity in PS 1.

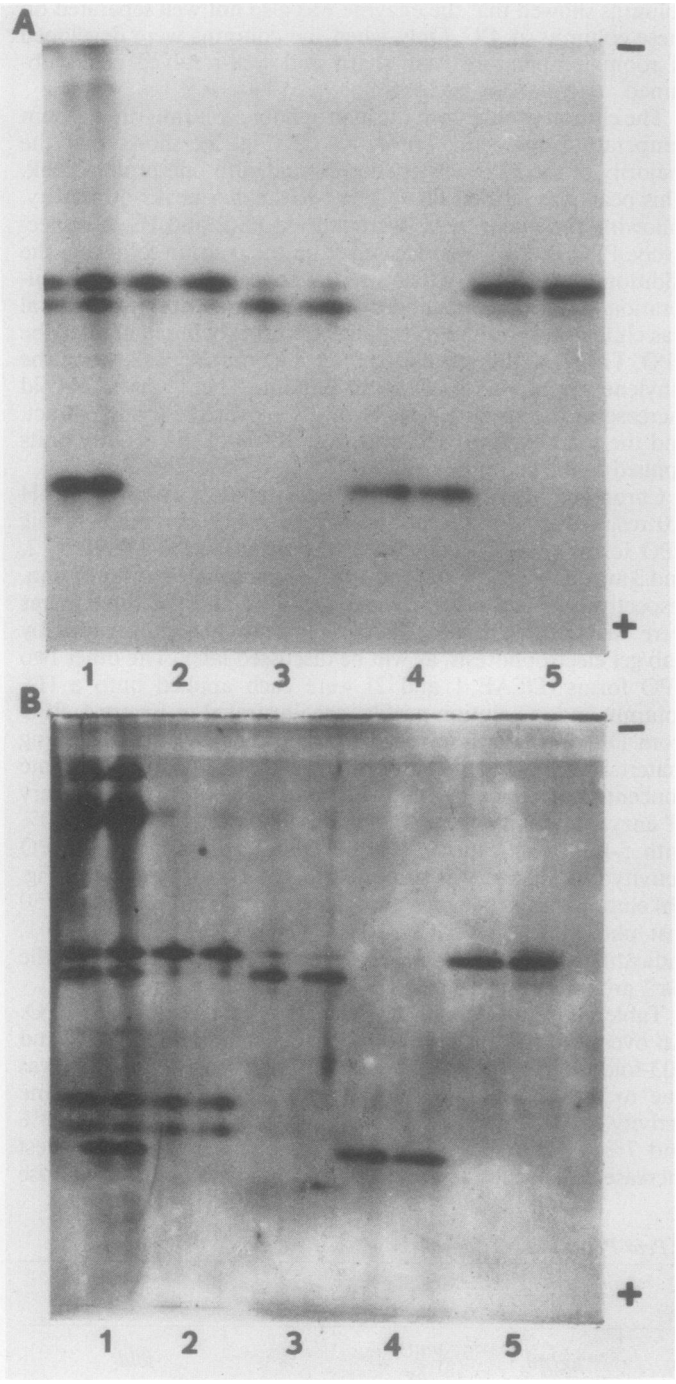

FIG. 5. Polyacrylamide electrophoresis on 7% slab gels of pear PPO during purification. A, Gel stained for PPO activity. B, Gel stained for protein. The enzyme samples were: (1), PS 1; (2), DEAE 1; (3), DEAE 2; (4), DEAE 3; (5), HA 1. Two wells were used for each sample.

column, suggesting that the use of this resin was important to the successful purification of d'Anjou pear PPO. Reproducibility of the entire procedure was excellent. Similar elution profiles were obtained for all of the chromatographic columns from trials with several pear nitrogen powders.

Electrophoresis. After the isolation of PPO, the purity of the preparations and the multiplicity of the enzyme were examined by polyacrylamide slab gel electrophoresis. Results of the various runs are shown in Figure 5A for the enzyme stain and Figure 5B for the protein stain. Samples 1 through 5 represent the progression in purification. After the Phenyl Sepharose column (Fig. 5, sample 1), three active PPO bands were present on the enzyme-stained gel and these were separated on the DEAE-cellulose and HA columns.

The silver-stained gel (Fig. 5B) revealed the presence of several other proteins in the eluate of the PS column, including two very prominent bands with low relative mobilities. Sample 2 represents the most active PPO peak from the DEAE column (DEAE 1) and, although the two large bands at the top of the gel had been removed, two minor protein bands that were not PPO remained. These were eliminated after the HA column, leaving a pure, active PPO band at a relative mobility of 0.41 (sample 5). DEAE 3, relative mobility 0.73 (sample 4), was also pure, although it had 3 to 4 times less enzyme activity than HA 1. The third PPO fraction, relative mobility 0.43 (DEAE 2), is free from other protein contaminants but had a trace of HA 1 present that was not removed even after an HA column.

Enzyme Stability. Stability of the purified PPO extract (HA 1) was monitored for 120 d after purification during storage at 4°C in the elution solution from the HA column (36 mM phosphate, 0.72 M KCl, pH 7.0). Within the first 15 d, 25% of the initial activity of the extract was lost, but the activity remained at that level for 120 d. This stability made it possible to store the extract at refrigeration temperatures and continually use the purified extract for 2 months after purification with no detrimental effects. Initially, the enzyme extracts were frozen and stored at −40°C; however, 50% of the original activity was lost during the freeze-storage-thaw cycle.

Effect of DMSO. The effect of DMSO on enzyme activity was studied using the most active peak eluted from the Phenyl Sepharose column (PS 1), and Figure 6 details the results. DMSO concentrations below 50% in the enzyme mixture significantly increased the PPO activity over the control, with the maximum level obtained at 5% DMSO. At 80% DMSO, however, enzyme activity was decreased 70%.

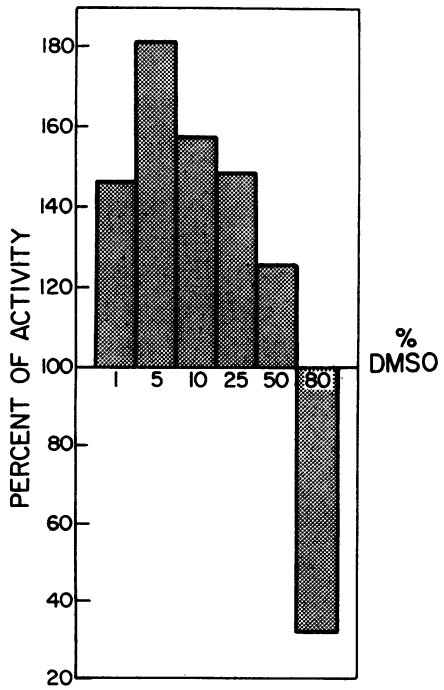

FIG. 6. Effect of DMSO on the enzyme activity of d'Anjou pear PPO. Activity measurements were performed with PS 1. 100% activity represents the enzyme activity in the absence of DMSO.

DISCUSSION

Detergent extraction, using Triton X-100, increased the amount of PPO activity that could be extracted from D'Anjou pears. Several other researchers have reported similar results with detergents (11, 13, 26). The increase in enzyme activity has been attributed to an activation due to the detergent (11), and to the disruption of chloroplast membranes to release more enzyme (21). Natural ripening of fruit renders PPO in a more soluble form than is observed in the green fruit. The pears used in this study were green and unripe. Therefore, it is possible that the detergent treatment was necessary to release and solubilize enzyme which was associated with membranous material. Triton X-100 also extracted the green pigment from the pear skins. However, when the extract was applied onto a hydrophobic column, all of the pigment remained on top of the column, while the enzyme could be eluted in several clear and colorless fractions, indicating that the enzyme was not in a tight complex with the Chl. Vaughan *et al.* (26) recommended the use of cetyltrimethylammonium bromide (Cetavlon), a cationic detergent, in the extraction of PPO from spinach beet since it did not liberate the Chl pigments. The effect of temperature on the elution of PPO from the Phenyl Sepharose, DEAE, and hydroxylapatite columns was an interesting observation. The results showed that when chromatography of PPO was carried out at 4°C, the enzyme could not be eluted from the Phenyl Sepharose column, while on the other columns PPO was eluted in the void volume. These results are in contradiction to the usual behavior of proteins. Low temperatures generally favor adsorption to ion-exchange columns (22) and decrease adsorption in hydrophobic interaction chromatography (14).

Hydrophobic chromatography, using Phenyl Sepharose CL-4B resin, provided a rapid partial purification of d'Anjou pear PPO. There was excellent reproducibility when the column was developed at room temperature and with Triton X-100 in the crude extract. The enzyme was eluted in a single major, protein peak, clearly separated from the bulk of the other 280-nm absorbing material. There was good separation and purification (24-fold) of PPO in this one step. Further work in our laboratory has also shown that Phenyl Sepharose CL-4B was useful in the partial purification of strawberry PPO (27). Results from the hydrophobic column indicate an increase in the specific activity and a recovery of more than 100% of the PPO activity from the crude extract for pear PPO (Table I). Occasionally, the column yield was close to 200% indicating the removal of an inhibitory substance or an activation of the enzyme. These observations are consistent with those reported by Wissemann and Lee (28) and Flurkey and Jen (8) for grape and peach PPO, respectively, purified on a hydrophobic column. These investigators found yields of PPO activity of over 180% in comparison with the crude extract. The remainder of the purification of pear PPO was performed using DEAE-cellulose and hydroxylapatite chromatography. Both of these columns are commonly used in other reported PPO purification procedures (9, 12, 24).

Polyacrylamide slab gel electrophoresis showed HA 1 and DEAE 3 to be pure when the gels were stained for protein by the silver stain procedure. HA 2 was free from other contaminating proteins, except for a trace of HA 1 PPO. An overall purification fold of 148, 103, and 7 was achieved for HA 1, HA 2, and DEAE 3, respectively. The low purification fold for DEAE 3 is a reflection of the low specific activity of this fraction. The observation that different enzyme forms of PPO have different specific activities has been previously reported (12, 24).

Few reports have been published on PPO preparations purified to apparent homogeneity from plants (1, 4, 12, 26), and only three researchers used fruits (9, 16, 24). Rivas and Whitaker (24) reported a purification procedure for Bartlett pears that yielded two enzyme fractions that were homogeneous by electrophoresis with a final 20- and 37-fold increase in specific activity; however, the extracts were tanned. A purification fold of over 900 was obtained by Golbeck and Cammarata (12) with spinach PPO. But generally the purification values are closer to 100 or even lower (1, 9). The values for specific activity and purification fold are based on enzyme activity and protein levels, and unless similar methods for protein and active PPO determination are used by all researchers, it is difficult to compare the various procedures based on these values. Unfortunately, the phenolic compounds that also cause problems in purifying native proteins from plants also interfere in several protein assay procedures (17). Several of the above workers reported having difficulty with tanning of their enzyme extracts. Only one of these researchers reported that the initial extract was free from tanning and found the enzyme to be homogeneous by SDS electrophoresis and sedimentation velocity studies (26).

Purified pear PPO was found to be stable for up to 4 months at 4°C in a solution of 36 mM Na-phosphate, 0.72 M KCl, pH 7.0; 25% of the enzyme activity was lost initially, and after 15 d the activity stabilized and remained at that level for 120 d. When the enzyme extracts were stored at −40°C, 50% of the original activity was lost after 2 months of storage. These results are in direct contrast to those reported by Smith and Montgomery (25) for a crude d'Anjou pear PPO extract. In their study, the enzyme was most stable at pH 5.0 and lost no activity when stored for 3 months at −40°C. Flurkey and Jen (9) found no significant loss in PPO activity when their purified preparations were stored for 2 months at −15°C in 20% glycerol, sucrose, or ethylene glycol solutions. However, these authors noticed a gradual loss in activity when the purified samples were stored at 4°C. Ionic strength (0.72 M KCl) was relatively high in the purified pear PPO extract which appeared to stabilize the enzyme. Previous work on PPO has shown that activity increased with an increase in the ionic strength of the buffer (24).

Studies on the effect of DMSO on PPO activity were started when DMSO was investigated as a possible component of the elution buffer for the hydrophobic column. This solvent has been recommended for proteins requiring a more hydrophobic environment, to decrease the polarity of the solution (5). Figure 6 illustrates that DMSO significantly increased the enzyme activity of PPO over the control. DMSO possesses a lone pair of electrons and forms a strong association with proton donors, such as water (18). The compatibility of PPO with DMSO suggests that the enzyme is active in a hydrophobic environment. Other substances which decrease the polarity of a solution are sucrose, glycerol, and ethylene glycol (5), all of which increases the stability of peach PPO at concentrations of 20% (9).

The information provided in this report on the purification of pear PPO will make it possible to study this enzyme in more detail. Currently, studies employing the purified d'Anjou pear PPO to determine mol wt, multiplicity, isoelectric point, and amino acid composition are in progress. Purified PPO preparations are also being used in our laboratory to study the mode of inhibition of PPO by SO_2.

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