AN ABSTRACT OF THE THESIS OF

NAMROD DAVID BENJAMIN (Name) for the DOCTOR OF PHILOSOPHY (Degree)

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Title: PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASES OF ROYAL ANNE CHERRIES (PRUNUS AVIUM L.)

Abstract approved: Morris W. Montgomery

The polyphenol oxidase (PPO) system of Royal Anne cherries was fractionated and the properties of these fractions were investigated. Optimum conditions for the extraction of PPO were investigated using buffer, polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), acetone powder, Dowex 1-X8 and protamine sulfate. The extraction procedure, which resulted in the highest purification, involved the mixing of 4 g of freeze-dried cherry powder with 6 ml of 20% aqueous PEG followed by the addition of 9 ml of cold acetone. The precipitate obtained from centrifugation for 5 min at 31,000 G (4°C) was washed with 6 ml of acetone and extracted with 100 ml of 0.05 M acetate, pH 5.6, for 12 min. After filtering through nylon cloth, the extract was centrifuged at 27,000 G for 10 min (4°C). The supernatant was designated as the PEG-acetone extract. This procedure was reproducible and decreased the phenolic content of the enzyme extract. The

specific activity of the enzyme was further increased by acetone precipitation and dialysis against 1 mM phosphate buffer, pH 6.2.

To study PPO in greater detail, the acetone precipitate of PEG-acetone extract was separated into three fractions (S1, S2, S3) by column chromatography with Sephadex G-100. The profile of cherry PPO on Sephadex G-100 suggested that it was composed of more than one enzyme of different molecular size. Separation of the acetone precipitate on diethylaminoethyl (DEAE)-cellulose with potassium phosphate concave gradient at pH 6.2 resulted in two fractions (DE1 and DE2) indicating that cherry PPO was composed of enzymes which have different surface charge densities. Fractions DE1 and DE2 were 200 and 180-fold, respectively, purer than the PEG-acetone extract. Polyacrylamide-gel electrophoresis of the individual fraction tubes from column chromatography demonstrated the presence of three PPO groups consisting of 11 bands. These groups were the same as those observed in the PEG-acetone extract. S1 and S2 consisted of the slow and intermediate moving groups while S3 contained the fast moving group. DE1 was composed of the first two groups, while DE2 contained the fast moving group. These bands suggest that Royal Anne cherry PPO was composed of isozymes. Treatment of acetone precipitate with sodium dioctylsulfosuccinate and $CaCl_2$ did not change the electrophoretic pattern. The pH optima for these fractions ranged from 7.3 to 7.8, while the acetone precipitate had

pH 7.0. Heat inactivation of Royal Anne cherry PPO follows first order kinetics. The half lives of DE1, DE2, and acetone precipitate were 1.9, 2.7 and 8.0 min, respectively. However, at room temperature acetone precipitate had a half life of four and one-half days. Michaelis constants (K_m) were 43, 53, and 46 mM catechol for DE1, DE2, and acetone precipitate, respectively. V_{max} was 1000 units/ml for both DE1 and DE2 and 760 units/ml for the acetone precipitate.

Six o-diphenols and three monophenols and various concentrations of inhibitors were used to characterize the fractions obtained from DEAE-cellulose chromatography. Differences in substrate specificity with pyrogallol and 4-methyl catechol were shown between DE1 and DE2. In other respects, these fractions were similar in substrate specificity in that they oxidized only o-diphenols and not monophenols. On polyacrylamide-gel electrophoresis, the three groups were active toward o-diphenols and p-cresol. It was concluded that this difference might be attributed to chromagen p-phenylenediamine which may act as an activator for the oxidation of p-cresol. Group A was specific for pyrogallol and group C for 4-methyl catechol. Inhibitor studies indicated that all three groups were inhibited by diethyldithiocarbamate (DEDTC), $K_2S_2O_3$, dithiothreitol and KCN. *In vivo* studies demonstrated that dipping bruised cherries in oleic acid followed by treatment with DEDTC inhibited subsequent browning.

This finding suggests the possible commercial use of DEDTC in production of brined cherries.

Purification and Characterization of Polyphenol Oxidases of Royal Anne Cherries (*Prunus avium* L.)

by

Namrod David Benjamin

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Dean of Graduate School

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PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASES OF ROYAL ANNE CHERRIES (*PRUNUS AVIUM* L.)

INTRODUCTION

Maraschino cherry production is an important industry in the United States. According to the National Cherry Growers (Anonymous, 1970), United States is listed as the second largest sweet cherry producer, after West Germany. Of the annual production of 124, 700 short tons, 45% is brined in sulfur dioxide-hydrated lime solution, 15% canned, 38% sold fresh and 2% sold frozen (Anonymous, 1970). In 1970, Oregon was the leading sweet cherry producing state in the nation followed by California, Washington and Michigan, respectively (Anonymous, 1970).

Since labor costs make up about three-fourths of the production costs of maraschino cherries, cherry growers are beginning to mechanize the harvesting of cherries (Norton, 1962). During mechanical harvesting, the tree is mechanically shaken causing the fruit to strike other fruit, branches and the collection canvas. This causes physiological injury to the cherry, which results in a rapid change in color in the injured area by the formation of dark brown or reddish pigments. Following mechanical harvesting, color changes could amount to 20% or more of the cherry crop. This results in a reduction of the grade of the cherries and a decrease in the economic return to the cherry grower. Therefore, the major problem in the

production of high quality maraschino cherries is the removal or prevention of the brown spots which impart undesirable appearance to the final product.

This browning has been attributed to the action of an enzyme called polyphenol oxidase (EC 1.10.3.1) (International Union of Biochemistry, 1964) on phenolic substrates to form quinones. These quinones are further oxidized to dark brown pigment generally known as melanin.

The mechanism of this enzyme in the Royal Anne cherry is not well understood and the only acceptable means to prevent browning in cherries is to use a high concentration of sodium chloride (Sioud and Luh, 1966), sucrose (Schwimmer, 1969), or sulfur dioxide (Underkofler, 1968).

To assist with these problems, the present work was undertaken to:

1) determine the optimum condition for the extraction and assay of the enzyme;
2) purify the enzyme using fractionation, precipitation, column chromatography and electrophoresis;
3) study the physical and chemical properties of the enzyme catalyzing the browning reaction;
4) characterize the purified fractions according to their pH optimum, heat inactivation, substrate and inhibitor

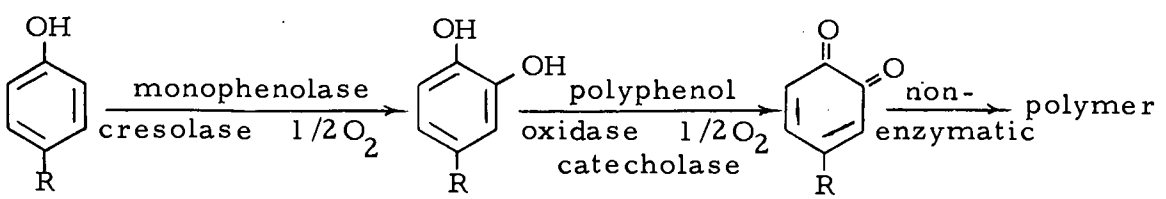
specificities and kinetic properties;

5) apply the information to inhibition of browning of mechanically harvested cherries.

REVIEW OF LITERATURE

Enzymic oxidation of mono- and di-phenolic compounds has been known to biochemists since the report of Bertrand (1894). Polyphenol oxidase was classified as an o-diphenol:O_2-oxidoreductase, EC 1.10.3.1 and p-diphenol oxidase as p-diphenol:O_2-oxidoreductase, EC 1.10.3.2 by the Commission on Enzymes of the International Union of Biochemistry (1965). Hayaishi (1962) and Hayaishi and Nozaki (1969) classified the di-phenolase as oxidases, and the mono-phenolase, or cresolase, as oxygenases. These enzymes are also known under several trivial names such as phenolase, phenol oxidase, catechol oxidase, tyrosinase, and cresolase. In this report these enzymes will be referred to as polyphenol oxidases (PPO).

Color changes caused by physiological injury to fruit have been attributed to the action of PPO on phenolic substrates. The mechanism of this enzyme in cherries is not well understood. Corse (1964) defined enzymatic browning as the reaction of an enzyme (or multiple enzymes) that oxidizes catechol derivatives to o-quinones. Frequently, the catechol derivatives are formed enzymatically from monophenols by hydroxylation to give o-diphenols. The nature of substituent groups



on the benzene ring influences the reactivity.

Three components are required for the browning reaction, the enzyme, the phenolic substrate, and molecular oxygen. According to Corse (1964), it is possible that the enzyme combines with oxygen and this complex combines with phenolic substrates.

Effect of pH and Temperature on PPO Activity

El-Tabey and Cruess (1949) reported an optimum pH of 6.5 to 7.0 for the PPO of apricot with catechol as the substrate. Apple PPO was found to have two pH optima, 5.2 and 7.3, with the activity at pH 5.2 several times greater than that at pH 7.3 (Shannon and Pratt, 1967). An optimum pH of 7.0 was observed for eggplant PPO (Rhoades and Chen, 1968), McFarlin cranberry PPO (Chan and Yang, 1971) and bean leaf PPO (Racusen, 1970). In a more recent report, Wong et al. (1971) separated the PPO of peaches into four components having pH optima of 6.8, 6.5, 7.2 and 7.0. Brown and Ward (1958) showed pH 6.8 as the pH optimum for mouse tyrosinase. From these data, the pH optima of various PPO systems appear to be near neutrality.

Bodine et al. (1944) reported the temperature range from 55° to 70°C produces maximum activity of tyrosinase from the egg of grasshoppers while exposures to higher temperatures resulted in inactivation. In an experiment conducted to determine the heat of inactivation of PPO in fruit purees, Dimick et al. (1951) measured inactivation as

a function of time, temperature, and pH. The rate of inactivation at a constant temperature was found to be different for each fruit and to follow first order kinetics. Heat inactivation of cranberry PPO was also found to follow first order kinetics and the activation energy for the inactivation of this enzyme was observed to be 27.7 Kcal/mole (Chan and Yang, 1971). Wong et al. (1971) reported different heat stabilities for each of the four components of peach PPO.

Substrate Specificity of PPO

Phenolic acids, the substrate necessary for browning, are of widespread occurrence in plants and most of them are phytotoxic (Pridham, 1960), but probably harmless to the plant because, in the natural state, they are glycosides or esters. The enzyme, or enzymes, which catalyze the oxidation of these phenolic acids vary from fruit to fruit and their substrate specificity also varies.

Onslow (1920) showed that peaches contain both oxidizing enzymes and certain phenolic compounds. Later, Reyes and Luh (1960) found the PPO extracted from peach was highly active toward catechol. Samisch and Cruess (1934) tested the catalytic activity of apricot extract on various phenolic substrates and found that the extract was able to catalyze only the oxidation of catechol and pyrogallol but not tyrosine, resorcinol, quinol, or phloroglucinol. These authors concluded that this PPO was specific for o-diphenol and did

not oxidize monophenols. Activity of PPO from apples was studied toward catechol, pyrogallol, resorcinol, p-cresol, phenol, and quaiacol (Ponting and Joslyn, 1948). This enzyme showed maximum activity for catechol and did not oxidize either hydroquinone or tyrosine. In contrast to this, Durkee and Poapst (1965) found that most of the phenolic acids present in core tissues and ripe seeds of McIntosh apples were potential substrates for crude apple PPO. Using other phenolic compounds, Shannon and Pratt (1967) found that esculetin and dihydroquercetin were substrates for apple PPO. Quercetin, quercitrin, rutin, cyanidin chloride, phloroglucinol and resorcinol were neither substrates nor inhibitors. On the other hand, phloroglucinol and resorcinol showed a synergistic action toward apple PPO, which could have been due to their nucleophilic properties. These authors hypothesized that the conjugated system in flavones prevents these compounds from being apple PPO substrates (Shannon and Pratt, 1967).

Griffiths (1959) pointed out that the browning of bananas is an enzymatic oxidation of dopa. This enzyme system was studied more extensively by Palmer (1963), who found that the enzyme catalyzed the oxidation of a variety of o-diphenols with maximum activity toward dopamine. Monophenols such as p-cresol, o-cresol, tyrosine, and tyramine were not oxidized by this enzyme.

Alberghina (1964) reported a PPO purified from potato tuber

slices which differed from those previously described. This enzyme was highly specific for chlorogenic acid and 4-methyl catechol with less activity toward dopa and catechol. Alberghina (1964) also found that the activity of the enzyme in the crude extract toward catechol was similar to the activity with chlorogenic acid, while in the purified preparation, the activity shifted in favor of chlorogenic acid and was inhibited by a high concentration of catechol.

Knapp (1965) observed that avocado PPO rapidly oxidized catechol, catechin and nordihydroguaiaretic acid. By using thin-layer gel filtration, Dizik and Knapp (1970) were able to separate avocado PPO into five fractions. Electrophoresis was applied to the most active fraction and revealed four to five isozymes with different specificities toward the substrates. They concluded that the activity of one of the isozymes was responsible for the activity of the crude and purified extract, since it was most active toward all the substrates used and developed a color much sooner than other PPO components.

An extract from eggplant was able to catalyze the oxidation of diphenol substrates (catechol, dopamine, caffeic acid) with higher activity toward dopamine and no activity toward monophenols (Rhoades and Chen, 1968).

Racusen (1970) found bean leaf PPO to be specific for polyphenols such as pyrogallol, caffeic acid and dopa, but did not show activity toward hydroquinone and tyrosine. Based on these findings, it seems

likely that the natural substrates for the PPO of broad bean would be the caffeic acid and flavanoid derivatives rather than dopa from tyrosine. In contrast to this, a preparation of PPO from leaves of spinach beet was shown to catalyze the hydroxylation of p-coumaric acid after a lag period (Vaughan and Butt, 1970).

Wong et al. (1971) investigated various phenolic compounds as possible substrates of clingstone peach isozymes. All of the isozymes had activity toward o-diphenols with higher specific activity for catechol and o-catechin. Monophenols and m-diphenols were not oxidized by these isozymes.

Brown and Ward (1958) prepared mammalian tyrosinase from Harding-Passey mouse. Three active components were isolated by chromatography on DEAE-cellulose. All of the isolated components were active toward tyrosine and dopa, with four times more activity with dopa. Their experimental results suggest that the same enzyme may be responsible for the oxidation of both diphenols and monophenols.

Mechanism of PPO Oxidation

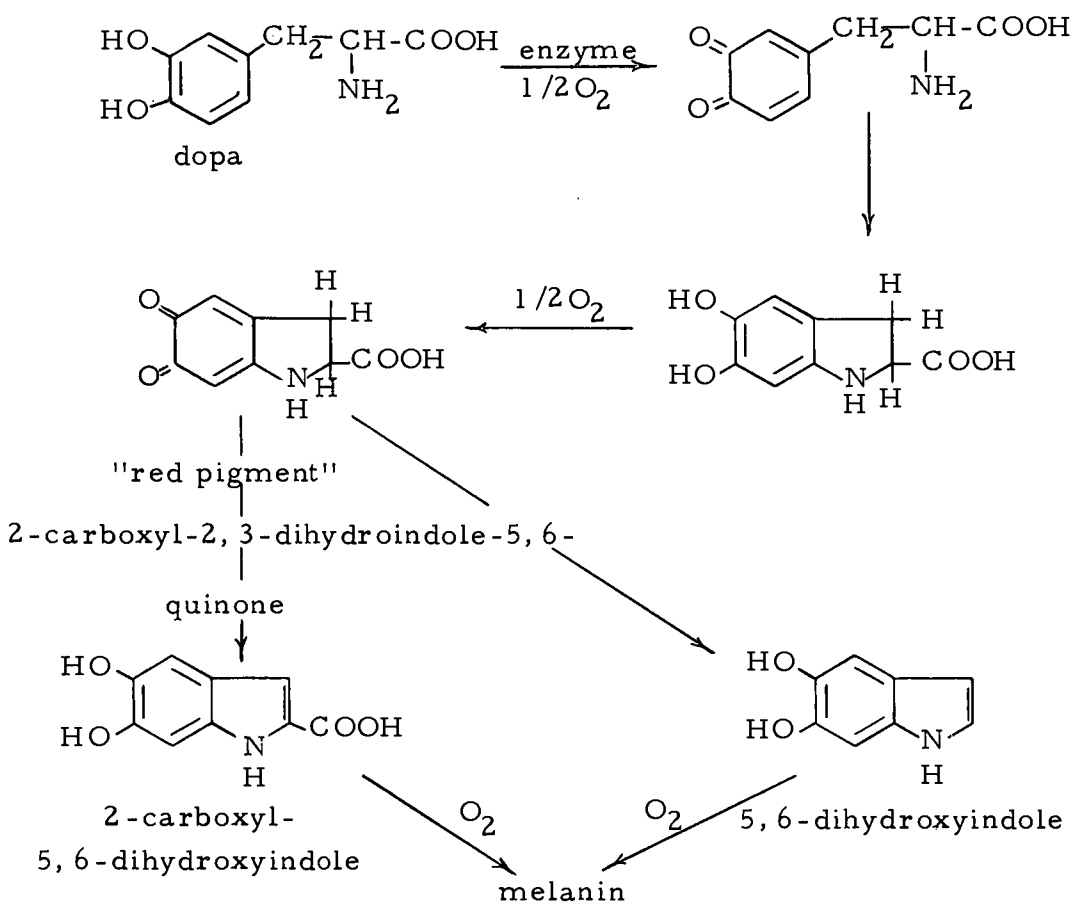
Monophenolase activity may be due to oxygen transfer, while diphenolase activity may be due to oxygen transfer, one electron transfer, or two electron transfer (Hayaishi and Nozaki, 1969). The mode of connection of these activities is a matter of controversy.

Several proposed mechanisms related to these activities, such as hydroxylation of monophenol, could take place nonenzymatically due to the presence of highly reactive o-quinone which is formed by the enzymatic oxidation of o-diphenol (Kertesz and Zito, 1962). In contrast to this Nelson and Dawson (1944), Mason (1966), and Long and Alben (1968) concluded that during the induction period there is a slow enzymatic conversion of monophenol to o-quinone and a slow nonenzymatic reduction of the o-quinone to an o-diphenol until the o-diphenol concentration is sufficient to allow rapid oxidation of the monophenol. Recently, Vaughan and Butt (1970) suggested that the presence of small amounts of o-dihydric phenol interact with enzymic copper to produce a reactive species toward hydroxylation of monophenols. Also there could be two active sites in the enzyme, one for cuprous and one for cupric and each could function separately (Dressler and Dawson, 1960).

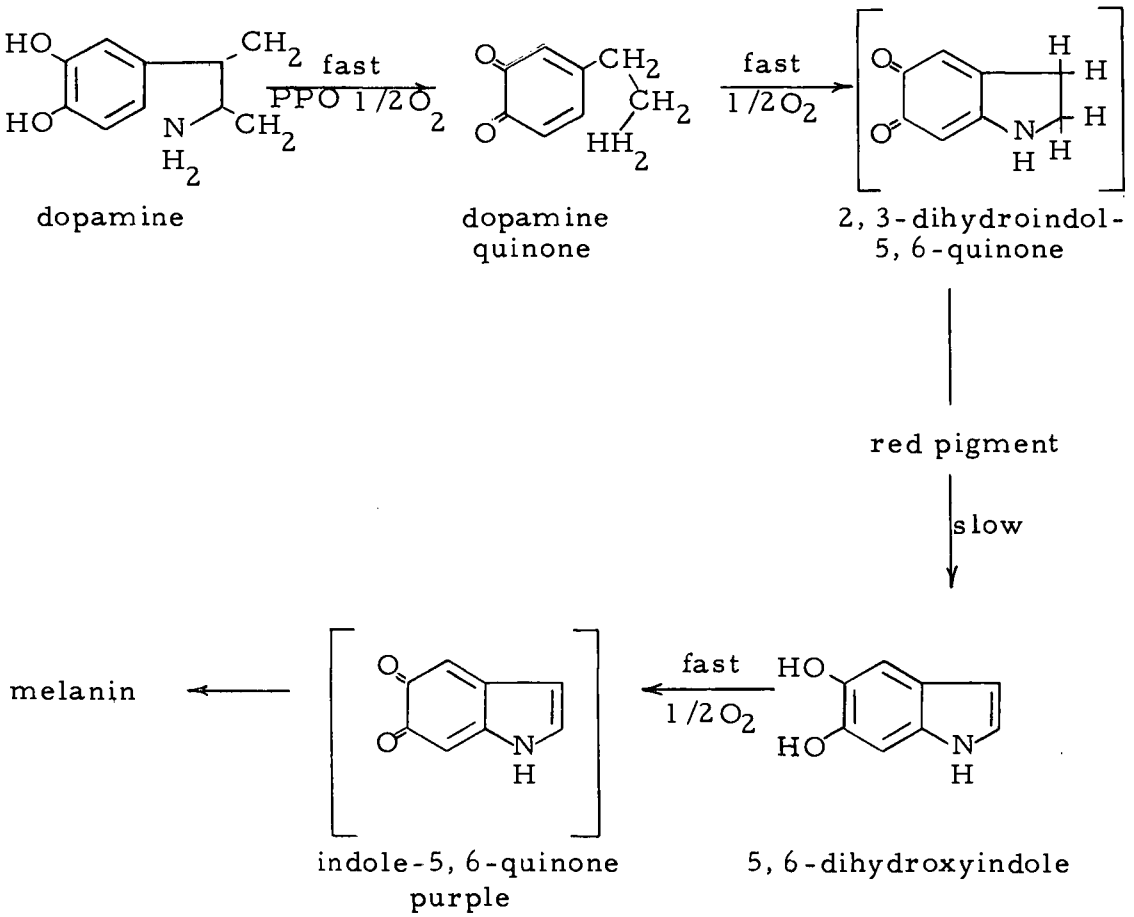
There are two distinct sites of activity on the molecule of mammalian tyrosinase (Shimao, 1962), one of which is responsible for the oxidation of dopa and another where tyrosine is bound but not oxidized during the first phase of the reaction (Shimao, 1962).

More recent findings propose that PPO is a mixture of two phenolases, one for catalyzing the oxidation of o-diphenol and the other catalyzing the hydroxylation of monophenols (Macrae and Duggleby, 1968).

Two other mechanisms have been proposed for the enzymatic oxidation of diphenols. Raper (1938) proposed the following mechanism for the enzymic conversion of 3, 4-dihydroxyphenylalanine (dopa) to synthetic dopa melanin:



Based on spectrochemical evidence, Palmer (1963) proposed the following reaction mechanism for the oxidation of dopamine by banana PPO:



A number of attempts have been made to implicate these enzymes in respiratory metabolism, by postulating physiologically significant transfer of electrons from reduced coenzymes to quinones (Wosilait and Nason, 1954). In 1937 an experiment was conducted in vitro to construct a system showing the ability of this enzyme to transfer

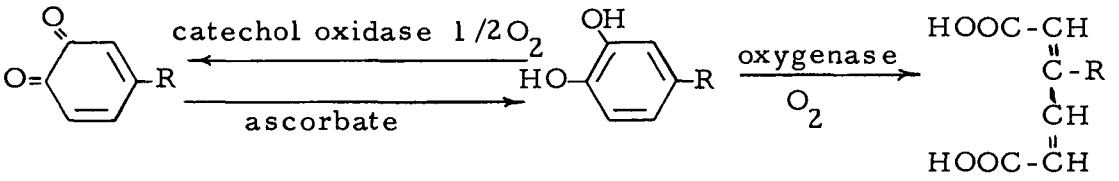
electrons from $NADPH_2$ to oxygen by Kubowitz (1937), but no evidence was found that it could function in the intact cell. Alberghina and Servettaz (1962) showed that such a system did exist when they found the presence of NADP-chlorogenic acid oxidoreductase and chlorogenic acid oxidase in potato tuber tissue.

Inhibitor Specificity

Enzymatic browning occurs when three components, enzyme, substrate, and oxygen, are brought together. Preventing any one of these from reacting will prevent browning. Natural control exists in many fruits. For instance, in Sunbeam peach the enzyme is present but no substrate. On the other hand, most berries and citrus fruits do not have the enzyme but contain the substrate (Underkofler, 1968). Another means of controlling enzymatic browning is the blanching process but this is not applicable to many fruits due to the adverse effect of heat on their texture.

Three different methods of modifying catechol oxidase substrates and preventing browning have been suggested. Finkle and Nelson (1963) showed o-methylation of catechol compounds by o-methyl transferase in apple juice and suggested the dipping of cut fruits into an alkaline buffer solution, which may accomplish the o-methylation reaction (Nelson and Finkle, 1964). Kelly and Finkle (1969) showed that catechol oxidase substrates in apple juice were

modified by the action of ring cleaving oxygenases (protocatechuate-3, 4-dioxygenase) in the presence of a reducing agent. Based on alternative enzymic oxidation paths of catechol substrates, Kelly and Finkle (1969) proposed the following mechanism:



According to Bedrosian et al. (1960) and Racusen (1970), borate was found to form a borate-o-diphenol complex with catechol. This modified substrate would be acted upon enzymatically at a much slower rate. Inhibition of bean leaf PPO by borate was the reversible type being reversed by mannitol. Mannitol forms a complex with the borate releasing the o-diphenol for enzymic oxidation (Racusen, 1970). Lee and Aronoff (1967) demonstrated the borate inhibition of an enzyme important in the conversion of carbohydrate to phenols in plants. In this connection, it is interesting to recall that a major symptom of borate deficiency in plants is the massive accumulation of phenolic compounds (Reed, 1947).

The initial effect of ascorbic acid on the reaction between mushroom PPO and tyrosine was found by Krueger (1950) to be that of pro-oxidant and later as an anti-oxidant. Kertesz (1952) showed that the oxidation rate of PPO in the presence of ascorbic acid was dependent

on the concentration of the cupric ion and suggested that ascorbic acid affects the specific structure of the enzyme in which copper was involved. The pro-oxidant effect of ascorbic acid could be due to the oxidation of ascorbic acid by ionic copper. Ascorbic acid also had a denaturing effect on the enzyme and the presence of copper ions resulted in the formation of colored condensation products (Baruah and Swain, 1953). Reyes and Luh (1962) found that both ascorbic acid and isoascorbic acid were equally effective in inhibiting the darkening of catechol by PPO from Freestone peaches and were unable to decolorize the polymerized product. The addition of ascorbic acid to the growth medium prolonged the lag period of p-diphenol oxidase extracted from the culture of Polyporus versicolor (Bocks, 1967). He also found that the addition of copper sulfate increased the activity of p-diphenol oxidase.

PPO was not inhibited by sulfhydryl reagents (Barron and Singer, 1945). Bolin et al. (1964) tried to preserve fresh peeled apples by adding $NaHSO_3$, which inhibited the browning. Embs (1969) showed that bisulfite does not react with the enzyme itself, but forms a complex with o-quinone which results in a colorless product. In the inhibition studies conducted by Wong et al. (1971) on the peach isozymes, $NaHSO_3$, l-ascorbic acid, and glutathione delayed the increase in absorbance at 420 nm due to the reduction of o-benzoquinone back to catechol. The induction period varied with the concentration of

reducing agent, and three isozymes were inhibited by these reducing agents but one was not. Phloroglucinol was a competitive inhibitor of peach PPO (Reyes and Luh, 1960). Shannon and Pratt (1967) showed that ferulic acid, fisetin, rhamnetin, and p-coumaric acid inhibited apple PPO.

Copper has been reported to be an essential part of PPO prepared from various plants and animals. It was also found to be essential for the enzymatic activity of mammalian PPO (Lerner et al., 1950). Inhibition of this enzyme by the thio compounds in some instances was reversed by the addition of cupric ion. Swain et al. (1966) showed that metal chelator, diethyldithiocarbamate (DEDTC) forms a complex with the copper of the PPO and inhibits the enzyme.

Partially purified PPO from potato and two kinds of French grape were found to degrade pure anthocyanin in vitro, and this degradation was inhibited by DEDTC, KCN, and thio urea (Segal and Segal, 1969). Prabhakaran et al. (1969) reported that DEDTC penetrates the bacillus and completely suppressed the activity of PPO of Mycobacterium leprae. Prabhakaran et al. (1969) also found that human leprosy bacilli possess a PPO property and DEDTC inhibits this enzyme.

Vaughan and Butt (1970) found that the hydroxylation of p-coumaric acid catalyzed by PPO from the leaves of spinach beet was inhibited by DEDTC. With a low concentration of inhibitor, or with

the addition of o-diphenols before the substrate, hydroxylation recovered after a lag period (Vaughan and Butt, 1970). No reversal of DEDTC inhibition was observed by these authors during catechol oxidation, therefore they indicated competition between a product of hydroxylation (o-diphenols) and DEDTC for the enzymic copper. A similar observation was reported by Mayer and Friend (1960), when a reversible inhibition of p-cresol oxidation by DEDTC occurred with sugar beet chloroplast PPO. However, Pierpoint (1966) suggested a mechanism contrary to the above, in that the o-quinone produced by the oxidation of chlorogenic acid with tobacco PPO reacts with DEDTC. Grncarevic and Hawker (1971) showed that if the grape berry skins were dipped in an oil emulsion, then treated with DEDTC, the browning reaction was inhibited.

Purification of PPO

During extraction of enzymes from plant systems, the phenolic compounds interfere with the isolation and usually result in the formation of melanoprotein, which inhibits many enzymes (Loomis and Battaile, 1966). Since the concentration and type of phenolic compounds vary in plants, any one procedure cannot be expected to be equally effective for the extraction of enzymes from all plants. Loomis and Battaile (1966) found that in the extraction of PPO from plants rich in phenolic compounds, the phenols should be separated

from the proteins first. This prevents oxidation of the phenols, which if not removed would combine reversibly with proteins by hydrogen bonding and irreversibly by oxidation (Loomis and Battaile, 1966). To overcome the difficulties of extracting enzyme from plants, Jones et al. (1965) and Loomis and Battaile (1966) studied the effect of insoluble polyvinylpyrrolidone (PVP) in binding with phenols. This was further investigated by Anderson and Sowers (1968) to determine the optimal conditions for bonding of plant phenols to insoluble PVP in tobacco leaves. Lam and Shaw (1970) compared the effect of insoluble PVP and Dowex 1-X8 anion exchange resin for the removal of phenolics from plant extracts and concluded that Dowex 1-X8 was more effective. In an experiment conducted by Badran and Jones (1965), comparing polyethylene glycol (PEG) with insoluble PVP, PEG was found to be more effective for extracting PPO.

The molecular weight for PPO has been reported by various workers. Dizik and Knapp (1970) found the molecular weight for the PPO fractions from avocado to range from 14,000 to 400,000. Using the sedimentation-diffusion method, Kertesz and Zito (1965) reported a range of 100,000 to 128,000 for mushroom phenolase. By using the light-scattering method, they found a value of 133,000 $\pm$ 10,000 which is in good agreement with that obtained by the sedimentation-diffusion method.

Shimao (1962) used Harding-Passey mouse melanoma to prepare

soluble mammalian PPO and found that almost all of the PPO activity was precipitated with $(NH_4)_2SO_4$ between 0.45 and 0.60 saturation. An attempt to separate the two components of this PPO system by $(NH_4)_2SO_4$ precipitation was not successful.

Using sedimentation techniques, treatment with $(NH_4)_2SO_4$ has been shown to convert a mushroom PPO preparation from one to two components (Kertesz and Zito, 1962). Upon storage in the cold room for two days, the two components were converted to one component. Kertesz and Zito (1962) ascribed this change to a polymerization-depolymerization reaction.

Sephadex G-100 gel filtration has been used by many investigators to purify PPO from various sources. Nakamura et al. (1966), using Sephadex G-100, found that mushrooms harvested in autumn gave one peak of PPO activity while spring harvested mushrooms gave two peaks. Upon storage of these two fractions at $4^{\circ}C$, rechromatography on Sephadex G-100 revealed a different ratio of the components. Nakamura et al. (1966) suggested that this apparent change in molecular weight indicated a possible polymerization process during storage. A similar phenomenon was observed by Khandobina and Geraskina (1969) in an experiment carried out on healthy and infected carrots. They found that infected carrots showed induced synthesis of PPO with a lower molecular weight and separation on Sephadex G-100 revealed two peaks of PPO activity. In healthy carrots, PPO

was eluted earlier and showed only one peak of activity. Gaspar et al. (1969), using Sephadex G-100, was able to detect one peak for PPO from Lens and Pisum roots.

PPO present in apple peel was separated into two fractions by DEAE-cellulose column chromatography (Walker and Hulme, 1966). These two fractions exhibited similar substrate specificities. However, Shannon and Pratt (1967) were able to observe only one fraction of the PPO activity from apple extract using paper electrophoresis. Mushroom PPO was purified and separated by DEAE-cellulose chromatography and starch-gel electrophoresis into high catecholase and high cresolase fractions (Smith and Krueger, 1962). Based on their data, Smith and Krueger (1962) concluded that the high cresolase enzyme was multi-component in nature. These authors suggested that the multi-components were naturally occurring and were not the result of degradation or fragmentation of a single enzyme. Smith and Krueger (1962) also showed that the five components of mushroom PPO activity could be separated from an extract by chromatography on hydroxyapatite columns. One of their active fractions moved as a single substance during starch-gel electrophoresis, but the other fractions were heterogeneous. Pomerantz (1963) separated and partially purified two PPO from hamster melanoma on DEAE-cellulose and starch-gel electrophoresis. The two components had the same pH optima and kinetic properties with several substrates

and inhibitors.

Multiple Forms of PPO

Since the development of polyacrylamide-gel electrophoretic techniques (Raymond and Weintraub, 1959), many enzyme systems have been separated into numerous molecular forms. The term isozyme has been used to describe these different molecular forms. Wilkinson (1966) defined isozymes as "enzymically active proteins, catalyzing the same reaction and occurring in the same species, but differing in certain of their physico-chemical properties." Recently the term conformers has been used by Kaplan (1968) to describe a group of multiple forms of enzymes which have the same amino acid sequence but exist in varying conformations. This difference in conformation may cause differences in exposed charges. Variation in electrophoretic migration or differential behavior toward ion exchange resins could arise from these differences in exposed charges. Genetic and physiological significance of isozymes is not understood for the majority of isozyme systems, although the concept of isozymes has been known to biologists and biochemists for several decades (Wilkinson, 1966). An intensive review of the isozymes of lactic dehydrogenase and malate dehydrogenase has been published by Kaplan (1968).

Several mechanisms have been proposed to account for multiple

molecular forms of PPO. Based on his results, Jolley (1966) suggested that different degrees of polymerization of like subunits or combination of unlike subunits would be one of the mechanisms for the existence of isozymes. On the other hand, a single gene could encode a polypeptide chain, which may degrade into a series of isozymes (Markert, 1968). A further possibility would be the presence of conformational isozymes.

The multiplicity of PPO has been studied by several researchers. Constantinides and Bedford (1967), using polyacrylamide-gel electrophoresis, showed that PPO extracted from mushroom was composed of nine distinct multiple forms. Three of these multiple forms were active toward l-tyrosine, the rest were active toward dl-dopamine. They also found that potatoes contained 11 bands, while apples had only three bands of PPO activity toward dopa. With bean leaf PPO, Racusen (1970) obtained three isozymes with gradient electrophoresis, but only one fraction on DEAE-cellulose chromatography.

Smith and Krueger (1962) found five active components when a crude extract of mushroom PPO was chromatographed on hydroxyapatite. However, Bouchilloux et al. (1963) obtained only four active components. Since the $(NH_4)_2SO_4$ fraction between 66 and 70% saturation was not used in their work, Bouchilloux et al. (1963) suggested that this could account for the difference in the number of

active components. Dissociation of these PPO isozymes into subunits occurred in the presence of sodium dodecylsulfate (SDS). These results confirmed the hypothesis that subunits are involved in the PPO structure (Bouchilloux et al., 1963). Robb et al. (1964) and Swain et al. (1966), using anionic detergent (sodium dioctylsulfosuccinate), found an activation of broad bean leaf PPO after one minute of treatment followed by a loss in activity, which was thought to be due to dissociation into subunits. The activation of the latent PPO by anionic detergent has been related to changes in tertiary structure (Robb et al., 1964).

Jolley (1966) showed that the multiple forms of mushroom PPO appear to consist primarily of simple polymers. In his paper he discussed the conditions under which interconversion of isozymes can take place and also the nature of bonds involved in quaternary structure. At moderate temperatures, ionic strength, and enzyme concentration, the interconversion was slow. Association was favored by high enzyme concentration or treatment with $CaCl_2$, while dissociation was stimulated by high ionic strength, higher temperature ($50^{\circ}C$), SDS and EDTA. Urea (8 M) did not dissociate the enzyme but caused inactivation. In later work, Jolley et al. (1969) pointed out that perhaps divalent cations (Ca^{++} or Mg^{++}) bind to mushroom PPO drawing the subunits together and allowing other attractive forces to take over.

Based on his data, Sussman (1961) concluded that the two PPO

enzymes from Neurospora crassa differed mainly in their secondary and tertiary structures and not in their active center. He used the unfolding agents, urea and formamide, and found that these substances had a great inhibitory effect on PPO. Based on the above reports, it can be concluded that PPO exists in multi-molecular forms, and separation of these forms is possible by different chromatographic techniques.

MATERIALS AND METHODS

Source of Fruit

Royal Anne cherries (Prunus avium L.) were obtained from an orchard in Salem, Oregon on June 30, 1969. About 200 lbs of cherries were hand picked, placed in polyethylene bags, iced, and brought to the pilot plant. Stems and pits were removed before the cherries were sharp frozen at -30°C and freeze-dried. During freeze-drying, the temperature of the cherries did not exceed 32°C to preserve enzymatic activity. Elapsed time between picking and freezing was approximately 2 hr. After flushing with N_2 and under a pressure of 27 inches of Hg, the dried fruit was sealed in No. 1-1/2 cans. The cans were stored at -23°C until used.

Reagents

dl-3, 4-Dihydroxyphenylalanine (dopa), 4-methyl catechol, p-cresol, sodium dioctylsulfosuccinate, and 2, 3-dihydroxynaphthalene were purchased from K & K Laboratories, Inc. Epinephrine, thiourea, sodium diethyldithiocarbamate, dithiothreitol, tyramine, tyrosine, pyrogallol, and chlorogenic acid were purchased from Sigma. Pyrocatechol was obtained from Eastman Organic Chemicals, and p-phenylenediamine from Matheson Coleman & Bell.

PPO Assay Procedure

Enzyme activity was determined by measuring the increase in absorbance at 400 nm with a Beckman DB recording spectrophotometer with a chart speed of 1 in/min. For the assay, the reference cuvette contained 2 ml 0.2 M phosphate buffer, pH 7.0, and 1 ml 10 mM catechol and the sample cuvette contained 1 ml 0.2 M phosphate buffer and 1 ml 10 mM catechol. The reaction was started by adding 1 ml of enzyme solution to the sample cuvette. Mixing in the cuvette was accomplished with a plastic perforated plunger to allow air to be incorporated evenly within the mixture. The temperature of the reaction was 30°C (Vaughan and Butt, 1970; Chan and Yang, 1971; Wong et al., 1971) and readings were recorded immediately after mixing. Under these conditions linearity was maintained with several different substrates for 2 min. One unit of enzyme activity was defined as the amount of enzyme that causes a change in absorbance of 0.001 per min. Specific activity was expressed as units of enzyme activity/mg of protein.

Protein Estimation

The spectrophotometric method of Warburg and Christian (1941) and Kalcker (1947) was used in the determination of protein concentration. The formula used was:

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

Analysis of Total Phenolic Content

The Folin-Denis colorimetric method described by A. O. A. C. (1960) was used in evaluating the different extraction methods on the basis of their phenolic content. Tannic acid was used to prepare the standard curve. One ml of enzyme solution was pipetted into 75 ml of glass-distilled water in a 100 ml volumetric flask and 5 ml of Folin-Denis reagent and 10 ml of saturated sodium carbonate were added. The volume was made up to 100 ml with distilled water and the solution was allowed to stand for 30 min. A photo Lumerton colorimeter was used to determine the absorbance at 650 nm.

Extraction and Preparation of Enzyme Powder

In preliminary experiments on extraction of Royal Anne cherry PPO using a buffer, a dark brown solution with low activity was obtained. Therefore, an attempt was made using different methods of extraction with different binding agents to obtain a crude extract of higher activity. Evaluation of each method was based on: protein, E_{280}/E_{260}, tannins, and PPO activity. All purification steps were carried out in a cold room at $5^{o}C$ and all reagents were prepared using glass-distilled water unless otherwise specified.

Crude Powder

Freeze-dried cherries were ground in a stainless steel Waring Blendor for 2 min with liquid nitrogen, placed in a brown bottle, sealed, covered with aluminum foil and placed in a desiccator containing calcium chloride under vacuum. The desiccator was stored in the cold room at 5°C.

Buffer Extract

Four g of the crude powder were mixed with 100 ml of 0.05 M acetate buffer, pH 5.6, and gently stirred in an ice bath for 12 min, squeezed through nylon cloth, and centrifuged at 27,000 G for 10 min at 4°C. The supernatant solution constituted the buffer extract which was used for evaluation.

Acetone Powder Extract

Ten g of crude powder were blended with 200 ml of cold acetone (-23°C) in a stainless steel Waring Blendor for 1 min. The slurry was filtered under suction in a Buchner funnel through Whatman No. 1 filter paper. The wet cake was washed three times with 200 ml acetone and air dried after the last washing. The powder was then stored in a desiccator under vacuum at 4°C. The enzyme extract was prepared from the powder as previously described for the buffer extract.

Insoluble Polyvinylpyrrolidone (PVP) Extract

This method was developed and modified by Loomis and Battaile (1966). Polyclar AT (insoluble PVP) was purified by boiling 10 min in 10% HCl and washed with glass-distilled water until free of Cl^-. A thin paste was prepared by mixing 5 g of treated PVP with 100 ml of 0.2 M phosphate buffer, pH 7.0, with 0.25 M sodium ascorbate. The thin paste was chilled in an ice bath and allowed to stand for 24 hr. Various levels of PVP were used to determine the most efficient level required to remove most of the phenolic compounds without inhibiting the enzyme.

Acetone-PVP Extract

Five g of crude powder were blended with 100 ml of cold acetone ($-23^{\circ}C$) and 1 g of PVP in a Waring Blendor for 1 min. After filtering with a Buchner funnel under suction, the residue was washed twice with acetone. The enzyme extract was prepared from the powder as previously described for the buffer extract.

Anion Exchange Resin Extract

Lam and Shaw (1970) used Dowex 1-X8 to remove polyphenolic compounds from flax straw. Therefore, an experiment was conducted to determine if the use of this anion exchange resin would be beneficial

in the extraction of PPO from cherries. Dowex 1-X8 chloride resin (400 mesh) was washed several times with glass-distilled water and equilibrated with buffer overnight. The buffer was decanted and fresh buffer was added to give approximately a 10% (w/v) solution. Four g of crude powder were added to 100 ml of 10% ion exchanger to give approximately a 4% (w/v) mixture. The mixture was stirred for 2 min, squeezed through nylon cloth and centrifuged in the cold room at 27,000 G for 10 min. The supernatant constituted the resin extract which was used for the activity determinations.

Polyethylene Glycol (PEG)
Acetone Extract

PEG forms a complex with phenolic compounds and has been used by several researchers (Badran and Jones, 1965; Arakji, 1968; Chan and Yang, 1971; Wong et al., 1971) in isolating enzymes. This method was extensively studied and modified to minimize the excess PEG in the extract solution which interfered with the protein determination at 280 nm. All steps were carried out in an ice bath unless otherwise specified.

Six ml of freshly prepared 20% aqueous PEG (20,000) was added to a centrifuge tube and chilled in an ice bath for 15 min. Four g of one-day-old crude powder were added and made into a thick paste and allowed to stand for 3 min. This paste was mixed with 9 ml

cold acetone and stirred gently with a glass rod until a white creamy substance formed on the surface, usually 1 min. The supernatant (31, 000 G for 5 min) was discarded and the precipitate was rinsed with one volume cold acetone. After the addition of 100 ml 0.05 M acetate buffer, pH 5.6, to the precipitate, the mixture was stirred in an ice bath for 12 min, squeezed through nylon cloth, and centrifuged at 27, 000 G for 10 min. The clear supernatant solution constituted the PEG-acetone extract as shown in Figure 1.

Acetone Precipitation of PEG-Acetone Extract

Three hundred ml cold acetone was added to 200 ml of freshly prepared PEG-acetone extract enzyme, stirred moderately with a glass rod for 2 min and allowed to stand for 90 min in an ice bath. The supernatant was decanted and discarded while the cloudy solution was centrifuged at 20, 000 G for 10 min. The supernatant was discarded and the precipitate was dissolved in a minimum volume of glass-distilled water to give a 20-fold concentration.

Sephadex Chromatography

Sephadex G-100 (Pharmacia), particle size of 40-120 μ, was prepared as described by the manufacturer (Technical Data Sheet No. 6). The gel was suspended in 1 liter of 50 mM acetate buffer, pH 4.5, and left at room temperature for three days. During this

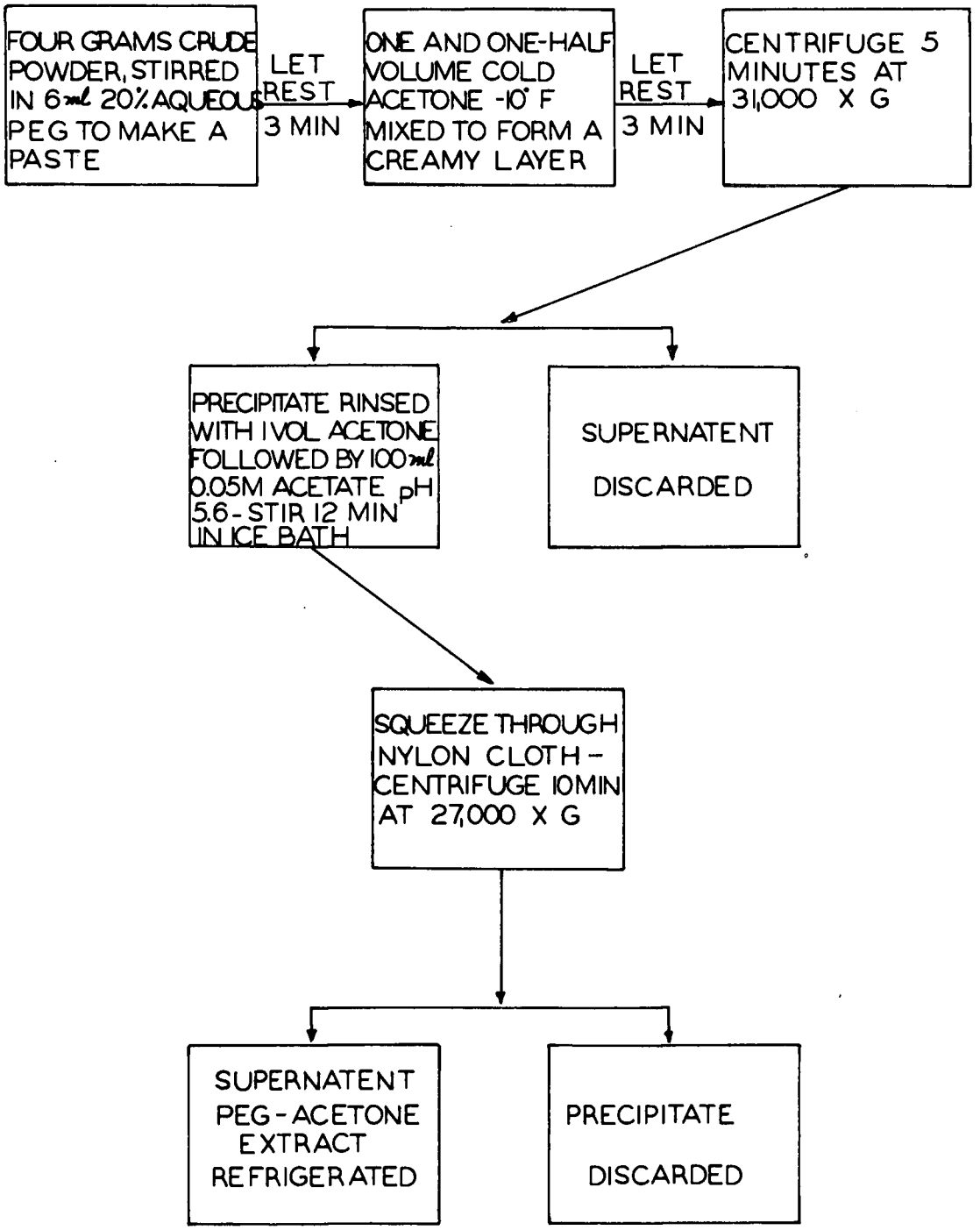


Figure 1. Flow sheet for extraction of PPO.

period, the gel was stirred and decanted several times to remove fine particles and soluble impurities. A Pharmica column (2.5 x 100 cm) was packed by filling the column with 50 mM acetate buffer, pH 4.5, and connecting to a reservoir which contained the gel. The gel was allowed to flow under gravity with a head pressure not in excess of 30 cm. The packed column was equilibrated with three bed volumes of buffer at a flow rate of 12-15 ml/hr. The uniformity of the column bed and the void volume was determined with Blue Dextran 2000.

Acetone precipitate was dissolved in 20 ml of 50 mM acetate buffer, pH 4.5, and dialyzed overnight against the same buffer. Ten ml of this solution was applied to the column. The outlet of the column was connected to a flow-through cell of a Gilson UV absorption meter monitored at 280 nm and recorded with a Texas Instruments "rectiriter" recorder. Ten ml fractions were collected with a LKB fraction collector equipped with an automatic fraction marker. The fractions were placed in ice and assayed for PPO activity and protein concentration on a Beckman DB spectrophotometer. Fractions containing PPO activity were combined and concentrated by lyophilization and redissolved to make a 20-fold concentration for further separation by electrophoresis.

Ion-Exchange Chromatography

Pre-swollen microgranular diethylaminoethyl (DEAE)-cellulose

with a capacity of 1.0 meq/g was obtained from Reeve Angel, Inc., and prepared according to Whatman Advanced Ion-exchange Cellulose-Laboratory Manual. One hundred fifty g of the ion exchanger were suspended in 1 mM phosphate buffer, pH 6.2 (25 ml per dry g) and adjusted to pH 6.2 with the 0.5 M acid component of the buffer. After 10 min, the buffer was decanted and the exchanger was resuspended in the buffer. This step was repeated six times until the pH of the supernatant liquor was exactly the same pH as the buffer. To remove the gases, the pH of the ion exchanger was maintained below 4.5 with the 0.5 M acid component of the buffer, then degassed by stirring the slurry in a stoppered suction flask under vacuum. Fine particles were removed by suspending the ion exchanger in a 1 liter graduated cylinder filled with glass-distilled water (6 ml/g exchanger). After 1 hr, the fines were removed and the exchanger was equilibrated with 1 mM phosphate buffer at pH 6.2.

A column (Pharmica 2.5 x 50 cm) was packed by the same procedure as the Sephadex column, except that it was packed at room temperature. The packed column was equilibrated overnight with starting buffer (1 mM phosphate buffer, pH 6.2) at 4^{o}C. pH of the effluent was monitored until it was the same as that of the starting buffer. A sample applicator was placed on the top of the bed and 8 ml of enzyme preparation (10-fold PEG-acetone precipitate dialyzed against starting buffer) were carefully pipetted into the sample

applicator. When all the sample had entered the cellulose, the sample was rinsed with three portions of 5 ml of starting buffer, followed by 20 ml of buffer. The column was connected to the buffer reservoir through a pump set at 50 ml/hr. Absorbance of the eluate was recorded and 10 ml fractions were collected.

Six chambers of a Buchler Varigrad were used to generate a concave elution gradient. Zero, 30, 60, 50, 40, and 100 ml of final buffer (1 M phosphate, pH 6.2), respectively, were placed in each of the chambers. Starting buffer was added to make the volume in each chamber 100 ml. After one bed volume of starting buffer had passed through the column, the gradient was started. At the end of the chromatographic run, the DEAE-cellulose was re-equilibrated by eluting with 100 ml of 0.5 M KH_2PO_4, and with the starting buffer until pH had returned to 6.2.

Electrophoretic Procedure

Discontinuous vertical gel electrophoresis was performed to study Royal Anne cherry PPO. The method used was described by Petropakis (1969) and Taylor (1970). A Model EC-470 with a power supply Model EC-454 (EC Apparatus Corporation, Philadelphia, Pa.) was used. Assembly and operation of this equipment is described by EC Apparatus Corporation Technical Bulletin 128. Spacer buffer, 0.062 M Tris-HCl, pH 6.7, was prepared by dissolving 22.5 g Tris

and 12 ml of concentrated HCl in 3 liters of glass distilled water, and adjusting to pH 6.7 with dilute HCl. Running buffer, 0.38 M Tris-HCl, pH 9.0, was prepared by dissolving 138 g Tris and 12 ml concentrated HCl in glass distilled water to make a final volume of 3 liters. Electrode buffer, 0.0165 M Tris-0.039 M glycine, pH 8.75, was prepared by dissolving 11.6 g glycine and 8 g Tris to make 4 liters of buffer.

The outer edges of the gaskets of the cell were sealed with 0.5% agarose before assembling the cell. A 20% plug gel was prepared by dissolving 20 g of Cyanogum-41 (95% acrylamide and 5% N, N'-methylenebisacrylamide) in 100 ml running buffer and filtering through Whatman No. 1 filter paper. After adding 0.1 ml TMED (N, N, N', N'-tetramethylethylenediamine), a small drop of Tween 80 (approximately 0.02 ml), and 0.1 g ammonium persulfate, the solution was stirred gently to avoid air bubbles. Approximately 75 ml of this solution was carefully poured into the cell at a 45° angle. To obtain a smooth surface, 5 ml of distilled water was pipetted over the gel solution and allowed to stand in this position for 15 min to polymerize.

Running gel solution (10%) and spacer gel (4%) were prepared in a similar manner as the plug gel, except spacer gel used spacer buffer. After the plug gel had polymerized, the water was removed and the cell was placed in a vertical position. Running gel solution was poured between the cooling plates to a level of 2.5 cm from the

top of the inner cooling plate and 5 ml distilled water was pipetted to the surface. When polymerized, the water was removed and the cell was placed in a horizontal position, filled with spacer gel and the slot former was inserted. After polymerization, the excess gel above the slot former was removed. The cell was placed in a vertical position and 2 liters of electrode buffer were poured into the electrode compartments. Temperature was maintained at $0^{\circ}C$ throughout the electrophoretic run by circulating cold water through the coolant channels of the cell.

A syringe was used to place 150 µl of sample containing 10% sucrose and a small quantity of bromphenol blue into each slot. Electrophoresis was run at 200 V until the sample was completely stacked at the spacer gel-running gel interface. The voltage was increased to 400 V and maintained at this level until the dye had traveled to the end of the running gel. On completion of the electrophoretic run, the gel slab was removed with a gel scoop and cut into eight strips for detecting enzyme and staining for protein.

PPO activity in the gel strips was detected by immersing the strip in 100 ml 10 mM catechol-0.2 M phosphate, pH 7.0, containing 0.05% p-phenylenediamine for 10 min followed by 100 ml 1 mM ascorbic acid for 5 min. After 48 hr in distilled water, the strips were stored in 30% ethyl alcohol. The bands were stable for more than two months at room temperature. Gel patterns were photographed

with a Polaroid MP-3 camera, type 55 P/N Polaroid film, blue filter and fluorescent light box for illumination.

Several electrophoretic runs were conducted to study substrate specificity and the effect of inhibitors. The gel strips were incubated in 100 ml (1 mM) inhibitor solutions for 1 hr at room temperature. After incubation period, the inhibitor solutions were poured out and replaced with 100 ml 0.2 M phosphate buffer, pH 7.0, containing 10 mM catechol and 0.05% p-phenylenediamine for 15 min.

RESULTS

During the extraction of PPO by conventional techniques (buffer extract), the phenolics in the cherry tissue were exposed to the action of PPO resulting in the formation of brown polymers (Webb, 1966). These products appeared to inhibit the enzyme, since the activity of the supernatant was decreased. Therefore, many extraction methods were tested to achieve an enzyme preparation of higher activity and to remove most of the phenolics without inhibiting the enzyme.

Since insoluble PVP had been used for the separation of peppermint PPO (Loomis and Battaile, 1966), various levels of PVP were used to determine if cherry PPO could be extracted by this method. Data presented in Table 1 show that 0.5 g of PVP/g of crude powder resulted in an increase in the activity of PPO extracted. In addition the protein was decreased, which yielded an increase in the specific activity of the extract. Higher quantities of PVP showed a decrease in the activity and specific activity extracted from the crude powder.

Previous workers (Badran and Jones, 1965; Chan and Yang, 1971; Wong et al., 1971) have found that PEG, a polymer of ethylene oxide, formed an insoluble complex with phenols, which can be used to remove phenolics from plant enzyme extracts. Table 2 shows 6 ml

Table 1. Effect of PVP on the extraction of PPO from cherries.[a]

PVP[b] (g)	Activity (units/ml)	Absorbance 280 (nm)	Absorbance 260 (nm)	Protein (mg/ml)	Specific activity (units/mg)
0.0	150	7.9	8.0	5.5	27
0.5	163	6.0	5.9	4.3	38
0.8	98	5.8	5.9	4.1	24
1.0	90	5.5	5.4	4.0	23
1.5	80	5.0	4.8	3.7	22
2.0	75	4.5	4.4	3.3	23

[a]The results are the average of two trials on different crude powder preparations.

[b]PVP used per g of crude powder.

Table 2. Effect of PEG on the extraction of PPO from cherries.[a]

20% Aqueous PEG (ml/100 ml)	Activity (units/ml)	Absorbance 280 (nm)	Absorbance 260 (nm)	Protein (mg/ml)	Specific activity (units/mg)
0	105	10.5	12.8	5.8	18
2	105	7.2	8.0	4.5	23
4	122	6.4	7.6	3.7	33
6	122	5.0	6.0	2.8	44
8	112	5.5	6.4	3.2	35
10	110	7.1	8.0	4.4	25
12	90	8.0	9.3	4.7	19
14	85	8.4	9.6	5.1	17

[a]The results are the average of two trials on different crude powder preparations.

of 20% aqueous PEG per 4 g of enzyme crude powder was the most effective in yielding an extract with the highest specific activity. However, it was noted from these data that the absorbance at 260 nm was higher than the absorbance at 280 nm, which would indicate that a considerable quantity of PEG remained in the extract. By washing the precipitated PPO with acetone before buffer extraction, most of the PEG could be removed.

Results of the various extraction methods are presented in Table 3. These data reveal that the extraction of the crude powder with buffer (0.05 M acetate, pH 5.6) resulted in low activity and specific activity with a high content of phenolic compounds. Presence of PVP in the buffer reduced the phenolic compounds substantially and increased the activity with a resulting increase in specific activity. When an acetone powder was prepared from the crude powder, the phenolic content was also decreased and the activity was increased over that of the PVP-buffer. Addition of PVP to the extracting buffer did not reduce the phenolic content, nor did it greatly increase the specific activity of the preparation. The use of PEG in the buffer for extracting PPO from the crude cherry powder gave the highest activity of any of the methods used and also a low phenolic content. However, a considerable amount of PEG remained in the extract as shown by the high absorbance at 280 and 260 nm. When the PEG extract was washed with acetone and subsequently

Table 3. Extraction of cherry PPO by various methods.[a]

Extraction method	Activity (units/ml)	Absorbance 280 (nm)	Absorbance 260 (nm)	Protein (mg/ml)	Specific activity (units/mg)	Phenolic content (μg tannic acid/ml)
Buffer	200	6.6	6.8	4.5	44	165
PVP + buffer	373	5.5	5.8	3.7	101	95
Acetone powder + buffer	420	6.0	6.5	3.9	108	90
Acetone powder + PVP + buffer	425	5.5	5.9	3.6	118	92
PEG + buffer	540	12.8	13.0	8.9	61	75
PEG - acetone + buffer	450	5.5	6.5	3.2	141	65
Dowex 1 - X8 + buffer	210	2.5	2.3	1.9	111	52

[a]The results are the average of three trials on different crude powder preparations.

extracted with buffer, both the UV absorbance and phenolic content were reduced. This resulted in the highest specific activity. The use of Dowex 1-X8 in the extracting buffer did not appear to increase the activity, although this resin appeared to be the best for removal of phenolic compounds from the preparation. Since it also removed the enzyme a low specific activity was obtained. From these data the best extraction method was the PEG-acetone treatment followed by extraction with buffer. This preparation was used in the following work and is referred to as PEG-acetone extract.

Assay of Royal Anne Cherry PPO

To determine the effect of enzyme concentrations on the initial velocity of the reaction, various amounts of the PEG-acetone extract were added to the assay mixture, replacing buffer. The results in Figure 2 show that the velocity of the reaction was directly proportional to the volume of enzyme used. This linear relationship indicates that the Lambert-Beer law was applicable and, therefore, the assay method was suitable for the study of Royal Anne cherry PPO.

Protamine Sulfate Treatment

Protamine sulfate has been used in crude enzyme extracts to precipitate nucleic acids and their anionic biopolymers (Felix, 1960). The method used to prepare the protamine sulfate solution was that of

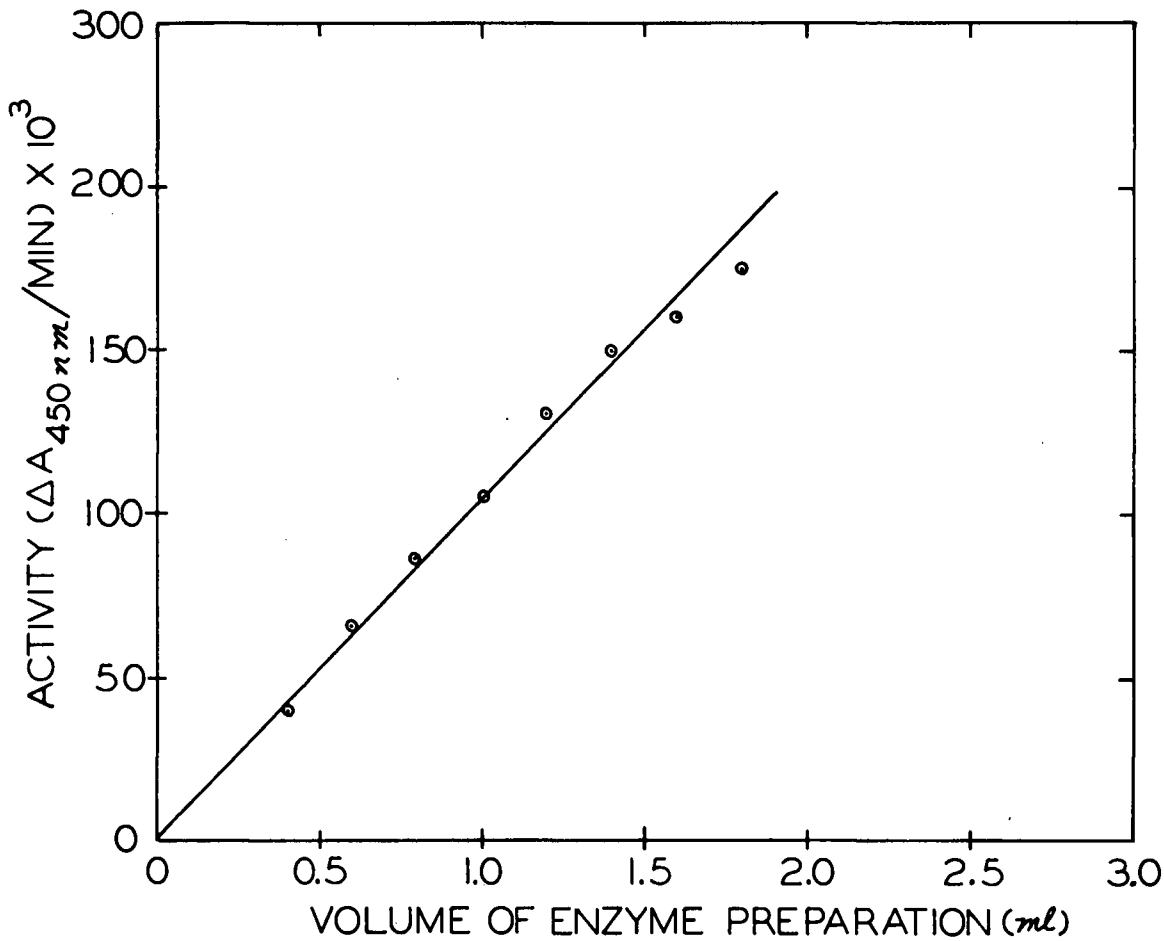


Figure 2. Effect of enzyme concentration on rate of oxidation of catechol.

Veerabhadrappa and Montgomery (1971). Results in Table 4 show, however, that even a small amount of protamine sulfate decreased the specific activity of the enzyme; therefore, the use of protamine sulfate did not provide a means of obtaining a more purified preparation of cherry PPO.

Ammonium Sulfate Precipitation

During early work on purification, difficulty was encountered in solubilizing the lyophilyzed PEG-acetone extract. The lyophilyzed preparation did not enter the Sephadex gel, which may have been due to the high concentration of salts and proteins. Therefore, ammonium sulfate precipitation was used to fractionate and concentrate the PEG-acetone extract. The amount of ammonium sulfate required at different levels of saturation was calculated from a table in Dixon and Webb (1964). After centrifuging, fractions were dissolved in a minimum volume of 0.05 M acetate buffer, pH 4.5, to give a ten-fold concentration and dialyzed against this buffer for 12 hr with two changes of buffer. These preparations were stored at $-20^{\circ}C$ for further studies.

Data presented in Table 5 show that 75% of the total PPO was precipitated at 10% saturation. However, during fractionation, difficulty was encountered in solubilizing the precipitate at 10% saturation. The precipitate was clear except for small flake-like

Table 4. Effect of protamine sulfate on PPO activity.[a]

% Protamine sulfate in extract	Activity (units/ml)	Absorbance 280 (nm)	Absorbance 260 (nm)	Protein (mg/ml)	Specific activity (units/mg)
0.0	145	3.9	4.7	2.2	66
0.1	95	3.5	4.2	2.0	48
0.2	55	3.0	3.8	1.5	37
0.3	55	3.0	3.7	1.6	34
0.4	52	2.9	3.5	1.6	33
0.6	51	2.8	3.4	1.6	32
0.8	50	2.7	3.2	1.6	31
1.0	46	2.5	3.1	1.3	35

[a]The results are the average of two trials on different crude powder preparations.

Table 5. Ammonium sulfate fractionation of Royal Anne cherry PPO.

Saturation (%)	Activity (units /ml)	Total (units)	Yield (%)	Absorbance 280 (nm)	Absorbance 260 (nm)	Specific activity (units /mg)
0[a]	270	6750	100.0	3.1	3.7	153
10	204	5100	75.0	0.3	0.4	1457
20	22	550	8.1	0.8	0.8	39
30	14	350	5.1	0.1	0.1	200
40	Npt.[b]	-	-	-	-	-
60	65	1625	24.0	0.5	0.5	186
75	30	750	11.1	0.1	0.1	429
90	15	375	5.5	0.1	0.1	214
100	0	-	-	-	-	-

[a]PEG-acetone extract

[b]No precipitation

particles which were not solubilized immediately. Centrifuging removed the particles but resulted in a loss of activity. At 20% and 30% saturation, a light brown precipitate was obtained and at 40%, no precipitate was observed, while at 60% and 75%, a thin layer of floating material was formed. Further saturation did not yield any significant activity. The enzyme precipitated at 10% saturation had a specific activity several fold higher than other levels of ammonium sulfate.

Effect of Dialysis

Enzyme preparations from PEG-acetone extract, ammonium sulfate fractionation, and acetone precipitation were dialyzed against 2 liters of 0.05 M acetate buffer, pH 4.5, for 12 hr with two changes of buffer. Effect of this dialysis was studied by determining the activities before and after dialysis in all preparations. Data presented in Table 6 show a sharp decrease in absorbing material at 280 and 260 nm after dialysis of the acetone precipitate. Dialysis increased the specific activity in both the PEG-acetone extract and the acetone precipitate, but caused no change in the specific activity of the ammonium sulfate fraction. The acetone precipitate revealed a 8.3-fold purification, while the ammonium sulfate showed a 3.6-fold increase in purification after dialysis. These data show that some inhibitory substances present in the extract were removed by dialysis.

Table 6. Effect of dialysis on PPO activity.[a]

Treatment	Activity (units/ml)	Absorbance 280 (nm)	Absorbance 260 (nm)	Protein (mg/ml)	Specific activity (units/mg)	Purification (fold)
Before dialysis						
PEG-acetone extract	375	3.25	4.10	1.68	223	-
10-75% sat. $(NH_4)_2So_4$	500	0.85	0.83	0.62	806	3.6
Acetone precipitate	425	2.50	3.30	1.18	360	1.6
After dialysis						
PEG-acetone extract	457	1.20	1.10	0.93	491	2.2
10-75% sat. $(NH_4)_2So_4$	410	0.70	0.68	0.51	804	3.6
Acetone precipitate	464	0.35	0.34	0.25	1856	8.3

[a]The results are the average of four trials of different crude powder preparations.

pH Stability

The pH stability of PPO was studied to determine the pH of buffers to use in column chromatography. The activity of the enzyme at different pH values is shown in Figure 3. Maximum stability appears to be at pH 4.5. These data indicate that cherry PPO activity was more stable at pH levels lower than 5.6, in contrast to the rate of pigment formation and the sequences leading to melanin formation, which increase as pH was increased above 5.6 (Mason, 1948). A striking observation was that samples adjusted with H^+ or OH^- ions between pH 3.9 and 6.0 had higher activity than the control. The experiment was repeated four times and in all cases, this phenomenon was noticed. This increase in activity could have been due to the activation of the PPO with H^+ or OH^- ions. Swain et al. (1966) found a similar increase in the activity of broad bean PPO when the pH was altered by H^+ or OH^- ions. Although the optimum stability was at pH 4.5-4.7, they concluded that at neutrality the prosthetic groups of the enzyme were masked by the tertiary structure and alteration of this structure by a change of pH or an anionic detergent resulted in a more active enzyme.

Gel Filtration

The elution profile of PPO from Sephadex G-100 showed three

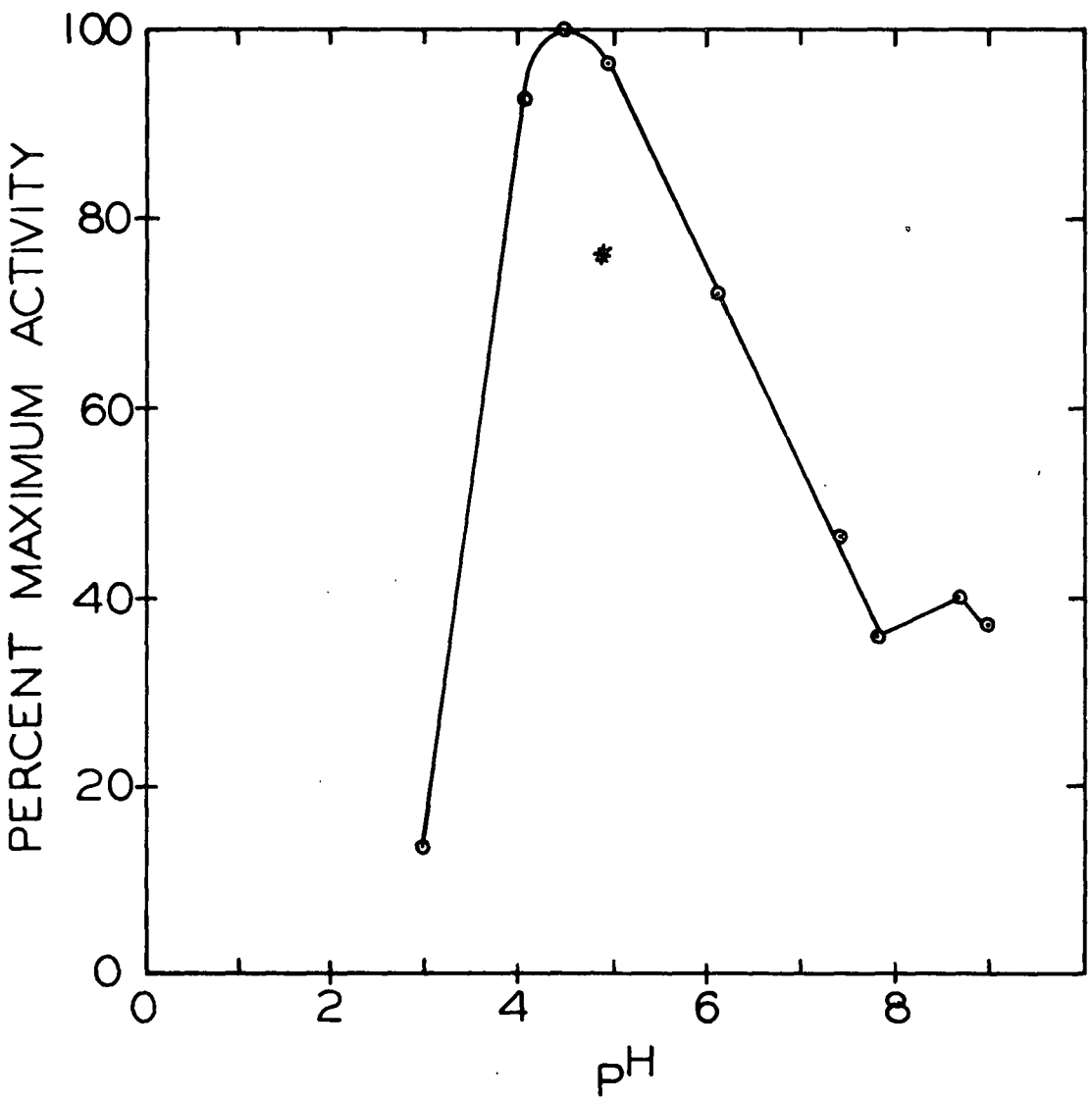


Figure 3. pH stability of Royal Anne cherry PPO. The results are the average of four different trials of crude powder preparation. * - control.

fractions (Figure 4). Tubes 12-15 correspond to fraction S1. Tubes 16-19 were pooled and were called fraction S2. Tubes 22-25 were designated as fraction S3. The fractions were concentrated by lyophilization and kept in the refrigerator at -20°C for further studies.

Since specific activites of fractions S1 and S2 were not increased by gel filtration, no purification occurred after acetone precipitation (Table 7). After filtration fraction S3, which had only 4% of the total activity of the acetone precipitate, showed a 63-fold purification over the PEG-acetone extract (Table 7). These results suggest that S1, which was eluted first from the Sephadex gel, had the highest relative molecular size followed by S2 and S3, respectively. Further studies were made using G-150 and G-200 in an attempt to separate the peaks further, but the separation was not improved and low specific activities resulted.

Ion-Exchange Chromatography

Studies were made using microgranular carboxymethyl (CM)-cellulose to separate Royal Anne cherry PPO but no separation could be detected. An attempt was then made to increase the separation of S1 by chromatography on a microgranular DEAE-cellulose column, but no increase in purification was obtained. Therefore, column chromatography using microgranular DEAE-cellulose was conducted on PPO prepared from the acetone precipitate. After trying several

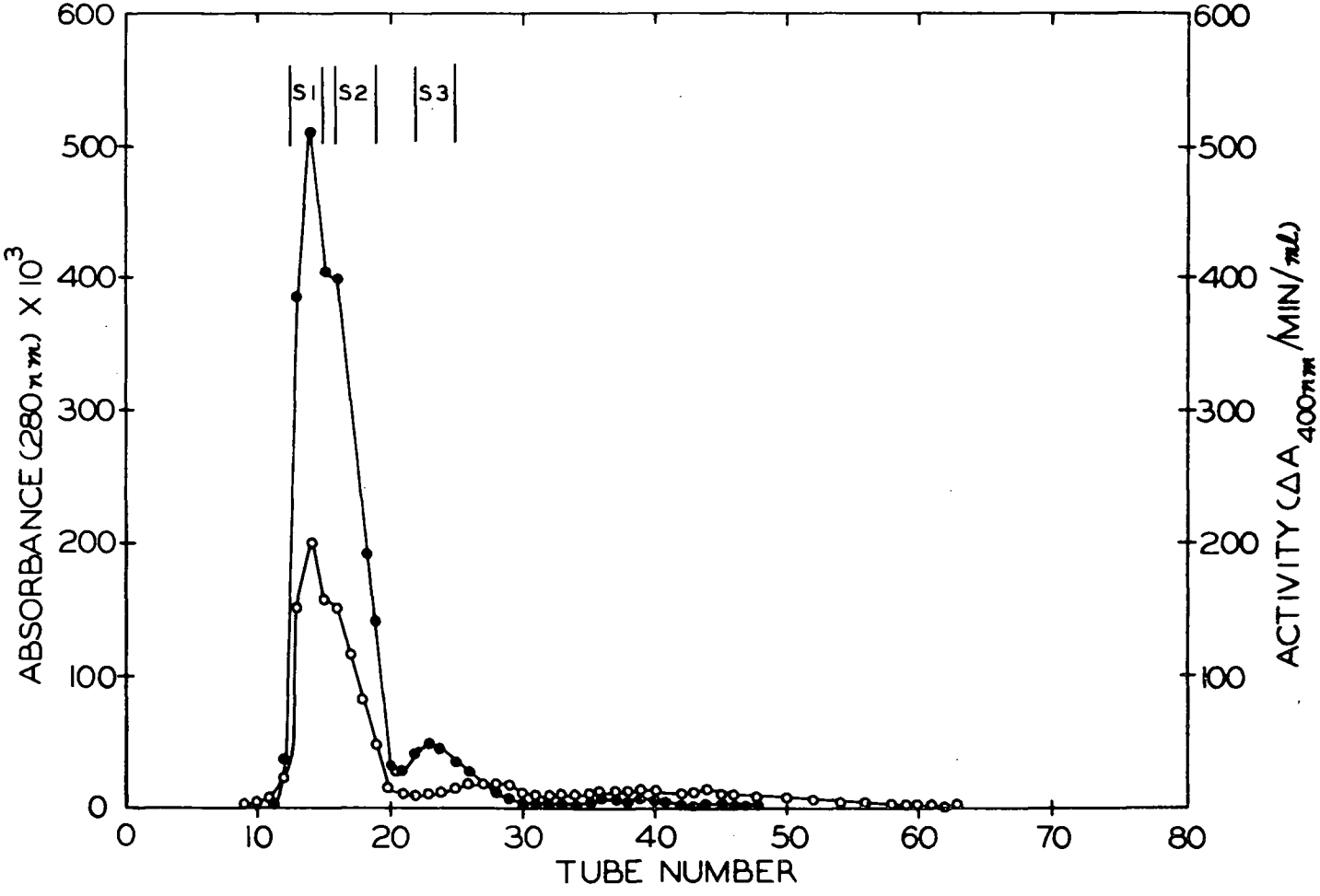


Figure 4. Elution profile of PPO on Sephadex G-100. Equilibrated and eluted with 0.05 M acetate buffer, pH 4.5, flow rate 12 ml per hr at 5°C. -●-●-●-, PPO activity (units/ml); -o-o-o-, abosrbance at 280 nm.

Table 7. Purification of PPO from Royal Anne cherry with Sephadex G-100.

Purification step	Volume (ml)	Total activity (units x 10^{-3})	Total protein (mg)	Specific activity[a] (units/mg x 10^{-3})	Yield (%)	Purification (fold)
1. PEG-acetone extract	100	25.5	240.0	0.1		
2. Acetone precipitate[b]	10	48.5	12.0	4.0	190	40
3. Sephadex G-100						
fraction S1	35	17.9	4.9	3.7	70	37
fraction S2	40	16.0	4.0	4.0	62	40
fraction S3	40	1.9	0.3	6.3	7	63

[a] Most active tube of each fraction.

[b] Acetone precipitate dialyzed against acetate buffer 0.05 M pH 4.5.

different gradients, optimal separation was obtained with the gradient presented in Figure 5. Two fractions designated DE1 (tubes 32-35) and DE2 (tubes 36-39) were obtained.

Forty-five and 33% of total activity of the acetone precipitate was found in DE1 and DE2, respectively (Table 8). DE1 resulted in a 200-fold purification over the PEG-acetone extract, while DE2 was purified 180-fold. The fractions were concentrated by lyophilization and stored at -20°C for further studies.

pH Optimum for PPO Activity

The effect of pH on Royal Anne cherry PPO activity was determined by using the PEG-acetone extract and fractions DE1 and DE2. Three different buffers were used, each at 0.2 M; acetate buffer for pH 4.0 to 5.6, phosphate buffer for pH 6.0 to 8.0, and Tris-HCl buffer for pH 8.0 to 9.0. Catechol was used as the substrate. The preliminary results indicated that the pH optimum with the PEG-acetone extract was 7.0 and assays were performed at this pH.

Results of this more detailed study are presented in Figure 6. They show a bell-shaped curve for the PEG-acetone extract with a maximum at pH 7.0. On the other hand both fractions, DE1 and DE2, demonstrated a broad plateau from pH 7.3 to 7.8.

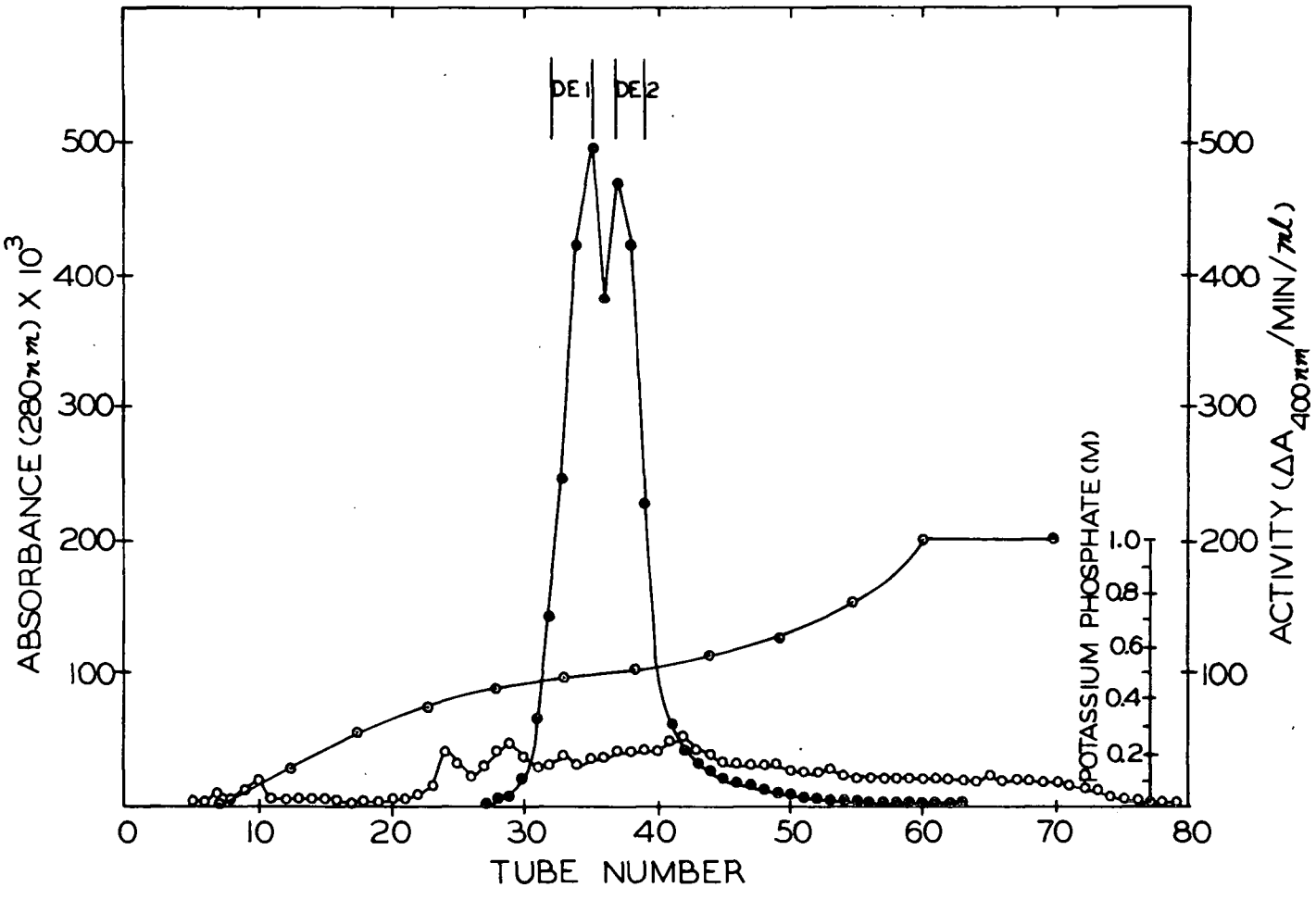


Figure 5. Chromatography of PPO on DEAE-cellulose. Eluted with a concave gradient elution, potassium phosphate buffer, pH 6.2, flow rate 50 ml per hr at 5°C. -●-●-●-, PPO activity (units/ml); -o-o-o-, absorbance at 280 nm.

Table 8. Purification of Royal Anne cherry PPO by DEAE-cellulose chromatography.

Purification step	Volume (ml)	Total activity (units x 10^{-3})	Total protein (mg)	Specific activity[a] (units/mg x 10^{-3})	Yield (%)	Purification (fold)
1. PEG-acetone extract	80	20.4	192	0.1		
2. Acetone precipitate[b]	8	43.2	8.5	5.0	211	50
3. DEAE-cellulose						
fraction DE1	40	19.8	1.0	20.0	97	200
fraction DE2	30	14.4	0.8	18.0	70	180

[a]Most active tube of each fraction.

[b]Acetone precipitate dialyzed against phosphate buffer 1 mM, pH 6.2.

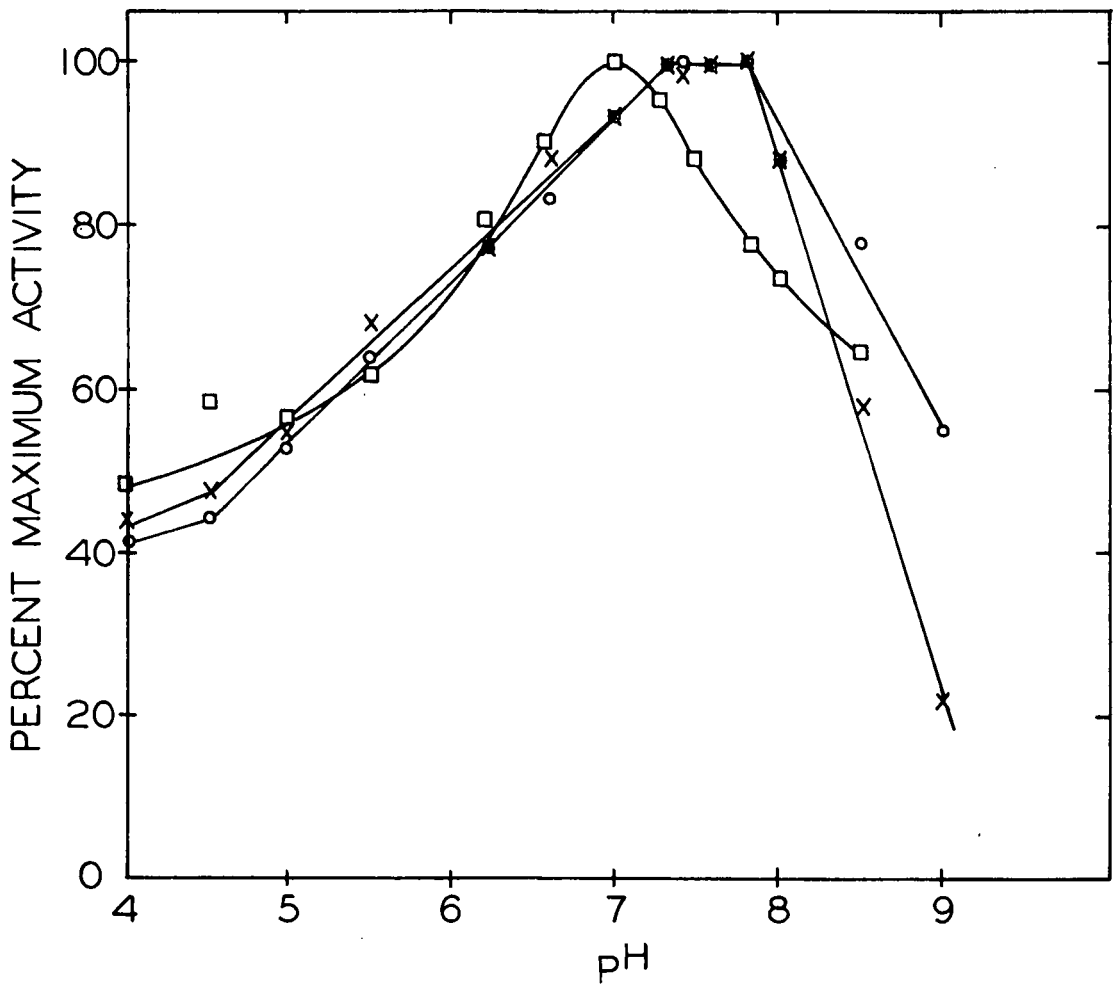


Figure 6. Effect of pH on PPO activity. -□-□-□- , PEG-acetone extract; -x-x-x-, DE1; -o-o-o-, DE2. Acetate buffer for pH 4.0 - 6.0, phosphate buffer, pH 6.0 - 8.0; and Tris-HCl, 8.0 - 9.0.

Effect of Substrate Concentration

The effect of substrate concentration on the initial rate of reaction was determined for each fraction using catechol as substrate. Michaelis constants (K_m) and maximal velocities (V_{max}) are shown in Table 9. These were calculated from S/v vs S plots (Figure 7). PPO from acetone precipitate, DE1 and DE2 fractions had similar K_m values. The maximal velocity was the same for DE1 and DE2 and higher than the acetone precipitate.

Table 9. Michaelis constants and maximum velocities of fractions of Royal Anne cherry PPO.

PPO	K_m	V_{max}
Acetone precipitate	46	766
DE1	43	1000
DE2	53	1000

Substrate Specificity

Various monophenols and o-diphenols were used to determine the substrate specificity of the acetone precipitate and the pooled fractions from DEAE-cellulose chromatography. Protein concetration of all three fractions was adjusted to approximately 0.1

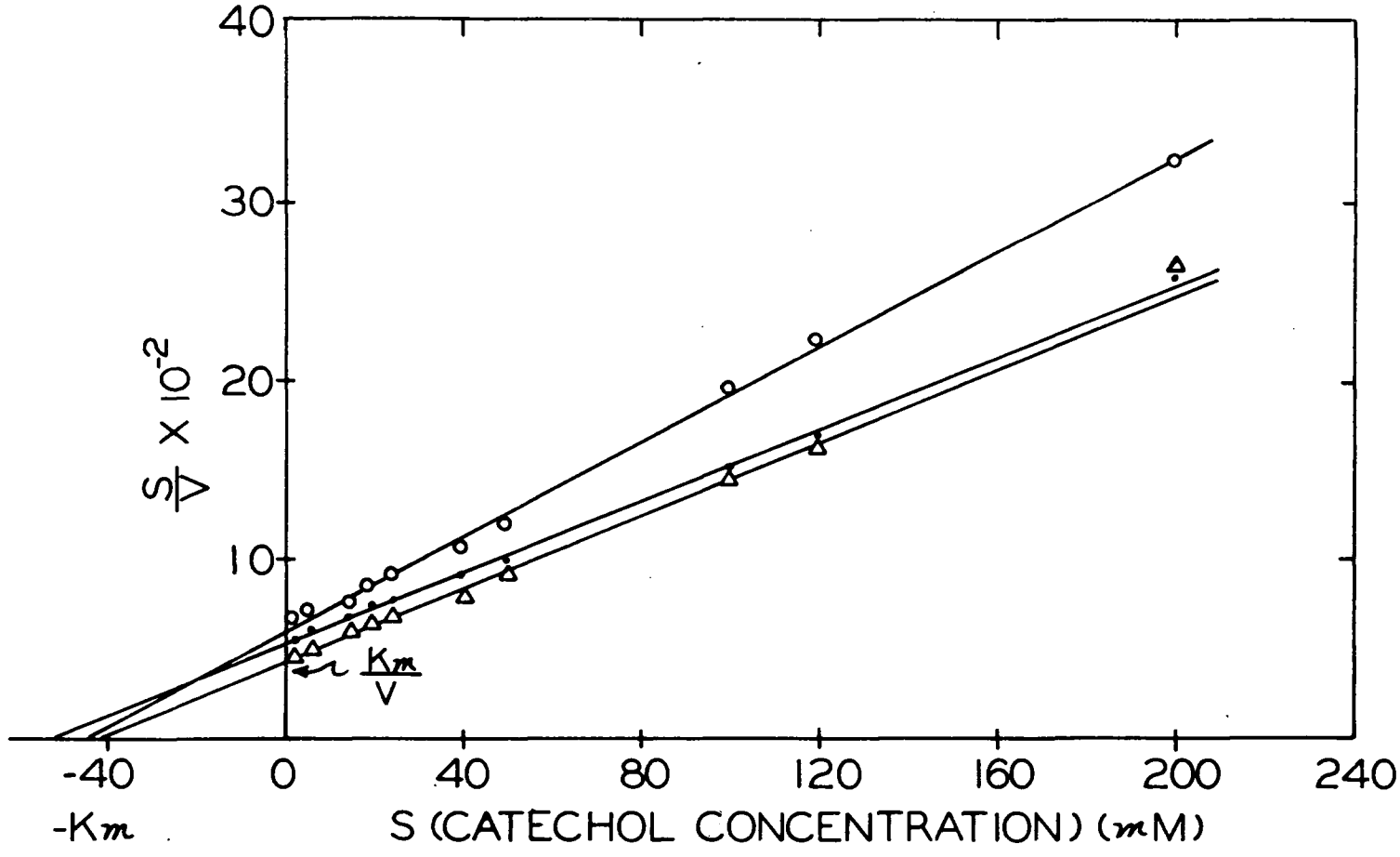


Figure 7. Plot of S/v vs. S for Royal Anne cherry PPO activity on catechol. -o-o-o-, acetone ppt.; -Δ-Δ-Δ-, DE1; -●-●-●-, DE2.

mg/ml. Rate of reaction for the different substrates was measured at appropriate wavelengths, which were determined 5 min after the start of the reaction.

Data presented in Table 10 reveal that all the fractions were active toward o-diphenols but not with monophenols. These results indicate that Royal Anne cherry PPO cannot catalyze the oxidation of monophenol to o-diphenol. Of the substrates used in this study, pyrogallol was oxidized most rapidly by the acetone precipitate and fraction DE1 while fraction DE2 oxidized 4-methyl catechol most rapidly. This indicates that the substrate specificity of DE1 and DE2 was different. Hence, these two fractions were composed of different enzymes. All of the fractions showed intermediate activity toward catechol and lower activity with the other o-diphenols.

Inhibitor Studies

Several compounds known to reduce the enzymic browning of fruits were used as inhibitors of the Royal Anne cherry PPO fractions. Using catechol as substrate, sodium diethyldithiocarbamate (DEDTC), dithiothreitol, and $K_2S_2O_3$ all caused a delay in the start of the reaction (induction period). Inhibition by the various compounds was calculated from the rate of change in absorbance at 400 nm immediately following the induction period, which ranged from 2 to 5 min. The two fractions and the acetone precipitate showed quite similar

Table 10. Specificity of Royal Anne cherry PPO toward different substrates.

Substrate	Wavelength (nm)	Activity (units/ml)			Ratio of activity
		Acetone ppt.	DE1	DE2	(DE1/DE2)
catechol	400	355	220	150	1.5
pyrogallol	334	715	490	175	2.8
4-methyl catechol	400	650	305	390	0.8
dopa	460	205	80	60	1.3
chlorogenic acid	400	225	165	110	1.5
l-epinephrine	470	205	140	100	1.4
tyramine	472	0	0	0	0
l (-) tyrosine	472	0	0	0	0
p-cresol	400	0	0	0	0

behavior toward various inhibitors (Table 11). All of the compounds used in this study inhibited all of the fractions of the cherry PPO. Sodium chloride was used at a concentration of 1000 times greater than the other compounds and showed only slight inhibition of PPO. All of the other inhibitors, except thiourea, showed 100% inhibition of all the fractions at the highest concentrations used. Dithiothreitol and DEDTC revealed particular ability to inhibit PPO at reasonably low concentrations.

Stability

Heat inactivation of the purified fractions of Royal Anne cherry PPO was studied. Five ml of enzyme solution were placed in a pre-warmed test tube at 75°C. Enzyme solutions were withdrawn at various intervals and assayed for residual activity. The rate of decrease of PPO activity at a constant temperature appears to follow first order kinetics up to 7 min (Figure 8). After this time, fractions DE1 and DE2 deviate from linearity. Although fractions DE1 and DE2 behaved similarly in their pH optima, they showed different heat stabilities. Half-lives of DE1, DE2 and acetone precipitate at 75°C were 1.9, 2.7 and 8 min, respectively (Figure 8).

Stability of PPO during storage of the enzyme solution was also determined. Results in Figure 9 show that the loss of activity of PPO is first order up to five days, then stabilizes at room temperature.

Table 11. Effect of different inhibitors on PPO fractions DE1, DE2, and acetone precipitate.

Inhibitor	Concentration	Acetone ppt. inhibition (%)	DE1 inhibition (%)	DE2 inhibition (%)
NaCl	100 mM	0	0	0
	333	14	26	16
	400	18	32	19
Sodium diethyl-dithiocarbamate	83 μM	61	77	68
	166	93	97	99
	333	100	100	100
$K_2S_2O_3$	83 μM	54	43	48
	166	100	100	100
Dithiothreitol	67 μM	50	43	45
	83	100	100	100
	166	100	100	100
Thiourea	33 μM	36	30	32
	166	48	66	71
	333	57	86	87
KCN	33 μM	40	39	45
	166	100	100	100

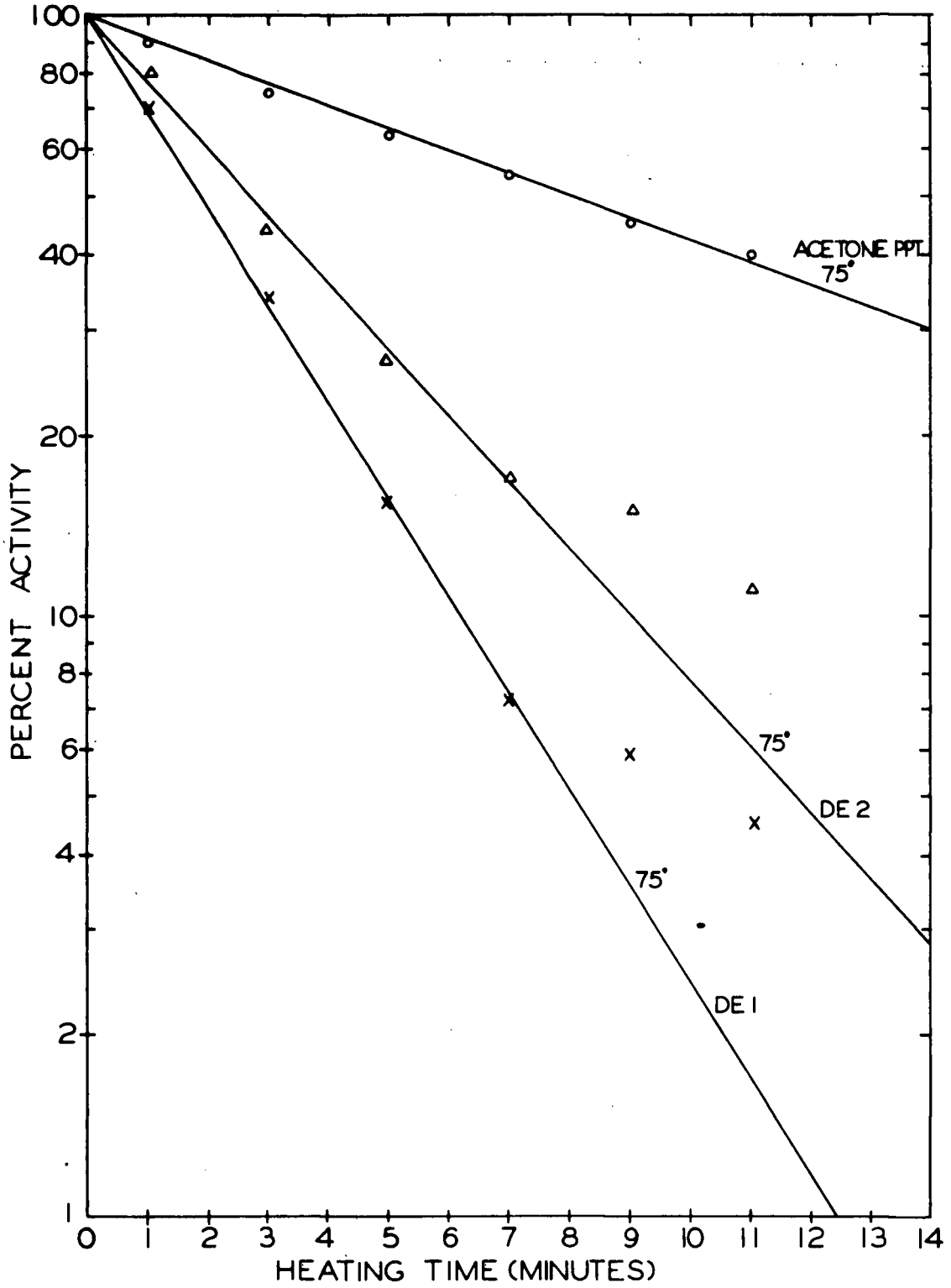


Figure 8. Heat inactivation of Royal Anne cherry PPO.

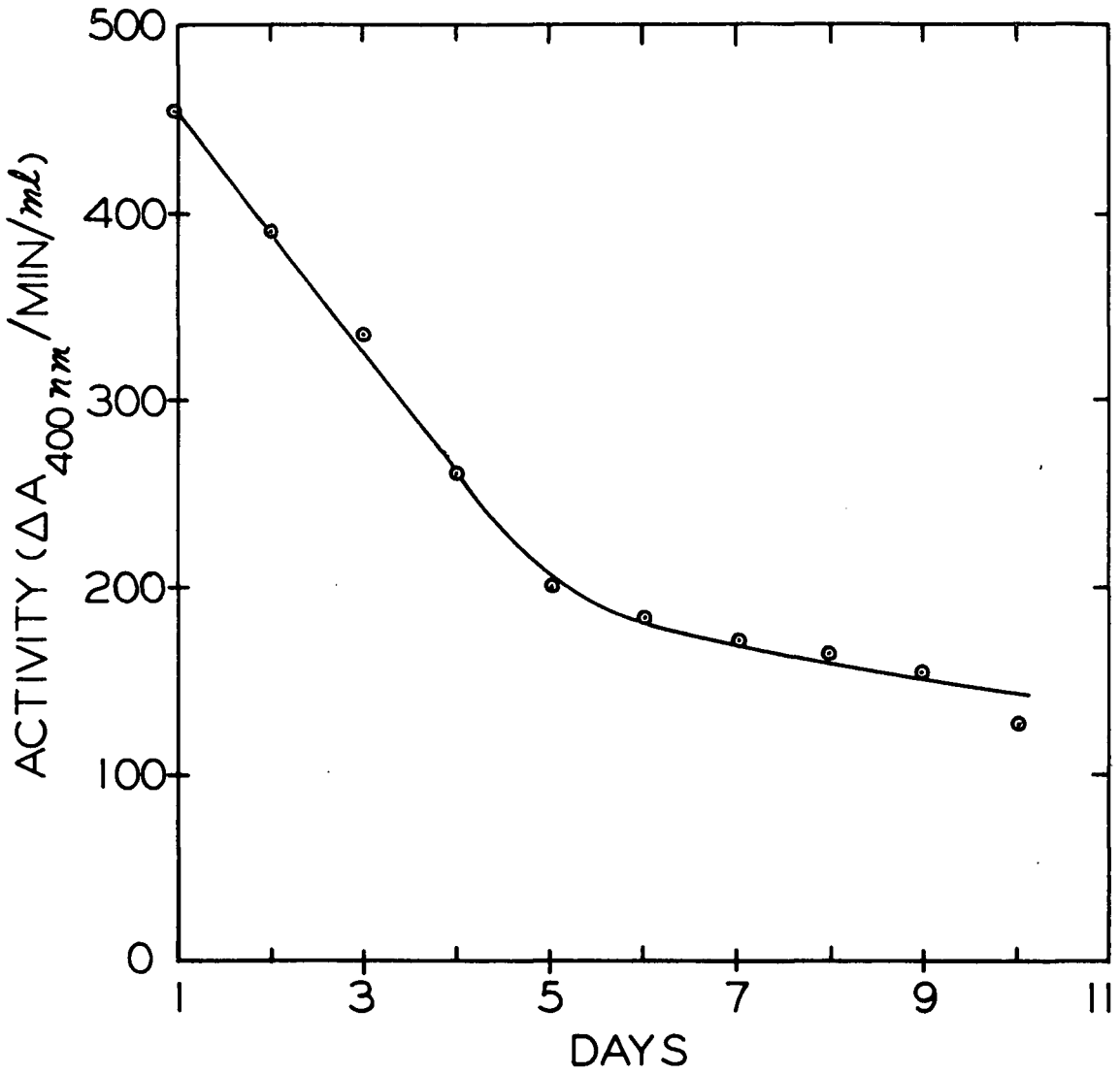


Figure 9. Loss of PPO activity with time at room temperature.

The half-life was about four and one-half days at room temperature.

Changes in the Absorption Spectrum of Reaction Mixture

The absorption spectrum of the enzyme solution from the acetone precipitate shows a typical maximum at 280 nm (Figure 10). Protein concentration of the enzyme solution was 0.13 mg/ml. Figure 10 also shows a spectrum for catechol at 1 mM having a sharp peak at 275 nm. No other peaks were observed in the ultraviolet or visible spectrum.

The absorption spectrum of the product of reaction between PPO and catechol in phosphate buffer, pH 7.0, was determined with a Beckman DB spectrophotometer at various times after initiation of the reaction. Maximal absorption at 400 nm occurred after 5 min. This is in agreement with the data for eggplant PPO (Rhoades and Chen, 1968) and McFarlin cranberry PPO (Chan and Yang, 1971). Changes in the absorption spectra of the reaction mixture at 1, 10, 20, and 40 min after initiation of the reaction are presented in Figure 11. These data reveal that as the reaction proceeded there was a decrease in absorbance at 400 and 230 nm and an increase at 290 nm. This indicates that the initial reaction product, which absorbed at 400 nm, reacted further to form a substance that absorbed at 290 nm. The decrease in absorbance at 400 nm could account for the observed decrease in the rate of the PPO reaction at 400 nm after 3 or 4 min.

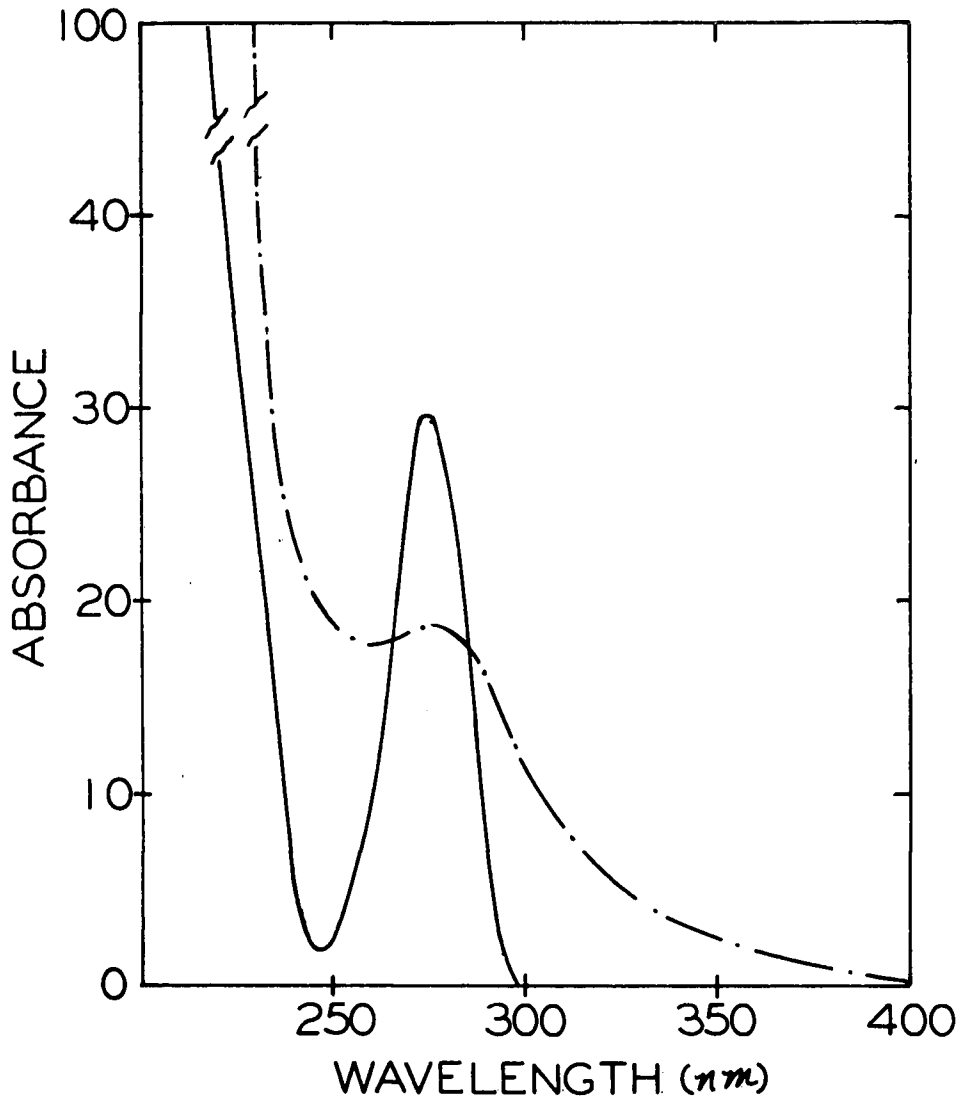


Figure 10. Absorption spectra of catechol and PPO preparation. ____, catechol; -.-.-, PPO.

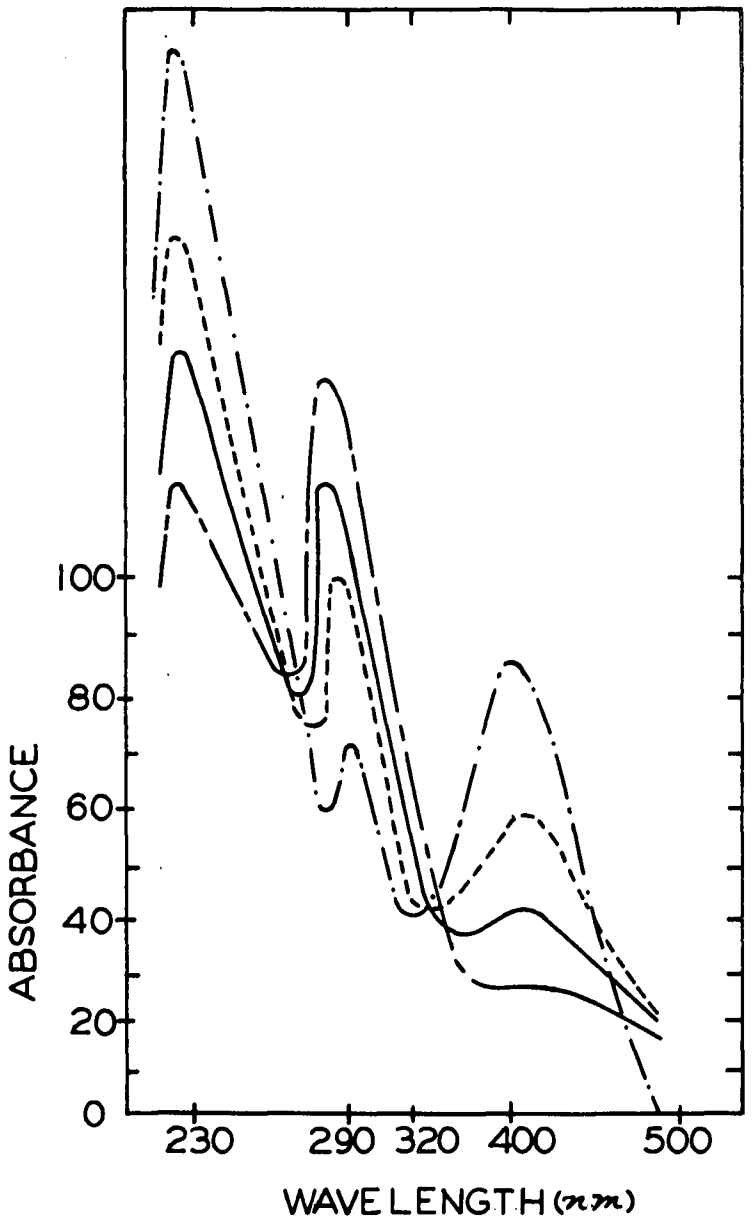


Figure 11. Absorption spectra of PPO assay mixture at various times after addition of the enzyme. -.-.-., 1 min; ----, 10 min; —, 20 min; — — —, 40 min.

Figure 12. Electrophoretic patterns of PEG-acetone extract (1), acetone ppt. (2), bromophenol blue (3), and no sample (4).

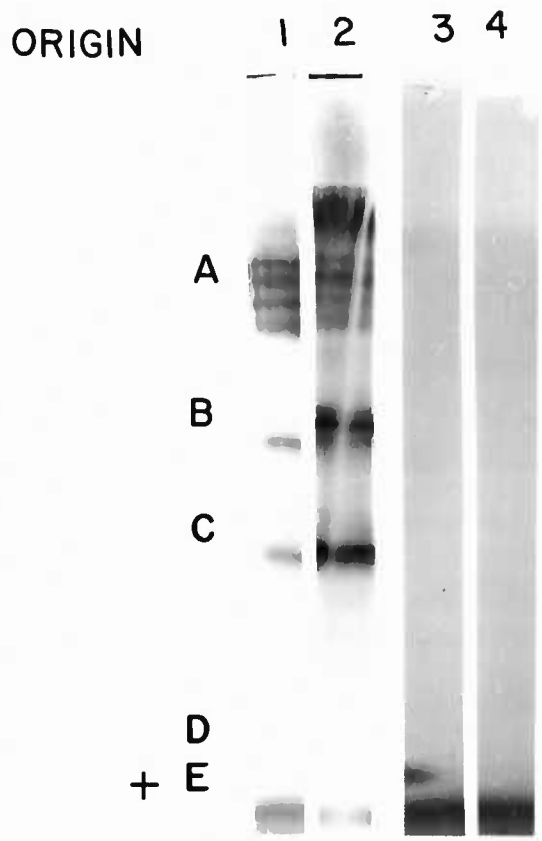
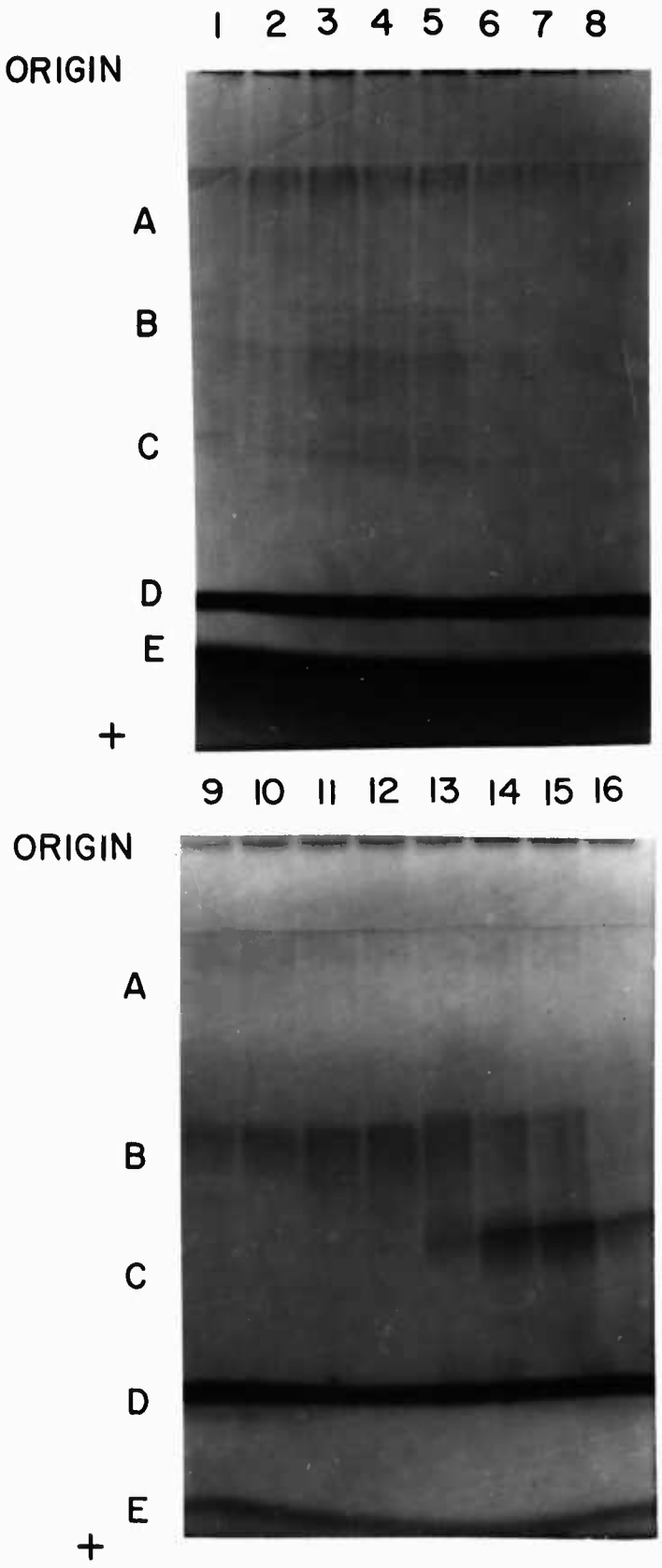

ORIGIN
1
2
3
4
A
B
C
D
+ E

Figure 13. Electrophoretic patterns of fractions from Sephadex G-100 (1-8), and DEAE-cellulose (9-16) column chromatography.

1-8 represent tubes 12-19, from Sephadex G-100 respectively.

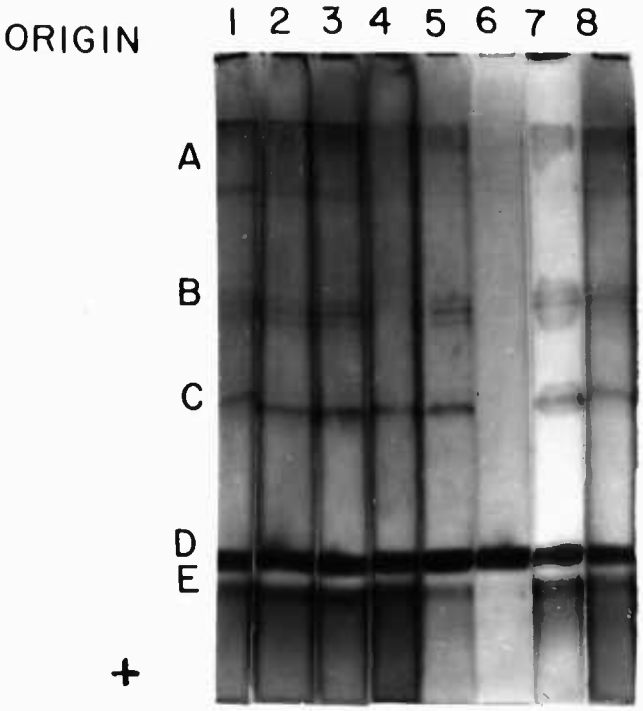
9-16 represent tubes 32-39, from DEAE-cellulose, respectively.

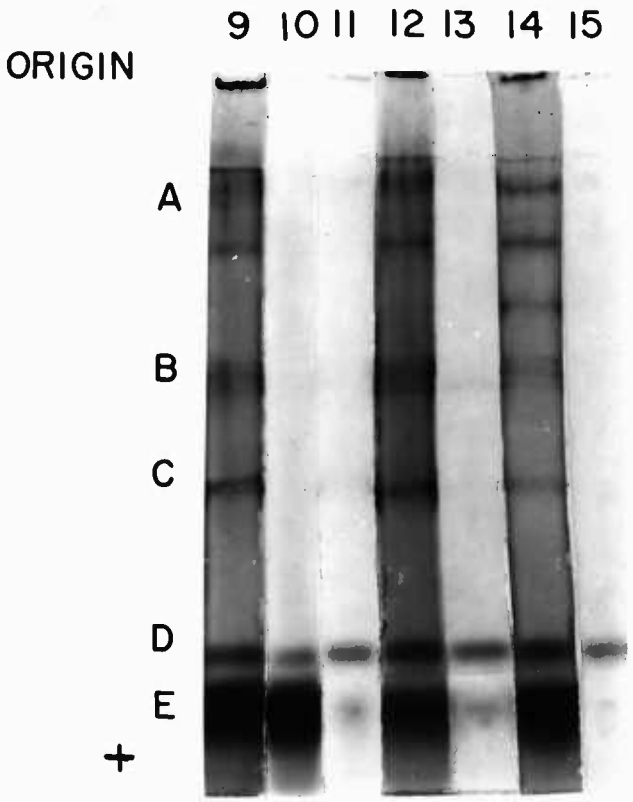

1 2 3 4 5 6 7 8
ORIGIN
A
B
C
D
E
+
9 10 11 12 13 14 15 16
ORIGIN
A
B
C
D
E
+

column. Although these bands appear very light, on the original gels, it was evident that there was a tendency for group A to be concentrated in the first portion of the Sephadex peak while Group C was more dominant in the last portion of Sephadex fractions. This indicates that Sephadex separated the PPO molecules according to their molecular size. Molecules of lesser size were retarded to a greater extent on the Sephadex column and migrated faster on the gel. Also noted on the original gels was that group B contained four bands and group C had three bands.

Strips 9-16 in Figure 13 are fractions 32-39 of the DEAE-cellulose column. Here again there was a fractionation of the groups with the first fractions containing group A, the next fractions group B, and the last fractions group C. Since group A was either of larger size or had less surface-charge density on the molecule, it migrated slower on the gel electrophoresis and was retarded less on the DEAE-cellulose column. In contrast to this, group C, which was retarded the greatest on the DEAE-cellulose column, was probably the most highly charged and/or smallest molecular size, since it migrated at the fastest rate on the gel.

Strips 1-8 Figure 14 show the use of different substrates with the polyacrylamide-gel electrophoresis. Catechol, chlorogenic acid, dopa and p-cresol served as substrates for groups A, B, and C with the following chromogenic patterns: dark brown, light yellow, deep red, and red, respectively. With 10 mM pyrogallol no activity was evident in the strip (Figure 14-6), however at 100 mM band one group


ORIGIN
1 2 3 4 5 6 7 8
A
B
C
D
E
+


ORIGIN
9 10 11 12 13 14 15
A
B
C
D
E
+

A oxidized pyrogallol to a yellow chromogen. Group C was the only group of cherry PPO isozymes that showed activity toward 4-methyl catechol and this was revealed as a red color. p-Cresol was oxidized by the enzyme on the gel, but did not serve as a substrate in the spectrophotometric assay. Pomerantz and Warner (1967) demonstrated an activator function for dopa in tyrosine hydroxylation catalyzed by hamster melanoma PPO. In agreement to this, Long and Alben (1968) reported that with a spectrophotometric assay the presence of an activator (4-methyl catechol) was necessary for the oxidation of p-cresol by mushroom PPO. While in the absence of this activator, the enzyme was inactive toward p-cresol. When 4-methyl catechol was added to the spectrophotometric assay used in this work, cherry PPO also oxidized p-cresol. This activity was greater than with 4-methyl catechol alone. It is not known from these trials if the 4-methyl catechol was an activator for the p-cresol or vice versa. In the gel, the p-phenylenediamine may have acted as an activator for the oxidation of p-cresol.

To determine if the isozymes of cherry PPO could be dissociated into subunits, the acetone precipitate preparation was dialyzed against a 0.25% sodium dioctylsulfosuccinate for 24 hours and also another portion was dialyzed against 10 mM $CaCl_2$ for 16 hours. Spectrophotometric assay showed no differences in the activity of these treated portions from that of the control. Strips 2 and 3 of

Figure 14 demonstrate that with catechol as substrate, no differences were noted in the gel electrophoretic patterns of these two treatments. This study indicates that there was no dissociation into subunits under the conditions used.

Strips 9-15 of Figure 14 are the results of various inhibitors on the activity of PPO. These gels were exposed to a 10 mM inhibitor for one hour before being placed in the catechol solution. Electrophoretic patterns reveal that DEDTC, $K_2S_2O_3$, dithiothreitol, and KCN inhibited all three groups, while thiourea and 2, 3-dihydroxynaphthalene did not inhibit these groups. All bands within a given group appeared to behave similarly toward the inhibitors.

Since these inhibitors showed an induction period in the spectrophotometric assay, the gels were immersed in distilled water for 12 hours. Results from these studies suggest that the inhibition was reversible and after the time in the water, the PPO activity was restored.

In vivo Studies

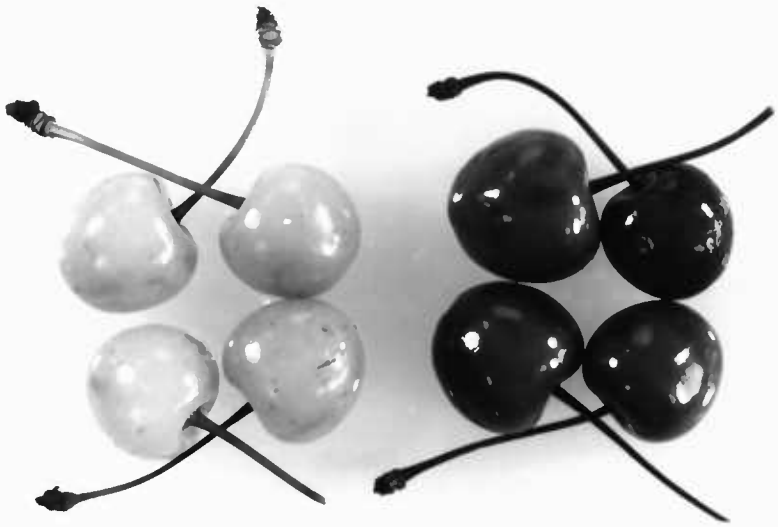
Table 12 shows the results of Royal Anne cherries that have been bruised by dropping ten feet and treated with different inhibitors. Combination of citric acid and NaCl treatment resulted in a desirable color (yellow) but had the disadvantage of lowering the pH to 1.9, which caused a large number of cracks in the cherries. $K_2S_2O_3$ was

Table 12. Evaluation of Royal Anne cherries that have been bruised and treated with various inhibitors.

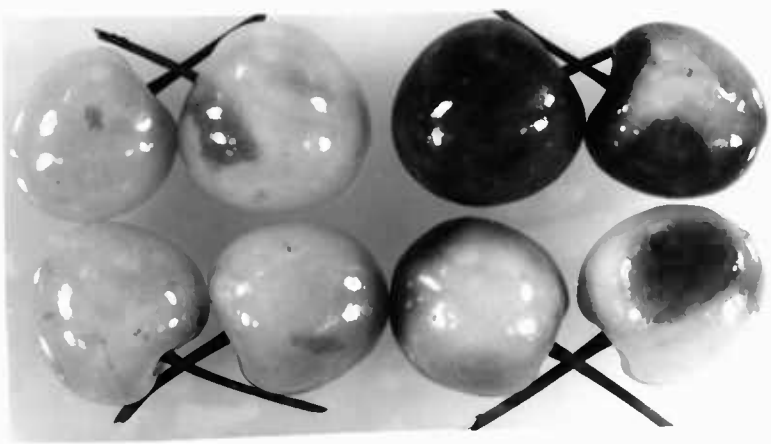
Treatment[a]	Color of skin		Texture	pH
	2 weeks	6 weeks		
control	browning close to the top and the bruised area	dark brown all over, 10% cracked	soft	4.3
Ascorbic acid (10 mM)	browning of the bruised area	slight browning, 50% cracked	soft	5.3
Thiourea (10 mM)	slight browning of the bruised area	brown with cracking and water pockets	soft	4.2
Citric acid (100 mM) NaCl (500 mM)	yellow to brown with cracks skin rupture	light brown, 50% cracked, water pockets	very soft	1.9
$K_2S_2O_3$[a] (10 mM)	light yellow and light brown around the bruised area	light yellow, brown around the bruised area, 10% cracked	soft	4.3
DEDTC (10 mM)	browning of the bruised area, a precipitate	brown	soft	7.3
Dipped in 0.1% oleic acid emulsion for 5 min; placed in 2.5% DEDTC	yellow precipitate, unpleasant odor	light yellow	very firm	8.3

[a]0.1% sodium benzoate added as preservative.
[b]After one day, most of the bleaching was complete.

superior in bleaching most of the anthocyanin pigment, but left brown blemishes and a soft texture. DEDTC was partially successful, but it did not appear to penetrate the waxy layer on the outside of the cherries. After dipping the cherries in a 0.1% oleic acid solution for 5 min, followed by 2.5% aqueous DEDTC containing 0.5% potassium sorbate as preservative, DEDTC reduced both browning and softening. This treatment apparently accelerated the penetration of DEDTC, which caused a bleaching of the anthocycnin pigments during one week at room temperature. There was a firming in the cherry texture with no formation of solution pockets or cracks. The pH of the DEDTC solution was 8.3. In Figure 15 are photographs taken six weeks after treatment which show the brown blemish areas in the $K_2S_2O_3$ treated sample, while the DEDTC treated sample is free from these blemishes.



DIETHYLDITHIOCARBAMATE CONTROL



$K_2S_2O_3$ (1×10^{-2} M) DEDTC (1×10^{-2} M)

DISCUSSION

While this work was in its later stages, Wong et al. (1971) published a similar study on the PPO system of the clingstone peach. These workers were able to isolate PPO from the peach with use of PEG followed by acetone precipitation and chromatography on DEAE-cellulose. These fractions were then separated by disc-electrophoresis and demonstrated some properties that were similar to the enzymes isolated from Royal Anne cherries.

Although PVP has been used in many cases for binding of phenolic compounds from plant enzyme extracts, it was found that PEG protected PPO better than the PVP in this study. This was probably due to lower binding of the enzyme by PEG than PVP. Walker and Hulme (1965) have reported that PVP in high concentrations will bind protein as well as the polyphenolic compounds of plant tissue. One of the problems encountered in the present study was the removal of PEG from the enzyme extract after the polyphenolics had been bound. This problem was solved by washing the enzyme with acetone. These results are in agreement with those of Badran and Jones (1965) who concluded that PEG was a more effective phenolic binding agent than PVP for extracting PPO from the core of green banana. Arakji (1968) also found that PEG-acetone treatment of McFarlin cranberry PPO was better than treatment with PVP.

Protamine sulfate has been used in the purification of the esterases from green beans and peas in this laboratory (Veerabhadrappa and Montgomery, 1971). It was somewhat surprising that protamine sulfate did not remove a larger quantity of the 260 nm absorbing material than was evident from the data presented in Table 4. Although ammonium sulfate precipitation of cherry PPO was abandoned in favor of acetone precipitation of the PEG-acetone extract, the precipitation of PPO by ammonium sulfate appears to have some merit. The preparation could be easily concentrated with a low level of ammonium sulfate. The nature of the insoluble material resulting from the precipitation of the enzyme at 10% saturation of ammonium sulfate was not investigated further; however, ammonium sulfate may have denatured some of the proteins or caused a complexing of PPO with some other material in the enzyme extract.

Dialysis appeared to be beneficial in the removal of PEG or other 260 and 280 nm absorbing material and a necessary step in the purification of the cherry PPO activity before separation by column chromatographic techniques.

Three fractions of Royal Anne cherry PPO were separated by gel filtration with Sephadex G-100 (Figure 5). This observation differed from work cited in the literature which indicated no separation of PPO by this procedure (Kertesz and Zito, 1962;

Nakamura et al., 1966; Gaspar et al., 1969). The separation profile of cherry PPO on the Sephadex G-100 column indicates that these enzymes have similar molecular size. Since the use of gels with higher exclusion limits did not provide better separation, cherry PPO may complex with much larger molecules. However, the separation that was obtained with Sephadex G-100 suggests that the cherry PPO system contains more than one enzyme of different molecular sizes.

Since cherry PPO was eluted at the void volume as one peak on CM-cellulose column chromatography, these enzymes had a negative net charge at pH 4.5. However, separation of PPO on DEAE-cellulose column chromatography was rewarding. The separation of two components indicates that the PPO was composed of enzymes having different surface charge densities. This observation is in agreement with the data for PPO extracted from potato (Patil and Zucker, 1965), apple (Walker and Hulme, 1965), and banana (Palmer, 1963). Purification and specific activity which was obtained on the DEAE-cellulose column, was higher than that reported by Wong et al. (1971) for the PPO of peach.

Results from electrophoretic studies show that the cherry PPO is composed of three groups of electrophoretically separable components. Comparison of the results of gel filtration and ion exchange chromatography with the electrophoretic patterns suggests that the PPO from Royal Anne cherry not only differ in molecular size but

also in their net charge. The order of elution of the three fractions (S1, S2, S3) from Sephadex G-100 was in opposite order of their mobility on polyacrylamide-gel electrophoresis. These data suggest that the first groups had the highest relative molecular size with the second and third group of intermediate and smallest molecular size, respectively.

The pH optima of cherry PPO fractions were similar to those reported by others in studies on apple (Shannon and Pratt, 1967), McFarlin cranberry (Chan and Yang, 1971) and more recently in peach (Wong et al., 1971). The difference in pH optimum for the PEG-acetone extract and the DEAE-cellulose fractions would indicate that either a purification had taken place on the DEAE-cellulose column with the loss of a fraction that had a lower pH optimum, or that chromatography on DEAE-cellulose caused a possible shift in the ionic environment of PPO which resulted in a change in the pH optimum.

Results of the substrate specificity experiment showed that the cherry PPO were specific for the o-diphenols but did not oxidize monophenols. This compares favorably with most of the other work on the substrate specificity of plant PPO. In contrast to this, mushroom and potato PPO had been found to oxidize both mono- and o-diphenols. Brown and Ward (1958) showed that mouse tyrosinase oxidized both tyrosine and dopa but the rate of dopa oxidation was four

times greater than that of tyrosine. Differences in substrate specificities between DE1 and DE2 toward pyrogallol and 4-methyl catechol indicate that these two enzymes were different. As shown later, the substrate specificity may have been due to the charge differences and/or size variations in these two fractions. However, the substrate specificity of these two fractions was similar toward other substrates that were used since they oxidized only o-diphenols and not monophenols. Since monophenolase is less stable than o-diphenolase (Mason, 1956), the acetone precipitate was used to determine if monophenolase activity had been lost during the chromatography. No monophenolase activity was observed in the acetone precipitate, even though differences in the rate of oxidation toward substrates were noted between the acetone precipitate and DEAE-cellulose fractions. When p-cresol was used as the substrate, the two fractions and the acetone precipitate did not show activity for 20 min, but when a mixture of 4-methyl catechol and p-cresol was used, activity was observed. This observation confirmed the results of Long and Alben (1968). However, when polyacrylamide-gel electrophoretic strips were treated with p-cresol, all three groups oxidized p-cresol. Based on the work of Nelson and Dawson (1944), Mason (1966), and Long and Alben (1968), there is a slow enzymatic conversion of monophenol to o-quinone and a slow non-enzymatic reduction of the o-quinone to an o-diphenol until the

o-diphenol concentration is sufficient to allow the rapid oxidation of monophenol. From the above observation and results of this study, it was concluded that possibly p-phenylenediamine acts as activator for the reaction. A striking observation from gel electrophoretic strips was that group A was specific for pyrogallol and group C was specific for 4-methyl catechol.

Michaelis constants (K_m) for Royal Anne cherry PPO were slightly different from that of the Bartlett pear (Tate et al., 1964) but were 71-88 fold different from the PPO of tobacco root (Sizler and Evans, 1958). These differences may be attributed to different sources of the enzymes.

Although it has been known for quite some time that sodium chloride will retard the browning of cherries during processing, data presented in Table 11 indicate that many other compounds could be better inhibitors of cherry PPO. The use of DEDTC as the complexing agent for the copper of PPO appears to have some merits (Swain et al., 1966; Vaughan and Butt, 1970; Grncarevic and Hawker, 1971). Data presented in this report show that DEDTC inhibited both the cherry PPO and the browning due to bruising of the cherries. These observations agree with the work of Swain et al. (1966), Segal and Segal (1969), Prabhakaran et al. (1969), Grncarevic and Hawker (1971) and Wong et al. (1971), who found that DEDTC would inhibit the PPO of the broad bean, grapes, Mycobacterium leprae, grape berries

and peaches, respectively. Data presented in Figure 14 show that DEDTC, $K_2S_2O_3$, dithiothreitol, and KCN completely inhibited all three groups. However, groups A and C were less sensitive than B toward DEDTC and dithiothreitol after a 12 hr induction period. Group A was more resistant than B and C toward KCN and $K_2S_2O_3$. These variations in the properties of the individual groups suggest the presence of more than one PPO with different sensitivity toward different inhibitors.

DEDTC has the possibility of use in the commercial production of brine cherries for making maraschino cherries. However, this requires further investigation to determine the proper concentration and effects on the quality of the final product. Combining DEDTC with SO_2 may be the most suitable treatment for the industrial production of maraschino cherries with better quality and less loss of fruit. DEDTC has been used in Australia to retard browning during the dehydration process of Sultana grape berries (Grncarevic and Hawker, 1971). However, the possibility of toxicity cannot be ruled out and it should be investigated.

Changes in the absorption spectrum of the reaction mixture of the catechol with the cherry PPO would indicate that the absorbance at 400 nm was a measure of initial product of the oxidation and that this product was further oxidized to a substance which absorbs at 290 nm. As the 290 nm absorbing material was formed the product absorbing at

400 nm and the catechol which absorbed at 275 nm were reduced. This may suggest a mechanism by which the o-quinone combines with unreacted catechol to form new compounds which absorb at 290 nm. The nature of this new compound was not investigated and is unknown at this time.

Since Markert and Appella (1961) considered the term "isozyme" to be applicable to multiple forms of enzymes differing in specific properties, the name isozyme can be designated to Royal Anne cherry PPO. PPO that are different in their activity toward substrates and inhibitors and other properties have been called isozymes (Walker and Hulme, 1965; Wong et al., 1971). The largest number of isozymes observed in this work was 11 which agrees with that of Constantinides and Bedford (1967) with potato PPO.

Some workers reported that under certain conditions the isozymes of PPO were interconvertible. Bouchilloux et al. (1963) concluded that subunits were involved in the tyrosinase structure and the combination of the subunits in different ways may lead to multiple forms. Jolley et al. (1969) reported that association and dissociation could take place under varying conditions of pH, ionic strength, heat, ionic detergents, and protein concentration. Jolley and Mason (1965) suggested that polymerization of like and unlike subunits and conformation changes of a single protein were possible reasons for multiplicity of PPO in mushroom. However, data reported in Figure 14 show that

sodium dioctylsulfosuccinate did not cause dissociation of the cherry PPO. This may have been due to the lack of the detergent in the electrode and gel buffers. However, similarities between the samples treated with detergent and those not treated would indicate that, if dissociation had occurred, reassociation had taken place to form the same bands as were present before the treatment with the detergent. However, full exploration of the multiplicity of Royal Anne cherry PPO requires purification of the individual fractions and a detailed study of their physico-chemical properties.

SUMMARY AND CONCLUSIONS

This investigation was undertaken to study the PPO of Royal Anne cherries and to apply the results of this study to minimize the undesirable enzymatic browning in mechanically harvested cherries. An optimum condition for the extraction and assay of Royal Anne cherry PPO was determined. This method was found to be reproducible and has given preparations of high specific activity in a reasonable yield.

For further purification of the enzyme, fractionation with ammonium sulfate, precipitation with acetone, column chromatography followed by polyacrylamide-gel electrophoresis was used. The result of the purification showed that Royal Anne cherry PPO consists of isozymes possessing few differences in their substrate and inhibitor properties.

The following findings were obtained from these experiments:

1. The specific activity of the enzyme was increased by the acetone precipitation and dialysis against phosphate buffer.
2. Three fractions (S1, S2, S3) were obtained from chromatography on Sephadex G-100.
3. DEAE-cellulose chromatography with a concave gradient elution resulted in two fractions, DE1 and DE2.
4. Subsequent analysis of the individual fraction tubes from cellulose chromatography by polyacrylamide-gel

electrophoresis demonstrated the presence of three groups with 11 bands. DE1 consisted of the slow and intermediate moving groups, while the fast migrating group was in DE2.

5. The three groups of Royal Anne cherry PPO were not only different in molecular size but also differ in their net charges.

6. Isozymes of PPO were not artifacts nor degraded from a single entity.

7. Fractions obtained from ion exchange chromatography were active toward o-diphenols but not monophenols.

8. K_m values were 43, 53, and 46 mM catechol for DE1, DE2 and the acetone precipitate, respectively. V_{max} was the same for both DE1 and DE2 with a value of 1000 units/ml and 760 units/ml for the acetone precipitate.

9. Optimum pH for the fractions DE1 and DE2 ranged from 7.3 to 7.8, whereas, the acetone precipitate had an optimum pH of 7.0.

10. Heat inactivation at 75°C of Royal Anne cherry PPO follows first order kinetics with a half-life of DE1 and DE2 of 1.9 and 2.7 min, respectively. The acetone precipitate had a half-life of 8 min at 75°C. At room temperature PPO from acetone precipitate had a half-life of four and one-half days.

11. Inhibitor studies indicated the presence of sensitive and resistant components of PPO activity in each fraction. DEDTC inhibited PPO in vitro. After pretreatment with oleic acid, DEDTC inhibited browning in fresh cherries. Further studies are needed to determine the optimum conditions for inhibition of browning in mechanically harvested cherries.

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