AN ABSTRACT OF THE THESIS OF

Denise Myrtle Smith for the degree of Master of Science

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Title: Methods of Extraction and Characterization of Polyphenol Oxidase from d'Anjou Pears (Pyrus communis L.)

Abstract approved:: Dr. Morris W. Montgomery

Using phenolic scavengers different methods were examined to determine the optimum conditions for extracting polyphenol oxidase (PPO) from d'Anjou pears. Efficiency of the extraction was evaluated based on browning of the extract, PPO activity per g of pear tissue, absorption scan of 410 nm to 240 nm, and the isozyme patterns produced by polyacrylamide gel electrophoresis.

Variability in pear samples was reduced by standardized harvesting and preservation techniques. Adequate preservation was achieved by initially quick freezing the pears in liquid nitrogen, then sealing in cryovac bags, and storing at -40°C. To prevent chemical reactions during maceration of the pear tissue the frozen samples were pulverized in the presence of liquid nitrogen, and stored under liquid nitrogen until used. For extraction the pear powder was added to the desired adsorbent-buffer combination.

A water extract of pear PPO proved unsatisfactory. PPO artifacts were produced in the water extract which were represented as additional bands on electrophoresis. An acetone powder technique did not remove

enough of the endogenous phenolics to prevent browning of the extract. The following adsorbents, arranged in the order of increasing efficiency, improved the extraction of PPO when used at or above the level specified: PVPP (0.75 g dry weight/g tissue), XAD-4 (1.0 g dry weight/g tissue), AG 1-X8 (0.5 g dry weight/g tissue), and AG 2-X8 (0.5 g dry weight/g tissue). All adsorbents were used in the hydrated form. Optimum activity was extracted within a pH range of 5.6 to 5.9. Anion exchange resins were particularly effective in removing hydroxycinnamic acid compounds. It was recommended that PVPP be used in combination with the polystyrene resins to aid in the adsorption of leucoanthocyanins.

The characteristics of PPO extracted with AG 2-X8 (1.0 g dry weight/g pear tissue) were examined. Three PPO isozymes were identified with relative mobilities of 0.510, 0.703, and 0.749. The pH optimum for activity was 5.1, and the enzyme was most stable at pH 5.0. When adjusted to pH 5.0 the PPO extract lost 11% of its original activity in 11 days when stored at 4°C. When the adjusted PPO extract was stored for 3 months at -40°C no change in activity or electrophoretic pattern was noted. Under the conditions studied neither XAD-4, AG 1-X8, nor AG 2-X8 adsorbed PPO. The gel electrophoresis procedure did not produce artifacts which could be identified erroneously as PPO.

# Methods of Extraction and Characterization of Polyphenol Oxidase from d'Anjou Pears (*Pyrus Communis* L.)

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Denise Myrtle Smith

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Head of Department of Food Science and Technology

Dean of Graduate School

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# METHODS OF EXTRACTION AND CHARACTERIZATION OF POLYPHENOL OXIDASE FROM D'ANJOU PEARS (PYRUS COMMUNIS L.)

## INTRODUCTION

Pear production is an important industry in the Pacific Coast states. Of the 727,000 tons of pears produced in 1978, 94% of the total crop was grown in California, Oregon, and Washington. Oregon alone produced 21% of the total (Anonymous, 1979).

Since 1936 pears have been Oregon's leading fruit crop in terms of cash farm income. In 1978 this amounted to $32,974,000, an increase of $9,274,000 in 2 years (Anonymous, 1979). Bartlett pears were produced in the largest quantity, followed by the winter pear varieties d'Anjou, Bosc, and Comice.

Enzymic browning is a serious problem for pear producers and processors. Labor costs are responsible for a large portion of the production costs, but mechanical harvesting cannot be implemented as pear tissue is very readily bruised. Pears are commonly stored for a few days to as long as 8 months before being shipped to the fresh market or processed. During this time enzymes are active and any tissue damage which occurs may lead to browning. Damage to the cuticular layer of the pears by packing house belts, which results in browning, is a common problem when pears are sold on the fresh market. Other pears may be canned, dried, or processed into juices or concentrates. Processing procedures must be rapid as browning is initiated as soon as the pear is peeled, cored, or pulped. In all cases enzymic browning may decrease consumer appeal, lower the grade, and otherwise

result in substantial economic losses. Unfortunately little is known about the mechanism of enzymic browning and few methods have been completely successful in controlling this problem.

Enzymic browning results from physiological injury, when the cell structure of the pear is disrupted. Endogenous phenolics are oxidized to quinones or to semiquinone radicals by the enzyme polyphenol oxidase (EC 1.14.8.1). The quinones produced are further oxidized and polymerized to melanin, the pigment which is responsible for brown discoloration.

To gain further insight into enzymic browning this study was undertaken to determine an optimum procedure for extracting polyphenol oxidase from d'Anjou pears and to characterize the extract for future research. d'Anjou pears were used as they are a major winter pear variety in Oregon and are increasing in popularity.

# LITERATURE REVIEW

Enzymic browning of plant tissue has been attributed to ascorbic acid oxidase, peroxidase, polyphenol oxidase, and other oxidoreductases. It is now generally accepted that the majority of enzymic browning is due to an enzyme system variously known as polyphenol oxidase, phenolase, catechol oxidase, or tyrosinase. The Commission on Enzymes of the International Union of Biochemistry has recently reclassified this enzyme as EC 1.14.8.1 monophenol monooxygenase, combining EC 1.10.3.1 o-diphenol:oxygen oxidoreductase and EC 1.10.3.2. p-diphenol:oxygen oxidoreductase into one enzyme classification (Mayer and Harel, 1979). This enzyme will be referred to as polyphenol oxidase (PPO) in this thesis.

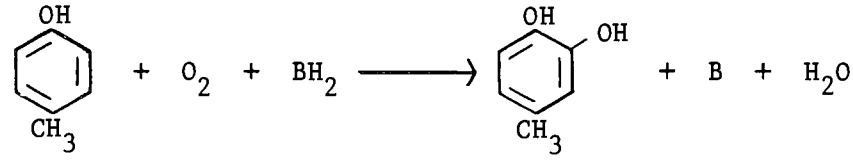
PPO reacts with certain phenolic substrates in the plant tissue to cause discoloration or browning after physiological injury. Browning may also occur as part of the normal life cycle (Matthew and Parpia, 1971). Enzymic browning is often considered detrimental in fruit and vegetable processing, but there are exceptions. Browning is considered essential in the manufacture of black tea, coffee, cocoa, and cider.

## Reactions of PPO

The names cresolase and catecholase have been applied to the enzyme as PPO has been shown to catalyze two separate types of reactions. Cresolase activity involves the hydroxylation of monophenols such as p-cresol to o-diphenols. The dehydrogenation of o-diphenols to o-quinones is known as catecholase activity, as catechol is a common laboratory

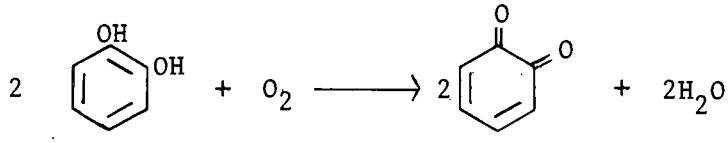
substrate. A copper prosthetic group is involved in both reactions (Mason, 1955). Recent work has shown the copper atoms to occur in pairs (Strothkamp et al., 1976). The reaction mechanism is not known with certainty, but the following generalized scheme can be shown:

Cresolase activity




p-cresol reducing agent or diphenol 4-methyl catechol

Catecholase activity




catechol o-benzoquinone

Mason (1956) and Nelson and Dawson (1944) postulated that a reducing agent was necessary to reduce the copper of the prosthetic group from cupric to the cuprous form to induce cresolase activity. Thus, cresolase activity is characterized by an induction period which continues until sufficient o-diphenol can be produced. The induction period can be eliminated by the addition of o-diphenol or other reducing agent. A nonenzymic hypothesis for cresolase activity is less favored. In this scheme it was suggested that the o-quinone produced by enzymic oxidation of o-diphenol causes the hydroxylation of the monophenol (Kertesz, 1952; Kertesz and Zito, 1962). Upon purification cresolase activity will often disappear and is rarely demonstrated in fruit PPO extracts (Scott, 1975).

Catecholase activity is most responsible for fruit browning as a major portion of the phenolics in fruit are o-diphenols. The o-quinones formed are highly reactive compounds and undergo oxidative polymerization to yield the brownish pigment, melanin. O-quinones also react readily with amino acids and protein to enhance this discoloration (Matthew and Parpia, 1971).

## Properties of PPO

Kertesz and Zito (1965) first reported a molecular weight of 32,500 for mushroom PPO, but later revised their figure to 118,000 - 128,000. Jolley et al. (1969) found mushroom PPO was composed of four subunits with a molecular weight of 32,400 for the monomer. Strothkamp et al. (1976) later reported mushroom PPO composed of a heavy subunit, H (MW 43,000) and a light subunit, L (MW 13,400) which was active in the ratio $L_2H$. PPO of tea shoots have a molecular weight of 144,000 ±16,000 (Gregory and Bendall, 1966). Harel and Mayer (1968) reported three different molecular weight fractions for apple PPO of 30,000 - 40,000, 60,000 - 70,000, and 120,000 - 130,000. The molecular weight of pear PPO has not been reported.

In most plant tissues studied the pH for optimum activity ranges from 5.0 to 7.0, with a loss of activity below 3.0 (Scott, 1975). The pH optimum for Bartlett pear PPO has been reported as 6.2 (Tate et al., 1964) and 4.0 (Rivas and Whitaker, 1973). Halim and Montgomery (1978) reported a pH optimum of 7.0 for d'Anjou pear PPO. The pH for optimum stability during heating trials was found to be between 5.0 and 7.0 for pear puree (Scott, 1975). The pH optimum for peach PPO isozymes

has been reported to range between 6.5 and 7.0 (Wong et al., 1971) and to be pH 7.0 for bananas (Palmer, 1963). The pH optimum for apples has been reported as 5.1 for both chloroplast (Harel et al., 1965) and mitochondrial (Walker and Hulme, 1965) PPO fractions.

PPO is generally considered to be a soluble enzyme (Anderson, 1968), but has been reported bound to chloroplasts, mitochondria and other cell organelles (Mayer and Harel, 1979). Mason (1955) postulated that PPO was found associated with cell organelles only after extraction, as a result of the affinity of PPO for the hydrophobic areas of cell membrane lipids. Sanderson (1964) presented evidence for the solubility of tea PPO and refuted the reports of Takeo and Uritani (1965) and Li and Bonner (1947) who claimed tea shoot PPO was structurally bound. Sanderson (1964) found that the distribution of enzyme activity between the precipitate and supernatant fractions was a function of the ratio of polycaprolaktam powder to plant material used during extraction, with a ratio of 1:1 (w/w) sufficient to maintain the enzyme in soluble form. He suggested the occurrence of bound PPO was due to the precipitation of protein by bound phenolics which was prevented when polycaprolaktam powder was used to adsorb the endogenous phenolics.

The substrate specificity of PPO is diverse. Most fruit PPO extracts were reported active only on o-diphenols and were unable to catalyze hydrogenation of monophenols. Walker (1964) noted similar activities between apple and pear extracts, with PPO being most active on o-diphenols and only slightly active with the monophenol, p-cresol. Tate et al. (1964) reported Bartlett pear PPO active only on

o-diphenolic compounds with no activity toward m- or p-diphenolic compounds. Rivas and Whitaker (1973) found only o-diphenolase activity with no detectable monophenolase or laccase activity in their Bartlett pear PPO preparation. Halim and Montgomery (1978) reported similar results with d'Anjou pear PPO. In all cases, pear PPO was most active on catechol and chlorogenic acid. Cherry (Benjamin and Montgomery, 1973) and peach (Wong et al., 1971) PPO exhibited activity only on o-diphenols. Palmer (1963) reported that banana PPO was active only on o-diphenolic compounds, whereas Montgomery and Sgarbieri (1975) found limited activity with tyrosine.

## PPO Activity Measurements

PPO activity has commonly been measured by spectrophotometric, chronometric, manometric, and polarographic methods (Bendall and Gregory, 1963). A comparison of these methods has been published by Mayer et al. (1966). Another less widely used method involves measurement of the release of tritiated water during hydroxylation of tyrosine-3,5-$^{3}H$ (Pomeranz, 1966). To obtain accurate activity measurements with any method it has been essential to monitor the initial rate as the products of the PPO reaction react with each other, unchanged substrate, oxygen, and protein, to alter further activity measurements (Walker, 1975).

A Warburg apparatus measures oxygen uptake by PPO in the manometric method. The chronometric method monitors the coupled oxidation of ascorbic acid in the presence of substrate by indotitration, spectrophotometer, or by detection of the appearance of quinone with an

external indicator (Bendall and Gregory, 1963). In both techniques difficulty was found in maintaining linearity with increasing concentrations of enzyme. Initial rate measurements have been reported inaccurate when using the Warburg apparatus (Mayer et al., 1966).

The spectrophotometric method has been used most frequently and has proved adequate for comparative studies of PPO activity in different fruit varieties (Walker, 1962). This method measures the increase in absorptivity of the pigments produced from the corresponding o-diphenol. Walker (1975) recommended that activities between various substrates not be compared as the pigments produced absorb at different wavelengths and have different molar absorptivities. Recently, coupling reactions have been used to measure the quinone formed (Esterbauer et al., 1977). Mayer and Harel (1979) noted that oxygen concentration was often below that necessary to saturate the enzyme when the spectrophotometric method was used. Initial rate measurements may last less than 30 sec due to the instability of the product (Mayer et al., 1966).

The polarographic method, utilizing an oxygen electrode, has often been considered the method of choice (Bendall and Gregory, 1963; Mayer et al., 1966; Walker, 1975). The magnitude of the current flow which occurs when oxygen is reduced at a polarized platinum electrode is proportional to the concentration of oxygen in the medium. Thus, the rate of oxygen consumption by PPO can be measured. Results may vary due to barometric pressure changes as the partial pressure of oxygen in the reaction medium is affected by this factor (Yellow Springs Instrument Co., 1978).

## PPO Isozymes

Isozymes are defined as multiple molecular forms of enzymically active proteins, catalyzing the same reaction and occurring in the same species, but differing in certain physical, chemical, and kinetic properties (Markert and Moller, 1959). Little is known about the genetic or physiological significance of isozymes, but they appear to allow an enzyme to function optimally under different physiological conditions, either at different sites within a cell or tissue, or in distinct metabolic sequences (Markert, 1968). Isozymes may be produced by two or more genes or a single gene, and may result from one or more of the following: genetic code mutations, post-translational modifications, binding of small molecules, nonspecific aggregation, conformational changes, or multimer formation by identical or nonidentical subunits (Kaplan, 1968). Artifacts caused by enzyme isolation techniques are often mistakenly identified as isozymes (Markert, 1968).

Multiple forms of PPO have been reported in mushrooms (Mallette and Dawson, 1949; Bouchilloux et al., 1963), apples (Harel et al., 1965; Walker and Hulme, 1966), bananas (Montgomery and Sgarbieri, 1975), peaches (Wong et al., 1971), pears (Rivas and Whitaker, 1973; Halim and Montgomery, 1978), and many other plant tissues.

Highly purified preparations of mushroom PPO (Bouchilloux et al., 1963; Jolley and Mason, 1965; Kertesz and Zito, 1965) and apple PPO (Harel and Mayer, 1968) contained several isozymes which exhibited association-dissociation phenomena when treated by certain physical or chemical methods. This multiplicity was explained as resulting from

various degrees of combination of like or unlike subunits, or from the formation of conformers (Jolley and Mason, 1965; Harel and Mayer, 1968). VanHolde (1966) has reported that a protein will tend to associate if it contains 30% or more of certain hydrophobic amino acid residues. Since PPO does contain ca. 30% of the required hydrophobic residues, Jolley et al. (1969) suggested an equilibrium may exist between single and multichain forms. Whether this phenomenon occurs in the intact tissue is not known (Harel and Mayer, 1968; Jolley et al., 1969).

The possibility of artifacts resulting from the isolation procedure must be considered (Bouchilloux et al., 1963). Gregory and Bendall (1966) obtained four soluble yellow fractions during an intermediate stage of purification of tea PPO. On further purification two forms of PPO were recovered from each of the four semipurified extracts. Gregory and Bendall (1966) concluded that the four yellow fractions represented various complexes of PPO with phenolic products and were artifacts of the isolation procedure.

Brown and Wright (1963) noted changes in the mobility and quantity of protein bands during electrophoresis of milk proteins incubated with tea polyphenols. Fields and Tyson (1973) reported similar changes in the electrophoretic patterns of peroxidase isozymes isolated from flax due to phenolic binding. Loomis (1978) was able to generate new isozymes when several purified proteins were incubated with allyl isothiocyanate, and has established that artifacts of horseradish peroxidase were produced during extraction, due to glucosinolates present in the tissue.

## Composition of d'Anjou Pears

Watt and Merril (1950) reported pear tissue to be composed of 82.7% water, 0.7% protein, and 0.4% fat. The next year Strachen et al. (1951) published the composition of mature d'Anjou pear tissue, excluding seeds, core, and skin as listed in Table 1.

Table 1. Composition of d'Anjou pears.

| | |
|---|---|
| pH | 4.1 |
| total solids | 15.8% |
| total acid (as anhydrous citric) | 0.28% |
| total sugar | 9.72% |
| pectin (as calcium pectate) | 0.71% |
| tannin | 0.022% |
| total ash | 0.30% |

## Pear Phenolic Compounds

Phenolic compounds, which are of widespread occurrence in fruit, can be broadly classified as either hydroxycinnamic acid or flavonoid compounds. The hydroxycinnamic acid derivatives have the general structure $C_6C_3$ and are also known as phenylpropanes. Flavonoids possess the $C_6C_3C_6$ skeleton of diphenylpropanes. B-ring hydroxylation is most common at the 3' and 4' positions, followed by hydroxylation at the 4' position only (Herrmann, 1976). In the intact cell these compounds are found as glycosides or esters, rather than as the aglycone (Williams, 1957).

The concentrations of phenolics in fruit tissue vary due to variety, size of fruit, degree of ripeness, growing conditions, and

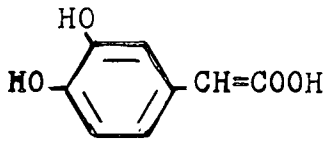
location in the plant (Williams, 1957; Herrmann, 1976). Within a class of plants, such as the Pomoidiae, similarities exist in gross phenolic composition, however differences are seen in trace phenolic constituents (Williams, 1957).

Hydroxycinnamic acid compounds found in pear tissue include chlorogenic and isochlorogenic acids (Bradfield et al., 1952), p-coumarylquinic acids, and caffeic acid (Cartwright et al., 1955). Flavonoid compounds include catechin and epicatechin (Siegleman, 1955), leucoanthocyanins (Joslyn and Peterson, 1956), quercetin and isoquercetin (Sioud and Luh, 1966), with smaller quantities of kaempferol (Herrmann, 1976), and isorhamnetin (Duggan, 1969). Arbutin has also been identified in pear tissue (Durkee et al., 1968). The structures of these compounds are shown in Figure 1.

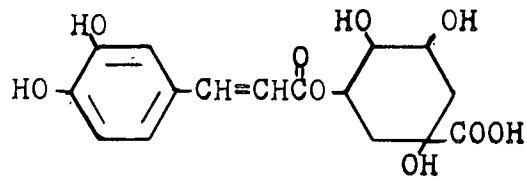
Sioud and Luh (1966) reported the phenolic fraction of canned Bartlett pear puree to contain 32.5% leucoanthocyanin, 32.5% catechin and epicatechin, 23.8% chlorogenic acid, 10% quercitrin, and less than 1% quercetin and p-coumarylquinic acids. Ury (1964) examined the difficulties involved in measuring the leucoanthocyanin content of Bartlett pears by the method of Swain and Hillis (1959) and recommended methods which could be implemented to improve the extraction and quantitation of leucoanthocyanins.

Chlorogenic acid has been implicated as the major browning substrate in pear tissue (Weurman and Swain, 1953; Walker, 1964; Tate et al., 1964). Randive and Haard (1971) found the browning tendency of four different pear varieties to correlate well with the total phenolic content, especially chlorogenic acid and catechin. Siegelman

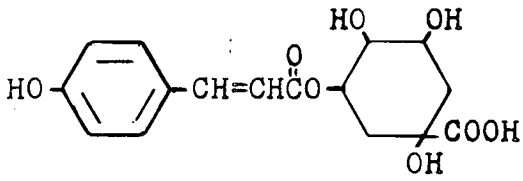
Hydroxycinnamic acid compounds:



caffeic acid

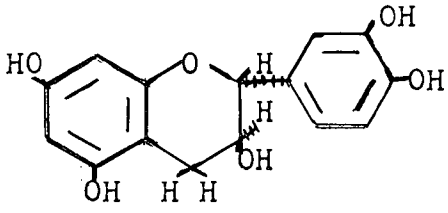


chlorogenic acid

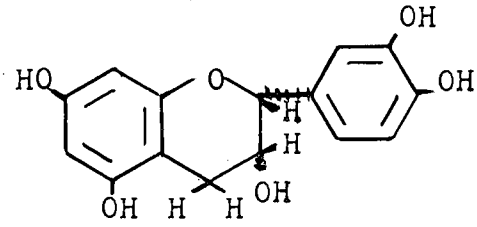


p-coumarylquinic acid

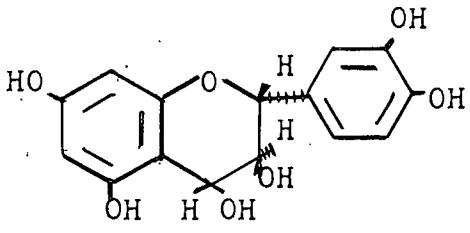
Flavonoid compounds:



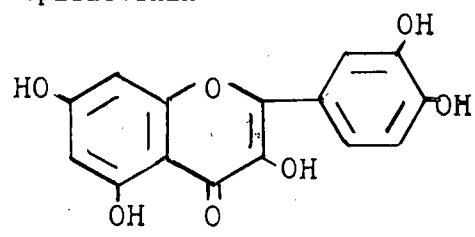
catechin



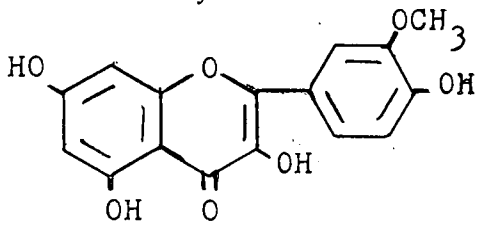
epicatechin



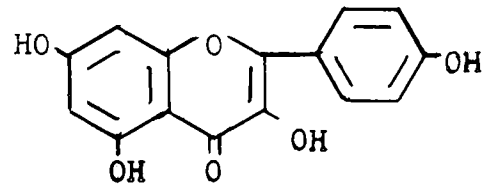
leucoanthocyanidin



quercetin



isorhamnetin



kaempferol

Figure 1. Structures of some phenolic compounds found in pears.

(1955) reported epicatechin and catechin to be the principle browning substrates in Bartlett pear skin, agreeing with the fact that larger quantities of flavonol glycosides were found in the skin than in the pulp, as their formation depends on the presence of light (Herrmann, 1976).

The phenolic content of pears changes considerably on ripening. The level of total phenolics is at the highest concentration during early stages of growth and drops rapidly to reach a constant level just before harvest (Hulme and Rhodes, 1971). Chlorogenic acid follows a similar pattern. Randive and Haard (1971) reported an increase in the total phenolic content of pear pulp on ripening when compared to the phenolic content immediately after harvest.

## Plant Enzyme Extractions

A plant cell is composed of a small amount of cytoplasm separated by membranes from a large central vacuole and a rigid cell wall. Along with lipids and nucleic acids present in both plant and animal tissue, plants may contain vacuole acids, starch, pectin, phenolic compounds, and often other secondary metabolites, such as monoterpenes and isothiocyanates. When the cell is ruptured during extraction, mixing of all these constituents occurs. Many of these compounds can react with protein to cause various modifications (Loomis, 1974). As a result, special techniques are necessary to isolate plant proteins.

The successful isolation of plant enzymes has been the subject of several reviews. Loomis and Battaile (1966) discussed the problems involved in isolating plant enzymes and recommended the use of certain

polymers which act as phenolic scavengers. Anderson (1968) extended the review of Loomis and Battaile (1966) by including chemical inhibitors and reducing agents which could be used to inactivate PPO or prevent phenolic oxidation. In another review Loomis (1974) presented a wide variety of isolation techniques available to overcome the special problems of plant tissues. Rhodes (1977) examined the problems of extraction and stabilization of plant enzymes and described several purification techniques.

## Protein - Phenolic Interactions

Phenolic compounds, their corresponding quinones, and resultant polymerized products can react with protein in a plant tissue extract. O-quinones produced as metabolic intermediates are some of the most reactive compounds in living tissue and are least restricted in respect to chemical interreactions (Mason, 1955). Loomis (1974) classified these reactions into four main categories which include: hydrogen bonding, covalent coupling, ionic interactions, and hydrophobic interactions.

Strong hydrogen bonds are formed between isolated phenolic hydroxyl groups and the oxygen of the peptide bond. Binding decreases at high pH due to the ionization of hydroxyl groups (Loomis and Battaile, 1966). It is generally agreed that tannins interact with protein by multiple hydrogen bonds (Loomis and Battaile, 1966; VanSumere et al., 1975), often causing precipitation (Haslam, 1974) and interfering in the digestion of protein (Synge, 1975).

Quinones can undergo a variety of covalent coupling reactions with

protein, especially 1,4 addition. Early work on the mechanism has been reviewed in detail by Mason (1955). Pierpoint (1969) found the thiol group of cysteine to be particularly reactive. Other reactive residues include the ε-amino group of lysine, the α-amino group, especially the terminal α-amino group of peptides and proteins, and the imino group of proline. Cross-linking of protein often results. Covalent coupling is the principle cause of browning in plant tissues and extracts, and may reduce the nutritional value of the protein (Synge, 1975).

At high pHs salt linkages may occur between basic amino acid residues and phenolic hydroxyl groups, due to the high pK value of phenolic hydroxyls. Ionic bond formation may also occur at or below neutral pHs due to the negatively charged carboxyl group of certain phenylpropanoid compounds (Loomis, 1974). Also, hydrophobic bonding may occur between the aromatic ring structure of phenolics and the hydrophobic regions of protein (Loomis, 1974). Upon denaturation this effect may become more severe.

All of these reactions serve to modify a protein such that its native properties and structure are altered. Often the modification is severe enough to cause enzyme inactivation (Alberghina, 1964; Anderson and Rowan, 1966). Loomis and Battaile (1966) mentioned a variety of cases where phenolics have interfered in the isolation of plant enzymes.

## Phenolic Adsorbents

Since all plant materials contain different types and quantities of phenolic compounds and other secondary products, no universal method has been devised to produce a satisfactory enzyme extract. Reducing agents, enzyme inhibitors, and phenolic scavengers have been used singularly or in combination to improve an extraction (Loomis and Battaile, 1966; Anderson, 1968). Many polymers are available which act as phenolic scavengers when added to the extraction medium. These include: nylon 66 (McFarlane and Bayne, 1961), bovine serum albumin (Loomis and Battaile, 1966), polycaprolaktam powder (Nylon 6) (Sanderson, 1964), polyethylene glycol (Badran and Jones, 1965), insoluble polyvinylpyrrolidone (PVPP, insoluble PVP) (Loomis and Battaile, 1966), Amberlite XAD resins (Loomis, 1974; Loomis et al., 1979), and anion exchange resins (Lam and Shaw, 1970). The use of PVPP, XAD, and anion exchange resins will be reviewed here.

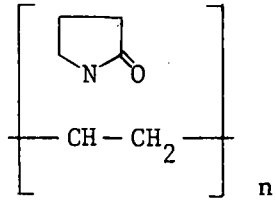
### Insoluble PVP

Gustavson (1954) reported soluble PVP to have the capacity to precipitate both condensed and hydrolyzable tannins from solution. Soluble PVP has been used successfully in the preparation of mitochondria from plant tissues (Hulme et al., 1964, Jones et al., 1964), but could not be used for the preparation of PPO because it was found to inhibit the enzyme (Harel et al., 1964; Walker and Hulme, 1965).

An early use for insoluble PVP was in the chillproofing of beer, as it was more easily removed than soluble PVP (McFarlane and Bayne,

1961). Loomis and Battaile (1966) were pioneers in the use of insoluble PVP for enzyme extractions. Using peppermint, they were able to identify mevalonic kinase, alkaline phosphatase, and glutamyl transferase activities which had been inactivated when other extraction techniques had been used. Using similar techniques with PVPP, Loomis and Battaile (1966) were able to extract soluble protein from apple fruit and Canadian thistle leaves. Although several enzymes had previously been extracted and characterized from tea leaves, Coggon et al. (1973) were able to produce greatly improved extracts which resulted in more accurate characterization of peroxidase, PPO, and gallate esterase activities in tea by incorporating PVPP into the extraction medium. Kelly and Adams (1977) identified peroxidase, esterase, and α-terpineol dehydrogenase from juniper leaves in extracts prepared with PVPP in combination with a buffer containing certain inhibitory compounds. Soluble PPO activity was found in bananas (Montgomery and Sgarbieri, 1975) and pears (Halim and Montgomery, 1978) using PVPP.

Insoluble PVP is a high molecular weight polymer with the general structure:



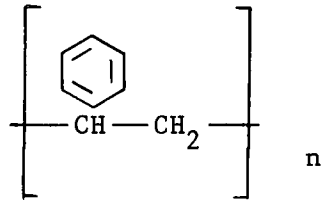

PVPP forms hydrogen bonded complexes between the polymer oxygen and isolated hydroxyl groups of phenolic compounds (Loomis and Battaile, 1966; Anderson and Sowers, 1968). Anderson and Sowers (1968) and Loomis (1974) reviewed some of the conditions necessary for optimum

binding between PVPP and phenolic compounds. Anderson and Sowers (1968) found PVPP ineffective at high pH due to the ionization of phenolic hydroxyl groups and reported pH 3.5 as optimum for PVPP-phenolic binding. Loomis (1974) reported PVPP effective at neutral to acidic pHs and pointed out that the actual difference in binding between pH 6.0 and 3.5 as demonstrated by Anderson and Sowers (1968) was very small.

PVPP has a limited capacity to adsorb chlorogenic acid. Anderson and Sowers (1968) attributed this to the limited number of hydroxyl groups on chlorogenic acid and suggested possible competition between intramolecular bond formation and PVPP-phenol bond formation. Gray (1978) found insoluble PVPP to have high affinity for quercetin, catechin, and proanthocyanin, and low affinity for chlorogenic acid at pH 7.5. Loomis et al. (1979) reported a low affinity of PVPP for tyrosine, with increased affinity for chlorogenic acid and quercetin at pH 6.5.

Amberlite XAD Resins

Amberlite XAD-2 and XAD-4 (Rohm and Haas Co.) are non-ionic, macroreticular adsorbents made of polystyrene and divinylbenzene cross-linked to form microspheres. These microspheres are fused together into a spherical agglomerate of 20-50 mesh beads with a total surface area of 725 $m^2/g$ for XAD-4 (Rohm and Haas Co., 1978) and 290-330 $m^2/g$ for XAD-2 (Rohm and Haas Co., 1975). The resins are highly aromatic in nature and adsorb hydrophobic compounds strongly. The general structure of the XAD adsorbents is:



Loomis (1974) reported that these compounds bind plant phenolic compounds and other hydrophobic or surface active materials which may otherwise bind protein during enzyme extractions. Loomis et al. (1979) have recently reviewed the use of polystyrene polymers in plant enzyme isolations.

Croteau et al. (1973) successfully used XAD-4 to remove monoterpenes from plant extracts. XAD-4 and PVPP in combination produced clear extracts from potato tubers, apple fruit, English walnut hulls, and horseradish root and removed the odor of isothiocyanate from the horseradish root extract (Loomis et al., 1979).

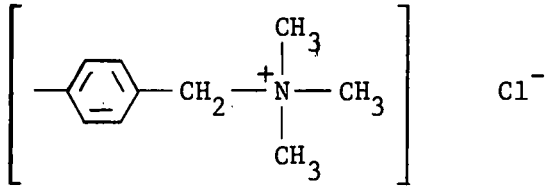
XAD-2 was reported ineffective in adsorbing proanthocyanins, while having a higher affinity for hydroxycinnamic acid derivatives than PVPP at pH 7.5 (Gray, 1978). XAD-4 was found to adsorb chlorogenic acid more efficiently than PVPP, whereas quercetin was adsorbed at about the same level (Loomis et al., 1979). XAD-4 was more efficient than XAD-2 for adsorbing catechol (Loomis et al., 1979). Neither bovine serum albumin nor horseradish peroxidase was bound by XAD-4 (Loomis et al., 1979).

Anion Exchange Resins

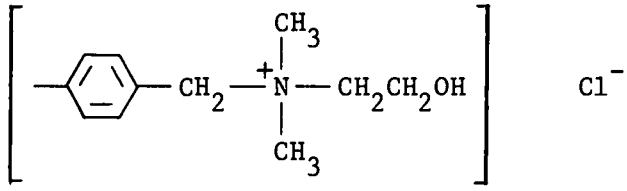
Anion exchange resins, based on the copolymerization of styrene and divinylbenzene and containing quaternary ammonium exchange groups, are commonly used as phenolic adsorbents. The strong-base resins,

Dowex 1 and 2 (Dow Chemical Co.), incorporate two types of ionizable functions:

Dowex I



Dowex II



Lam and Shaw (1970) were able to demonstrate PPO and peroxidase activity when Dowex 1 was used to adsorb the phenolic material from flax. Dowex 1 was later used successfully for the extraction of peroxidase isozymes from flax (Fields and Tyson, 1973), and to examine the isozyme patterns of commercial tobacco plants (Gray et al., 1974). Gross et al. (1975) used a combination of Dowex 1 and PVPP to examine hydroxycinnamate:coenzyme A ligase from several plants.

The adsorption capacity of anion exchange resin is due mainly to hydrophobic and ionic bonding (Moore and Stein, 1951). Hydrogen bonding may be involved (Olsson et al., 1976), especially in the case of Dowex 2 (Loomis et al., 1979). Gray (1978) found Dowex 1 to adsorb chlorogenic acid, quercetin, and catechin well, but not proanthocyanidin at pH 7.5. Loomis et al. (1979) have reported Dowex 1 and 2 to be more efficient in adsorbing chlorogenic acid and quercetin than PVPP or XAD-4 at pH 6.5. Dowex 2 was more effective than Dowex 1.

Anion and cation exchange resins have been reported to bind protein (Fasold et al., 1961). Lam and Shaw (1970) recommended that anion

exchange resins not be used for extracting enzymes with low isoelectric points, as highly negatively charged proteins may be adsorbed. Fasold et al. (1961) stated the binding was due to reaction of the hydrophobic matrix of the resin with partially unfolded proteins. Boardman and Partridge (1955) suggested binding by the carboxyl groups of their resin with protein, while Loomis et al. (1979) mentioned possible binding of the carboxyl groups of protein with certain resins.

# MATERIALS AND METHODS

## Preparation of Samples

d'Anjou pears (Pyrus communis L.) were obtained from the Mid-Columbia Experiment Station, Hood River, Oregon. Preliminary trials were performed with pears grown during the 1977 season and stored at -1°C for 8 months. Upon shipment to Corvallis, the pears were quartered, cored, frozen in liquid nitrogen, sealed in cryovac bags, and stored at -40°C until used. Later experiments were performed on pears grown during the 1978 season which had been handpicked from the same tree. The pears had a pressure test of 19 psi. The pears were brought to Corvallis and any pear which deviated greatly in size or other physical characteristic was discarded. The next day the pears were frozen as described above.

## Assay of PPO Activity

Enzyme activity was measured by either a spectrophotometric or polarographic method. A Perkin Elmer spectrophotometer, Model 550, attached to a recorder was used to measure the increase in absorbance at 410 nm. The reference cuvette contained 2.5 ml of 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0, and 0.5 ml of 0.1 M catechol in 0.01 M citrate - 0.02 M phosphate buffer, pH 3.6. Sample cuvette contained 2.0 ml of 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0, 0.5 ml of 0.1 M catechol in 0.01 M citrate - 0.02 M phosphate buffer, pH 3.6, and 0.5 ml enzyme preparation. The reaction was carried out at 24°C. Initial velocity was determined from the linear portion of the curve

within 60 sec of initiation of the reaction. One unit of enzyme activity was defined as that amount of enzyme which caused a change in absorbance of 0.001 per min.

Polarographic measurements were performed using the YSI Model 53 Biological Oxygen monitor equipped with a Clark type electrode. The reaction chamber was held at 30°C by a Lauda K2/2 constant temperature circulator. The instrument was standardized to 100% using air-saturated distilled water. Two ml of 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0, and 0.5 ml of the enzyme solution were added to the reaction chamber and allowed to stand for 3 min to achieve temperature equilibration. To initiate the reaction 0.5 ml of 0.1 M catechol in 0.01 M citrate - 0.02 M phosphate buffer, pH 3.6, was injected down the access groove. The linear portion of the curve was used to measure initial rates of oxygen consumption. Oxygen consumption was expressed as percent loss of oxygen per min per g of pear tissue or converted to nmols of oxygen consumed per min per g of pear tissue, based on the oxygen content of air-saturated water at 30°C.

## Absorption Scan of PPO Extract

Absorption scans were performed in the range of 410 nm to 230 nm using a Perkin Elmer spectrophotometer, Model 550, at a scan speed of 120 nm/min. The reference cuvette contained 3 ml of 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0. The sample cuvette contained 2.0 ml of 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0, plus 1.0 ml of enzyme solution.

## Discontinuous Polyacrylamide Gel Electrophoresis

Anionic disc gel electrophoresis was performed following the general principles described by Davis (1964) and Williams and Reisfeld (1964). The actual procedure used was described by Halim and Montgomery (1978) with some modifications. The constant voltage power supply was EC-Model 454 (EC Apparatus Corp.).

The following stock solutions were prepared and stored at 4°C.

1. Electrode buffer: 0.0165 M Tris - 0.039 M glycine, pH 8.75; 11.6 g glycine was mixed with ca. 8.0 g of Tris in 4.0 ℓ of distilled water.
2. Spacer buffer: 0.062 M Tris - HCl, pH 6.7; 22.5 g of Tris, 1.5 ml of N,N,N',N'-tetramethylethylenediamine (TEMED, Eastman Kodak) and ca. 12.0 ml of concentrated HCl were combined in 2.0 ℓ of distilled water. The pH was adjusted with 1.0 N HCl and made up to a final volume of 3.0 ℓ with distilled water.
3. Running buffer: 0.038 M Tris - HCl, pH 9.0; 138.0 g Tris, 3.0 ml TEMED, and ca. 12.0 ml of concentrated HCl were combined in 2.0 ℓ of distilled water. The pH was adjusted with 1.0 N HCl and brought to a final volume of 3.0 ℓ.

The following solutions were prepared immediately before use.

1. Running gel solution, 7% (w/v): 2.45 g of Cyanogum 41 (95% acrylamide and 5% bisacrylamide, EC Apparatus Corp.) was dissolved in 35.0 ml of running buffer and filtered through Whatman No. 1 filter paper. Polymerization was catalyzed by adding 0.035 g ammonium persulfate (AP) to the solution.

2. Spacer gel solution, 4% (w/v): The AP catalyzed gel was prepared by dissolving 0.8 g of Cyanogum 41 in 20.0 ml of spacer buffer and filtering through Whatman No. 1 filter paper. Polymerization was initiated by adding 0.01 g of AP. The riboflavin catalyzed gel was made by dissolving 0.64 g of Cyanogum 41 in 14.0 ml of spacer buffer, adding 2.0 ml of 0.004% (w/v) riboflavin solution and filtering.

The gels were prepared in glass tubing 0.5 cm I.D. and 12.0 cm in length. Tubes were cleaned in nitric acid and soaked in 0.01% Photoflo (Eastman Kodak Co.). One end of each tube was sealed with parafilm.

Immediately after preparation the running gel solution was pipetted into each tube to a height of 9.0 cm. A small amount of isopropyl alcohol was layered on the top of each column (Cunningham et al., 1978). Gelation occurred in ca. 20 min as indicated by a faint opalescence. The alcohol was removed and the tubes rinsed well with distilled water and dried.

Next, the spacer gel solution was prepared and pipetted on top of the running gel to a height of 1.0 cm. The tubes were overlaid with isopropyl alcohol. If the solution was catalyzed by riboflavin a fluorescent lamp was placed within 10.0 cm of the tubes. In either case polymerization was effected in ca. 20 min.

Before electrophoresis the enzyme solution was run through a 1.0 cm by 10.0 cm column of Bio-Gel P-6 (Bio-Rad Laboratories) prepared as described by Cooper (1977) to remove residual phenolics. The enzyme

solution was then mixed with 10% sucrose and a small amount of bromophenol blue. This was layered on top of the spacer gel with a syringe in 50 µl to 400 µl quantities to obtain constant PPO activity levels in each tube.

The power supply was connected such that the cathode (-) lead was attached to the upper chamber and the anode (+) lead to the lower chamber. Electrophoresis was carried out at 4°C. An initial current of 100 V (ca. 2 ma/tube) was applied until the sample stacked at the surface of the running gel (ca. 45 min), then increased to 200 V for the remainder of the run (ca. 2 hr). On completion of the run the gels were removed by carefully forcing water between the glass tube and gel with a water filled syringe.

## Detection of PPO Isozymes

To detect PPO activity the gels were immersed in 10.0 mM catechol in 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0, 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 2 accessory. Gels were scanned at 520 nm, 0.2 mm slit width, with a scan speed of 6 cm/min.

## Cleaning of Phenolic Adsorbents

### Insoluble PVP

Insoluble PVP (GAF Corp.) was cleansed of impurities following the procedure suggested by Loomis (1974). PVPP was added to 10% HCl to form a thick slurry and stirred continuously while boiling for 10 min.

The PVPP was then placed in a large Buchner funnel lined with bolting silk and rinsed with distilled water. This was continued until the rinse water was free of chloride ions as indicated by the lack of a white precipitate when tested with a 1% silver nitrate solution. The PVPP was dried at 60°C and before use was hydrated overnight in the extraction buffer.

Amberlite XAD-4

Amberlite XAD-4 (Rohm and Haas Co.) was cleaned following procedures described by Loomis et al. (1979). XAD-4 was rinsed several times in distilled water to remove fines, followed by washing in 3 volumes of acetone. Next, the XAD was placed in a large Soxhlet extractor and extracted in acetone for 7 days. It was then washed in 3 volumes of 10% HCl, followed by rinsing in distilled water until the effluent was free of chloride ions, as determined by the silver nitrate precipitation test. The resin was kept moist until use.

Anion Exchange Resins

AG 1-X8, 200-400 mesh, and AG 2-X8, 200-400 mesh (Bio-Rad Laboratories) were prepared for use following the recommendations of Cooper (1977). The resin was rinsed in distilled water to remove fines, washed twice with 95% ethanol and twice in 2.0 N HCl. Next, the resin was boiled in 2.0 N HCl, followed by rinsing with 2.0 N acetic acid (three times), 1.0 M sodium acetate (three times), and 0.1 M acetic acid. Finally, the resin was washed in distilled water until the pH of the effluent was the same as distilled water. The resin was stored

hydrated at 4°C and before use was equilibrated overnight in the extraction buffer.

## General Extraction Procedure

Samples were prepared fresh each day. The frozen pear quarters were chilled in liquid nitrogen until brittle and ground in the presence of liquid nitrogen in a large Waring Blendor (Model CB-5) or in an analytical mill (Model A10, Tekmar Co.). The suspension of fine powder produced was poured into a Dewar flask and stored in liquid nitrogen until use.

Most experimental extractions were performed by mixing 2.5 g pear powder with a specified amount of adsorbent in 20.0 or 25.0 ml of distilled water or appropriate buffer. Since the amount of water in each adsorbent varied, the weight of adsorbent specified was based on dry weight, but only hydrated adsorbents were used during extraction. This mixture was gently stirred by a magnetic stirring apparatus for 4 min.

To remove cell debris and phenolic adsorbents the extract was first crudely filtered through glass wool. The extract was then centrifuged at 14,500 xG for 10 min in a Sorvall RC5 Superspeed centrifuge at 0°C or filtered through a juice extractor (Oster Model 361), the basket of which was lined with glass fiber filter paper (Reeve Angel). The resultant clear extract was kept in ice, or frozen at -40°C.

## Acetone Powder Extraction

An acetone powder extraction as described by Benjamin and Montgomery (1973) was investigated. The procedure was performed in the cold room at 4°C. Ten g of pear powder was blended with 200 ml cold acetone (-23°C) for 1 min in a stainless steel Waring Blendor. The resultant slurry was filtered under suction through Whatman No. 1 filter paper using a Buchner funnel. The residue was washed four times with 200 ml cold acetone, dissolved in 50 ml of 0.2 M acetate buffer, pH 5.0, and refiltered using Whatman No. 1 filter paper.

## PPO Adsorption Test

To determine if XAD-4, AG 1-X8, or AG 2-X8 adsorb PPO during extraction the following experiment was devised. PPO was extracted with PVPP (2.5 g PVPP/2.5 g pear tissue/25.0 ml of 0.05 M acetate 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 saved for electrophoresis. The remaining extract was divided into two 10.0 ml aliquots. One g of the appropriate hydrated adsorbent was added to each aliquot which was mixed gently for 3 min, centrifuged, and analyzed as above. Electrophoresis was performed on both the first and second extract.

# RESULTS AND DISCUSSION

## Preservation of Samples

To adequately preserve the pear samples it was necessary to prevent physiological changes and protein-phenolic interactions throughout the storage period. Mellenthin and Wang (1974) reported changes in total phenolics and PPO activity in d'Anjou pears stored at -1°C over an eight week period. Browning often occurs in freeze dried samples due to intermixing of cellular constituents, thus indicating phenolic oxidation (Loomis, 1974). Tate et al. (1964) found no darkening of pears sealed under a vacuum and frozen at -26°C after 2 years storage. Quick freezing of plant tissue was recommended as slow freezing may result in the destruction of cellular integrity due to the formation of large ice crystals (Potter, 1973). Consequently, pear samples were quick frozen in liquid nitrogen, sealed under an atmosphere of nitrogen in cryovac bags, and stored at -40°C. No browning of the pear tissue was visible over the 8 month storage period.

## Water Extract of Pear PPO

A water extract of the pear tissue was evaluated to demonstrate the detrimental effects of phenolic compounds on PPO. The pH of the extract was 4.2. Activity loss was rapid as more than 60% of the original activity was lost within 7 hr of extraction (Figure 2). Browning was observed visually within 30 min of extraction. Changes in the absorption scan of the extract were observed (Figure 3). The increase in absorbance at 410 nm over the 8 hr period indicated browning of the

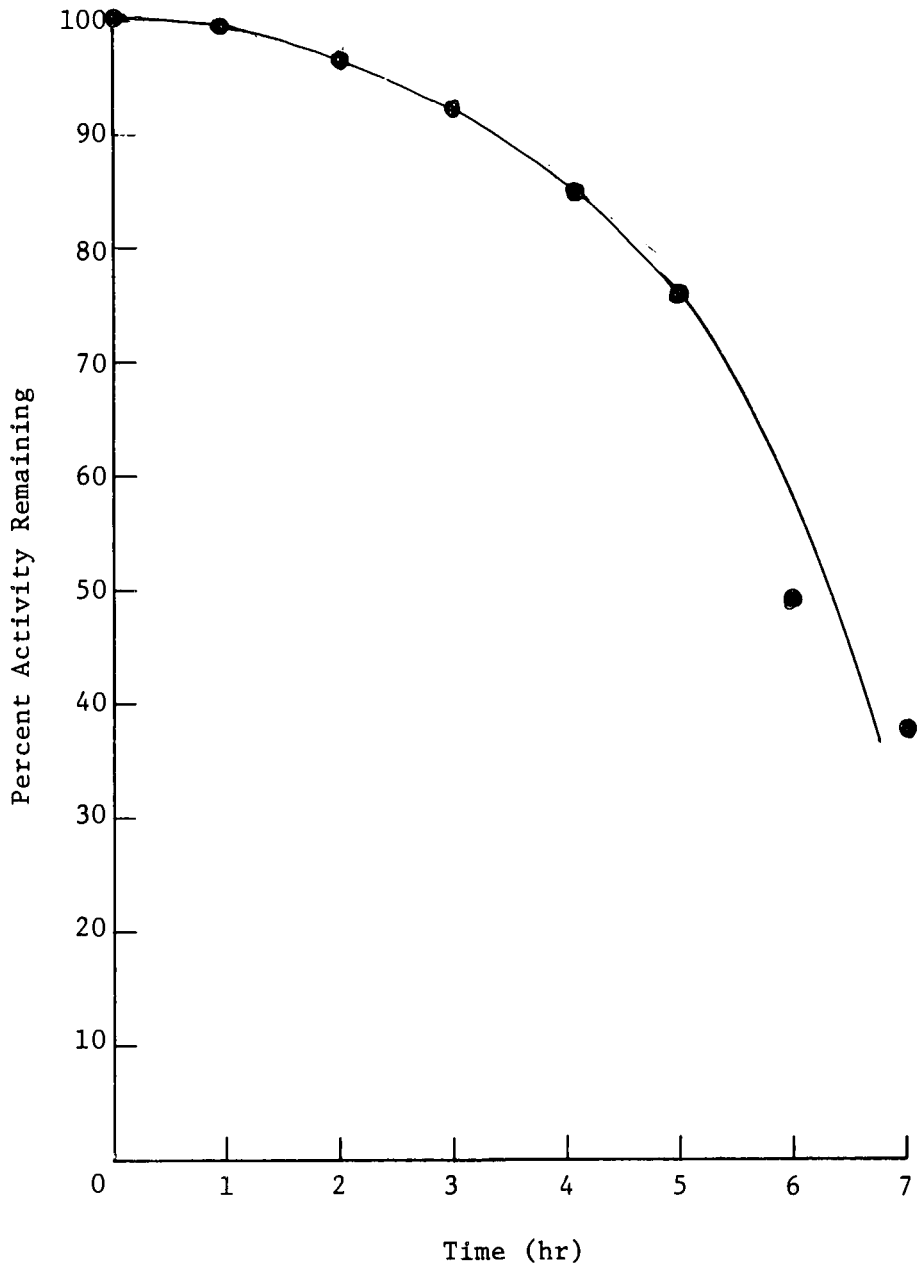



Figure 2. PPO activity loss in water extract of pear tissue stored at 4°C for 7 hr. Monitored by oxygen electrode. Average of three trials.

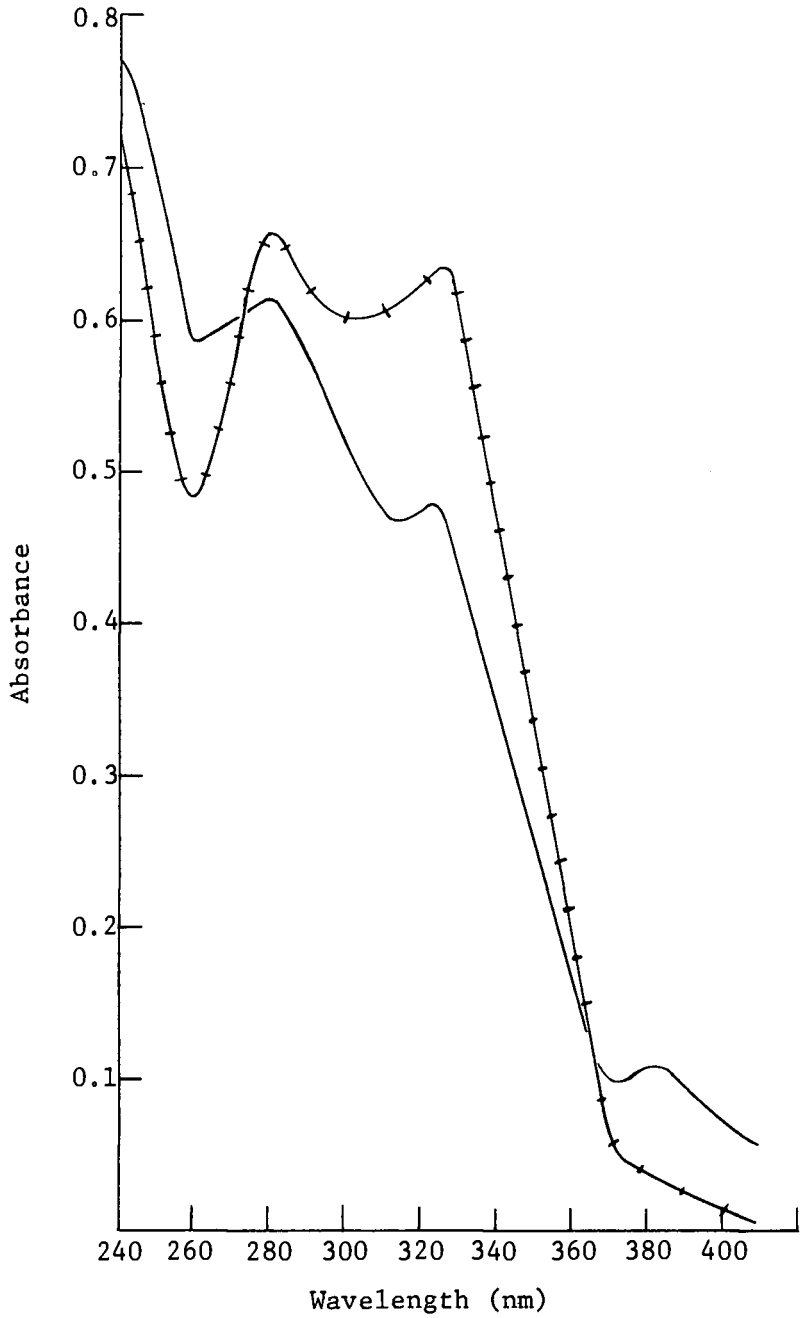



Figure 3. Changes in the absorption scan of a water extract of PPO. Samples stored at 4°C for 8 hr. +++, 5 min after extraction; ——, 8 hr after extraction.

extract. A decrease in peak height at 324 nm and 280 nm, with subsequent increases at 260 nm and 240 nm was noted over the observation period. Scans of commercially purified chlorogenic acid (Sigma) and D-catechin (Sigma) are shown in Figures 4 and 5, respectively. Similar changes in the absorbance spectra were noted when these solutions were made alkaline to promote phenolic oxidation. The browning, loss of activity, and absorbance changes were indicative of reactions between PPO and endogenous pear phenolic compounds present in the water extract.

Three regions of PPO activity, containing many distinct PPO bands, were identified when the extract was subjected to electrophoresis (Figure 6a). In later experiments when the phenolic compounds were removed with XAD-4 (1g/g tissue) the number of apparent isozymes decreased (Figure 6b). Additional PPO bands were produced in the XAD-4 extract upon the addition of chlorogenic acid (3 mM) or pear tissue (2.5% w/v) in which the enzymes had been inactivated by heat treatment (Figure 6c,d). Materials were added to the XAD-4 extract, pH 5.0, and incubated for 30 min at 23°C. It was also noted that the PPO activity immediately after extraction in water extracts was higher than the PPO activity present in corresponding adsorbent-treated extracts. The decrease in PPO activity and number of isozymes in the adsorbent-treated extracts was indicative of the removal of native phenolic substrates. Endogenous phenolic substrates if not removed during extraction of PPO can react with the protein to produce additional isozymes which were not present in the intact tissue. This phenomenon has been documented by several authors (Brown and Wright, 1963; Fields and Tyson, 1973; Loomis, 1978).

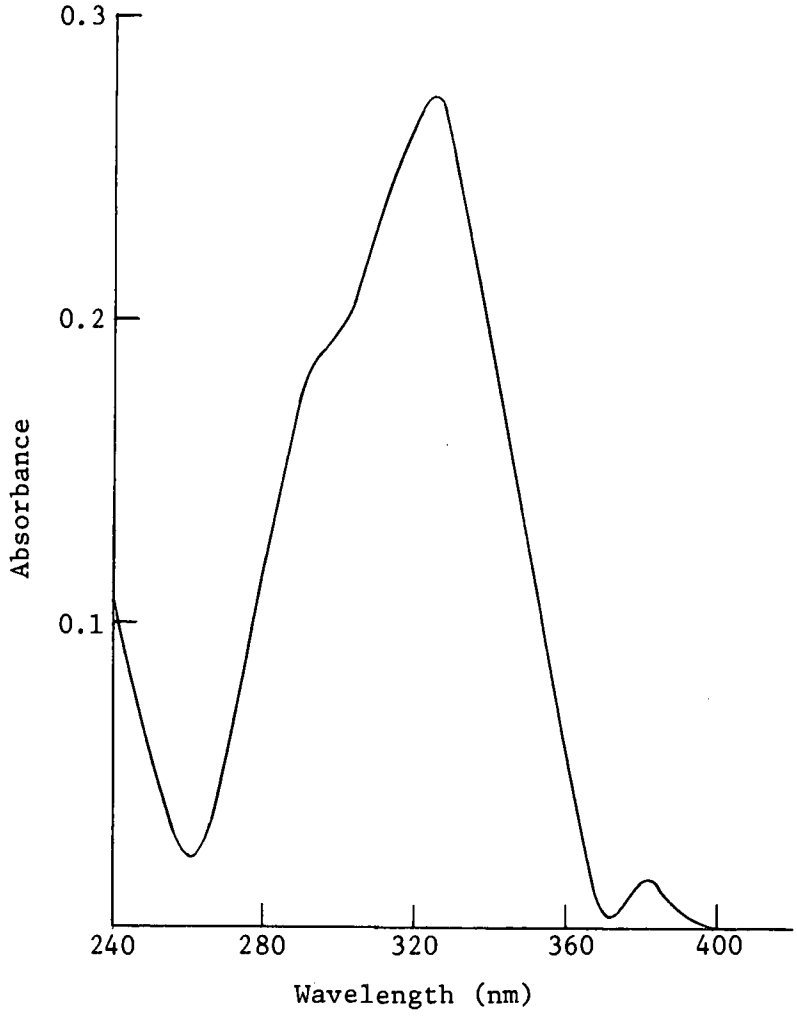



Figure 4. Absorbance scan of chlorogenic acid (1.5 mM) in distilled water.

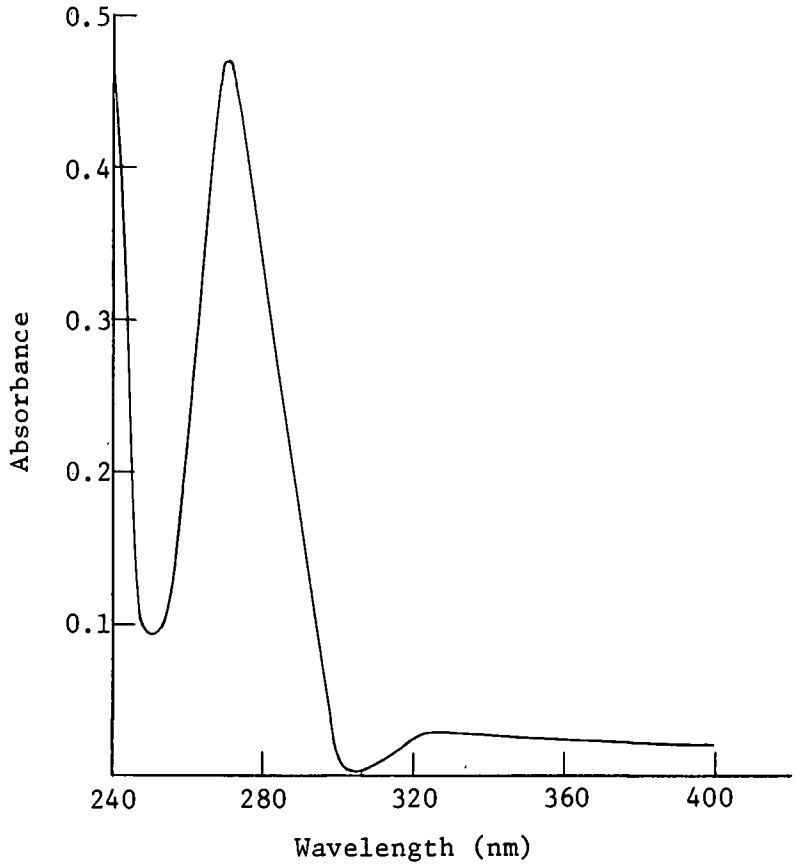



Figure 5. Absorbance scan of D-catechin (0.4 mM) in distilled water.

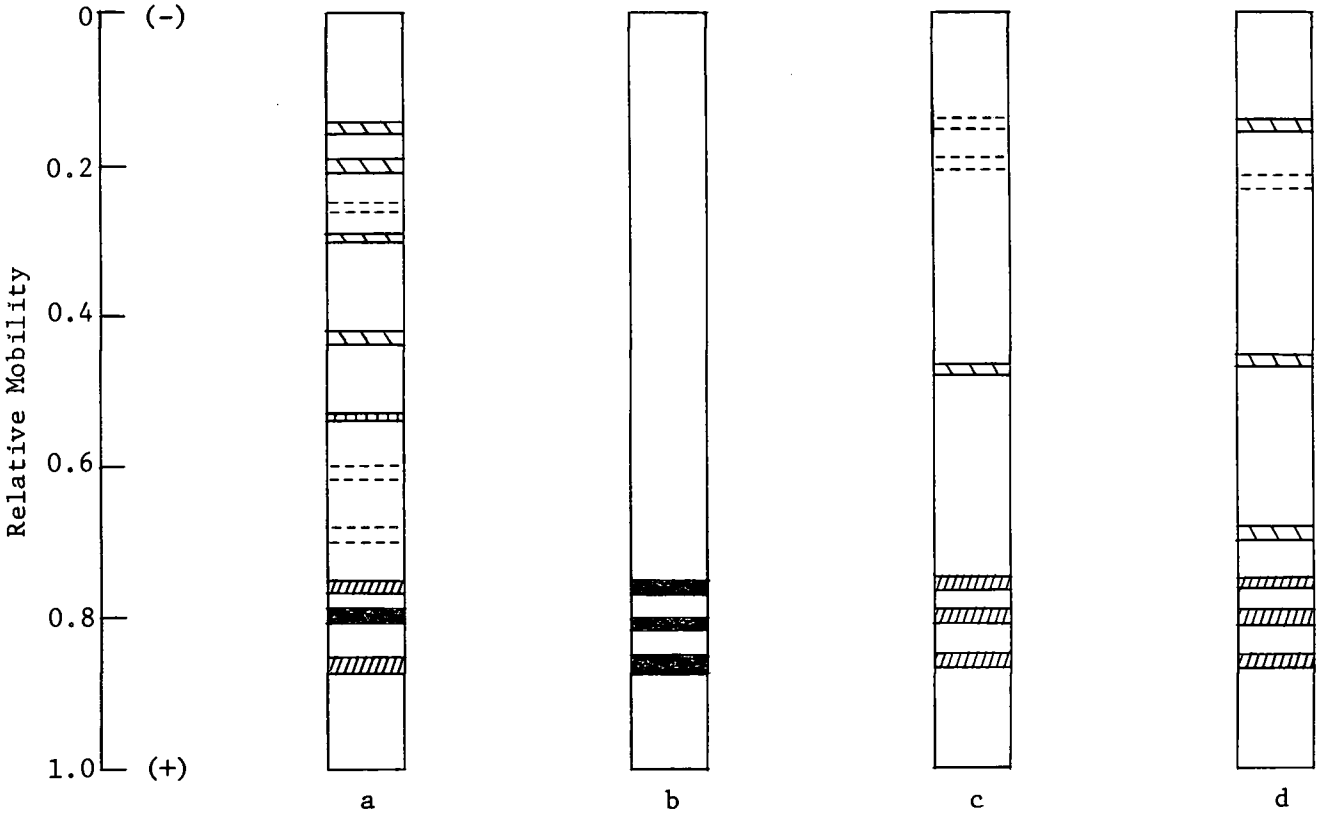



Figure 6. Representative electrophoretic patterns of pear PPO. (a) water extract (b) XAD-4 extract (1 g/g tissue), (c) 3 mM chlorogenic acid added to the XAD-4 extract, (d) 2.5% pear powder (enzymes inactivated) added to XAD-4 extract. Substrates incubated with PPO extract at 23°C, pH 5.0 for 30 min before electrophoresis. Relative mobility based on migration of bromophenol marker.

## Acetone Powder Extraction

Most of the PPO extraction procedures reported in the literature involve the homogenization of plant tissue in cold acetone, followed by acetone washings of the protein. The enzyme is precipitated while most of the phenols are extracted into the acetone (Bendall and Gregory, 1963). Therefore, an acetone powder extract was investigated to determine the value of this type of extract for pear PPO. After several trials using the procedure of Benjamin and Montgomery (1973) the technique was abandoned as all extracts became slightly brown on standing. When acetone was used to extract PPO from tobacco (Clayton, 1959), mushroom (Kertesz and Zito, 1957; Bouchilloux et al., 1963), tea (Bendall and Gregory, 1963), potato (Patil and Zucker, 1965), and peach (Wong et al., 1971) a yellowish colored enzyme solution was reported. Bendall and Gregory (1963) attributed this to reactions of PPO with residual phenolic compounds remaining after extraction and warned of possible modification of native protein.

## PVPP Extractions

Several reports have been published recommending the use of phenolic adsorbents when extracting enzymes from plant tissue containing endogenous phenolic compounds (Loomis and Battaile, 1966; Anderson, 1968; Rhodes, 1977). PVPP has been used successfully due to its ability to hydrogen bond to phenolics and prevent their interaction with protein (Loomis and Battaile, 1966; Coggon et al., 1973; Montgomery and Sgarbieri, 1975; Flurkey and Jen, 1978).

To determine the amount of PVPP necessary to extract PPO from the pear, various levels were tested (Table 2). At levels of 0.75 g PVPP/g tissue and above the extracts remained clear on standing. A slight increase in activity was noted with increasing levels of PVPP which may be attributed to a concentration effect produced during hydration of the dried PVPP. The 280/260 absorbance ratio was indicative of the removal of some phenolic compounds, but the 280/240 absorbance ratio below 1.000 and the peak at 324 nm indicated residual phenolic compounds (Loomis, 1978). The 324 nm peak suggested the presence of hydroxycinnamic acid compounds in the extract, most likely chlorogenic acid as it is present in high concentrations in the pear (Sioud and Luh, 1966). This corresponds to results of Anderson and Sowers (1968) who reported PVPP unable to bond chlorogenic acid efficiently. The number of bands on electrophoresis was variable, indicating possible modification of the native proteins by phenolic compounds.

The greatest level of PPO activity occurred when the extraction medium was buffered between pH 5.4 and 5.9 (Table 3). Levels above pH 6.0 were not investigated as autoxidation of phenolic compounds has been reported above this pH (Bendall and Gregory, 1963). Results indicated a level of 0.75 g PVPP/g tissue or above at a pH of 5.4 to 5.9 produced the optimum extract using PVPP.

The PPO activity in these initial PVPP extracts was low when compared with XAD-4 and anion exchange resin extracts (Tables 2, 4, and 6). This was attributed to the presence of soluble PVP which was not removed during cleaning. Soluble PVP has been reported to inhibit PPO (Harel et al., 1964; Walker and Hulme, 1965). Loomis and Battaile

Table 2. Characteristics of PPO preparations extracted with different levels of PVPP.

| PVPP[a] (g) | Browning[b] | Activity (units/ml) | Activity g tissue (units/g) | Absorbance 280/260 | Absorbance 280/240 | Absorbance 324 peak[c] | PPO bands |
|---|---|---|---|---|---|---|---|
| 0.25 | +++[d] | - | - | - | - | - | - |
| 0.50 | + | 235 | 2350 | 1.097 | 0.888 | + | 5-7 |
| 0.75 | - | 256 | 2560 | 1.128 | 0.856 | + | - |
| 1.00 | - | 278 | 2780 | 1.067 | 0.792 | + | 5-7 |
| 1.25 | - | 286 | 2860 | 1.028 | 0.819 | + | - |

[a]PVPP used per g of tissue in 25.0 ml 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0.

[b]Browning designated as severe, +++; moderate, ++; slight, +; none, -.

[c]Designates presence or absence of an absorbance peak at 324 nm.

[d]When browning was severe no further experimentation was performed.

Table 3. Effect of pH on the extraction of PPO with PVPP.[a]

| pH | Activity[b] g tissue (units/g) | Percent of maximum activity |
|---|---|---|
| 4.0 | 1900 | 73 |
| 4.4 | 2100 | 90 |
| 4.8 | 2130 | 91 |
| 5.4 | 2240 | 100 |
| 5.9 | 2230 | 100 |

[a]Extracts in 25.0 ml 0.1 M citrate - 0.2 M phosphate buffer at the appropriate pH, with 1.0 g PVPP/g tissue.

[b]Activity measured at pH 5.0.

(1966) reported that trichloroacetic acid precipitated soluble PVP. This same phenomenon was noted when PVPP extracts were treated with 0.75% potassium ferrocyanide. Upon more rigorous cleaning of PVPP the activity of all extracts increased proportionally.

## XAD-4 Extractions

XAD-4 has been recommended for use during plant enzyme extractions as its hydrophobic nature allows it to remove phenolic compounds from solution via their aromatic ring structure (Loomis, 1974). Table 4 shows the characteristics of PPO extracted at various levels of XAD-4. XAD-4 was used in hydrated form which contains ca. 50% water. At least 1.0 g XAD-4/g tissue was necessary to prevent browning. PPO activity was reduced at higher levels of XAD-4, probably due to the combined effect of dilution by the hydrated resin and to removal of endogenous substrate which could participate in enzymic and nonenzymic reactions. Like PVPP, the 280/260 absorbance ratio was indicative of the removal of phenolics, but some remained in solution as the 280/240 absorbance ratio was below 1.000 and the 324 nm peak was present. Electrophoresis revealed five to seven bands at low levels of XAD-4, whereas at a level of 1 g XAD-4/g tissue only three PPO bands were present. Consequently, XAD-4 may be a more efficient adsorbent of pear phenolics than PVPP. Loomis *et al.* (1979) have emphasized that each plant tissue is unique.

A pH of 5.0 to 5.9 was necessary in the extraction buffer to produce PPO extracts with optimum activity (Table 5). The reduced activity at lower pH values may be attributed to slight denaturation and subsequent exposure of hydrophobic amino acid residues to the resin resulting

Table 4. Characteristics of PPO preparations extracted with different levels of XAD-4.

| XAD-4[a] (g) | Browning[b] | Activity (units/ml) | Activity g tissue (units/g) | Absorbance 280/260 | Absorbance 280/240 | Absorbance 324 peak[c] | PPO bands |
|---|---|---|---|---|---|---|---|
| 0.25 | +++[d] | - | - | - | - | - | - |
| 0.50 | ++ | 1220 | 12200 | 1.070 | 0.715 | + | 5-7 |
| 0.75 | + | 1200 | 12000 | 1.038 | 0.734 | + | - |
| 1.00 | - | 930 | 9300 | 1.020 | 0.770 | + | 3 |
| 1.50 | - | 940 | 9400 | 0.960 | 0.545 | + | 3 |

[a]XAD-4 used per g of tissue in 25.0 ml 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0

[b]Browning designated as severe, +++; moderate, ++; slight, +; none, -.

[c]Designates presence or absence of absorbance peak at 324 nm.

[d]When browning was severe no further experimentation was performed.

Table 5. Effect of pH on the extraction of PPO with XAD-4.[a]

| pH | Activity[b] g tissue (units/g) | Percent of maximum activity |
|---|---|---|
| 4.0 | 1840 | 26 |
| 4.6 | 6270 | 88 |
| 5.0 | 7000 | 100 |
| 5.6 | 7030 | 100 |
| 5.9 | 6970 | 99 |

[a]Extracted with 1.0 g XAD-4/g tissue in 25.0 ml 0.1 M citrate - 0.2 M phosphate buffer at the appropriate pH.

[b]PPO activity measured at pH 5.0.

in protein adsorption or to the reduced solubility of PPO at its isoelectric point. The isoelectric point of tea PPO has been reported to be pH 4.1 (Coggon et al., 1973).

## Extraction with Anion Exchange Resins

Anion exchange resins have been used successfully in plant enzyme isolations (Lam and Shaw, 1970; Gray and Kekwick, 1973; Fields and Tyson, 1973) due to their ability to complex with phenolic compounds through hydrophobic, ionic, and hydrogen bond mechanisms (Moore and Stein, 1951; Olsson et al., 1976). Gray (1978) reported Dowex 1 to have a high affinity for chlorogenic acid, while Loomis et al. (1979) found both Dowex 1 and 2 efficient in removing quercetin and chlorogenic acid from solution, Dowex 1 being more efficient than Dowex 2. In this study anion exchange resins were investigated in hopes of eliminating the 324 nm absorbance peak present in the absorption scans of XAD-4 and PVPP extracts.

The anion exchange resin AG 1-X8 (Bio-Rad Laboratories), synonymous with Dowex 1-X8 (Dow Chemical Co.), produced a clear extract at lower levels of resin than either XAD-4 or PVPP (Table 5). The resin was used in hydrated form which contained ca. 50% water. Browning was eliminated at 0.50 g AG 1-X8/g tissue, and at this level and above the activity was constant. The absorption spectrum of the PPO extract was improved as the 280/260 absorbance ratio was consistently greater than 1.000 and the 324 nm peak was eliminated, but the 280/240 absorbance ratio was still below 1.000. Three PPO bands were resolved on electrophoresis when the enzyme was extracted with 0.50 g AG 1-X8/g tissue and

above. As with XAD-4, the pH of the extraction buffer needed to be above 5.0 to produce maximum activity in the extracts (Table 6).

Characteristics of PPO extracted with various levels of the anion exchange resin AG 2-X8(Bio-Rad Laboratories) are shown in Table 7. AG 2-X8 is synonymous with Dowex 2-X8 (Dow Chemical Co.). Browning was prevented at 0.50 g AG 2-X8/g tissue and above, and PPO activity was constant in the clear extracts. The absorbance scans showed improvement as the 280/240 absorbance ratio was near to or above 1.000 at 1.5 and 1.00 g AG 2-X8, respectively. Three isozymes were detected on electrophoresis. A pH above 5.6 in the extraction buffer produced maximum activity in the extracts (Table 9). Higher PPO activities might have been noted in Table 7 if the pH of the extraction medium had been 5.6 instead of 5.0.

## Effect of Polystyrene Resins on PPO

Nonspecific binding of proteins to ion exchange resin has been reported (Fasold *et al.*, 1961). Lam and Shaw (1970) warned of the possible adsorption of proteins with low isoelectric points to ion exchange resins. Experiments were performed to determine if PPO was adsorbed by XAD-4, AG 1-X8, or AG 2-X8 upon extraction. The results are shown in Table 10. Assuming a 5% dilution factor due to the hydrated resins, no decrease in PPO activity or absorption at 280 nm was noted. An improvement in the 280/260 ratio and a decrease in the 324 nm peak were noted when either AG 1-X8 or AG 2-X8 was added to the PVPP cleaned extract, probably due to the adsorption of additional phenolic compounds. Upon electrophoresis no change in the number,

Table 6. Characteristics of PPO preparations extracted with different levels of AG 1-X8.

| AG 1-X8[a] (g) | Browning[b] | Activity (units/ml) | Activity g tissue (units/g) | Absorbance 280/260 | Absorbance 280/240 | 324 peak[c] | PPO bands |
|---|---|---|---|---|---|---|---|
| 0.10 | + | 834 | 6672 | 1.047 | 0.598 | + | 7 |
| 0.50 | - | 796 | 6368 | 1.217 | 0.881 | - | 3 |
| 0.75 | - | 786 | 6288 | 1.157 | 0.870 | - | - |
| 1.00 | - | 756 | 6048 | 1.124 | 0.880 | - | 3 |
| 1.50 | - | 770 | 6160 | 1.088 | 0.894 | - | 3 |

[a]AG 1-X8 used per g of tissue in 20.0 ml 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0.

[b]Browning designated as severe, +++; moderate, ++; slight, +; none, -.

[c]Designates the presence or absence of an absorbance peak at 324 nm.

Table 7. Effect of pH on the extraction of PPO with AG 1-X8.[a]

| pH | Activity[b] g tissue (units/g) | Percent of maximum activity |
|---|---|---|
| 3.9 | 1408 | 25 |
| 4.4 | 3616 | 65 |
| 5.0 | 5480 | 100 |
| 5.6 | 5496 | 100 |

[a]Extracted with 1.0 g AG 1-X8/g tissue in 20.0 ml 0.1 M citrate - 0.2 M phosphate buffer at the appropriate pH.

[b]PPO activity measured at pH 5.0.

Table 8. Characteristics of PPO preparations extracted with different levels of AG 2-X8.

| AG 2-X8[a] (g) | Browning[b] | Activity (units/ml) | Activity / g tissue (units/g) | Absorbance 280/260 | Absorbance 280/240 | 324 peak[c] | PPO bands |
|---|---|---|---|---|---|---|---|
| 0.25 | + | 460 | 3680 | 1.034 | 0.569 | - | - |
| 0.50 | - | 518 | 4144 | 1.103 | 0.869 | - | - |
| 1.00 | - | 500 | 4000 | 1.283 | 1.007 | - | 3 |
| 1.50 | - | 505 | 4040 | 1.283 | 0.957 | - | - |

[a]AG 2-X8 used per g of tissue in 20.0 ml 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0.

[b]Browning designated as severe, +++; moderate, ++; slight, +; none, -.

[c]Designates the presence or absence of an absorbance peak at 324 nm.

Table 9. Effect of pH on the extraction of PPO with AG 2-X8.[a]

| pH | Activity[b] g tissue (units/g) | Percent of maximum activity |
|---|---|---|
| 3.3 | 192 | 5 |
| 3.6 | 672 | 18 |
| 4.0 | 1024 | 27 |
| 4.6 | 3000 | 80 |
| 5.0 | 3264 | 87 |
| 5.6 | 3744 | 100 |
| 5.9 | 3720 | 100 |

[a]Extracted with 1.0 g AG 2-X8/g tissue in 20.0 ml 0.1 M citrate - 0.2 M phosphate buffer at the appropriate pH.

[b]PPO activity measured at pH 5.0.

Table 10. Effect of polystyrene resins on extraction of PPO.

| Extract | Activity g tissue (units/g) | Absorbance (nm) 280 | 280/260 | 324 peak[b] |
|---|---|---|---|---|
| PVPP | 3180 | 0.280 | 0.962 | + |
| plus XAD-4 | 3000(3150)[a] | 0.272(0.286) | 0.956 | + |
| PVPP | 2700 | 0.285 | 0.930 | + |
| plus AG 1-X8 | 2640(2772) | 0.270(0.284) | 0.970 | - |
| PVPP | 2630 | 0.255 | 1.000 | + |
| plus AG 2-X8 | 2500(2630) | 0.240(0.252) | 1.065 | - |

[a]Figures in parentheses are corrected for the approximately 5% dilution due to the addition of the hydrated resin.

[b]Designates presence or absence of an absorbance peak at 324 nm.

location, or intensity of the PPO bands was detected.

The results agree in part with those of Loomis et al. (1979) who reported no adsorption of horseradish peroxidase or bovine serum albumin by XAD-4 resin. Due to the hydrophobic binding sites of bovine serum albumin they concluded it would be very unlikely for any other protein to be bound by hydrophobic interactions to either XAD-4 or ion exchange resin. Loomis et al. (1979) did not rule out the possibility of the carboxyl group of protein binding to the functional group of ion exchange resin. It appears, at least with the PPO preparation used in this investigation, that there was no adsorption of PPO by the polystyrene resins by either ionic or hydrophobic forces.

## Variability in Pear Samples

The preceding experiments were performed with pears harvested commercially in 1977 and stored for 9 months at -1°C before being frozen as samples. Variations between pears were great and it was difficult to compare results of individual experiments. In hopes of reducing this variability new pear samples were obtained which had been handpicked from a single tree upon reaching maturity during the 1978 season. Any pear which deviated greatly in size or other physical characteristic was discarded before freezing. This procedure did reduce the variability between pears, and subsequent experiments were performed with them.

Even though the pears were harvested from the same orchard, differences were noted between the 1977 and 1978 samples. The efficiency of the adsorbents differed, and the absorbance ratios and

electrophoretic patterns were changed. The activity in the 1978 samples was reduced. Variations were attributed to several factors, such as varying environmental conditions during growth, and physiological changes occurring during the 9 month storage of the 1977 pears.

## Comparison of Extraction Methods

Using the newly acquired pears, extracts were made using all the adsorbents previously studied (Table 11). PVPP was used in combination with the polystyrene resins as it was reported to be a more efficient scavenger of leucoanthocyanins (Gray, 1978). The activity per g of pear tissue was probably the most accurate method for comparison of PPO activity (Loomis, 1978) and was found constant in all extracts except those containing PVPP. A slight decrease in PPO activity was noted, and may be attributed to some residual PVP in solution. The activity of all extracts, except PVPP (1 g/g tissue), were linear with increasing concentrations of extract when measured by spectrophotometric and polarographic methods, as demonstrated by an AG 2-X8 extract in Figure 7. Residual phenolic compounds in the PVPP extracts may have prevented a linear representation. The absorbance ratios (Table 11) indicate anion exchange resins as the most efficient adsorbents of pear phenolics, as the 324 nm peak was reduced and the 280/240 absorbance ratio improved when compared to PVPP and XAD-4 extracts. Figure 8 compares the absorbance scans of an XAD-4 and AG 2-X8 extract of PPO. The electrophoretic patterns of all the extracts are shown in Figure 9. The AG 2-X8 extracts, with or without PVPP, exhibited three PPO bands which were constant and reproducible. The major band occurred at a Rm of

Table 11. Comparison of PPO extracts prepared using different adsorbents.[a]

| Adsorbent | Level (g/g tissue) | Activity (units/ml) | Activity / g tissue (units/g) | Absorbance 280/260 | Absorbance 280/240 | 324 peak[b] | PPO bands |
|---|---|---|---|---|---|---|---|
| PVPP | 1.0 | 225 | 2250 | 1.279 | 0.867 | + | 5-7 |
| XAD-4 | 1.0 | 241 | 2410 | 1.306 | 0.926 | + | 5 |
| XAD + PVPP | 1.0, 1.0 | 228 | 2280 | 1.250 | 0.842 | + | 5 |
| AG 1-X8 | 1.0 | 243 | 2430 | 1.338 | 1.066 | - | 4 |
| AG 1-X8 + PVPP | 1.0, 1.0 | 223 | 2230 | 1.320 | 0.935 | - | 4 |
| AG 2-X8 | 1.0 | 241 | 2410 | 1.339 | 1.088 | - | 3 |
| AG 2-X8 + PVPP | 1.0, 1.0 | 231 | 2310 | 1.301 | 1.009 | - | 3 |

[a]All extracts were prepared in 25.0 ml 0.1 M acetate buffer, pH 5.6.

[b]Designates presence or absence of an absorbance peak at 324 nm.

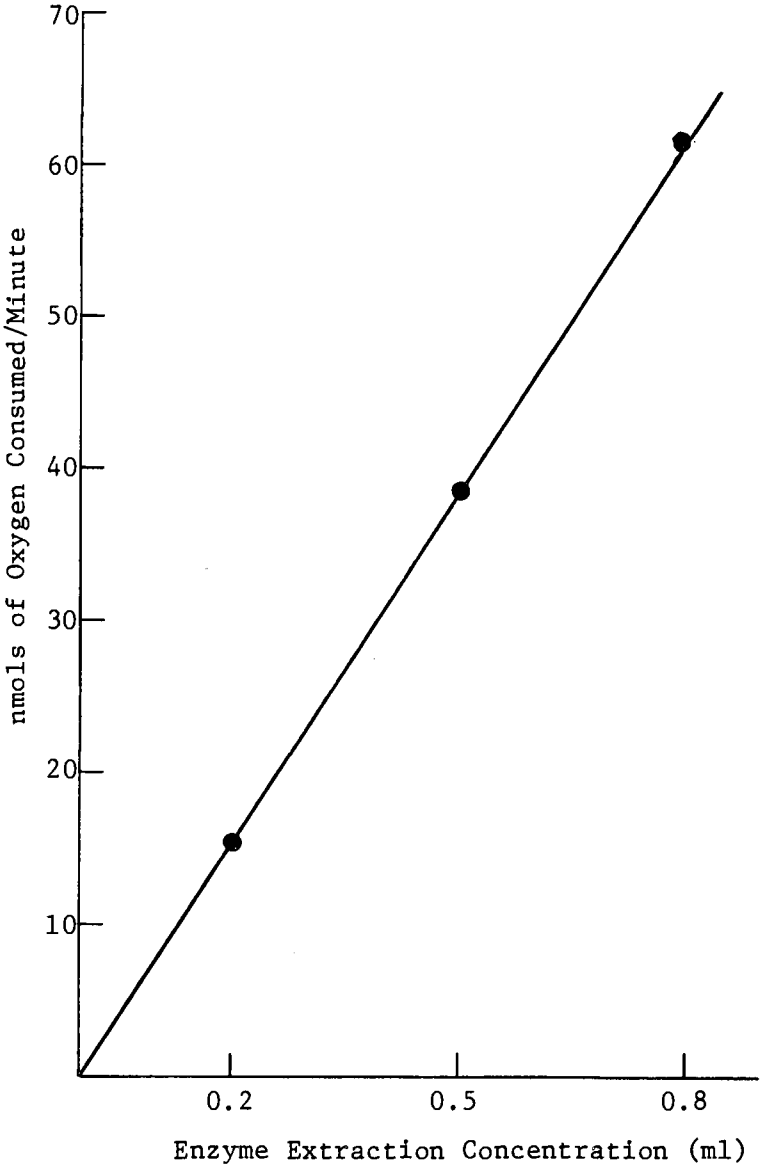



Figure 7. Effect of enzyme extract concentration on PPO activity. Measured using oxygen electrode, catechol as substrate, AG 2X8 (2.5 g tissue/2.5 g AG 2X8/25 ml of .05 M acetate buffer, pH 5.6).

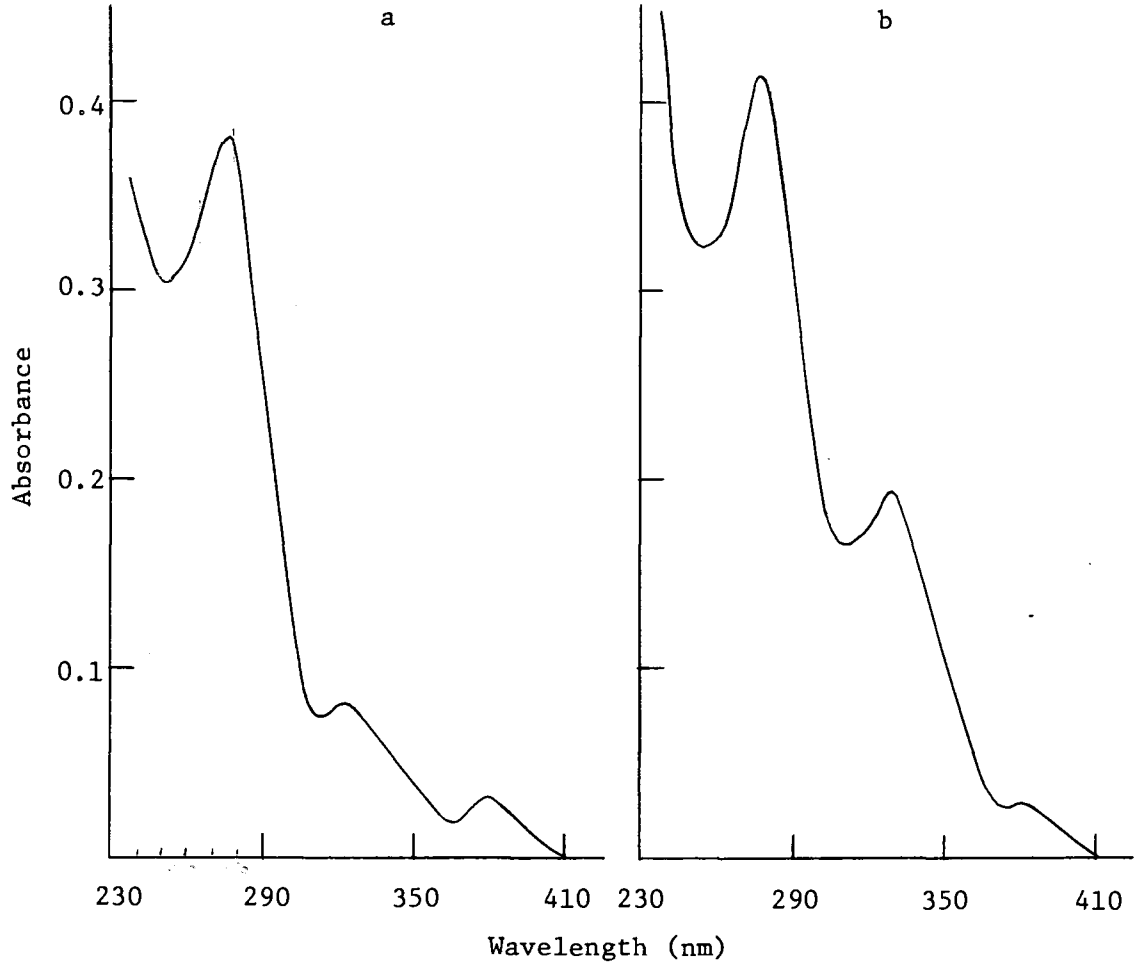



Figure 8. Absorbance scans of PPO extracts. (a) AG 2X8 (1 g/g tissue), (b) XAD-4 (1 g/g tissue).

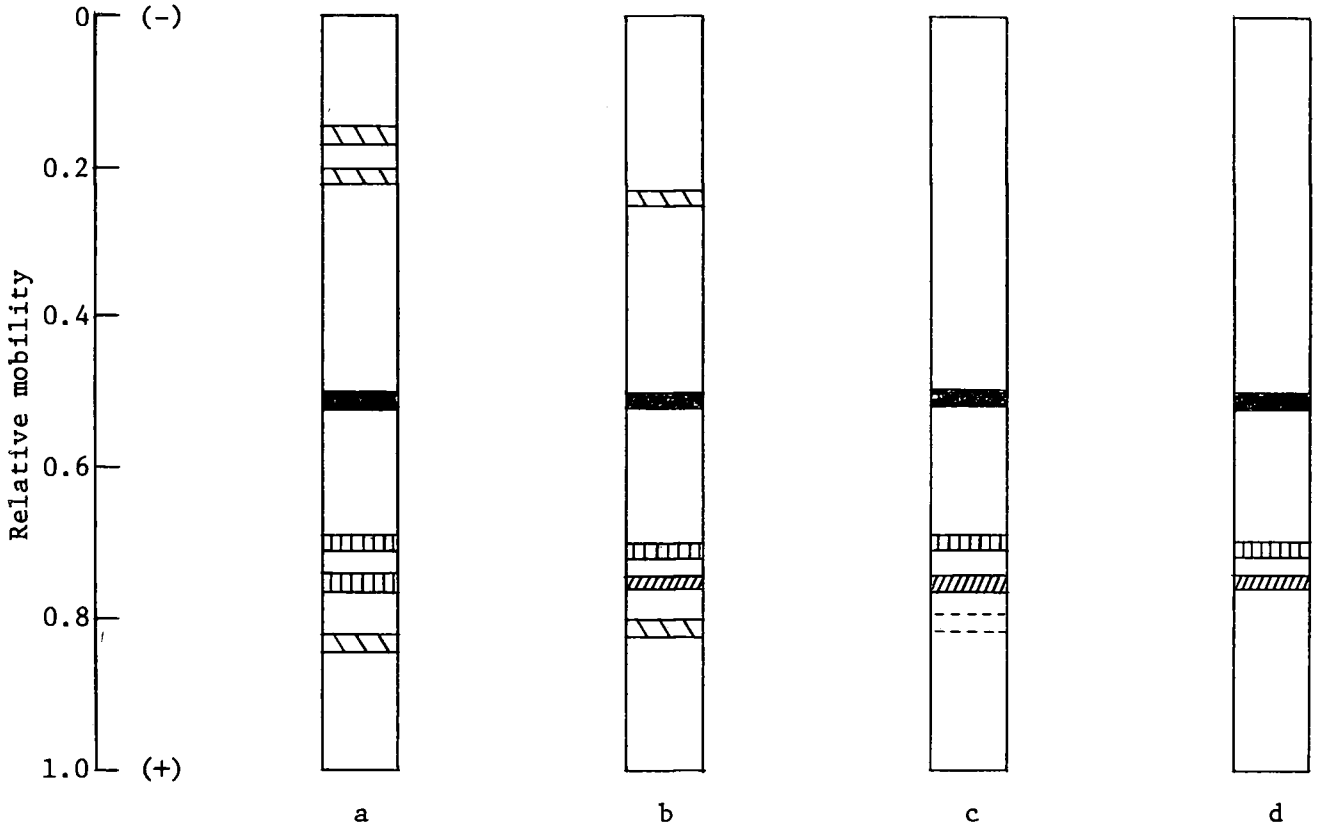



Figure 9. Electrophoretic patterns of pear PPO extracted with different adsorbents. (a) PVPP (1 g/g tissue), (b) XAD-4 (1 g/g tissue), (c) AG 1X8 (1 g/g tissue), (d) AG 2X8 (1 g/g tissue). Relative mobility based on migration of bromophenol blue marker dye.

0.510, with lighter bands at 0.703 and 0.749. The lack of PPO bands of lower Rm, such as those seen with PVPP and XAD-4 extracts, may indicate the elimination of PPO artifacts of higher apparent molecular weight resulting from phenolic coupling.

Results indicate that AG 2-X8 was capable of producing a superior extract of d'Anjou pear PPO. Even though PVPP did not appear to improve the extraction when used in combination with the polystyrene resins, its use is advised. Using the leucoanthocyanin test of Swain and Hillis (1959) it was determined that PVPP (0.75 g/g tissue) removed all the leucoanthocyanins when used during extraction, whereas AG 1-X8 (1.0 g/g tissue) removed approximately 40% and XAD-4 (1.0 g/g tissue) removed less than 10% of the pear leucoanthocyanins when compared to a water extract control. The remaining experiments were performed with a PPO preparation extracted in the presence of AG 2-X8 (2.5 g resin/ 2.5 g tissue/20 ml 0.05 M acetate buffer, pH 5.6).

## pH Optimum for PPO Activity

The pH optimum for d'Anjou pear PPO activity was determined in 0.1 M citrate - 0.2 M phosphate buffer using both catechol and chlorogenic acid as substrates. A pH of 5.1 was determined optimum (Figure 10). Above pH 6.5 it was necessary to include a blank, as autoxidation of the substrate occurred with corresponding uptake of oxygen. Results are in contrast to those of Halim and Montgomery (1978) who reported a pH optimum of 7.0 for d'Anjou pear PPO.

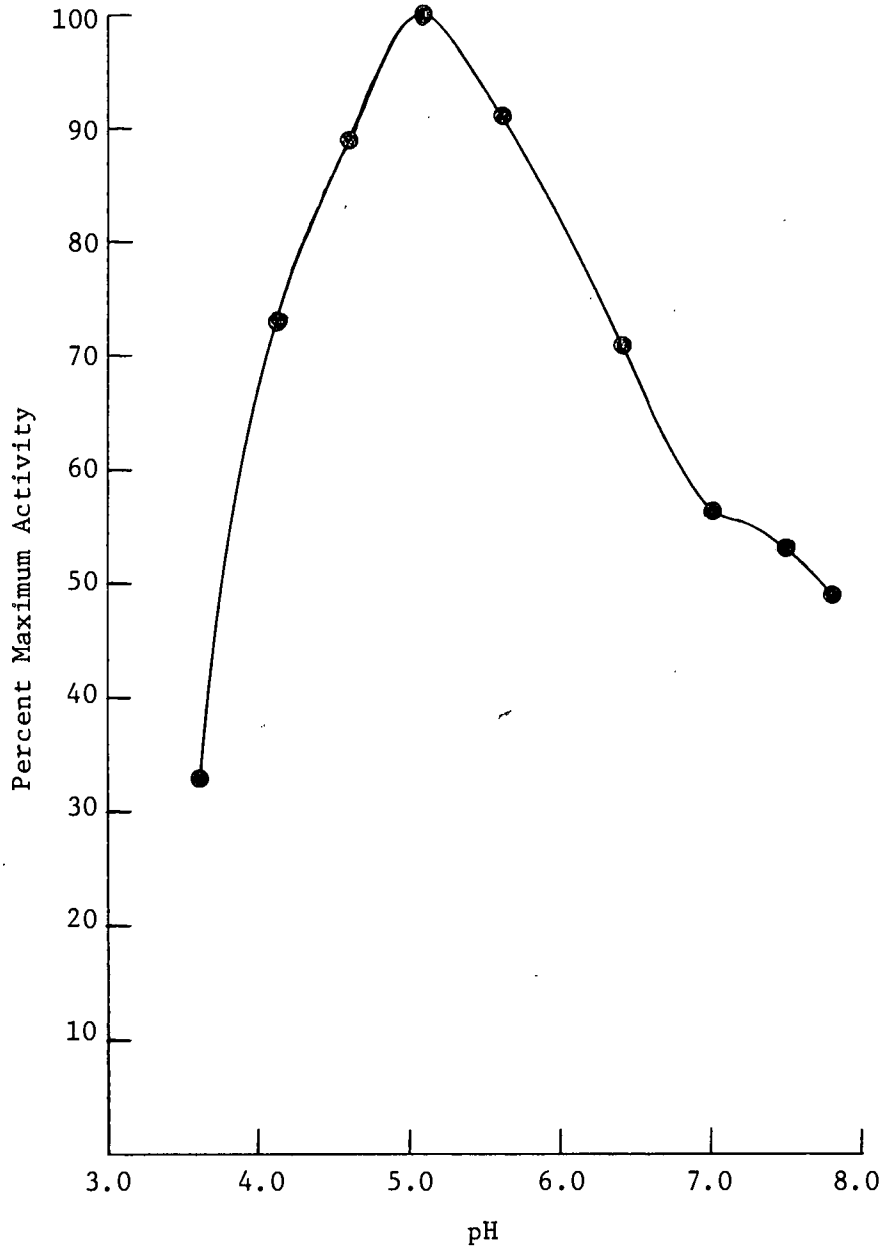



Figure 10. pH optimum for activity of pear PPO. Measured using oxygen electrode, catechol as substrate. Average of three trials.

## Stability of PPO

The stability of the PPO extract stored at various pH values was studied. An extract was separated into aliquots, adjusted with HCl or NaOH to a pH within the range of 2.0 to 6.5, brought to constant volume with distilled water, and stored at 4°C. Activity was measured 5 min, 8 hr, and 24 hr after pH adjustment. The results are shown in Figure 11. PPO appears to be most stable at pH 5.0. The sample acidified to pH 2.0 lost activity instantly. The instability at pH 6.5 may be due to the autoxidation of residucal phenolic compounds, such as leucoanthocyanins, and their reaction with protein. The results agree with Rhodes (1977) who reported that the pH for optimum activity was often the pH of greatest stability.

The stability of the PPO extract adjusted to pH 5.0 was studied during storage at -40°C and 4°C. At -40°C no loss of PPO activity was detected over the 3 month storage period and there were no changes in the electrophoretic patterns. The results of the 4°C storage study indicated an 11% loss of activity during the 11 day storage period (Figure 12). The experiment was terminated after 11 days due to microbial growth.

## Validity of Electrophoresis

Several experiments were performed to determine if the brown bands detected on electrophoresis were in fact PPO and not artifacts of the electrophoretic procedure. Autoxidation of residual phenolics could result in artifact formation when the PPO extract was applied to the

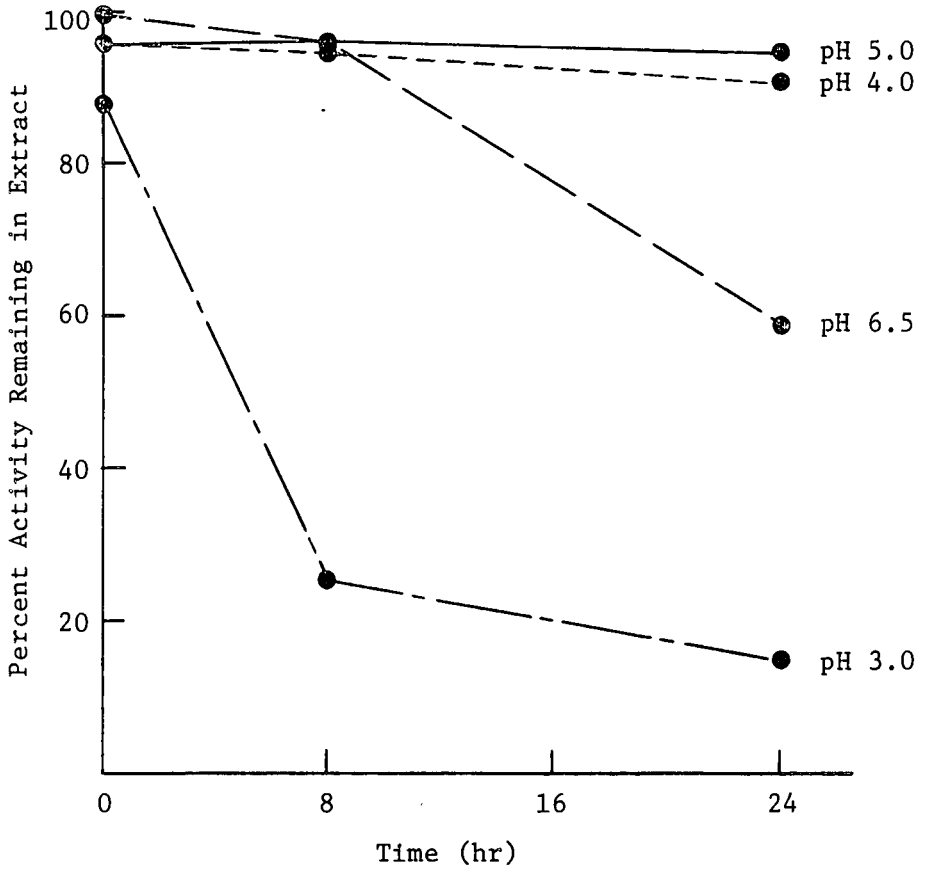



Figure 11. pH stability of PPO at 4°C over a 24 hr period. Monitored with oxygen electrode. Results are the average of 3 trials.

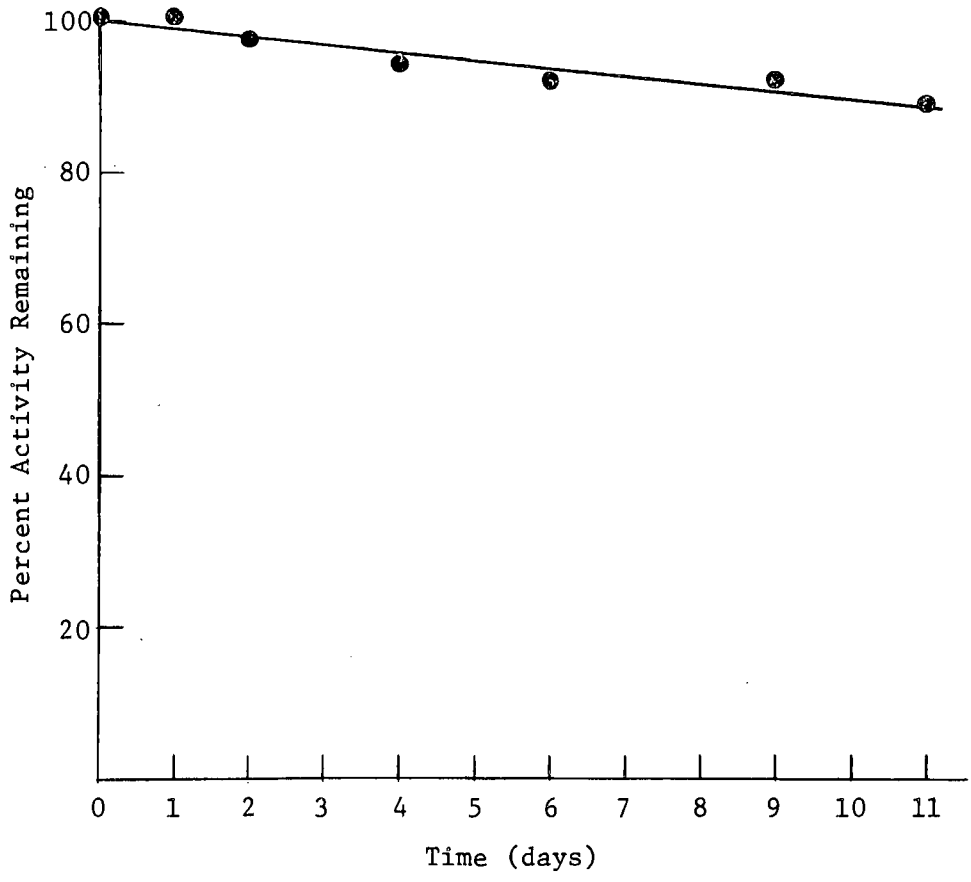



Figure 12. PPO stability at 4°C over an 11 day period. Extract adjusted to pH 5.0. Results are the average of 3 trials.

top of the gel column, due to the high pH of the electrode buffer. To aid in the removal of the residual phenolics the PPO extract was run through a column of Bio-Gel P-6 (Bio-Rad Laboratories) before electrophoresis. No change in the electrophoretic band patterns occurred, but in all cases a slight improvement in the 280/240 ratio was noted.

Gels polymerized with ammonium persulfate often contain residual peroxides (Cooper, 1977). To determine if these compounds cause modification of PPO, spacer gels were polymerized with either ammonium persulfate or riboflavin. No differences were found in the gel patterns, agreeing with the results of Kahn (1976).

Sheen (1972) warned of possible peroxidase activity when the gels were developed in catechol or caffeic acid due to the residual peroxides produced by ammonium persulfate. To find if peroxidase was responsible for any of the brown bands an AG 2-X8 extract was subjected to electrophoresis and the resultant gels scanned at 405 nm before development and at 520 nm after development in catechol. The gels were scanned at 405 nm to detect the presence of the heme group of peroxidase. Two peaks were resolved when the gel was scanned at 405 nm, but these did not correspond to the three PPO peaks detected at 520 nm (Figure 13).

Another experiment was devised to determine if the brown bands resulted from enzymic or nonenzymic oxidation of catechol. Gels were placed in 90°C 0.1 M citrate - 0.2 M phosphate buffer, pH 5.0, for 5 min to inactivate any enzymes resolved on electrophoresis of the PPO extract. No bands were produced when developed in catechol, thus indicating that enzymic activity was responsible for the color development.

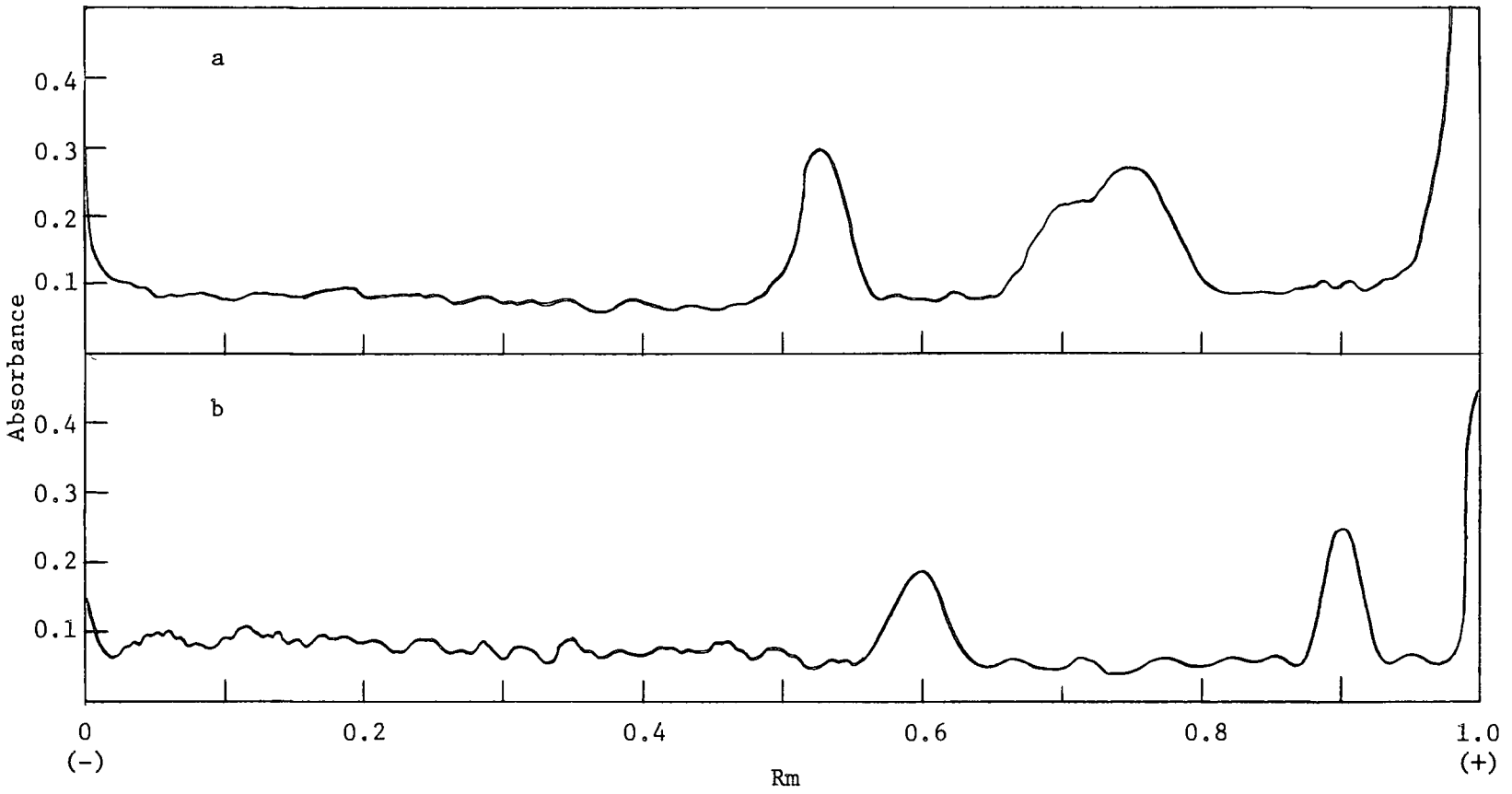



Figure 13. Gel scans of PPO extract. (a) 520 nm scan after development in catechol, (b) 405 nm scan scan before development. Peak at Rm 1.0 is due to bromophenol blue marker dye.

# SUMMARY

In order to prevent modification of PPO by endogenous phenolic compounds several extraction methods were investigated to determine the optimum conditions for extracting PPO from d'Anjou pears. The method producing the superior extraction was then used to extract PPO for further characterization of pH optimum for activity and stability.

The study yielded the following results:

1. Differences in PPO characteristics noted between pears was minimized by standardized harvesting and preservation procedures. Adequate preservation was effected by quick freezing the pear tissue in liquid nitrogen, sealing in cryovac bags, and storing at -40°C.
2. Chemical reactions in the macerated pear tissue were prevented by pulverizing the frozen sample in a Waring Blendor or analytical mill containing liquid nitrogen, and storing the resultant fine powder under a layer of liquid nitrogen until use.
3. A water extract of pear PPO was unsatisfactory as demonstrated by browning, loss of activity, changes in the absorbance spectrum, and a large number of artifactual isozymes in the extract.
4. An acetone powder extraction technique proved inadequate as browning of the PPO extract occurred.
5. PVPP (0.75 g/g tissue), XAD-4 (1.0 g/g tissue), AG 1-X8 (0.50 g/g tissue), and AG 2-X8 (0.50 g/g tissue) improved the extraction of PPO by acting as phenolic scavengers. Constant activity was produced in all extracts when extracted at a pH between 5.6 and 6.0.

6. Anion exchange resins were particularly effective in removing hydroxycinnamic acid compounds.
7. AG 2-X8 produced a superior extract containing three PPO isozymes detected by gel electrophoresis with the following Rm values: 0.510, 0.703, and 0.749.
8. Neither XAD-4, AG 1-X8, nor AG 2-X8 was found to adsorb PPO extracted under the conditions described in this thesis.
9. The pH optimum for activity was 5.1 when either catechol or chlorogenic acid was used as substrate. d'Anjou pear PPO was most stable at pH 5.0. At 4°C, 11% of the PPO activity was lost in 11 days. At -40°C no PPO activity was lost and no changes in the electrophoretic patterns occured over a 3 month storage period.
10. PPO isozymes detected on the polyacrylamide gels were not artifacts produced by the electrophoretic procedure.

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