AN ABSTRACT OF THE THESIS OF

David Husein Halim   for the degree of   Master of Science

in Food Science and Technology presented on _September 17, 1976_

Title: POLYPHENOL OXIDASE OF d'ANJOU

PEARS (PYRUS COMMUNIS L.)

Abstract approved: _____

Dr. Morris W. Montgomery

Stability, substrate and inhibitor specificity and electrophoretic

properties of a crude polyphenol oxidase (PPO) preparation extracted

from d'Anjoy pears (Pyrus communis L.) were investigated. Levels

of polyvinylpyrrolidone and pH of buffer for extraction were found

to affect the specific activity of the extracted enzyme. An extract

prepared with 1.5 g insoluble PVP per 15 g fresh tissue in acetate

buffer (pH 5.6) resulted in the highest PPO specific activity of the

crude extract. Addition of PVP did not affect the electrophoretic

patterns of PPO isozymes. The pH optimum of PPO occurs at 7.0.

Heat inactivation of PPO followed first order kinetics and approxi-

mately 50% of PPO activity was inactivated after heating for 11.7,

6.25, 2.25 and 1.1 min at temperature of 70°, 75°, 80° and 85°C,

respectively.

The crude PPO enzyme was active towards o-dihydroxyphenols,

but inactive towards monophenols. Disc electrophoresis on 7%

polyacrylamide gels revealed eight active isozymes towards catechol, 4-methylcatechol, chlorogenic acid, caffeic acid, dopamine, d-catechin and DL-dopa. Similar electrophoretic patterns were observed with all substrates. No differences in the band patterns were observed between a fresh crude PPO preparation, a frozen crude extract and a dialyzed extract when catechol was used as substrate. L-cysteine, diethyldithiocarbamate, thiourea, meta-bisulfite, cyanide, mercaptoethanol and ascorbic acid inhibited the enzyme activity. L-cysteine and diethyldithiocarbamate were the most effective inhibitors.

Polyphenol Oxidase of d'Anjou
Pears (<u>Pyrus</u> <u>communis</u> L.)

by

David Husien Halim

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed September 1976

Commencement June 1977

APPROVED:

_____
Associate Professor of Food Science and Technology
                in charge of major

_____
Head of Department of Food Science and Technology

_____
Dean of Graduate School

Date thesis is presented _September 17, 1956_

Typed by Opal Grossnicklaus for <u>David Husien Halim</u>

ACKNOWLEDGMENT

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

POLYPHENOL OXIDASE OF d'ANJOU PEARS (PYRUS COMMUNIS L.)

INTRODUCTION

Fruits undergo rapid, undesirable color changes (brown discoloration) following mechanical or physiological injury during harvesting, storage, packing and transportation. Brown discoloration of fruits is of major importance to both the producer and the consumer. The unattractive appearance of discolored fruit adversely affects the consumer acceptance of the fruits.

Brown discoloration of fresh fruit was found to be caused by combination of physical injury, followed by the oxidation of phenolic compounds of fruits by the enzyme polyphenol oxidase (EC 1.10.3.1) to quinones which undergo polymerization and further oxidation to impart the characteristic brown discoloration. Pears, like many other fruits, are susceptible to enzymic browning. The major utilization of winter pears has been for fresh market and only a few have been used in processed products. d'Anjou pears were chosen for this study because one of the major problems of the pear industry is friction discoloration of fresh fruits and a rapid browning of pear juice concentrates. This results in a reduction of the grade or quality of the pears or pear products and causes an economical loss to the producers and processors. An understanding of the enzyme is

necessary if a more effective way of controlling or preventing the browning is to be obtained. Polyphenol oxidases in peaches, grapes, banana, apples and Bartlett pears have been investigated but no work has been done with d'Anjou pears. In view of the importance of this enzyme in the browning of the fruit, the present study was undertaken to determine the conditions for the enzyme extraction, and to characterize the enzyme according to pH optimum, heat inactivation, substrate and inhibitor specificities and electrophoretic properties.

REVIEW OF LITERATURE

Polyphenol Oxidase

Many fruits undergo discoloration or browning following mechanical or physiological injury during postharvest storage, handling, and processing. The natural color of fruits or fruit products may be destroyed or masked by the formation of an unattractive dark brown or blackish pigment (Joslyn and Ponting, 1951). Cause of the color change has been attributed to the action of an enzyme called polyphenol oxidase (PPO) on phenolic substrates. The precise pathway for phenol oxidation by the PPO remains uncertain although it has been accepted generally to proceed by the following scheme:
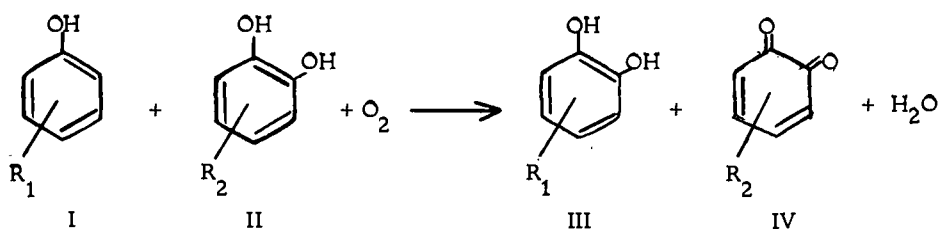
According to this scheme, three types of reactions are brought about by PPO. A phenolic substrate(I) is hydroxylated in the ortho-position, yielding a 1, 2-benzene-diol (III), often referred to as o-diphenolic compound. Second, the enzyme oxidizes a substrate (II) to a 3, 5-cyclo-hexadiene-1, 2-dione (IV) which is commonly called quinone or 1, 2-benzoquinone. Finally molecular oxygen is reduced, with one atom being incorporated in the catecholic product and the other

combining with hydrogen to form water (Nelson and Dawson, 1944).

The enzyme is believed to have two distinct types of active sites. One binds aromatic substrates such as phenol and catechol, while the other has an affinity for oxygen and metal binding agents such as cyanide (Duckworth and Coleman, 1970).

PPO exhibits activity toward a variety of aromatic substrates and subsequently, it has received numerous trivial names. Among these are phenolase, catecholase, polyphenol oxidase, tyrosinase and cresolase. The Commission of Enzymes of the International Union of Biochemistry has established a method for the systematic classification and naming of enzymes, including a numbering system (1961). The name and number assigned to PPO is o-diphenol:oxygen oxido-reductase (EC 1.10.3.1). In this study, this enzyme will be referred to as polyphenol oxidase or PPO. PPO contains copper as its prosthetic group and according to Kubowitz (1938), the copper is present in the enzyme as the bivalent ion.

The PPO system has been shown to contain numerous isozymes. Using polyacrylamide gel electrophoresis, Constantinides and Bedford (1967) showed that the enzyme system in the tissues of mushroom, potatoes, and apples exhibited the phenomenon of multiple forms. The mushroom PPO system was found to consist of at least nine distinct bands with 3,4-dihydroxyphenylalanine (DOPA) as substrate, potatoes showed at least 11 bands while apples showed

at least three multiple forms of DOPA oxidizing activity. Wong et al. (1971) detected four isozymes in extracts from acetone powder of the clingstone peach. Montgomery and Sgarbieri (1975) reported that banana PPO exists in multiple forms. They detected nine, eight and ten PPO isozymes in the interior pulp, exterior pulp, and the peel of banana, respectively, with catechol as substrate. Kahn (1976) has recently shown that avocado PPO also exists in multiple forms; six active isozymes were detected with DL- dopa, 4-methyl-catechol, catechol, caffeic acid and chlorogenic acid.

Isolation of Enzyme from Fruit

In the isolation of enzymes from fruit, the fruit tissue is disrupted or damaged and the compartmentalization is destroyed. Phenolics in the tissue are exposed to the action of PPO, resulting in the formation of brown pigments. The quinones thus formed react with proteins to cause a partial or complete inhibition of the enzymes. The inhibition of fruit enzymes by phenolic compounds has been described by Walker and Hulme (1965), Dilley (1966) and Badran and Jones (1965).

Loomis and Battaile (1965) discussed the effects of plant phenolic compounds on the isolation of plant enzymes. In the extraction of enzymes from plants rich in phenolic compounds, these authors found that the phenolics must be separated from the protein to prevent

the oxidation of phenols to quinones by PPO. If the quinones were allowed to form, they would combine reversibly with the protein by hydrogen bonding or irreversibly by covalent condensation. Enzymes which have undergone these reactions are inactivated.

The general approach in isolating fruit enzymes is to remove phenolic compounds and other secondary products as quickly as possible to prevent the formation of covalent complexes. Several polymeric absorbents are available which can be used to remove non-covalently bound phenols by competititive binding. The polymeric compound, polyvinylpyrrolidone (PVP), which has been shown to rapidly hydrogen bond to the phenolics, is either essential or highly beneficial in isolating enzymes from plants or fruits. Gustavson (1963) postulated that hydrogen bonding was the main reaction between PVP and the OH-groups of phenolic compounds as shown in Figure 1.

Many enzymes combine with their substrates through hydrogen bonding with -OH and -SH groups. When sufficient PVP is present during the enzyme isolation procedures, the phenolics may be seques-tered preferentially in solution without interfering with enzyme reac-tions. If, however, excess PVP is present, the PVP may inhibit competitively or non-competitively the enzyme systems under study. Jones et al. (1965) found that for mature apple fruit, a concentration of 1 % PVP was the best compromise, giving near maximum enzyme

activity. Chan and Yang (1971) reported that the enzyme extracts

of cranberry obtained by using a buffered-PVP gave the highest

specificity activity. Benjamin and Montgomery (1973) showed that

0. 5 g of PVP/g of crude acetone powder of cherry reduced the phe-

nolic compounds substantially and increased the activity of cherry

PPO. Also, in the banana, Montgomery and Sgarbieri (1975) found

that about 15% more PPO activity could be extracted with the use of

PVP.
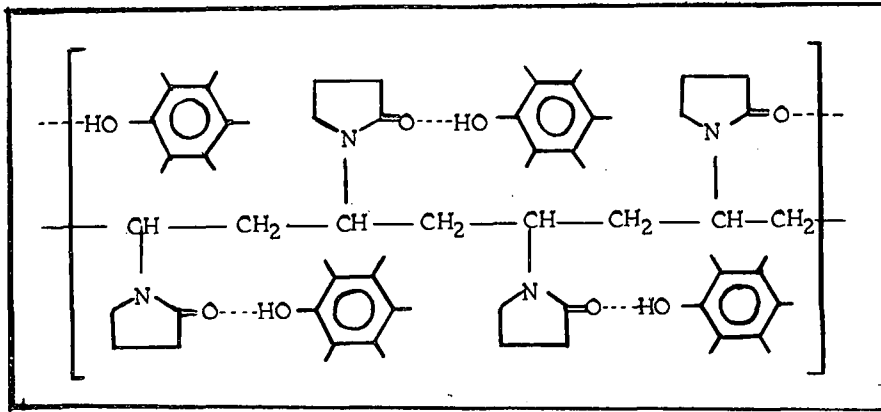
Figure 1. Postulated hydrogen bonding of phenols to PVP.

Numerous phenolic substances that could possibly bind with

PVP in plant systems have been reported. Sioud and Luh (1966)

identified the presence of leucoanthocyanidins, d-catechin, epi-

catechin, chlorogenic acids, caffeic acid in Bartlett pear puree.

They found that catechin and chlorogenic acid constitute about 90%

of the total phenolic content of Bartlett pears. Earlier, Seigelman

(1955) had reported that epicatechin and catechin were the main

endogenous pear browning substrates. Walker (1964) later reported

that chlorogenic acid was the chief phenolic substrate involved in the

pear browning reaction. Chlorogenic acid has also been found in

many fruits, including apples (Hulme, 1957; Siegelman, 1955), apri-

cots (El-Sayed and Luh, 1965) and peaches (Luh et al., 1967). Struc-

tures of some of the phenolic compounds which serve as substrates

for PPO are shown in Figure 2.

Assay of PPO

Enzyme activity may be estimated by manometric, polaro-

graphic, chronometric, and spectrophotometric methods. Mono-

metric and polarographic methods measure oxygen uptake during

enzymic reaction by use of a Warburg apparatus and O_2-sensitive

electrode, respectively. Chronometric method determines the reduc-

tion of the oxidized phenolic substrate by ascorbic acid and the spec-

trophotometric method determines the initial rate of the formation

of oxidation products. A critical comparison of methods has been

discussed by Mayer et al. (1966). For determining the activity of

PPO in fruit, the spectrophotometric method was found to be a simple,

quick and reproducible procedure. This simple technique has proven

to be adequate for comparative studies of the browning levels of differ-

ent fruit varieties (Walker, 1962).
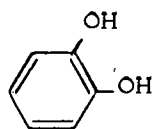
Catechol   4-Methylcatechol   Chlorogenic acid
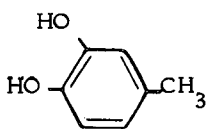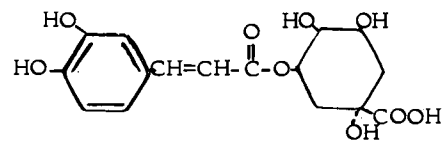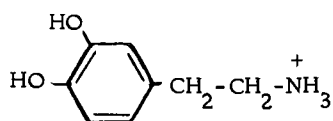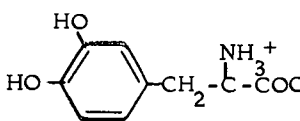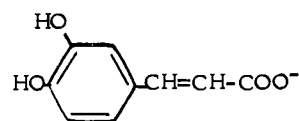
Dopamine   DL-Dopa   Caffeic acid
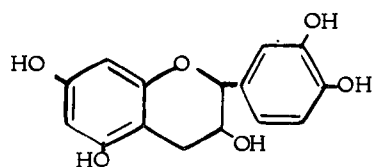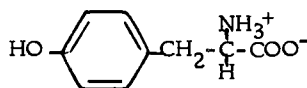
Catechin   L-Tyrosine
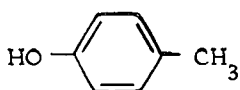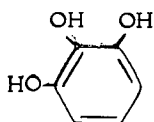
p-Cresol   Pyrogallol

Figure 2. Structure of phenolic compounds.

pH Optima of PPO in Fruits

Widely different pH optima have been reported for various fruit PPO. The pH optimum of banana PPO on dopamine was found to be 7.0 (Palmer, 1963). Cranberry PPO was also reported to have the optimum pH at 7.0 (Chan and Yang, 1971). Reyes and Luh (1960) reported that the optimum PPO activity of the freestone peach ranged between pH 5.9 to 6.3. In a more recent report, the optimum pH of clingstone peach PPO was shown to be 6.2 (Luh and Phithakpol, 1972). Shanon and Pratt (1967) found apple PPO to have two pH optima, 5.2 and 7.3, with the activity at pH 5.2 being several times greater than that at pH 7.3. PPO of apricot had maximum catecholase activity at a pH range of 5.0 to 6.0 (Soler et al., 1965) The optimal activity with catechol as substrate for Bartlett pear PPO was found to be pH 6.2 (Tate et al., 1964).

Heat Inactivation of PPO

Several investigators have studied the heat inactivation of PPO in various fruits. Dimick et al. (1951) reported data on the heat inactivation of PPO in apples, apricots, peaches, pears and grapes. They determined that the rate of inactivation of PPO at a constant temperature was different for each fruit and followed first order kinetics. The time for inactivation of 90% of the PPO activity at

75°C was as follows: pear, 390 sec; apricot, 80 sec; grape, 27 sec; peach, 12 sec; and apple, 7.5 sec. The rate of inactivation at 75°C was most rapid in apple, and slowest in pear. Chan and Yang (1971) reported that 90% of the cranberry PPO was inactivated by heating for 1.32 min at 70°C, 7.05 min at 60°C and 15 min at 50°C with the heat inactivation of the enzyme following first order kinetics. Heat inactivation of cherry PPO at 75°C followed first order kinetics for 7 min (Benjamin and Montgomery, 1973). In a comparative study between fruits and vegetables, Yankov (1963) reported that the PPO of fruits was more stable than that of vegetables.

Substrate Specificity of PPO

Most fruit PPO show specificity for o-diphenolic substrates. Cresolase activity, which catalyzes the oxidation of monophenol to diphenol, is rarely found in fruit. The substrate specificities of PPO vary from fruit to fruit. Tate et al. (1964) studied the PPO of Bartlett pears and found that only those substrates with an o-dihydroxy configuration were attacked by the enzyme. The pear PPO rapidly oxidized catechol, chlorogenic acid, caffeic acid and d-catechin. More recently, Rivas and Whitaker (1973) also indicated that the Bartlett pear PPO possessed only o-diphenol activity but no cresolase or laccase activity as indicated by lack of activity toward p-coumaric acid, ferulic acid, recorsinol and phloroglucinol. They found that

chlorogenic acid, d-catechin, pyrocatechol and 4-methylcatechol were all rapidly oxidized.

In apple PPO, the maximum activity of the compound tested occurred with hydrocaffeic acid, followed by chlorogenic acid, iso-chlorogenic acid, caffeic acid, 3,4-dihydroxyphenylacetic and 3,4-di-hydrobenzoic acid (Walker, 1964a). Walker (1964b) found that pear and apple PPO had similar substrate specificities.

Wong et al. (1971) studied the PPO of clingstone peaches and reported that all of the isozymes had activity toward o-diphenols but none toward monophenols and m-diphenols even in the presence of a small amount of the o-diphenol, catechol. Peach PPO was found to be most active toward d-catechin, followed by catechol, chloro-genic acid, and caffeic acid and moderately active toward dopamine (Luh and Phithakpol, 1972). Samisch and Cruess (1934) found that the apricot extract was only active toward catechol and pyrogallol, but not tyrosine or phloroglucinol and concluded that apricot PPO was specific only for o-diphenols. Benjamin and Montgomery (1973) investigated various monophenolic and o-diphenolic compounds to determine the substrate specificity of Royal Ann cherries and indi-cated that cherry PPO cannot catalyze the oxidation of monophenol to o-diphenol. Of the diphenolic substrates used in their study, pyrogallol and 4-methylcatechol were oxidized most rapidly.

Montgomery and Sgarbieri (1975) studied banana PPO and found

that it was most active with o-diphenols and showed a decrease in

activity as the substituent group on the ring was increased in size.

The o-diphenols which appeared to be better substrates for banana

PPO were catechol, 4-methylcatechol, pyrogallol and dopamine. All

of these substrates either lack or have a short chain on the ring in the

4-position. They also pointed out that the side chain attached the

diphenol ring and the length of the side chain may affect the action of

PPO on the substrate as noted by the difference in the activity between

dopamine and Dopa, 4-methylcatechol and caffeic acid.

Inhibitors of PPO

Generally the inhibitors of PPO can be classified into three

groups according to their mode of action; a) complexing with or

substituting for the prosthetic group of the enzyme, b) interacting

with products of the reaction, and c) complexing with the substrate.

Only a limited number of PPO inhibitors have been considered accep-

table on grounds of safety and/or expense as practical for use in

controlling enzymic browning in foods or food products.

Many compounds containing sulfur have been widely used to

prevent discoloration in fruits. These include sulfurous acid and its

salts (bisulfites and metabisulfites), cysteine and glutathione. The

mechanism of the inhibition of enzymic browning by SO_2 was studied

by Embs and Markakis (1965) with mushroom PPO on catechol at

pH 6. 5. They showed that inhibition was not due to the removal of oxygen by oxidation of sulphite to sulphate, but was due partly to inactivation of the enzyme itself and partly to the formation of o-quinone sulphite. The onset of browning was delayed until all the SO_2 had been consumed by these reactions and then proceeded at a rate dependent on the residual activity of the enzyme.

Inhibition of enzymic browning by cysteine has been reported by several researchers who suggested that cysteine inhibits the oxidation of o-dihydroxyphenols by combining with quinone which is formed during the reaction to form a colorless cysteine phenolic complex (Bouchilloux et al. , 1960; Walker, 1964; Muneta and Waldradt, 1968). Walker and Reddish (1965) reported that small amounts of cysteine inhibited the browning of apple products and the inhibition was of much lohger duration and did not induce any undesirable off-flavors. They found that cysteine was effective only if the thiol/phenolic ratio was greater than unity. Less than equimolar amounts of thiol did not prevent browning. At higher concentrations, however, the cysteine reacted with the phenolic compound to produce a red colored complex. Recently, the PPO of garland chrysanthemum was studied by Maruyama et al. (1975) who reported that inhibition of PPO system by cysteine may be due to the formation of the substrate (chlorogenic acid) and cysteine complex.

Ascorbic acid is another widely used inhibitor of enzymic

browning. It prevents browning by reducing the o-benzoquinone back to the original o-diphenol compound as rapidly as it is formed by donating two hydrogens and is itself thus oxidized to dehydro-ascorbic acid (Ponting and Joslyn, 1948). Reyes and Luh (1962) found that both ascorbic acid and isoascrobic acid appeared to be equally effective in inhibiting the browning reaction catalyzed by PPO from freestone peaches. Control of enzymic browning during the manufacture of opalescent apple juice and other similar fruit products is commonly achieved by addition of ascorbic acid, because it does not impart undesirable flavor or odors, blends easily with natural fruit flavors and increases the vitamin C content. However, this inhibition reaction can only continue while there is an excess of ascorbic acid. When it has been fully utilized, browning occurs.

The reaction of cysteine and ascorbic acid is shown in Figure 3.

Diphenol $\xrightarrow{\text{O}_2}$ Quinone $\xrightarrow{\text{ascorbic acid}}$ Diphenol + Dihydroascorbic acid

Quinone $\xrightarrow{\text{cysteine}}$ Cysteine-phenolic complex (colorless)
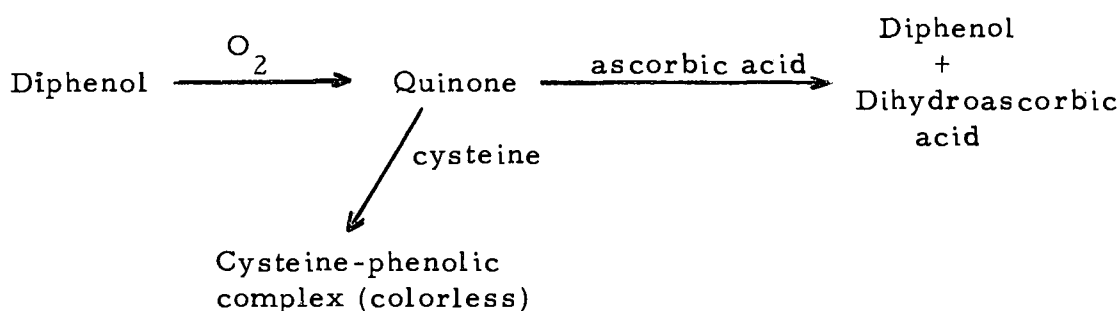
Figure 3. Role of cysteine and ascorbic acid in phenolic browning.

Diethyldithiocarbamate (DIECA) was found to be an efficient inhibitor of PPO (Pierpoint, 1966), because it chelates copper. The complexing reaction appears to withdraw the copper ion from the PPO active site making the enzyme inoperative. Kahn (1975) reported that DIECA strongly inhibited the PPO activity of each of three avocado varieties.

Another potent inhibitor of PPO is mercaptobenzothiozole (MBT). Palmer and Roberts (1967) reported that very low concentrations of MBT inhibited PPO although inhibition could be reversed by adding equally low concentrations of the cupric ion. These workers demonstrated that two moles MBT complexed with one mole cupric ion and suggested that the compound combined with copper at the active center of PPO, thus forming an inactive complex. Wang and Mellenthin (1974) studied the inhibition of friction discoloration of d'Anjou pears by MBT and found that treatment with MBT inhibited the discoloration of the fruits. Their study provided evidence that the inhibition of the browning by MBT was through inactivation of pear PPO.

MATERIALS AND METHODS

Source of Fruit

d'Anjou pears (<u>Pyrus communis</u> L.) used in this study were obtained from Mid-Columbia Experiment Station in Hood River, Oregon. The pears were stored at $-1^{\circ}C$ until used in this study.

Extraction of PPO

The method for the extraction of enzyme using insoluble poly-vinylpyrrolidone (PVP) as developed by Loomis and Battaile (1966) and modified by Montgomery and Sgarbieri (1975) was applied to pears. Fifteen grams of fresh pears were cut quickly into thin slices, and blended with 30 ml of cold 0.05 M acetate buffer (pH 5.6) containing 1.5 g of PVP (Sigma Chemical Co., pharmaceutical grade) in a Waring blendor for 15 sec. Following extraction, the slurry was centrifuged at 12,000 x G for 10 min at $4^{\circ}C$. The supernatant (re-ferred to as the crude PPO extract) was decanted carefully through glass wool and stored in a small stoppered glass bottle at $-30^{\circ}C$ until used.

Enzyme Activity Measurement

PPO activity was determined by measuring the initial rate of brown color formation as indicated by an increase in absorbance at

410 nm. A Beckman DB recording spectrophotometer was employed throughout the study. Each sample was assayed in duplicate, the sample cuvette contained 2.0 ml of 10 mM catechol (freshly prepared in 0.2 M potassium phosphate buffer, pH 7.0), 0.8 ml of 0.05 M phosphate buffer (pH 7.0) and 0.2 ml of enzyme solution. Reference cuvette (blank) contained 2.0 ml of the same substrate solution and 1.0 ml of 0.05 M phosphate buffer (pH 7.0).

The reaction was carried out at 21°C. Initial velocity was estimated from the linear slopes of the curve (first 60 sec) obtained on the recorder. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min. Specific activity was expressed as units of enzyme activity per mg of protein.

Protein Determination

The concentration of protein was determined by spectrophotometric method of Warburg and Christian (1941) and Kalckar (1947). The protein concentration was calculated by the following equation:

$$\text{Protein concentration (mg/ml)} = 1.45 \, A_{280} - 0.74 \, A_{260}$$

where A_{280} and A_{260} are absorbance at 280 and 260 nm, respectively.

Heat Inactivation of PPO

For heat treatment of PPO, 10 ml of crude enzyme solution was pipetted into a preheated test tube in a water bath. After each heating interval, 0.6 ml of the sample was withdrawn, chilled immediately in an ice bath and then assayed for the remaining activity of PPO.

Substrate Specificity

The effect of substrate on PPO was studied. All substrates were commercial grade (Sigma Chemical Co.) and were used without further purification. For activity assay, 0.2 ml of enzyme solution was mixed rapidly into 2.6 ml of substrate (freshly prepared in 0.2 M phosphate buffer, pH 7.0) and 0.2 ml of 0.05 M phosphate buffer (pH 7.0) in the reaction cuvette. The rate of reaction was recorded at 410 nm.

Effect of Inhibitors

To determine the effect of inhibitors, reactions containing 2.6 ml of 10 mM catechol, 0.2 ml of enzyme solution were run at $21^{\circ}C$ in the presence or absence of 0.2 ml of inhibitors at various concentrations. The reaction was followed at 410 nm and the rate of reaction was determined from the slope of the reaction curve following any delay in change in absorbance at 410 nm due to the inhibitors.

Discontinuous Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to Davis (1964) and Montgomery and Sgarbieri (1975). The electrophoresis was conducted in an apparatus constructed by Sullivan (1976) as described by Davis (1964), with a power supply Model EC-454 (EC-Apparatus Corp.).

Buffers and solutions requried for this procedure were prepared as follows:

Ru:ining gel buffer (pH 9. 0, 0. 38 M Tris-HCl) was prepared by dissolving 138 g of Tris and 12 ml of concentrated HCl in 2000 ml of distilled water. After addition of most of the concentrated HCl, the pH was adjusted to 9. 0 with 1. 0 N HCl. Final volume was made up to three liters with distilled water.

Spacer buffer (pH 6. 7, 0. 062 M Tris-HCl) was prepared by using 22. 5 g of Tris and 12 ml of concentrated HCl to make three liters of this buffer. Preparation and adjustment of pH were carried out as described above.

Electrode buffer (pH 8. 75, 0. 0165 M Tris-0. 039 M glycine) was prepared by dissolving 11. 6 g of glycine and 8. 0 g of Tris in distilled water to make up four liters of the buffer.

The above solutions were kept in tightly sealed bottles to avoid atmospheric contamination and stored at the room temperature (21°C)

with the exception of the electrode buffer which was stored at 4°C.

Running gel solution was prepared by dissolving 2. 1 g of Cyanogum 41 (95% acrylamide and 5% bisacrylamide, EC-Apparatus Corp.) in 30 ml running buffer to make a 7% solution. 0. 03 ml of N, N, N', N'-tetramethylethylediamine (TEMED) was added prior to filtration through Whatman #1 paper.

Spacer gel solution was prepared by dissolving 0. 8 g of Cyanogum 41 in spacer buffer to make 20 ml of 4% solution. 0. 01 ml of TEMED was added and the solution filtered.

Due to the possible instability, the running gel and spacer gel solution were freshly prepared immediately before use.

The polyacrylamide column was prepared in glass tubes of 0. 5 cm (i. d.) and 12 cm in length. The tubes were placed vertically in a tube rack, with one end sealed with parafilm. To catalyze gel polymerization, 30 ml of running gel solution was mixed with 0. 03 g ammonium persulfate (AP) and carefully pipetted into each tube to a height of 8 cm. Distilled water was layered gently and carefully over the surface of the gel solution to eliminate the minicus. The tubes were allowed to stand undisturbed until the solution gelled (ca. 15-20 min) as indicated by a faint opalescence in the gels. After gel polymerization, the water was removed. Twenty mls of spacer gel solution was mixed with 0. 02 g AP and pipetted gently on top of running gel to ca. 1. 5 cm in height and allowed to polymerize (ca. 15-20 min).

Once the gels were prepared, the parafilm was removed and the gels were allowed to stand for another 30 min before electrophoresis. The tubes were inserted into the upper buffer chamber and this chamber was lowered in place so that the gel tubes were immersed ca. 4 cm in the buffer of the lower chamber (ca. 500 ml). Any air bubbles in the bottom of gels were removed.

Crude enzyme solution (150 µl) containing 10% sucrose and a small amount of bromophenol blue (used as a marker) was introduced with a syringe directly onto the top of spacer gel. The power supply was connected, cathode to upper chamber and anode to lower chamber. An electric current of 100 V (ca. 2 ma/tube) was applied initially until the sample was stacked in spacer gel and had moved to the surface of the running gel (ca. 45 min). Current was then increased to 200 V for the remainder of the electrophoretic run (ca. 2 hrs). The run was stopped when the marker dye (bromophenol blue) was about 1 cm from the end of the tube. All electrophoretic runs were carried out at 4°C. After completion of the run, the gels were removed from the tubes by using 2 ml syringe with a stainless steel needle (ca. 15 cm). The syringe was filled with water and the needle was slowly pushed and moved spirally along the inside wall of the tube. The gels were placed in test tubes.

Detection of PPO

The number and location of PPO following electrophoresis separation was determined by immersing the gels in 15 ml of 10 mM catechol solution containing 0.05% of phenylenediamine for ca. 20 min to intensify the coloration of bands. To stabilize the color of the stained bands and to decrease the non-specific background color, the gels were stored in 10 mM ascorbic acid for ca. 5 min. The bands were stable for more than one hr. Each gel was scanned at 410 nm in Gilford scanning densitometer to detect the number and location of PPO bands.

Substrate Specificity

Several electrophoretic runs were conducted to study substrate specificity. The gels were incubated in a number of substrates for 20 min. The method of detection of PPO activity toward these substrates was carried out as described above.

Inhibitor Studies

To determine the effect of inhibitors, the gels were incubated for 1 hr in 15 ml of inhibitor solutions, which had been freshly prepared in 0.2 M phosphate buffer (pH 7.0). PPO bands were revealed by treatment with 10 mM catechol solution (ca. 15 ml) for 20-30 min.

RESULTS AND DISCUSSION

Enzyme Extraction

The insoluble polymer, polyvinylpyrrolidone (PVP), has been

used for the extraction of soluble enzymes from plant tissues (Loomis

and Battaile, 1966; Coggen et al., 1973; Montgomery and Sgarbieri,

1975). PVP is very effective in removing the phenolic compounds

and the procedure is relatively easy and simple. In this study, there-

fore, the PVP-buffered extraction procedure was used to obtain crude

enzyme extracts from fresh pear tissues.

In preliminary experiments, extracts prepared with low levels

of PVP in phosphate buffer (pH 7. 0) had low PPO activity and were

brown. Hence, a study was made to determine the proper pH of the

buffer and the levels of PVP required for the extraction.

Effect of Buffer pH on the PPO Activity During Extraction

To determine the effect of pH, 15 g of freshly sliced pears were

extracted in cold 0. 05 M buffer at pH values from 5. 0 to 7. 0 in the

presence of 1. 0 g of PVP. The results are shown in Figure 4. These

data show that the maximum activity was extracted at pH 5. 6 in an

acetate buffer. Above or below this pH, the activity of PPO decreased,

especially between pH 6. 5 and 7. 0. Another striking observation was

that the extract prepared with phosphate buffer had lower activity than
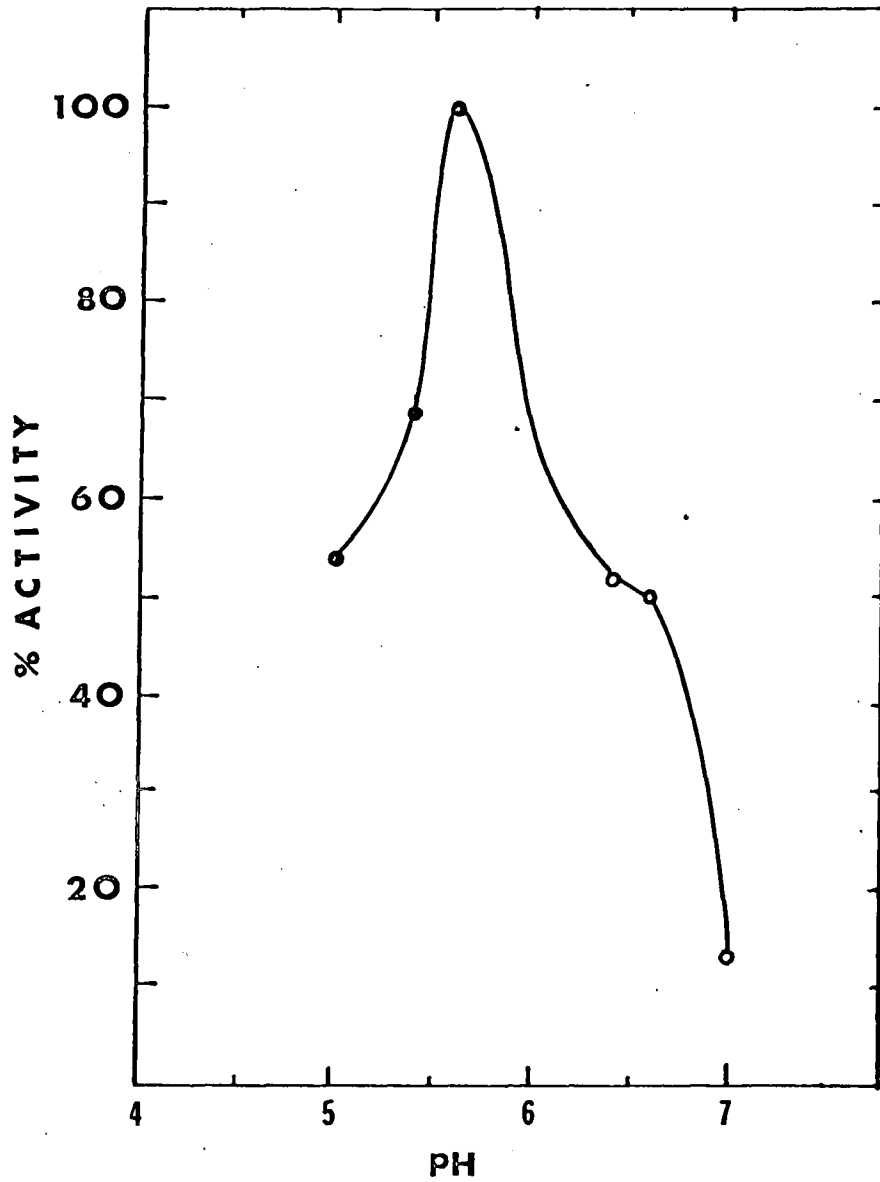
Figure 4. Effect of pH on extraction of crude d'Anjou pear PPO activity. -●-, acetate buffer; -o-, phosphate buffer.

that prepared with acetate buffer. The experiment was repeated three times and in all cases this phenomenon was noted. The reason for these findings is not known. It is open to speculation whether neutral or slightly acidic pH values caused an ionization or oxidation of phenolic compounds which resulted for ineffective adsorption of phenols by PVP as described by Andersen and Sowers (1968) or some other factor caused a reduced extraction at neutral pH.

Effect of PVP on the PPO Activity

To determine the proper levels of PVP required for the extraction, fresh sliced pears were extracted in the presence of various levels of PVP. The extracts prepared without PVP were browner in color, whereas the color of the extracts progressed from brownish or yellowish to a clear slight yellowish color as the level of PVP was increased.

Results presented in Table 1 indicate that although the extracts prepared with the addition of PVP were not as high in PPO activity as the control, specific activity at the PVP level of 1.25 to 1.5 g were higher. The extract prepared with 1.5 g of PVP yielded the highest specific activity (25% above that of the control) and a lighter colored extract. With lower or higher levels of PVP, the extracts were either browner or had lower activities or specific activities. A decrease in activity of PPO with higher levels of PVP was also

reported by Benjamin and Montgomery (1973). The inhibition of

PPO by excess PVP has been reported also by other workers (Harel

et al., 1964; Walker and Hulme, 1966). Since the supernatant of the

PVP-buffered extract had a higher specific activity than the extract

without PVP, all extractions were made with 1.5 g of PVP per 15 g

of tissue in 30 ml of cold 0.05 M acetate buffer (pH 5.6).

To further investigate the effect of PVP on electrophoretic pat-

terns of pear PPO, 150 μl of each extract prepared without PVP or

with PVP at various levels were applied to polyacrylamide gel electro-

phoresis. Similar PPO isozyme patterns were obtained in all of these

samples. No differences in the number and location of the bands were

noted in the electrophoretic patterns. This finding, similar to that

reported by Montgomery and Sgarbieri (1975) on the extraction of

banana PPO, suggests that PPO was not affected by the presence of

PVP.

Table 1. Effect of PVP on the extraction of crude d'Anjou pear PPO

PVP (g)	Activity (units/ml)*	A_{280}	A_{260}	Protein (mg/ml)	Spec. Activity (units/mg)
0.0	600	5.7	5.9	3.9	154
0.75	300	4.0	3.8	3.0	100
1.0	355	4.3	4.2	3.1	114
1.25	450	3.4	3.2	2.6	180
1.50	500	3.5	3.3	2.6	192
2.0	200	3.4	3.2	2.6	77

*One unit = 0.001 A/min at 410 nm

Preservation of Enzyme Extract

During studies on ways to preserve crude PPO extracts, difficulty was encountered in obtaining a desirable freeze-dried crude powder. When fresh crude extracts were subjected to freeze drying, a dark brown polymer-like sample was obtained. In addition, difficulty was encountered in removing the dried sample from the freeze drying flask. Therefore, dialyzing or partially purifying the extract by ammonium sulfate fractionation was used in an attempt to improve the quality of freeze dried samples.

In dialysis, 20 ml of crude extracts were dialyzed against 1000 ml of 0.05 M acetate buffer (pH 4.5) overnight (ca. 16 hr) at 4°C. The buffer was changed three times.

Prior to freeze drying, the effect of dialysis of the extract was tested and the results are shown in Table 2. These data indicate that dialysis increased the specific activity of the extract about 60%. This higher specific activity of the extract was largely due to a reduction in absorbing material at 280 and 260 nm after dialysis. Some of the impurities or inhibitory substance present in the extract were probably removed by dialysis.

Attempts to use the dialyzed extract for freeze drying did not greatly improve the quality of the freeze dried preparation. It remained brown and difficult to remove. Therefore, ammonium sulfate

precipitation was used to fractionate and concentrate the crude extract

before freeze drying. After addition of the ammonium sulfate, the

solutions were stirred for 20 min at $4^{o}C$ and centrifuged at 14,000 x G

for 10 min. The precipitate was redissolved in cold 0.05 M acetate

buffer (pH 5.6) to give a two-fold concentration and dialyzed for 12 hr

with three changes of buffer.

Table 2. Effect of dialysis on crude d'Anjou pear PPO activity.

Treatment	Activity (units/ml)	A_{280}	A_{260}	Protein (mg/ml)	Spec. Activity (units/mg)
Before dialysis	500	5.8	6.2	3.8	131
After dialysis	410	3.2	3.5	2.0	205

The results of ammonium sulfate fractionation of PPO extract

are presented in Table 3. The fraction obtained at 20% saturation had

a higher activity than other levels. During fractionation, it was noted

that at 10 to 40% saturation, a light brownish precipitate was obtained,

while at 50%, 60% and 80%, a very thin layer of light brownish floating

material was formed and at 70% and 90% saturation, no precipitate

was evident. The fraction obtained at 20% saturation was used to

prepare a freeze dried sample. Again, the attempt failed to give a

desirable freeze dried powder. The failure of the attempts to obtain

freeze dried crude powder was disappointing. The reason for this

failure is not clear.

Table 3.   Ammonium sulfate fractionation of crude d'Anjou pear PPO extract.

Saturation	Activity (units/ml)	Total (units)	Yield (%)	A_{280}	A_{260}	Protein (mg/ml)	Spec. Activity (units/mg)
0.0	600	30,000	100	2.8	3.1	1.8	340
10	350	17,500	58.0	1.3	1.4	0.8	437
20	525	26,250	87.5	1.2	1.3	0.7	750
30	150	7500	25.0	0.9	1.0	0.6	250
40	100	5000	16.7	0.6	0.7	0.4	250
50	60	3000	10.0	0.4	0.4	0.3	200
60	50	2500	8.3	0.5	0.5	0.3	167
70	npt	--	--	--	--	--	--
80	50	2500	8.3	0.4	0.5	0.3	166
90	npt	--	--	--	--	--	--

As the result of this failure, the crude extract was preserved by freezing at -30°C. The activity of these frozen extracts was checked periodically and remained stable for at least two months without showing any noticeable browning during storage. Therefore, all experiments in these studies were conducted by using the frozen crude enzyme extract.

pH Optimum of d'Anjou Pear PPO

The effect of pH on crude d'Anjou pear PPO activity with catechol as substrate is shown in Figure 5 and Table 4. These data reveal that the pH optimum of the crude PPO enzyme occurs at pH 7.0. The pH curve
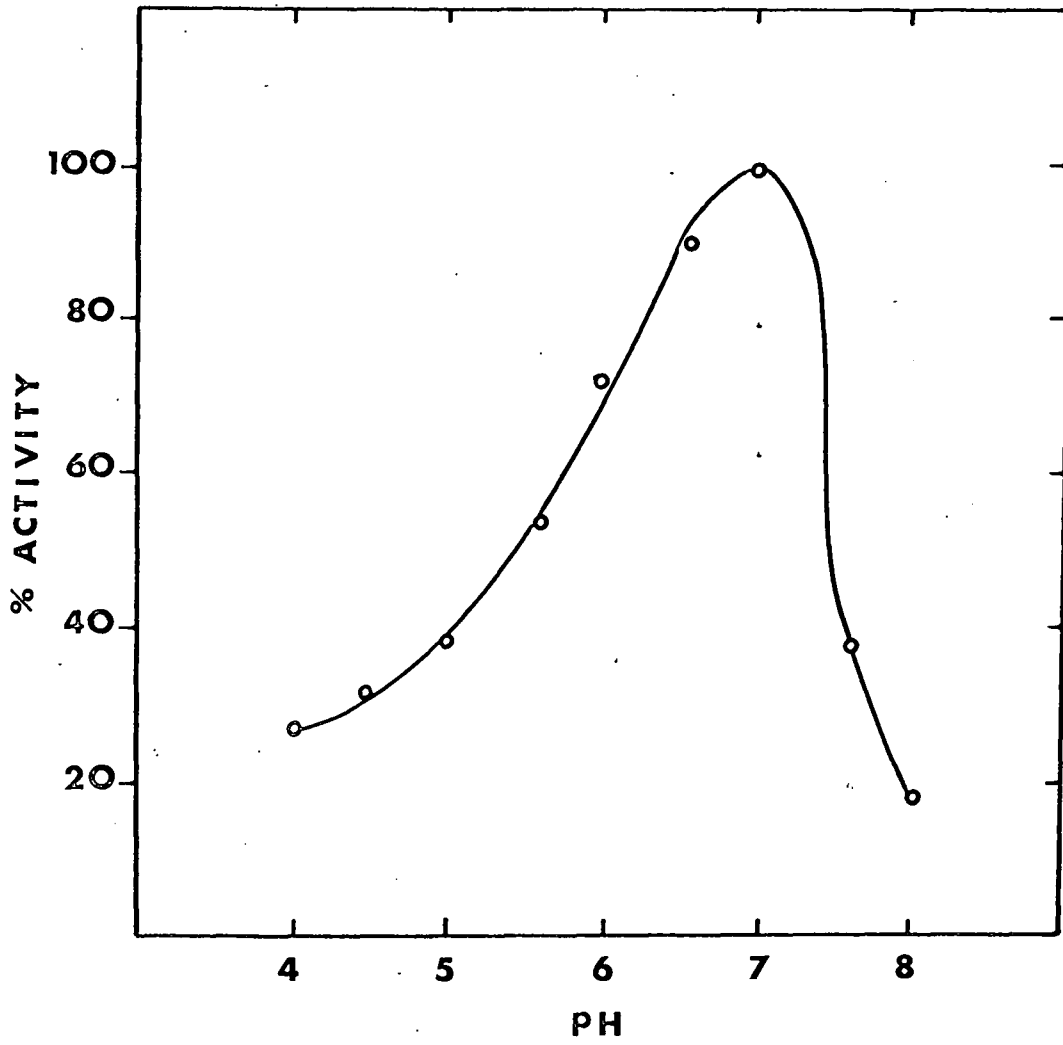
Figure 5.   pH optimum of crude d'Anjou pear PPO.

is characterized by a rapid decrease in activity at alkaline pH values.
Since the pH of the pears was 4.25, the activity in the fruit was ca.
30% of that at pH 7.0. The pH optimum of PPO system in fruits has
been shown to be most active at or near neutral pH values. A maxi-
mum activity at pH 7.0 was found in apple (Betrosian et al., 1960),
apricot and prunes (Joslyn and Ponting, 1951), cranberry (Chan and
Yang, 1971). Tate et al. (1964) reported that pH optimum of Bartlett
pear PPO occurred at pH 6.2. In ripe clingstone peaches, Wong et al.
(1971) found four PPO isozymes which had pH optima at 6.8, 6.5,
7.2 and 7.0.

Table 4. Effect of pH on crude d'Anjou pear PPO.

pH	Activity (units/ml)	Percent maximum activity
4.0	150	27
4.5	175	32
5.0	210	38
5.6	300	55
6.0	400	73
6.6	500	91
7.0	550	100
7.6	210	38
8.0	100	18

Acetate buffer for pH 4.0 - 5.6
Phosphate buffer, pH 5.6 - 7.0
Tris-HCl, 7.0-8.0

Heat Inactivation of d'Anjou Pear PPO

The results of heat inactivation of PPO at 70°, 75°, 80° and 85°C are shown in Figure 6 and Table 5. As expected, the rate of heat inactivation was greater with increasing temperature and followed first order kinetics. As shown in Figure 6, approximately 50% of crude PPO enzyme activity was inactivated after heating 11.7, 6.25, 2.25, and 1.1 min at temperatures of 70°, 75°, 80° and 85°C, respectively. Several researchers (Dimick et al., 1951; Chan and Yang, 1971; Benjamin and Montgomery, 1973) have reported the rate of heat inactivation of PPO of a number of fruits also followed first order kinetics. Dimick et al. (1951) reported that the time required to reduce 90% of Bartlett pear PPO activity at 70°C was 6.5 min. In a comparative study between fruits and vegetables, PPO of fruits were more stable than those of vegetables (Yankov, 1963; Jankow, 1963).
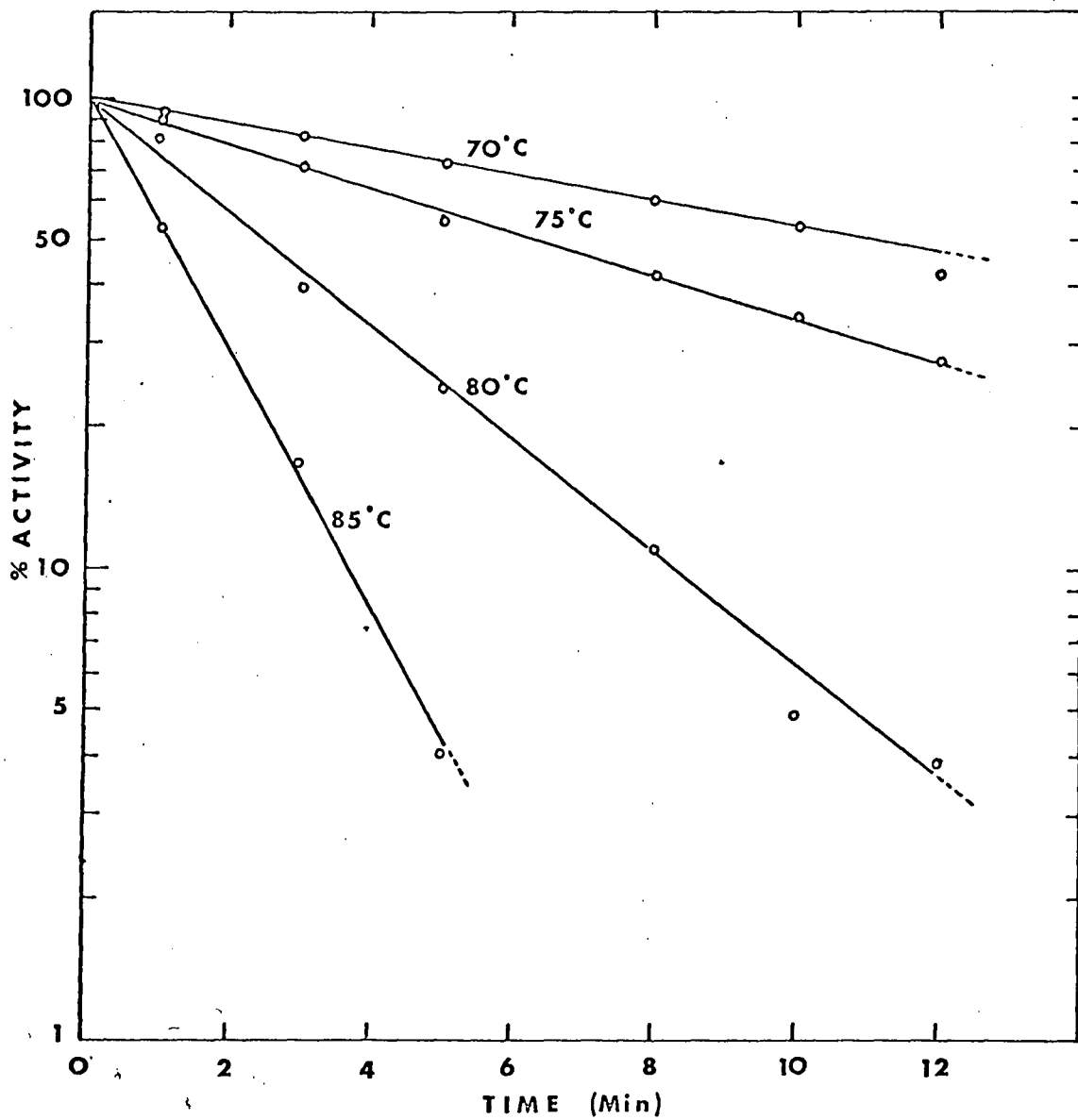
Figure 6. Heat inactivation of crude d'Anjou pear PPO.

Table 5. Heat inactivation of crude d'Anjou pear PPO.

Time (min)	Percent Activity			
	70°C	75°C	80°C	85°C
0	100.0	100.0	100.0	100.0
1	92.0	90.0	81.0	52.0
3	83.0	71.5	39.0	16.5
5	73.0	54.5	24.5	4.0
8	61.0	42.5	11.0	0
10	54.0	34.5	4.90	0
12	42.5	28.0	3.90	0
15	32.0	19.5	0	0

Effect of Storage on the Stability of
d'Anjou Pear PPO

The stability of pear crude PPO extracts during storage was studied. Fifty mls of the extract was kept in a small stoppered Erlenmeyer flask at 4°C and 21°C for a week. Samples were taken each day for assay to determine the loss of PPO activity with time. The results are shown in Figure 7 and Table 6. As shown in Figure 7, the PPO activity decreased sharply during the first two days at 21°C, then decreased slowly as the storage was prolonged. Plot of log percent enzyme activity remaining vs. time revealed that pear PPO followed first order kinetics upon storage at 21°C. At 4°C, the PPO appeared to be relatively stable over a two-day period, since no loss of PPO activity was observed. Thereafter the activity began to decline slowly. These findings suggest that there may be some differences between the stability of the isozymes at low temperature (4°C).
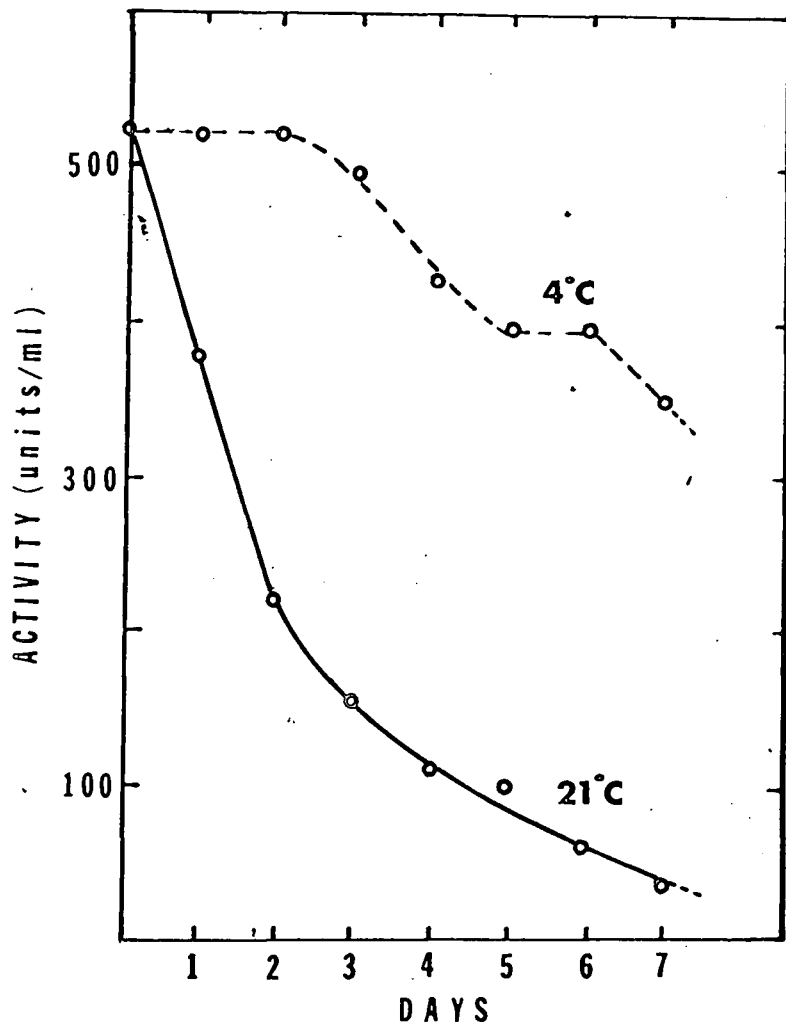
Figure 7. Stability of crude d'Anjou pear PPO during
storage.

Table 6. Effect of storage on the stability of crude
d'Anjou pear PPO.

Temperature (°C)	Time (Day)	Activity (units/ml)
4	0	525
	1	525
	2	525
	3	500
	4	430
	5	400
	6	400
	7	350
21	0	525
	1	380
	2	220
	3	155
	4	110
	5	100
	6	60
	7	40

Substrate Specificity

Eight o-diphenols and two monophenols were tested in a study

of substrate specificity of the crude enzyme preparation. Results of

these studies are shown in Table 7 and reveal that pear PPO had

activity toward o-diphenols but not toward monophenols. This latter

observation was indicated by lack of activity toward p-cresol and

L-tyrosine. The enzyme was found to be more reactive toward

catechol, 4-methylcatechol, chlorogenic acid, followed by caffeic

acid, dopamine, d-catechin, DL-dopa, and pyrogallol in a descending

order.

Montgomery and Sgarbieri (1975) studied the substrate specifi-

city of banana PPO and found that there was a marked difference

in the activity between dopamine and DL-dopa, 4-methylcatechol and

caffeic acid. They reported that the side chain attached to the

diphenol ring and the length of the side chain may affect the action

of banana PPO on the substrate. In this study (Table 7), there was

a general decrease in activity as the size of the side chain was in-

creased, however, no correlation between the size of the side chain

and the relative activity was apparent. For example, pear PPO had

more activity toward chlorogenic acid than caffeic acid, which has a

smaller side chain.

PPO that have only o-diphenolase activity have been reported

from peaches (Wong et al. , 1971; Luh and Phithakpol, 1972), apples

(Stelzig et al. , 1972) Bartlett pears (Tate et al., 1964; Rivas et al., ,

1973) and cherries (Benjamin and Montgomery, 1973).

Table 7. Substrate specificity of crude d'Anjou pear PPO.

Substrate	Concentration (mM)	Activity (units/ml)
Catechol	10	605
4-Methyl catechol	10	360
Chlorogenic acid	5	345
Dopamine	10	160
DL-Dopa	10	122
Pyrogallol	10	107
Caffeic acid	10	200
d-Catechin	10	150
L-Tyrosine	2. 5	0
p-Cresol	2. 5	0

The comparison of the specificity activities among different

substrates was only approximate, since the extinction coefficient of

the products were not identical at 410 nm.

Inhibitor Studies

Effect of a number of inhibitors on the pear PPO was studied.

Using catechol as substrate, a lag period of 75, 75, 55, 60 and 50 sec

was observed when the inhibitors L-cysteine, diethyldithiocarbamate (DIECA), metabisulfite, mercaptoethanol and ascorbic acid, respectively, were used at 1 mM. This lag period was presumably due to a temporary reduction of the product of enzymic oxidation by these compounds. The percentage of inhibition caused by these inhibitors at four different concentrations was determined from the rate of change in absorbance at 410 nm after the lag period. These data are shown in Table 8.

All of the inhibitors used in this study inhibited PPO activity with the extent of inhibition being dependent on the concentrations of the compounds used. All the compounds except thiourea, cyanide, and sodium chloride showed 100% inhibition on PPO activity at 10 mM concentration. L-cysteine and DIECA appeared to be the most effective inhibitors; they were able to inhibit the activity from about 40% to 60% at 1 mM concentration, while all other inhibitors, except sodium chloride showed inhibition of 20% to 30%. Sodium chloride was found to be the least potent inhibitor used, showing only a slight inhibition of PPO at high concentrations. Similar findings were also reported by Benjamin and Montgomery (1973) on cherry PPO.

Table 8. Effect of inhibitors on crude d'Anjou pear PPO.

Inhibitor	Conc. (mM)	Percent Inhibition
L-Cysteine	10	100.0
	1	57
	0.1	17
	0.01	4
Sodium-Diethyldithio carbamate	10	100.0
	1	42
	0.1	12
	0.01	7
Thiourea	10	96
	1	27
	0.1	10
	0.01	4
Potassium-Metabisulfite	10	100.0
	1	27
	0.1	7
	0.01	4
Potassium-Cyanide	10	85
	1	21
	0.1	8
	0.01	0
Mercaptoethanol	10	100.0
	1	28
	0.1	3
	0.01	0
Ascorbic acid	10	100
	1	25
	0.1	8
	0.01	2
Sodium Chloride	10	12
	1	8
	0.1	0
	0.01	0

Gel Electrophoresis Studies

Polyacrylamide gel electrophoresis was used to detect pear PPO isozymes. Samples of fresh crude extract, frozen crude extract and dialyzed extract were analyzed by 7% gels. Under test conditions, eight active isozymes were separated by electrophoresis and detected with catechol as substrate. Similar isozyme patterns were obtained in each of the samples. This suggests that no conformational changes that were detectable by electrophoresis occurred either by freezing or dialyzing the enzyme preparation. Hence, it can be concluded that storing the crude extracts in the frozen state (-30°C) did not affect the conformation of pear PPO isozymes.

Densitometer scan (Figure 8) at 410 nm of a gel was used to provide a record of the number and location of the PPO isozymes. Relative migration (Rm) of the band was determined relative to the bromophenol blue marker dye. The pattern of enzyme activities in the gel reveals that band d and f were the most active as indicated by the highest color intensity. Bands a, e, g, and h had similar color intensity and bands b and c appear to be least active. The color development of these bands was much slower than others and less intense.
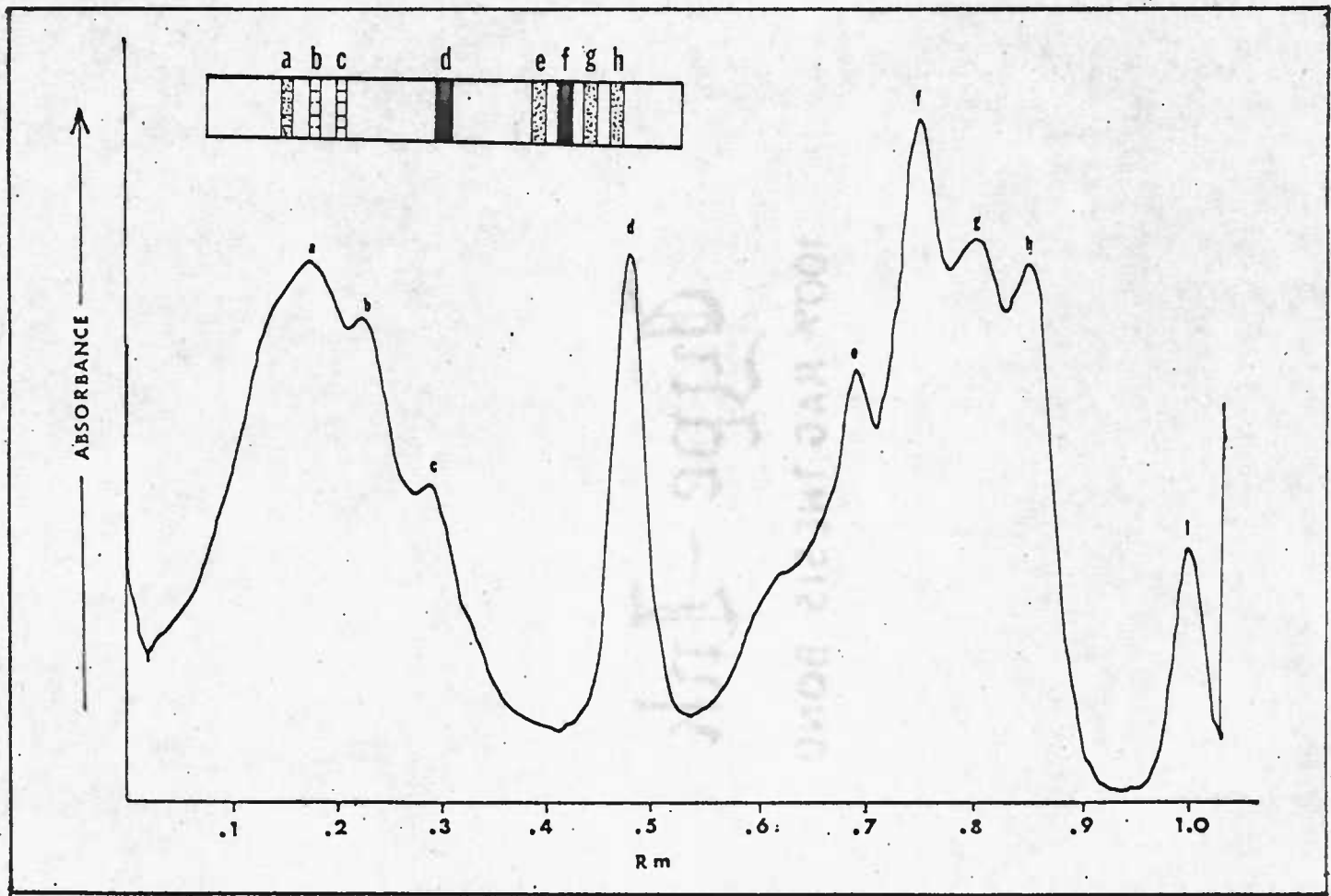
Figure 8. Detection of d'Anjou pear PPO activity in polyacrylamide gel at 410 nm in a Gilford scanning densitometer.

Substrate Specificity of d'Anjou Pear PPO Isozymes

Activity of the pear PPO isozymes with different substrates was tested in an attempt to differentiate between the relative activity of the isozymes. Electrophoretic patterns of PPO activity towards different substrates were determined by incubating the gels in substrate solutions of 10 mM concentration. The results are presented in Figure 9. Similar to many other fruits, pear PPO is only active towards o-diphenol substrates, no bands showed activity towards tyrosine and cresol. Electrophoretic patterns of the relative activities of the isozymes toward catechol, 4-methylcatechol, chlorogenic acid, dopamine, DL-dopa, caffeic acid, and d-catechin were found to be similar. However, the color intensity of the bands was characteristic for each substrate and time needed for development varied from 10 to 20 min. Catechol and caffeic acid had chromogenic pattern of dark brown to brownish color; 4-methylcatechol, dopamine and DL-dopa had red to reddish blue; and chlorogenic acid, d-catechin and pyrogallol had yellow to pale yellow. In each case, bands d and f appeared to be most active, followed by the moderately active bands of a, e, g and h, and the less active bands, b and c.

The triphenolic pyrogallol was slowly oxidized by only the isozyme represented by band d. Addition of the third hydroxy group to the benzene ring probably is significant in determining the activity of PPO to pyrogallol.
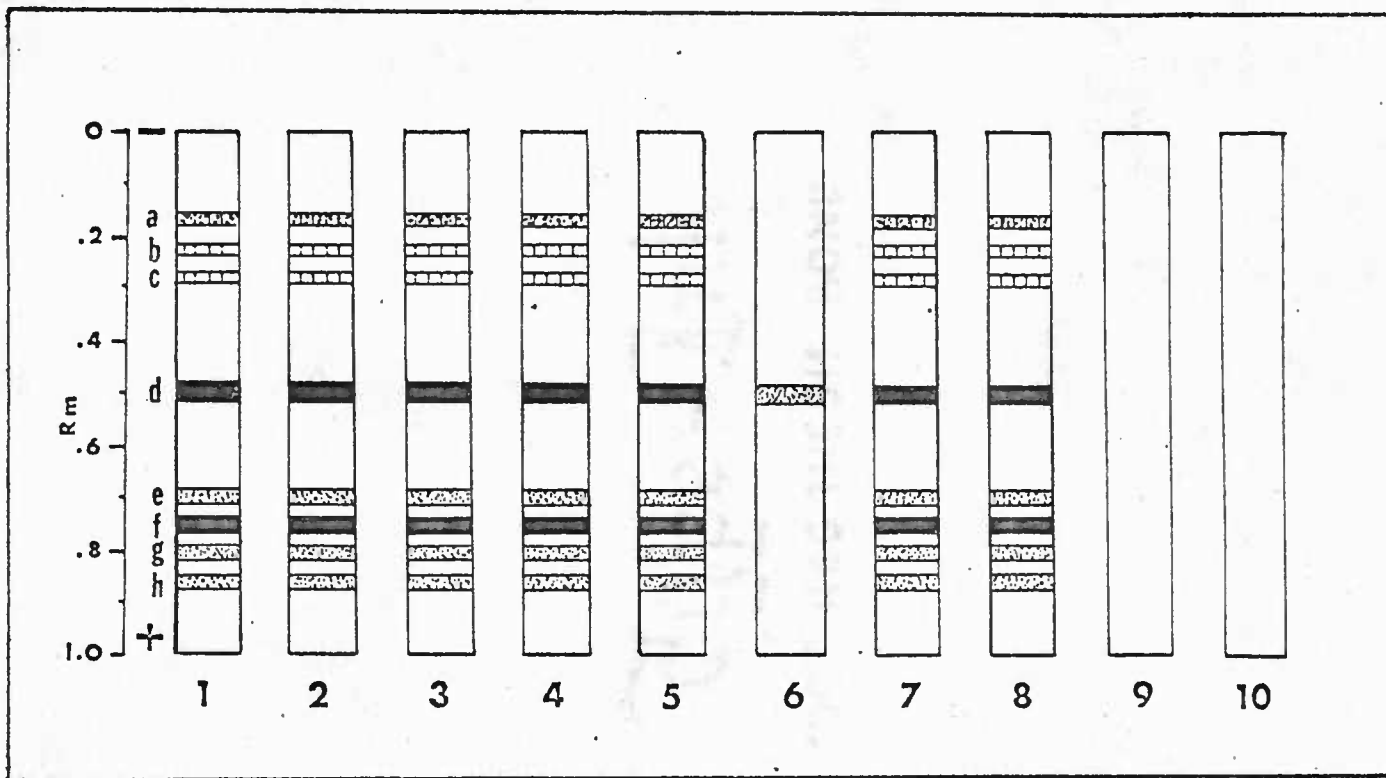
Figure 9. Electrophoretic patterns of d'Anjou pear PPO with different substrates (10 mM). 1, catechol; 2, 4-methylcatechol; 3, chlorogenic acid; 4, dopamine; 5, DL-dopa; 6, pyrogallol; 7, caffeic acid; 8, d-catechin; 9, L-tyrosine; 10, p-cresol.

Inhibitor Studies by Gel Electrophoresis

Effect of inhibitors on the activity of PPO isozymes was studied by incubating the gels in 1 mM and 10 mM inhibitors solution (ca. 15 ml) for 1 hr before being placed in the 10 mM catechol solution. In this study, inhibitors at 10 mM were very effective in inhibiting the PPO isozymes. With the exception of thiourea, all the inhibitors used completely inhibited the PPO isozymes in the gels. No active bands were observed in the gels that have been treated with L-cysteine, DIECA, metabisulfite, cyanide, mercaptoethanol and ascorbic acid. In the case of thiourea, the eight active bands remained, but the color intensity of bands was reduced to indicate partial inhibition.

Electrophoretic patterns of PPO isoenzymes toward different inhibitors at 1 mM concentration are shown in Figure 9. L-cysteine, DIECA, thiourea, metabisulfite, mercaptoethanol and ascorbic acid showed similar inhibitor specificities, i.e., the electrophoretic patterns appear to be similar to that of the control (with no inhibitor), but the relative intensities of the bands were somewhat reduced. The intensities of the slow moving bands a, b, and c were particularly reduced, which suggests that these isozymes were more sensitive to the inhibitors. Inhibition caused by L-cysteine appeared to be less effective than DIECA, which differs from spectrophotometric assay, where L-cysteine showed a greater inhibition than DIECA. The explanation

for such differences could be due to the rate of permeability of the gel by the inhibitor or dilution of the inhibitor by the buffers in the gel. These findings suggest that the concentration of inhibitors is critical for complete inhibiton of PPO isozymes.

As can be seen in Figure 10, the PPO isozymes were very sensitive to cyanide even at 1 mM concentration. Again, this finding was different from the spectrophotometric assay. This phenomenon is difficult to explain. However, it may be attributed to the rate of penetration of the gel by the compound.
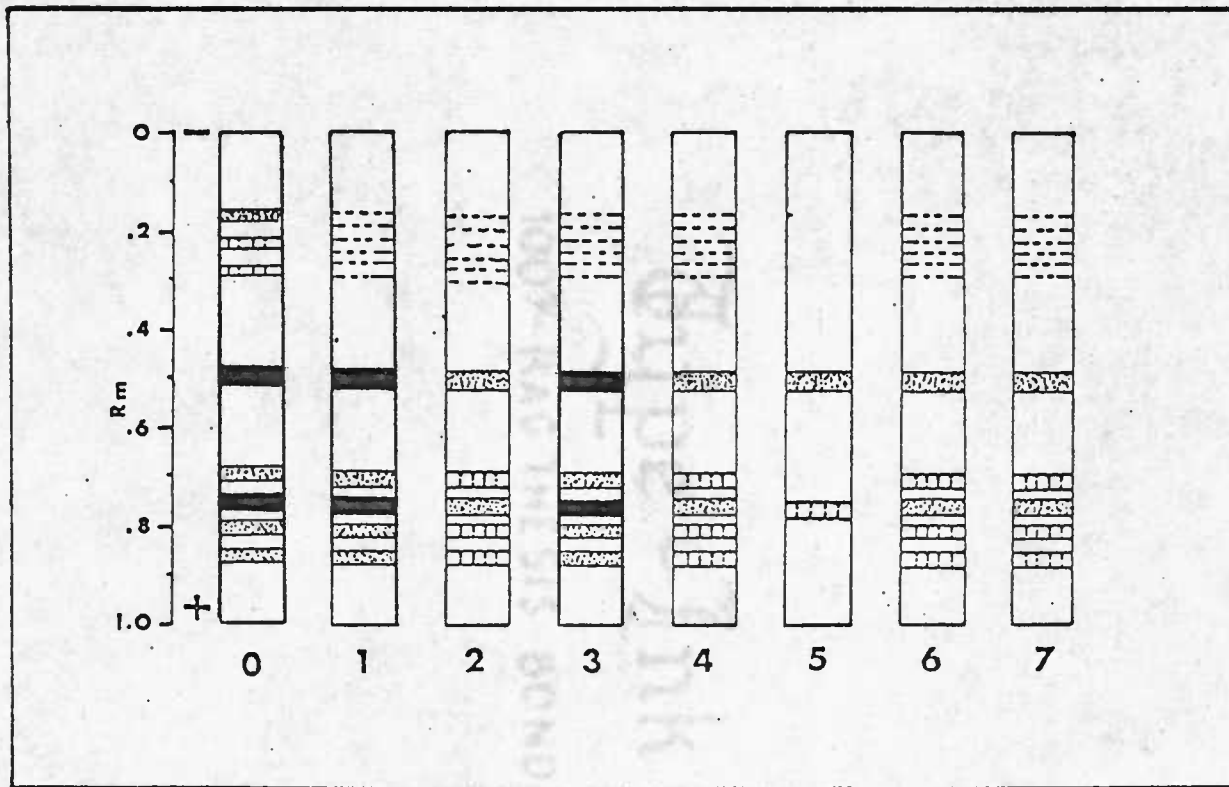
Figure 10.   Electrophoretic patterns of d'Anjou pear PPO toward different
inhibitors (1 mM).   0, control (no inhibitor); 1, L-cysteine; 2,
DIECA; 3, thiourea; 4, metabisulfite; 5, cyanide; 6, mercapto-
ethanol; 7, ascorbic acid.

SUMMARY AND CONCLUSIONS

Crude PPO enzyme extracts of d'Anjou pears were prepared and studied. The optimum conditions of enzyme extraction were determined using PVP-buffered extraction procedure. The stability toward pH and temperature, substrate and inhibitor specificities, and electrophoretic properties of this crude enzyme preparation were investigated. From this study the following conclusions can be made:

1. The phenol binding agent, polyvinylpyrrolidone (PVP), increased the PPO specific activity in the PVP-buffered extract. In addition, the electrophoretic patterns of pear PPO did not appear to be affected by the presence of PVP.

2. The pH optimum of pear PPO is 7.0.

3. Heat inactivation of pear PPO was determined to follow first order kinetics, with approximately 50% destruction at 70°, 75°, 80° and 85°C requiring 11.7, 6.25, 2.25 and 1.1 min, respectively.

4. Pear PPO was found to be active only on phenolic substrates having an o-diphenolic configuration. This activity showed a general decrease as the size of side chain was increased.

5. Inhibitor studies revealed that the pear PPO was relatively sensitive to most inhibitors tested with the exception of sodium chloride which was least potent. Sensitivity of PPO toward

different inhibitors was found to be dependent on the concentration of the compound.

6. Polyacrylamide gel electrophoresis of fresh crude PPO extract, frozen crude extract and dialyzed PPO extract showed similar electrophoretic patterns. Eight isozymes were detected with catechol as substrate.

BIBLIOGRAPHY

√ Andersen, R. A. and J. A. Sowers. 1968. Optimum conditions for bonding of plant phenols to insoluble polyvinylpyrrolidone. Phytochemistry. 7:293-301.

Anderson, J. W. 1968. Extraction of enzymes and subcellular organelles from plant tissues. Phytochemistry 7:1973-1988.

Badran, A. M. and D. E. Jones. 1965. Polyethylene glycols-tannins interaction in extracting enzymes. Nature 206:622-624.

Benjamin, N. D. and M. W. Montgomery. 1972. Purification and characterization of polyphenol oxidases of Royal Anne cherries. (Prunus avium L.). Ph. D. thesis. Corvallis, Oregon State University. 93 numb. leaves.

Benjamin, N. D. and M. W. Montgomery. 1973. Polyphenol oxidases of Royal Anne cherries purification and characterization. Journal of Food Science 38:799-806.

Betrosian, K., M. P. Steinburg and A. I. Nelson. 1960. Effect of borates and other inhibitors on enzymic browning in apple tissue. II. Mechanism. Food Technology 14:480-483.

Bouchilloux, S. and A. Mayrargue-Kodja. 1960. Combination des thiols avec les quinone se formant au cours de la melanogenese. Bull. Soc. Chim. Biol. 42:1045-1048.

Burroughs, L. 1974. Browning control in fruit juices and wines. Chemistry and Industry 21:718-720.

Chan, H. T. and H. Y. Yang. 1971. Identification and characterization of some oxidizing enzymes of the McFarlin cranberry. Journal of Food Science 35:169-173.

√ Chung, J. I. and B. S. Luh. 1972. Brown discoloration in canned peaches in relation to polyphenoloxidase activity and anthocyanin. Confructa 17(1):8-15.

Coggen, P., G. A. Moss and G. W. Sanderson. 1973. Tea catechol oxidase isolation, purification and kinetic characterization. Phytochemistry 12:1947-1955.

Constantinides, S. M. and C. L. Bedford. 1967. Multiple forms of polyphenol oxidase. Journal of Food Science 32:446-450.

Davis, B. J. 1964. Disc electrophoresis II. Method and application to human serum proteins. Annals of the New York Academy of Science. 121:404-427.

Dilley, D. R. 1966. Purification and properties of apple fruit malic enzyme. Plant Physiology 41:214-220.

Dimick, K. P., J. O. Ponting and B. Makower. 1951. Heat inactivation of polyphenol oxidase in fruit purees. Food Technology 6: 237-241.

Duckworth, H. W. and J. E. Coleman. 1970. Kinetic properties of Mushroom tyrosinase. Journal of Biological Chemistry 245: 1613-1625.

El-Sayed, A. S. and B. S. Luh. 1965. Polyphenol compounds in canned aprictos. Journal of Food Science 30:1016-1018.

Embs, R. J. and P. Markakis. 1965. The mechanism of sulfite inhibitor of browning caused by polyphenoloxidase. Journal of Food Science 30(5):753-758.

Gustavson, K. H. 1963. The linkage of vegetable tannins to the -CON- group of polyvinylpyrrolidone. Leder 14:27-34. (Chem. Abstract 59, 1855g).

Henze, R. E. 1956. Inhibitor of enzymatic browning of chlorogenic solution with cysteine and glutathione. Science 123:1174-1175.

Hulme, A. C. 1957. Some aspects of the biochemistry of apple and pear fruits. Advances in Food Research 8:297-395.

Jankow, C. I. 1963. Thermal inactivation of oxidase in fruits and vegetables. Lebensm-Ind. 10:23-27. (Chem. Abstract 63, 7578a).

Jen, J. J. and K. R. Kahler. 1974. Characterization of polyphenol oxidase in peaches grown in Southeast. HortScience 9(6):590-591.

Jones, J. O., A. C. Hulme and L. S. C. Wooltorton. 1965. The use of polyvinylpyrrolidone in the isolation of enzymes from apple fruits. Phytochemistry 4:659-676.

√ Joslyn, M. A. and J. D. Ponting. 1951. Enzyme-catalyzed oxidative browning of fruit products. Advances in Food Research 3:1-44.

Kahn, Varda. 1975. Polyphenol oxidase activity and browning of three avocado varieties. Journal of the Science of Food and Agriculture 26:1319-1324.

Kahn, Varda. 1976. Polyphenol oxidase isoenzymes in avocado. Phytochemistry 15:267-272.

Kalckar, H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. Journal of Biochemistry 167:461-462.

Kubowitz, F. 1938. Cleavage and resynthesis of polyphenol oxidase and of hemocyanin. Biochemistry Z. 299:32-35.

Loomis, W. D. and J. Battaile. 1966. Plant phenolic compounds and the isolations of plant enzymes. Phytochemistry 5:423-438.

Loomis, W. D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods in Enzymology 31:528-544.

√ Luh, B. S. and B. Phithakpol. 1972. Characteristic of polyphenol oxidase related to browning in cling peaches. Journal of Food Science 37:264-268.

Maruyama, E., M. Fujita and T. Kajita. 1975. The browning of Food. III. Polyphenol oxidase of garland chrysanthemum. Kaseigaku Zasshi 26(5):346-350.

Mayer, A. M., E. Harel and R. Ben-Shaul. 1966. Assay of catechol oxidase and critical comparison of methods. Phytochemistry 5: 783-789.

Mellenthin, W. M. and C. Y. Wang, 1974. Friction discoloration of d'Anjou pears in relation to fruit size, maturity, storage and polyphenol oxidase activities. HortScience 9(6):592-593.

Montgomery, M. W. and V. C. Sgarbieri. 1975. Isozymes of banana polyphenol oxidase. Phytochemistry 14:1245-1249.

⩗ Muneta, P. and J. Walradt. 1968. Cysteine inhibition of enzymatic blackening with polyphenol oxidase from potatoes. Journal of Food Science 33:606-608.

Nelson, J. M. and C. R. Dawson. 1944. Tyrosinase. Advances in Enzymology. 4:99-152.

Palmer, J. K. and J. B. Roberts. 1967. Inhibition of banana by 2-mercaptobenzothiozole. Science 157:200-202.

Palmer, J. K. 1963, Banana polyphenol oxidase preparation and properties. Plant Physiology 38:508-513.

Pierpoint, W. S. 1966. The enzymatic oxidation of chlorogenic acid and some reactions of the quinone produced. Biochemistry Journal 98:567-580.

Ponting, J, D. and M.A.Joslyn.1948. Ascorbic acid oxidation and browning in apple tissue extracts. Arch. Biochemistry. 19:47-63.

Ranadive, A. S. and N. F. Haard. 1971. Changes in polyphenolics on ripening of selected pear varieties. Journal of Science of Food and Agriculture 22:86-89.

Reyes, P. and B. S. Luh. 1960. Characteristics of browning enzymes in Fay Elberta freestone peaches. Food Technology 14:570-575.

Rivas, N. J. and J. R. Whitaker. 1973. Purification and some properties of two polyphenol oxidases from Bartlett pears. Plant Physiology 52:501-507.

Samish, R. and W V. Cruess. 1934. Enzymatic darkening in apricots. Proceedings of the American Society for Horticultural Science. 31:28-31.

Shannon, C. T. and D. E. Pratt. 1967. Apple polyphenol oxidase activity in relation to various phenolic compounds. Journal of Food Science 32:479-483.

Siegelman, H. W. 1955. Detection and identification of polyphenol oxidase substrates in apple and pear skin. Archives of Biochemistry and Biophysics 56:97-102.

Sioud, F. B. and B. S. Luh. 1966. Polyphenolic compounds in pear purees. Food Technology 20:534-538.

Soler, A., F. S. Garcia and J. A. Lozano. 1965. Effect of pH, time and temperature on phenolase from apricots. Rev. Agroquim Technol Alimentos 5(3):353-358. (Chem. Abstract 64, 11488a).

Stelzig, D. A., S. Akhtar and S. Ribiero. 1972. Catechol oxidase of Red Delicious apple peel. Phytochemistry 11:535-539.

Sullivan, D. P. 1976. Some visceral proteases of albacore tuna fish (Thunnus Alalunga). M.S. thesis. Corvallis, Oregon State University. 56 numb. leaves.

Tate, J. N., B. S. Luh and G. K. York. 1964. Polyphenol oxidase in Bartlett pears. Journal of Food Science 29:829-836.

Walker, J. R. L. 1964a. Enzymic browning of apples. II. Properties of apply polyphenolases. Australian Journal of Biological Science 17:360-364.

Walker, J. R. L. 1964b. The polyphenoloxidase of pear fruit. Australian Journal of Biological Science 17:575-577.

Walker, J. R. L. and C. E. S. Reddish. 1964. The use of cysteine to prevent browning in apple products. Journal of Science of Food and Agriculture 15:902-904.

Walker, J. R. L. and A. C. Hulme. 1965. Inhibition of the phenolases from apple peel by polyvinylpyrrolidone. Phytochemistry 4:677-685.

Walker, J. R. L. 1975. Enzymic browning in foods: a review. Enzyme Technology Digest 4(3):89-100.

Walker, J. R. L. and Wilson. 1975. Studies on the enzymic browning of apples; inhibition of apple $\underline{o}$-diphenol oxidase by phenolic acids. Journal of Science of Food and Agriculture 26:1825-1831.

Wang, C. Y. and W. M. Mellenthin. 1973. Relationship of friction to phenolic compounds in d Anjou pears. HortScience 8(4): 321-323.

Wang, C. Y. and W. M. Mellenthin. 1974. Inhibition of friction discoloration on d'Anjou pears by 2-mercaptobenzothiozole. HortScience 9(3):196.

Warburg, O. and W. Christian. 1941. Isolierung und kristulisation des garungsferments enolase. Biochem. Z. 320:384-421.

Wong, T. C., B. S. Luh and J. R. Whitaker. 1971. Isolation and characterization of polyphenol oxidase isozymes of clingstone peach. Plant Physiology 48:19-23.

Yankov, S. I. 1963. Heat inactivation of oxidizing enzymes of some fruits and vegetables. Izv. Vyssh Uch. Zaved Pishch Tekhnol. (Chem. Abstr. 59, 7851g.)