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IMPROVED METHODS FOR THE EXTRACTION OF POLYPHENOL OXIDASE FROM d'ANJOU PEARS

DENISE M SMITH and M W MONTGOMERY

Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331 U S A

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Abstract—The conditions for extracting polyphenol oxidase (PPO, monophenol monooxygenase, EC 1 14 18 1) from d'Anjou pears have been studied Water extracts of pear PPO contained artefacts which were present as additional bands on polyacrylamide-gel electrophoresis Buffer extracts of an acetone powder did not remove sufficient endogenous phenolics to prevent browning of the extract The following phenolic absorbents, arranged in order of increasing efficiency, reduced the formation of artefacts in extracts of PPO PVPP, Amberlite XAD-4, Bio-Rad AG 1-X8, and Bio-Rad AG 2-X8 Greatest activity was extracted within a pH range of 5 6–5 9 Anion exchange resins were particularly effective in removing phenolics XAD-4, AG 1-X8, or AG 2-X8 did not adsorb PPO and reduced the electrophoretically separable bands of PPO activity from 11 in water extracts to 3 The properties of the crude PPO were also studied

INTRODUCTION

Extraction of enzymes from plant sources is complicated by the presence of endogenous phenolic compounds, which are oxidized to the corresponding quinones or semiquinone radicals by the enzyme, polyphenol oxidase (PPO) [1] These phenolic compounds interact with enzymes and change the characteristics of these enzymes [2] As a consequence, this reaction must be prevented during extraction of plant enzymes Problems related to the isolation of plant enzymes have been the subject of several reviews [2–6]

The purpose of this research was to investigate several methods of extracting PPO from d'Anjou pears to determine the most efficient technique for binding phenolic compounds during extraction A water extract, a buffered extract of acetone powder and extracts using the phenol adsorbents polyvinylpolypyrrolidone (PVPP), Amberlite XAD-4, and the ion-exchange resins Bio-Rad AG 1-X8 and AG 2-X8 were investigated

RESULTS

Water extract of pear PPO

A water extract of the pear tissue had a pH of 4 2 and at 4° lost 60% of the PPO activity 7 hr after extraction Visible browning of the extract occurred within 30 min and absorbance (A) at 410 nm increased for 8 hr, indicating formation of polymerized phenolic compounds This increase in A at 410 nm was accomplished by a decrease in A at 324 and 280 nm and an increase at 260 and 240 nm over the 8 hr observation period Similar spectral changes were noted in alkaline aqueous solutions of chlorogenic acid and D-catechin Browning, loss of activity, and absorbance changes of the pear extracts with time were indicative of reactions between PPO and endogenous pear phenolic compounds present in the water extract

Electrophoresis of the water extract revealed three regions of PPO activity containing 11 PPO bands (Fig 1a) In later experiments when the phenolic compounds were removed with XAD-4 (1 g/g tissue), the number of apparent isoenzymes was decreased to 3 (Fig 1b) Additional PPO bands were produced in the XAD-4 extract upon the addition of chlorogenic acid (3 mM) (Fig 1c) or pear tissue (2 5% w/v) (Fig 1d) in which the enzymes had been inactivated by heat treatment Thus endogenous phenolic substrates, if not removed during extraction of PPO, can react with the protein to produce additional isoenzymes which were not present in the intact tissue This phenomenon has been

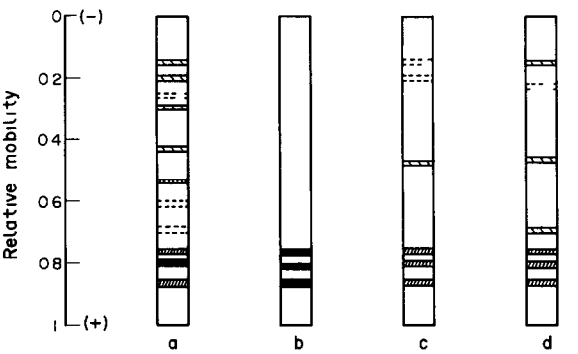

Fig. 1 Representative electrophoretic patterns of pear PPO (a) Water extract, (b) XAD-4 extract (1 g dry wt/g fresh tissue), (c) 3 mM chlorogenic acid added to the XAD-4 extract, (d) 2 5% pear powder (enzymes inactivated) added to XAD-4 extract The substrates in c and d were incubated with PPO extract at 23°, pH 5, for 30 min before electrophoresis Relative mobility based on migration of bromophenol marker dye The extracts were from pears of a different season than those used in Fig 2

documented by several authors ([7–9], Loomis, W D, personal consultation)

Acetone powder extraction

An acetone powder extract was investigated to determine the value of this type of extraction for pear PPO After several trials the technique was abandoned, as all the extracts became slightly brown overnight at 4° Similar observations have been noted by others [10–14]

Extractions with phenolic adsorbents

PVPP has been used successfully during plant enzyme extractions due to its ability to hydrogen bond to phenolics and prevent phenol–protein interaction [2, 3, 15–17] Optimum extraction of PPO activity occurred at 0 75 g PVPP/g tissue or above, and at a pH of 5 4–5 9 Under these conditions, extracts remained clear on standing overnight at 4° Lower levels of PVPP resulted in browning of the extract on standing The presence of a peak at 324 nm and variability in the isoenzyme bands indicated the presence of residual phenolic compounds The 324 nm peak suggested the presence of hydroxycinnamic acids in the extract, most likely chlorogenic acid, which is present in high concentrations in the pear [18] This agrees with the results of Anderson and Sowers [19], who reported that PVPP is unable to bond chlorogenic acid efficiently

XAD-4 had been recommended for use during plant enzyme extractions to remove phenolic compounds from solution by hydrophobic adsorption [2, 6] At least 1 g XAD-4/g tissue was necessary to prevent visible browning, however, a peak at 324 nm indicated the presence of phenolics in solution Electrophoresis revealed five to seven PPO bands when less than 1 g XAD-4/g tissue was used during extraction, whereas at a level of 1 g XAD-4/g tissue or higher, only three bands were observed Consequently, XAD-4 was a more efficient adsorbent of pear phenolics than PVPP A pH of 5 0–5 9 was necessary in the extraction buffer for extraction of maximum activity

Anion exchange resins have been used successfully in plant enzyme isolation [8, 20, 21] due to their ability to complex with phenolic compounds through hydrophobic, ionic and hydrogen bonding mechanisms [22, 23] When 0 5 g AG 1-X8/g tissue or above was used during PPO extraction, browning was eliminated, the extracted activity was constant, and the 324 nm peak was eliminated Three PPO bands were resolved on electrophoresis under these conditions As with XAD-4, extraction buffers above pH 5 produced maximum activity in the extracts Browning was prevented at 0 5 g AG 2-X8/g tissue and above, and the PPO activity was constant in these clear extracts Three bands of PPO activity were detected on electrophoresis A pH above 5 6 in the extraction buffer produced maximum activity in the extracts

Non-specific binding of proteins by ion exchange resins at low pH has been reported [24] and Lam and Shaw [20] warned of the possible adsorption of proteins with low isoelectric points on ion exchange resins The absence of the 324 nm peak was noted when either AG 1-X8 or AG 2-X8 was added to the extract prepared with PVPP, with no decrease in the PPO activity or A at 280 nm Upon electrophoresis of the polymer-treated extracts, no change in the number, location, or intensity of the PPO bands was detected

These results agree, in part, with those of Loomis *et al* [6]

Comparison of extraction methods

Extracts were made using all the adsorbents previously studied (Table 1) PVPP was used in combination with the polystyrene resins, as it has been reported to be a more efficient adsorbent of leucoanthocyanins [25] The activities of all extracts, except the PVPP extract, were linear with increasing concentrations of extract when measured by spectrophotometric and polarographic methods (see Experimental) The peak at 324 nm was eliminated in those extracts prepared with the polystyrene resins Extracts prepared with AG 2-X8, with or without PVPP, exhibited three PPO bands which were constant and reproducible (Fig 2) The lack of PPO bands of low relative migration, such as those seen with PVPP and XAD-4 extracts, indicate the elimination of PPO artefacts of higher apparent M_r and/or charge masking resulting from phenolic coupling

Table 1 Comparison of PPO extracts prepared using different adsorbents*

Absorbents used†	Activity‡ (units/mg)	A 324 (nm) peak§	No of PPO bands
PVPP	2 25	+	5–7
XAD-4	2 41	+	5
PVPP + XAD-4	2 28	+	5
AG 1-X8	2 43	–	4
PVPP + AG 1-X8	2 23	–	3
AG 2-X8	2 41	–	3
PVPP + AG 2-X8	2 31	–	3

*All extracts were prepared in 25 ml of 0 1 M NaOAc buffer, pH 5 6

†All adsorbents were added at a level of 1 g dry wt/g fresh tissue

‡One unit of PPO activity is equal to ΔA of 0 001 at 410 nm/min per mg fr wt at 24° See Experimental for details of assay and extraction procedure

§Designates presence or absence of an A peak at 324 nm in the extract

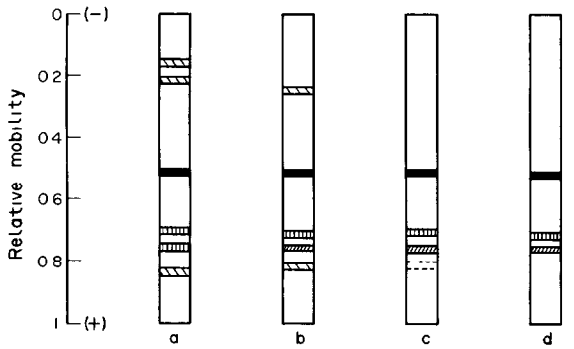

Fig 2 Electrophoretic patterns of pear PPO extracted with different adsorbents (a) PVPP, (b) XAD-4, (c) AG 1-X8, (d) AG 2-X8 The level of adsorbents was 1 g dry wt/g fresh tissue Relative mobility based on migration of bromophenol blue marker dye The extracts were the same as those in Table 1 and were from pears from a different season than those used in Fig 1

Even though PVPP did not appear to improve the extraction of PPO activity when used in combination with the polystyrene resins, the use of PVPP is advisable When the leucoanthocyanin test of Swain and Hillis [26] was used, PVPP (0 75 g/g tissue) removed almost all the leucoanthocyanins during extraction, whereas AG 1-X8 (1 g/g tissue) removed *ca* 40% and XAD-4 (1 g/g tissue) removed less than 10% of the pear leucoanthocyanins, when compared to a water extract control

Characteristics of pear PPO

These experiments were performed with a PPO preparation extracted in the presence of AG 2-X8 (1 g resin/g tissue/8 ml 0 05 M HOAc buffer, pH 5 6) A pH optimum of 5 1 was obtained with half optimum activities at pH 3 7 and 7 7 for d'Anjou pear PPO activity, determined with the O_2 electrode with either catechol or chlorogenic acid as substrate The results are in contrast with those of Halim and Montgomery [27], who reported a pH optimum of 7 0 for a crude extract of d'Anjou pear PPO prepared with PVPP, but are broadly in agreement with others [28] These observations suggest that the binding of phenols to pear PPO causes the pH optimum to increase, possibly due to masking of charges at or near the active site This masking of charges on PPO would account for the additional electrophoretic bands observed in pear extracts prepared in the absence of polystyrene resins (Fig 1a) In the pH range of 2 0–6 5, PPO was most stable at pH 4 0 over a 24 hr period at 4° The enzyme was unstable at pH 6 5 At pH 2 the enzyme lost activity before the assay could be performed, while at pH 3 0 more than 80% of the original activity was lost in 24 hr Stability of the PPO extract adjusted to pH 5 0 and stored at −40° showed no loss of activity over 3 months and there were no changes in the electrophoretic patterns This is in contrast to the results with PPO of spinach chloroplasts where the PPO dimer yielded a higher M_r protein after freezing [29] At 4° the extract lost 11% of the PPO activity during the 11 day storage period

DISCUSSION

The presence of multiple forms of PPO in pear extracts [27, 28] as well as in extracts from other sources [1, 14, 16, 17, 30–35] is well known Many of these multiple forms are thought to be artefacts [1, 36] which are caused by partial release from membranes [31], association–dissociation reactions [33], partial denaturation, fragmentation, proteolysis, activation of latent forms, and interaction with phenols [1] From the information presented in this paper, if proper precautions are not taken during the extraction of pear PPO, artefacts are generated during the extraction process Since these artefacts are reduced or eliminated if PVPP and polystyrene resins are used in the extraction media, it appears that the artefacts are due to the interaction of phenols and/or other endogenous substances with the PPO [6] Whether the three electrophoretically separable PPO bands observed in the pear extracts prepared with AG 2-X8 (Fig 2) are true isoenzymes (i e differing only in primary structure) or are artefacts caused by one of the above factors is not known

A note of caution should be given The procedures and materials used in the preparation of the pear extracts of PPO activity studied in this work should not be used blindly for the preparation of PPO extracts from other sources Unpublished work in this laboratory (Wesche Ebeling, P A E, personal communication) suggests that a combination of equal parts of PVPP, XAD-4 and fruit powdered in liquid N_2 is optimum for extraction of PPO from strawberries Thus, each system investigated will require optimization, not only for the adsorbents to be used, but also the concentration of the adsorbent in the extraction media

EXPERIMENTAL

d'Anjou pears (*Pyrus communis* L) were obtained from the Mid-Columbia Experiment Station, Hood River, Oregon Preliminary trials were performed with pears that had been stored at −1° for 8 months at Hood River Upon shipment to the Corvallis laboratory, the pears were quartered, cored, frozen in liquid N_2, sealed in cryovac bags, and stored at −40° until used Later experiments were with pears handpicked from the same tree and brought to the laboratory, and any pear which deviated greatly in size or other apparent physical characteristics was discarded After storage at −1° overnight, the pears were frozen as described above

Analytical methods Enzyme activity was measured by either a spectrophotometric or a polarographic method The spectrophotometric assay was used to measure the increase in A at 410 nm The reference cuvette contained 2 5 ml of the standard buffer (0 1 M K citrate–0 2 M KPi buffer, pH 5) and 0 5 ml 0 1 M catechol prepared in standard buffer diluted 10-fold The sample cuvette contained 2 ml of the standard buffer, 0 5 ml 0 1 M catechol in the diluted standard buffer and 0 5 ml enzyme preparation The initial velocity at 24° was determined from the linear portion of the curve within 60 sec of initiation of the reaction One unit of enzyme activity was that amount of enzyme which caused a change in A of 0 001/min

Polarographic measurements were performed using a Clark type O_2 electrode at 30° standardized with air-saturated distilled H_2O 2 ml of the standard buffer and 0 5 ml of the enzyme soln were added to the reaction chamber and allowed to equilibrate for 3 min To initiate the reaction, 0 5 ml 0 1 M catechol in the diluted standard buffer was injected The linear portion of the curve was used to measure initial rates of O_2 consumed/min per g of pear tissue, based on the O_2 content of air-saturated H_2O at 30°

Disc electrophoresis was performed with an electrode buffer at pH 8 75 as described in ref [27] Before electrophoresis the enzyme soln was passed through a 1 × 10 cm column of Bio-Gel P-6 (Bio-Rad Laboratories) prepared as described in ref [37] The eluant was layered on top of the spacer gel in 50–400 μl quantities to obtain constant PPO activity in each tube To detect PPO activity, the gels were developed in 10 mM catechol in standard buffer containing 0 05 % *p*-phenylenediamine for *ca* 30 min Relative migration (*Rm*) and relative intensity were determined either visually or with a Beckman Acta CIII spectrophotometer equipped with a gel scanner Gels were scanned at 520 nm, 0 2 mm slit width, with a scan speed of 6 cm/min

Materials Insoluble PVPP (GAF Corp) was acid-washed as suggested in ref [2] Amberlite XAD-4 (Rohm and Haas Co) was cleaned following procedures described in ref [6] AG 1-X8, 200–400 mesh, and AG 2-X8, 200–400 mesh (Bio-Rad Laboratories), were prepared for use following the recommendations in ref [37] All resins were equilibrated overnight in the appropriate buffer before use Glass distilled H_2O was used throughout

Extraction procedures Samples were prepared fresh each day The frozen pear quarters were chilled in liquid N_2 until brittle and ground in the presence of liquid N_2 in a large Waring Blendor (Model CG-5) or in an analytical mill (Model A10, Tekmar Co) The suspension of fine powder was poured into a Dewar flask and stored in liquid N_2 until used Most experimental extractions were performed by mixing 2 5 g pear powder with a specific amount of adsorbent in 20 or 25 ml of H_2O or appropriate buffer Since the amount of H_2O in each adsorbent varied, the wt of adsorbent specified was based on dry wt, but only hydrated resins were used during extraction This mixture was stirred gently with a magnetic stirrer for 4 min and filtered through glass wool before centrifugation at 14 500 *g* for 10 min at 0° or filtered through a juice extractor (Oster model 316), the basket of which was lined with glass fiber filter paper The resultant clear extract was kept in ice H_2O until used

The Me_2CO powder extraction procedure used has been described in ref [30]

PPO adsorption test PPO was extracted with PVPP (1 g PVPP/1 g pear tissue/10 ml of 0 05 M NaOAc buffer at pH 5 6) as outlined in the general extraction procedure PPO activity and the adsorption scan were recorded and a portion of the extract was saved for electrophoresis The remaining extract was divided into 2 10-ml aliquots 1 g dry wt of the appropriate hydrated adsorbent was added to each aliquot which was mixed gently for 3 min, centrifuged, and analysed as above

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